STEM CELL RESEARCH – STATE OF ART, REVISED CONCEPTS AND PERSPECTIVES

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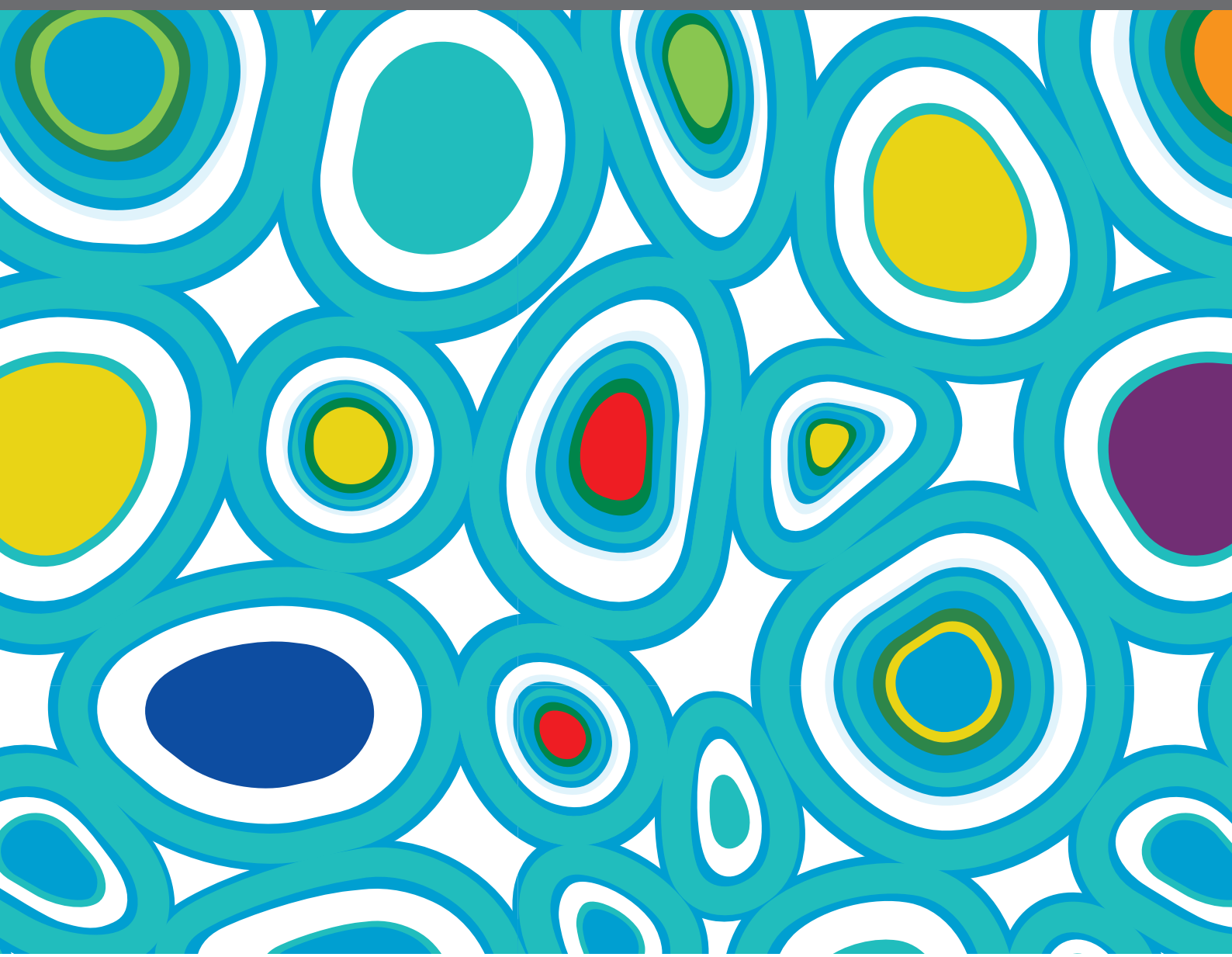

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STEM CELL RESEARCH – STATE OF ART, REVISED CONCEPTS AND PERSPECTIVES

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HYPOTHESIS AND THEORY
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Similarities Between Embryo Development and Cancer Process Suggest New Strategies for Research and Therapy of Tumors: A New Point of View

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Here, I propose that cancer stem cells (CSCs) would be equivalent to para-embryonic stem cells (p-ESCs), derived from adult cells de-re-programmed to a ground state. p-ESCs would differ from ESCs by the absence of genomic homeostasis. A p-ESC would constitute the cancer cell of origin (i-CSC or CSC0), capable of generating an initial tumor, corresponding to a pre-implantation blastocyst. In a niche with proper signals, it would engraft as a primary tumor, corresponding to a post-implantation blastocyst. i-CSC progeny would form primary pluripotent and slow self-renewing CSCs (CSC1s), blocked in an undifferentiated state, corresponding to epiblast cells; CSC1s would be tumor-initiating cells (TICs). CSC1s would generate secondary CSCs (CSC2s), corresponding to hypoblast cells; CSC2s would be tumor growth cells (TGCs). CSC1s/CSC2s would generate tertiary CSCs (CSC3s), with a mesenchymal phenotype; CSC3s would be tumor migrating cells (TMCs), corresponding to mesodermal precursors at primitive streak. CSC3s with more favorable conditions (normoxia), by asymmetrical division, would differentiate into cancer progenitor cells (CPCs), and these into cancer differentiated cells (CDCs), thus generating a defined cell hierarchy and tumor progression, mimicking somito-histo-organogenesis. CSC3s with less favorable conditions (hypoxia) would delaminate and migrate as quiescent circulating micro-metastases, mimicking mesenchymal cells in gastrula morphogenetic movements. In metastatic niches, these CSC3s would install and remain dormant in the presence of epithelial/mesenchymal transition (EMT) signals and hypoxia. But, in the presence of mesenchymal/epithelial transition (MET) signals and normoxia, they would revert to self-renewing CSC1s, reproducing the same cell hierarchy of the primary tumor as macro-metastases. Further similarities between ontogenesis and oncogenesis involving crucial factors, such as ID, HSP70, HLA-G, CD44, LIF, and STAT3, are strongly evident at molecular, physiological and immunological levels. Much experimental data about these factors led to considering the cancer process as ectopic rudimentary

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ontogenesis, where CSCs have privileged immunological conditions. These would consent to CSC development in an adverse environment, just like an embryo, which is tolerated, accepted and favored by the maternal organism in spite of its paternal semi-allogeneicity. From all these considerations, novel research directions, potential innovative tumor therapy and prophylaxis strategies might, theoretically, result.

Keywords: HSP70, HLA-G, ESCs, MSCs, CSCs, tumor hierarchy/immunoevasion/therapy/prophylaxis

INTRODUCTION

Nearly 30 years ago, I proposed that cancer stem cells (CSCs) would be cells blocked at early steps of their genic program, with reiterated expression of embryonic factors responsible for malignant characters and loss of differentiated factors for terminal genomic homeostasis: thus, CSCs would be equivalent to para-embryonic stem cells (p-ESCs) (Manzo, 1989). The main aspects of stem cells (SCs) are self-renewal, pluripotency (Liu et al., 2007) and the need for a niche (Plaks et al., 2015) with proper stereotrophic factors (space, oxygen) and persistent specific signals (ACTIVIN-A, BMP, WNT, LIF, FGF, TGFb) (Okita and Yamanaka, 2006; Xiao et al., 2006).

ESCs arise from the inner cell mass (ICM) of mammalian pre-implantation blastocyst (Henderson et al., 2002; Ginisa et al., 2004; **Figure 1B**); they can self-renew symmetrically and indefinitely, maintain the widest pluripotency and generate all cell lineages of the body. This phenomenon requires defined transcription factors (TFs) specifically expressed in SCs, such as OCT4, SOX2, NANOG, STAT3, KLF4, c-MYC et al., that together constitute a pluripotency gene regulatory network (PGRN) (HaKashyap et al., 2009; Do et al., 2013; Festuccia et al., 2013). Human ESCs (hESCs) and human embryos express comparable stage-specific embryonic antigens (Henderson et al., 2002) and can differentiate into the trophectoderm (TE) by BMP4 (Xu et al., 2002; **Figure 1B**). hESCs are epithelial cells (Ullmann et al., 2006), but during *in vitro* differentiation they can acquire a mesenchymal phenotype (Eastham et al., 2007).

MSCs (mesenchymal stem cells) have a mesenchymal phenotype and markers (Ullmann et al., 2006; Eastham et al., 2007; Thiery et al., 2009). MSCs, in Matrigel, grow at the periphery of hESC clusters, have an undifferentiated phenotype and preserve potential expression of pluripotency TFs such as NANOG and OCT4. This indicates that ESCs can undergo epithelial–mesenchymal transition (EMT) without loss of pluripotency, which would be expressed after mesenchymal–epithelial transition (MET) (Ullmann et al., 2006; Thiery et al., 2009). Cells with mesenchymal features largely lie at the primitive streak (PS) in the embryo and in the tumor stroma (Thiery et al., 2009; Nishimura et al., 2012; **Figure 1C**).

CSCs are tumor cells that are able to generate all the cell types present in the primary tumor and to form metastases, with identical cell types and hierarchy (Marjanovic et al., 2013; Cabrera et al., 2015). CSCs are a small portion of the tumor mass (Collins et al., 2005; Liu et al., 2014) and are often distinct in tumor-initiating cells (TICs) and tumor migrating cells (TMCs) (Hermann et al., 2007; Biddle et al., 2011). TICs have an epithelial phenotype and are able to grow in an anchorage-independent way, to produce spheroids *in vitro* by self-renewal and to initiate tumor development. TMCs have a mesenchymal phenotype, are free, migrating, invasive and generally quiescent, but are able to generate metastases (Dieter et al., 2011; Brabletz, 2012; Liu et al., 2014).

Therefore, cells with ESC, MSC, and CSC features are at the basis of both embryo development and cancer process (**Figure 2**).

THE TUMOR PROCESS AS ECTOPIC RUDIMENTARY ONTOGENESIS

Cell of Origin (CSC0): Initial Cancer Stem Cell (i-CSC) as a Reprogrammed Para-ESC

Reprogramming would be the main mechanism for genesis and proliferation of the initial i-CSC (CSC0): it has been shown that somatic cell reprogramming requires a MET at its initiation (Li et al., 2010; Samavarchi-Tehrani et al., 2010), with subsequent reversal to a self-renewal and pluripotency state (Silva et al., 2008; Nichols and Smith, 2009; Gillich et al., 2013). Thus, I propose that i-CSCs would be equivalent to ESCs, with one important difference: while an ESC has an integral genic program, an i-CSC would have a program impaired in terminal genome homeostasis (Katoh, 2007; Silva et al., 2008). In this model, an i-CSC would be a p-ESC (**Figure 3**). At a molecular level, iCSC genesis would presumably require the following steps in the original cell: (a) De-programming (de-differentiation), by impairment of the systems of genomic homeostasis, carried out by growth modulators (p16, p21, p53, p27, E2A, pRb) (Prabhu et al., 1997; Ouyang et al., 2002; Menendez et al., 2010; Tapia and Schöler, 2010) and/or by specific autocrine and paracrine signal pathways (WNT, BMP, LIF, FGF, ERK, TGFb) (Sato et al., 2004; Katoh, 2007; Scheel et al., 2011; Chen et al., 2015), with a consequent differentiation loss and reversal to a previous more primitive (mesenchymal/epithelial/undifferentiated) state (Silva et al., 2008; Schwitalla et al., 2013). (b) Stable PGRN reactivation, by de-regulation of the activity of defined genes (NANOG, OCT4, SOX2, STAT3, ID), with reacquisition of self-renewal and pluripotency (Silva et al., 2008; Do et al., 2013; Yin et al., 2015). (c) Reprogramming, through ID1 gene expression, that prevents i-CSC/CSC0s (p-ESCs) and their direct progeny from normally differentiating ("blocking event") (Ying et al., 2003; Wang et al., 2012; Nair et al., 2014). ID proteins associate with ubiquitous E proteins, preventing their DNA binding and differentiation activity (Ruzinova and Benezra, 2003; Wang

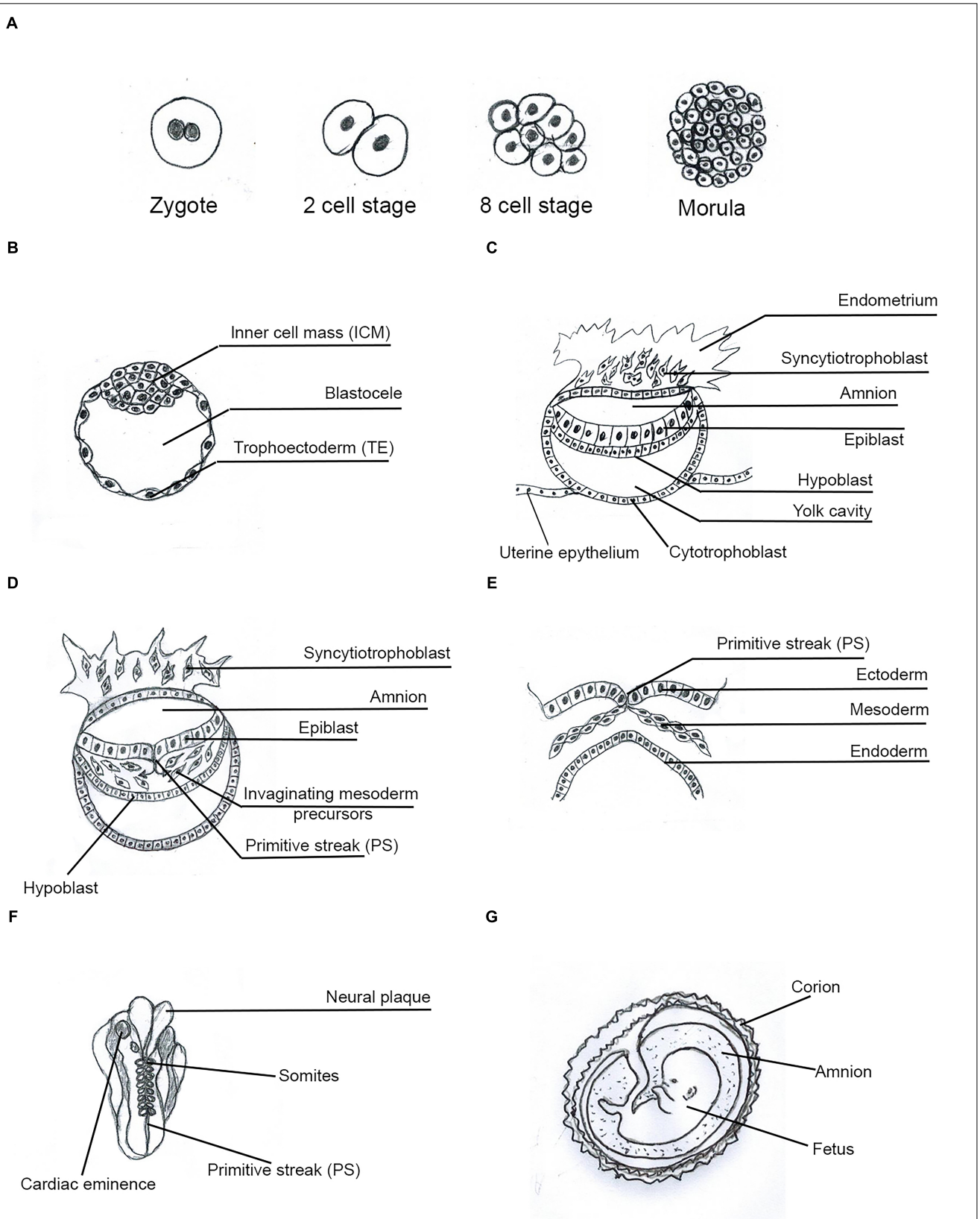

FIGURE 1 | Human Embryo development. Main phases and structures of the embryogenesis process. **(A)** Zygote to morula transition; **(B)** pre-implantation blastocyst; **(C)** implanted blastocyst; **(D)** early gastrula; **(E)** late gastrula; **(F)** somito-histo-organogenesis; **(G)** fetal growth-differentiation.

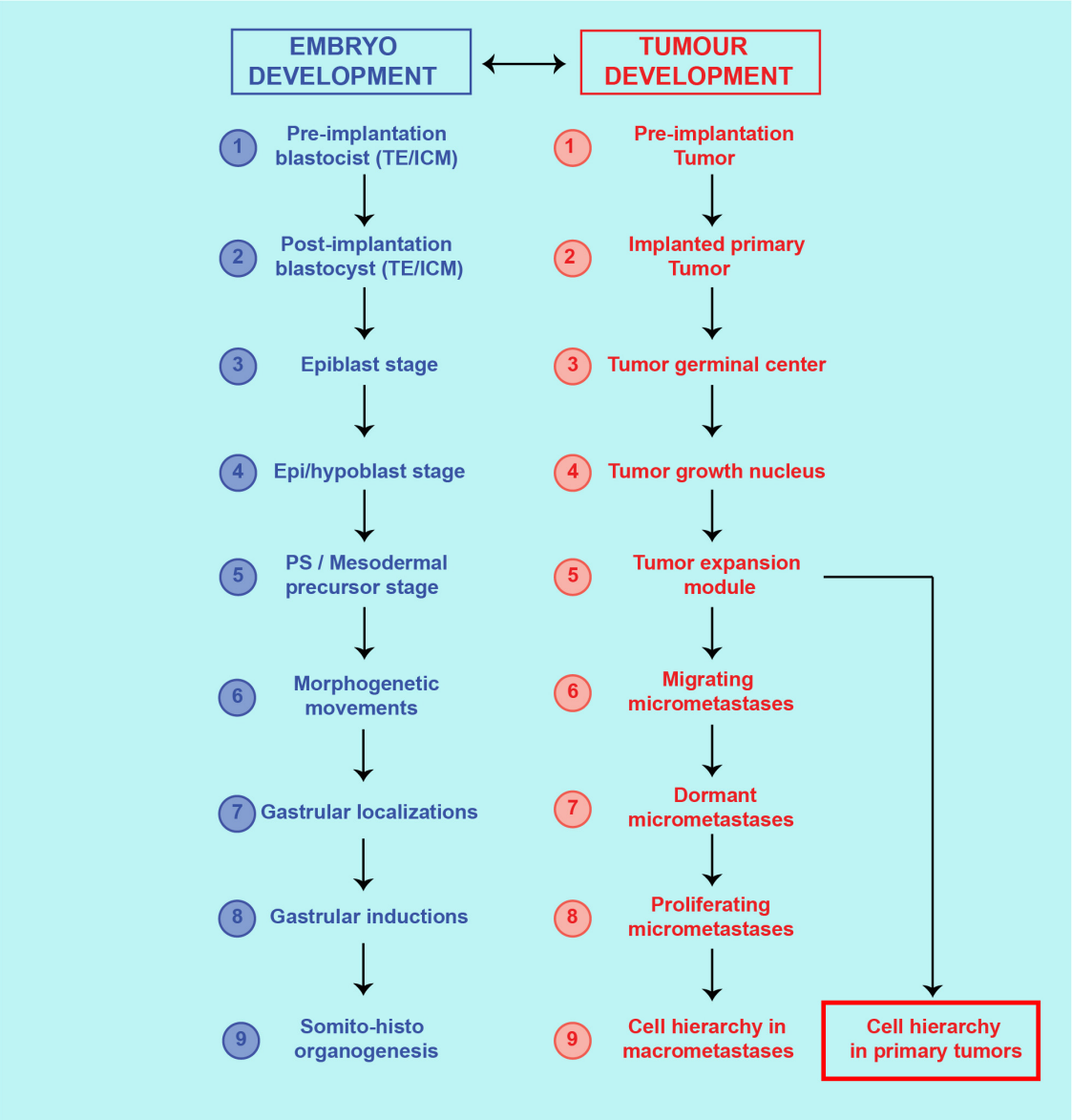

FIGURE 2 | Theoretical similarities between cancer process and ontogenetic development. Correspondence of steps and structures between the cancer process and embryo development.

and Baker, 2015). ID1 constitutive expression determines neo-angiogenesis, survival, anti-apoptosis and invasion/migration, both in cancer and embryo development.

Thus, I suggest an iCSC/p-ESC model for the tumor initiation.

Primary Cancer Stem Cells (CSC1s) as Epiblast Cells

Once generated, i-CSC/CSC0s would survive and symmetrically proliferate early in an anchorage-independent way. Due to its presumed nature as a p-ESC, an i-CSC/CSC0, like an ESC, could generate all cell lineages of the body and, therefore, an initial structure similar to a pre-implantation blastocyst *in vivo* (**Figure 1B**), or a tumorsphere *in vitro* (**Figure 3**; Johnson et al., 2013), that is able to implant in a surrounding microenvironment (niche) (Stewart et al., 1992), where ID1 proteins would synchronize stemness and anchorage to the niche (Niola et al., 2012). Here, the p-ESC direct progeny would proliferate in an anchorage-dependent way, with an autocrine symmetric mechanism, generating primary cancer stem cells (CSC1s), presumably corresponding to epiblast cells (**Figure 1C**). CSC1s would form a "tumor germinal center" that continuously feeds the neo-forming tumor (**Figures 2**, **3**). CSC1s would be blocked in a pluripotent undifferentiated/epithelial state (p-ESCs/p-Epi-SCs) (Nichols and Smith, 2009; Gillich et al., 2013), owing to NANOG, OCT4, SOX2, and STAT3 (PGRN)

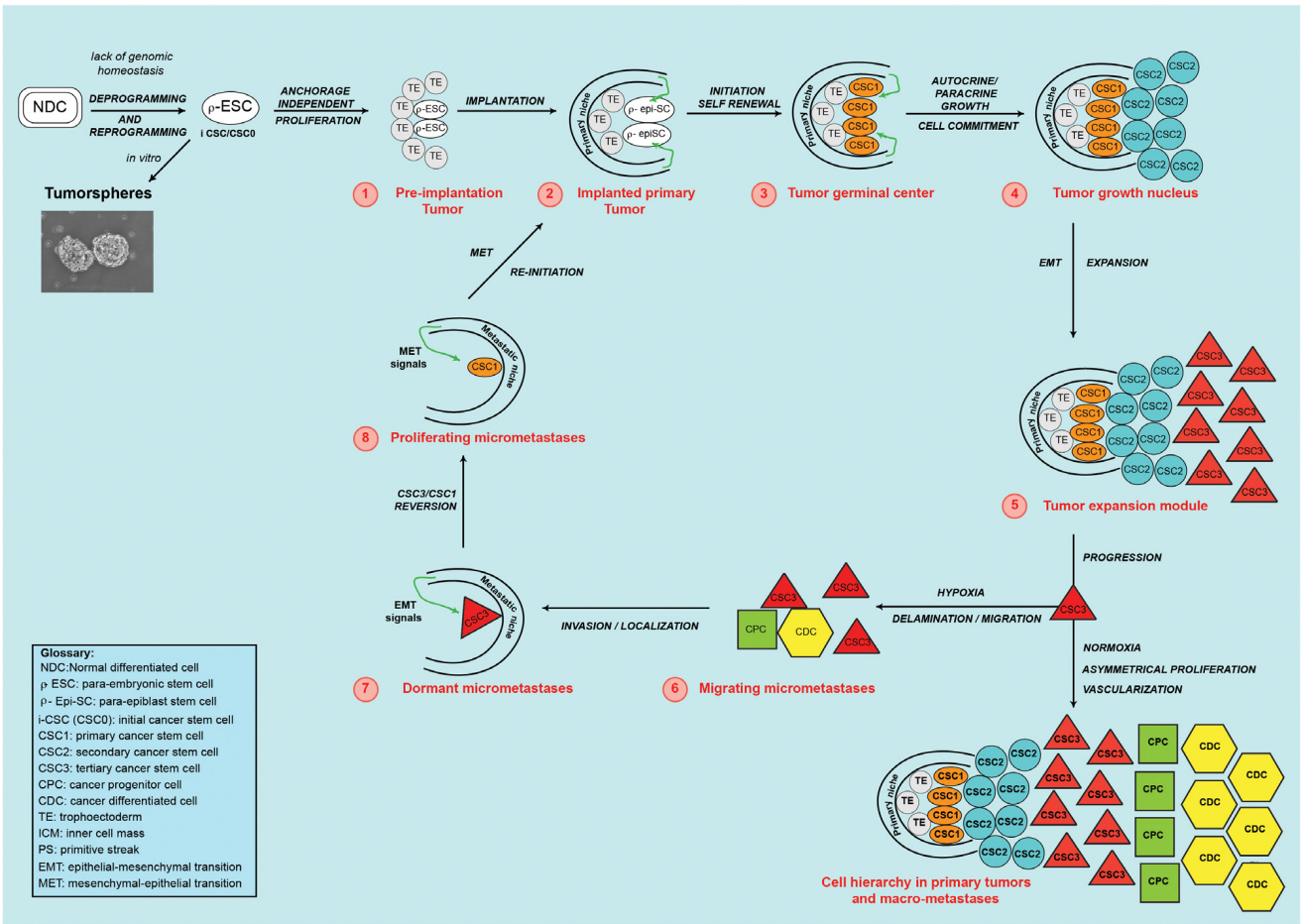

FIGURE 3 | Theoretical cancer process. A normal differentiated cell (NDC), in the absence of genomic homeostasis, would be de-programmed and re-programmed to a para-ESC (p-ESC), constituting the initial cancer stem cell (i-CSC or CSC0). **(1)** An i-CSC, by anchorage-independent proliferation, would generate p-ESC progeny, forming an initial tumor *in vivo* (1-red) (or a tumorsphere *in vitro*), corresponding to a pre-implantation blastocyst (TE/ICM). **(2)** The initial tumor would install in a niche with proper factors and develop a primary tumor, mimicking an implanted blastocyst in the endometrium. **(3)** By PGRN activity, primary undifferentiated, pluripotent, slow self-renewing CSCs (CSC1s/TICs) would arise. CSC1s, epigenetically blocked in a ground/primed state, corresponding to the epiblast state, would form a "tumor germinal center," continuously feeding the tumor. **(4)** Through autocrine/paracrine growth, from CSC1s, committed non-self-renewing secondary CSCs (CSC2s) would arise. CSC1s and CSC2s, together, would form a "tumor growth nucleus" corresponding to the epiblast/hypoblast state. **(5)** From CSC1s/CSC2s, tertiary CSCs (CSC3s), with a mesenchymal phenotype (EMT), would be generated. CSC3s would be able to migrate and invade adjoining sites, mimicking delamination of mesodermal precursors at the primitive streak. CSC1s, CSC2s, and CSC3s, together, would form a "tumor expansion module". In the growing primary tumor, favorable conditions (normoxia) would induce CSC3s to proliferate asymmetrically and generate CPCs and, then, CDCs. This would determine a CSC1-CSC2-CSC3-CPC-CDC cell hierarchy and tumor progression, thus mimicking a partial, rudimentary somito-histo-organogenesis process. **(6)** In parallel, unfavorable conditions (hypoxia) would induce CSC3s to migrate as circulating micro-metastases, mimicking the gastrula morphogenetic movements. **(7)** In distant niches with proper EMT signals, CSC3s in the niche would locate as quiescent micro-metastases, mimicking embryonic locations of mesodermal cells at somitogenesis sites. **(8)** Micro-environmental proper MET signals, would induce mesenchymal CSC3 to revert, into epithelial self-renewing CSC1s mimicking gastrular induction (TICs). Self-renewing CSC1s, as TICs, would form a new tumor germinal center and re-initiate the tumor process in metastatic sites, repeating the same steps and reproducing the same cell types and hierarchy of the primary tumor. Tumorsphere figure was adapted from Bond et al. (2013).

overexpression (Wen et al., 2010; Miyanari and Torres-Padilla, 2012), or to other genomic conditions inducing ID1 constitutive expression (Ruzinova and Benezra, 2003; Ying et al., 2003; Nair et al., 2014). CSC1s would express self-renewal and pluripotency markers (ALDH1, CD44, CD133) (Wakamatsu et al., 2012). The number of CSC1s would be low, stable and strictly controlled by the "niche contact", as a limiting factor. ALDH1$^+$ CD44$^+$ Ki67$^+$ CSCs, detected in a central position in mammary tumors, might be hypothetical CSC1s, constituting about 0.084% of the tumor mass (Liu et al., 2014). CSC1s would be TICs, like the identified small population of "slow-cycling melanoma cells," essential for continuous tumor growth (Roesch et al., 2010), or the "long-term renewing" TICs (LT-TICs), that drive tumor feed and metastasis formation in colon cancer (Dieter et al., 2011).

Thus, I propose that tumor initiation and growth might be continuously sustained by cells (CSC1s) with features (pluripotency and self-renewal) typical of epiblast cells.

Secondary Cancer Stem Cells (CSC2s) as Hypoblast Cells

When niche contact becomes limiting, only one CSC1 daughter could retain maternal place and phenotype, while the other could acquire a new epithelial phenotype (CSC2) (Ezashi et al., 2005; Quail et al., 2012). CSC2s would correspond to hypoblast cells (**Figure 1C**) and therefore they would not have self-renewal and pluripotency, differently to CSC1s. However, CSC2s might, eventually, revert to CSC1s when a niche contact becomes available (Ezashi et al., 2005; Quail et al., 2012). The CSC2 phenotype could occur via the LIF-STAT3-RAS-MAPK-ERK-MYC-ID2 pathway, by NANOG inhibition (Kunath et al., 2007; Yamanaka et al., 2010), ID1/2 switching, loss of stemness/pluripotency (Itahana et al., 2003; Park et al., 2013) and acquisition of fast paracrine growth (Iavarone et al., 1994). Hypothetical CSC2s might be the CSCs with an ALDH1$^+$ CD44$^-$ Ki67$^+$ profile detected in mammary tumors (Liu et al., 2014). The "tumor transient-amplifying cells" (T-TACs) with limited or no self-renewal in human colon cancer, and the "rapidly proliferating main population" surrounding the previously mentioned "slow-cycling melanoma cells" (Roesch et al., 2010; Dieter et al., 2011) also could be hypothetical CSC2s. The CSC2 number would be related to the niche volume, nutriments and oxygen quantities (Ezashi et al., 2005; Mohyeldin et al., 2010; Quail et al., 2012): in mammary carcinomas, ALDH1$^+$ CD44$^-$ Ki67$^+$ CSCs (CSC2s) constitute about 5.54% of the tumor mass and lie in a sub-central site, physically distinct from ALDH1$^+$ CD44$^+$ Ki67$^+$ CSCs (CSC1s) (Liu et al., 2014). CSC1s and CSC2s, together, would form a presumable "tumor growth nucleus" responsible for tumor expansion (**Figure 3**).

Thus, I think that tumor growth might result from an autocrine/paracrine signaling-driven proliferation of cells (CSC2s) with features typical of hypoblast cells.

Tertiary Cancer Stem Cells (CSC3s) as Mesoderm Precursor Cells

When the niche microenvironment becomes limiting for growth, certain stereotrophic factors, such as hypoxia, and particular autocrine or paracrine signals (LIF, STAT3, TGFb, WNT, NOTCH) (Ezashi et al., 2005; Mohyeldin et al., 2010; Scheel et al., 2011) could induce CSC1s/CSC2s to generate a new phenotype (CSC3), that is able to migrate, invade and search for more favorable survival conditions elsewhere (Sahlgren et al., 2008; Conley et al., 2012; Tiwari et al., 2012). This phenotype would have mesenchymal features, that would result as a downstream effect of the STAT3-RAS-MAPK-ERK-MYC pathway, regulating ID3/E47 interactions and promoting tumor cell migration and invasion (Bain et al., 2001) through expression of mesenchymal genes such as MMPs, SNAIL1, TWIST1, and PRRX1. The CSC3 phenotype would be the result of an EMT that recalls the EMT of mesoderm precursors at the PS (**Figures 1D,E**). STAT3-SNAIL1 would confer delamination, migration and invasive properties to CSC3s, as TMCs (Barrallo-Gimeno and Nieto, 2005; Thiery et al., 2009; Kamran et al., 2013; Yin et al., 2015). TWIST1 would increase their invasive properties and, together with PRRX1, would favor installation as micro-metastases in metastatic niches (Yang et al., 2008; Eckert et al., 2011; Tran et al., 2011; Ocaña et al., 2012), thus presumably mimicking the onset of somitogenesis at gastrulation sites. ALDH1$^-$ CD44$^+$ Ki67$^-$ CSCs, shown within mammary tumors and located in a peripheral position, at the invasive front, would be presumable CSC3s, constituting about 12.87% of the tumor mass (Liu et al., 2014). CSC1s, CSC2s, and CSC3s, together, would form a "tumor progression module" (**Figures 2**, **3**). I hypothesize that, here, CSC3s with more favorable stereotrophic conditions (normoxia) would become TWIST1$^+$ CSC3s, that is able to install and proliferate asymmetrically via ID3/E47, generating more differentiated oligopotent cancer progenitor cells (CPCs). CPCs, in turn, would yield further differentiated cancer cells (CDCs) (Ezashi et al., 2005; Cakouros et al., 2010; Mohyeldin et al., 2010). The resulting CSC1s/CSC2s/CSC3s/CPCs/CDCs, together, would mimic an ectopic, rudimentary somito-histo-organogenesis process (**Figure 1F**), and would account for the cell hierarchic heterogeneity in tumor progression (**Figure 2**), where CSCs would be surrounded and protected by CPCs and CDCs (Marjanovic et al., 2013; Liu et al., 2014; Cabrera et al., 2015; **Figure 3**). On the contrary, CSC3s with less favorable stereotrophic conditions (hypoxia) would undergo EMT, becoming SNAIL1$^+$ CSC3s, that would be induced to delaminate and migrate as quiescent circulating micro-metastases (Ezashi et al., 2005; Sahlgren et al., 2008; Mohyeldin et al., 2010; Conley et al., 2012; Tiwari et al., 2012; **Figure 3**).

Thus, I propose that tumor progression might occur by appearance of cells (CSC3s) with features of mesoderm precursor cells, that are able to migrate, invade and colonize new sites.

Micro-Metastases and Macro-Metastases as Localization, Induction, and Growth of Gastrula Migrating Cells

After installation as micro-metastases in defined metastatic sites (Arvelo et al., 2016), by specific niche signals, SNAIL1$^+$ CSC3s would undergo MET (Chaffer et al., 2006; Brabletz, 2012; Ocaña et al., 2012; Teo et al., 2014), reverting to self-renewing CSC1s (TICs), that are able to finally generate macro-metastases (**Figures 2**, **3**). Development of macro-metastases would occur via a hypothetical bidirectional genic system TWIST1/ID3/E47/ID1/PRRX1/CD44/PGRN, balanced by TGFb (Gupta et al., 2007; Bourguignon et al., 2012; Ocaña et al., 2012; Stankic et al., 2013; Teo et al., 2014; Wang and Baker, 2015). This system would form a genic switch point in (re)programming: back, for stemness (MET) via PRRX1-CD44-PGRN-ID1 and, forward, for growth-differentiation via ID3-E47-TWIST1 (Stankic et al., 2013). This would lead, in macro-metastases, simultaneously to self-renewal, angiogenesis and differentiation growth, like in the primary tumor. Presumably, PRRX1 downregulation would suppress the mesenchymal state, restoring the epithelial state (MET) through PGRN reactivation via CD44-ID1. It has been shown that downregulation of PRRX1 expression is necessary for micro-metastases to undergo MET and generate macro-metastases (Brabletz, 2012; Ocaña et al., 2012; Hirata et al., 2015), conferring TIC properties to quiescent CSC3s via an ID3/E47/ID1 switch (O'Brien et al., 2012; Hirata

et al., 2015; Wang and Baker, 2015; **Figure 3**). ID1 and ID3 are necessary for TIC functions in the genesis of both primary tumors and metastases, sustaining proliferation in early stages via p21 (Gupta et al., 2007; O'Brien et al., 2012). Moreover, ID1 and ID3 are required for angiogenesis and vascularization of tumor xenografts (Lyden et al., 1999), necessary for macro-metastasis development. TGFb/ID1 signals promote metastatic colonization via a MET, antagonizing TWIST1 EMT (Gupta et al., 2007; Stankic et al., 2013) in normoxic metastatic sites, but not in hypoxic primary tumor sites (Mohyeldin et al., 2010), where EMT is governed by SNAIL1 (Stankic et al., 2013). I hypothesize that EMT/MET switching could occur through ID3/E47/ID1 balance (Bain et al., 2001; Cubilio et al., 2013; Bohrer et al., 2015; Wang and Baker, 2015) and that it could involve ID3b and ID1b isoforms, generated by alternative splicing of the ID1 and ID3 genes (Deed et al., 1996; Tamura et al., 1998).

CRUCIAL MOLECULAR FACTORS COMMON TO EMBRYOS AND CANCER

Pre-implantation Embryos/Initial Tumors (Initiation)

Pre-implantation embryos lie in unfavorable nutriment and microenvironment conditions. Thus, it is necessary they reach and are installed in a niche that is able to supply proper stereotrophic factors for survival. To this end, molecules such as HSP70, HLA-G, ID, and LIF are crucial both for embryos and cancer.

ID Proteins - ID1, ID2, ID3, and ID4 proteins are highly expressed in normal ontogenetic development, where their function is associated with the primitive proliferative phenotype, and regulate differentiation associating to ubiquitous E proteins (Ruzinova and Benezra, 2003; Wang and Baker, 2015). Numerous studies indicate that ID1, ID2, and ID3 have an oncogenic function, whereas ID4 promotes the survival of adult SCs, differentiation and/or differentiation time (Patel et al., 2015). ID proteins are key regulators of CSCs and tumor aggressiveness (Lasorella et al., 2014). During distinct stages of breast metastases, ID proteins mediate phenotypic switching of CSCs (Stankic et al., 2013) and control CSC niches in an autocrine/paracrine way (Niola et al., 2012; Nair et al., 2014). ID1 has multiple roles in cancer progression, such as implantation in primary and metastatic niches, angiogenesis, CSC survival, chemoresistance, growth, apoptosis inhibition and activation of WNT signaling (Ling et al., 2006; Niola et al., 2012; Nair et al., 2014). In particular, the ID1b isoform has been shown to maintain cell quiescence, confer self-renewal and CSC-like properties, and impair malignancy, inhibiting proliferation and angiogenesis (Tamura et al., 1998; Nguewa et al., 2014; Manrique et al., 2015). Thus, ID1b could be related to slow symmetrical self-renewal, niche anchorage and pluripotency maintenance. The ID1a isoform, on the contrary, could be related to fast asymmetrical self-renewal, angiogenesis and evolutionary growth (Lyden et al., 1999), allowing ID1/ID2 switching, with overcoming of pluripotency and subsequent lineage commitment, like in epi/hypoblast segregation. ID2 enhances cell proliferation by binding pRb, an inhibitor of cell cycle progression, and is directly repressed by p53 (Iavarone et al., 1994; Paolella et al., 2011). A WNT-bCAT signal increases ID2 expression level and the incidence of CSC-like phenotype, mediating the effects of hypoxia on the breast and colorectal CSC hierarchy (Rockman et al., 2001; Dong et al., 2016). I hypothesize that ID2 would be involved in overcoming stemness/pluripotency, thus leading to cell commitment and fast autocrine/paracrine growth, via LIF-STAT3-RAS-MAPK-ERK-MYC (Park et al., 2003; Pankaj et al., 2013), with ERK blocking NANOG-ID1 (Itahana et al., 2003; Kunath et al., 2007; Park et al., 2013). ID1/ID2 switching would occur via ID1-WNT/bCAT-ID2, induced by hypoxia (Rockman et al., 2001), and might be related to the epi/hypoblast and CSC1/CSC2 transitions. Moreover, ID2 expression might lead, via RAS-MAPK-ERK, to the activation of ID3 genes (Bain et al., 2001), with subsequent ID2/ID3 switching and mesenchymal gene expression, namely to EMT. Because the RAS-MAPK-ERK cascade regulates ID3/E2A (E47) interaction (Bain et al., 2001), and ID3/E47 balance would determine stem/precursor differentiation (Bohrer et al., 2015), and because ID3 interacts with ID1 (Wang and Baker, 2015), I hypothesize that ID3/E47/ID1 balance would be related to the potential stemness of mesenchymal phenotypes, such as MSCs and CSC3s (O'Brien et al., 2012; Cubilio et al., 2013; Teo et al., 2014; Bohrer et al., 2015). In more detail, the ID3a isoform could be related to overcoming of the CSC3 SNAIL1^{+} mesenchymal state and to the stem/progenitor switch via TWIST1 (Cakouros et al., 2010; Tran et al., 2011; Bohrer et al., 2015). ID3b isoforms, on the contrary, would be related to the maintenance of the SNAIL1^{+} mesenchymal state of dormant CSC3s and, thus, to a potential EMT/MET switch for reversion to the CSC1 (TIC) phenotype. Indeed, ID3b inhibits vascular formations (Deed et al., 1996; Forrest et al., 2004) and, thus, the possibility of macro-metastasis development, maintaining dormancy. ID4 acts as an ID1/ID2/ID3 inhibitor and promotes E47 binding and transcriptional activity in a differentiation direction (Umetani et al., 2004; Yun et al., 2004; Sharma et al., 2015). ID4 is a potential tumor suppressor (Umetani et al., 2004; Carey et al., 2009) and suppresses MMP2-mediated invasion of glioblastoma-derived cells by direct inactivation of TWIST1 (Rahme and Israel, 2015). I hypothesize that ID4 might be related to terminal differentiation growth, via E47, and to global genomic homeostasis (Umetani et al., 2004; Sharma et al., 2015). Thus, ID proteins seem to be crucial factors both in cancer and embryos.

HSP70 (heat shock protein 70 kDa) molecules are precociously expressed in ontogenesis, from zygotic gene activation, through blastulation, implantation, gastrulation, and organogenesis to fetal maturation (**Figures 1A–G**) (Bensaude et al., 1983; Luft and Dix, 1999). In early ontogenesis and oncogenesis, HSP70 could be a first system of protection and survival for pre-implantation embryos, as well as for initial tumors. Protective action of HSP70 for pre-implantation embryos might occur at an endocellular level for preventing apoptosis (Samali and Cotter, 1996); in effect, HSP70 knockdown renders embryonic cells weaker and apoptotic (Neuer et al., 1998). A small amount of HSP70.1-3 is necessary for pre-implantation embryogenesis

(Luft and Dix, 1999). HSP70 pre-implantation expression includes a constitutive component (HSC70 and HSP70.1-3) from zygote to four-blastomer morula, and a component (HSP70.1-2) inducible by heat and chemical agents in four- to eight-blastomer morula and in blastocyst (**Figures 1A,B**) (Luft and Dix, 1999). In adult normal tissues, HSP70s are normally absent, except for transient expression in normal mitosis (Stangl et al., 2011).

HSP70 deregulated overexpression is associated with tumor transformation. In human tumor cells, global profiling of the surface proteome has revealed HSP70.1-2 abundance (Stangl et al., 2011). HSP70s are highly expressed in various tumor cell types, thus rendered resistant to adverse microenvironments and chemotherapy (Shu and Huang, 2008). In human melanoma cell lines, HSP70.1-2 constitutive expression occurs (Dressel et al., 1998). During tumor development, HSP70s can be expressed on the cell surface or exported in the circulation (Shu and Huang, 2008). Human tumor cell lines of colon, breast, lungs and melanomas bind anti-HSP70.1 monoclonal antibodies (mAbs) (Stangl et al., 2011); highly metastatic tumors, but not their primary or poorly malignant counterparts, express membrane HSP70 (mHSP70) (Stangl et al., 2011). mHSP70^{+} tumors actively release lipidic vesicles (exosomes) with an HSP70^{+} surface (Stangl et al., 2011). Therefore, I suggest that HSP70s might constitute a first important system of protection and survival in early ontogenesis and oncogenesis.

HLA-G (human leukocyte antigen-G) molecules could be a second important system of protection and survival both for initial embryos and cancer. HLA-Gs appear to be evolutionally and genetically linked to HSP70s: heat shock, a major inductor of HSP70.1-2 expression, and arsenite chemical shock also induce HLA-G expression in tumor cell lines (Ibrahim et al., 2000; Yao et al., 2005). In human pre-implantation embryos, HLA-Gs are expressed as several isoforms, including HLA-G1 and HLA-G5. HLA-G1s are already expressed in two- to eight-blastomer embryos and in all blastocysts as membrane-bound molecules (mHLA-G), whereas, HLA-G5s are expressed only from the blastocyst onwards, as soluble forms (sHLA-G) secreted in biological liquids, or generated by mHLA-G shedding (Yao et al., 2005). HLA-Gs circulating in biological fluids might also be associated with extracellular vesicles (EVs) (Rebmann et al., 2016), containing too antigens, ligands, receptors, cytokines, GFs, mRNAs, and miRNAs (da Silva Nardi et al., 2016). Human pre-implantation embryos express and secrete HLA-Gs, the level of which might be predictive of their implantation capacity (Yao et al., 2005). HLA-Gs orchestrate the early interaction of human trophoblasts with the maternal niche (Gregori et al., 2015). HLA-Gs are expressed early in human ICM and ESCs but, later, mainly in invasive TE, and no more in ICM; after implantation, HLA-Gs are expressed in hypoblast, but no longer in epiblast; yolk-sac mesoderm, endothelial cells of developing vessels, mesenchymal cells and progenitor cells express sHLA-Gs (Hunt et al., 2005; Yao et al., 2005; Verloes et al., 2011) (**Figure 1**). During pregnancy, sHLA-Gs can be detected in maternal serum. In adult normal tissues, HLA-G constitutive expression is mainly restricted to the fetal extravillous trophoblasts (EVTs), which invade the maternal decidua, rich in NK cells and macrophages (Tilburgs et al., 2015).

Tumor and mesenchymal cells also secrete HLA-Gs in EVs (Yen et al., 2009; Burrello et al., 2016). Tumor cells utilize EVs for dictating a defined phenotype to surrounding cells (Naito et al., 2017). Recent data show that tumor EVs contain molecules for intercellular communications (da Silva Nardi et al., 2016; Rebmann et al., 2016), that act on and impair the recipient immune cells, favoring immune evasion, initiation, tumor development, angiogenesis, invasion, metastasis, CSC and EMT preservation and chemoresistance (Sheu and Shih, 2010; Kosaka, 2016). HLA-G expression has been shown in 22/33 primary tumor tissues of human ovarian carcinoma, but not in normal tissue (Lin et al., 2007), and in 30% of surgically removed melanoma lesions (Yan et al., 2005). In lung cancer, HLA-G1/5 upregulation associates with a high-grade histology, HLA-Ia loss and immunosuppressive IL10 production (Urosevic et al., 2001). Increased HLA-G expression correlates with immune evasion during colorectal cancer progression (Fukushima et al., 1998) and in gastric cancer (Du et al., 2011). These data might, thus, indicate that, in general, in embryos and cancer, HLA-Gs and HSP70s might act not only as protective shields in potentially adverse environments (Sheu and Shih, 2010), but also as means for their invasion and colonization (Yao et al., 2005). More in detail, it seems that HLA-G1 is preferentially expressed precociously and in more peripheral embryonic structures in an essentially protective scope, whereas HLA-G5 is produced later and in invasive embryonic structures and in tumor metastatic cells, in order to favor protection, invasion, installation and development in the host (Rouas-Freiss et al., 2007; Sheu and Shih, 2010). HLA-Gs are induced by hypoxia via HIF-1a (Rebmann et al., 2003) and are upregulated by IL10 with autocatalytic feedback (Urosevic et al., 2001, 2002; Stankic et al., 2013). HLA-Gs induce IL6 production (Urosevic et al., 2002), and thus activation of the gp130-STAT3 pathway, regulating proliferation, invasion, migration and angiogenesis (Kamran et al., 2013). In my opinion, all these data suggest that HLA-G molecules could be key inter-players and shared actors in the initiation and maintenance of both embryogenesis and carcinogenesis. Particularly, HLG together to HSP70, would represent an essential system for protection and survival of both embryo and tumor.

Peri-Implantation Embryos/Primary Tumors (Implantation and Growth)

Beyond a certain stage, a blastocyst might develop further only if a supply of nutrients is possible from the outer environment: this would implicate for the embryo the necessity of implantation in the maternal uterus, and for initial tumors in a niche. Blastocyst implantation occurs thanks to the structure and properties of the syncytiotrophoblast (**Figures 1C,D**; Park et al., 2003) and to a parallel maternal endometrium condition, suited to accepting implantation. The process requires some crucial interacting factors, such as LIF/LIFr, IL6/IL6r, IL10, IL11, GP130, JAK, and STAT3, and correlated signal pathways, leading to the switch from anchorage-independent to anchorage-dependent growth, both in embryos and cancer.

LIF/LIF-r, IL6/IL6-r, gp130-JAK-STAT3 - In peri-implantation, LIF (leukemia inhibitory factor) is crucial: in LIF knockout mice, blastocyst implantation does not occur; in women, LIF strongly increases in the implantation window (Sherwin et al., 2002; Aghajanova, 2004). Uterine expression of LIF coincides with the onset of blastocyst implantation, and this depends on maternal expression of LIF (Bhatt et al., 1991; Cheng et al., 2001; Sherwin et al., 2002; Yue et al., 2015). LIF is essential for inducing a receptive uterus, but not for embryogenesis (Chen et al., 2000). LIF and LIFr expression in the human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation (Cullinam et al., 1996). In implantation, LIF carries on its biological functions mainly by activation and regulation of the JAK-STAT3, AKT, ERK1-2, and MAPK signal pathways, inducing expression of integrin α5β1, that realizes implantation, endothelial proliferation and endometrial vascularization (Cheng et al., 2001; Sherwin et al., 2002; Park et al., 2003). LIF-gp130-STAT3 is also linked to HLA-G expression through IL6 and IL10 (Urosevic and Dummer, 2003; Yue et al., 2015). In initial embryos, HLA-G expression is a fundamental prerequisite for obtaining pregnancy: indeed, in *in vitro*-fertilized human embryos, only those whose culture surnatant contains sHLA-G are able to perform implantation (Fuzzi et al., 2002; Alizadeh et al., 2016).

A LIF signal is also expressed at high levels in a wide spectrum of human cancers, including melanomas, skin, kidney, prostate, pancreas and breast cancer, where cell proliferation is stimulated by paracrine and autocrine pathways, as in embryo implantation (Cullinam et al., 1996; Kellokumpu-Lehtinen et al., 1996). The amount of LIF secreted by a tumor seems to regulate cancerogenesis (Guo et al., 2015); LIF overexpression in breast cancer patients is significantly associated with an unfavorable rate of survival without relapses (Guo et al., 2015). High expression of LIFr identifies very malignant melanocytic lesions at an early stage, and it is a crucial condition for the nevus/implanted melanoma transition (Guo et al., 2015). Analysis of 441 melanomas and 90 nevi showed low LIFr expression for all nevus stages, whereas the presence of this receptor starts to increase in dysplastic nevi, with significantly higher expression in primary melanomas (implantation), and even higher in metastatic melanomas (Guo et al., 2015), suggesting a striking correlation between LIF/LIFr expression and oncogenesis. LIFr knockdown inhibits melanoma cell migration in wound-healing tests (Guo et al., 2015). In general, LIFr activation can promote metastasis and increase the invasion potential of solid tumors (Guo et al., 2015). In human colorectal cancer cells and in solid tumors, hypoxia is an important factor inducing LIF mRNA expression, mediated mainly by HIF-2a (Yue et al., 2015). TGFb also induces expression of LIF mRNA; LIF induction is important to maintain the self-renewal of glioma-initiating cells and prevent their differentiation (Yue et al., 2015). LIF binds to human breast cancer cells and stimulates their proliferation (Estrov et al., 1999). I hypothesize that in the tumor process, LIF/LIFr might be at the basis of: (a) implantation of the primary tumor (CSC1s) via gp130-JAK-STAT3-ID1, with self-renewal and pluripotency (Niola et al., 2012); (b) subsequent paracrine growth (CSC1s/CSC2s) via gp130-JAK-STAT3-RAS-MAPK-ERK-MYC-WNT-ID2 (Cheng et al., 2001; Park et al., 2003); (c) genesis of cells with a mesenchymal phenotype (CSC3s) via CD44-STAT3-RAS-SNAIL1-MMPs-ID3-TWIST1 (Bain et al., 2001; Cheng et al., 2006; Su et al., 2011; Zeilstra et al., 2014; Yan et al., 2015). All these data clearly indicate that LIF/LIFr and related signaling pathways are crucial in cancer initiation, implantation, growth and diffusion, in a way that recalls their function in embryo development.

Post-implantation Embryos/Metastatic Tumors (Progression)

In the post-implantation embryo, at the PS, cells with a mesenchymal phenotype appear, migrating between epiblast and hypoblast cells (**Figure 1D**). The mesenchymal phenotype also characterizes metastatic TMCs (Sahlgren et al., 2008; Thiery et al., 2009; Ocaña et al., 2012). The main factors that correlate with the embryonic mesenchymal state, tumor metastases and progression include HLA-G, HSP70, hypoxia, STAT3, CD44, SNAIL1, TWIST1, PRRX1, TGFb, WNT/bCAT, ID, and MMPs. Mesenchymal progenitors derived from hESCs (EMPs) express surface HLA-G1 (Yen et al., 2009). Mesenchymal cells and progenitor cells also express HLA-Gs. IL10 selectively induces HLA-G expression in human invasive trophoblasts and monocytes (Moreau et al., 1999). In mesenchymal CSCs of kidney cancer, HLA-Gs and EVs enhance metastasis and progression (Grange et al., 2015). Highly metastatic tumors show the presence of membrane HSP70s (Stangl et al., 2011). In progression of many cancer types, migration is stimulated by the LIF-STAT3 pathway, constitutively activated, that regulates proliferation, migration, angiogenesis and metastasis (Kamran et al., 2013; Guo et al., 2015). The mesenchymal phenotype is highly characterized by the surface marker CD44.

CD44 is a cell-surface glycoprotein constituting a signal platform that regulates the expression of genes related to cell-matrix adhesion, migration, proliferation, survival and differentiation in development (Okamoto et al., 1999; Williams et al., 2013; Yan et al., 2015). The main CD44 receptor is hyaluronic acid (HA), a component of the extracellular matrix (ECM), that envelops tumor cells and bulk, regulating proliferation and motility in cancer progression and metastasis (Chanmee et al., 2015; Avnet and Cortini, 2016). The CD44-STAT3 complex induces Cyclin D1, MMP9, HIF-a2, c-MYC, TWIST1, cytoskeleton remodeling (Su et al., 2011; Yan et al., 2015) and activation of RAS signaling (Cheng et al., 2006). Expression of CD44s (standard) is ubiquitous, while CD44v (variant) isoforms seem restricted to aggressive tumors (Zeilstra et al., 2014). CD44s/CD44v switching is a critical event during EMT (Yan et al., 2015).

CD44v (v3, v6, v8) isoforms are CSC markers and have a crucial role in regulation of stemness, self-renewal, tumor initiation, metastasis and chemoresistance (Bourguignon et al., 2012; Chanmee et al., 2015; Yan et al., 2015; Avnet and Cortini, 2016). A positive feedback loop couples RAS activation and CD44v isoform expression (Cheng et al., 2006).

CD44/osteopontin (the main component of metastatic niches) interaction activates NANOG-STAT3, OCT4-SOX2-NANOG and c-MYC, namely PGRN, and it is induced by hypoxia via HIF-a1 (Krishnamachary et al., 2012; Pietras et al., 2014). CD44 transcription and cell growth are suppressed by p53 (Godar et al., 2008). Thus, I hypothesize that CD44v could be a sort of molecular trigger that is able to directly activate PGRN in the initial MET for reprogramming (Su et al., 2011; Yan et al., 2015) and, thus, a crucial factor in the generation of signaling for the switch between mesenchymal and epithelial states, usually occurring both in embryos and cancer.

IMMUNOLOGICAL ASPECTS COMMON TO EMBRYOS AND CANCER: HOST TOLERANCE AND FAVORING

HSP70s and HLA-Gs are able to strongly influence many important functions of the immune system. Membrane HSP70s affect NK cell cytotoxicity by acting as recognition/activator ligands (Luft and Dix, 1999; Stangl et al., 2008), whereas HLA-Gs are able to inhibit practically all immune system components (NK, T, DC, and B cells) (Hofmeister and Weiss, 2003; Hunt et al., 2005; Gros et al., 2008; Loumagne et al., 2014; Gregori et al., 2015; Tilburgs et al., 2015; Lopatina et al., 2016). In ESCs and pre-implantation embryos, mHLA-G and sHLA-G block all the uterine immune cells, binding the KIR2DL4 inhibitory receptors on NK cells, and ILT2 (LILRB1) and ILT4 (LILRB2) inhibitor receptors on all the leukocytes and macrophages (Hofmeister and Weiss, 2003; Li et al., 2004; Verloes et al., 2011; Tilburgs et al., 2015; Rebmann et al., 2016). Both membrane and soluble HLA-Gs would confer immune-suppressive properties to the cells producing them, in various ways: (a) For HLA-G1, direct interaction with ILT2 and ILT4, determining $CD8^+$ T cell apoptosis, NK cell immobilization, and impairment of monocyte/DC differentiation, with a high production of IL4 and IL10 immunosuppressive cytokines (Rebmann et al., 2003; Urosevic and Dummer, 2003; Gros et al., 2008; Gregori et al., 2015; Rebmann et al., 2016). Mesenchymal progenitors derived from hESCs (EMPs) strongly suppress NK and T cells by HLA-G1 (Yen et al., 2009). (b) For induction of cascade signaling and biological activity in the host cells, thanks to the factors contained in EVs, internalized by trogocytosis or pinocytosis (Rouas-Freiss et al., 2007). CSCs cross-talk with MSCs through EVs containing mRNA and miRNA, identified as the main factors responsible for the phenotypic changes induced in the cells receiving EVs (Lopatina et al., 2016). (c) For amplification of mechanisms and conditions favorable to the cells releasing EVs, with further involvement of other surrounding cells. In human neuroblastomas, tumor cells instruct monocytes to produce and release sHLA-G (Rouas-Freiss et al., 2003). HLA-G transfer from antigen-presenting cells (APCs) to activated T cells has been reported in embryos, with reversion of these cells to a regulator phenotype blocking the allo-immune response (Rebmann et al., 2016). A co-culture of activated decidual NK (dNK) cells or peripheral NK (pNK) cells with EVTs results in mHLA-G initial acquisition within 18 h, and complete acquisition in 36 h (Tilburgs et al., 2015).

In tumors, HLA-Gs transfer quickly from APCs or cancer cells to T and NK cells, and convert these cells into temporarily suppressor $HLA\text{-}G^+$ cells (Rebmann et al., 2003). Once acquired by NK cells, HLA-Gs are degraded, and pNK cells revert to their previous cytotoxic phenotype (Tilburgs et al., 2015). Both HLA-G5 and HLA-G1 endocytosed by NK cells lead to NFkB pathway activation and, finally, to transcription of immunosuppressor (IL10) and pro-angiogenetic (IL6) factors (Rebmann et al., 2016). It has been shown that pNK cells might acquire via trogocytosis HLA-Gs from a transfected melanoma M8 cell line, and that, after acquisition, NK cells are no longer cytotoxic and are unable to realize immune synapses (Lesport et al., 2009; Tilburgs et al., 2015). In gastric cancer, HLA-G overexpression associates with immune escape and correlates with a local increase of T regulatory cells (TREGs) (Du et al., 2011). NK cytolysis depends on the amount of HLA-G1 expressed, that in malignant tumors can go from 0 to 100%, with complete NK inhibition, reduced local and systemic immunosurveillance and tumor progression (Rebmann et al., 2003; Chen et al., 2013). HLA-G1s and HLA-G5s have an additive suppressor effect on NK cytolysis dependent on their level, but HLA-G5 is a more potent inhibitor (Zhang et al., 2014). Within a population of $HLA\text{-}G^-$ tumor cells, few $HLA\text{-}G^+$ cells have significant immune inhibitory effects (Lesport et al., 2009). HLA-G upregulation also occurs through factors such as cytokines (IL10), stress and chemotherapeutic demethylating agents (Rouas-Freiss et al., 2003; Yan et al., 2005). IL10 upregulates HLA-G, that induces an immunosuppressive Th2 profile (Urosevic et al., 2002; Rebmann et al., 2003), with further IL10 increase in a vicious circle (Moreau et al., 1999). Interaction of $HLA\text{-}G^+$ cells with NK cells also enhances IL6 production, that induces angiogenesis and SC proliferation via gp130-JAK-STAT3-OCT4. The inhibiting effects of HLA-G on NK cells are eliminated by the action of IL15, IL2 and IL12 (Tilburgs et al., 2015). CSC immune evasion also occurs through shedding of MIC-A/B, HSP70 and HLA-G1 (Ames et al., 2015). It has been shown that highly metastatic tumors, but not their primary or poorly malignant counterparts, are $mHSP70^+$, capable of shedding (Stangl et al., 2011). It is presumable that a high number of molecules such as HSP70, HLA-G, and MIC-A/B released in circulation could lead to ectopic activation and subsequent neutralization of immune cells, without these coming into contact with CSC targets (Stangl et al., 2011), thus favoring diffusion of metastasis.

CURRENT ANTICANCER THERAPEUTIC APPROACHES IN AGREEMENT WITH THE I-CSC/P-ESC MODEL

Several current partially successful approaches for cancer therapy are in agreement with the i-CSC/p-ESC model, targeting crucial factors common to embryos and tumors, such as HSP70, HLA-G, ID, LIF/LIFr, and CD44. CSCs seem to be preferential targets for NK cells through upregulation of antigens (HSP70, MIC-A/B, FAS, DR5) induced by stress, by which NK cells are capable

of targeting (only) quiescent, non-proliferating cells (Ames et al., 2015). Thus, in solid tumors, slow-renewing or quiescent CSCs might be more easily killed by NK cells after depletion of proliferating non-CSCs (CPCs) through anti-proliferative therapies (Ames et al., 2015).

HLA-Gs are a valid target in cancer therapy (Lin and Yan, 2015; Zhang et al., 2018); cytotoxicity studies show that HLA-Gs drastically inhibit lysis of human ovarian carcinoma cells, with subsequent immune evasion, and that lysis can be restored by the conformational anti-HLA-G mAb 87G (Tilburgs et al., 2015). Interestingly, dNK cells in culture with IL2, IL12, and IL15 lose the acquired surface HLA-Gs by internalization and degradation, reacquiring cytolytic activity (Tilburgs et al., 2015). In breast cancer patients treated with NACT (neo-adjuvant chemotherapy), high levels of HLA-Gs/EVs before NACT correlate to tumor progression and the presence of circulating tumor stem-like cells, releasing EVs, while high sHLA-G levels (presumably from tumor lysis) indicate a better clinical outcome (König et al., 2016). HLA-G expression is induced in the melanoma cell line OCM-1A after treatment with 5-aza-2'-deoxycytidine (Yan et al., 2005).

Anticancer therapeutic approaches based on endocellular and exocellular HSP70s have been carried out (Didelot et al., 2007; Shu and Huang, 2008; Jego et al., 2010). It has been reported that $mHSP70^+$ tumors actively release lipid vesicles (exosomes) with an $HSP70^+$ surface, and that they can attract already activated NK cells (but not resting NK cells) (Stangl et al., 2011), causing their ectopic degranulation and neutralization. The TKD peptide (14 amino acids common to all HSP70s), combined with IL2 low doses, has been found to stimulate NK cell migratory and cytolytic activity against $HSP70^+$ tumor cells (Stangl et al., 2011). A murine antibody anti-TKD (cmHsp70.1 mAb) binds all human vital tumor cell lines of colon, breast, lungs and melanomas; thus, HSP70s could be an immune-therapeutic target in a wide spectrum of tumor types (Shu and Huang, 2008; Stangl et al., 2011). In fact, cmHsp70.1 mAb injected into mice with CT26 colon tumors can significantly reduce the bulk of $mHSP70^+$ tumors and increase survival via ADCC induction, that can be further enhanced by NK cells pre-activated with TKD/IL2 (Stangl et al., 2011).

ID genes and proteins are a promising target in cancer therapy for inhibiting tumor cells and their supply in the blood (Benezra, 2001; Fong et al., 2004; Iavarone and Lasorella, 2006). ID protein inhibition by a peptide aptamer induces cell cycle arrest and apoptosis in ovarian cancer cells (Mern et al., 2010a). Suppression of invasion and metastasis in aggressive salivary and breast cancer cells is targeted through inhibition of ID1 expression (Fong et al., 2003; Murase et al., 2016). Inactivation of ID1 genes induces sensitivity of prostate cancer cells to chemotherapeutic drugs (Wong et al., 2008). Co-suppression of ID1 and ID3 results in a significant reduction in the proliferation rate, invasiveness and anchorage-independent growth, reduced angiogenesis and increased apoptosis in small-cell lung cancer (Chen et al., 2014), and significantly reduces the ability of gastric cancer cells to form peritoneal metastases (Tsuchiya et al., 2005). Moreover, ID1 and ID3 knockdown inhibits the metastatic potential of pancreatic cancer, both for proliferation and migration (Shuno et al., 2010). Targeting ID1 and ID3 by a specific peptide aptamer induces E-box promoter activity, cell cycle arrest and apoptosis in breast cancer cells (Mern et al., 2010b). TGFb receptor inhibitors target the $CD44^{high}/Id1^{high}$ glioma-initiating cells in human glioblastoma (Anido et al., 2010). It has been shown that ID2 knockdown by an inhibitor of WNT-bCAT signaling markedly suppresses the formation of CSC spheres *in vitro*, and metastases *in vivo* (Rockman et al., 2001), by inhibition of CSC-like phenotypes (Jang et al., 2015).

LIFr expression is a crucial condition for nevus/implanted melanoma transition; LIFr knockdown inhibits migration of melanoma cells in wound-healing tests; thus, LIFr could be a potential target for developing therapies in initial tumor interventions (Guo et al., 2015). Neutralizing antibodies knock down activity or expression of LIF and reduce *in vitro* the stem cell-like properties of murine slow-growing CSCs (American Association for Cancer Research, 2012).

CD44 is a biomarker and therapeutic target in CSCs (Su et al., 2011; Yan et al., 2015), and CD44v is a promising approach for elimination of CSCs (Jin et al., 2006; Orian-Rousseau and Ponta, 2015; Yan et al., 2015). In breast cancer, cells with an EMT phenotype can be inhibited by Abs specific for CD44 (Yan et al., 2015); knockdown of CD44 induces differentiation of breast CSCs and is a promising differentiation therapy (Pham et al., 2011). CD44 targeting reduces tumor growth and prevents post-chemotherapy relapse of human breast cancer xenografts (Marangoni et al., 2009). Inhibition of CD44v3 and v6 by A5G27 peptide copolymer blocks tumor invasion and metastatic colonization (Zaiden et al., 2017). DNA vaccination with CD44v isoforms reduces mammary tumor local growth and lung metastases (Wallach-Dayan et al., 2008).

Immunological approaches to target CSCs have been carried out, and through CSC vaccination, significant antitumor immunity can be conferred (Ning et al., 2012; Pan et al., 2015). For improving the efficacy of breast cancer treatment, a combination therapy has been used, that permits targeting of both CSC-like and bulk tumor cells (Wang et al., 2016). Thus, it is evident that many current therapeutic strategies are addressed to target crucial factors, common to embryos and cancer, at the basis of this i-CSC/p-ESC model.

DISCUSSION AND CONCLUSION: PROPOSALS FOR INNOVATIVE CANCER THERAPY AND PROPHYLAXIS STRATEGIES

Considerations for a Proper Antitumor Therapeutic Strategy

From the numerous experimental data reported above, it is evident that embryo and tumor development occurs in very similar physio-pathological conditions of immune tolerance by the host, which accepts and even favors them. Anticancer

therapies carried out so far, targeting separately the factors HLA-G, ID, LIF, HSP70, and CD44, have got important positive, but only partial and non-resolutive, results. This limit might be due to several factors: (a) a particular hierarchic tumor structure, immunologically protective for CSCs, surrounded by CPCs and CDCs (Nishimura et al., 2012; Marjanovic et al., 2013; Liu et al., 2014; Ames et al., 2015; Cabrera et al., 2015); (b) interconversion among CSCs (CSC1/CSC2/CSC3/CSC1) via ID1, ID2 and ID3 proteins (Hermann et al., 2007; Biddle et al., 2011; Dieter et al., 2011; Brabletz, 2012; Quail et al., 2012; Do et al., 2013; Cabrera et al., 2015); (c) the presence of HLA-G and HSP70 on the surface of CSCs and CPCs, with impairment of various immune surveillance mechanisms, NK activity in particular (Samali and Cotter, 1996; Rouas-Freiss et al., 2007; Sheu and Shih, 2010; Stangl et al., 2011); (d) anti-proliferative chemotherapies, that only eliminate actively proliferating CCs (CSC2s and CPCs), but spare slow-renewing CSCs (CSC1s) in niches, or quiescent circulating CSCs (CSC3s), and non-proliferating CDCs (Ames et al., 2015; Wang et al., 2016); (e) release into the circulation of a high quantity of HSP70 by shedding, exosomes or chemotherapeutic necrosis (Shu and Huang, 2008; Stangl et al., 2011), attracting ectopically activated NK cells and neutralizing them at a distance from CSC targets (Stangl et al., 2011); (f) release into the circulation, by shedding, EVs or chemotherapeutic necrosis, of high amounts of HLA-G, that is able to block any immune mechanism, or even instruct normal cells to favor the tumor (Hofmeister and Weiss, 2003; Li et al., 2004; Rouas-Freiss et al., 2007; Gros et al., 2008; Loumagne et al., 2014; Lopatina et al., 2016); (g) negative collateral effects of anti-angiogenetic therapies that, by blocking tumor vascularization, might induce hypoxia and a subsequent increase of metastatic CSCs (CSC3s) (Conley et al., 2012).

General Suggestions for a Potential Multi-Target Multistep Cancer Therapy

On the basis of the previous considerations, I suggest some general indications for a multistep multi-target therapeutic strategy. To this end, I think that it would be necessary to make use of "micro-antibodies" (mi-Abs), which are artificial, chemically synthetized short chains of amino acids, copied from fully functional natural antibodies (Heap et al., 2005). Their small size allows them to leave the circulation quickly and reach specific target sites in the tissues, normally unapproachable for mAbs. mi-Abs are poorly immunogenic and do not stimulate an immune response versus the host. mi-Abs show neutralizing properties versus viruses, such as HIV, infecting cells *in vitro* (Fujii, 2009). All these mi-Ab properties might consent the following antitumor therapeutic strategy, leading to progressive back-dismantling of the tumor hierarchic organization, selectively targeting the diverse tumor cell populations in a defined multistep sequence: (a) co-culture of autologous peripheral blood mononuclear cells (PBMCs) with IL15, IL12 and IL2, to restore them from the effects of HLA-G (Tilburgs et al., 2015) and dispose of HLA-G-free PBMCs, to be used in next steps; (b) tumor biopsy for the selection of the various populations: differentiated non-proliferating cells (CDCs), non- or slow-proliferating SCs (CSC1s and CSC3s), actively proliferating cells (CSC2s and CPCs); (c) co-culture of HLA-G-free PBMCs with each selected tumor population, and "in toto" tumor populations, previously treated with anti-HLA-G and anti-HSP70 mi-Abs, allowing *in vitro* PBMC sensitization without conditioning; (d) anti-HLA-G and anti-HSP70 prophylaxis of the subject by specific mi-Abs; (e) reinfusion of anti-CDC-sensitized PBMCs and/or chemotherapeutic treatment, to reduce the tumor bulk and expose the internal cell populations (Ames et al., 2015; Wang et al., 2016), thus making them more approachable in the next steps; (f) chemotherapeutic anti-proliferative treatment of the subject to eliminate fast-proliferating CSC2s and CPCs, upon protection with anti-HLA-G and anti-HSP70 neutralizing mi-Abs; (g) treatment of the subject with anti-CD44v and anti-ID1/ID3 mi-Abs to neutralize circulating CSC3s from an eventual CSC3/CSC1 transition (MET), and CSC1s in niches from anchorage and self-renewal (Marangoni et al., 2009; Chen et al., 2014; Zaiden et al., 2017); (h) treatment of the subject with anti-LIF/LIFr and anti-ID1/2 mi-Abs to prevent or block CSC1 implantation in niches and angiogenesis, CSC migration by activation of the LIF-STAT3 pathway, and a possible CSC1/CSC2/CSC3 transition (EMT) (Rockman et al., 2001; American Association for Cancer Research, 2012; Guo et al., 2015; Jang et al., 2015); (i) treatment of the subject with anti-HLA-G, anti-HSP70, anti-CD44, anti-ID1/ID2/ID3 and anti-LIF/LIFr mi-Abs for general prophylaxis; (ii) final treatment with "in toto" sensitized HLA-G-free PBMCs to restore natural immunological and physiological conditions in the host.

Considerations and Suggestions for Potential Cancer Prophylaxis

On the basis of the i-CSC/p-ESC model, it might be logically and biologically possible to use anti-HSP70/HLA-G (Qa-2 in mouse)/ID/LIF-LIFr/CD44 mi-Abs for antitumor prophylaxis. Periodical treatments with mi-Abs at defined times and in defined ways could protect healthy subjects from arising tumors (i-CSC/CSC0s) or implanting initial tumors (CSC1s). The validity of this hypothesis might be tested in appropriate animal models, inoculating before, together and after mi-Ab treatments: (a) CSCs of various human tumor types, in nude mice; (b) CSCs of various mouse tumor types in normal autologous mice. Verifying tumor development, an eventual absence of tumors with the treatment before or together with CSC inoculation would indicate a preventive effect of the mi-Abs while, with the subsequent treatment, it could also indicate a drainage effect of the mi-Abs versus initial tumors. The use of mi-Abs for periodical prophylaxis could be evaluated in relation to the subject age, in general. In particular, in women, it could be considered in relation to an eventual pregnancy, because all the mi-Ab tumor targets are crucial embryonic factors.

In conclusion, I hope that this work might make a valid contribution for a better vision of the cancer, and that it might stimulate the interest of others to debate and verify some validity of the ideas expressed, as well as to test the therapeutic potential of the suggested proposals.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REFERENCES

Aghajanova, L. (2004). Leukemia inhibitory factor and human embryo implantation. *Ann. N. Y. Acad. Sci.* 1034, 176–183. doi: 10.1196/annals.1335.020

Alizadeh, N., Mosaferi, E., Farzadi, L., Majidi, J., Monfaredan, A., Yousefi, B., et al. (2016). Frequency of null allele of human leukocyte antigen-G (HLA-G) locus in subjects to recurrent miscarriage. *Int. J. Reprod. Biomed.* 14, 459–464. doi: 10.29252/ijrm.14.7.4

American Association for Cancer Research (2012). *Leukemia Inhibitory Factor May be a Promising Target Against Pancreatic Cancer. Public Release*. Available at: https://www.eurekalert.org/pub_releases/2012-06/aafc-lif061412.php

Ames, E., Canter, R. J., Grossenbacher, S. K., Mac, S., Chen, M., Smith, R. C., et al. (2015). NK cells preferentially target tumor cells with a cancer stem cell phenotype. *J. Immunol.* 195, 4010–4019. doi: 10.4049/jimmunol.1500447

Anido, J., Saez-Borderias, A., Gonzalez-Junca, A., Rodon, L., Folch, G., Carmona, M. A., et al. (2010). TGF-beta receptor inhibitors target the CD44(high)/Id1(high) glioma-initiating cell population in human glioblastoma. *Cancer Cell* 18, 655–668. doi: 10.1016/j.ccr.2010.10.023

Arvelo, F., Sojo, F., and Cotte, C. (2016). Tumor progression and metastasis. *Ecancermedicalscience* 10:617. doi: 10.3332/ecancer.2016.617

Avnet, S., and Cortini, M. (2016). Role of pericellular matrix in the regulation of cancer stemness. *Stem Cell Rev.* 12, 464–475. doi: 10.1007/s12015-016-9660-x

Bain, G., Cravatt, C. B., Loomans, C., Alberola-Ila, J., Hedrick, S. M., and Murre, C. (2001). Regulation of the helix-loop-helix proteins, E2A and Id3, by the Ras-ERK MAPK cascade. *Nat. Immunol.* 2, 165–171. doi: 10.1038/84273

Barrallo-Gimeno, A., and Nieto, M. A. (2005). The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development* 132, 3151–3161. doi: 10.1242/dev.01907

Benezra, R. (2001). The Id proteins: targets for inhibiting tumor cells and their blood supply. *Biochim. Biophys. Acta* 1551, F39–F47. doi: 10.1016/S0304-419X(01)00028-2

Bensaude, O., Babinet, C., Morange, M., and Jacob, F. (1983). Heat shock proteins, first major products of zygotic gene activity in mouse embryo. *Nature* 305, 331–333. doi: 10.1038/305331a0

Bhatt, H., Brunet, L. J., and Stewart, C. L. (1991). Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc. Natl. Acad. Sci. U.S.A.* 88, 11408–11412. doi: 10.1073/pnas.88.24.11408

Biddle, A., Liang, X., Gammon, L., Fazil, B., Harper, L. J., Emich, H., et al. (2011). Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. *Cancer Res.* 71, 5317–5326. doi: 10.1158/0008-5472.CAN-11-1059

Bohrer, C., Pfurr, S., Mammadzada, K., Schildge, S., Plappert, L., Hils, M., et al. (2015). The balance of Id3 and E47 determines neural stem/precursor cell differentiation into astrocytes. *EMBO J.* 34, 2804–2819. doi: 10.15252/embj.201591118

Bond, W. S., Akinfenwa, P. Y., Perlaky, L., Hurwitz, M. Y., Hurwitz, R. L., and Chévez-Barrios, P. (2013). Tumorspheres but not adherent cells derived from retinoblastoma tumors are of malignant origin. *PLoS ONE* 8:e63519. doi: 10.1371/journal.pone.0063519

Bourguignon, L. Y., Wong, G., Earle, C., and Chen, L. (2012). Hyaluronan-CD44v3 interaction with Oct4-Sox2-Nanog promotes mir-302 expression leading to self-renewal, clonal formation, and cisplatin resistance in cancer stem cells from head and neck squamous cell carcinoma. *J. Biol. Chem.* 287, 32800–32824. doi: 10.1074/jbc.M111.308528

Brabletz, T. (2012). EMT and MET in metastasis: where are the cancer stem cells? *Cancer Cell* 22, 699–701. doi: 10.1016/j.ccr.2012.11.009

Burrello, J., Monticone, S., Gai, C., Gomez, Y., Kholia, S., and Camussi, G. (2016). Stem cell-derived extracellular vesicles and immune-modulation. *Front. Cell. Dev. Biol.* 4:83. doi: 10.3389/fcell.2016.00083

Cabrera, M. C., Hollingsworth, R. E., and Hurt, E. M. (2015). Cancer stem cell plasticity and tumor hierarchy. *World J. Stem Cells* 7, 27–36. doi: 10.4252/wjsc.v7.i1.27

Cakouros, D., Raices, R. M., Gronthos, S., and Glackin, C. A. (2010). Twist-ing cell fate: mechanistic insights into the role of twist in lineage specification/differentiation and tumorigenesis. *J. Cell. Biochem.* 110, 1288–1298. doi: 10.1002/jcb.22651

Carey, J. P., Asirvatham, A. J., Galm, O., Ghogomu, T. A., and Chaudhary, J. (2009). Inhibitor of differentiation 4 (Id4) is a potential tumor suppressor in prostate cancer. *BMC Cancer* 9:173. doi: 10.1186/1471-2407-9-173

Chaffer, C. L., Brennan, J. P., Slavin, J. L., Blick, T., Thompson, E. W., and Williams, E. D. (2006). Mesenchymal-to-epithelial transition facilitates bladder cancer metastasis: role of fibroblast growth factor receptor-2. *Cancer Res.* 66, 11271–11278. doi: 10.1158/0008-5472.CAN-06-2044

Chanmee, T., Ontong, P., Kimata, K., and Itano, N. (2015). Key roles of hyaluronan and its CD44 receptor in the stemness and survival of cancer stem cells. *Front. Oncol.* 5:180. doi: 10.3389/fonc.2015.00180

Chen, B. G., Xu, D. P., Lin, A., and Yan, W. H. (2013). NK cytolysis is dependent on the proportion of HLA-G expression. *Hum. Immunol.* 74, 286–289. doi: 10.1016/j.humimm.2012.12.005

Chen, D., Forootan, S. S., Gosney, J. R., Forootan, F. S., and Ke, Y. (2014). Increased expression of ID1 and ID3 promotes tumorigenicity by enhancing angiogenesis and suppressing apoptosis in small cell lung cancer. *Genes Cancer* 5, 212–225.

Chen, H., Guo, R., Zhang, Q., Guo, H., Yang, M., Wu, Z., et al. (2015). Erk signaling is indispensable for genomic stability and self-renewal of mouse embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 112, E5936–E5943. doi: 10.1073/pnas.1516319112

Chen, J. R., Cheng, J. G., Shatzer, T., Sewell, L., Hernandez, L., and Stewart, C. L. (2000). Leukemia inhibitory factor can substitute for nidatory estrogen and is essential for inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinology* 141, 4365–4372. doi: 10.1210/endo.141.12.7855

Cheng, C., Yaffe, M. B., and Sharp, P. A. (2006). A positive feedback loop couples Ras activation and CD44 alternative splicing. *Genes Dev.* 20, 1715–1720. doi: 10.1101/gad.1430906

Cheng, J. G., Chen, J. R., Hernandez, L., Alvord, W. G., and Stewart, C. L. (2001). Dual control of LIF expression and LIF receptor function regulate Stat3 activation at the onset of uterine receptivity and embryo implantation. *Proc. Natl. Acad. Sci. U.S.A.* 98, 8680–8685. doi: 10.1073/pnas.151180898

Collins, A. T., Berry, P. A., Hyde, C., Stower, M. J., and Maitland, N. J. (2005). Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res.* 65, 10946–10951. doi: 10.1158/0008-5472.CAN-05-2018

Conley, S. J., Gheordunescu, E., Kakarala, P., Newman, B., Korkaya, H., Heath, A. N., et al. (2012). Antiangiogenic agents increase breast cancer stem cells via the generation of tumor hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2784–2789. doi: 10.1073/pnas.1018866109

Cubilio, E., Diaz-Lopez, A., Cuevas, E. P., Moreno-Bueno, G., Peinado, H., Montes, A., et al. (2013). E47 and ID1 interplay in epithelial-mesenchymal transition. *PLoS One* 8:e59948. doi: 10.1371/journal.pone.00948

Cullinam, E. B., Abbondanzo, S. J., Anderson, P. S., Pollard, J. W., Lessey, B. A., and Stewart, C. L. (1996). Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3115–3120. doi: 10.1073/pnas.93.7.3115

da Silva Nardi, F., König, L., Wagner, B., Giebel, B., Santos Manvailer, L. F., and Rebmann, V. (2016). Soluble monomers, dimers and HLA-G-expressing extracellular vesicles: the three dimensions of structural complexity to use HLA-G as a clinical biomarker. *HLA* 88, 77–86. doi: 10.1111/tan.12844

Deed, R. W., Jasiok, M., and Norton, J. D. (1996). Attenuated function of a variant form of the helix-loop-helix protein, Id-3, generated by an alternative splicing mechanism. *FEBS Lett.* 393, 113–116. doi: 10.1016/0014-5793(96)00868-X

Didelot, C., Lanneau, D., Brunet, M., Joly, A. L., De Thonel, A., Chiosis, G., et al. (2007). Anti-cancer therapeutic approaches based on intracellular and extracellular heat shock proteins. *Curr. Med. Chem.* 14, 2839–2847. doi: 10.2174/092986707782360079

Dieter, S. M., Ball, C. R., Hoffmann, C. M., Nowrouzi, A., Herbst, F., Zavidij, O., et al. (2011). Distinct types of tumor-initiating cells form human colon cancer tumors and metastases. *Cell Stem Cell* 9, 357–365. doi: 10.1016/j.stem.2011.08.010

Do, D. V., Ueda, J., Messerschmidt, D. M., Lorthongpanich, C., Zhou, Y., Feng, B., et al. (2013). A genetic and developmental pathway from STAT3 to the OCT4-NANOG circuit is essential for maintenance of ICM lineages in vivo. *Genes Dev.* 27, 1378–1390. doi: 10.1101/gad.221176.113

Dong, H. J., Jang, G. B., Lee, H. Y., Park, S. R., Kim, J. Y., Nam, J. S., et al. (2016). The Wnt/β-catenin signaling/Id2 cascade mediates the effects of hypoxia on the hierarchy of colorectal-cancer stem cells. *Sci. Rep.* 6:22966. doi: 10.1038/srep22966

Dressel, R., Johnson, J. P., and Günther, E. (1998). Heterogeneous patterns of constitutive and heat shock induced expression of HLA-linked HSP70–1 and HSP70–2 heat shock genes in human melanoma cell lines. *Melanoma Res.* 8, 482–492. doi: 10.1097/00008390-199812000-00002

Du, L., Xiao, X., Wang, C., Zhang, X., Zheng, N., Wang, L., et al. (2011). Human leukocyte antigen-G is closely associated with tumor immune escape in gastric cancer by increasing local regulatory T cells. *Cancer Sci.* 102, 1272–1280. doi: 10.1111/j.1349-7006.2011.01951.x

Eastham, A. M., Spencer, H., Soncin, F., Ritson, S., Merry, C. L., and Stern, P. L. (2007). Epithelial-mesenchymal transition events during human embryonic stem cell differentiation. *Cancer Res.* 67, 11254–11262. doi: 10.1158/0008-5472.CAN-07-2253

Eckert, M. A., Lwin, T. M., Chang, A. T., Kim, J., Danis, E., Ohno-Machado, L., et al. (2011). Twist1-induced invadopodia formation promotes tumor metastasis. *Cancer Cell* 19, 372–386. doi: 10.1016/j.ccr.2011.01.036

Estrov, Z., Samal, B., Lapushin, R., Kellokumpu-Lehtinen, P., Sahin, A. A., Kurzrock, R., et al. (1999). Leukemia inhibitory factor binds to human breast cancer cells and stimulates their proliferation. *J. Interferon Cytokine Res.* 15, 905–913. doi: 10.1089/jir.1995.15.905

Ezashi, T., Das, P., and Roberts, R. M. (2005). Low O2 tensions and the prevention of differentiation of hES cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 4783–4788. doi: 10.1073/pnas.0501283102

Festuccia, N., Osorno, R., Wilson, V., and Chambers, I. (2013). The role of pluripotency gene regulatory network components in mediating transitions between pluripotent cell states. *Curr. Opin. Genet. Dev.* 23, 504–511. doi: 10.1016/j.gde.2013.06.003

Fong, S., Debs, R. J., and Desprez, P. Y. (2004). Id genes and proteins as promising targets in cancer therapy. *Trends Mol. Med.* 10, 387–392. doi: 10.1016/j.molmed.2004.06.008

Fong, S., Itahana, Y., Sumida, T., Singh, J., Coppe, J. P., Liu, Y., et al. (2003). Id-1 as a molecular target in therapy for breast cancer cell invasion and metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13543–13548. doi: 10.1073/pnas.2230238100

Forrest, S. T., Barringhaus, K. G., Perlegas, D., Hammarskjold, M. L., and McNamara, C. A. (2004). Intron retention generates a novel Id3 isoform that inhibits vascular lesion formation. *J. Biol. Chem.* 279, 32897–32903. doi: 10.1074/jbc.M404882200

Fujii, I. (2009). Beyond antibodies: generation of conformationally constrained peptides for molecular-targeting therapy. *Yakugaku Zasshi* 129, 1303–1309. doi: 10.1248/yakushi.129.1303

Fukushima, Y., Oshika, Y., Nakamura, M., Tokunaga, T., Hatanaka, H., Abe, Y., et al. (1998). Increased expression of human histocompatibility leukocyte antigen-G in colorectal cancer cells. *Int. J. Mol. Med.* 2, 349–351. doi: 10.3892/ijmm.2.3.349

Fuzzi, B., Rizzo, R., Criscuoli, L., Noci, I., Melchiorri, L., Scarselli, B., et al. (2002). HLA-G expression in early embryos is a fundamental prerequisite for the obtainment of pregnancy. *Eur. J. Immunol.* 32, 311–315. doi: 10.1002/1521-4141(200202)32:2<311::AID-IMMU311>3.0.CO;2-8

Gillich, A., Bao, S., and Surani, M. A. (2013). Reversion of mouse postimplantation epiblast stem cells to a naïve pluripotent state by modulation of signalling pathways. *Methods Mol. Biol.* 1074, 15–29. doi: 10.1007/978-1-62703-628-3-2

Ginisa, I., Luoa, Y., Miuraa, T., Thiesb, S., Brandenbergerb, R., Gerecht-Nirc, S., et al. (2004). Differences between human and mouse embryonic stem cells. *Dev. Biol.* 269, 360–380. doi: 10.1016/j.ydbio.2003.12.034

Godar, S., Ince, T. A., Bell, G. W., Feldser, D., Donaher, J. L., Bergh, G., et al. (2008). Growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. *Cell* 134, 62–73. doi: 10.1016/j.cell.2008.06.006

Grange, C., Tapparo, M., Tritta, S., Deregibus, M. C., Battaglia, A., Gontero, P., et al. (2015). Role of HLA-G and extracellular vesicles in renal cancer stem cell-induced inhibition of dendritic cell differentiation. *BMC Cancer* 15:1009. doi: 10.1186/s12885-015-2025-z

Gregori, S., Amodio, G., Quattrone, F., and Panina-Bordignon, P. (2015). HLA-G orchestrates the early interaction of human trophoblasts with the maternal niche. *Front. Immunol.* 6:128. doi: 10.3389/fimmu.2015.00128

Gros, F., Cabillic, F., Toutirais, O., Maux, A. L., Sebti, Y., and Amiot, L. (2008). Soluble HLA-G molecules impair natural killer/dendritic cell crosstalk via inhibition of dendritic cells. *Eur. J. Immunol.* 38, 742–749. doi: 10.1002/eji.200736918

Guo, H., Cheng, Y., Martinka, M., and McElwee, K. (2015). High LIFr expression stimulates melanoma cell migration and is associated with unfavorable prognosis in melanoma. *Oncotarget* 6, 25484–25498. doi: 10.18632/oncotarget.4688

Gupta, G. P., Perk, J., Acharyya, S., de Candia, P., Vivek Mittal, V., Todorova-Manova, K., et al. (2007). ID genes mediate tumor reinitiation during breast cancer lung metastasis. *Proc. Natl. Acad. Sci. U.S.A.* 194, 19506–19511. doi: 10.1073/pnas.0709185104

HaKashyap, V., Rezende, N. C., Scotland, K. B., Shaffer, S. M., Persson, J. L., Gudas, L. J., et al. (2009). Regulation of stem cell pluripotency and differentiation involves a mutual regulatory circuit of the NANOG, OCT4, and SOX2 pluripotency transcription factors with polycomb repressive complexes and stem cell micro-RNAs. *Stem Cells Dev.* 18, 1093–1108. doi: 10.1089/scd.2009.0113

Heap, C. J., Wang, Y., Pinheiro, T. J., Reading, S. A., Jennings, K. R., and Dimmock, N. J. (2005). Analysis of a 17-amino acid residue, virus-neutralizing microantibody. *J. Gen. Virol.* 86, 1791–1800. doi: 10.1099/vir.0.80812-0

Henderson, J. K., Draper, J. S., Baillie, H. S., Fishel, S., Thomson, J. A., Moore, H., et al. (2002). Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells* 20, 329–337. doi: 10.1634/stemcells.20-4-329

Hermann, P. C., Huber, S. L., Herrler, T., Aicher, A., Ellwart, J. W., Guba, M., et al. (2007). Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. *Cell Stem Cell* 1, 313–323. doi: 10.1016/j.stem.2007.06.002

Hirata, H., Sugimachi, K., Takahashi, Y., Ueda, M., Sakimura, S., Uchi, R., et al. (2015). Downregulation of PRRX1 confers cancer stem cell-like properties and predicts poor prognosis in hepatocellular carcinoma. *Ann. Surg. Oncol.* 22(Suppl. 3), S1402–S1409. doi: 10.1245/s10434-014-4242-0

Hofmeister, V., and Weiss, E. H. (2003). HLA-G modulates immune responses by diverse receptor interactions. *Semin. Cancer Biol.* 13, 317–323. doi: 10.1016/S1044-579X(03)00022-1

Hunt, J. S., Petroff, M. G., McIntire, R. H., and Ober, C. (2005). HLA-G and immune tolerance in pregnancy. *FASEB J.* 19, 681–693. doi: 10.1096/fj.04-2078rev

Iavarone, A., Garg, P., Lasorella, A., Hsu, J., and Israel, M. A. (1994). The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. *Genes Dev.* 8, 1270–1284. doi: 10.1101/gad.8.11.1270

Iavarone, A., and Lasorella, A. (2006). ID proteins as targets in cancer and tools in neurobiology. *Trends Mol. Med.* 12, 588–594. doi: 10.1016/j.molmed.2006.10.007

Ibrahim, E. C., Morange, M., Dausset, J., Carosella, E. D., and Paul, P. (2000). Heat shock and arsenite induce expression of the nonclassical class I histocompatibility HLA-G gene in tumor cell lines. *Cell Stress Chaperones* 5, 207–218. doi: 10.1379/1466-1268(2000)005<0207:HSAAIE>2.0.CO;2

Itahana, Y., Singh, J., Sumida, T., Coppe, J. P., Parrinello, S., Bennington, J. L., et al. (2003). Role of Id-2 in the maintenance of a differentiated and noninvasive phenotype in breast cancer cells. *Cancer Res.* 63, 7098–7105.

Jang, G. B., Kim, J. Y., Cho, S. D., Park, K. S., Jung, J. Y., Lee, H. Y., et al. (2015). Blockade of Wnt/β-catenin signaling suppresses breast cancer metastasis by inhibiting CSC-like phenotype. *Sci. Rep.* 5:12465. doi: 10.1038/srep12465

Jego, G., Hazoumè, A., Seigneuric, R., and Garrido, C. (2010). Targeting heat shock proteins in cancer. *Cancer Lett* 332, 275–285. doi: 10.1016/j.canlet.2010.10.014

Jin, L., Hope, K. J., Zhai, Q., Smadja-Joffe, F., and Dick, J. E. (2006). Targeting of CD44 eradicates human acute myeloid leukemic stem cells. *Nat. Med.* 12, 1167–1174. doi: 10.1038/nm1483

Johnson, S., Chen, H., and Lo, P. K. (2013). In vitro tumor-sphere formation assays. *Bio.Protoc.* 3:e325. doi: 10.21769/BioProtoc.325

Kamran, M. Z., Patil, P., and Gude, R. P. (2013). Role of STAT3 in cancer metastasis and advances. *Biomed. Res. Int.* 2013:421821. doi: 10.1155/2013/421821

Katoh, M. (2007). Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis. *Stem Cell Rev.* 3, 30–38. doi: 10.1007/s12015-007-0006-6

Kellokumpu-Lehtinen, P., Talpaz, M., Harris, D., Van, Q., Kurzrock, R., and Estrov, Z. (1996). Leukemia inhibitory factor stimulates breast, kidney and prostate cancer cell proliferation by paracrine and autocrine pathways. *Int. J. Cancer* 66, 515–519. doi: 10.1002/(SICI)1097-0215(19960516)66:4<515::AID-IJC15>3.0.CO;2-6

König, L., Kasimir-Bauer, S., Hoffmann, O., Bittner, A. K., Wagner, B., Manvailer, L. F., et al. (2016). The prognostic impact of soluble and vesicular HLA-G and its relationship to circulating tumor cells in neoadjuvant treated breast cancer patients. *Hum. Immunol.* 77, 791–799. doi: 10.1016/j.humimm.2016.01.002

Kosaka, N. (2016). Decoding the secret of cancer by means of extracellular vesicles. *J. Clin. Med.* 5:E22. doi: 10.3390/jcm5020022

Krishnamachary, B., Penet, M. F., Nimmagadda, S., Mironchik, Y., Raman, V., Solaiyappan, M., et al. (2012). Hypoxia regulates CD44 and its variant isoforms through HIF-1a in triple negative breast cancer. *PLoS One* 7:e44078. doi: 10.1371/journal.pone.0044078

Kunath, T., Saba-El-Leil, M. K., Almousailleakh, M., Wray, J., Meloche, S., and Smith, A. (2007). FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development* 134, 2895–2902. doi: 10.1242/dev.02880

Lasorella, A., Benezra, R., and Iavarone, A. (2014). The ID proteins: master regulators of cancer stem cells and tumour aggressiveness. *Nat. Rev. Cancer* 14, 77–91. doi: 10.1038/nrc3638

Lesport, E., Baudhuin, J., LeMaoult, J., Sousa, S., Doliger, C., Carosella, E. D., et al. (2009). Human melanoma cells secreting human leukocyte antigen-G5 inhibit natural killer cell cytotoxicity by impairing lytic granules polarization toward target cell. *Hum. Immunol.* 70, 1000–1005. doi: 10.1016/j.humimm.2009.07.019

Li, L., Baroja, M. L., Majumdar, A., Chadwick, K., Rouleau, A., Gallacher, L., et al. (2004). Human embryonic stem cells possess immune-privileged properties. *Stem Cells* 22, 448–456. doi: 10.1634/stemcells.22-4-448

Li, R., Liang, J., Ni, S., Zhou, T., Qing, X., Li, H., et al. (2010). A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. *Cell Stem Cell* 7, 51–63. doi: 10.1016/j.stem.2010.04.014

Lin, A., and Yan, W. H. (2015). Human leukocyte antigen-G (HLA-G) expression in cancers: roles in immune evasion, metastasis and target for therapy. *Mol. Med.* 21, 782–791. doi: 10.2119/molmed.2015.00083

Lin, A., Yan, W. H., Xu, H. H., Gan, M. F., Cai, J. F., Zhu, M., et al. (2007). HLA-G expression in human ovarian carcinoma counteracts NK cell function. *Ann. Oncol.* 18, 1804–1809. doi: 10.1093/annonc/mdm356

Ling, M. T., Wang, X., Zhang, X., and Wong, Y. C. (2006). The multiple roles of Id-1 in cancer progression. *Differentiation* 74, 481–487. doi: 10.1111/j.1432-0436.2006.00083.x

Liu, N., Lu, M., Tian, X., and Han, Z. J. (2007). Molecular mechanisms involved in self-renewal and pluripotency of embryonic stem cells. *Cell Physiol.* 211, 279–286. doi: 10.1002/jcp.20978

Liu, S., Cong, Y., Wang, D., Sun, Y., Deng, L., Liu, Y., et al. (2014). Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts. *Stem Cell Rep.* 2, 78–91. doi: 10.1016/j.stemcr.2013.11.009

Lopatina, T., Gai, C., Deregibus, M. C., Kholia, S., and Camussi, G. (2016). Cross talk between cancer and mesenchymal stem cells through extracellular vesicles carrying nucleic acids. *Front. Oncol.* 6:125. doi: 10.3389/fonc.2016.00125

Loumagne, L., Baudhuin, J., Favier, B., Montespan, F., Carosella, E. D., and Rouas-Freiss, N. (2014). In vivo evidence that secretion of HLA-G by immunogenic tumor cells allows their evasion from immunosurveillance. *Int. J. Cancer* 135, 2107–2117. doi: 10.1002/ijc.28845

Luft, J. C., and Dix, D. J. (1999). HSP70 expression and function during embryogenesis. *Cell Stress Chaperones* 4, 162–170. doi: 10.1379/1466-1268(1999)004<0162:HEAFDE>2.3.CO;2

Lyden, D., Young, A. Z., Zagzag, D., Yan, W., Gerald, W., O'Reilly, R., et al. (1999). Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumor xenografts. *Nature* 401, 670–677. doi: 10.1038/44334

Manrique, I., Nguewa, P., Bleau, A. M., Nistal-Villan, E., Lopez, I., Villalba, M., et al. (2015). The inhibitor of differentiation isoform Id1b, generated by alternative splicing, maintains cell quiescence and confers self-renewal and cancer stem cell-like properties. *Cancer Lett.* 356, 899–909. doi: 10.1016/j.canlet.2014.10.035

Manzo, G. (1989). Phylogenesis–ontogenesis–oncogenesis. *Med. Hypotheses* 30, 245–257. doi: 10.1016/0306-9877(89)90033-9

Marangoni, E., Lecomte, N., Durand, L., de Pinieux, G., Decaudin, D., Chomienne, C., et al. (2009). CD44 targeting reduces tumour growth and prevents post-chemotherapy relapse of human breast cancers xenografts. *Br. J. Cancer* 100, 918–992. doi: 10.1038/sj.bjc.6604953

Marjanovic, N. D., Weinberg, R. A., and Chaffer, C. L. (2013). Cell plasticity and heterogeneity in cancer. *Clin. Chem.* 59, 168–179. doi: 10.1373/clinchem.2012.184655

Menendez, S., Camus, S., and Izpisua Belmonte, J. C. (2010). p53: guardian of reprogramming. *Cell Cycle* 9, 3887–3891. doi: 10.4161/cc.9.19.13301

Mern, D. S., Hasskarl, J., and Burwinkel, B. (2010a). Inhibition of Id proteins by a peptide aptamer induces cell-cycle arrest and apoptosis in ovarian cancer cells. *Br. J. Cancer* 103, 1237–1244. doi: 10.1038/sj.bjc.6605897

Mern, D. S., Hoppe-Seyler, K., Hoppe-Seyler, F., Hasskarl, J., and Burwinkel, B. (2010b). Targeting Id1 and Id3 by a specific peptide aptamer induces E-box promoter activity, cell cycle arrest, and apoptosis in breast cancer cells. *Breast Cancer Res. Treat.* 124, 623–633. doi: 10.1007/s10549-010-0810-6

Miyanari, Y., and Torres-Padilla, M. E. (2012). Control of ground-state pluripotency by allelic regulation of Nanog. *Nature* 483, 470–473. doi: 10.1038/nature10807

Mohyeldin, A., Garzon-Muvdi, T., and Quinones-Hinojosa, A. (2010). Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell* 7, 150–161. doi: 10.1016/j.stem.2010.07.007

Moreau, P., Adrian-Cabestre, F., Menier, C., Guiard, V., Gourand, L., Dausset, J., et al. (1999). IL-10 selectively induces HLA-G expression in human trophoblasts and monocytes. *Int. Immunol.* 11, 803–811. doi: 10.1093/intimm/11.5.803

Murase, R., Sumida, T., Kawamura, R., Onishi-Ishikawa, A., Hamakawa, H., McAllister, S. D., et al. (2016). Suppression of invasion and metastasis in aggressive salivary cancer cells through targeted inhibition of ID1 gene expression. *Cancer Lett.* 377, 11–16. doi: 10.1016/j.canlet.2016.04.021

Nair, R., Teo, W. S., Mittal, V., and Swarbrik, A. (2014). ID proteins regulate diverse aspects of cancer progression and provide novel therapeutic opportunities. *Mol. Ther.* 22, 1407–1415. doi: 10.1038/mt.2014.83

Naito, Y., Yoshioka, Y., Yamamoto, Y., and Ochiya, T. (2017). How cancer cells dictate their microenvironment: present roles of extracellular vesicles. *Cell. Mol. Life Sci.* 74, 697–713. doi: 10.1007/s00018-016-2346-3

Neuer, A., Mele, C., Liu, H. C., Rosenwaks, Z., and Witkin, S. S. (1998). Monoclonal antibodies to mammalian heat shock proteins impair mouse embryo development in vitro. *Hum. Reprod.* 3, 987–990. doi: 10.1093/humrep/13.4.987

Nguewa, P., Manrique, I., Diaz, R., Redrado, M., Parrondo, R., Perez-Stable, C., et al. (2014). Id-1B, an alternatively spliced isoform of the inhibitor of differentiation-1, impairs cancer cell malignancy through inhibition of proliferation and angiogenesis. *Curr. Mol. Med.* 14, 151–162. doi: 10.2174/1566524013666131203100643

Nichols, J., and Smith, A. (2009). Naive and primed pluripotent states. *Cell Stem Cell* 4, 487–492. doi: 10.1016/j.stem.2009.05.015

Ning, N., Pan, Q., Zheng, F., Teitz-Tennenbaum, S., Egenti, M., Yet, J., et al. (2012). Cancer stem cell vaccination confers significant antitumor immunity. *Cancer Res.* 72, 1853–1864. doi: 10.1158/0008-5472

Niola, F., Zhao, X., Singh, D., Castano, A., Sullivan, R., Lauria, M., et al. (2012). Id proteins synchronize stemness and anchorage to the niche of neural stem cells. *Nat. Cell Biol.* 14, 477–487. doi: 10.1038/ncb2490

Nishimura, K., Semba, S., Aoyagi, K., Sasaki, H., and Yokozaki, H. (2012). Mesenchymal stem cells provide an advantageous tumor microenvironment for the restoration of cancer stem cells. *Pathobiology* 79, 290–306. doi: 10.1159/000337296

O'Brien, C. A., Kreso, A., Ryan, P., Hermans, K. G., Gibson, L., Wang, Y., et al. (2012). ID1 and ID3 regulate the self-renewal capacity of human colon cancer-initiating cells through p21. *Cancer Cell* 21, 777–792. doi: 10.1016/j.ccr.2012.04.036

Ocaña, O. H., Córcoles, R., Fabra, A., Moreno-Bueno, G., Acloque, H., Vega, S., et al. (2012). Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer PRRX1. *Cancer Cell* 22, 709–724. doi: 10.1016/j.ccr.2012.10.012

Okamoto, I., Kawano, Y., Tsuiki, H., Sasaki, J., Nakao, M., Matsumoto, M., et al. (1999). CD44 cleavage induced by a membrane-associated metalloprotease plays a critical role in tumor cell migration. *Oncogene* 18, 1435–1446. doi: 10.1038/sj.onc.1202447

Okita, K., and Yamanaka, S. (2006). Intracellular signaling pathways regulating pluripotency of embryonic stem cells. *Curr. Stem Cell Res. Ther.* 1, 103–111. doi: 10.2174/157488806775269061

Orian-Rousseau, V., and Ponta, H. (2015). Perspectives of CD44 targeting therapies. *Arch. Toxicol.* 89, 3–14. doi: 10.1007/s00204-014-1424-2

Ouyang, X. S., Wang, X., Ling, M. T., Wong, H. L., Tsao, S. W., and Wong, Y. C. (2002). Id-1 stimulates serum independent prostate cancer cell proliferation through inactivation of p16(INK4a)/pRB pathway. *Carcinogenesis* 23, 721–725. doi: 10.1093/carcin/23.5.721

Pan, Q., Li, Q., Liu, S., Ning, N., Zhang, X., Xu, Y., et al. (2015). Concise review: targeting cancer stem cells using immunological approaches. *Stem Cells* 33, 2085–2092. doi: 10.1002/stem.2039

Pankaj, S., Sudha Saryu, M., and Satish Kumar, G. (2013). LIF-STAT signaling and trophoblast biology. *JAKSTAT* 2:e25155. doi: 10.4161/jkst.25155

Paolella, B. R., Havrda, M. C., Mantani, A., Wray, C. M., Zhang, Z., and Israel, M. A. (2011). p53 directly represses Id2 to inhibit the proliferation of neural progenitor cells. *Stem Cells* 29, 1090–1101. doi: 10.1002/stem.660

Park, H. J., Hong, M., Bronson, R. T., Israel, M. A., Frankel, W. N., and Yun, K. (2013). Elevated Id2 expression results in precocious neural stem cell depletion and abnormal brain development. *Stem Cells* 31, 1010–1021. doi: 10.1002/stem.1351

Park, J. I., Strock, C. J., Ball, D. W., and Nelkin, B. D. (2003). The Ras/Raf/MEK/extracellular signal-regulated kinase pathway induces autocrine-paracrine growth inhibition via the leukemia inhibitory factor/JAK/STAT pathway. *Mol. Cell Biol.* 23, 543–554. doi: 10.1128/MCB.23.2.543-554.2003

Patel, D., Morton, D. J., Carey, J., Havrda, M. C., and Chaudhary, J. (2015). Inhibitor of differentiation 4 (ID4): from development to cancer. *Biochim. Biophys. Acta* 1855, 92–103. doi: 10.1016/j.bbcan.2014.12.002

Pham, P. V., Phan, N. L., Nguyen, N. T., Truong, N. H., Duong, T. T., Le, D. V., et al. (2011). Differentiation of breast cancer stem cells by knockdown of CD44: promising differentiation therapy. *Transl. Med.* 9:209. doi: 10.1186/1479-5876-9-209

Pietras, A., Katz, A. M., Ekstrom, E. J., Wee, B., Halliday, J. J., Pitter, K. L., et al. (2014). Osteopontin-CD44 signaling in the glioma perivascular niche enhances cancer stem cell phenotypes and promotes aggressive tumor growth. *Cell Stem Cell* 14, 357–369. doi: 10.1016/j.stem.2014.01.005

Plaks, V., Kong, N., and Werb, Z. (2015). The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell* 16, 225–238. doi: 10.1016/j.stem.2015.02.015

Prabhu, S., Ignatova, A., Park, S. T., and Sun, X. H. (1997). Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and ID proteins. *Mol. Cell Biol.* 17, 5888–5896. doi: 10.1128/MCB.17.10.5888

Quail, D. F., Taylor, M. J., and Postovit, L. M. (2012). Microenvironmental regulation of cancer stem cell phenotypes. *Curr. Stem Cell Res. Ther.* 7, 197–216. doi: 10.2174/157488812799859838

Rahme, G. J., and Israel, M. A. (2015). Id4 suppresses MMP2-mediated invasion of glioblastoma-derived cells by direct inactivation of Twist1 function. *Oncogene* 34, 53–62. doi: 10.1038/onc.2013.531

Rebmann, V., König, L., Nardi Fda, S., Wagner, B., Manvailer, L. F., and Horn, P. A. (2016). The potential of HLA-G-bearing extracellular vesicles as a future element in HLA-G immune biology. *Front. Immunol.* 7:173. doi: 10.3389/fimmu.2016.00173

Rebmann, V., Regel, J., Stolke, D., and Grosse-Wilde, H. (2003). Secretion of sHLA-G molecules in malignancies. *Semin. Cancer Biol.* 13, 371–377. doi: 10.1016/S1044-579X(03)00028-2

Rockman, S. P., Currie, S. A., Ciavarella, M., Vincan, E., Dow, C., Thomas, R. J., et al. (2001). Id2 is a target of the beta-catenin/T cell factor pathway in colon carcinoma. *J. Biol. Chem.* 276, 45113–45119. doi: 10.1074/jbc.M107742200

Roesch, A., Fukunaga-Kalabis, M., Schmidt, E. C., Zabierowski, S. E., Brafford, P. A., Vultur, A., et al. (2010). A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. *Cell* 141, 583–594. doi: 10.1016/j.cell.2010.04.020

Rouas-Freiss, N., Moreau, P., Menier, C., and Carosella, E. D. (2003). HLA-G in cancer: a way to turn off the immune system. *Semin. Cancer Biol.* 13, 325–336. doi: 10.1016/S1044-579X(03)00023-3

Rouas-Freiss, N., Moreau, P., Menier, C., LeMaoult, J., and Carosella, E. D. (2007). Expression of tolerogenic HLA-G molecules in cancer prevents antitumor responses. *Semin. Cancer Biol.* 17, 413–421. doi: 10.1016/j.semcancer.2007.07.003

Ruzinova, M. B., and Benezra, R. (2003). Id proteins in development, cell cycle and cancer. *Trends Cell Biol.* 13, 410–418. doi: 10.1016/S0962-8924(03)00147-8

Sahlgren, C., Gustafsson, M. V., Jin, S., Poellinger, L., and Lendahl, U. (2008). Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc. Natl. Acad. Sci. U.S.A.* 105, 6392–6397. doi: 10.1073/pnas.0802047105

Samali, A., and Cotter, T. G. (1996). Heat shock proteins increase resistance to apoptosis. *Exp. Cell Res.* 223, 163–170. doi: 10.1006/excr.1996.0070

Samavarchi-Tehrani, P., Golipour, A., David, L., Sung, H. K., Beyer, T. A., Datti, A., et al. (2010). Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. *Cell Stem Cell* 7, 64–77. doi: 10.1016/j.stem.2010.04.015

Sato, N., Meijer, L., Skaltsounis, L., Greengard, P., and Brivanlou, A. H. (2004). Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat. Med.* 10, 55–63. doi: 10.1038/nm979

Scheel, C., Eaton, N. E., Li, S. H. J., Chaffer, C. L., Reinhardt, F., Kah, K. J., et al. (2011). Paracrine and autocrine signals induce and maintain mesenchymal and stem cell states in the breast. *Cell* 145, 926–940. doi: 10.1016/j.cell.2011.04.029

Schwitalla, S., Fingerle, A. A., Cammareri, P., Nebelsiek, T., Goktuna, S. I., Ziegler, P. K., et al. (2013). Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* 152, 25–38. doi: 10.1016/j.cell.2012.12.012

Sharma, P., Chinaranagari, S., and Chaudhary, J. (2015). Inhibitor of differentiation 4 (ID4) acts as an inhibitor of ID-1, -2 and -3 and promotes basic helix loop helix (bHLH) E47 DNA binding and transcriptional activity. *Biochimie* 112, 139–150. doi: 10.1016/j.bochi.2015.03.006

Sherwin, J. R. A., Smith, S. K., Wilson, A., and Sharkey, A. M. (2002). Soluble gp130 is up-regulated in the implantation window and shows altered secretion in patients with primary unexplained infertility. *J. Clin. Endocrinol. Metab.* 87, 3953–3960. doi: 10.1210/jcem.87.8.8766

Sheu, J., and Shih, I. E. (2010). HLA-G and immune evasion in cancer cells. *Med. Assoc.* 109, 248–257. doi: 10.1016/S0929-6646(10)60050-2

Shu, C. W., and Huang, C. M. (2008). HSP70s: from tumor transformation to cancer therapy. *Clin. Med. Oncol.* 2, 335–345. doi: 10.4137/CMO.S475

Shuno, Y., Tsuno, N. H., Okaji, Y., Tsuchiya, T., Sakurai, D., Nishikawa, T., et al. (2010). ID1/ID3 knockdown inhibits metastatic potential of pancreatic cancer. *J. Surg. Res.* 161, 76–82. doi: 10.1016/j.jss.2008.10.031

Silva, J., Barrandon, O., Nichols, J., Kawaguchi, J., Theunissen, T. W., Smith, A., et al. (2008). Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS Biol.* 6:e253. doi: 10.1371/journal.pbio.0060253

Stangl, S., Gehrmann, M., Riegger, J., Kuhs, K., Riederer, I., Sievert, W., et al. (2011). Targeting membrane heat-shock protein 70 (HSP70) on tumors by cmHSP70.1 antibody. *Proc. Natl. Acad. Sci. U.S.A.* 108, 733–738. doi: 10.1073/pnas.1016065108

Stangl, S., Gross, C., Pokley, A. G., Asea, A. A., and Multhoff, G. (2008). Influence of HSP70 and HLA-E on the killing of leukemic blasts by cytokine/HSP70 peptide activated human natural killer (NK) cells. *Cell Stress Chaperones* 13, 221–230. doi: 10.1007/s12192-007-0008-y

Stankic, M., Pavlovic, S., Chin, Y., Brogi, E., Padua, D., Norton, L., et al. (2013). TGF-β-Id1 signaling opposes Twist1 and promotes metastatic colonization via a mesenchymal-to-epithelial transition. *Cell Rep.* 5, 1228–1242. doi: 10.1016/j.celrep.2013.11.014

Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F., et al. (1992). Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* 359, 76–79. doi: 10.1038/359076a0

Su, Y. J., Lai, H. M., Chang, Y. W., Chen, J. Y., and Lee, J. L. (2011). Direct reprogramming of stem cell properties in colon cancer cells by CD44. *EMBO J.* 30, 3186–3199. doi: 10.1038/emboj.2011.211

Tamura, Y., Sugimoto, M., Ohnishi, K., Sakai, T., and Hara, E. (1998). Differential activity of a variant form of the human Id-1 protein generated by alternative splicing. *FEBS Lett.* 436, 169–173. doi: 10.1016/S0014-5793(98)01117-X

Tapia, N., and Schöler, H. R. (2010). P53 connects tumorigenesis and reprogramming to pluripotency. *J. Exp. Med.* 207, 2045–2048. doi: 10.1084/jem.20101866

Teo, W. S., Nair, R., and Swarbrick, A. (2014). New insights into the role of ID proteins in breast cancer metastasis: a MET affair. *Breast Cancer Res.* 16, 305. doi: 10.1186/bcr3654

Thiery, J. P., Acloque, H., Huang, R. J. Y., and Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell* 139, 871–890. doi: 10.1016/j.cell.2009.11.007

Tilburgs, T., Evans, J. H., Crespo, A. C., and Strominger, J. L. (2015). The HLA-G cycle provides for both NK tolerance and immunity at the maternal-fetal interface. *Proc. Natl. Acad. Sci. U.S.A.* 112, 13312–13317. doi: 10.1073/pnas.1517724112

Tiwari, N., Gheldof, A., Tatari, M., and Christofori, G. (2012). EMT as the ultimate survival mechanism of cancer cells. *Semin. Cancer Biol.* 22, 194–207. doi: 10.1016/j.semcancer.2012.02.013

Tran, D. D., Corsa, C. A., Biswas, H., Aft, R. L., and Longmore, G. D. (2011). Temporal and spatial cooperation of snail1 and twist1 during epithelial-mesenchymal transition predicts for human breast cancer. *Mol. Cancer Res.* 9, 1644–1657. doi: 10.1158/1541-7786.MCR-11-0371

Tsuchiya, T., Okaji, Y., Tsuno, N. H., Sakurai, D., Tsuchiya, N., Kawai, K., et al. (2005). Targeting ID1 and ID3 inhibits peritoneal metastasis of gastric cancer. *Cancer Sci.* 96, 784–790. doi: 10.1111/j.1349-7006.2005.00113.x

Ullmann, U., In't Veld, P., Gilles, C., Sermon, K., De Rycke, M., Van de Velde, H., et al. (2006). Epithelial-mesenchymal transition process in human embryonic stem cells cultured in feeder-free conditions. *Mol. Hum. Reprod.* 13, 21–32. doi: 10.1093/molehr/gal091

Umetani, N., Takeuchi, H., Fujimoto, A., Shinozaki, M., Bilchik, A. J., and Hoon, D. S. B. (2004). Epigenetic inactivation of ID4 in colorectal carcinomas correlates with poor differentiation and unfavorable prognosis. *Clin. Cancer Res.* 10, 7475–7483. doi: 10.1158/1078-0432.CCR-04-0689

Urosevic, M., and Dummer, R. (2003). HLA-G and IL-10 expression in human cancer–different stories with the same message. *Semin. Cancer Biol.* 13, 337–342. doi: 10.1016/S1044-579X(03)00024-5

Urosevic, M., Kurrer, M. O., Kamarashev, J., Mueller, B., Weder, W., Burg, G., et al. (2001). Human leukocyte antigen G up-regulation in lung cancer associates with high-grade histology, human leukocyte antigen class I loss and interleukin-10 production. *Am. J. Pathol.* 159, 817–824. doi: 10.1016/S0002-9440(10)61756-7

Urosevic, M., Willers, J., Mueller, B., Kempf, W., Burg, G., and Dummer, R. (2002). HLA-G protein up-regulation in primary cutaneous lymphomas is associated with interleukin-10 expression in large cell T-cell lymphomas and indolent B-cell lymphomas. *Blood* 99, 609–617. doi: 10.1182/blood.V99.2.609

Verloes, A., Van de Velde, H., LeMaoult, J., Mateizel, I., Cauffmann, G., Horn, P. A., et al. (2011). HLA-G expression in human embryonic stem cells and preimplantation embryos. *J. Immunol.* 186, 2663–2671. doi: 10.4049/jimmunol.1001081

Wakamatsu, Y., Sakamoto, N., Oo, H. Z., Naito, Y., Uraoka, N., Anami, K., et al. (2012). Expression of cancer stem cell markers ALDH1, CD44 and CD133 in primary tumor and lymph node metastasis of gastric cancer. *Pathol. Int.* 62, 112–119. doi: 10.1111/j.1440-1827.2011.02760.x

Wallach-Dayan, S. B., Rubinstein, A. M., Hand, C., Breuer, R., and Naor, D. (2008). DNA vaccination with CD44 variant isoform reduces mammary tumor local growth and lung metastasis. *Mol. Cancer Ther.* 7, 1615–1623. doi: 10.1158/1535-7163.MCT-07-2383

Wang, L. H., and Baker, N. E. (2015). E proteins and ID proteins: helix-loop-helix partners in development and disease. *Dev. Cell* 35, 269–280. doi: 10.1016/j.devcel.2015.10.019

Wang, T., Narayanaswamy, R., Ren, H., and Torchilin, V. P. (2016). Combination therapy targeting both cancer stem-like cells and bulk tumor cells for improved efficacy of breast cancer treatment. *Cancer Biol. Ther.* 17, 698–707. doi: 10.1080/15384047

Wang, Z., Oron, E., Nelson, B., Razis, S., and Ivanova, N. (2012). Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. *Cell* 10, 440–454. doi: 10.1016/j.stem.2012.02.016

Wen, J., Park, J. Y., Park, K. H., Chung, H. W., Bang, S., Park, S. W., et al. (2010). Oct4 and Nanog expression is associated with early stages of pancreatic carcinogenesis. *Pancreas* 39, 622–626. doi: 10.1097/MPA.0b013e3181c75f5e

Williams, K., Motiani, K., Giridhar, P. V., and Kasper, S. (2013). CD44 integrates signaling in normal stem cell, cancer stem cell and (pre)metastatic niches. *Exp. Biol. Med.* 238, 324–338. doi: 10.1177/1535370213480714

Wong, Y. C., Zhang, X. M., Ling, M. T., and Wang, X. H. (2008). Inactivation of ID-1 gene induces sensitivity of prostate cancer cells to chemotherapeutic drugs. *Adv. Exp. Med. Biol.* 617, 565–572. doi: 10.1007/978-0-387-69080-3_58

Xiao, L., Yuan, X., and Sharkis, S. J. (2006). Activin A maintains self-renewal and regulates fibroblast growth factor, Wnt, and bone morphogenic protein pathways in human embryonic stem cells. *Stem Cells* 24, 1476–1486. doi: 10.1634/stemcells.2005-0299

Xu, R. H., Chen, X., Li, D. S., Li, R., Addicks, G. C., Glennon, C., et al. (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat. Biotechnol.* 20, 1261–1264. doi: 10.1038/nbt761

Yamanaka, Y., Lanner, F., and Rossant, J. (2010). FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. *Development* 137, 715–724. doi: 10.1242/dev.043471

Yan, W. H., Lin, A. F., Chang, C. C., and Ferrone, S. (2005). Induction of HLA-G expression in a melanoma cell line OCM-1A following the treatment with 5-aza-2'-deoxycytidine. *Cell Res.* 15, 523–531. doi: 10.1038/sj.cr.7290376

Yan, Y., Zuo, X., and Wei, D. (2015). Concise review: emerging role of CD44 in cancer stem cells: a promising biomarker and therapeutic target. *Stem Cells Transl. Med.* 4, 1033–1043. doi: 10.5966/sctm.0048

Yang, M. H., Wu, M. Z., Chiou, S. H., Chen, P. M., Chang, S. Y., Liu, C. J., et al. (2008). Direct regulation of TWIST by HIF-1alpha promotes metastasis. *Nat. Cell Biol.* 10, 295–305. doi: 10.1038/ncb1691

Yao, Y. Q., Barlow, D. H., and Sargent, I. L. (2005). Differential expression of alternatively spliced transcripts of HLA-G in human preimplantation embryos and inner cell masses. *J. Immunol.* 175, 8379–8385. doi: 10.4049/jimmunol.175.12.8379

Yen, B. L., Chang, C. J., Liu, K. J., Chen, Y. C., Hu, H. I., Bai, C. H., et al. (2009). Human embryonic stem cell-derived mesenchymal progenitors possess strong immunosuppressive effects toward natural killer cells as well as T lymphocytes. *Stem Cells* 27, 451–456. doi: 10.1634/stemcells.2008-0390

Yin, X., Zhang, B. H., Zheng, S. S., Gao, D. M., Qiu, S. J., Wu, W. Z., et al. (2015). Coexpression of gene Oct4 and Nanog initiates stem cell characteristics in hepatocellular carcinoma and promotes epithelial-mesenchymal transition through activation of Stat3/Snail signaling. *J. Hematol. Oncol.* 8:23. doi: 10.1186/s13045-015-0119-3

Ying, Q. L., Nichols, J., Chambers, I., and Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 115, 281–292. doi: 10.1016/S0092-8674(03)00847-X

Yue, X., Wu, L., and Hu, W. (2015). The regulation of leukemia inhibitory factor. *Cancer Cell Microenviron.* 2:e877. doi: 10.14800/ccm.877

Yun, K., Mantani, A., Garel, S., Rubenstein, J., and Israel, M. A. (2004). Id4 regulates neural progenitor proliferation and differentiation in vivo. *Development* 131, 5441–5448. doi: 10.1242/dev.01430

Zaiden, M., Feinshtein, V., and David, A. (2017). Inhibition of CD44v3 and CD44v6 function blocks tumor invasion and metastatic colonization. *J. Control Release* 257, 10–20. doi: 10.1016/j.conrel.2017.01.021

Zeilstra, J., Joosten, S. P., van Andel, H., Tolg, C., Berns, A., Snoek, M., et al. (2014). Stem cell CD44v isoforms promote intestinal cancer formation in Apc (min) mice downstream of WNT signaling. *Oncogene* 33, 665–670. doi: 10.1038/onc.2012.611

Zhang, W. Q., Xu, D. P., Liu, D., Li, Y. Y., Ruan, Y. Y., Lin, A., et al. (2014). HLA-G1 and HLA-G5 isoforms have an additive effect on NK cytolysis. *Hum. Immunol.* 75, 182–189. doi: 10.1016/j.humimm.2013.11.001

Zhang, Y., Yu, S., Han, Y., Wang, Y., and Sun, Y. (2018). Human leukocyte antigen-G expression and polymorphisms promote cancer development and guide cancer diagnosis/treatment. *Oncol. Lett.* 15, 699–709. doi: 10.3892/ol.2017.7407

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Cortical Development and Brain Malformations: Insights From the Differential Regulation of Early Events of DNA Replication

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During the development of the cortex distinct populations of Neural Stem Cells (NSCs) are defined by differences in their cell cycle duration, self-renewal capacity and transcriptional profile. A key difference across the distinct populations of NSCs is the length of G1 phase, where the licensing of the DNA replication origins takes place by the assembly of a pre-replicative complex. Licensing of DNA replication is a process that is adapted accordingly to the cell cycle length of NSCs to secure the timed duplication of the genome. Moreover, DNA replication should be efficiently coordinated with ongoing transcription for the prevention of conflicts that would impede the progression of both processes, compromising the normal course of development. In the present review we discuss how the differential regulation of the licensing and initiation of DNA replication in different cortical NSCs populations is integrated with the properties of these stem cells populations. Moreover, we examine the implication of the initial steps of DNA replication in the pathogenetic mechanisms of neurodevelopmental defects and Zika virus-related microcephaly, highlighting the significance of the differential regulation of DNA replication during brain development.

Keywords: DNA replication, origins licensing, cortex development, neural stem cells, microcephaly

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INTRODUCTION

The neocortex is a complicated brain region characterized by excessive cell diversity as it is composed by multiple types of neuronal and glial cells assembled in networks that regulate the higher order functions. The cortex derives from the dorsal telencephalon located in the most anterior part of the neural tube through a strictly regulated process that involves diverse types of neural stem cells (NSCs) and more committed neural progenitors (NPCs) organized in discrete zones (Azzarelli et al., 2015; Agirman et al., 2017). Excitatory neurons are produced consecutively within the proliferating zones and subsequently their somata migrate radially to their final position in the cortical plate. Cortical connectivity is completed with the integration of cortical interneurons that are generated in the ventral telencephalon and migrate tangentially toward the developing cortex (Dwyer et al., 2016; Lim et al., 2018).

Coordinated action of morphogens and intrinsic signaling cues leads to the successive generation of the distinct types of NSCs and NPCs during cortical development. These populations differ on their self-renewal ability and differentiation potential. Numerous studies have identified

distinct morphological and molecular features that describe the diversity among NPCs (reviewed in Govindan and Jabaudon, 2017; Uzquiano et al., 2018).

DNA replication is a key cellular process that is coupled with growth and proliferation. Elucidating the differential regulation of DNA replication within the distinct populations of NPCs will expand our knowledge on their functional diversity. Interestingly, increasing evidence over the last decades supports the implication of defective DNA replication in the pathophysiology of cortical malformations like microcephaly (Jackson et al., 2014; Khetarpal et al., 2016). In this review, we will discuss the differential regulation of the initial steps of DNA replication, focusing on the NSCs of the early stages of cortical development and the impact of defective replication on normal development.

ESTABLISHMENT OF NEURAL STEM CELLS DURING EARLY CORTICAL DEVELOPMENT

The first population of NSCs that is established in the developing cortex are the neuroepithelial cells (NECs), while at the onset of neurogenesis NECs are replaced by apical radial glial cells (aRGs) that remain in contact with the apical membrane and form the ventricular zone (VZ), one of the main proliferating zones of the cortex. During mid neurogenesis a second proliferating zone is established basally of the VZ by intermediate progenitors, which delaminate from the apical membrane and are translocated to the subventricular zone (SVZ). Despite these primary populations, other minor groups of progenitors are also generated over the course of development like the short neural precursors (SNPs) that appear in the VZ and the outer RGs (oRGs) located in the upper boundaries of the SVZ. Here, we will focus on the populations of the apical NSCs that prevail the VZ during the initial stages of cortical generation (**Figure 1A**). A more detailed description of the NSCs and NPCs population of the developing cortex are reviewed by Götz and Huttner (2005), Taverna et al. (2014), and Uzquiano et al. (2018).

Neuroepithelial cells consist the first population that reside in the walls of the neural tube. These cells are highly polarized, possessing a basal process that is attached to the basal lamina and an apical process attached to the apical surface in contact with the neural tube lumen (Arai and Taverna, 2017). NECs sense and respond to signaling cues from the cerebrospinal fluid through a primary cilium that protrudes from their apical membrane into the lumen (Guemez-Gamboa et al., 2014). The nuclei of NECs move across the apical-basal distance in a cell cycle dependent way; mitosis occurs always in the apical surface of the neural tube and as the cell progresses to the G1 phase the nucleus is moving radially toward the basal side. During DNA replication the nucleus reaches the basal membrane and migrates to the opposite direction until it will be repositioned to the apical surface for a new division. This process is known as interkinetic nuclear migration (INM) and gives to the neuroepithelium the appearance of a pseudostratified epithelium (Miyata et al., 2014). NECs exhibit a relatively short cell cycle (∼12 h) that correlates with the high proliferation potential of these cells and permits the establishment of the initial pool of NSCs through symmetric self-renewing divisions (Takahashi et al., 1995; Borrell and Calegari, 2014).

Upon the onset of neurogenesis at embryonic day (E)10.5 in murine development and until E12.5, NECs are gradually transformed to aRGs, which constitute the main population of NSCs during cortical development. aRGs are also polarized cells exhibiting an apical and a basal process that span the radial axis of the developing cortex and maintain their primary cilium that projects into the newly formed telencephalic vesicles (Arai and Taverna, 2017). Similar to NECs, aRGs undergo INM during a cell cycle but the movement of their nucleus is restricted within the VZ (Miyata et al., 2014; Bertipaglia et al., 2017). SNPs that constitute a subtype of aRGs also appear in the VZ during that stage. SNPs remain in contact with the apical surface of the cortex through their apical process, however, their basal process does not span until the basal membrane but is constrained to the VZ (Stancik et al., 2010). The transition of NECs to aRGs is critical during brain development as it defines the switch from a self-renewing to a neurogenic state. The establishment of aRGs is defined by the elongation of the cell cycle (∼17 h) owed primarily to an increase of the G1 phase length, which doubles during mid-neurogenesis and covers more than 60% of aRGs cell cycle (Noctor et al., 2002; Calegari et al., 2005). Elongation of the G1 phase allows the action of fate determinants and the respective response of the cells (Matsuzaki and Shitamukai, 2015).

During cortical development, extrinsic signaling cues derived from the local environment coordinate the proliferation and fate commitment of NSCs. Various fate determinants and signaling pathways impact not only in the genetic program of NSCs by regulating the expression of specific factors, but also in their intrinsic features like their cell cycle and chromatin dynamics (Borrell and Calegari, 2014; Albert et al., 2017). Activation of the Notch pathway was identified to mediate NECs to aRGs transition, when an activated form of the Notch1 receptor was introduced in the telencephalon of E9.5 embryos and promoted radial glia identity (Gaiano et al., 2000). Upon establishment of aRGs, Notch signaling promotes cell cycle progression and maintenance of this population (Ohtsuka et al., 2001). Notably, the fibroblast growth factor (FGF) signaling pathway has key roles during aRGs specification and maintenance and thus controls neuronal output and proper cortical formation (Sahara and O'Leary, 2009; Rash et al., 2013). Interestingly, it has been shown that FGF signaling promotes proliferation of NPCs by directly decreasing the G1 length, suggesting an interplay between fate determinants and cell cycle regulation (Lukaszewicz et al., 2002).

AN OVERVIEW OF DNA REPLICATION LICENSING AND INITIATION STEPS IN EUKARYOTIC CELLS

Within a complete cell cycle the genetic information must be faithfully duplicated and pass intact to the progeny. Moreover, in the level of multicellular organisms, replication must be coordinated with cell fate decisions that are mediated by complicated transcriptional programs. DNA replication is

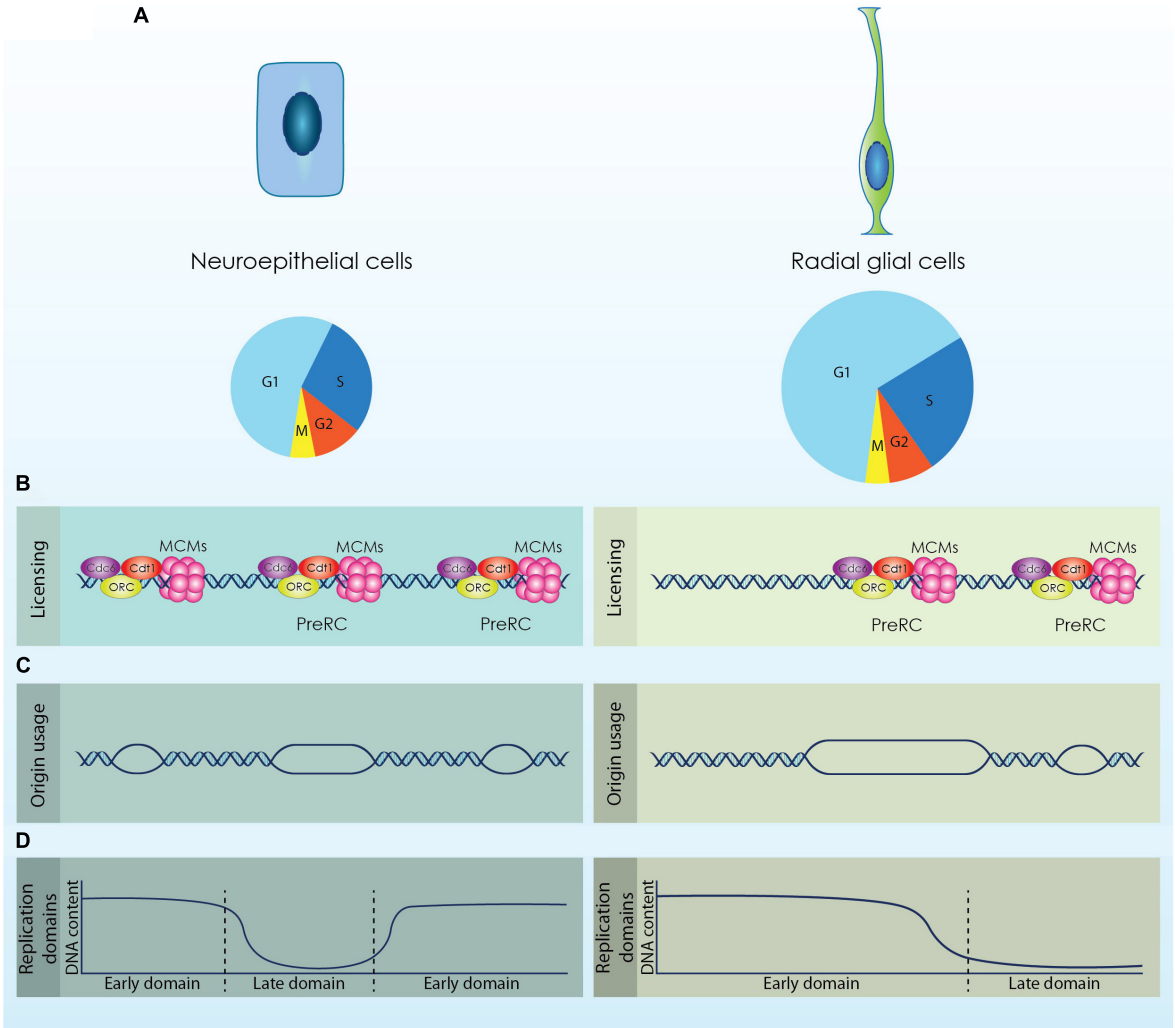

FIGURE 1 | The diverse populations of neural stem cells (NSCs) that emerge during cortical development exhibit distinct cell cycle kinetics and differential regulation of the DNA replication licensing and initiation processes. **(A)** Neuroepithelial cells are characterized by a shorter cell cycle compared to Radial Glial cells that permits their fast proliferation. (**B**; Licensing) During the G1 phase origins of replication are licensed by the formation of a competent pre-replicative complex (Pre-RC). Higher expression of licensing factors (ORC, Cdc6, Cdt1 and MCMs) is required for the efficient licensing of origins in NECs that exhibit a shorter G1 phase. (**C**; Origin usage) Increased formation of Pre-RCs facilitates the usage of more origins for the accurate completion of DNA replication that further causes a reduction of the available dormant origins. RGs activate less origins of replication compared to NECs to complete the duplication of their genome. (**D**; Replication Domains) DNA is replicated in well-defined segments constituted by multiple origins that fire synchronously in distinct time points throughout S phase. Rapid proliferating cells complete DNA replication in smaller segments, while cells with longer G1 phase exhibit fewer but larger replication domains.

organized at multiple levels to ensure that genome duplication will be completed within the available time and over- or under-duplication that threatens genome integrity will be avoided. In eukaryotic cells, DNA replication initiates from multiple sites along the genome known as replication origins. Initiation of replication is strictly regulated by a two-step process that involves the formation of a multiprotein complex termed pre-replicative complex (Pre-RC) on the potential origins and is called origins' licensing, followed by the sequential activation of a subset of origins known as origins' firing (reviewed in Symeonidou et al., 2012; Fragkos et al., 2015).

During the G1 phase replication origins are licensed by the sequential recruitment of proteins that will form an inactive pre-RC (**Figure 1B**; Licensing). The Origin Recognition Complex (ORC1-6) is the first factor that recognizes and binds to the origins serving as a docking site for the association of the rest of the licensing proteins. Following, the licensing factors Cdc6 and Cdt1 interact with the bound ORCs and promote the loading of the Minichromosome Maintenance complex (MCM) resulting in a competent pre-RC (Champeris Tsaniras et al., 2014). The MCM complex consists of six subunits (MCM2 to MCM7) with an ATP dependent DNA helicase activity and is loaded to chromatin as an inactive head-to-head double hexameric ring that encircles double-stranded DNA. To ensure the timed duplication of the genome, cells license more origins than the origins that require and therefore load more MCM complexes into chromatin, as

reduced MCM loading makes the cells sensitive to replication stress (Ibarra et al., 2008). Firing of origins occurs during the G1/S transition with the binding of the CDC45 and GINS to MCM2-7 that will form a stable CMG (CDC45-MCMs-GINS) complex with helicase activity. From that point, multiple replication factors are recruited to the activated origins to form the replisome, which is consisted of an active helicase subunit and DNA polymerases and is moving along the DNA to complete replication (Sun et al., 2015).

Upon entry to S phase multiple mechanisms negatively regulate licensing by phosphorylating the licensing factors and thus preventing the re-assembly of the Pre-RC (reviewed in Siddiqui et al., 2013). Phosphorylated CDT1 is targeted for degradation by the E3 ubiquitin ligase SCF-Skp2, while the chromatin-bound fraction of CDT1 is ubiquitinated by the CRL4-Cdt2 (Li et al., 2003; Nishitani et al., 2006). In metazoan an additional mechanism for CDT1 regulation is mediated by Geminin. During the S, G2 and M phases of the cell cycle Geminin binds to CDT1 and thus prevents its association with other licensing factors. Once mitotis is completed Geminin is deactivated permitting a new round of origins licensing (Caillat and Perrakis, 2012). CDC6 is also phosphorylated upon its release from chromatin and is translocated to the cytoplasm (Delmolino et al., 2001). Finally, in mammalian cells, the ORC1 subunit is specifically dissociated from chromatin due to ubiquitination and part of it is degraded (Li and DePamphilis, 2002).

REGULATION OF THE LICENSING FACTORS DURING THE SHORT G1 PHASE OF NECs

Neuroepithelial cells are characterized by an unusual cell cycle structure compared to the populations of neural stem and progenitor cells that are generated later during the development of the cortex. NECs exhibit a relatively small cell cycle while the length of the G1 phase is gradually increased as these cells are transformed to a RGs (Takahashi et al., 1995; Calegari et al., 2005). The short cell cycle length ensures the fast proliferation of NECs and the establishment of an initial pool of NSCs within a defined period during embryogenesis. Therefore, origin licensing during DNA replication must be accurately completed in the restricted cell cycle duration of NECs in order to secure faithful duplication of the genome. Consequently, a question that is raised is whether NECs employ different mechanisms to regulate licensing of DNA replication than other populations of NSCs or NPCs.

Neuroepithelial cells exhibit comparable cell cycle kinetics with Embryonic stem cells (ESCs), as both populations have similar cell cycle duration. It is also established that a short G1 phase is an intrinsic property of multipotency as both in ESCs and NECs G1 length increases upon fate commitment (Calegari et al., 2005; White and Dalton, 2005). The potential to *in vitro* differentiate ESCs to NPCs offers a suitable system for monitoring modifications in the licensing of DNA replication along with the progressive elongation of the cell cycle and neural fate commitment. It has been shown that ESCs express and maintain higher levels of CDT1 and CDC6 compared to differentiated cells to secure sufficient licensing and timed initiation of DNA replication (Fujii-Yamamoto et al., 2005; Ballabeni et al., 2011). Moreover, increased expression of licensing factors in hESCs mediates rapid MCM loading to chromatin, which facilitates the licensing of a "sufficient" number of origins within their short G1 phase. Interestingly, neuronal differentiation entailed with reduced expression of licensing factors and G1 elongation was sufficient to reduce the loading rate of MCM proteins (Matson et al., 2017).

These observations suggest that NECs might also require similar adaptations in the licensing of DNA replication due to their shortened G1 phase, while these features are probably absent from more committed NPCs defined by a longer G1 (**Figure 1B**; Licensing). Analyses of NPCs derived from different developmental stages are required to establish the differential regulation of licensing.

BALANCE BETWEEN ORIGINS USAGE AND DORMANT ORIGINS CONTRIBUTES TO CORTICAL INTEGRITY

Eukaryotic cells license a greater number of origins during G1 phase compared to the origins that will fire to complete genome duplication. Some of the licensed origins that are not activated remain dormant and fire to cover unreplicated regions when the progression of the initially formed replication forks is impeded. Interestingly, reduction of dormant origins (DOs) challenges the successful completion of DNA replication compromising genome stability (Alver et al., 2014; Shima and Pederson, 2017). NPCs that carry the hypomorphic allele $MCM4^{chaos}$, revealed significant decrease in DOs and exhibit increased DNA damage and reduced proliferation *in vitro*. Interestingly, reduction in DOs had also a direct effect in brain development, as embryos homozygous for the $MCM4^{chaos}$ allele showed a thinner cortex during mid-embryogenesis due to increased cell death of intermediate NPCs resulted in defective generation of neurons (Ge et al., 2015). Accordingly, deletion of the MCM2 subunit using an inducible CreERT2 system resulted in impaired neurogenesis in the adult brain caused by a significant decrease in NSCs of the SVZ (Pruitt et al., 2007). It thus appears that reduction in the number of licensed origins that eliminates the abundance of DOs, leads to DNA damage probably because of unresolved replication structures and causes eventually the apoptosis of NSCs.

During the expansion phase of the developing cortex, NSCs utilize a high number of origins during DNA replication due to their short cell cycle (Ge et al., 2015). However, this inherent mechanism sensitizes NSCs against exogenous stress as it restricts the available DOs that could be activated as a response to stress. As the cell cycle length of NSCs is progressively increased, more DOs are available and NSCs become less sensitive to genotoxic stress. It can be therefore speculated that there is a threshold of available DOs to safeguard accurate genome duplication of NSCs (**Figure 1C**; Origin usage). Additional data are required to establish this elegant mechanism that safeguards genomic

stability of cortical NSCs and secures structural and functional integrity of the cortex.

REPLICATION TIMING MODIFICATIONS PERMIT RAPID ADAPTATION TO DEVELOPMENTAL CUES DURING CORTICAL DEVELOPMENT

In eukaryotic cells initiation of DNA replication is dispersed throughout the S phase resulting in chromosomal segments that are replicated in defined points, known as replication domains (Rivera-Mulia and Gilbert, 2016; Takebayashi et al., 2017). Replication timing of most domains is conserved among different cell types, however, specific domains that are subjected to developmental control have been also identified. Comprehensive analyses of *in vitro* systems that recapitulate the progressive fate commitment of mouse and human ESCs confirmed the relation between replication timing and gene expression and showed that changes in the timing of replication coordinate with transcriptional activation (Hiratani et al., 2010; Rivera-Mulia et al., 2015).

Upon commitment of ESCs toward the neuronal lineage, the 20% of the genome is subjected to replication timing modifications. These modifications include mainly consolidation of replication domains that lead to fewer but larger segments of coordinated replication (**Figure 1D**; Replication domains) (Hiratani et al., 2008). Coordination between transcription and replication is critical as conflicts between the two machineries would lead to defective gene expression and moreover to genomic instability (Lin and Pasero, 2012; García-Muse and Aguilera, 2016). During cortical development, NSCs are subjected to a strict developmental program that defines their transcriptional profile. Completion of DNA replication in larger segments permits the rapid adaptation of the transcriptional program by minimizing the possibility of collisions (**Figure 1B**; Replication domains). Thus, dynamic regulation of replication timing in NSCs is critical for an effective response to the solid developmental program that is required during cortex formation.

IMPAIRED REGULATION OF DNA REPLICATION RESULTS IN BRAIN MALFORMATIONS

Genetic or environmental factors that limit the proliferation potential of stem or progenitor cells during embryogenesis result in a variety of developmental abnormalities in human (Faheem et al., 2015; Ernst, 2016). Perturbed regulation of DNA replication, leading to a significant decrease in proliferating cells, has been already associated not only with developmental retardation but also with brain malformations like microcephaly (**Table 1**) (Mazouzi et al., 2014; Khetarpal et al., 2016). The distinctive features of NSCs regarding the licensing and initiation of DNA replication are critical for their rapid proliferation, required during the initial stages of brain development, highlighting the sensitivity of the brain to defected DNA replication.

Meier-Gorlin Syndrome (MGS) is of the main syndromes that has set the link between licensing of DNA replication and brain development. MGS is an autosomal recessive type of primordial dwarfism due to developmental retardation, characterized by proportionate growth deficits, incomplete patellae formation and typical facial features (de Munnik et al., 2015). Severity of the symptoms varies across MGS patients and, interestingly, most severe cases are additionally diagnosed with microcephaly (Bicknell et al., 2011b; Burrage et al., 2015). Genetic analyses on reported cases of MGS revealed that biallelic mutations in the genes that form the pre-RC (ORC1, ORC4, ORC6, CDT1 and CDC6) are implicated in the syndrome (Bicknell et al., 2011a; de Munnik et al., 2012). *De novo* mutations in the licensing inhibitor Geminin were also identified in a group of patients (Burrage et al., 2015). The implication of DNA replication in MGS was further supported when, recently, mutations in the genes of CDC45 and MCM5 that form an active CMG complex were associated with the syndrome (Fenwick et al., 2016; Vetro et al., 2017). Studies performed in patient derived cell lines indicate delayed entry to S phase and decreased proliferation caused by insufficient licensing of DNA replication (Bicknell et al., 2011b; Burrage et al., 2015). Similar results indicating reduced proliferation were also obtained when mutated alleles previously identified in MGS patients were studied in heterologous systems (Guernsey et al., 2011).

The microcephaly observed in MGS patients emphasizes the role of proper licensing of DNA replication during brain development (**Figure 2**). It is evident that impaired licensing severely compromises the rapid proliferation of NSCs resulting in insufficient generation of the initial NSCs pool and eventually in incomplete development. However, the mechanism that underlies the decreased proliferation of NSCs in MGS remains elusive. Proliferating NSCs accumulate higher levels of licensing factors due to their shorter G1 phase, consequently, in the case of the MGS where the expression of these factors is reduced, decreased formation of Pre-RCs and under licensing occur. Given that proliferating NSCs utilize more replication origins to complete genome duplication, under licensing has a direct effect on the progression of the S phase. Alternatively, the reduction of NSCs that is observed in MGS patients could be appointed to defected gene expression. Dynamic regulation of the replication timing in NSCs ensures the implementation of the respective transcriptional program, thus discordant DNA replication due to impaired licensing could affect normal gene expression resulting in brain malformations.

Environmental factors may further affect the proliferation of NSCs. Infectious agents that target NSCs, disrupt the early stages of cortical development resulting in neurological phenotypes. Due to its recent outbreak, Zika virus (ZIKV) is one of the most known aetiologies of infectious microcephaly, however, congenital infections including the cytomegalovirus or the parasite *Toxoplasma gondii* have been also associated with microcephaly (Devakumar et al., 2018). The implication of ZIKV in congenital microcephaly was established when its RNA was detected in the brain tissues of microcephalic fetuses as well as in

TABLE 1 | Genes linked to microcephaly that encode proteins involved in DNA replication.

Disease	Phenotype	Genes	Functions	Reference
Meier-Gorlin Syndrome	Microcephaly, primordial dwarfism and hypoplastic patella	*ORC1, ORC4, ORC6, CDT1* and *CDC6*	Pre-Replicative Complex formation	de Munnik et al., 2015
		Geminin	Re-licensing inhibitor	Burrage et al., 2015
		CDC45, MCM5	CMG complex establishment	Fenwick et al., 2016; Vetro et al., 2017
Bloom syndrome	Microcephaly, short stature and cancer predisposition	*BLM*	Replication fork stability and resolution of replication intermediates	German et al., 2007; Guo et al., 2007
Seckel syndrome (ATR/ATRIP-related)	Microcephaly, primordial dwarfism and dysmorphic facial features	*ATR*, ATR-Interacting Protein	Response to replication stress and stabilization of stalled forks	Goodship et al., 2000; Ogi et al., 2012; Mokrani-Benhelli et al., 2013
Microcephalic Primordial Dwarfism	Microcephaly and primordial dwarfism	*DONSON*	Replication fork stability	Reynolds et al., 2017

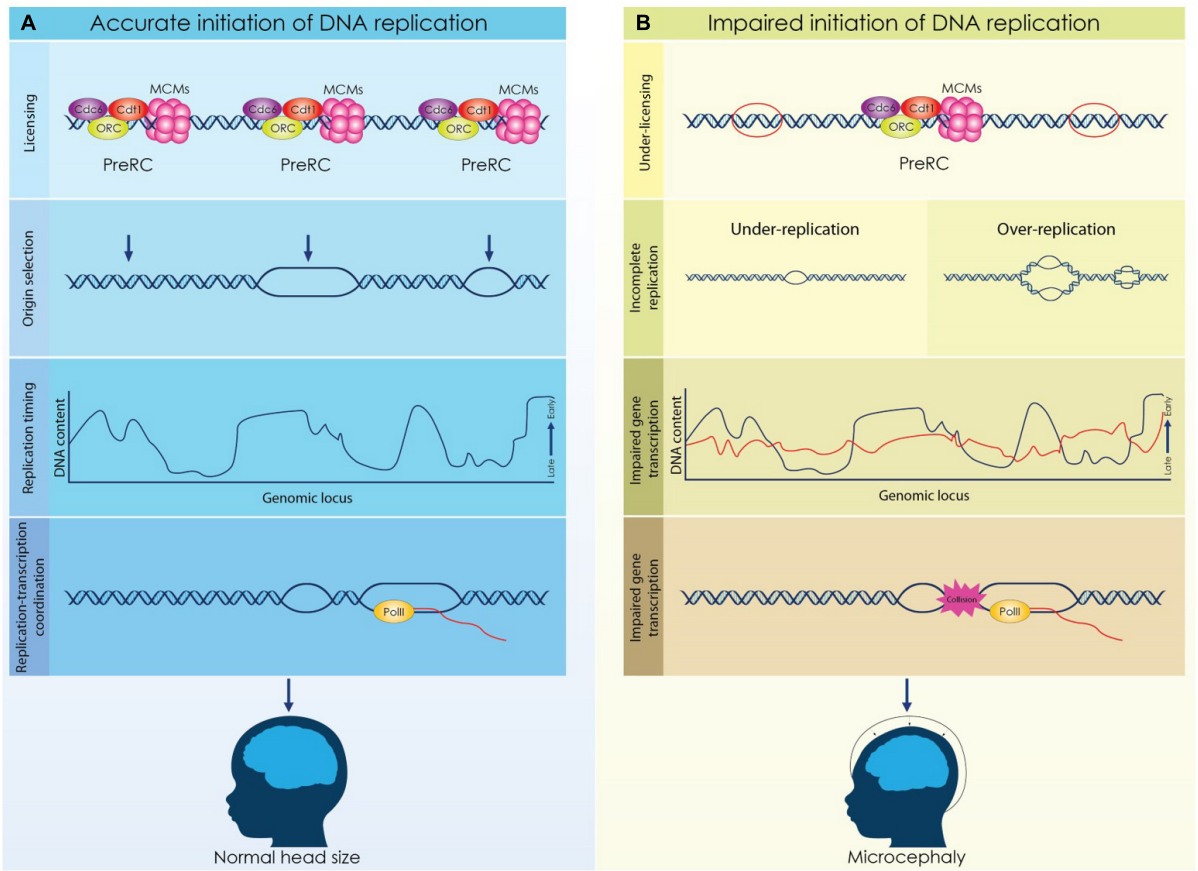

FIGURE 2 | Licensing, origin selection and replication timing are dynamically regulated during cortical development to secure the formation of a functional cortex. **(A)** Origins of replication are licensed during the G1 phase by the formation of a competent pre-replicative complex (Pre-RC). A fraction of the licensed origins is selected during the S phase to establish a bidirectional replication fork while not activated origins remain dormant. DNA is replicated in well-defined chromosomal segments, that are duplicated in distinct time points throughout S phase. Establishment of a characteristic replication timing profile permits the coordination of replication and transcriptional machineries. **(B)** Impaired licensing and initiation of DNA replication lead to defective brain development and microcephaly. Reduced expression of licensing factors results in decrease of licensed origins that further causes incomplete initiation of DNA replication. Aberrant licensing directly affects the successful duplication of the genome due to under-replication or over-replication. Moreover, deviation of the normal replication timing due to defective licensing, can lead to impaired gene expression and possibly, to conflicts between replication and transcription. Under these conditions, the proliferation of NSCs is compromised resulting in severe brain malformations.

the amniotic fluid of their infected mothers (Driggers et al., 2016). Initial studies on hiPSCs derived NPCs and brain organoids showed that proliferating NPCs are the main population of cells targeted by ZIKV, while differentiated neurons are mildly affected (Garcez et al., 2016; Tang et al., 2016). Interestingly, analysis of the cell cycle profiles showed that infected NPCs are accumulated to the S phase resulting in reduced proliferation and eventually apoptosis (Tang et al., 2016). Moreover, the

brains from established animal models for ZIKV infection also exhibited a decrease in proliferating NPCs due to altered cell cycle kinetics (Li et al., 2016; Wu et al., 2016). In line with these results, transcriptomics analysis of infected NPCs confirmed the downregulation of genes involved in cell cycle and specifically DNA replication progression (Zhang et al., 2016). The possible effects of ZKV in the normal progression of DNA replication is a very intriguing scenario that could explain the preference of the virus toward proliferating NSCs.

CONCLUSION AND PERSPECTIVES

During the last decade the importance of the dynamic regulation of DNA replication during organismal development has been established (Nordman and Orr-Weaver, 2012; Champeris Tsaniras et al., 2014; Hua and Orr-Weaver, 2017). In the present review we describe differences in the processes of initiation of DNA replication between different populations of NSCs. NECs exhibit a short cell cycle with a short G1 phase similar to ESCs. For that reason they will require increased levels of licensing factors to facilitate the higher number of licensed origins required in order to cope with their accelerated cell cycle. Consequently, the number of the available DOs is reduced in rapid proliferating NECs increasing the risk of replication stress and genotoxic insults that would compromise the survival of NSCs. More data regarding the formation of licensing and initiation of DNA replication in NECs and RGs are needed to establish the above hypothesis which is mainly based on our knowledge on ESCs.

Moreover, adaptations in the number of licensed origins and in the replication timing profile permit the integration of the active transcriptional program that operates in NSCs during cortical development. How the plasticity of DNA replication initiation is regulated within the complicated environment of the developing brain and whether it is determined by signaling cues or it is an inherent feature of NSCs remain important questions.

Mutations in the genes that express licensing factors have been associated with developmental retardation characterized by microcephaly, stressing the significance of efficient licensing and accurate initiation of DNA replication during brain development. A future direction will be the establishment of animal models for impaired-licensing syndromes, that will permit the detailed analysis of brain development under these conditions. Further insights into the response of NSCs upon DNA replication stress will be critical to our understanding of the pathophysiology of neurodevelopmental syndromes. Accordingly, the possible effects of ZKV in the normal progression of DNA replication is a very intriguing scenario that could explain the preference of the virus in early proliferating NSCs. Deciphering the role of the differential regulation of DNA replication will provide new grounds for research on the mechanisms leading to brain malformations like the hereditary or infectious microcephaly.

AUTHOR CONTRIBUTIONS

AK provided the study material and wrote the manuscript. ZL performed proofreading of the manuscript and approved final version of the manuscript. ST wrote the manuscript, approved final version of the manuscript, and provided financial support.

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REFERENCES

Agirman, G., Broix, L., and Nguyen, L. (2017). Cerebral cortex development: an outside-in perspective. *FEBS Lett.* 591, 3978–3992. doi: 10.1002/1873-3468.12924

Albert, M., Kalebic, N., Florio, M., Lakshmanaperumal, N., Haffner, C., Brandl, H., et al. (2017). Epigenome profiling and editing of neocortical progenitor cells during development. *EMBO J.* 36, 2642–2658. doi: 10.15252/embj.201796764

Alver, R. C., Chadha, G. S., and Blow, J. J. (2014). The contribution of dormant origins to genome stability: from cell biology to human genetics. *DNA Repair.* 19, 182–189. doi: 10.1016/j.dnarep.2014.03.012

Arai, Y., and Taverna, E. (2017). Neural progenitor cell polarity and cortical development. *Front. Cell. Neurosci.* 11:384. doi: 10.3389/fncel.2017.00384

Azzarelli, R., Hardwick, L. J. A., and Philpott, A. (2015). Emergence of neuronal diversity from patterning of telencephalic progenitors. *Wiley Interdiscip. Rev. Dev. Biol.* 4, 197–214. doi: 10.1002/wdev.174

Ballabeni, A., Park, I.-H., Zhao, R., Wang, W., Lerou, P. H., Daley, G. Q., et al. (2011). Cell cycle adaptations of embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19252–19257. doi: 10.1073/pnas.1116794108

Bertipaglia, C., Gonçalves, J. C., and Vallee, R. B. (2017). Nuclear migration in mammalian brain development. *Semin. Cell Dev. Biol.* 82, 57–66. doi: 10.1016/j.semcdb.2017.11.033

Bicknell, L. S., Bongers, E. M., Leitch, A., Brown, S., Schoots, J., Harley, M. E., et al. (2011a). Mutations in the pre-replication complex cause Meier-Gorlin syndrome. *Nat. Genet.* 43, 356–359. doi: 10.1038/ng.775

Bicknell, L. S., Walker, S., Klingseisen, A., Stiff, T., Leitch, A., Kerzendorfer, C., et al. (2011b). Mutations in ORC1, encoding the largest subunit of the origin recognition complex, cause microcephalic primordial dwarfism resembling Meier-Gorlin syndrome. *Nat. Genet.* 43, 350–355. doi: 10.1038/ng.776

Borrell, V., and Calegari, F. (2014). Mechanisms of brain evolution: regulation of neural progenitor cell diversity and cell cycle length. *Neurosci. Res.* 86, 14–24. doi: 10.1016/j.neures.2014.04.004

Burrage, L. C., Charng, W.-L., Eldomery, M. K., Willer, J. R., Davis, E. E., Lugtenberg, D., et al. (2015). De novo GMNN mutations cause autosomal-dominant primordial dwarfism associated with Meier-Gorlin Syndrome. *Am. J. Hum. Genet.* 97, 904–913. doi: 10.1016/j.ajhg.2015.11.006

Caillat, C., and Perrakis, A. (2012). Cdt1 and geminin in DNA replication initiation. *Subcell. Biochem.* 62, 71–87. doi: 10.1007/978-94-007-4572-8_5

Calegari, F., Haubensak, W., Haffner, C., and Huttner, W. B. (2005). Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. *J. Neurosci. Off. J. Soc. Neurosci.* 25, 6533–6538. doi: 10.1523/JNEUROSCI.0778-05.2005

Champeris Tsaniras, S., Kanellakis, N., Symeonidou, I. E., Nikolopoulou, P., Lygerou, Z., and Taraviras, S. (2014). Licensing of DNA replication, cancer, pluripotency and differentiation: an interlinked world? *Semin. Cell Dev. Biol.* 30, 174–180. doi: 10.1016/j.semcdb.2014.03.013

de Munnik, S. A., Bicknell, L. S., Aftimos, S., Al-Aama, J. Y., van Bever, Y., Bober, M. B., et al. (2012). Meier-Gorlin syndrome genotype-phenotype studies: 35 individuals with pre-replication complex gene mutations and 10 without molecular diagnosis. *Eur. J. Hum. Genet. EJHG* 20, 598–606. doi: 10.1038/ejhg.2011.269

de Munnik, S. A., Hoefsloot, E. H., Roukema, J., Schoots, J., Knoers, N. V. A. M., Brunner, H. G., et al. (2015). Meier-Gorlin syndrome. *Orphanet J. Rare Dis.* 10:114. doi: 10.1186/s13023-015-0322-x

Delmolino, L. M., Saha, P., and Dutta, A. (2001). Multiple mechanisms regulate subcellular localization of human CDC6. *J. Biol. Chem.* 276, 26947–26954. doi: 10.1074/jbc.M101870200

Devakumar, D., Bamford, A., Ferreira, M. U., Broad, J., Rosch, R. E., Groce, N., et al. (2018). Infectious causes of microcephaly: epidemiology, pathogenesis, diagnosis, and management. *Lancet Infect. Dis.* 18, e1–e13. doi: 10.1016/S1473-3099(17)30398-5

Driggers, R. W., Ho, C.-Y., Korhonen, E. M., Kuivanen, S., Jääskeläinen, A. J., Smura, T., et al. (2016). Zika virus infection with prolonged maternal viremia and fetal brain abnormalities. *N. Engl. J. Med.* 374, 2142–2151. doi: 10.1056/NEJMoa1601824

Dwyer, N. D., Chen, B., Chou, S.-J., Hippenmeyer, S., Nguyen, L., and Ghashghaei, H. T. (2016). Neural stem cells to cerebral cortex: emerging mechanisms regulating progenitor behavior and productivity. *J. Neurosci.* 36, 11394–11401. doi: 10.1523/JNEUROSCI.2359-16.2016

Ernst, C. (2016). Proliferation and differentiation deficits are a major convergence point for neurodevelopmental disorders. *Trends Neurosci.* 39, 290–299. doi: 10.1016/j.tins.2016.03.001

Faheem, M., Naseer, M. I., Rasool, M., Chaudhary, A. G., Kumosani, T. A., Ilyas, A. M., et al. (2015). Molecular genetics of human primary microcephaly: an overview. *BMC Med. Genomics* 8:S4. doi: 10.1186/1755-8794-8-S1-S4

Fenwick, A. L., Kliszczak, M., Cooper, F., Murray, J., Sanchez-Pulido, L., Twigg, S. R. F., et al. (2016). Mutations in CDC45, encoding an essential component of the pre-initiation complex, cause Meier-Gorlin syndrome and craniosynostosis. *Am. J. Hum. Genet.* 99, 125–138. doi: 10.1016/j.ajhg.2016.05.019

Fragkos, M., Ganier, O., Coulombe, P., and Méchali, M. (2015). DNA replication origin activation in space and time. *Nat. Rev. Mol. Cell Biol.* 16, 360–374. doi: 10.1038/nrm4002

Fujii-Yamamoto, H., Kim, J. M., Arai, K., and Masai, H. (2005). Cell cycle and developmental regulations of replication factors in mouse embryonic stem cells. *J. Biol. Chem.* 280, 12976–12987. doi: 10.1074/jbc.M412224200

Gaiano, N., Nye, J. S., and Fishell, G. (2000). Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* 26, 395–404. doi: 10.1016/S0896-6273(00)81172-1

Garcez, P. P., Loiola, E. C., Madeiro da Costa, R., Higa, L. M., Trindade, P., Delvecchio, R., et al. (2016). Zika virus impairs growth in human neurospheres and brain organoids. *Science* 352, 816–818. doi: 10.1126/science.aaf6116

García-Muse, T., and Aguilera, A. (2016). Transcription-replication conflicts: how they occur and how they are resolved. *Nat. Rev. Mol. Cell Biol.* 17, 553–563. doi: 10.1038/nrm.2016.88

Ge, X. Q., Han, J., Cheng, E.-C., Yamaguchi, S., Shima, N., Thomas, J.-L., et al. (2015). Embryonic stem cells license a high level of dormant origins to protect the genome against replication stress. *Stem Cell Rep.* 5, 185–194. doi: 10.1016/j.stemcr.2015.06.002

German, J., Sanz, M. M., Ciocci, S., Ye, T. Z., and Ellis, N. A. (2007). Syndrome-causing mutations of the *BLM* gene in persons in the Bloom's Syndrome Registry. *Hum. Mutat.* 28, 743–753. doi: 10.1002/humu.20501

Goodship, J., Gill, H., Carter, J., Jackson, A., Splitt, M., and Wright, M. (2000). Autozygosity mapping of a seckel syndrome locus to chromosome 3q22. 1-q24. *Am. J. Hum. Genet.* 67, 498–503. doi: 10.1086/303023

Götz, M., and Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat. Rev. Mol. Cell Biol.* 6, 777–788. doi: 10.1038/nrm1739

Govindan, S., and Jabaudon, D. (2017). Coupling progenitor and neuronal diversity in the developing neocortex. *FEBS Lett.* 591, 3960–3977. doi: 10.1002/1873-3468.12846

Guemez-Gamboa, A., Coufal, N. G., and Gleeson, J. G. (2014). Primary cilia in the developing and mature brain. *Neuron* 82, 511–521. doi: 10.1016/j.neuron.2014.04.024

Guernsey, D. L., Matsuoka, M., Jiang, H., Evans, S., Macgillivray, C., Nightingale, M., et al. (2011). Mutations in origin recognition complex gene ORC4 cause Meier-Gorlin syndrome. *Nat. Genet.* 43, 360–364. doi: 10.1038/ng.777

Guo, R.-B., Rigolet, P., Ren, H., Zhang, B., Zhang, X.-D., Dou, S.-X., et al. (2007). Structural and functional analyses of disease-causing missense mutations in Bloom syndrome protein. *Nucleic Acids Res.* 35, 6297–6310. doi: 10.1093/nar/gkm536

Hiratani, I., Ryba, T., Itoh, M., Rathjen, J., Kulik, M., Papp, B., et al. (2010). Genome-wide dynamics of replication timing revealed by in vitro models of mouse embryogenesis. *Genome Res.* 20, 155–169. doi: 10.1101/gr.099796.109

Hiratani, I., Ryba, T., Itoh, M., Yokochi, T., Schwaiger, M., Chang, C.-W., et al. (2008). Global reorganization of replication domains during embryonic stem cell differentiation. *PLoS Biol.* 6:e245. doi: 10.1371/journal.pbio.0060245

Hua, B. L., and Orr-Weaver, T. L. (2017). DNA replication control during *Drosophila* development: insights into the onset of S phase, replication initiation, and fork progression. *Genetics* 207, 29–47. doi: 10.1534/genetics.115.186627

Ibarra, A., Schwob, E., and Méndez, J. (2008). Excess MCM proteins protect human cells from replicative stress by licensing backup origins of replication. *Proc. Natl. Acad. Sci. U.S.A.* 105, 8956–8961. doi: 10.1073/pnas.0803978105

Jackson, A. P., Laskey, R. A., and Coleman, N. (2014). Replication proteins and human disease. *Cold Spring Harb. Perspect. Biol.* 6:a013060. doi: 10.1101/cshperspect.a013060

Khetarpal, P., Das, S., Panigrahi, I., and Munshi, A. (2016). Primordial dwarfism: overview of clinical and genetic aspects. *Mol. Genet. Genomics MGG* 291, 1–15. doi: 10.1007/s00438-015-1110-y

Li, C., Xu, D., Ye, Q., Hong, S., Jiang, Y., Liu, X., et al. (2016). Zika virus disrupts neural progenitor development and leads to microcephaly in mice. *Cell Stem Cell* 19, 120–126. doi: 10.1016/j.stem.2016.04.017

Li, C.-J., and DePamphilis, M. L. (2002). Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S-to-M transition in the cell division cycle. *Mol. Cell. Biol.* 22, 105–116. doi: 10.1128/MCB.22.1.105-116.2002

Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. (2003). The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *J. Biol. Chem.* 278, 30854–30858. doi: 10.1074/jbc.C300251200

Lim, L., Mi, D., Llorca, A., and Marín, O. (2018). Development and functional diversification of cortical interneurons. *Neuron* 100, 294–313. doi: 10.1016/j.neuron.2018.10.009

Lin, Y.-L., and Pasero, P. (2012). Interference between DNA replication and transcription as a cause of genomic instability. *Curr. Genomics* 13, 65–73. doi: 10.2174/138920212799034767

Lukaszewicz, A., Savatier, P., Cortay, V., Kennedy, H., and Dehay, C. (2002). Contrasting effects of basic fibroblast growth factor and neurotrophin 3 on cell cycle kinetics of mouse cortical stem cells. *J. Neurosci. Off. J. Soc. Neurosci.* 22, 6610–6622. doi: 10.1523/JNEUROSCI.22-15-06610.2002

Matson, J. P., Dumitru, R., Coryell, P., Baxley, R. M., Chen, W., Twaroski, K., et al. (2017). Rapid DNA replication origin licensing protects stem cell pluripotency. *eLife* 6:e30473. doi: 10.7554/eLife.30473

Matsuzaki, F., and Shitamukai, A. (2015). Cell division modes and cleavage planes of neural progenitors during mammalian cortical development. *Cold Spring Harb. Perspect. Biol.* 7:a015719. doi: 10.1101/cshperspect.a015719

Mazouzi, A., Velimezi, G., and Loizou, J. I. (2014). DNA replication stress: causes, resolution and disease. *Exp. Cell Res.* 329, 85–93. doi: 10.1016/j.yexcr.2014.09.030

Miyata, T., Okamoto, M., Shinoda, T., and Kawaguchi, A. (2014). Interkinetic nuclear migration generates and opposes ventricular-zone crowding: insight into tissue mechanics. *Front. Cell. Neurosci.* 8:473. doi: 10.3389/fncel.2014.00473

Mokrani-Benhelli, H., Gaillard, L., Biasutto, P., Le Guen, T., Touzot, F., Vasquez, N., et al. (2013). Primary microcephaly, impaired DNA replication, and genomic instability caused by compound heterozygous *ATR* mutations. *Hum. Mutat.* 34, 374–384. doi: 10.1002/humu.22245

Nishitani, H., Sugimoto, N., Roukos, V., Nakanishi, Y., Saijo, M., Obuse, C., et al. (2006). Two E3 ubiquitin ligases, SCF-Skp2 and DDB1-Cul4, target human Cdt1 for proteolysis. *EMBO J.* 25, 1126–1136. doi: 10.1038/sj.emboj.7601002

Noctor, S. C., Flint, A. C., Weissman, T. A., Wong, W. S., Clinton, B. K., and Kriegstein, A. R. (2002). Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J. Neurosci. Off. J. Soc. Neurosci.* 22, 3161–3173. doi: 10.1523/JNEUROSCI.22-08-03161.2002

Nordman, J., and Orr-Weaver, T. L. (2012). Regulation of DNA replication during development. *Development* 139, 455–464. doi: 10.1242/dev.061838

Ogi, T., Walker, S., Stiff, T., Hobson, E., Limsirichaikul, S., Carpenter, G., et al. (2012). Identification of the first ATRIP–deficient patient and novel mutations in ATR define a clinical spectrum for ATR–ATRIP Seckel Syndrome. *PLoS Genet.* 8:e1002945. doi: 10.1371/journal.pgen.1002945

Ohtsuka, T., Sakamoto, M., Guillemot, F., and Kageyama, R. (2001). Roles of the basic helix-loop-helix genes hes1 and hes5 in expansion of neural stem cells of the developing brain. *J. Biol. Chem.* 276, 30467–30474. doi: 10.1074/jbc.M102420200

Pruitt, S. C., Bailey, K. J., and Freeland, A. (2007). Reduced Mcm2 expression results in severe stem/progenitor cell deficiency and cancer. *Stem Cells* 25, 3121–3132. doi: 10.1634/stemcells.2007-0483

Rash, B. G., Tomasi, S., Lim, H. D., Suh, C. Y., and Vaccarino, F. M. (2013). Cortical gyrification induced by fibroblast growth factor 2 in the mouse brain. *J. Neurosci.* 33, 10802–10814. doi: 10.1523/JNEUROSCI.3621-12.2013

Reynolds, J. J., Bicknell, L. S., Carroll, P., Higgs, M. R., Shaheen, R., Murray, J. E., et al. (2017). Mutations in DONSON disrupt replication fork stability and cause microcephalic dwarfism. *Nat. Genet.* 49, 537–549. doi: 10.1038/ng.3790

Rivera-Mulia, J. C., Buckley, Q., Sasaki, T., Zimmerman, J., Didier, R. A., Nazor, K., et al. (2015). Dynamic changes in replication timing and gene expression during lineage specification of human pluripotent stem cells. *Genome Res.* 25, 1091–1103. doi: 10.1101/gr.187989.114

Rivera-Mulia, J. C., and Gilbert, D. M. (2016). Replicating large genomes: divide and conquer. *Mol. Cell* 62, 756–765. doi: 10.1016/j.molcel.2016.05.007

Sahara, S., and O'Leary, D. D. M. (2009). Fgf10 regulates transition period of cortical stem cell differentiation to radial glia controlling generation of neurons and basal progenitors. *Neuron* 63, 48–62. doi: 10.1016/j.neuron.2009.06.006

Shima, N., and Pederson, K. D. (2017). Dormant origins as a built-in safeguard in eukaryotic DNA replication against genome instability and disease development. *DNA Repair.* 56, 166–173. doi: 10.1016/j.dnarep.2017.06.019

Siddiqui, K., On, K. F., and Diffley, J. F. X. (2013). Regulating DNA replication in eukarya. *Cold Spring Harb. Perspect. Biol.* 5:a012930. doi: 10.1101/cshperspect.a012930

Stancik, E. K., Navarro-Quiroga, I., Sellke, R., and Haydar, T. F. (2010). Heterogeneity in ventricular zone neural precursors contributes to neuronal fate diversity in the postnatal neocortex. *J. Neurosci. Off. J. Soc. Neurosci.* 30, 7028–7036. doi: 10.1523/JNEUROSCI.6131-09.2010

Sun, J., Shi, Y., Georgescu, R. E., Yuan, Z., Chait, B. T., Li, H., et al. (2015). The architecture of a eukaryotic replisome. *Nat. Struct. Mol. Biol.* 22, 976–982. doi: 10.1038/nsmb.3113

Symeonidou, I.-E., Taraviras, S., and Lygerou, Z. (2012). Control over DNA replication in time and space. *FEBS Lett.* 586, 2803–2812. doi: 10.1016/j.febslet.2012.07.042

Takahashi, T., Nowakowski, R. S., and Caviness, V. S. (1995). The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. *J. Neurosci. Off. J. Soc. Neurosci.* 15, 6046–6057. doi: 10.1523/JNEUROSCI.15-09-06046.1995

Takebayashi, S., Ogata, M., and Okumura, K. (2017). Anatomy of mammalian replication domains. *Genes* 8:E110. doi: 10.3390/genes8040110

Tang, H., Hammack, C., Ogden, S. C., Wen, Z., Qian, X., Li, Y., et al. (2016). Zika virus infects human cortical neural progenitors and attenuates their growth. *Cell Stem Cell* 18, 587–590. doi: 10.1016/j.stem.2016.02.016

Taverna, E., Götz, M., and Huttner, W. B. (2014). The cell biology of neurogenesis: toward an understanding of the development and evolution of the neocortex. *Annu. Rev. Cell Dev. Biol.* 30, 465–502. doi: 10.1146/annurev-cellbio-101011-155801

Uzquiano, A., Gladwyn-Ng, I., Nguyen, L., Reiner, O., Götz, M., Matsuzaki, F., et al. (2018). Cortical progenitor biology: key features mediating proliferation versus differentiation. *J. Neurochem.* 146, 500–525. doi: 10.1111/jnc.14338

Vetro, A., Savasta, S., Russo Raucci, A., Cerqua, C., Sartori, G., Limongelli, I., et al. (2017). MCM5: a new actor in the link between DNA replication and Meier-Gorlin syndrome. *Eur. J. Hum. Genet. EJHG* 25, 646–650. doi: 10.1038/ejhg.2017.5

White, J., and Dalton, S. (2005). Cell cycle control of embryonic stem cells. *Stem Cell Rev.* 1, 131–138. doi: 10.1385/SCR:1:2:131

Wu, K.-Y., Zuo, G.-L., Li, X.-F., Ye, Q., Deng, Y.-Q., Huang, X.-Y., et al. (2016). Vertical transmission of Zika virus targeting the radial glial cells affects cortex development of offspring mice. *Cell Res.* 26, 645–654. doi: 10.1038/cr.2016.58

Zhang, F., Hammack, C., Ogden, S. C., Cheng, Y., Lee, E. M., Wen, Z., et al. (2016). Molecular signatures associated with ZIKV exposure in human cortical neural progenitors. *Nucleic Acids Res.* 44, 8610–8620. doi: 10.1093/nar/gkw765

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REVIEW
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Chaperones and Beyond as Key Players in Pluripotency Maintenance

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Pluripotency is orchestrated by distinct players and chaperones and their partners have emerged as pivotal molecules in proteostasis control to maintain stemness. The proteostasis network consists of diverse interconnected pathways that function dynamically according to the needs of the cell to quality control and maintain protein homeostasis. The proteostasis machinery of pluripotent stem cells (PSCs) is finely adjusted in response to distinct stimuli during cell fate commitment to determine successful organism development. Growing evidence has shown different classes of chaperones regulating crucial cellular processes in PSCs. Histones chaperones promote proper nucleosome assembly and modulate the epigenetic regulation of factors involved in PSCs' rapid turnover from pluripotency to differentiation. The life cycle of pluripotency proteins from synthesis and folding, transport and degradation is finely regulated by chaperones and co-factors either to maintain the stemness status or to cell fate commitment. Here, we summarize current knowledge of the chaperone network that govern stemness and present the versatile role of chaperones in stem cells resilience. Elucidation of the intricate regulation of pluripotency, dissecting in detail molecular determinants and drivers, is fundamental to understanding the properties of stem cells in order to provide a reliable foundation for biomedical research and regenerative medicine.

Keywords: pluripotency, chaperone, stem cells, histone, stemness

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INTRODUCTION

Pluripotency is an important and unique feature attributed to specific types of cells, and can be defined as the ability of cells to replicate indefinitely in the absence of senescence (self-renewal) while retaining the differentiation potential, or the ability to differentiate into all cells of an organism (Martello and Smith, 2014). Embryonic stem cells (ESCs) are classified as pluripotent stem cells (PSCs), and represent great possibilities for research and cell therapy. ESCs can be obtained from the inner cell mass (ICM) of preimplantation blastocysts. The establishment of cultures of ESCs *in vitro* (Evans and Kaufman, 1981; Martin, 1981; Martello and Smith, 2014) brought about unquestionable advances in scientific research, as the starting point for several works that sought to explore the molecular mechanisms that maintain pluripotency. In 2006, a state of ESC-like, achieved from the reprogramming of differentiated adult cells was described, referred to as induced pluripotent stem cells (iPSCs). Reprogramming of the cells was possible through the induction of specific transcription factors (TFs), OCT4, SOX2, c-MYC, and KLF4 (Takahashi and Yamanaka, 2006). OCT4, NANOG, and SOX2 are considered key factors for the maintenance of PSCs *in vivo* and *in vitro*, forming a pluripotency core that, with additional TF and cofactors, regulates pluripotency in an expanded transcriptional network (Wang et al., 2006; Kim et al., 2008).

Although many studies have been conducted to understand the exact mechanisms by which the TFs and additional factors regulate pluripotency, much remains to be elucidated. Studies are still being conducted to understand TFs individual and integrated functioning. By unmasking pluripotency mechanisms, it will be possible to use PSCs more safely and harness their therapeutic potential, also serving as a model to understand early development, important cellular processes and diseases. Besides transcriptional regulation, other mechanisms are being recently discussed as relevant to understanding the maintenance of pluripotency in stem cells, such as chromatin conformation and proteome quality control assisted by molecular chaperones. In this review we discuss aspects of PSCs maintenance, such as TFs regulation and chromatin conformation in PSCs, as well as the relationship of chaperones, co-chaperones and ubiquitin-proteasome system (UPS) with the control of TF levels and pluripotency in PSCs. A comprehensive and integrated understanding of the events – from transcription, translation to post-translational processes – that govern pluripotency is needed to answer questions that remain unanswered in the field of PSCs.

PLURIPOTENCY MAINTENANCE MECHANISMS

Pluripotent stem cells are regulated by a series of interconnected cellular processes that pass through the transcription, translation, and final destination of proteins through different post-translational modifications. The state of chromatin conformation is important for the exposure or concealment of regulatory regions in DNA. Regulatory regions are possible targets of several TFs, that will associate in a specific way in the DNA molecule and regulate the transcription of several genes.

Several studies described the essentiality of the TFs OCT4, SOX2, and NANOG to pluripotency. These TFs form an interconnected self-regulating core, cooperatively associating with their own promoters and co-occupying more than 300 targets in an integrated manner, finely regulating their own and also their targets expression, repressing differentiation genes and activating pluripotency (Boyer et al., 2005; Wang et al., 2006). Other TFs (referred hereafter as expanded core) associated to the main pluripotency core were described, such as STAT3, SMAD1, DAX1, REX1, ZPF281, among others (Kim et al., 2008; Bharathan et al., 2017; Collier et al., 2017; Trusler et al., 2018). In 2006, the publication of Takahashi and Yamanaka's (2006) landmark work, describing the acquiring of PSCs from the induction of four major TFs – OCT4, SOX2, c-MYC, and KLF4 – profoundly impacted stem cell research, allowing the field of development to dispose of a new and effective tool for pluripotency studies.

Although pluripotency is controlled by the expression of a network of TFs, the levels of this expression must be highly regulated in order to maintain the pluripotent state. PSCs balance between self-renewal and differentiation potential. Some researches (briefly reviewed in Torres-Padilla and Chambers, 2014), have already shown that the levels of TFs associated with pluripotency may vary in ESCs. Even in cells of the same colony, the expression of certain factors can be heterogeneous and transient. In addition to transcriptional regulation, the levels of TFs can be modulated according to translational and degradation rates and post-translational modifications.

In order to maintain PSCs undifferentiated, a cytokine member of the IL6 superfamily, named leukemia inhibitory factor (LIF), is used in cell culture (Nicola and Babon, 2015). Briefly, in the signal transduction cascade, LIF couples with gp130 receptors and activates JAKs in the cell interior which, in turn, will activate STAT3 through phosphorylation (Huang et al., 2017). Although LIF is not essential for the maintenance of these cells *in vivo* (Stewart et al., 1992), and is not solely responsible for the maintenance of pluripotency and self-renewal *in vitro*, it is an important tool in the culture of ESCs and iPSCs. Later in this review we will discuss how STAT3 relates to an important protein complex of chaperones and co-chaperones, acting in the maintenance of pluripotency in stem cells *in vitro*.

Interestingly, the existence of different statuses of pluripotency has been reported, naïve and primed, with great variety in transcriptional and epigenetic profile (reviewed in Nichols and Smith, 2009; Hackett and Azim Surani, 2014). The primed state, as the name suggests, is a state more prone to differentiation when compared to the naïve state (Marks et al., 2012), although in both states the cells remain expressing pluripotency core TFs. In addition to these classically established states, the existence of other levels for pluripotency has recently been hypothesized. As recently proposed by Smith, pluripotency can be seen as a progression through very early different developmental stages. The author emphasizes the need for a formative pluripotency state between the naïve and primed, in which cells acquire abilities to change their genomic and epigenetic profile to proceed in the course of cell-fate commitment (Smith, 2017). While some authors defend great transcriptional heterogeneity between primed and naïve states (Marks et al., 2012), other discuss that, although the two populations have their specific transcriptional signatures, this heterogeneity is expressed in low levels between the states (Messmer et al., 2019). Much still needs to be studied in order to establish a response to these controversies. Studies exploring these states *in vivo* may contribute to the understanding of their existence as part of the development of organisms, or as artifacts of cell culture.

Pluripotent stem cells require elevated protein synthesis for continuous replication and thus, enhanced mechanisms of proteome quality control like elevated chaperone and proteasome activities is essential to avoid senescence and maintain stemness. The viability of stem cells critically depends on the ability to maintain protein homeostasis to adapt continuously the cellular proteome to extrinsic and intrinsic variations. The capacity of stem cells to sense and respond to changing conditions and stress is critical for normal cell growth, development and organism viability. The complexity of the proteome requires interconnected quality-control processes to meet the dynamic needs of the cell. The protein homeostasis (proteostasis) network (PN) ensures the balance of the proteome by coordinating protein synthesis, folding and conformational maintenance; and protein degradation. PN is achieved by an orchestrated system of proteins, including molecular chaperones and their regulators, which help proteins to reach its functionally active conformation,

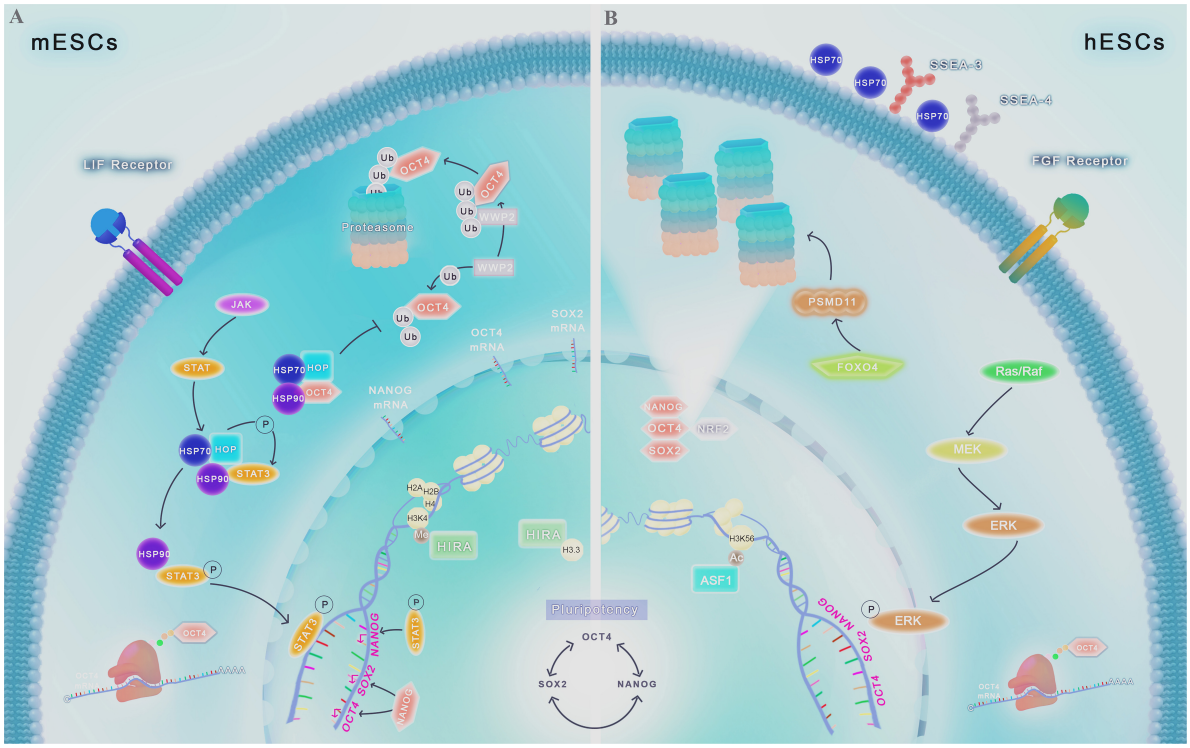

FIGURE 1 | Chaperome regulation and proteostasis network in ESCs. Scheme shows molecular pathways ranging from gene transcription to protein degradation involved in pluripotency control. The interconnected self-regulating nuclear core formed by OCT4, SOX2, and NANOG is essential for the maintenance of stemness. **(A)** In mESCs, HIRA is abundantly associated with promoter regions of developmentally regulated genes, being responsible for H3.3 deposition and enrichment, co-localizing with the transcriptional active form of methylated H3K4. Chaperone protein HSP90 and its partner HOP are engaged in key intracellular signaling pathways in PSCs, including LIF/JAK/STAT3. HSP90-HOP complex participates actively in the phosphorylation and translocation of STAT3 to the nucleus, leading to the transcription of pluripotency core factors. HSPs complexes can also prevent OCT4 degradation by proteasome. Proteasome-related proteins, such as WWP2, acting as E3 ligases or by other mechanisms, lead to TFs degradation by UPS, controlling its levels and maintaining proteostasis balance in these cells. **(B)** In hESCs, FGF2, used to culture these cells, activate the signaling cascade mediated by Ras/MEK/ERK and p-ERK translocation to the nucleus, favoring the expression of pluripotency genes. Acetylation of H3K56 by ASF1 regulates de expression of pluripotency genes. Unlike differentiated cells, HSP70 is present in the cell surface of hESCs, colocalizing with known pluripotency markers such as SSEA3 and SSEA4. Upregulation of the protein FOXO4 leads to the increase of the 19S proteasome subunit PSMD11, resulting in more functional proteasome subunits formed and increased activity of the UPS. The TF NRF2 upregulation is also associated with the increase in functional proteasome subunits, and also is associated with expression of the pluripotency TFs OCT4, SOX2, and NANOG.

without being part of its final structure. In addition, the UPS exerts a post-transcriptional control on the levels of proteins, such as TFs, which is important to pluripotency maintenance (**Figures 1**, **2**; Okita and Nakayama, 2012).

Considering the fine mechanics of chromatin conformation control, the importance of PN for the maintenance of cellular functions, both in health and in diseases, the increased expression of PN elements in PSCs, as well as an increased activity of PN in these cells, many studies have been conducted to understand the control of pluripotency from the perspective of these events. **Tables 1**, **2** summarize different molecules, addressed in this review, involved in the pluripotency control.

TFs CORE REGULATION BY UBIQUITIN-PROTEASOME SYSTEM

Regardless of the mediation of chaperones, an important fraction of polypeptides usually exhibit errors in folding or refolding (Schubert et al., 2000); consequently, they are identified and disposed by proteolytic degradation, to avoid accumulation of potentially toxic aggregate species. One major protein degradation pathway is the UPS. The UPS performs coordinated activities of enzymes that conjugate the polypeptide co-factor, ubiquitin, to proteins and tags them for degradation by an ATP-dependent process that involves three enzymes, E1 (Ub-activating enzyme), E2s (Ub-carrier or conjugating proteins) and the key E3-ligases (Ub-protein ligase) (Schubert et al., 2000; Lecker et al., 2006). The labeled proteins are identified by the 26S proteasome, which degrades them to small peptides (Lecker et al., 2006).

The protein levels of pluripotency TFs must be finely regulated for the maintenance of PSCs specific properties. It has been shown that both downregulation and upregulation beyond the required levels of some TFs, such as OCT4 (Rodriguez et al., 2008; Zafarana et al., 2009), leads to differentiation in specific tissues or impairment in stem cell identity. The UPS is one of

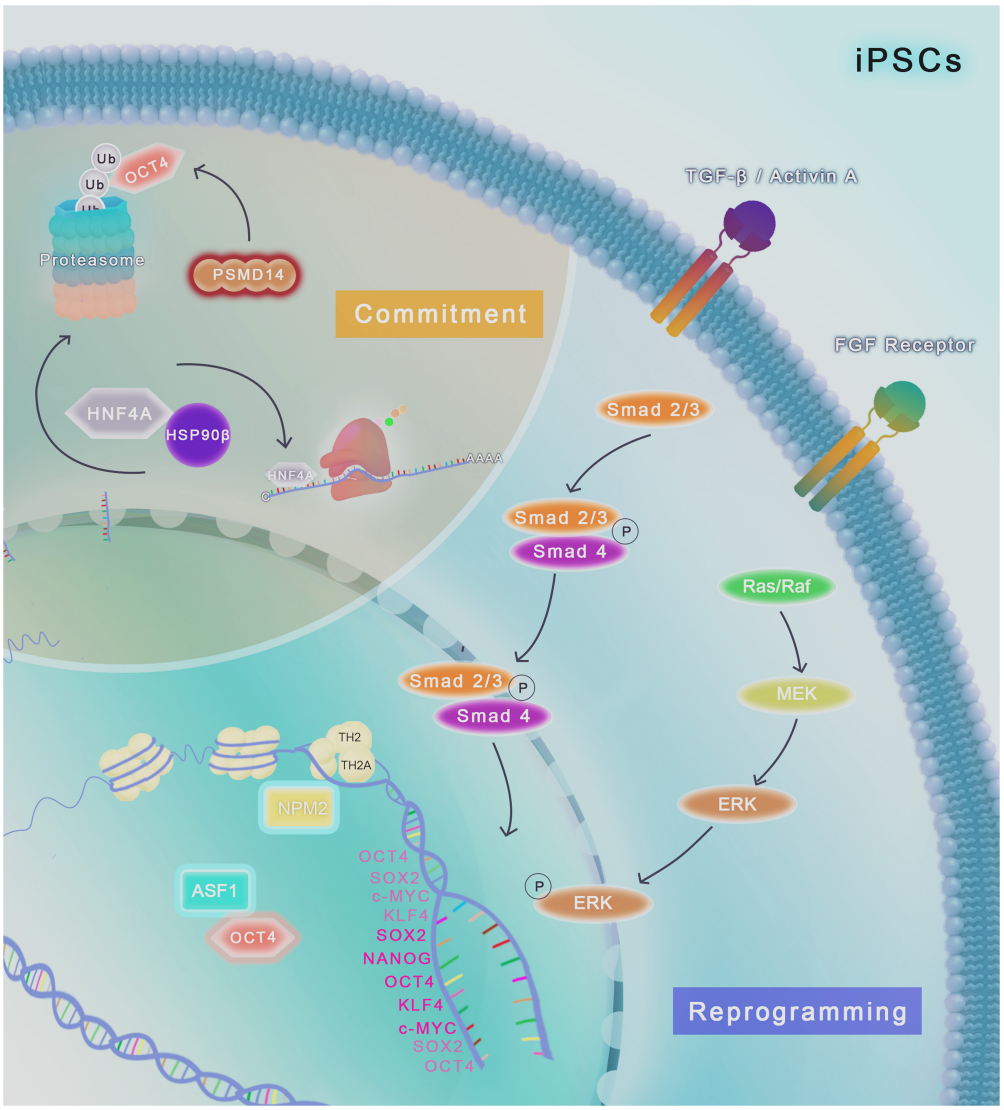

FIGURE 2 | Chaperome regulation and proteostasis network in human iPSCs. TGF-β/Activin A and FGF2/Ras/MEK/ERK pathways are required for the maintenance of iPSCs in culture conditions. The histone chaperone NPM2 binds to the histone variants TH2A and TH2 and improve the reprogramming of human fibroblast into iPSCs modulated by OCT4, SOX2, KLF4 and c-MYC, generating more naïve human iPSCs compared to factors induction alone. ASF1a histone chaperone upregulation, together with OCT4, also has an important role in reprogramming of human fibroblast. Cell fate commitment (highlighted in light brown) involves the induction of different specific pathways that can lead to differentiation into various cell types. The molecular chaperone HSP90β physically binds to HNF4A and control the protein turnover of these client, modulating differentiation of iPSCs to endoderm-derived hepatic progenitor cells. Downregulation (represented as a red glow around the molecule) of the proteasome-related protein PSMD14, a 26S proteasome subunit, impairs the deubiquitylation of OCT4, leading to its degradation in the proteasome and impairment of pluripotency.

the main post-translational mechanisms for regulating the levels of these proteins. The selectivity of proteins and aggregations for ubiquitin system presents a relevant participation of chaperones and co-chaperones, more specifically from the HSP70 family, for example HSC70 and the co-chaperone BAG3 (Arndt et al., 2010). In this section, we highlight classical and recent findings that explore the role of UPS in the control of TFs levels, essential for PSCs maintenance.

Investigating the total profile of ubiquitinated proteins in mESCs and iPSCs, Buckley et al. (2012) revealed that NANOG and OCT4 are regularly ubiquitinated, with the levels of this modification varying throughout the self-renewal process, a phenomenon that is not observed in differentiated cells (Buckley et al., 2012). The work demonstrates how these TFs are finely regulated for the maintenance of pluripotency properties in these cells, and suggests the great importance of UPS for self-renewal. It is known that elevated proteasome activity is somehow essential not only for the control of TFs levels, but also for expression of genes associated with pluripotency, cell proliferation and cell cycle progression. Defects in the proteasome leads to malfunction

TABLE 1 | Major classes of histone chaperones and their function in stemness of different PSCs models.

Chaperone		Function in PSCs biology	References
HIRA		Highly expressed in the promoters of developmentally regulated genes in mESCs	Goldberg et al., 2010
		Differentiation of mESCs in hemogenic endothelium	Banaszynski et al., 2013; Scambler et al., 2015
		Pluripotency maintenance of hESCs, promoting isocitrate dehydrogenase genes (IDHs) transcription	Zhu et al., 2017
		Developmental reprogramming – deposition of paternal core histone and reactivation of maternal genome in mice	Lin et al., 2014
DAXX/ATX		Telomeric deposition (immortalization) in mESCs	Elsässer et al., 2015
ASF1		Differentiation during murine early embryogenesis and gonad development	Messiaen et al., 2016
		Pluripotency maintenance in hESCs	Gonzalez-Muñoz et al., 2014
		Pluripotency maintenance in mESCs	Tan et al., 2013
		Reprogramming of human fibroblasts into iPSCs	Gonzalez-Muñoz et al., 2014
CAF-1		Early developmental arrest and early gastrulation of mESCs	Filipescu et al., 2013; Akiyama et al., 2011; Houlard et al., 2006; Hatanaka et al., 2015
		Reprogramming of mouse fibroblasts into iPSCs	Cheloufi et al., 2015
		Pluripotency maintenance during blastomeric stage in mice	Yankulov, 2015
FACT		Proliferation and neural differentiation of mESCs	Mylonas and Tessarz, 2018
		Associates with OCT4 and regulates mESCs pluripotency Survival during early blastocyst stage of mESCs	Cao et al., 2003; Ding et al., 2012; Gaspar-Maia et al., 2009; Pardo et al., 2010
		Reprogramming into iPSCs	Shakya et al., 2015; Shen et al., 2018
HMGA2		mESCs specific DNA repair mechanism.	Yu et al., 2014
NPM2		Reprogramming of human fibroblast into iPSCs; Improvement of murine cells reprogramming using only KLF4 and OCT4	Fernández-Rivero et al., 2016; Shinagawa et al., 2014
NPM3		Proliferation of mESCs	Motoi et al., 2008
SPT6		Pluripotency maintenance of mESCs	Robert, 2017
SET	SETα	Proliferation of hESCs	Edupuganti et al., 2017
	SETβSETP	Differentiation of hESCs	

of all of the above processes, including a G2/M arrest in hESCs and iPSCs (Jang et al., 2014). Several research groups have been exploring the specific effects of UPS-associated proteins on the regulation of TFs in PSCs.

Upon the downregulation of PSMD14, a 26S proteasome non-ATPase subunit, the loss of OCT4 expression is observed, which is linked to an apparent dysfunction of the deubiquitinating enzymatic activity of PSMD14 (**Figure 2**; Buckley et al., 2012). Further, although the modulation of the E3 ligase protein FBXW7 has no direct effects on OCT4, NANOG and SOX2 expression, it has a negative effect on the protein stability of c-MYC, an important factor linked to the differentiation potential in PSCs (Buckley et al., 2012). NRF2 is a TF whose activation leads to increased levels of several proteasome subunits. NRF2 activity is increased in hESCs (**Figure 1B**) and iPSCs and loss of this activity results in reduced levels of OCT4, SOX2, and NANOG and also impairs proliferation, indicating a role of this protein in self-renewal (**Figure 1B**; Jang et al., 2014). Additionally, NRF2 colocalizes with the mentioned core TFs (e.g., OCT4, SOX2, NANOG) during differentiation, and its activation by pharmacological or molecular techniques prevents their degradation during differentiation process in hESCs (Jang et al., 2014).

The proteasome activity is enhanced in hESCs and iPSCs, with a loss of activity being observed as the cells differentiate, with a concomitant increase in differentiation factors, such as PAX6, as well as a decrease in the expression of pluripotency TFs (Vilchez et al., 2012). Furthermore, the transcription factor FOXO4 has been shown to be an important modulator of proteasome activity in hESCs, since it regulates the expression PSMD11, a 19S proteasome subunit. The increased expression of PSMD11 is sufficient to increase the number of functional proteasome complexes formed, increasing proteasomal activity (**Figure 1B**; Vilchez et al., 2012). Interestingly, PSMD11 levels are somehow related with other pluripotency proteins. Decreased levels of L1TD1, an RNA binding protein, correlates with decreased levels of PSMD11 (Emani et al., 2015). Pharmacological inhibition of proteasome activity leads to decrease in L1TD1 and SOX2 in hESCs (Emani et al., 2015). However, the exact mechanisms by which these molecules interact with each other, and how they interact with OCT4, SOX2, NANOG, and other pluripotency TFs still needs to be better studied.

The COP9 signalosome (CSN) is an important protein complex that prevents protein degradation, and was previously implicated in pluripotency maintenance (Chia et al., 2010). Experiments using knockdown (KD) mESCs for COPS2 protein, a specific subunit of the CSN, showed that COPS2 regulates protein stability of NANOG by its independent and direct interaction with the α-helixes 2 and 3 of NANOG's homeobox domain, preventing its degradation by the proteasome

TABLE 2 | List of chaperones and proteasome-related proteins and their function associated to protein homeostasis and pluripotency control in different PSCs models.

Proteins and families		Function in PSCs biology	References
Chaperome	HSP90	Pluripotency maintenance and mesoderm differentiation of mESCs	Bradley et al., 2012
		STAT3 translocation to nucleus and NANOG negative regulation in mESC	Setati et al., 2010
		Endoderm differentiation of iPSCs	Jing et al., 2017
	HOP	STAT3 expression and phosphorylation and NANOG expression in mESCs	Longshaw et al., 2009
		Murine embryonic survival	Beraldo et al., 2013
	HSP70	Surface marker of pluripotency in hESCs	Alekseenko et al., 2012
		Differentiation of mESCs	Baharvand et al., 2008; Battersby et al., 2007; Saretzki et al., 2007
		Differentiation and survival of iPSCs	Brodarac et al., 2015
		Early differentiation of hESCs and mESCs	Park et al., 2011
	HSP60	OCT4 expression, proliferation, self-renewal and survival of mESCs	Seo et al., 2018
	HSP40	mESCs differentiation into smooth muscle cells Endoderm differentiation marker	Wong et al., 2014; Wang and Gudas, 1988
	HSP27	NANOG inactivation and neuronal differentiation of human placenta-derived cells	Cheng et al., 2016
Proteasome related	PSMD14	OCT4 regulation in mESCs and iPSCs	Buckley et al., 2012
	FBXW7	Negative regulation of c-MYC protein stability in mESCs and iPSCs	
	NRF2	Pluripotency maintenance in hESCs and iPSCs	Jang et al., 2014
	PSMD11	Functional proteasome complexes formation in hESC and iPSC	Vilchez et al., 2012
	F0X04	PSMD11 expression regulator in hESCs and iPSC	
	L1TD1	Downregulation leads to decrease in SOX2 and PSMD11 of hESC	Emani et al., 2015
	C0PS2	NANOG protein stability regulator of mESCs	Zhang et al., 2016
	WWP2	Promotes OCT4 and SOX2 proteasome degradation in mESCs	Xu et al., 2004, 2009, Fang et al., 2014
	SET7	SOX2 methylation and proteasome degradation promotion in mESCs Transcriptional activity inhibition in mESCs	Fang et al., 2014
	AKT1	SOX2 phosphorylation and proteasome degradation prevention in mESCs	
	UBR5	Proteostasis machinery regulator in hESCs and iPSCs	Koyuncu et al., 2018
	FBXW8	Polyubiquitynates NANOG and mESCs	Kim et al., 2014
	USP21	NANOG protein stabilization in mESCs and hESCs	Jin et al., 2016; Liu et al., 2017
	USP26	NANOG and SOX2 genes inhibition in hESCs	Ning et al., 2017

(Zhang et al., 2016). Further, the levels of NANOG remain unaltered after the replacement of four lysine residues for arginine in its C-terminal domain and subsequent KD of COPS2, indicating this region is a strong candidate for ubiquitination, serving as a signalization for UPS degradation (Zhang et al., 2016).

More specifically, it has been previously demonstrated that OCT4 can be post-translationally modified by ubiquitin in both mESCs and hESCs. WWP2 protein was identified as the first to post-translationally modify OCT4, functioning as an E3 ligase (Xu et al., 2004). WWP2 promotes degradation of OCT4 in a dosage-dependent and also enzymatic activity-dependent manner through the 26S proteasome (**Figure 1A**), since OCT4 protein level progressively decreases with the increase of WWP2 expression level, and WWP2 silencing (by iRNA and shRNA) elevates OCT4 levels (Xu et al., 2009). Further, OCT4 has been shown to suffer SUMOylation at lysine residue 118 (SUMO-1 acceptor site) in mESCs, and the disruption of this modification can lead to the degradation of OCT4 by 26 proteasome and consequent impairment in self-renewal (Zhang et al., 2007).

Interestingly, evidence shows that WWP2 also plays a role in the regulation of SOX2. WWP2 HECT domain recognizes methylation on lysine 119 (K119me) of SOX2, modification that stimulates SOX2 ubiquitination and subsequent degradation via proteasome pathway (Fang et al., 2014). Treatment with MG132, a potent proteasome inhibitor, led to prominently increased levels of SOX2 K119me. The methylation in SOX2 is catalyzed by the enzyme SET7 which, intriguingly, also inhibits the transcriptional activity of SOX2 (Fang et al., 2014). On the other hand, in ESCs, direct phosphorylation of SOX2 at threonine 118 by AKT1 is a prevalent protection mechanism acting in SOX2 protein stability (Jeong et al., 2010), and inhibits methylation by SET7 at K119, stabilizing SOX2 levels, being crucial in aspects such as self-renewal and differentiation potential (Fang et al., 2014). The mechanism of AKT-mediated phosphorylation as a protector for degradation by the UPS has also been recently identified in esophageal cancer stem cells, where phosphorylation of SOX2 on threonine 116 protects the degradation mediated by the ubiquitin E3 ligase UBR5 (Wang et al., 2019). It is interesting to note that UBR5 has also been identified as an important factor

in the regulation of proteostasis in hESCs and iPSCs. UBR5 is upregulated in hESCs and iPSCs derived from Huntington disease patients (Koyuncu et al., 2018). Both protein and mRNA levels are downregulated as these cells undergo differentiation (Koyuncu et al., 2018), indicating an important role of this protein in PSCs in health as well as disease. Given that PSCs and cancer stem cells share several features, the importance of UBR5 and other E3 ligases regulating the levels of TFs in PSCs should be further studied.

NANOG was previously described as a target for polyubiquitylation by the F-box protein family member FBXW8, and consequent degradation by proteasome (Kim et al., 2014). When phosphorylated by ERK1, NANOG binds to FBXW8, and experiments using mESCs and hESCs overexpressing FBXW8 resulted in a decrease in the half-life of endogenous NANOG. On the other hand, evaluation of alkaline phosphatase expression, a known pluripotency indicator, in FBXW8 KD cells, indicated decreased differentiation (Kim et al., 2014). The results suggest a key role of FBXW8, as a E3 ligase, in the regulation of NANOG levels in PSCs, by controlling its degradation.

The role of deubiquitinases (DUBs) started to be investigated in the context of proteostasis maintenance in PSCs. In brief, DUBs remove ubiquitin from protein substrates in order to maintain their targets stability. A recent study in mESCs, scanning for potential deubiquitinases regulators of NANOG, found that deubiquitinase ubiquitin-specific protease 21 (USP21) stabilizes NANOG protein levels removing K48-linked polyubiquitylation (Liu et al., 2017). USP21 directly interacts with NANOG through its ubiquitin carboxyl-terminal hydrolase domain (UCH), and the phosphorylation of USP21 by ERK1 prevents this binding, leaving this site exposed for ubiquitination, ultimately leading to NANOG degradation by the UPS (Jin et al., 2016). Another USP protein was recently reported as an indirect regulator for pluripotency TFs expression. USP26 inhibits expression of pluripotency core genes by physically binding to the members of the Protein Regulator of cytokinesis 1 (PRC1) complex, CBX4, and CBX6, preventing K48-linked polyubiquitination in these targets (Ning et al., 2017). The accumulation of CBX4 and CBX6 inhibits the expression of the SOX2 and NANOG genes, increasing the occupancy of their promoters, leading to reduction in pluripotency (Ning et al., 2017).

A summary of the proteasome-related proteins presented in this section and their function in PSCs can be found in **Table 2**. The evidence presented here points out the great relevance of studying the UPS in the context of PSCs, both as a possible model to better understand this system in health and diseases, and as a means of understanding the mechanisms governing the unique biology of these cells. Although many studies have been, and are still being conducted to explore these aspects, much remains to be described. It will be very interesting to follow what will be done in order to better understand the direct or indirect relationship between the great number of molecules involved in the UPS with the regulation of pluripotency TFs levels, or with other mechanisms important for PSCs maintenance, such as cell cycle control or self-renewal.

HISTONE CHAPERONES IN PSCs

As mentioned before, several factors affect the expression of pluripotency genes, and epigenetic modulations of transcription have been broadly studied to gain insight into the mechanisms which rule the rapid proliferation and turnover of pluripotent cells into differentiation. In the context of chaperone proteins, histone chaperones have recently been identified as important factors in regulating pluripotency. Here, we discuss the importance of histone chaperones in the modulation of transcription in PSCs, mainly ESCs, and their role in maintaining pluripotency through epigenetic modifications.

Chromatin in eukaryotes is organized in complexes called nucleosomes composed of 147 pairs of bases of DNA associated with a core of small basic proteins, the histones, which form an octamer of two copies of each protein H2A, H2B, H3, and H4, binding a linker histone H1 (Biterge and Schneider, 2014; Tessarz and Kouzarides, 2014). Histones can be canonical, essentially expressed during S-phase and incorporated to the nucleosome during DNA replication, or replacement histones (known as histone variants) which are incorporated into chromatin by specific histone chaperones during the cell cycle and can interact with several chromatin modifiers modulating the chromatin conformation (Biterge and Schneider, 2014). Beyond architectural functions, variant histones can regulate transcription, DNA repair and replication through covalently modifications at their flexible N- or C-terminal tails and globular domains, modulated by chromatin-modifying enzymes, which lead to a more open conformation of chromatin and allow DNA interaction with several molecules, including TFs (Strahl and Allis, 2000; Choi and Howe, 2009).

Histone chaperones are molecules that associate with histones and present an important role in histones dynamics. These chaperones are responsible for the transferring of histones to the DNA, and they can modulate histones modifications as acetylation and methylation or remodeling nucleosomes during transcription, among other important functions. Histone chaperones can work single, as the chaperone anti-silencing function 1 (ASF1), or form complexes as chromatin assembly factor 1 (CAF1) and the facilitates chromatin transcription (FACT), presenting relevant roles in post-translational histone modifications (De Koning et al., 2007; Tessarz and Kouzarides, 2014).

Embryonic stem cells present rapid changes in transcription associated with transition from pluripotency to a more differentiated state, therefore their chromatin is characterized by an open state with a less condensed structure and predominance of variant histone modified post-translationally (i.e., methylation and acetylation of H3K4) involved in transcription activation (Gaspar-Maia et al., 2011). One of the most important mechanisms by which ESCs maintain the open chromatin state is through the deposition of specific histone variants, for example H3.3, which is located at the −1 position in promoters of genes expressed in ESCs, and is commonly associated with more active transcription and decreased methylation of H3K9, a mark of condensed chromatin (heterochromatin) (Goldberg et al., 2010; Schlesinger et al., 2017). The chaperone

histone regulator A (HIRA) is responsible for H3.3 deposition in pluripotent cells during replication and co-localizes with the transcriptional active form of histone H3K4 methylated (Goldberg et al., 2010), playing important roles in pluripotency and differentiation (**Figure 1A**). Moreover, HIRA is highly expressed in the promoters of developmentally regulated genes in ESCs and is necessary for H3.3 enrichment at genome-wide transcriptionally active and repressed genes in mESCs (**Figure 1A**) and neural precursor cells (Goldberg et al., 2010).

HIRA is also required in mESC for the formation of H3.3 complex with polycomb repressive complex 2 (PRC2), which control gene transcription during lineage commitment in these cells through trimethylation of lysine 27 on histone H3 (H3K27me3) (Banaszynski et al., 2013). This complex is responsible for the proper establishment of H3K27me3 at the promoters of developmentally regulated genes and in bivalent domains, characterized by the presence of the variant histones H3K4me3 (activation-associated) and H3K27me3 (repression-associated) (Bernstein et al., 2006; Banaszynski et al., 2013). HIRA can also modulate hESCs self-renewal through its interaction with prohibitin (PHB) promoting transcription of isocitrate dehydrogenase genes (IDHs), leading to the production of α-ketoglutarate, which in turn participates in metabolic processes that support pluripotency of hESCs (Zhu et al., 2017).

Runt-related transcription factor 1 (RUNX1) is essential for hematopoietic cells transition and HIRA can modulate RUNX1 targets participating of mESCs differentiation process to hemogenic endothelium through its interaction with RUNX1 and deposition of H3.3 variant (Scambler et al., 2015). Additionally, H3.3 deposition by HIRA during early embryogenesis is required for developmental reprogramming, since the loss of HIRA in female mice impairs the deposition of paternal core histone and compromise the reactivation of maternal genome (Lin et al., 2014).

Transcriptional regulator ATRX (ATRX) is a member of SNF2 family of chromatin remodeling factors and presents histone chaperone activity, forming a chromatin-remodeling complex with the death domain-associated protein DAXX. ATRX and DAXX associate with H3.3 in a HIRA-independent manner modulating H3.3 deposition at telomeres and repression of telomeric RNA in mESCs (Goldberg et al., 2010). DAXX functions as an H3.3-specific chaperone in mESCs assembling H3.3/H4 tetramers on DNA templates, and the ATRX–DAXX complex modulates the remodeling of H3.3-containing nucleosomes bounding to telomeric chromatin or pericentric repeats (Lewis et al., 2010). Interestingly, it was demonstrated that DAXX and ATRX complex are responsible for H3.3 incorporation in transposable elements containing long terminal repeats, which present regions enriched with both H3.3 and H3K9me3, those able to regulate adjacent and endogenous genes in mESCs (Elsässer et al., 2015).

Anti-silencing function 1 is the most conserved chaperone of histone 3 and 4 and is associated with nucleosome assembly, transcriptional downregulation and DNA damage response (Thuret et al., 2005). In murine, two paralogous genes *ASF1a* and *ASF1b* are present; ASF1a depletion is embryonic lethal while ASF1b was correlated with differentiation potential during early embryonic stages and gonad development (Messiaen et al., 2016). Additionally, it has been demonstrated that ASF1 binds histones H3.1 and H3.3 in mESCs (Lewis et al., 2010). In hESCs, ASF1a can affect the expression of pluripotency genes through the acetylation of H3K56, a histone state that represents the epigenetic mark of hESC (**Figure 1B**; Gonzalez-Muñoz et al., 2014). Interestingly, when the TFs NANOG, SOX2 and OCT4 bind to their target gene promoters it is common to observe the presence of the histone variant H3K56ac at these locations and, since ASF1 is required for the acetylation of H3K56, ASF1 depletion leads to H3K56ac decrease, and, consequently reduces expression of pluripotency markers and promotes differentiation (Tan et al., 2013). Moreover, it was demonstrated that the induced expression of both ASF1a and OCT4 in human dermal fibroblasts is able to reprogram these cells into undifferentiated iPSCs (**Figure 2**; Gonzalez-Muñoz et al., 2014).

Chromatin assembly factor-1 (CAF-1) complex is formed by 3 subunits P150, P60, and P48/RbAp48a and is related to processes involved in DNA synthesis and repair (De Koning et al., 2007), and also chromatin remodeling in ESCs (Houlard et al., 2006). CAF-1 is the histone chaperone that mediates H3 deposition during S-phase, associates with H3.1, transports H3.3 in the absence of DAXX in mESC and its dominant negative leads to developmental arrest before 16-cell stage (Lewis et al., 2010; Filipescu et al., 2013). The lack of one of CAF-1 subunits (P150) induces the complete loss of H3.1 and H3.2 and impairs mouse blastocysts stage development, which present modified cellular structures such as nuclear elongation and the absence of heterochromatin foci (Akiyama et al., 2011). Houlard and Filipescu, in their respective studies, demonstrated that CAF-1 associated with histone H3.2 is responsible for heterochromatin proper architecture in early development, it may contribute to gene expression during this period of development, and is also required for early gastrulation (Houlard et al., 2006; Filipescu et al., 2013). The depletion of CAF-1 subunit P150 in mESCs affects the structure of heterochromatin, which is not observed in somatic cells, leading to its decondensation and the loss of clustering and mislocalization of pericentric heterochromatin domains (Houlard et al., 2006). Moreover, CAF-1 knockdown increases H2AX phosphorylation and the developmental arrest of mouse embryos, avoiding retrotransposons appropriate silencing and damaging genome integrity, since CAF-1 is responsible for modulating the replacement of H3.3 for H3.1/3.2 and leads to the deposition of other repressive histones as H3K9me3, H3K9me2, H3K27me3, and H4K20me3 in preimplantation mouse embryos (Hatanaka et al., 2015).

Cheloufi and colleagues recently demonstrated the participation of CAF-1 in the reprogramming of mouse fibroblasts into iPSCs, since the negative modulation of CAF-1 expression produces a decrease in somatic cells heterochromatin domains, increasing the binding of SOX2 to pluripotency specific targets and somatic cellular plasticity (Cheloufi et al., 2015; Cheloufi and Hochedlinger, 2017). Interestingly, CAF-1 suppression also allows ESCs to acquire characteristics of an early developmental

state, resembling totipotent 2-cell (2C) blastomeric stage of the preimplantation embryo (Yankulov, 2015).

The histone chaperone FACT is a complex composed by two subunits, SSRP1 and SPT16, and participates in transcription elongation (Mylonas and Tessarz, 2018). In mESC, FACT depletion causes a mis-activation of transcription start sites, which deregulates developmental and replication-associated genes leading to an increase in proliferation concomitant with neural commitment (Mylonas and Tessarz, 2018). FACT has been associated with OCT4 and its depletion damages mESC pluripotency and survival during early blastocyst stage (Cao et al., 2003; Gaspar-Maia et al., 2009; Pardo et al., 2010; Ding et al., 2012). Shakya et al. (2015) demonstrated that OCT4 recruits FACT complex, binds specifically SPT16 subunit of FACT in transcription sites to promote the removal of H3 histone from critical pluripotency targets such as OCT4, SOX2, and NANOG during reprograming. Moreover, it was demonstrated that the depletion of SPT16 subunit of FACT in mouse fibroblasts impair cellular reprograming and iPSCs generation (Shen et al., 2018).

Several other histone chaperones have presented important functions on ESCs biology and somatic cells reprograming. The mammalian high-mobility group AT-hook 2 (HMGA2) are highly expressed in ESCs and not translated in somatic normal cells, working as a replication fork chaperone stabilizing its integrity, in a DNA repair mechanism. During the proliferation of highly replicating cells as ESCs or cancer cells, HMGA2 expression is irrelevant, however, when forks are arrested; HMGA2 binds with high affinity to branched DNA, stabilizing stalled forks and preventing DNA mutations (Yu et al., 2014).

Another histone chaperone, nucleoplasmin-2 (NPM2), was recently associated with human dermal fibroblasts reprogramming. NPM2 associated with its histone variants TH2A and TH2 can improve the reprogramming modulated by OCT4, SOX2, KLF4, and c-MYC, generating iPSCs in a more naïve state compared to the classical TFs alone (**Figure 2**; Shinagawa et al., 2014; Fernández-Rivero et al., 2016). NPM2 phosphorylated form can also improve murine reprograming inducing an open chromatin structure and leading to reprogramming using only KLF4 and OCT4 (Shinagawa et al., 2014). Another NPM, the histone chaperone nucleoplasmin 3 (NPM3) is highly expressed in murine PSCs compared to differentiated cells and works as a chromatin remodeling protein, which modulates mESCs replication capacity, increasing proliferation (Motoi et al., 2008).

SPT6 is a histone chaperone and works as a transcription elongation factor in mESCs, modulating negatively H3K27 acetylation and methylation through its interaction with PRC2 core subunits, which maintains pluripotency and avoids differentiation mediated by the accumulation of H3K27me3 deposition at ESC critical super-enhancers (Robert, 2017). SET is a histone chaperone, which expression is modulated by OCT4, and is expressed during human early development and presents two isoforms, SETα and SETβ, each one controlling ESCs proliferation and differentiation, respectively (Edupuganti et al., 2017).

Finally, it is well known that PSCs present rapid turnovers to maintain an undifferentiated state or for entry into cellular commitment. Epigenetic mechanisms of gene activation that depends on histone variants allow these rapid turnovers observed in stem cells, especially PSCs, and histone chaperones are determinant for the refined work of histones modifications. Briefly, here we described how histones chaperones present relevant roles on development, pluripotency maintenance and somatic cell reprograming, as their main function is to modulate these essential epigenetic changes related to histones modifications and deposition, which have great participation on the activation and repression of TFs. **Table 1** compile the main histone chaperones and their role in pluripotency and differentiation. In addition to the histone chaperones, other chaperones are closely involved to maintain the pluripotent state, and their functions in PSCs biology will be explored in the next sessions.

PN AND HEAT SHOCK CHAPERONES IN PSCs

Elements of the PNs are enhanced in PSCs, such as chaperones and co-chaperones. Chaperones are classified in heat-shock proteins (HSPs) such as small heat shock proteins (sHSPs), HSP60, HSP70, HSP90, and HSP100. HSP70 and HSP90 are the main families of chaperones induced in response to cellular stress and they act along TFs known as heat shock factors (HSF), which regulate several genes encoding chaperones (Hartl et al., 2011; Saibil, 2013).

The heat shock factor 1 (HSF1) creates a dormant complex with HSP90 and HSP70 under balanced conditions, however, in the presence of misfolded proteins and cytotoxic protein aggregation, chaperones detach from HSF1, which then induces members of the PN to decrease production of new clients (Saibil, 2013). When the proteotoxic stress is reduced, the HSF1 complex reassembles and the system returns to balance. Likewise, other stress-inducible chaperones are expressed in the cytosol and within organelles to create arrangements that keep non-native proteins in solution and control *de novo* folding to establish functional structures (Saibil, 2013). HSPs are abundantly expressed in PSCs than in terminally differentiated cells, providing enhanced stress response competence for PSCs which is essential for the maintenance of stemness (Saretzki, 2004).

Both protein quality control and the maintenance of the PN are indispensable for cellular biology and for the health of whole organism. Important additional pathways such as the cytosolic stress response and the unfolded protein response (UPR) are part of this complex that contributes to circumvent the accumulation of misfolded molecules (Arndt et al., 2010). However, the PN is prone to failure, despite its regulatory mechanisms, and it opens a gap for pathological protein aggregate deposits. It is considered that aggregate formation confers on the disease protein a toxic gain of function (Dimant et al., 2012). Deficiencies in proteostasis related to aggregation have been shown to facilitate the development of several illnesses,

including neurodegeneration, diabetes, cystic fibrosis, and cancer (Kakkar et al., 2014). The importance of specific families of chaperones and co-chaperones in the context of pluripotency will be addressed in subsequent sessions.

HSP90-HOP-HSP70 Complex

Heat shock protein 90 (HSP90) is a highly conserved stress response protein in eukaryotic cells, being the most abundant protein in unstressed cells, modulates the activity, turnover and trafficking of several proteins and participates of signal transduction. HSP90 forms heterocomplexes with different proteins through the formation of a complex composed by HSP90 and another four chaperones – HSP70, HSP Organizing Protein (HOP), HSP40, and p23 – binding to several clients as steroid receptors. The assembly requires HSP90 and HSP70 bound by HOP and incorporates HSP40 and p23 during the process, assisting the folding and refolding of naïve proteins for properly active conformation (Pratt and Toft, 2003). This chaperone machinery is ATP-dependent, in which HSP90 binds to ATP and p23 stabilizes the complex. Both HSP70 and HSP40 also interact after ATP binding and HOP brings together both complexes preparing the chaperone machinery to receive a substrate, that can be a receptor, a protein kinase or a TF, among other proteins associated with signaling transduction (Pratt and Toft, 2003). The HSF are TFs responsible for increasing heat shock proteins expression during stress conditions and, on the other hand, HSP90 complex is responsible for sequester HSFs in normal conditions avoiding stress response activation in a feedback loop (Nichols et al., 1998).

Several studies support the importance of HSP90-HOP-HSP70 complex on PSCs biology. Recent data from our group determined that HOP, HSP70, and HSP90 are found as cargo of extracellular vesicles (implicated in intercellular communication) of mESCs, suggesting their participation in processes related to development, since the microenvironment is important for pluripotency maintenance through the modulation of several signaling pathways (Cruz et al., 2018a,b).

Constitutive HSP90 is required for mouse embryos development and maintenance of pluripotency via HOP-STAT3 interaction (**Figure 1A**); moreover, ESCs express a specific conjunct of types of chaperones related to this complex as HSP70 protein 4 (HSPA4) and HSP90β (HSPCB). In addition, stem cells including ESCs present an increase in the expression of HSP70 protein 5 (HSPA5), HSP70 protein 8 (HSPA8), and HOP (Fan, 2012). HSP70 protein 8 (HSPA8) is highly expressed on the surface of hESCs (**Figure 1B**) and co-expressed with specific stem cells markers, for example stage-specific embryonic antigen 3 and 4 (SSEA3/4) (Son et al., 2005).

HSP90 has been described in literature as an essential molecule for pluripotency maintenance. The inhibition of HSP90 expression decreases OCT4, NANOG and pSTAT3 levels and leads to mESCs differentiation, preferentially to the mesoderm layer. HSP90 associates with OCT4 and NANOG to protect them from ubiquitin proteasome degradation (**Figure 1A**) and is also able to modulate OCT4 mRNA levels supporting pluripotency maintenance (Bradley et al., 2012). During heat shock in hESCs, there is a hyperactivation of the MAP kinases and hESCs starts to differentiate through the increase of the HSF1 transcription factor, HSP90 releases HSF1 to answer the stress stimuli and HSF1 in turn binds to OCT4 promoter region leading to its repression (Byun et al., 2013).

Setati et al. (2010) described that HSP90 is responsible for STAT3 translocation to the nucleus during the activation of the JAK-STAT3 pathway, which occurs through LIF stimuli during mESCs self-renewal (**Figure 1A**). STAT3 translocation to the nucleus by HSP90 leads to STAT3 association with the NANOG promoter region modulating pluripotency of mESCs (Okumura et al., 2011). Negative modulation of NANOG transcription occurs through constitutive HSP90 sequester by TRIM8, which impairs its association with STAT3 and, consequently translocation to the nucleus, avoiding pluripotency signaling via LIF pathway (Setati et al., 2010). One of HSP90 clients is the transcription factor HNF4A, their binding in iPSCs is able to modulate specifically differentiation from these to endoderm-derived hepatic progenitor cells (Jing et al., 2017).

HSP90 partner HOP is essential for mouse development since its knockout (KO) is embryonic lethal and HOP KO mouse do not reach E10.5 development stage (Beraldo et al., 2013). HOP was suggested to modulate STAT3 phosphorylation and, through its binding to HSP90, participate in STAT3 translocation to the nucleus, being another important chaperone in mESC pluripotency by LIF/JAK/STAT3 signaling pathway (**Figure 1A**; Longshaw et al., 2009). Longshaw et al. (2009) demonstrated STAT3 accumulation into the cytoplasm, decreased phosphorylation and STAT3 mRNA levels after HOP depletion in mESC. Moreover, HOP knockdown mESCs presented a decrease in NANOG mRNA levels and decreased pluripotency observed in the formation of embryoid bodies (EBs).

HSP70 is constitutively expressed and its expression can be inducted during stress. In hESCs, constitutive HSP70 expression is slightly increased compared to somatic cells and is located into the cytoplasm and nucleus independent of differentiation status. However, in hESCs HSP70 can also be found on cell surface (**Figure 1B**), which is not observed in somatic cells, suggesting HSP70 is a possible surface marker for pluripotent cells (Alekseenko et al., 2012).

The HSPS from the family HSP70 HSPA1A (HSP70A1), HSPA1B (HSP70B1), and HSPA9A (HSP70A9, mortalin) are highly expressed in mESCs compared to differentiated cells and are associated with increased resistance of these cells against genotoxic stress (Saretzki, 2004). Additionally, it was demonstrated that during differentiation of some mESC lines there is a decrease in expression of both HSP70 and its partner HOP (Baharvand et al., 2008).

Recently, it was demonstrated that upregulation of HSP70 in iPSCs caused by stress as hypoxia promotes survival through the inhibition of apoptosis pathways, however, it also inhibits STAT3 phosphorylation leading to differentiation (**Figure 2**) and increased resistance to stress, since iPSCs are highly sensitive to conditions adverse to homeostasis (Brodarac et al., 2015). HSP70 participates in the early differentiation modulated by epigenetic factor histone deacetylase (HDAC) of mESCs and hESCs, through the activation of JNK and PI3K/AKT pathways (Park et al.,

2011). In mESC, HSP70 expression is enriched by the MAPK signaling pathway though JNK, ERK, and p38 modulating several responses to stress related to cell survival and anti-apoptosis processes (Nishitai and Matsuoka, 2008).

Differentiation of mESCs into neurogenic EBs (NEBs) leads to a decrease in the expression of mortalin, a chaperone from the family of HSP70 (HSP70A9) (Battersby et al., 2007). Another protein from HSP70 family, HSPA1b, also presents a significant decrease in its expression during differentiation and its remarkable that this downregulation occurs and is detectable before the expression of differentiation markers, supporting a role for HSP70 proteins in modulating differentiation (Saretzki et al., 2007).

HSP60, HSP40, and Small HSPs Families

HSP60, also known as HSPD1, is a mitochondrial chaperonin, involved in the synthesis and transportation of mitochondrial proteins from the cytoplasm to the mitochondria (Cappello et al., 2008). HSP60 is able to interact with different HSPs, such as HSP10, forming a complex that mediates protein folding (Okamoto et al., 2017), and with mitochondrial HSP70 (HSP70A), also known as mortalin, that have a role in cell proliferation and stress (Wadhwa et al., 2006). Studies shows that HSP60 have an important role in the biology of pluripotent cells. HSP60 deficiency in progenitor cells from the intestinal crypt compartment induces mitochondrial dysfunction, which leads to a decrease in stemness and cell proliferation (Berger et al., 2016). In mESCs, HSP60 expression decreases with cell differentiation, and its depletion caused a decrease in OCT4 expression, inhibiting proliferation and self-renewal, and increasing apoptosis in a caspase-3 independent-manner (Seo et al., 2018). Besides that, HSP60 knockdown also decreased EBs size and increased S-phase cells in mESCs (Seo et al., 2018). As seen in mouse cells, rabbit ESCs (rESCs) HSP60 levels are also increased when compared to differentiated cells, suggesting that proteins from the HSP family might have an important role in maintaining the undifferentiated status of embryonic cells (Intawicha et al., 2013). Interestingly, studies in cancer showed that *HSP60* is a target gene of c-MYC (Tsai et al., 2008), but is also able to induce c-MYC expression, as *HSP60* overexpression increased the proteins levels of c-MYC (Yan F.Q. et al., 2015).

HSP40 can interact with HSP70 and modulate its ATPase activity through stabilizing its interaction with subtracts (Prinsloo et al., 2009). HSP47, also known as SERPINH1, is required for the proper assembly of triple-helical procollagen molecules, and is highly expressed during mESC differentiation into smooth muscle cells (SMC) (Wong et al., 2014). Depletion of HSP47 leads to a decrease in the differentiation of mESC to mSMC and, in the same way, an overexpression of HSP47 leads to an increase in differentiation (Wong et al., 2014). HSP47 was identified as a marker for endodermal differentiation in mouse teratoma (Wang and Gudas, 1988). In bovine embryos, all HSP40 family members are upregulated during degeneration in an anti-apoptotic system, while normal blastocysts highly express HSP70 family members as HSPA5 and HSPA8 (Zhang et al., 2011). Both HSP40 and HSP47 are involved in the development of mouse limbs, along with other chaperones including from HSP70 family and small HSPs (Zhu et al., 2010; Yan F.Q. et al., 2015). A study using Glioblastoma Multiforme stem cells showed that HSP47 is capable of modulating TGF-β, inducing cell survivability, self-renewal, and increasing the number of stem cell-like cells within the tumor (Jing et al., 2017). Still, more studies elucidating the possible roles of the HSP40 family in the maintenance of stem cells are necessary.

The small HSP family is composed out of HSP of low molecular weight. Small HSPs present relevant roles in mouse development, for example HSP10, HSP22, and HSP25 that are involved in murine limb development (Zhu et al., 2010; Yan Z. et al., 2015). In adipose tissue-derived stem cells, HSP32, also known as hemeoxygenase-1, has its expression increased after the cells are left in 43°C for 1 h, which in turn increases the prevalence of live cells after a frozen-thawn cycle, reducing oxidative stress damage (Shaik et al., 2017). Besides that, a study in Chronic Myeloid Leukemia, a hematopoietic stem cell disease, shows that HSP32 is capable of increasing the survivability of those cells, through protection against apoptosis (Mayerhofer et al., 2008). Another small HSP with an interesting role in stemness is HSP27, also known as heat shock protein beta-1 (HSPB1). A study shows a downregulation of HSP27 after mESC differentiate into NEB (Prinsloo et al., 2009). Besides that, HSP27 is capable of regulating STAT3 expression, with their expression levels correlating directly in prostate cancer cells (Rocchi et al., 2005). HSP27 is also able to regulate EIF4E and eIF4G levels in the first trimester human placentae, indicating that HSP27 could alter placental protein translation (Shochet et al., 2016). HSP27 also seems to have an important role in neuronal differentiation, with its overexpression leading stem cells to arrest neuronal differentiation, and its expression decreasing as embryonic neuronal development occurred *in vivo* (Cheng et al., 2016). In placenta-derived multipotent cells (PDMCs) HSP27 inhibition leads to NANOG cleavage mediated by caspase-3 activation (Cheng et al., 2016).

In zebrafish development both HSP27 family members, HSPB7 and HSPB12, are modulated by GATA4 protein and modulate cardiac development, since their depletion leads to a disruption in normal cardiac morphogenesis perturbing Kuppfer's vesicle morphology (Rosenfeld et al., 2013). Indeed, HSPB7 is highly expressed in cardiac cells and its depletion in mouse lacks the normal development of the heart, leading to embryo lethality before embryonic day 12.5 (Wu et al., 2017). HSPB1 (HSP27 family) also presents an important role in zebrafish development modulating the growth of myofibrils of skeletal muscle (Middleton and Shelden, 2013). In avian blastoderms, the expression of the sHSP HSP25 is required during the embryo development for the expression of pluripotency genes and for resistance against apoptosis (Hwang et al., 2016).

In light of all these findings, it is remarkable that the participation of heat shock proteins in the biology of PSCs. HSPs can modulate several signaling cascades including pluripotency essential pathways. They also modulate both expression and localization of TFs and relevant proteins, and their expression is essential for the development of the embryos of many species. Together, these studies demonstrate the importance of stress response and HSPs function in development and pluripotency maintenance of PSCs.

PERSPECTIVES

A better overview of the nature of pluripotency holds great promise for therapeutic approaches. Unveiling the exact molecular mechanisms that govern the ability of ESCs and iPSCs to differentiate in all cell types to give rise to an adult organism is essential to exploit these cells both in regenerative medicine and to model human diseases pathogenesis including cancer and neurodegenerative disorders.

A regulatory core (NANOG, OCT4, and SOX2) and other TFs, epigenetic regulators and specific signaling pathways are key elements to orchestrate pluripotency. Although these well-established TFs are of undeniable importance for pluripotency maintenance, the search for additional TFs that are able to interact with core factors to regulate processes related to pluripotency should be enhanced, and the role of an expanded core has just begun to be dissected. Moving forward to the debate on the importance of TFs for pluripotency and stemness regulation, this will be a fruitful area for further research.

As presented here, emerging evidence points out the network plasticity of chaperones controlling the activities of key players involved in pluripotency maintenance. Chaperones play roles from shaping chromatin dynamic to controlling transcriptional regulation of pluripotency genes, to the assistance of proper folding of these genes when translated into proteins in different PSCs models (**Figures 1**, **2**). The broad spectrum of chaperones activities in the essential processes of stemness control reveals that stem cells exhibit intrinsic proteostasis mechanism, which can be included as a component of the pluripotency regulatory network. In somatic cells a collapse in proteostasis underlies several diseases including neurodegenerative disease and cancer, conversely, PSCs exhibit the remarkable capacity to correct and repress proteome imbalance, indicating that additional investigation is required for an in-depth understanding of enhanced protein homeostasis in stem cell biology.

This review brings together classical and recent research on the control of pluripotency, going through broad important cellular processes related to this unique and promising feature. The diversity of these processes embraces different levels of cellular regulation and shows how complex is the understanding of the pluripotency context.

AUTHOR CONTRIBUTIONS

CF and RI conceived the presented idea and proof outline, did the literature review, organized the tables, and wrote distinct topics. MP did the literature review and wrote a topic. MM-E did the literature review, wrote a topic, and prepared the illustration (art-**Figures 1**, **2**). ML conceived the presented idea and proof outline, wrote topics, and reviewed the manuscript.

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REFERENCES

Akiyama, T., Suzuki, O., Matsuda, J., and Aoki, F. (2011). Dynamic replacement of histone H3 variants reprograms epigenetic marks in early mouse embryos. *PLoS Genet.* 7:e1002279. doi: 10.1371/journal.pgen.1002279

Alekseenko, L. L., Zemelko, V. I., Zenin, V. V., Pugovkina, N. A., Kozhukharova, I. V., Kovaleva, Z. V., et al. (2012). Heat shock induces apoptosis in human embryonic stem cells but a premature senescence phenotype in their differentiated progeny. *Cell Cycle* 11, 3260–3269. doi: 10.4161/cc.21595

Arndt, V., Dick, N., Tawo, R., Dreiseidler, M., Wenzel, D., Hesse, M., et al. (2010). Chaperone-assisted selective autophagy is essential for muscle maintenance. *Curr. Biol.* 20, 143–148. doi: 10.1016/j.cub.2009.11.022

Baharvand, H., Fathi, A., Gourabi, H., Mollamohammadi, S., and Salekdeh, G. H. (2008). Identification of mouse embryonic stem cell-associated proteins. *J. Proteome Res.* 7, 412–423. doi: 10.1021/pr700560t

Banaszynski, L. A., Wen, D., Dewell, S., Whitcomb, S. J., Lin, M., Diaz, N., et al. (2013). Hira-dependent histone H3.3 deposition facilitates prc2 recruitment at developmental loci in ES cells. *Cell* 155, 107–120. doi: 10.1016/j.cell.2013.08.061

Battersby, A., Jones, R. D., Lilley, K. S., McFarlane, R. J., Braig, H. R., Allen, N. D., et al. (2007). Comparative proteomic analysis reveals differential expression of Hsp25 following the directed differentiation of mouse embryonic stem cells. *Biochim. Biophys. Acta Mol. Cell Res.* 1773, 147–156. doi: 10.1016/j.bbamcr.2006.08.030

Beraldo, F. H., Soares, I. N., Goncalves, D. F., Fan, J., Thomas, A. A., Santos, T. G., et al. (2013). Stress-inducible phosphoprotein 1 has unique cochaperone activity during development and regulates cellular response to ischemia via the prion protein. *FASEB J.* 27, 3594–3607. doi: 10.1096/fj.13-232280

Berger, E., Rath, E., Yuan, D., Waldschmitt, N., Khaloian, S., Allgäuer, M., et al. (2016). Mitochondrial function controls intestinal epithelial stemness and proliferation. *Nat. Commun.* 7:13171. doi: 10.1038/ncomms13171

Bernstein, B. E., Mikkelsen, T. S., Xie, X., Kamal, M., Huebert, D. J., Cuff, J., et al. (2006). A Bivalent Chromatin Structure Marks Key Developmental Genes in Embryonic Stem Cells. *Cell* 125, 315–326. doi: 10.1016/j.cell.2006.02.041

Bharathan, S. P., Manian, K. V., Aalam, S. M. M., Palani, D., Deshpande, P. A., Pratheesh, M. D., et al. (2017). Systematic evaluation of markers used for the identification of human induced pluripotent stem cells. *Biol. Open* 6, 100–108. doi: 10.1242/bio.022111

Biterge, B., and Schneider, R. (2014). Histone variants: key players of chromatin. *Cell Tissue Res.* 356, 457–466. doi: 10.1007/s00441-014-1862-4

Boyer, L. A., Tong, I. L., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., et al. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122, 947–956. doi: 10.1016/j.cell.2005.08.020

Bradley, E., Bieberich, E., Mivechi, N. F., Tangpisuthipongsa, D., and Wang, G. (2012). Regulation of embryonic stem cell pluripotency by heat shock protein 90. *Stem Cells* 30, 1624–1633. doi: 10.1002/stem.1143

Brodarac, A., Saric, T., Oberwallner, B., Mahmoodzadeh, S., Neef, K., Albrecht, J., et al. (2015). Susceptibility of murine induced pluripotent stem cell-derived cardiomyocytes to hypoxia and nutrient deprivation. *Stem Cell Res. Ther.* 6, 1–19. doi: 10.1186/s13287-015-0057-6

Buckley, S. M., Aranda-Orgilles, B., Strikoudis, A., Apostolou, E., Loizou, E., Moran-Crusio, K., et al. (2012). Regulation of pluripotency and cellular reprogramming by the ubiquitin-proteasome system. *Cell Stem Cell* 11, 783–798. doi: 10.1016/j.stem.2012.09.011

Byun, K., Kim, T. K., Oh, J., Bayarsaikhan, E., Kim, D., Lee, M. Y., et al. (2013). Heat shock instructs hESCs to exit from the self-renewal program through

negative regulation of OCT4 by SAPK/JNK and HSF1 pathway. *Stem Cell Res.* 11, 1323–1334. doi: 10.1016/j.scr.2013.08.014

Cao, S., Bendall, H., Hicks, G. G., Nashabi, A., Sakano, H., Shinkai, Y., et al. (2003). The high-mobility-group box protein SSRP1/T160 is essential for cell viability in day 3.5 mouse embryos. *Mol. Cell. Biol.* 23, 5301–5307. doi: 10.1128/mcb.23.15.5301-5307.2003

Cappello, F., Conway De Macario, E., Marasà, L., Zummo, G., and Macario, A. J. L. (2008). Hsp60 expression, new locations, functions and perspectives for cancer diagnosis and therapy. *Cancer Biol. Ther.* 7, 801–809. doi: 10.4161/cbt.7.6.6281

Cheloufi, S., Elling, U., Hopfgartner, B., Jung, Y. L., Murn, J., Ninova, M., et al. (2015). The histone chaperone CAF-1 safeguards somatic cell identity. *Nature* 528, 218–224. doi: 10.1038/nature15749

Cheloufi, S., and Hochedlinger, K. (2017). Emerging roles of the histone chaperone CAF-1 in cellular plasticity. *Curr. Opin. Genet. Dev.* 46, 83–94. doi: 10.1016/j.gde.2017.06.004

Cheng, Y. C., Huang, C. J., Lee, Y. J., Tien, L. T., Ku, W. C., Chien, R., et al. (2016). Knocking down of heat-shock protein 27 directs differentiation of functional glutamatergic neurons from placenta-derived multipotent cells. *Sci. Rep.* 6, 1–15. doi: 10.1038/srep30314

Chia, N. Y., Chan, Y. S., Feng, B., Lu, X., Orlov, Y. L., Moreau, D., et al. (2010). A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. *Nature* 468, 316–320. doi: 10.1038/nature09531

Choi, J. K., and Howe, L. J. (2009). Histone acetylation: truth or consequences? *Biochem. Cell Bio* 87, 139–150.

Collier, A. J., Panula, S. P., Schell, J. P., Chovanec, P., Plaza Reyes, A., Petropoulos, S., et al. (2017). Comprehensive cell surface protein profiling identifies specific markers of human naive and primed pluripotent states. *Cell Stem Cell* 20, 874.e7–890.e7. doi: 10.1016/j.stem.2017.02.014

Cruz, L., Arevalo Romero, J. A., Brandão Prado, M., Santos, T. G., and Hohmuth Lopes, M. (2018a). Evidence of extracellular vesicles biogenesis and release in mouse embryonic stem cells. *Stem Cell Rev. Reports* 14, 262–276. doi: 10.1007/s12015-017-9776-7

Cruz, L., Romero, A. A. J., Iglesia, P. R., and Lopes, H. M. (2018b). Extracellular vesicles: decoding a new language for cellular communication in early embryonic development. *Front. Cell Dev. Biol* 6:94. doi: 10.3389/fcell.2018.00094

De Koning, L., Corpet, A., Haber, J. E., and Almouzni, G. (2007). Erratum: histone chaperones: an escort network regulating histone traffic (nature structural and molecular biology (2007) 14, (997-1007)). *Nat. Struct. Mol. Biol.* 14, 997–1007. doi: 10.1038/nsmb1318

Dimant, H., Ebrahimi-Fakhari, D., and McLean, P. J. (2012). Molecular chaperones and co-chaperones in parkinson disease. *Neuroscientist* 18, 589–601. doi: 10.1177/1073858412441372

Ding, J., Xu, H., Faiola, F., Ma'Ayan, A., and Wang, J. (2012). Oct4 links multiple epigenetic pathways to the pluripotency network. *Cell Res.* 22, 155–167. doi: 10.1038/cr.2011.179

Edupuganti, R. R., Harikumar, A., Aaronson, Y., Biran, A., Sailaja, B. S., Nissim-Rafinia, M., et al. (2017). Alternative SET/TAFI promoters regulate embryonic stem cell differentiation. *Stem Cell Rep.* 9, 1291–1303. doi: 10.1016/j.stemcr.2017.08.021

Elsässer, S. J., Noh, K.-M., Diaz, N., Allis, D., and Banaszynski, L. A. (2015). Histone H3.3 is required for endogenous retrovira element silencing in embryonic stem cells. *Science* 522, 240–244. doi: 10.1038/nature14345

Emani, M. R., Närvä, E., Stubb, A., Chakroborty, D., Viitala, M., Rokka, A., et al. (2015). The L1TD1 protein interactome reveals the importance of post-transcriptional regulation in human pluripotency. *Stem Cell Rep.* 4, 519–528. doi: 10.1016/j.stemcr.2015.01.014

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154–156. doi: 10.1038/292154a0

Fan, G.-C. (2012). Genetics of stem cells. *Part A Prog. Mol. Biol. Transl. Sci.* 111, 305–322. doi: 10.1016/B978-0-12-398459-3.00014-9

Fang, L., Zhang, L., Wei, W., Jin, X., Wang, P., Tong, Y., et al. (2014). A methylation-phosphorylation switch determines Sox2 stability and function in ESC maintenance or differentiation. *Mol. Cell* 55, 537–551. doi: 10.1016/j.molcel.2014.06.018

Fernández-Rivero, N., Franco, A., Velázquez-Campoy, A., Alonso, E., Muga, A., and Prado, A. (2016). A quantitative characterization of nucleoplasmin/histone complexes reveals chaperone versatility. *Sci. Rep.* 6, 1–14. doi: 10.1038/srep32114

Filipescu, D., Szenker, E., and Almouzni, G. (2013). Developmental roles of histone H3 variants and their chaperones. *Trends Genet.* 29, 630–640. doi: 10.1016/j.tig.2013.06.002

Gaspar-Maia, A., Alajem, A., Meshorer, E., and Ramalho-Santos, M. (2011). Open chromatin in pluripotency and reprogramming. *Nat. Rev. Mol. Cell Biol.* 12, 36–47. doi: 10.1038/nrm3036

Gaspar-Maia, A., Alajem, A., Polesso, F., Sridharan, R., Mason, M. J., Heidersbach, A., et al. (2009). Chd1 regulates open chromatin and pluripotency of embryonic stem cells. *Nature* 460, 863–868. doi: 10.1038/nature08212

Goldberg, A. D., Banaszynski, L. A., Noh, K. M., Lewis, P. W., Elsaesser, S. J., Stadler, S., et al. (2010). Distinct factors control histone variant H3.3 localization at specific genomic regions. *Cell* 140, 678–691. doi: 10.1016/j.cell.2010.01.003

Gonzalez-Muñoz, E., Yohanna, A.-E., Hasan, H. O., and Jose, B. C. (2014). Histone chaperone ASF1A is required for maintenance of pluripotency and cellular reprogramming. *Science* 345, 822–825. doi: 10.1126/science.1254745

Hackett, J. A., and Azim Surani, M. (2014). Regulatory principles of pluripotency: from the ground state up. *Cell Stem Cell* 15, 416–430. doi: 10.1016/j.stem.2014.09.015

Hartl, F. U., Bracher, A., and Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature* 475, 324–332. doi: 10.1038/nature10317

Hatanaka, Y., Inoue, K., Oikawa, M., Kamimura, S., Ogonuki, N., Kodama, E. N., et al. (2015). Histone chaperone CAF-1 mediates repressive histone modifications to protect preimplantation mouse embryos from endogenous retrotransposons. *Proc. Natl. Acad. Sci.* 112, 14641–14646. doi: 10.1073/pnas.1512775112

Houlard, M., Berlivet, S., Probst, A. V., Quivy, J. P., Héry, P., Almouzni, G., et al. (2006). CAF-1 is essential for heterochromatin organization in pluripotent embryonic cells. *PLoS Genet.* 2:e18. doi: 10.1371/journal.pgen.0020181

Huang, D., Wang, L., Duan, J., Huang, C., Tian, X., Zhang, M., et al. (2017). LIF-activated Jak signaling determines esrrb expression during late-stage reprogramming. *Biol. Open* 7:bio029264. doi: 10.1242/bio.029264

Hwang, Y. S., Ko, M. H., Kim, Y. M., Park, Y. H., Ono, T., and Han, J. Y. (2016). The avian-specific small heat shock protein HSP25 is a constitutive protector against environmental stresses during blastoderm dormancy. *Sci. Rep.* 6, 1–12. doi: 10.1038/srep36704

Intawicha, P., Wang, S. H., Hsieh, Y. C., Lo, N. W., Lee, K. H., Huang, S. Y., et al. (2013). Proteomic profiling of rabbit embryonic stem cells derived from parthenotes and fertilized embryos. *PLoS One* 8:e67772. doi: 10.1371/journal.pone.0067772

Jang, J., Wang, Y., Kim, H. S., Lalli, M. A., and Kosik, K. S. (2014). Nrf2, a regulator of the proteasome, controls self-renewal and pluripotency in human embryonic stem cells. *Stem Cells* 32, 2616–2625. doi: 10.1002/stem.1764

Jeong, C. H., Cho, Y. Y., Kim, M. O., Kim, S. H., Cho, E. J., Lee, S. Y., et al. (2010). Phosphorylation of Sox2 cooperates in reprogramming to pluripotent stem cells. *Stem Cells* 28, 2141–2150. doi: 10.1002/stem.540

Jin, J., Liu, J., Chen, C., Liu, Z., Jiang, C., Chu, H., et al. (2016). The deubiquitinase USP21 maintains the stemness of mouse embryonic stem cells via stabilization of Nanog. *Nat. Commun.* 7, 1–15. doi: 10.1038/ncomms13594

Jing, R., Duncan, C. B., and Duncan, S. A. (2017). A small-molecule screen reveals that HSP90β promotes the conversion of induced pluripotent stem cell-derived endoderm to a hepatic fate and regulates HNF4A turnover. *Development* 144, 1764–1774. doi: 10.1242/dev.146845

Kakkar, V., Meister-Broekema, M., Minoia, M., Carra, S., and Kampinga, H. H. (2014). Barcoding heat shock proteins to human diseases: looking beyond the heat shock response. *Dis. Model. Mech.* 7, 421–434. doi: 10.1242/dmm.014563

Kim, J., Chu, J., Shen, X., Wang, J., and Orkin, S. H. (2008). An extended transcriptional network for pluripotency of embryonic stem cells. *Cell* 132, 1049–1061. doi: 10.1016/j.cell.2008.02.039

Kim, S. H., Kim, M. O., Cho, Y. Y., Yao, K., Kim, D. J., Jeong, C. H., et al. (2014). ERK1 phosphorylates Nanog to regulate protein stability and stem cell self-renewal. *Stem Cell Res.* 13, 1–11. doi: 10.1016/j.scr.2014.04.001

Koyuncu, S., Saez, I., Lee, H. J., Gutierrez-Garcia, R., Pokrzywa, W., Fatima, A., et al. (2018). The ubiquitin ligase UBR5 suppresses proteostasis collapse in

pluripotent stem cells from Huntington's disease patients. *Nat. Commun.* 9, 1–22. doi: 10.1038/s41467-018-05320-3

Lecker, S. H., Goldberg, A. L., and Mitch, W. E. (2006). Protein degradation by the ubiquitin–proteasome pathway in normal and disease states. *J. Am. Soc. Nephrol* 17, 1807–1819. doi: 10.1681/ASN.2006010083

Lewis, P. W., Elsaesser, S. J., Noh, K.-M., Stadler, S. C., and Allis, C. D. (2010). Daxx is an H3.3-specific histone chaperone and cooperates with ATRX in replication-independent chromatin assembly at telomeres. *Proc. Natl. Acad. Sci.* 107, 14075–14080. doi: 10.1073/pnas.1008850107

Lin, C. J., Koh, F. M., Wong, P., Conti, M., and Ramalho-Santos, M. (2014). Hira-mediated H3.3 incorporation is required for DNA replication and ribosomal RNA transcription in the mouse zygote. *Dev. Cell* 30, 268–279. doi: 10.1016/j.devcel.2014.06.022

Liu, X., Yao, Y., Ding, H., Han, C., Chen, Y., Zhang, Y., et al. (2017). USP21 deubiquitylates nanog to regulate protein stability and stem cell pluripotency. *Signal Transduct. Target. Ther.* 2:16046. doi: 10.1038/sigtrans.2016.46

Longshaw, V. M., Baxter, M., Prewitz, M., and Blatch, G. L. (2009). Knockdown of the co-chaperone hop promotes extranuclear accumulation of stat3 in mouse embryonic stem cells. *Eur J Cell Biol.* 88, 153–166. doi: 10.1016/j.ejcb.2008.09.003

Marks, H., Kalkan, T., Menafra, R., Denissov, S., Jones, K., Hofemeister, H., et al. (2012). The transcriptional and epigenomic foundations of ground state pluripotency. *Cell* 149, 590–604. doi: 10.1016/j.cell.2012.03.026

Martello, G., and Smith, A. (2014). The nature of embryonic stem cells. *Annu. Rev. Cell Dev. Biol.* 30, 647–675. doi: 10.1146/annurev-cellbio-100913-013116

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78, 7634–7638. doi: 10.1073/pnas.78.12.7634

Mayerhofer, M., Gleixner, K. V., Mayerhofer, J., Hoermann, G., Jaeger, E., Aichberger, K. J., et al. (2008). Targeting of heat shock protein 32 (Hsp32)/heme oxygenase-1 (HO-1) in leukemic cells in chronic myeloid leukemia: a novel approach to overcome resistance against imatinib. *Blood* 111, 2200–2210. doi: 10.1182/blood-2006-11-055723

Messiaen, S., Guiard, J., Aigueperse, C., Fliniaux, I., Tourpin, S., Barroca, V., et al. (2016). Loss of the histone chaperone ASF1B reduces female reproductive capacity in mice. *Reproduction* 151, 477–489. doi: 10.1530/REP-15-0327

Messmer, T., von Meyenn, F., Savino, A., Santos, F., Mohammed, H., Lun, A. T. L., et al. (2019). Transcriptional heterogeneity in naive and primed human pluripotent stem cells at single-cell resolution. *Cell Rep.* 26, 815.e4–824.e4. doi: 10.1016/j.celrep.2018.12.099

Middleton, R. C., and Shelden, E. A. (2013). Small heat shock protein HSPB1 regulates growth of embryonic zebrafish craniofacial muscles. *Exp. Cell Res.* 319, 860–874. doi: 10.1016/j.yexcr.2013.01.002

Motoi, N., Suzuki, K. I., Hirota, R., Johnson, P., Oofusa, K., Kikuchi, Y., et al. (2008). Identification and characterization of nucleoplasmin 3 as a histone-binding protein in embryonic stem cells. *Dev. Growth Differ.* 50, 307–320. doi: 10.1111/j.1440-169X.2008.01034.x

Mylonas, C., and Tessarz, P. (2018). Transcriptional repression by FACT is linked to regulation of chromatin accessibility at the promoter of ES cells. *Life Sci. Alliance* 1, e201800085. doi: 10.26508/lsa.201800085

Nichols, J., and Smith, A. (2009). Naive and primed pluripotent states. *Cell Stem Cell* 4, 487–492. doi: 10.1016/j.stem.2009.05.015

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., et al. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 95, 379–391. doi: 10.1016/S0092-8674(00)81769-9

Nicola, N. A., and Babon, J. J. (2015). Leukemia inhibitory factor (LIF). *Cytokine Growth Factor Rev.* 26, 533–544. doi: 10.1016/j.cytogfr.2015.07.001

Ning, B., Zhao, W., Qian, C., Liu, P., Li, Q., Li, W., et al. (2017). USP26 functions as a negative regulator of cellular reprogramming by stabilising PRC1 complex components. *Nat. Commun.* 8, 1–10. doi: 10.1038/s41467-017-00301-4

Nishitai, G., and Matsuoka, M. (2008). Differential regulation of HSP70 expression by the JNK kinases SEK1 and MKK7 in mouse embryonic stem cells treated with cadmium. *J. Cell. Biochem.* 104, 1771–1780. doi: 10.1002/jcb.21743

Okamoto, T., Yamamoto, H., Kudo, I., Matsumoto, K., Odaka, M., Grave, E., et al. (2017). HSP60 possesses a GTPase activity and mediates protein folding with HSP10. *Sci. Rep.* 7:16931. doi: 10.1038/s41598-017-17167-7

Okita, Y., and Nakayama, K. I. (2012). UPS delivers pluripotency. *Cell Stem Cell* 11, 728–730. doi: 10.1016/j.stem.2012.11.009

Okumura, F., Okumura, A. J., Matsumoto, M., Nakayama, K. I., and Hatakeyama, S. (2011). TRIM8 regulates Nanog via Hsp90β-mediated nuclear translocation of STAT3 in embryonic stem cells. *Biochim. Biophys. Acta Mol. Cell Res.* 1813, 1784–1792. doi: 10.1016/j.bbamcr.2011.05.013

Pardo, M., Lang, B., Yu, L., Prosser, H., Bradley, A., Babu, M. M., et al. (2010). An expanded oct4 interaction network: implications for stem cell biology. *Devel. Dis. Cell Stem Cell* 6, 382–395. doi:10.1016/j.stem.2010.03.004

Park, J. A., Kim, Y. E., Seok, H. J., Park, W. Y., Kwon, H. J., and Lee, Y. (2011). Differentiation and upregulation of heat shock protein 70 induced by a subset of histone deacetylase inhibitors in mouse and human embryonic stem cells. *BMB Rep.* 44, 176–181. doi: 10.5483/BMBRep.2011.44.3.176

Pratt, W. B., and Toft, D. O. (2003). Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp. Biol. Med.* 228, 111–133. doi: 10.1177/153537020322800201

Prinsloo, E., Setati, M. M., Longshaw, V. M., and Blatch, G. L. (2009). Chaperoning stem cells: a role for heat shock proteins in the modulation of stem cell self-renewal and differentiation? *BioEssays* 31, 370–377. doi: 10.1002/bies.200800158

Robert, F. (2017). Spt6 gets in the way of polycomb to promote ESC pluripotency. *Mol. Cell* 68, 263–264. doi: 10.1016/j.molcel.2017.10.005

Rocchi, P., Beraldi, E., Ettinger, S., Fazli, L., Vessella, R. L., Nelson, C., et al. (2005). Increased Hsp27 after androgen ablation facilitates androgen-independent progression in prostate cancer via signal transducers and activators of transcription 3-mediated suppression of apoptosis. *Cancer Res.* 65, 11083–11093. doi: 10.1158/0008-5472.CAN-05-1840

Rodriguez, R. T., Velkey, J. M., Lutzko, C., Seerke, R., Kohn, D. B., O'Shea, K. S., et al. (2008). Manipulation of OCT4 levels in human embryonic stem cells results in induction of differential cell types. *Exp. Biol. Med.* 232, 1368–1380. doi: 10.3181/0703-rm-63

Rosenfeld, G. E., Mercer, E. J., Mason, C. E., and Evans, T. (2013). Small heat shock proteins Hspb7 and Hspb12 regulate early steps of cardiac morphogenesis. *Dev. Biol.* 381, 389–400. doi: 10.1016/j.ydbio.2013.06.025

Saibil, H. (2013). Chaperone machines for protein folding, unfolding and disaggregation. *Nat. Rev. Mol. Cell Biol.* 14, 630–642. doi: 10.1038/nrm3658

Saretzki, G. (2004). Stress defense in murine embryonic stem cells is superior to that of various differentiated murine cells. *Stem Cells* 22, 962–971. doi: 10.1634/stemcells.22-6-962

Saretzki, G., Walter, T., Atkinson, S., Passos, J. F., Bareth, B., Keith, W. N., et al. (2007). Downregulation of multiple stress defense mechanisms during differentiation of human embryonic stem cells. *Stem Cells* 26, 455–464. doi: 10.1634/stemcells.2007-0628

Scambler, P. J., Joseph, S., Syed, K. M., Dutta, D., and Majumder, A. (2015). Histone chaperone HIRA in regulation of transcription factor RUNX1. *J. Biol. Chem.* 290, 13053–13063. doi: 10.1074/jbc.m114.615492

Schlesinger, S., Kaffe, B., Melcer, S., Aguilera, J. D., Sivaraman, D. M., Kaplan, T., et al. (2017). A hyperdynamic H3.3 nucleosome marks promoter regions in pluripotent embryonic stem cells. *Nucleic Acids Res.* 45, 12181–12194. doi: 10.1093/nar/gkx817

Schubert, U., Antón, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W., and Bennink, J. R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* 404, 770–774. doi: 10.1038/35008096

Seo, N. H., Lee, E. H., Seo, J. H., Song, H. R., and Han, M. K. (2018). HSP60 is required for stemness and proper differentiation of mouse embryonic stem cells. *Exp. Mol. Med.* 50, e459. doi: 10.1038/emm.2017.299

Setati, M. M., Prinsloo, E., Longshaw, V. M., Murray, P. A., Edgar, D. H., and Blatch, G. L. (2010). Leukemia inhibitory factor promotes Hsp90 association with STAT3 in mouse embryonic stem cells. *IUBMB Life* 62, 61–66. doi: 10.1002/iub.283

Shaik, S., Hayes, D., Gimble, J., and Devireddy, R. (2017). Inducing heat shock proteins enhances the stemness of frozen–thawed adipose tissue-derived stem cells. *Stem Cells Dev.* 26, 608–616. doi: 10.1089/scd.2016.0289

Shakya, A., Callister, C., Goren, A., Yosef, N., Garg, N., Khoddami, V., et al. (2015). Pluripotency transcription factor oct4 mediates stepwise nucleosome demethylation and depletion. *Mol. Cell. Biol.* 35, 1014–1025. doi: 10.1128/mcb.01105-14

Shen, Z., Formosa, T., and Tantin, D. (2018). FACT inhibition blocks induction but not maintenance of pluripotency. *Stem Cells Dev.* 27, 1693–1701. doi: 10.1089/scd.2018.0150

Shinagawa, T., Takagi, T., Tsukamoto, D., Tomaru, C., Huynh, L. M., Sivaraman, P., et al. (2014). Histone variants enriched in oocytes enhance reprogramming to induced pluripotent stem cells. *Cell Stem Cell* 14, 217–227. doi: 10.1016/j.stem.2013.12.015

Shochet, G. E., Komemi, O., Sadeh-Mestechkin, D., Pomeranz, M., Fishman, A., Drucker, L., et al. (2016). Heat shock protein-27 (HSP27) regulates STAT3 and eIF4G levels in first trimester human placenta. *J. Mol. Histol.* 47, 555–563. doi: 10.1007/s10735-016-9699-7

Smith, A. (2017). Formative pluripotency: the executive phase in a developmental continuum. *Development* 144, 365–373. doi: 10.1242/dev.142679

Son, Y. S., Park, J. H., Kang, Y. K., Park, J.-S., Choi, H. S., Lim, J. Y., et al. (2005). Heat shock 70-kDa protein 8 isoform 1 is expressed on the surface of human embryonic stem cells and downregulated upon differentiation. *Stem Cells* 23, 1502–1513. doi: 10.1634/stemcells.2004-0307

Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Köntgen, F., et al. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* 359, 76–79. doi: 10.1038/359076a0

Strahl, B. D., and Allis, C. D. (2000). The language of covalent histone modifications. *Nature* 403, 41–45. doi: 10.1038/47412

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. doi: 10.1016/j.cell.2006.07.024

Tan, Y., Xue, Y., Song, C., and Grunstein, M. (2013). Acetylated histone H3K56 interacts with Oct4 to promote mouse embryonic stem cell pluripotency. *Proc. Natl. Acad. Sci.* 110, 11493–11498. doi: 10.1073/pnas.1309914110

Tessarz, P., and Kouzarides, T. (2014). Histone core modifications regulating nucleosome structure and dynamics. *Nat. Rev. Mol. Cell Biol.* 15, 703–708. doi: 10.1038/nrm3890

Thuret, J.-Y., Amigues, B., Mann, C., Agez, M., Guerois, R., Lautrette, A., et al. (2005). Structural basis for the interaction of Asf1 with histone H3 and its functional implications. *Proc. Natl. Acad. Sci.* 102, 5975–5980. doi: 10.1073/pnas.0500149102

Torres-Padilla, M.-E., and Chambers, I. (2014). Transcription factor heterogeneity in pluripotent stem cells: a stochastic advantage. *Development* 141, 2173–2181. doi: 10.1242/dev.102624

Trusler, O., Huang, Z., Goodwin, J., and Laslett, A. L. (2018). Cell surface markers for the identification and study of human naive pluripotent stem cells. *Stem Cell Res.* 26, 36–43. doi: 10.1016/j.scr.2017.11.017

Tsai, Y. P., Teng, S. C., and Wu, K. J. (2008). Direct regulation of HSP60 expression by c-MYC induces transformation. *FEBS Lett.* 582, 4083–4088. doi: 10.1016/j.febslet.2008.11.004

Vilchez, D., Boyer, L., Morantte, I., Lutz, M., Merkwirth, C., Joyce, D., et al. (2012). Increased proteasome activity in human embryonic stem cells is regulated by PSMD11. *Nature* 489, 304–308. doi: 10.1038/nature11468

Wadhwa, R., Takano, S., Kaur, K., Aida, S., Yaguchi, T., Kaul, Z., et al. (2006). Identification and characterization of molecular interactions between mortalin/mtHsp70 and HSP60. *Biochem. J.* 391(Pt 2), 185–190. doi: 10.1042/bj20050861

Wang, J., Rao, S., Chu, J., Shen, X., Levasseur, D. N., Theunissen, T. W., et al. (2006). A protein interaction network for pluripotency of embryonic stem cells. *Nature* 444, 364–368. doi: 10.1038/nature05284

Wang, S. Y., and Gudas, L. J. (1988). Protein synthesis inhibitors prevent the induction of laminin B1, collagen IV (α1), and other differentiation-specific mRNAs by retinoic acid in F9 teratocarcinoma. *cells. J. Cell. Physiol.* 136, 305–311. doi: 10.1002/jcp.1041360213

Wang, Z., Kang, L., Zhang, H., Huang, Y., Fang, L., Li, M., et al. (2019). AKT drives SOX2 overexpression and cancer cell stemness in esophageal cancer by protecting SOX2 from UBR5-mediated degradation. *Oncogene* 38, 5250–5264. doi: 10.1038/s41388-019-0790-x

Wong, M. M., Yin, X., Potter, C., Yu, B., Cai, H., Di Bernardini, E., et al. (2014). Over-expression of HSP47 augments mouse embryonic stem cell smooth muscle differentiation and chemotaxis. *PLoS One* 9:e86118. doi: 10.1371/journal.pone.0086118

Wu, T., Mu, Y., Bogomolovas, J., Fang, X., Veevers, J., Nowak, R. B., et al. (2017). HSPB7 is indispensable for heart development by modulating actin filament assembly. *Proc. Natl. Acad. Sci.* 114, 11956–11961. doi: 10.1073/pnas.1713763114

Xu, H., Wang, W., Li, C., Yu, H., Yang, A., Wang, B., et al. (2009). WWP2 promotes degradation of transcription factor OCT4 in human embryonic stem cells. *Cell Res.* 19, 561–573. doi: 10.1038/cr.2009.31

Xu, H. M., Liao, B., Zhang, Q. J., Wang, B. B., Li, H., Zhong, X. M., et al. (2004). Wwp2, An E3 ubiquitin ligase that targets transcription factor Oct-4 for ubiquitination. *J. Biol. Chem.* 279, 23495–23503. doi: 10.1074/jbc.M400516200

Yan, F. Q., Wang, J. Q., Tsai, Y. P., and Wu, K. J. (2015). HSP60 overexpression increases the protein levels of the p110α subunit of phosphoinositide 3-kinase and c-Myc. *Clin. Exp. Pharmacol. Physiol.* 42, 1092–1097. doi: 10.1111/1440-1681.12457

Yan, Z., Wei, H., Ren, C., Yuan, S., Fu, H., Lv, Y., et al. (2015). Gene expression of Hsps in normal and abnormal embryonic development of mouse hindlimbs. *Hum. Exp. Toxicol.* 34, 563–574. doi: 10.1177/0960327114555927

Yankulov, K. (2015). Totipotency in the absence of CAF-I: unhindered choices when the chaperone is out. *Nucleus* 6, 468–470. doi: 10.1080/19491034.2015.1121355

Yu, H., Lim, H. H., Tjokro, N. O., Sathiyanathan, P., Natarajan, S., Chew, T. W., et al. (2014). Chaperoning HMGA2 protein protects stalled replication forks in stem and cancer cells. *Cell Rep.* 6, 684–697. doi: 10.1016/j.celrep.2014.01.014

Zafarana, G., Avery, S. R., Avery, K., Moore, H. D., and Andrews, P. W. (2009). Specific knockdown of OCT4 in human embryonic stem cells by inducible short hairpin RNA interference. *Stem Cells* 27, 776–782. doi: 10.1002/stem.5

Zhang, B., Peñagaricano, F., Driver, A., Chen, H., and Khatib, H. (2011). Differential expression of heat shock protein genes and their splice variants in bovine preimplantation embryos. *J. Dairy Sci.* 94, 4174–4182. doi: 10.3168/jds.2010-4137

Zhang, W., Ni, P., Mou, C., Zhang, Y., Guo, H., Zhao, T., et al. (2016). Cops2 promotes pluripotency maintenance by stabilizing nanog protein and repressing transcription. *Sci. Rep.* 6, 4–12. doi: 10.1038/srep26804

Zhang, Z., Liao, B., Xu, M., and Jin, Y. (2007). Post-translational modification of POU domain transcription factor Oct-4 by SUMO-1. *FASEB J.* 21, 3042–3051. doi: 10.1096/fj.06-6914com

Zhu, Y., Zhu, J., Wan, X., Zhu, Y., and Zhang, T. (2010). Gene expression of sHsps, Hsp40 and Hsp60 families in normal and abnormal embryonic development of mouse forelimbs. *Toxicol. Lett.* 193, 242–251. doi: 10.1016/j.toxlet.2010.01.016

Zhu, Z., Li, C., Zeng, Y., Ding, J., Qu, Z., Gu, J., et al. (2017). PHB associates with the hira complex to control an epigenetic-metabolic circuit in human ESCs. *Cell Stem Cell* 20, 274.e7–289.e7. doi: 10.1016/j.stem.2016.11.002

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REVIEW
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Cardiomyogenesis Modeling Using Pluripotent Stem Cells: The Role of Microenvironmental Signaling

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Pluripotent stem cells (PSC) can be used as a model to study cardiomyogenic differentiation. *In vitro* modeling can reproduce cardiac development through modulation of some key signaling pathways. Therefore, many studies make use of this strategy to better understand cardiomyogenesis complexity and to determine possible ways to modulate cell fate. However, challenges remain regarding efficiency of differentiation protocols, cardiomyocyte (CM) maturation and therapeutic applications. Considering that the extracellular milieu is crucial for cellular behavior control, cardiac niche studies, such as those identifying secreted molecules from adult or neonatal tissues, allow the identification of extracellular factors that may contribute to CM differentiation and maturation. This review will focus on cardiomyogenesis modeling using PSC and the elements involved in cardiac microenvironmental signaling (the secretome – extracellular vesicles, extracellular matrix and soluble factors) that may contribute to CM specification and maturation.

Keywords: cardiomyocytes, pluripotent stem cell, secretome, cell differentiation, maturation

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INTRODUCTION

Pluripotent stem cells (PSC), both embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), show strong potential to proliferate and differentiate. PSC have already been successfully differentiated into a number of cell types, including cardiomyocytes (CM) (Murry and Keller, 2008). Key events that regulate lineage commitment can be reproduced *in vitro* and used as a model to study cardiomyogenesis, to generate CMs and produce clinically relevant cell populations, and to evaluate cardiac toxicity or model congenital abnormalities (Kehat et al., 2001; Xu et al., 2002; Laflamme et al., 2007; Kattman et al., 2011; Burridge et al., 2012). Despite the advances in this field, new challenges are emerging, mainly related to cardiac differentiation efficiency and the functional maturation of human PSC-derived cardiomyocytes (hPSC-CM).

This review discusses cardiac differentiation and hPSC-CM maturation approaches that use extracellular components of the cardiac microenvironment. Initially, an overview of hPSC cardiomyogenic differentiation protocols was described, indicating some of the essential signaling pathways that control CM commitment. However, the main focus is to explore the cardiac niche, its components and the strategies developed to mimic its complexity *in vitro*. After a brief description of important signals and interactions available in a tissue niche, we emphasize aspects related to cardiac extracellular matrix (ECM; composition or structure), soluble factors and extracellular vesicles (EVs) that could influence *in vitro* CM differentiation and maturation.

OVERVIEW OF HEART DEVELOPMENT

The heart is a complex muscular organ composed of several cell types, including CM, smooth muscle cells (SMC), endothelial cells (EC), cardiac fibroblasts (cFB), and cardiac progenitor cells (CPC). Although CM occupy most of the heart volume, they comprise only ∼40% of the total cells. The other 60% largely comprises EC and cFB, however, the percentage of each of them is still not certain (Banerjee et al., 2007; Bergmann et al., 2015; Pinto et al., 2016).

The heart is the first organ to become functional in the vertebrate embryo (Brand, 2003). Although the heart develops early, cardiogenesis is a highly regulated process involving differentiation and cellular specialization, spatial integration and coordination of several signaling pathways. Cardiac tissue is mostly derived from the mesodermal layer and the induction to the cardiomyogenic phenotype depends on signals derived from adjacent layers, such as endodermal and ectodermal cells (Wagner and Siddiqui, 2007; Sun and Kontaridis, 2018). The signaling factors modulated over heart development include members of bone morphogenetic proteins (BMPs), Activin and NODAL, fibroblast growth factor (FGF), and Wingless (Wnt) families (Brand, 2003; Wagner and Siddiqui, 2007; Liu and Foley, 2011; Brade et al., 2013; Sun and Kontaridis, 2018). In **Figure 1**, we briefly highlight some aspects of embryonic cardiac commitment that will be important to understand and support the *in vitro* differentiation protocols using PSC. The signaling pathways influencing the stages of cell differentiation and the cell markers expressed in these different stages are indicated (**Figure 1**). For more details about the morphogenesis, signaling pathways and factors involved in this process, see Wagner and Siddiqui (2007), Brade et al. (2013), Sylva et al. (2014), Paige et al. (2015), Sun and Kontaridis (2018).

IN VITRO DIFFERENTIATION OF hPSC

Cardiac cell fate specification occurs through progressive steps that we are currently able to reproduce at the laboratory. There are three major strategies to derive CM from hPSC: (1) inductive coculture, (2) embryoid bodies, and (3) monolayer cultures. **Table 1** summarizes these strategies and their main references [complete reviews can be found in Burridge et al. (2012), Mummery et al. (2012), Denning et al. (2016)].

The inductive coculture protocol uses visceral endoderm-like cells (END-2), which play an important role in signaling the adjacent mesoderm in developing embryos, to induce the differentiation of cardiogenic precursor cells. This protocol was developed by Mummery et al. (2003) and improved by them in Mummery et al. (2007). The convenience of the coculture is that it requires few cells and is less time-consuming. On the other hand, the efficiency of the protocol is very low, and it is not commonly used.

Three-dimensional aggregates, known as embryoid bodies (EBs), represent the first structure in which CM could be produced *in vitro*. Using a protocol to derive spontaneous differentiation, contracting structures containing CM and other germ layer derivatives were generated (Itskovitz-Eldor et al., 2000). These EBs could also be transferred to attachment culture plates and give rise to beating areas (Kehat et al., 2001; Zhang et al., 2009). Studies in animal models have helped with valuable information for the optimization of *in vitro* hPSC differentiation protocols (Marvin et al., 2001; Ueno et al., 2007). Cardiomyocyte derivation from hPSC can be manipulated and directed to cardiac lineage by specific elements, such as growth factors known to be involved in *in vivo* heart development (Vidarsson et al., 2010). The same signaling pathways mentioned above as essential for heart development are also used to modulate hPSC differentiation *in vitro*, such as BMP, Nodal, FGF, and Wnt (Filipczyka et al., 2007). Concentration and time of addition or removal of specific factors, such as BMP4, activin A and Wnt modulators, are critical for cardiac lineage specification and were adapted in the protocols to improve efficiency (Yang et al., 2008; Kattman et al., 2011; Ren et al., 2011). A similar idea was applied to the monolayer-based protocol, in which the use of growth factors and other molecules could improve efficiency and would not be interfered by the diffusional barrier present in EBs (Mummery et al., 2012). Using a defined serum-free medium supplemented with BMP4 and activin A, Laflamme et al. (2007) reported better efficiency in CM differentiation in the monolayer system compared to the EB method. Other adaptations were made (Lian et al., 2012; Burridge et al., 2014), including the use of more specific and low cost signaling small molecules, such as CHIR99021 (an inhibitor of glycogen synthase kinase 3), leading to the monolayer protocol becoming the most popular and routinely employed cardiogenic differentiation method (Denning et al., 2016). In addition, the ECM influence was also tested to improve differentiation efficiency. Combining ECM with growth factor signaling in a protocol that uses a double Matrigel layer, so-called matrix sandwich, CM could be generated with high purity (Zhang J. et al., 2012).

Advances in methodologies to direct cardiac differentiation helped to improve efficiency in the protocols, increasing the final percentage of CM (usually cardiac troponin T positive – cTnT^{+}). However, the purity of these populations is still a limitation in the field. Some approaches were developed to enrich CM, rather than other cell types. For example, genetic selection strategies were based on the expression of a drug resistance gene or reporter protein gene under the control of a cardiac-specific promoter (Xu et al., 2008; Kita-Matsuo et al., 2009; Elliott et al., 2011). Reporter protein genes could also be applied in flow cytometry sorting, as well as selection by markers from distinct stages of differentiation (Yang et al., 2008; Lin et al., 2012; Den Hartogh et al., 2015). Purification of CM using a Percoll gradient (Xu et al., 2002) and energy metabolism differences (Tohyama et al., 2013) were also established. In addition to the purity of final populations, another limitation of *in vitro* cardiogenic differentiation is that the currently available methods generate a heterogeneous CM population that includes a mix of subtypes, such as ventricular, atrial, pacemaker, and non-contractile cells (Kolanowski et al., 2017; Friedman et al., 2018). Strategies to derive specific cardiac cell subtypes are being developed and could help the demand for therapeutic applications of these cells (Zhang et al., 2011;

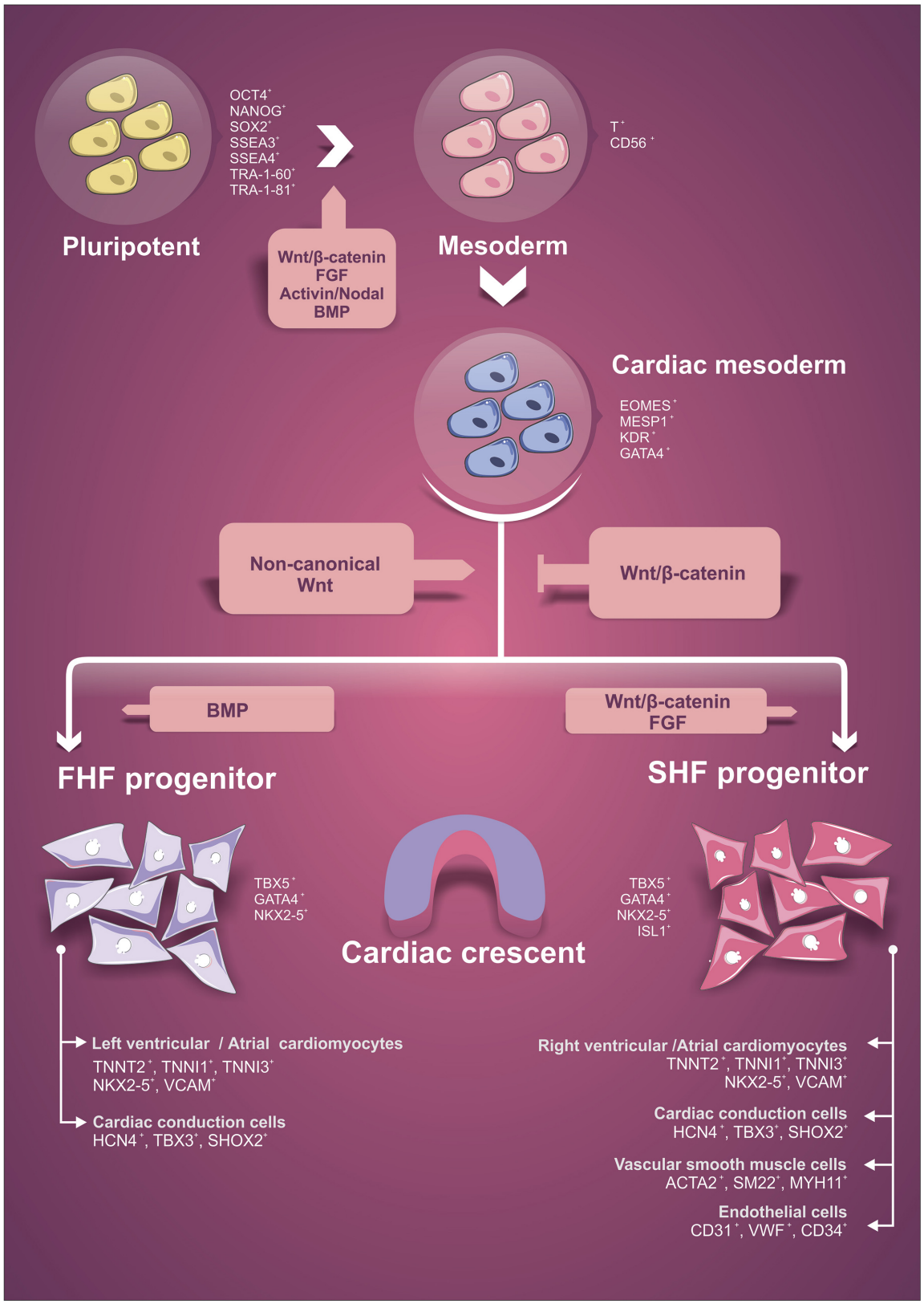

FIGURE 1 | Schematic representation of the initial steps of cardiac lineage commitment. Indication of signaling pathways that influence each differentiation stage and the specific cellular markers expressed during lineage differentiation. FHF, first heart field. SHF, second heart field.

TABLE 1 | Three major *in vitro* cardiac differentiation protocols.

Cardiac differentiation protocols		
Strategy	**Induction method**	**Main References**
Inductive coculture with END-2 *visceral endoderm-like cells*	direct coculture or END-2 conditioned medium	Mummery et al., 2003, 2007
Embryoid Body (EB)	spontaneous differentiation	Itskovitz-Eldor et al., 2000; Kehat et al., 2001; Zhang et al., 2009
	growth factors in defined media (GFs)	Yang et al., 2008; Kattman et al., 2011; Ren et al., 2011
Monolayer	growth factors in defined media (GFs)	Laflamme et al., 2007
	small molecules in defined media (SM)	Lian et al., 2012; Burridge et al., 2014

Their main references are indicated.

Karakikes et al., 2014; Devalla et al., 2015; Protze et al., 2016; Lee J.H. et al., 2017).

Another challenge in the field of hPSC cardiac differentiation is related to the maturity of hPSC-CM: most of the protocols generate immature CM. In recent years, a great number of studies have focused on investigating strategies to improve the maturation of hPSC-CM and make them more similar to adult CM (Sartiani et al., 2007; Lundy et al., 2013; Robertson et al., 2013), which are multinucleated (25–30%) with highly organized sarcomeres (I, A, and Z bands, M lines and intercalated disks), T-tubules, high expression of sarcomeric and ion channel genes, fatty acid β-oxidation metabolism, higher contractile force and upstroke and conduction velocities (Yang et al., 2014; Dunn and Palecek, 2018; Machiraju and Greenway, 2019). Among the approaches to achieve this purpose are the prolonged time for culture (e.g., 120–360 days), addition of hormones, metabolites or other soluble factors, mechanical or electrical stimulus, microtissue development, coculture with other cell types (such as SMC, cFB, and EC), stiffness regulation, and tridimensional (3D) cultures with biomaterials or ECM components (reviewed by Yang et al., 2014; Besser et al., 2018; Dunn and Palecek, 2018; Machiraju and Greenway, 2019).

Hence, cardiac differentiation efficiency and, consequently, the purity of the cell population and the immature phenotype of hPSC-CM are among the challenges regarding the use of these cells for drug discovery models, development studies, tissue engineering or cellular therapies. Considering the importance of the microenvironment for cellular behavior, we believe that knowledge about the cardiac niche and the use of strategies to mimic its specificities could improve (or create new) hPSC cardiac differentiation and maturation approaches. Since the coordination of all cellular processes including how the cells participate in the microenvironment is governed, inter alia, by gene expression, transcriptomic and proteomic analyses can contribute to the elucidation of cardiac niche specificities and also help to overcome the challenges of using hPSC-CM.

High-throughput studies have allowed the investigation of the global changes in gene expression at the transcriptional, post-transcriptional and protein levels, contributing with considerable insights about gene regulatory programs that are crucial to control cardiac tissue formation (**Table 2**). Distinct gene expression patterns drive PSC into specific cell types, consequently, the investigation of sequential stages during differentiation might help to discover essential molecules that determines the final destination of a cell. For instance, Paige et al. (2012) and, more recently, Liu et al. (2017) showed a temporal alteration in chromatin structure when analyzing distinct time-points of *in vitro* cardiomyogenesis. Changes in DNA methylation and post-transcriptional regulation were also described during cardiac differentiation and potentially participate in the modulation of gene regulatory programs (Tompkins et al., 2016; Fu et al., 2018; Pereira et al., 2019). Key regulators of cardiovascular development identified in those studies could be used to improve differentiation protocols, e.g., activating or inhibiting a specific signaling pathway. In addition, the identification of new cell surface proteins, such as the studies of den Hartogh et al. (2016) and Van Hoof et al. (2010), could provide new markers for development stages, cell lineage, cell subtype or maturation. Proteomic approaches also have highlighted important aspects regarding the cardiac commitment process, such as the metabolic and mitochondrial maturation described by Poon et al. (2015), the identification of metabolic and cytoskeletal proteins by Konze et al. (2017b) and the identification of proteins related to specific metabolic process, e.g., ketogenesis, described by Kim et al. (2019). Genes related to the ECM were also shown as modulated throughout the cardiac commitment process (den Hartogh et al., 2016; Pereira et al., 2019), and it will be discussed later in this review.

TISSUE MICROENVIRONMENT: COMPOSITION, INTERACTIONS, AND IMPORTANCE

Stem cells are modulated by microenvironmental cues in which they are embedded. This surrounding microenvironment is called the stem cell niche and is able to support cell maintenance and regulate the expansion or differentiation of stem cell populations. Thus, the stem cell niche was defined as an anatomical structure that includes cellular and acellular components, which integrates local and systemic factors that regulate stem cell behavior (Jones and Wagers, 2008). Since first being described by Schofield (1978), the niche concept has been expanded; it currently includes many components: the stem cell itself and tissue stromal cells; the ECM and related molecules; the secreted factors and extracellular vesicles (EVs); the blood vessels that carry systemic signals and cells (as immune regulators); the neural stimuli; oxygen concentration; and physical cues, such as shear stress (Jones and Wagers, 2008; Ferraro et al., 2010).

Cell signaling in the niche should be pictured as a result of multiple interactions and stimuli involving cell-to-cell connections, adhesive and de-adhesive ECM proteins, soluble trophic factors, and EVs. **Figure 2** depicts some of the interactions that might occur along with niche networking.

TABLE 2 | Summary of transcriptomic and proteomic studies based on hPSC cardiac differentiation.

Differentiation method	Time-points	High throughput method	Highlights	References
Monolayer in END-2 coculture	Days 0, 1, 3, 6, 9, and 12	Agilent Microarray	Identification and validation of time-dependent gene expression patterns	Beqqali et al., 2006
EB spontaneous differentiation	hESC, CM, and hF heart	Agilent Microarray	hESC-CM promoting recovery from cardiac ischemia reperfusion injury	Cao et al., 2008
EB in END-2 conditioned medium	hESC, EBs, CM-Day 21, fetal heart and adult heart	Illumina microarray	Evaluation of the biological relevance of uncharacterized genes	Xu et al., 2009
EB (GFs)	Days 0, 2, 5, 9, and 14	ChIP-seq and Affymetrix array	Temporal alterations in chromatin structure identify key regulators of cardiovascular development	Paige et al., 2012
EB (GFs) KDRlow/CD166 +	hESC or iPS, M-Day 6, Day 20: CM, SMC, EC	Illumina RNA-seq	Lineage-enriched genes and lncRNAs, RNA splicing isoforms	Li et al., 2015
Monolayer (SM/C)	hESC, D3, D4 and CM-D31	Illumina RNA-seq and MDB-seq	TFs, miRNAs, lncRNAs and methylome	Tompkins et al., 2016
Monolayer (GFs, SM/C) ∗ Day 3 MESP1 + sorting	Days 0, 3, 5, 7, 10, and 14	Illumina Microarray	Regulation of ECM components and new cell surface markers	den Hartogh et al., 2016
Monolayer cardiomyocyte differentiation kit (Thermo Fisher Scientific)	Days 0, 2, 4, and 30	RNA-seq and ATAC-seq	Mapping open chromatin patterns	Liu et al., 2017
Monolayer (GFs)	Days 0, 12 and 20	Illumina Microarray and ChIP-seq	Genetic and epigenetic changes and a role for NR2F2	Pursani et al., 2017
Monolayer (SM/C)	Days 0, 2, 5, 15, and 30	Illumina single-cell RNA-seq	Cardiomyocyte hypertrophy and maturation	Friedman et al., 2018
Monolayer (SM/C)	Days 0, 5, 14, and 45	Single-cell RNA-seq	Single-cell heterogeneity	Churko et al., 2018
EB (SM/C)	Days 0, 2, 5, 15 and 30	Illumina RNA-seq and RRB-seq	TFs, lincRNAs and DNA methylation changes	Fu et al., 2018
EB (GFs)	Days 0, 1, 4, 9, and 15	Illumina RNA-seq polysome	Polysomal RNAs and post-transcriptional regulation	Pereira et al., 2018
Monolayer (SM/C)	Days 0, 2, 5, and 14	RNA-seq and ATAC-seq	Interplay of local and global chromatin structure on gene regulation	Bertero et al., 2019
Monolayer in END-2 coculture	differentiated hESC, enriched populations of hESC-derived CM and primary hF CM	SILAC-based quantitative MS	Identification of cell surface proteins for antibody-based selection	Van Hoof et al., 2010
EB (GFs)	hESC, hESC-VCMs, hF-VCMs, and hA-VCMs.	2D-Differential-In-Gel Electrophoresis followed by MS	Metabolic and mitochondrial maturation	Poon et al., 2015
Monolayer (GFs, SM/C)	Days 0, 5, and 14	label-free quantitative	Identification of known and unknown regulatory proteins	Hofsteen et al., 2016
Monolayer (SM/C)	Days 0, 20, and 35	SILAC-labeled	Metabolic and cytoskeletal proteins	Konze et al., 2017b
Monolayer (SM/C)	Days 0, 7, and 15	SILAC upon PAL-based capture of sialylated glycoproteins (glyco-proteomic)	Global proteomic, sialo-glycoproteomic, and glycomic characterization	Konze et al., 2017a
Monolayer (SM/C)	Days 0, 5, and 15	Three-plex tandem mass tag labeling	Identification of proteins associated with branched chain amino acid degradation and ketogenesis	Kim et al., 2019

CM, cardiomyocytes; END-2, visceral endoderm-like cells; EB, embryoid body; EC, endothelial cells; GFs, growth factors; hF, human fetal; hA, human adult; iPSC, induced pluripotent cell; lncRNA, long non-coding RNA; M, multipotential cardiovascular progenitor cells; miRNA, microRNA; MS, mass spectrometry SMC, smooth muscle cells; SM/C, small molecules and chemical compounds; TFs, transcriptional factors; VCM, ventricular cardiomyocytes.

The complex netting of fibrillar proteins, proteoglycans and glycoproteins that compose the ECM acts in support and structure cells in the niches. In addition, ECM proteins and bioactive fragments released from the ECM by enzymatic degradation (the so-called matrikines) trigger cellular responses through interaction with cell receptors (Rozario and DeSimone, 2010; Ricard-Blum and Salza, 2014). Additionally, the ECM provides signals to the cells through its mechanical features (e.g., stiffness and elasticity) and its ability to function as a reservoir of growth factors and other bioactive elements. These secreted soluble factors may act locally or may diffuse throughout the niche, generating a concentration gradient (Jones and Wagers, 2008; Brizzi et al., 2012) that activates/inhibits signal pathways in the cells through autocrine/paracrine routes. Furthermore, another important player to consider on the niche site is the EVs that load different types of cargo (e.g., proteins,

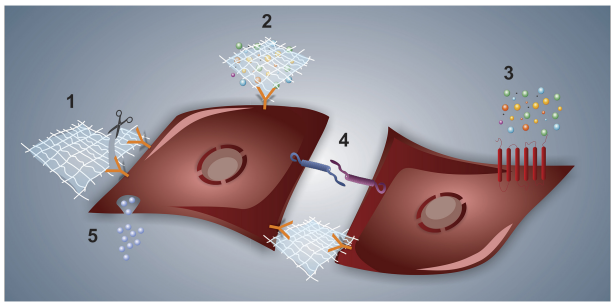

FIGURE 2 | Types of interactions at the cell–niche interface. In (1), a representative interaction between the cell and ECM is shown, whereas the matrikine-receptor interaction is pictured alongside the scissor, which represents matrix proteases. The ECM can also bind to and present a given trophic factor to the cell, as depicted in (2). The typical cytokine–receptor interaction is shown in (3). Cell-to-cell interactions are illustrated in (4), both by interacting membrane adhesive proteins and through ECM-integrin-mediated interaction. Finally, extracellular vesicle-driven signaling is represented in (5).

small RNAs, lipids) and function in intercellular communication (Stik et al., 2017; Durand et al., 2018).

Distinct stem cell populations are arranged in unique and specific tissue microenvironments (Xin et al., 2016), within which the way stem cells behave depends on the interactions between specific cells and niche elements. Regarding the heart, cardiogenic niches are highly dynamic, presenting different functionalities and characteristics according to the stage of development of the heart and its physiological state. During the early stages of development, the elements of the cardiogenic niche play roles related primarily to cell expansion and specification, controlling the size and shape of cardiac structures. Subsequently, the niche elements stimulate the completion of differentiation and maturation of cardiac cells (revised by Christalla et al., 2012). In the adult heart, however, the current understanding of the cardiac niches is limited. Fatih Kocabas et al. (2012) identified putative cardiac niches in the heart epicardium and sub-epicardium with low oxygen tensions and housing a metabolically distinct population of glycolytic progenitor cells. Future attempts to characterize the stem cell niches in the heart atria and apex, however, are controversial. Nevertheless, cardiac niches may contain quiescent stem cells and progenitors in addition to the influence of all other heart cells (e.g., EC, CM, and SMC) that interacts and communicate together secreting a wide range of ECM proteins and soluble factors (reviewed by Aguilar-Sanchez et al., 2018).

Besides cell-to-cell interactions, ECM and soluble trophic factors present in the tissue microenvironment, other important modulators to consider are the biomechanical and electrical stimuli that influence the differentiation and maturation of cardiomyocytes, and the correct formation of the heart. Mechanical forces present during cardiac development include shear and strain stress, flow forces (i.e., blood flow), pressure and stretch. These mechanical signals are sensed by cells and converted into intracellular signals that activated pathways that induce gene expression and cellular commitment. The perception and transmission of signals involve both cell–cell and cell–ECM interactions (reviewed by Lindsey et al., 2014; Andrés-Delgado and Mercader, 2016; Stoppel et al., 2016). The mechanical force dynamics in the development of the heart involves, e.g., cFB that secrete a great amount of ECM proteins influencing the stiffness and topography of the organ while cardiomyocytes improves expression of sarcomeric proteins increasing the contractile stress and tissue strength. As a result, the heart pumps more blood, which increases the flow forces (reviewed by Majkut et al., 2014). All these biomechanical forces are interconnected and influence different processes during heart formation, from the proliferation and differentiation of cardiomyocytes to the correct formation of valves and cardiac chambers. Considering the importance of mechanical and electrical signals in cardiac development, many approaches have been developed in an attempt to understand and mimic these signals *in vitro*, with the aim of improving cardiomyocyte differentiation or maturation (reviewed by Zhu et al., 2014; Stoppel et al., 2016; Besser et al., 2018).

Some possibilities to approach cell-niche signaling relies on the use of PSC cardiomyogenic modeling, culture of cardiac tissue explant/cardiospheres and isolated cell populations (e.g., cardiac progenitor cells). **Figure 3** summarizes the current strategies used to model cardiomyogenesis or cardiac cell/tissue behavior *in vitro* and ways to analyze the cardiac secretome (CS). The secretome comprises the complex set of secreted molecules/vesicles from cells to the extracellular space. At the cell–environment interface, the biological activity of the secretome is exerted by the modulation of signal transduction pathways that direct fundamental biological processes on cells, including cell fate and proliferation (Reus et al., 2016). Through the analysis of the ECM composition, soluble factors and EVs derived from cell and tissue cultures, we can search for essential signals and understand regulatory networks underlying human heart development. Regarding this issue, genomic and proteomic studies have provided important contributions (Reus et al., 2016; Wolling et al., 2018; Leitolis et al., 2019; Pereira et al., 2019). In the next sections, we will describe approaches to study the composition of cardiac secretome and discuss strategies that use the extracellular signals to induce PSC cardiac differentiation and maturation of PSC-CM.

EXPLORING THE CARDIAC ECM: COMPOSITION, TISSUE ENGINEERING STRATEGIES AND *IN VITRO* MODELING

Considering the actual challenges related to the use of PSC, the development of three-dimensional (3D) heart tissues constructs represents a strategy to improve *in vitro* models, both for studies concerning heart physiology and drug screening and to advance *in vivo* (translational) applications (Hirt et al., 2014; Ogle et al., 2016). However, recapitulating the complexity of cardiac tissue *in vitro* – structure, composition, and mechanical properties – is not an easy task. In this context, knowing the ECM, an important niche component, may have a relevant role in the development of new approaches to mimic heart tissue. Some methodologies use synthetic or natural biomaterials, singular ECM proteins or

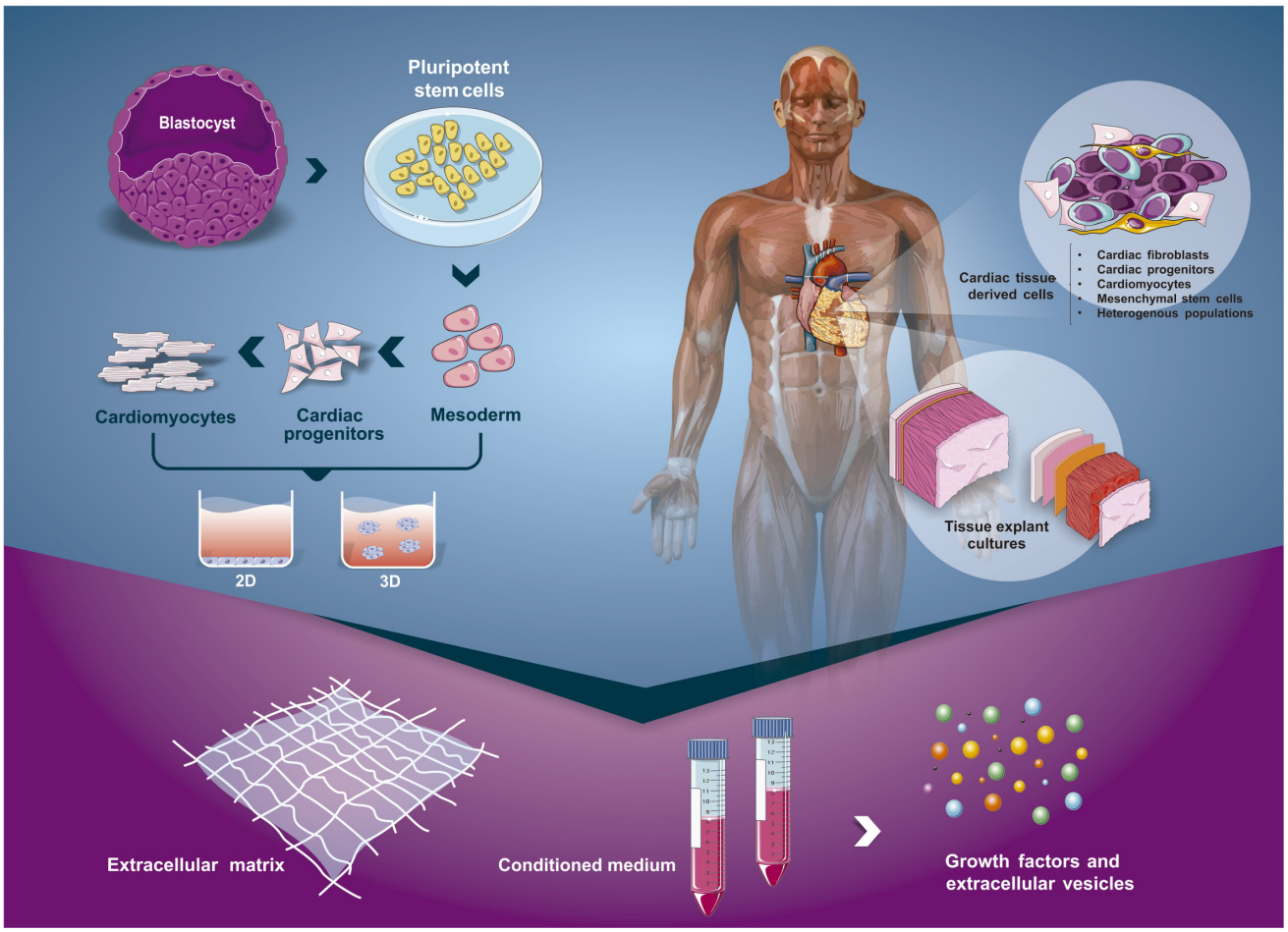

FIGURE 3 | Scheme of different approaches to isolate the cardiac secretome. Extracellular matrix, soluble factors and extracellular vesicles can be obtained from the cardiomyogenic developmental process (using hPSC) or from adult tissues through the use of different strategies. The process of hPSC cardiac differentiation can occur using 2D (monolayer) or 3D (embryoid bodies) cultures. On the other hand, with fragments of neonate or adult heart, we could isolate specific cell populations from the tissue (e.g., fibroblasts, cardiac progenitors) or culture the entire or parts of the cardiac tissue.

their combinations, and decellularized cardiac tissue (Smith et al., 2017; Zhang et al., 2017).

Cardiac ECM Composition

The ECM is not an inert scaffold restricted to supporting and structuring cells in tissues or organs but is also an important modulator of cellular responses either through its direct interaction with cell receptors, through the control of growth factor activities, controlling their diffusion or release, and transmitting mechanical signals (Rozario and DeSimone, 2010). ECM proteins comprise approximately 1–1.5% of the mammalian proteome called the "core matrisome," which is composed of approximately 300 proteins shared among glycoproteins, proteoglycans and many collagen types. In addition, there are a great number of ECM-associated proteins, including the group of mucins and lectins, enzymes, matrix metallopeptidases, and secreted factors, such as chemokines, interleukins, and growth factors (Hynes and Naba, 2012; Naba et al., 2012, 2016). Considering the diversity of ECM components, the content of ECM varies among tissues, phase of development and according to the pathophysiological state of an organ (Christalla et al., 2012; Bonnans et al., 2014; Kular et al., 2014).

The cardiac ECM composition and function have been studied over the last few years. Several collagen types, fibronectin, laminin, fibrillins, proteoglycans, such as perlecan, agrin and glypicans, and glycosaminoglycans, mainly hyaluronan, are among the constituents of heart ECM (Lockhart et al., 2011; Rienks et al., 2014). As a dynamic milieu, the expression of the heart ECM varies during cardiac development and, accordingly, development of the heart region. Furthermore, it was previously described that the deletion of some ECM proteins causes serious heart defects, many of them causing embryonic lethality, as reviewed by Lockhart et al. (2011). Another component present in the ECM, which is important in the development of the heart and in its response to injury, is matricellular proteins. Although these proteins do not have structural function, they interact with surface receptors, growth factors, and ECM, among others, aiming to integrate the signals of the microenvironment. Among

the members of this group are thrombospondin, tenascin, SPARC (acid secreted protein and cysteine rich), osteopontin, periostin and members of the CCN family (Frangogiannis, 2012, 2017; Rienks et al., 2014; Rienks and Papageorgiou, 2016).

Among heart cells, cFB are the main producers of cardiac ECM proteins. They are responsible for the ECM homeostasis, maintaining the balance between production and degradation of ECM proteins that are essential for the correct heart function. Despite its importance, there is still a lack of knowledge about the surface markers that could reveal their real molecular identity and, even, about functionality during the development of the heart. The cFBs are mainly derived from cells of the epicardium (with some contribution of the endothelium) that undergo epithelial-to-mesenchymal transition. In addition to ECM proteins, they secrete soluble factors (as will be discussed later in this review) and are also important in transducing and responding to electromechanical signals (Furtado et al., 2016; Civitarese et al., 2017; Tallquist and Molkentin, 2017). Interestingly, it was described that cFB expressed many specific cardiogenic genes, different of fibroblasts from other sources (Furtado et al., 2016). In adult heart, several factors can affect cFB activation, including response to injury. In this case, cFB can differentiate into myofibroblasts, which have increased proliferative and secretory capacity. The raise in the number of cFB and/or the deposition of ECM proteins causes fibrosis and, consequently, changes the rigidity of the tissue, the interaction between the cells, impairs myocyte contractility, oxygenation and metabolism (Tirziu et al., 2010; Martin and Blaxall, 2012; Fountoulaki et al., 2015), completely changing the cardiac microenvironment.

Originally, to better understand the role of cardiac ECM in embryonic development and elucidate its composition, the majority of studies used murine and other animal models (chicken, zebrafish) or were based on data related to congenital human cardiac diseases (Lockhart et al., 2011). Recently, one alternative is the use of hPSC cardiomyogenic differentiation to investigate ECM proteins secreted by cells. High-throughput analysis during hPSC differentiation showed that genes related to the ECM are modulated throughout the cardiac commitment process (den Hartogh et al., 2016; Pereira et al., 2019). Additionally, it was verified that during CM differentiation, total proteoglycan and hyaluronan decreased (Chan et al., 2010); meanwhile, using an endogenous optical signal, it was showed that elastin increased until day 9 of the murine EB differentiation protocol, and type I collagen (Col I) increased over 12 days (Thimm et al., 2015). Using a spontaneous differentiation protocol and isolating the beating areas, it was demonstrated that hESC-CM were surrounded by types I, IV, and XVIII collagens, laminin isoforms and fibronectin (FN) (van Laake et al., 2010).

In an attempt to characterize the CPC niche, Schenke-Layland et al. (2011) isolated fetal mouse and human hearts and identified a population of CPC that expressed $Isl1^{+}Flk1^{+}$. This population was delineated by a basement membrane with, primarily, collagen type IV (Col IV) and laminin, while FN and Col I were also present but more distant from it. Considering these results, it was shown that murine ESC (mESC) monolayer cultures with Col IV and laminin increase the number of CPC ($Flk1^{+}$), a number that was even higher in 3D Col IV cultures (Schenke-Layland et al., 2011).

Myocardial Tissue Scaffolds

More recently, the understanding of the native ECM composition and its role in cell behavior has advanced due to decellularization approaches (Crapo et al., 2011). Decellularization techniques aimed to remove all cellular components of an organ or tissue, maintaining the ECM proteins and structure (Crapo et al., 2011; Tang-Quan et al., 2018). This approach leads to distinct possibilities, including the development of new cardiac tissue engineering strategies, through the use of decellularized/recellularized organs in transplants, as well as allowing the characterization of the cardiac ECM in normal or pathological conditions. Over the last 10 years, different decellularization and recellularization strategies were developed and performed with murine (Ott et al., 2008; Carvalho et al., 2012; Lu et al., 2013; Wang et al., 2019), porcine (Ott et al., 2008; Weymann et al., 2014; Ferng et al., 2017; Lee P.-F. et al., 2017), bovine (Arslan et al., 2018) and even human (Sánchez et al., 2015; Garreta et al., 2016; Guyette et al., 2016) heart tissue (revised by Scarrit et al., 2015; Tang-Quan et al., 2018).

Regarding ECM characterization, histochemical and immunofluorescent analyses were performed to visualize the ECM structure and some of its components. Large-scale proteomic analysis through mass spectrometry of matrix proteins could be difficult, since they are poorly soluble as a result of their macromolecular nature, extensive posttranslational modifications and the tendency to form protein complexes (Chang et al., 2016; Lindsey et al., 2018). Nevertheless, many approaches have sought to improve the protocols for mass spectrometry ECM characterization, allowing the identification of heart ECM proteins and collaborating with advances in the area (Lindsey et al., 2018). The number of extracellular proteins identified varies according to the form of ECM solubilization, analysis and equipment used.

Different extracellular protein combinations were identified after decellularization processes in the infarcted area of the mouse left ventricle (De Castro Brás et al., 2013), in normal porcine myocardium (Perea-Gil et al., 2018), after ischemia/reperfusion porcine heart injury (Barallobre-Barreiro et al., 2012), from rat hearts (Nguyen et al., 2018) and others. Human cardiac tissues were also characterized under normal conditions (Guyette et al., 2016; Johnson et al., 2016; Robert et al., 2017). For example, Johnson et al. (2016) used ECM-target and non-target methodologies to quantify 43 proteins in decellularized samples and identified more than 200 proteins in the global approach; these researchers also verified some variation in ECM composition between different heart donors. Despite the differences, in general, the matrisome components found in greater quantity in decellularized cardiac ECM are collagen types, glycoproteins, such as fibronectin, and members of the basal membrane, such as laminins and perlecan.

Rat neonatal CM, EC (rat or human origin), hCPC, mesenchymal stem cells (MSC), hPSC and hPSC-CM or hPSC-CPC were some of the cells used for tissue recellularization (Scarrit et al., 2015; Tang-Quan et al., 2018). Using undifferentiated

hESC or hESC-derived mesendodermal cells, Ng et al. (2011) recellularized mouse hearts and, after 14 days under static culture conditions, cells began to express cardiac markers, such as cTnT, Nkx2.5, Myh6, and others. However, the cells were unable to contract, even *in vivo* (Ng et al., 2011). Lu et al. (2013) demonstrated that hiPSC-derived cardiac multipotent progenitors perfused into a decellularized mouse heart were able to migrate, proliferate and differentiate *in situ* into CM, SMC and EC, showing spontaneous contractions after 20 days, but they did not reach complete organ recellularization (Lu et al., 2013). Indeed, whole organ recellularization is an important barrier in cardiac tissue engineering. As a consequence, recent approaches include the use of cardiac patches with fragments/slices of decellularized matrices or its soluble form/hydrogel.

When undifferentiated mESC were cultured in slices of decellularized mouse hearts, they were able to express higher levels of cardiac markers in comparison with mESC cultured in slices of decellularized liver (Higuchi et al., 2013). Through the use of laser-cut sections of decellularized porcine myocardium, it was developed a system capable of maintaining hPSC-CM with organized sarcomeres and gap junctions that allowed the characterization of the biomechanical function of these EHT (Schwan et al., 2016). Pieces of decellularized rat heart were cultured with MSCs and hPSC-derived mature ventricular CM, which attached and formed a tissue-like structure with not only CM but also SMC and EC (Li et al., 2017). Other types of decellularized ECM were also tested both to support and differentiate PSC and to maintain PSC-CM. Hong et al. (2018) verified that decellularized mouse skeletal muscle was able to induce mESC to differentiate to a cardiac phenotype and supported mESC-CM (Hong et al., 2018). In addition, human placenta-derived hydrogel is another decellularized ECM that proved to be sufficient to maintain and generate more synchronized and electrically coupled hiPSC-CM (Francis et al., 2017).

Using human decellularized cardiac tissue, Oberwallner et al. (2015) showed that this ECM could support the mESC and iPSC and favored cardiac lineage differentiation (Oberwallner et al., 2015). More recently, slices of decellularized human heart cultured with PSC-CM presented spontaneous beating after 7–10 days, confirming cell–matrix interaction, demonstrated better electrophysiological response than in Matrigel and showed increased expression of cardiac ion channels in CM (Garreta et al., 2016). Additionally, Guyette et al. (2016), using two different tissue thicknesses and PSC-CM, showed spontaneous tissue contraction after 4–10 days, maintenance in culture for 60 or 120 days and the formation of a mechanical and electrical active myocardial tissue (Guyette et al., 2016).

Despite 3D approaches, an alternative is the use of hydrogels derived from decellularized ECM. Hydrogel prepared from decellularized porcine hearts combined with Col I, in proportion 75% ECM and 25% collagen, increased the number of cells expressing cTnT in comparison with those hydrogels with low ECM content (25%) or 100% collagen. This strategy also improved the maturation of hESC EBs, as demonstrated by the upregulation of connexin 43 (Cx43), the cardiac troponin I (cTnI) striation patterns, the improvement in the number of contracting cells and in the contraction amplitude (Duan et al., 2011). Fong et al. (2016) used hydrogels derived from decellularized bovine adult and fetal hearts in culture with hiPSC-CM in 2D (coating) and 3D (cardiac ECM + fibrinogen) approaches. These researchers demonstrated that 3D cultures with adult cardiac heart showed better improvement in CM maturation, mainly related to higher expression of mature cardiac genes (MYL2, Cx43, SERCA2a, and HCN4) and increased calcium signaling and kinetics compared with CM in 2D cultures (Fong et al., 2016). Together, these results indicate that native ECM provides a microenvironment capable of differentiating cells and providing a scaffold for the culture of CM, improving, at least in part, the maturation of these cells. These and other strategies used to induce differentiation and maturation of PSC-CM are summarized in **Table 3**.

Isolated ECM Components

In addition to the use of complex matrices and based on previous knowledge regarding cardiac ECM components, studies were performed with isolated ECM components. Many studies have used different combinations of ECM proteins to improve cardiac differentiation. hESC differentiated in a specific ratio of fibronectin (FN) and laminin (70:30) showed more differentiated cells than gelatin cultures, and its effects may be related to the integrin-mediated MEK/ERK signaling pathway (Sa et al., 2014). Furthermore, to optimize miPSC cardiac differentiation, Jung et al. (2015) investigated various combinations of ECM proteins. A systematic optimization indicated that a solution containing 61% Col I, 24% laminin-111 and 15% FN increased the number of cells expressing cTnT, MHCa, and α-actinin compared with suboptimal solutions (Jung et al., 2015). Additionally, it was demonstrated that the combination of ECM components, including FN, types I, III, and IV collagens, was important to allow hESC-derived cardiac progenitors to attach and survive (Lu et al., 2017). Recently, Yap et al. (2019), after verifying the higher expression of laminin-221 (LN-221) in adult cardiac tissue, developed a LN-221-based cardiac differentiation protocol, which reached more than 80% $TNNT2^{+}$ cells and presented high reproducibility with 2 different hESC lines. Additionally, the cardiac progenitors generated during this process were able to improve cardiac function in mice after myocardial infarction (Yap et al., 2019).

In addition to affecting PSC cardiac differentiation, niche components and structure could also be used to stimulate the maturation of PSC-CM. Then, studies have attempted to use both 2D or 3D cultures with synthetic or natural matrices to reach this goal. To generate a microenvironment favorable to CM maturation, hPSC-CM and non-CM cells (as FB, SMC, and EC) were seeded in a Col I gel localized around a template suture in a poly(dimethylsiloxane) (PDMS) channel – a platform called "biowire." Using this 3D structure associated with electrical stimulation, CMs increased their size, acquired a characteristic rod-like shape and an organized sarcomeric banding, presented a lower proliferative rate and improved Ca^{+2} handling properties. This finding indicates that the "biowire" platform with electrical stimulus (6 Hz) was able to induce hPSC-CM to a more mature phenotype (Nunes et al., 2013).

TABLE 3 | Summary of ECM approaches to improve PSC cardiomyogenic differentiation and/or PSC-CM maturation.

Decellularized heart ECM	Method	Cells	Highlights	References
Whole organ	Mouse ECM. Injected cells.	hESC or hESC-derived mesendodermal cells	After 14 days, both cell types expressed cardiac markers genes (cTnT, NKX2.5). No spontaneous contraction.	Ng et al., 2011
	Mouse ECM. Cells perfunded with growth factors.	hPSC-derived cardiac multipotent progenitors	Cells differentiate *in situ* in CM, SMC, and EC. Spontaneous contraction after 20 days. No complete recellularization.	Lu et al., 2013
	Human ECM. Injected cells. Human heart bioreactor.	hiPSC-CM	After 14 days, cells remained viable, integrate with matrix and showed a range of maturity. No complete recellularization.	Guyette et al., 2016
Slices	Mouse ECM. 60-μm thick slices.	mESC	Higher levels of cardiac markers in comparison with liver decellularized ECM.	Higuchi et al., 2013
	Porcine ECM. 150-μm thick slices. Laser-cut sheets.	hPSC-CM	Developed of an EHT for biomechanical characterization of PSC-CM. Cells presented organized sarcomeres and formed gap junctions.	Schwan et al., 2016
	Rat ECM.	MSCs and hPSC-derived ventricular CM	EHT with 75% CM and 25% MSC. After 2 weeks, cells formed a tissue-like structure with spontaneous contraction and CM, SMC and EC.	Li et al., 2017
	Human ECM from patients with end-stage non-ischemic dilated cardiopathy. 300-μm thick slices.	mESC, miPSC	Supported PSC proliferation. Increase expression of cardiac markers.	Oberwallner et al., 2015
	Human ECM. 400-μm thick slices.	PSC-CM	Spontaneous beating after 7–10 days. Better electrophysiological response. Uniform contraction, functional gap junctions. Increase expression of cardiac ion channels in CM.	Garreta et al., 2016
	Human ECM. 200-μm thick slices. Cardiac fiber bundles with 15 mm length, 2.5 mm diameter. Injected cells.	PSC-CM	Cells adhered, remained viable and functional. Spontaneous beating after 4–10 days. Maintenance in culture for 60–120 days. Formation of mechanical and electrical tissue.	Guyette et al., 2016
Hydrogel	Different hydrogels composition: 75%, 25% or 0% of porcine ECM.	hESC EBs	The hydrogel composed of 75% porcine ECM:25% collagen increase the number of cells cTnT + Improve expression of Cx43, number of contracting cells and contraction amplitude.	Duan et al., 2011
	2D (coating) or adult (cardiac patch) bovine adult and fetal ECM	hiPSC-CM	3D cultures with adult tissue showed higher expression of mature cardiac genes. Increase calcium signaling.	Fong et al., 2016
3D bioprinting	Porcine ECM. Different bioinks composition.	hCPC and/or MSC	Improve maturation of CPC. MSC + VEGF promoted vascular formation.	Jang et al., 2017
	Decellularized porcine ECM bioink. Custom digital light processing (DLP)-based scanningless and continuous 3D bioprinter.	hiPSC-CM	Improve expression of mature cardiac genes.	Yu et al., 2019
ECM preparations				
ECM proteins	Different proportions of fibronectin and laminin.	hESC	Ratio Fibronectin and Laminin (70:30) improve the number of differentiated cells (higher than 60% cTnI +).	Sa et al., 2014
	Systematic optimization of different ratios of type I collagen, laminin and fibronectin.	miPSC	Hydrogel composed of 61% type I collagen, 24% laminin-111 and 15% fibronectin increase number of cells (cTnT+).	Jung et al., 2015
	Different combinations of laminin, fibronectin, types I, III and IV collagens.	hESC- derived CPC	Combinations of ECM proteins improve CPC attachment and survival. Fibronectin, types I, III and IV collagens showed better results.	Lu et al., 2017
	Development of a Laminin-221 based cardiac differentiation protocol.	hESC	Combination of LN-521 + 221 matrix generated more CM (∼80%). High reproducibility confirmed by bulk and single-cell RNA-seq.	Yap et al., 2019

(Continued)

TABLE 3 | Continued

	Method	Cells	Highlights	References
	Biowire platform: Cells culture in type I collagen gel around a suture in a PDMS channel. Associate with electrical stimulation.	hPSC-CM and non-CM cells (e.g., FB, SMC, EC).	CM increase size, rod-like shape, organized sarcomeric banding, lower proliferative rate, improved Ca(+2) handling.	Nunes et al., 2013
Matrigel	Cells encapsulated in 3D cardiac strips composed of matrigel and type I collagen. Associated with mechanical cyclic stretch.	hESC-CM associated or not with non-CM cells	Conditions with non-CM cells improve more CM maturation. Cyclic stretch improved sarcomere size and expression of mature cardiac genes.	Zhang et al., 2017
	Cardiopatch hydrogel composed of matrigel, fibrinogen and cardiac media.	hPSC-CM	Improve expression of mature cardiac genes, sarcomeric banding, lower proliferative rate, more mature electrophysiology.	Shadrin et al., 2017
	Coating with fibronectin or matrigel in glass coverslips or PDMS membranes.	hPSC-CM	Matrigel in PDMS improve CM electrophysiology, number of binucleated cells, expression of sarcomere and myofilament markers.	Herron et al., 2016
	Matrigel Matress: 0.4–0.8 mm-thick of undiluted matrigel.	hiPSC-CM	CM developed rod-shape morphology, increase sarcomere size, upstroke velocity and expression of cardiac markers.	Feaster et al., 2015
	Cells culture in matrigel or hyaluronan-based hydrogel associated or not with pro-survival factors.	hiPSC-CM	*In vivo*, CM in matrigel presented a more mature phenotype.	Ogasawara et al., 2017
Fibrin	3D fibrin cardiac patch.	hESC-CM (SIRPA cells)	Improve sarcomere size, conduction velocity and expression of cardiac genes.	Zhang et al., 2013
	3D fibrin matrix.	hiPSC-CM	Increase sodium current density and upstroke velocity.	Lemoine et al., 2017
	Fibrin hydrogels associated with stretch and electrical stimulation	hiPSC-CM	Early-stage iPSC-CM associated with physical conditioning at an increasing intensity accelerated maturation, showed superior electrophysiological properties, CMs with increase cell size and sarcomere length.	Ronaldson-Bouchard et al., 2018

EBs, embryoid bodies; CM, cardiomyocytes; SMC, smooth muscle cells; EC, endothelial cells; VEGF, vascular endothelial growth factor; MSC, mesenchymal stem cells; CPC, cardiac progenitor cells.

Comparisons of 2D and 3D cultures were performed by other groups. Using a 3D fibrin-based cardiac patch, differentiated EBs were dissociated and seeded with different percentages of $SIRPA^+$ cells (CM marker) on the patch. Compared to monolayer cultures, 3D scaffolds augmented the conduction velocity, the size of sarcomeres and the expression of cardiac genes in hESC-CM (Zhang et al., 2013). Also, 3D cardiac strips produced through encapsulation of hESC-CM – with and without other niche cells (FB or MSC) – in Matrigel and Col I, associated with mechanical cyclic stretch, demonstrated potential in mature CM. Although all the conditions could generate a level of maturation in CM, those cultured with MSC or FB presented a more mature phenotype. Additionally, the use of cyclic stretching improved the sarcomere length and gene expression of maturation markers (Zhang et al., 2017).

Another platform of 3D construct is the "cardiopatch," a hydrogel composed of fibrinogen, Matrigel and cardiac media mixed with CM. During 5 weeks of differentiation, hPSC-CM presented sarcomeric banding (including M-band and T-tubules), enhanced expression of markers related to the cardiac maturation process (e.g., TNNI3, MYL2, CASQ2, and CKM), progressive decrease in cell proliferation, higher conduction velocity, among others (Shadrin et al., 2017). Comparing a 3D EHT generated with hiPSC-CM in a fibrin matrix with 2D monolayer cultures, Lemoine et al. (2017) demonstrated that the EHT strategy increased sodium current density and upstroke velocity, both values more similar to those from adult human CM (Lemoine et al., 2017). Furthermore, early-stage hiPSC-CM incorporated into fibrin hydrogels subjected to stretch and electrical stimulation (increase intensity training) accelerated CM maturation, confirmed by expression of mature cardiac makers, increase cell size and sarcomere length and mature-like electrophysiological responses (Ronaldson-Bouchard et al., 2018).

The Matrigel®, a commercially available protein mixture used in maintenance of PSC and cardiac differentiation protocol (Zhang J. et al., 2012), was also applied to different strategies, alone or in combination with specific ECM proteins. For example, Herron et al. (2016) tested different ECM combinations to improve the maturation of hPSC-CM: coating of FN or Matrigel in glass coverslips or PDMS membranes. Matrigel + PDMS promoted greater CM maturation. Parameters such as conduction and upstroke velocities, electrophysiological, number of binucleated and proliferated cells, expression of mature sarcolemal and myofilament markers resemble that of mature CM under optimal conditions (Herron et al., 2016). Another strategy developed was the "Matrigel Mattress." In this method, 0.4- to 0.8-mm-thick undiluted Matrigel® was prepared, and the hiPSC-CM cultured on this substrate developed a more

rod-shaped morphology, increased sarcomere length, upstroke velocity and expression of cardiac markers (Feaster et al., 2015).

This type of methodology could be combined with *in vivo* maturation. For example, hiPSC-CM was diluted on Matrigel with pro-survival factors or on a hyaluronan-based hydrogel associated or not with pro-survival factors and transplanted into an infarcted rat. After 4 weeks, the CM cultured on Matrigel + factors presented a more mature phenotype than the other groups (Ogasawara et al., 2017). Kadota et al. (2017) compared cells transplanted in neonatal rat hearts uninjured and infarcted adult rat hearts. In all situations, the hiPSC-CM engrafted and survived in rat hearts, but it was the adult tissue that promoted faster CM maturation.

Among the most recent strategies to produce an environment for CM maturation is the use of 3D printing technologies. For instance, 3D printing allows the building of specific patterns of 3D constructs (Ma et al., 2018) and the use of solubilized decellularized ECM as bioink to culture with CPC (Jang et al., 2017) or iPSC-CM (Noor et al., 2019; Yu et al., 2019) to improve maturation of the cells. Considering the results discussed in this study, we showed that mimic ECM microenvironmental signals using 3D cultures, combinations of ECM proteins or a natural scaffold are important modulators that influence cardiac differentiation or maturation.

CARDIAC SOLUBLE FACTORS: EFFECTS ON CARDIOMYOCYTE FATE AND BEHAVIOR

To maintain their functions properly, chemical signaling in the heart should be orchestrated by a variety of signals released by myocyte and non-myocyte cells. As mentioned previously, the set of those signals constituted by secreted soluble factors comprises the CS. CS include several bioactive molecules, such as growth factors, endocrine hormones, cytokines and peptides (Doroudgar and Glembotski, 2012). In addition, CS may also contain extracellular vesicles (EVs) that load different types of cargo, which may vary with biogenesis, cell type, and physiological conditions (Abels and Breakefield, 2016). Because of the complex interplay between cardiac factors, cell communication in the heart has not been fully elucidated to date. However, extensive evidence indicates that the secretome from cardiac cells may influence CM development and behavior.

One approach to studying and decipher the actors of cellular communication in the heart is the analysis of the conditioned medium from *in vitro* cultures of cardiac cells (**Figure 3**), such as cardiospheres (Sharma et al., 2015), cardiac resident cells (Zhang et al., 2015; Reus et al., 2016) and heart explant tissues (Schittini et al., 2010). In the last several years, mass spectrometry approaches have been used for signaling investigation. According to Lindoso et al. (2016), this approach provides an overview of proteins present in media and enables a measurement of protein level changes during normal or pathophysiological conditions. Recently, a study analyzed the secretome collected at seven different time points during *in vitro* CM differentiation. The authors found 1802 proteins significantly regulated during differentiation of which 431 are annotated as secreted. Numerous proteins that remarkably vary during the differentiation process affect the Wnt, TGFβ, Activin A, Nodal, BMP and FGF signaling pathways (Wolling et al., 2018). The identification of paracrine factors that modulate CM differentiation, proliferation and maturation may help the development or improvement of protocols for the *in vitro* differentiation of CM from PSC. In this section, we provide a broad overview of recent investigations of paracrine factors that affect CM with an emphasis on those released by the main types of cardiac cells. We also explore studies involving EVs and its influence in cardiac cell behavior.

Signaling From Cardiac Resident Cells

Endothelial Cells

EC constitute an important component of the heart. Recently, ECs were identified as one of the most abundant cell types in this organ (Pinto et al., 2016). Anatomically, these cells form a monolayer that covers the heart cavities and compose the vascular network that perfuses the myocardium. Therefore, factors secreted by ECs, such as NO, endothelin-1, angiotensin II (Ang II), prostaglandins, natriuretic peptides, adenyl purines, neuregulin-1, FGF, and VEGF, are able to affect heart function (Shah, 1996; Tirziu et al., 2010). Recently, the mediators of the EC-CM interaction involved in cardiac remodeling and regeneration were summarized in a review (Talman and Kivelä, 2018). In fact, the communication between ECs and CM is crucial for the maintenance of cardiac homeostasis, and the disruption in this signalization can result in pathophysiological conditions (Gogiraju et al., 2019). Furthermore, CM generation was demonstrated to be affected by the niche provided by ECs (Chen et al., 2010), and the ability of ECs to enhance the maturity of hPSC-CM was already verified (Caspi et al., 2007; Tulloch et al., 2011; Ravenscroft et al., 2016). Kivelä et al. (2019) also demonstrated that ECs regulate physiological CM growth via VEGFR2. Recently, a study generated a human cardiac microtissue through the co-differentiation of CM and ECs from PSC (Giacomelli et al., 2017). Interestingly, the inclusion of ECs (generated with a cardiac identity) and prolonged time in culture induced changes in CM gene expression associated with CM maturation. Among the EC-CM mediators, neuregulin-1 (NRG-1) is one of the key players in CM development (Rupert and Coulombe, 2015). Previous studies reported that NRG-1β/ErbB signaling was able to increase cardiomyogenesis in mESC and participate in cardiac subtype ("working-type" atrial or nodal) selection (Chen et al., 2013). Beyond the individual effects of NRG-1, this molecule also acts synergistically with IGF-1 to enhance proliferation and metabolic maturity in CM (Rupert and Coulombe, 2017). Other soluble factors released by ECs are able to drive cardiac differentiation. Endothelin-1 added to Nkx2.5 + CPC culture induced CPC differentiation into cardiac pacemaking cells (Zhang X. et al., 2012). This molecule has also been suggested to stimulate terminal differentiation of CM (Paradis et al., 2014). In addition, brain natriuretic peptide (BNP), a cardiac hormone secreted by EC, CM and cFB, stimulated CPC proliferation and CM differentiation, and these effects were demonstrated to occur via BNP binding to NPR-A and NPR-B, respectively (Bielmann et al., 2015).

Cardiac Fibroblasts

The cFBs are cells that produce connective tissues and are recognized as modulators of cardiac function, development and homeostasis (Zhang P. et al., 2012; Ivey and Tallquist, 2017). As mentioned previously, cFB are responsible for the synthesis of the major part of ECM proteins in the heart. Therefore, the main interplay between cFB-CM communication appears to occur through ECM molecules secreted by cFBs. These cells also secrete proteins related to cardiac development, such as FGFs, TGF, Ang II, interleukin-6 (IL-6), and IL-33 (Kakkar and Lee, 2010; Takeda and Manabe, 2011). In fact, conditioned medium from ventricular cFBs was demonstrated to induce Nkx2.5^{+} CPC differentiation in CM through Wnt pathway activation (Zhang et al., 2015). Similarly, our group investigated the effects of conditioned medium from CRSCs (a population that includes DDR-2^{+} cells) on progenitor cell (H9c2) behavior. The CRSC secretome was able to drive the proliferation and cardiac differentiation of H9c2 cells (Reus et al., 2016). Furthermore, the cFB conditioned medium was also reported to influence CM phenotype, including hypertrophy, expression of vimentin and electrophysiological changes (LaFramboise et al., 2007; Pedrotty et al., 2009). Recently, the secretome of cFB was investigated in normal and stressed (hypoxic) conditions (Cosme et al., 2017). The conditioned media was separated to obtain exosome and exosome-depleted fractions, and the results revealed almost 494 proteins differentially expressed between fractions and oxygen conditions. Indeed, culture conditions interfere with cFB secretion and consequently in the CM response. Furthermore, cFB can be activated in response to tissue injury and present a phenotype characterized by the expression of alpha-smooth muscle actin (α-SMA) (Hu and Phan, 2013). Cartledge et al. (2015) demonstrated hypertrophic effects on CM cocultivated with cFB, myofibroblast and myofibroblast-conditioned medium, which were related to TGF-β released from cFB. In fact, CM hypertrophy induced by cFB-paracrine factors has been extensively described (Booz et al., 1999; LaFramboise et al., 2007). In addition, in damaged myocardium, activated cFB also releases proinflammatory cytokines, such as cardiotrophin-1 (CT-1), a member of the IL-6 family. This molecule could enhance mouse IPS cardiomyogenic differentiation partly via the JAK2/STAT3/Pim-1 pathway and stimulate CM maturation (Liu et al., 2015).

Cardiac Progenitor Cells

Cardiac progenitor cells are a heterogeneous group of cells distributed throughout the heart. These cells are extensively studied mainly because of their potential effects on injured cardiac tissue (reviewed by Le and Chong, 2017). Originally, it was believed that after transplantation, CPC would be able to restore cardiac function through differentiation in CM, EC, and SMC (Torella et al., 2006; Bearzi et al., 2007). However, recent reports suggest that the regenerative effects induced by CPC occur through secreted paracrine factors (Khanabdali et al., 2016; Le and Chong, 2017). Many subtypes of cardiac stem cells have been reported (Chong et al., 2014), and apparently, the paracrine factors released are also diversified between them; however, the therapeutic effects occur with the different CPC populations. Recently, Torán et al. (2019) aimed to define a set of proteins specifically secreted by CPC. Authors isolated human CPC from myocardial samples and conducted a proteomic assay. The analysis identified a group of factors expressed at high to medium levels by CPC that included IL-1, GROa (CXCL1), CXCL6 (GCP2), and IL-8 (Torán et al., 2019). Similarly, considering the effects of CPC in myocardial recovery, Sharma et al. (2017) isolated cardiac progenitor cells from neonatal (nCPC) and adult patients (aCPC) and compared the functionality of their conditioned medium. After extensive characterization of CPC paracrine factors, differences were found in the secretion profile during development that affected the ability of conditioned medium to recover myocardial function. Both secretomes significantly induced proliferation and reduced apoptosis in CM; however, nCPC conditioned medium was more effective than aCPC (Sharma et al., 2017). A recent study also profiled the proteins that were secreted by CPC (Sca-1^{+}) derived from healthy and transgenic heart failure mice aiming to define the factors that are modulated in a failing heart microenvironment. The results showed that proteins usually associated with tissue regeneration, such as CSF1, COCA, IBP6, and TCPG, were found to be more abundant in transgenic samples than in healthy samples (Samal et al., 2018).

Extracellular Vesicles Signaling: Effects on Cardiomyocytes and Cardiac Tissue

In the heart, as well as in other tissues, protein secretion occurs in different ways: (1) direct release in the extracellular space through membrane-derived secretory vesicles; (2) translocation of cytosolic proteins across the plasma membrane; and (3) packaging in EVs (Reviewed by Doroudgar and Glembotski, 2012). The last mechanism is becoming more studied over the last several years. Since the discovery that EVs may harbor a varied content that includes not only proteins but also other active molecules (miRNAs, mRNA, DNA, and lipids) (Abels and Breakefield, 2016), the understanding of cell signaling was brought to a higher level of complexity. EVs have been isolated from different sources, including the previously mentioned cells: EC (Piryani et al., 2019), cFB (Borosch et al., 2017) and CPC (Barile et al., 2014), as well as other important components of the cardiac microenvironment, such as ECM (An et al., 2017), MSCs (Angulski et al., 2017), and CM (Liu et al., 2018). Recently, we isolated and characterized EVs from different regions of human heart tissue. Our results demonstrated that cardiac EVs contain a set of proteins advantageous for tissue regeneration approaches, and we verified their potential to modulate proliferation, wound healing, adhesion and angiogenesis differently depending on the target cell type (Leitolis et al., 2019). A systematic review compiled studies that investigated the cardioprotective characteristics of EVs (Wendt et al., 2018). In fact, EVs are able to regulate many biological activities, including those related to CM. Cardiac progenitor cell-derived EVs have been shown to stimulate migration and proliferation in AC16 CM (Hocine et al., 2019) and inhibit CM apoptosis (Barile et al., 2014) through a mechanism that involves PAPP-A

(pregnancy-associated plasma protein-A) (Barile et al., 2018). Similarly, Liu et al. (2018) showed that CM-derived EVs not only decreased CM apoptosis 24 h after rat infarction but also reduced arrhythmia and hypertrophy postinfarction (Liu et al., 2018). The hypertrophic effect on CM was also verified through cFB-derived exosomes in a paracrine mechanism by which Ang II intensifies its own signaling in CM (Lyu et al., 2013). In addition, apoptotic bodies (AB) also appear to modulate myocyte activity. CM-derived AB were able to stimulate proliferation and differentiation of CM precursors, as well as their frequency of contraction (Tyukavin et al., 2015). Beyond the studies conducted with EVs derived from the sources mentioned above, many studies have shown the biological effects of MSC-derived EVs (MSC-EVs). To date, many reports have demonstrated that MSC-EVs are able to exert protective effects in cardiovascular diseases, especially by the delivery of miRNA content to recipient cells. Recently, Moghaddam et al. (2019) summarized the cardioprotective exosomal miRNAs that include those secreted by MSCs. For instance, Wang et al. (2018) demonstrated that cardiac stem cells treated with MSC-derived exosomes containing miR-214 showed decreased apoptosis and reactive oxygen species (ROS) production after oxidative stress injury (Wang et al., 2018). Similarly, a recent study showed that exosomal miR-21-5p increased cardiac calcium handling and thereby contractility via the PI3K signaling cascade in human engineered cardiac tissue (Mayourian et al., 2018). Furthermore, a novel study showed that the coculture of iPSC-CM with MSCs modulates the functionality and maturation of CMs. These effects can be partially explained by the miRNA content in MSC-derived EVs (Yoshida et al., 2018). In fact, CM differentiation and maturation are closely related to miRNA regulation (White et al., 2016; Lock et al., 2018). For instance, let-7 family miRNAs were found to be highly upregulated during CM maturation, and let-7 members control CM metabolism, cell size and force contractility (Kuppusamy et al., 2015).

PERSPECTIVES

The elements from microenvironmental signaling, such as ECM proteins and paracrine factors, both secreted by cardiac resident cells, are biologically active molecules capable of affecting CM commitment, subtype specification, proliferation and maturation. These molecules are being tested in coculture experiments (CM plus non-myocyte cells), as well as in assays that use total secretome (conditioned medium), EVs, decellularized heart ECM or combinations of isolated ECM proteins. In most cases, the molecular mechanisms by which these molecules act have not been fully decoded.

Despite the progress in understanding the cardiac niche in different development stages, the modulation of extracellular signaling and how it governs cardiac commitment has not been thoroughly elucidated to date. The factors mentioned in this review, as well as others not explored to date, may be important tools for modulation of *in vitro* cardiomyogenesis, mainly regarding maturation of the developed CM. Hence, improving the functional characteristics of CM could potentiate their use in regenerative medicine and facilitate further advances in cell therapy and tissue engineering.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

Abels, E. R., and Breakefield, X. O. (2016). Introduction to extracellular vesicles: biogenesis, RNA cargo selection, content, release, and uptake. *Cell. Mol. Neurobiol.* 36, 301–312. doi: 10.1007/s10571-016-0366-z

Aguilar-Sanchez, C., Michael, M., and Pennings, S. (2018). Cardiac stem cells in the postnatal heart: lessons from development. *Stem Cells Int.* 2018, 1–13. doi: 10.1155/2018/1247857

An, M., Kwon, K., Park, J., Ryu, D. R., Shin, J. A., Lee Kang, J., et al. (2017). Extracellular matrix-derived extracellular vesicles promote cardiomyocyte growth and electrical activity in engineered cardiac atria. *Biomaterials* 146, 49–59. doi: 10.1016/j.biomaterials.2017.09.001

Andrés-Delgado, L., and Mercader, N. (2016). Interplay between cardiac function and heart development. *Biochim. Biophys. Acta Mol. Cell Res.* 1863, 1707–1716. doi: 10.1016/j.bbamcr.2016.03.004

Angulski, A. B. B., Capriglione, L. G., Batista, M., Marcon, B. H., Senegaglia, A. C., Stimamiglio, M. A., et al. (2017). The protein content of extracellular vesicles derived from expanded human umbilical cord blood-derived CD133+ and human bone marrow-derived mesenchymal stem cells partially explains why both sources are advantageous for regenerative medicine. *Stem Cell Rev. Reports* 13, 244–257. doi: 10.1007/s12015-016-9715-z

Arslan, Y. E., Galata, Y. F., Sezgin Arslan, T., and Derkus, B. (2018). Trans-differentiation of human adipose-derived mesenchymal stem cells into cardiomyocyte-like cells on decellularized bovine myocardial extracellular matrix-based films. *J. Mater. Sci. Mater. Med.* 29:127. doi: 10.1007/s10856-018-6135-4

Banerjee, I., Fuseler, J. W., Price, R. L., Borg, T. K., and Baudino, T. A. (2007). Determination of cell types and numbers during cardiac development in the neonatal and adult rat and mouse. *Am. Physiol.* 293, 1883–1891. doi: 10.1152/ajpheart.00514.2007

Barallobre-Barreiro, J., Didangelos, A., Schoendube, F. A., Drozdov, I., Yin, X., Fernández-Caggiano, M., et al. (2012). Proteomics analysis of cardiac extracellular matrix remodeling in a porcine model of ischemia/reperfusion injury. *Circulation* 125, 789–802. doi: 10.1161/CIRCULATIONAHA.111.056952

Barile, L., Cervio, E., Lionetti, V., Milano, G., Ciullo, A., Biemmi, V., et al. (2018). Cardioprotection by cardiac progenitor cell-secreted exosomes: role of pregnancy-associated plasma protein-A. *Cardiovasc. Res.* 114, 992–1005. doi: 10.1093/cvr/cvy055

Barile, L., Lionetti, V., Cervio, E., Matteucci, M., Gherghiceanu, M., Popescu, L. M., et al. (2014). Extracellular vesicles fromhuman cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function aftermyocardial infarction. *Cardiovasc. Res.* 103, 530–541. doi: 10.1093/cvr/cvu167

Bearzi, C., Rota, M., Hosoda, T., Tillmanns, J., Nascimbene, A., De Angelis, A., et al. (2007). Human cardiac stem cells. *PNAS* 104, 14068–14073.

Beqqali, A., Kloots, J., Ward-van Oostwaard, D., Mummery, C., and Passier, R. (2006). Genome-wide transcriptional profiling of human embryonic stem cells differentiating to cardiomyocytes. *Stem Cells* 24, 1956–1967. doi: 10.1634/stemcells.2006-0054

Bergmann, O., Zdunek, S., Felker, A., Salehpour, M., Alkass, K., Bernard, S., et al. (2015). Dynamics of cell generation and turnover in the human heart. *Cell* 161, 1566–1575. doi: 10.1016/j.cell.2015.05.026

Bertero, A., Fields, P. A., Ramani, V., Bonora, G., Yardimci, G. G., Reinecke, H., et al. (2019). Dynamics of genome reorganization during human cardiogenesis reveal an RBM20-dependent splicing factory. *Nat. Commun.* 10:1538. doi: 10.1038/s41467-019-09483-5

Besser, R. R., Ishahak, M., Mayo, V., Carbonero, D., Claure, I., and Agarwal, A. (2018). Engineered microenvironments for maturation of stem cell derived cardiac myocytes. *Theranostics* 8, 124–140. doi: 10.7150/thno.19441

Bielmann, C., Rignault-Clerc, S., Liaudet, L., Li, F., Kunieda, T., Sogawa, C., et al. (2015). Brain natriuretic peptide is able to stimulate cardiac progenitor cell proliferation and differentiation in murine hearts after birth. *Basic Res. Cardiol.* 110:455. doi: 10.1007/s00395-014-0455-454

Bonnans, C., Chou, J., and Werb, Z. (2014). Remodelling the extracellular matrix in development and disease. *Nat. Rev. Mol. Cell Biol.* 15, 786–801. doi: 10.1038/nrm3904

Booz, G. W., Dostal, D. E., and Baker, K. M. (1999). Paracrine actions of cardiac fibroblasts on cardiomyocytes: implications for the cardiac renin-angiotensin system. *Am. J. Cardiol.* 83, 44–47. doi: 10.1016/S0002-9149(99)00257-X

Borosch, S., Dahmen, E., Beckers, C., Stoppe, C., Buhl, E. M., Denecke, B., et al. (2017). Characterization of extracellular vesicles derived from cardiac cells in an in vitro model of preconditioning. *J. Extracell. Vesicles* 6:1390391. doi: 10.1080/20013078.2017.1390391

Brade, T., Pane, L. S., Moretti, A., Chien, K. R., and Laugwitz, K. (2013). Embryonic heart progenitors and cardiogenesis. *Cold Spring Harb. Perspect. Med.* 3, 1–18. doi: 10.1101/cshperspect.a013847

Brand, T. (2003). Heart development: molecular insights into cardiac specification and early morphogenesis. *Dev. Biol.* 258, 1–19. doi: 10.1016/S0012-1606(03)00112-X

Brizzi, M. F., Tarone, G., and Defilippi, P. (2012). Extracellular matrix, integrins, and growth factors as tailors of the stem cell niche. *Curr. Opin. Cell Biol.* 24, 645–651. doi: 10.1016/j.ceb.2012.07.001

Burridge, P. W., Keller, G., Gold, J. D., and Wu, J. C. (2012). Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. *Cell Stem Cell* 10, 16–28. doi: 10.1016/j.stem.2011.12.013

Burridge, P. W., Matsa, E., Shukla, P., Lin, Z. C., Churko, J. M., Ebert, A. D., et al. (2014). Chemically defined generation of human cardiomyocytes. *Nat. Methods* 11, 855–860. doi: 10.1038/nmeth.2999

Cao, F., Wagner, R. A., Wilson, K. D., Xie, X., Fu, J., Drukker, M., et al. (2008). Transcriptional and functional profiling of human embryonic stem cell-derived cardiomyocytes. *PLoS One* 3:e3474. doi: 10.1371/journal.pone.0003474

Cartledge, J. E., Kane, C., Dias, P., Tesfom, M., Clarke, L., Mckee, B., et al. (2015). Functional crosstalk between cardiac fibroblasts and adult cardiomyocytes by solublemediators. *Cardiovasc. Res.* 105, 260–270. doi: 10.1093/cvr/cvu264

Carvalho, J. L., de Carvalho, P. H., Gomes, D. A., and Goes, A. M. (2012). Characterization of decellularized heart matrices as biomaterials for regular and whole organ tissue engineering and initial in-vitro recellularization with ips cells. *J. Tissue Sci. Eng.* 11:2. doi: 10.4172/2157-7552.S11-002

Caspi, O., Lesman, A., Basevitch, Y., Gepstein, A., Arbel, G., Huber, I., et al. (2007). Tissue engineering of vascularized cardiac muscle from human embryonic stem cells. *Circ. Res.* 100, 263–272. doi: 10.1161/01.RES.0000257776.05673.ff

Chan, C. K., Rolle, M. W., Potter-Perigo, S., Braun, K. R., Van Biber, B. P., Laflamme, M. A., et al. (2010). Differentiation of cardiomyocytes from human embryonic stem cells is accompanied by changes in the extracellular matrix production of versican and hyaluronan. *J. Cell. Biochem.* 111, 585–596. doi: 10.1002/jcb.22744

Chang, C. W., Dalgliesh, A. J., López, J. E., and Griffiths, L. G. (2016). Cardiac extracellular matrix proteomics: challenges, techniques, and clinical implications. *Prot. Clin. Appl.* 10, 39–50. doi: 10.1002/prca.201500030

Chen, K., Bai, H., Arzigian, M., Gao, Y. X., Bao, J., Wu, W. S., et al. (2010). Endothelial cells regulate cardiomyocyte development from embryonic stem cells. *J. Cell. Biochem.* 111, 29–39. doi: 10.1002/jcb.22680

Chen, M., Bi, L. L., Wang, Z. Q., Zhao, F., Gan, X. D., and Wang, Y. G. (2013). Time-dependent regulation of neuregulin-1β/ErbB/ERK pathways in cardiac differentiation of mouse embryonic stem cells. *Mol. Cell. Biochem.* 380, 67–72. doi: 10.1007/s11010-013-1658-y

Chong, J. J. H., Forte, E., and Harvey, R. P. (2014). Developmental origins and lineage descendants of endogenous adult cardiac progenitor cells. *Stem Cell Res.* 13, 592–614. doi: 10.1016/j.scr.2014.09.008

Christalla, P., Hudson, J. E., and Zimmermann, W.-H. (2012). The cardiogenic niche as a fundamental building block of engineered myocardium. *Cells Tissues Organs* 195, 82–93. doi: 10.1159/000331407

Churko, J. M., Garg, P., Treutlein, B., Venkatasubramanian, M., Wu, H., Lee, J., et al. (2018). Defining human cardiac transcription factor hierarchies using integrated single-cell heterogeneity analysis. *Nat. Commun.* 9:4906. doi: 10.1038/s41467-018-07333-7334

Civitarese, R. A., Kapus, A., McCulloch, C. A., and Connelly, K. A. (2017). Role of integrins in mediating cardiac fibroblast–cardiomyocyte cross talk: a dynamic relationship in cardiac biology and pathophysiology. *Basic Res. Cardiol.* 112:6. doi: 10.1007/s00395-016-0598-6

Cosme, J., Guo, H., Hadipour-Lakmehsari, S., Emili, A., and Gramolini, A. O. (2017). Hypoxia-induced changes in the fibroblast secretome. exosome, and whole-cell proteome using cultured, cardiac-derived cells isolated from neonatal mice. *J. Proteome Res.* 16, 2836–2847. doi: 10.1021/acs.jproteome.7b00144

Crapo, P. M., Gilbert, T. W., and Badylak, S. F. (2011). An overview of tissue and whole organ decellularization processes. *Biomaterials* 32, 3233–3243. doi: 10.1016/j.biomaterials.2011.01.057

De Castro Brás, L. E., Ramirez, T. A., DeLeon-Pennell, K. Y., Chiao, Y. A., Ma, Y., Dai, Q., et al. (2013). Texas 3-Step decellularization protocol: looking at the cardiac extracellular matrix. *J. Proteomics* 86, 43–52. doi: 10.1016/j.jprot.2013.05.004

Den Hartogh, S. C., Schreurs, C., Monshouwer-Kloots, J. J., Davis, R. P., Elliott, D. A., Mummery, C. L., et al. (2015). Dual reporter MESP1 mCherry/w -NKX2-5 eGFP/w hESCs enable studying early human cardiac differentiation. *Stem Cells* 33, 56–67. doi: 10.1002/stem.1842

den Hartogh, S. C., Wolstencroft, K., Mummery, C. L., and Passier, R. (2016). A comprehensive gene expression analysis at sequential stages of in vitro cardiac differentiation from isolated MESP1-expressing-mesoderm progenitors. *Sci. Rep.* 6:19386. doi: 10.1038/srep19386

Denning, C., Borgdorff, V., Crutchley, J., Firth, K. S. A., George, V., Kalra, S., et al. (2016). Cardiomyocytes from human pluripotent stem cells: from laboratory curiosity to industrial biomedical platform. *Biochim. Biophys. Acta Mol. Cell Res.* 1863, 1728–1748. doi: 10.1016/j.bbamcr.2015.10.014

Devalla, H. D., Schwach, V., Ford, J. W., Milnes, J. T., El-Haou, S., Jackson, C., et al. (2015). Atrial-like cardiomyocytes from human pluripotent stem cells are a robust preclinical model for assessing atrial-selective pharmacology. *EMBO Mol. Med.* 7, 394–410. doi: 10.15252/emmm.201404757

Doroudgar, S., and Glembotski, C. C. (2012). The cardiomyokine story unfolds: investigating stress-induced protein secretion in the heart. *Trends Mol. Med.* 17, 207–214. doi: 10.1016/j.molmed.2010.12.003

Duan, Y., Liu, Z., O'Neill, J., Wan, L. Q., Freytes, D. O., and Vunjak-Novakovic, G. (2011). Hybrid gel composed of native heart matrix and collagen induces cardiac differentiation of human embryonic stem cells without supplemental growth factors. *J. Cardiovasc. Transl. Res.* 4, 605–615. doi: 10.1007/s12265-011-9304-0

Dunn, K. K., and Palecek, S. P. (2018). Engineering scalable manufacturing of high-quality stem cell-derived cardiomyocytes for cardiac tissue repair. *Front. Med.* 5:110. doi: 10.3389/fmed.2018.00110

Durand, C., Charbord, P., and Jaffredo, T. (2018). The crosstalk between hematopoietic stem cells and their niches. *Curr. Opin. Hematol.* 25, 285–289. doi: 10.1097/MOH.0000000000000438

Elliott, D. A., Braam, S. R., Koutsis, K., Ng, E. S., Jenny, R., Lagerqvist, E. L., et al. (2011). NKX2-5eGFP/w hESCs for isolation of human cardiac progenitors and cardiomyocytes. *Nat. Methods* 8, 1037–1040. doi: 10.1038/nmeth.1740

Feaster, T. K., Cadar, A. G., Wang, L., Williams, C. H., Chun, Y. W., Hempel, J. E., et al. (2015). Matrigel mattress. *Circ. Res.* 117, 995–1000. doi: 10.1161/CIRCRESAHA.115.307580

Ferng, A. S., Connell, A. M., Marsh, K. M., Qu, N., Medina, A. O., Bajaj, N., et al. (2017). Acellular porcine heart matrices: whole organ decellularization with 3D-bioscaffold & vascular preservation. *J. Clin. Transl. Res.* 3, 260–270. doi: 10.18053/jctres.03.201702.001

Ferraro, F., Celso, C. L., and Scadden, D. (2010). *Adult Stem Cels and Their Niches*. Boston, MA: Springer, 155–168.

Filipczyka, A. A., Passiera, R., Rochata, A., and Mummery, C. L. (2007). Regulation of cardiomyocyte differentiation of embryonic stem cells by extracellular signalling. *Cell. Mol. Life Sci.* 64, 704–718. doi: 10.1002/dvdy.20684

Fong, A. H., Romero-López, M., Heylman, C. M., Keating, M., Tran, D., Sobrino, A., et al. (2016). Three-dimensional adult cardiac extracellular matrix promotes maturation of human induced pluripotent stem cell-derived cardiomyocytes. *Tissue Eng. Part A* 22, 1016–1025. doi: 10.1089/ten.tea.2016.0027

Fountoulaki, K., Dagres, N., and Iliodromitis, E. K. (2015). Cellular communications in the heart. *Card. Fail. Rev.* 1, 64–68. doi: 10.15420/cfr.2015.1.2.64

Francis, M. P., Breathwaite, E., Bulysheva, A. A., Varghese, F., Rodriguez, R. U., Dutta, S., et al. (2017). Human placenta hydrogel reduces scarring in a rat model of cardiac ischemia and enhances cardiomyocyte and stem cell cultures. *Acta Biomater.* 52, 92–104. doi: 10.1016/j.actbio.2016.12.027

Frangogiannis, N. G. (2012). Matricellular proteins in cardiac adaptation and disease. *Physiol. Rev.* 92, 635–688. doi: 10.1152/physrev.00008.2011

Frangogiannis, N. G. (2017). The extracellular matrix in myocardial injury, repair, and remodeling. *J. Clin. Invest.* 127, 1600–1612. doi: 10.1172/JCI87491

Friedman, C. E., Nguyen, Q., Lukowski, S. W., Helfer, A., Chiu, H. S., Miklas, J., et al. (2018). Single-cell transcriptomic analysis of cardiac differentiation from human PSCs reveals HOPX-dependent cardiomyocyte maturation. *Cell Stem Cell* 23, 586.e–598.e. doi: 10.1016/j.stem.2018.09.009

Fu, K., Nakano, H., Morselli, M., Chen, T., Pappoe, H., Nakano, A., et al. (2018). A temporal transcriptome and methylome in human embryonic stem cell-derived cardiomyocytes identifies novel regulators of early cardiac development. *Epigenetics* 13, 1013–1026. doi: 10.1080/15592294.2018.1526029

Furtado, M. B., Nim, H. T., Boyd, S. E., and Rosenthal, N. A. (2016). View from the heart: cardiac fibroblasts in development, scarring and regeneration. *Development* 143, 387–397. doi: 10.1242/dev.120576

Garreta, E., de Oñate, L., Fernández-Santos, M. E., Oria, R., Tarantino, C., Climent, A. M., et al. (2016). Myocardial commitment from human pluripotent stem cells: rapid production of human heart grafts. *Biomaterials* 98, 64–78. doi: 10.1016/j.biomaterials.2016.04.003

Giacomelli, E., Bellin, M., Sala, L., van Meer, B. J., Tertoolen, L. G. J., Orlova, V. V., et al. (2017). Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells. *Development* 144, 1008–1017. doi: 10.1242/dev.143438

Gogiraju, R., Bochenek, M. L., and Schäfer, K. (2019). Angiogenic endothelial cell signaling in cardiac hypertrophy and heart failure. *Front. Cardiovasc. Med.* 6:20. doi: 10.3389/fcvm.2019.00020

Guyette, J. P., Charest, J. M., Mills, R. W., Jank, B. J., Moser, P. T., Gilpin, S. E., et al. (2016). Bioengineering human myocardium on native extracellular matrix. *Circ. Res.* 118, 56–72. doi: 10.1161/CIRCRESAHA.115.306874

Herron, T. J., Rocha, A. M., Da Campbell, K. F., Ponce-Balbuena, D., Willis, B. C., Guerrero-Serna, G., et al. (2016). Extracellular matrix–mediated maturation of human pluripotent stem cell–derived cardiac monolayer structure and electrophysiological function. *Circ. Arrhythmia Electrophysiol.* 9, 1–23. doi: 10.1161/CIRCEP.113.003638

Higuchi, S., Lin, Q., Wang, J., Lim, T. K., Joshi, S. B., Anand, G. S., et al. (2013). Heart extracellular matrix supports cardiomyocyte differentiation of mouse embryonic stem cells. *J. Biosci. Bioeng.* 115, 320–325. doi: 10.1016/j.jbiosc.2012.10.004

Hirt, M. N., Hansen, A., and Eschenhagen, T. (2014). Cardiac tissue engineering: state of the art. *Circ. Res.* 114, 354–367. doi: 10.1161/CIRCRESAHA.114.300522

Hocine, H. R., Brunel, S., Chen, Q., Giustiniani, J., San Roman, M. J., Ferrat, Y. J., et al. (2019). Extracellular vesicles released by allogeneic human cardiac stem/progenitor cells as part of their therapeutic benefit. *Stem Cells Transl. Med.* doi: 10.1002/sctm.18-0256 [Epub ahead of print].

Hofsteen, P., Robitaille, A. M., Chapman, D. P., Moon, R. T., and Murry, C. E. (2016). Quantitative proteomics identify DAB2 as a cardiac developmental regulator that inhibits WNT/β-catenin signaling. *Proc. Natl. Acad. Sci. U.S.A.* 113, 1002–1007. doi: 10.1073/pnas.1523930113

Hong, X., Yuan, Y., Sun, X., Zhou, M., Guo, G., Zhang, Q., et al. (2018). Skeletal extracellular matrix supports cardiac differentiation of embryonic stem cells: a potential scaffold for engineered cardiac tissue. *Cell. Physiol. Biochem.* 45, 319–331. doi: 10.1159/000486813

Hu, B., and Phan, S. H. (2013). Myofibroblasts. *Curr. Opin. Rheumatol.* 25, 71–77. doi: 10.1097/BOR.0b013e32835b1352

Hynes, R. O., and Naba, A. (2012). Overview of the matrisome–an inventory of extracellular matrix constituents and functions. *Cold Spring Harb. Perspect. Biol.* 4:a004903. doi: 10.1101/cshperspect.a004903

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., et al. (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol. Med.* 6, 88–95. doi: 10.1007/bf03401776

Ivey, M. J., and Tallquist, M. D. (2017). Defining the Cardiac fibroblast: a new hope. *Circ. J.* 80, 2269–2276. doi: 10.1253/circj.CJ-16-1003

Jang, J., Park, H. J., Kim, S. W., Kim, H., Park, J. Y., Na, S. J., et al. (2017). 3D printed complex tissue construct using stem cell-laden decellularized extracellular matrix bioinks for cardiac repair. *Biomaterials* 112, 264–274. doi: 10.1016/j.biomaterials.2016.10.026

Johnson, T. D., Hill, R. C., Dzieciatkowska, M., Nigam, V., Behfar, A., Christman, K. L., et al. (2016). Quantification of decellularized human myocardial matrix: a comparison of six patients. *Proteomics Clin. Appl.* 10, 75–83. doi: 10.1002/prca.201500048

Jones, D. L., and Wagers, A. J. (2008). No place like home: anatomy and function of the stem cell niche. *Nat. Rev. Mol. Cell Biol.* 9, 11–21. doi: 10.1038/nrm2319

Jung, J. P., Hu, D., Domian, I. J., and Ogle, B. M. (2015). An integrated statistical model for enhanced murine cardiomyocyte differentiation via optimized engagement of 3D extracellular matrices. *Sci. Rep.* 5, 1–13. doi: 10.1038/srep18705

Kadota, S., Pabon, L., Reinecke, H., and Murry, C. E. (2017). In Vivo maturation of human induced pluripotent stem cell-derived cardiomyocytes in neonatal and adult rat hearts. *Stem Cell Reports* 8, 278–289. doi: 10.1016/j.stemcr.2016.10.009

Kakkar, R., and Lee, R. T. (2010). Intramyocardial fibroblast myocyte communication. *Circ. Res.* 106, 47–57. doi: 10.1161/CIRCRESAHA.109.207456

Karakikes, I., Senyei, G. D., Hansen, J., Kong, C.-W., Azeloglu, E. U., Stillitano, F., et al. (2014). Small molecule-mediated directed differentiation of human embryonic stem cells toward ventricular cardiomyocytes. *Karakikes* 3, 18–31. doi: 10.5966/sctm.2013-0110

Kattman, S. J., Witty, A. D., Gagliardi, M., Dubois, N. C., Niapour, M., Hotta, A., et al. (2011). Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell* 8, 228–240. doi: 10.1016/j.stem.2010.12.008

Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., et al. (2001). Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J. Clin. Invest.* 108, 407–414. doi: 10.1172/JCI200112131

Khanabdali, R., Rosdah, A. A., Dusting, G. J., and Lim, S. Y. (2016). Harnessing the secretome of cardiac stem cells as therapy for ischemic heart disease. *Biochem. Pharmacol.* 113, 1–11. doi: 10.1016/j.bcp.2016.02.012

Kim, S., Jeon, J. M., Kwon, O. K., Choe, M. S., Yeo, H. C., Peng, X., et al. (2019). Comparative proteomic analysis reveals the upregulation of ketogenesis in cardiomyocytes differentiated from induced pluripotent stem cells. *Proteomics* 1800284, 1–12. doi: 10.1002/pmic.201800284

Kita-Matsuo, H., Barcova, M., Prigozhina, N., Salomonis, N., Wei, K., Jacot, J. G., et al. (2009). Lentiviral vectors and protocols for creation of stable hESC lines for fluorescent tracking and drug resistance selection of cardiomyocytes. *PLoS One* 4:e5046. doi: 10.1371/journal.pone.0005046

Kivelä, R., Hemanthakumar, K. A., Vaparanta, K., Robciuc, M., Izumiya, Y., Kidoya, H., et al. (2019). Endothelial cells regulate physiological cardiomyocyte growth via VEGFR2 -mediated paracrine signaling. *Circulation* 139, 2570–2584. doi: 10.1161/CIRCULATIONAHA.118.036099

Kocabas, F., Mahmoud, A. I., Sosic, D., Porrello, E. R., Chen, R., Garcia, J. A., et al. (2012). The hypoxic epicardial and subepicardial microenvironment. *J. Cardiovasc. Transl. Res.* 5, 654–665. doi: 10.1007/s12265-012-9366-9367

Kolanowski, T. J., Antos, C. L., and Guan, K. (2017). Making human cardiomyocytes up to date: derivation, maturation state and perspectives. *Int. J. Cardiol.* 241, 379–386. doi: 10.1016/j.ijcard.2017.03.099

Konze, S. A., Cajic, S., Oberbeck, A., Hennig, R., Pich, A., Rapp, E., et al. (2017a). Quantitative assessment of sialo-glycoproteins and n-glycans during cardiomyogenic differentiation of human induced pluripotent stem cells. *ChemBioChem* 18, 1317–1331. doi: 10.1002/cbic.201700100

Konze, S. A., Werneburg, S., Oberbeck, A., Olmer, R., Kempf, H., Jara-Avaca, M., et al. (2017b). Proteomic analysis of human pluripotent stem cell cardiomyogenesis revealed altered expression of metabolic enzymes and PDLIM5 isoforms. *J. Proteome Res.* 16, 1133–1149. doi: 10.1021/acs.jproteome.6b00534

Kular, J. K., Basu, S., and Sharma, R. I. (2014). The extracellular matrix: Structure, composition, age-related differences, tools for analysis and applications for tissue engineering. *J. Tissue Eng.* 5:204173141455711. doi: 10.1177/2041731414557112

Kuppusamy, K. T., Jones, D. C., Sperber, H., Madan, A., Fischer, K. A., Rodriguez, M. L., et al. (2015). Let-7 family of microRNA is required for maturation and adult-like metabolism in stem cell-derived cardiomyocytes. *Proc. Natl. Acad. Sci. U.S.A.* 112, E2785–E2794. doi: 10.1073/pnas.1424042112

Laflamme, M. A., Chen, K. Y., Naumova, A. V., Muskheli, V., Fugate, J. A., Dupras, S. K., et al. (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat. Biotechnol.* 25, 1015–1024. doi: 10.1038/nbt1327

LaFramboise, W. A., Scalise, D., Stoodley, P., Graner, S. R., Guthrie, R. D., Magovern, J. A., et al. (2007). Cardiac fibroblasts influence cardiomyocyte phenotype in vitro. *Am. J. Physiol. Physiol.* 292, C1799–C1808. doi: 10.1152/ajpcell.00166.2006

Le, T., and Chong, J. (2017). Cardiac progenitor cells for heart repair. *Cell Death Discov.* 2, 1–4. doi: 10.1038/cddiscovery.2016.52

Lee, J. H., Protze, S. I., Laksman, Z., Backx, P. H., and Keller, G. M. (2017). Human pluripotent stem cell-derived atrial and ventricular cardiomyocytes develop from distinct mesoderm populations. *Cell Stem Cell* 21, 179.e–194.e. doi: 10.1016/j.stem.2017.07.003

Lee, P.-F., Chau, E., Cabello, R., Yeh, A. T., Sampaio, L. C., Gobin, A. S., et al. (2017). Inverted orientation improves decellularization of whole porcine hearts. *Acta Biomater.* 49, 181–191. doi: 10.1016/j.actbio.2016.11.047

Leitolis, A., Suss, P. H., Roderjan, J. G., Angulski, A. B. B., da Costa, F. D. A., Stimamiglio, M. A., et al. (2019). Human heart explant-derived extracellular vesicles: characterization and effects on the in vitro recellularization of decellularized heart valves. *Int. J. Mol. Sci.* 20:1279. doi: 10.3390/ijms20061279

Lemoine, M. D., Mannhardt, I., Breckwoldt, K., Prondzynski, M., Flenner, F., Ulmer, B., et al. (2017). Human iPSC-derived cardiomyocytes cultured in 3D engineered heart tissue show physiological upstroke velocity and sodium current density. *Sci. Rep.* 7:5464. doi: 10.1038/s41598-017-05600-w

Li, B., Yang, H., Wang, X., Zhan, Y., Sheng, W., Cai, H., et al. (2017). Engineering human ventricular heart muscles based on a highly efficient system for purification of human pluripotent stem cell-derived ventricular cardiomyocytes. *Stem Cell Res. Ther.* 8, 1–18. doi: 10.1186/s13287-017-0651-x

Li, Y., Lin, B., and Yang, L. (2015). Comparative transcriptomic analysis of multiple cardiovascular fates from embryonic stem cells predicts novel regulators in human cardiogenesis. *Sci. Rep.* 5:9758. doi: 10.1038/srep09758

Lian, X., Zhang, J., Azarin, S. M., Zhu, K., Hazeltine, L. B., Bao, X., et al. (2012). Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nat. Protoc.* 8, 162–175. doi: 10.1038/nprot.2012.150

Lin, B., Kim, J., Li, Y., Pan, H., Carvajal-Vergara, X., Salama, G., et al. (2012). High-purity enrichment of functional cardiovascular cells from human iPS cells. *Cardiovasc. Res.* 95, 327–335. doi: 10.1093/cvr/cvs185

Lindoso, R. S., Sandim, V., Collino, F., Carvalho, A. B., Dias, J., da Costa, M. R., et al. (2016). Proteomics of cell-cell interactions in health and disease. *Proteomics* 16, 328–344. doi: 10.1002/pmic.201500341

Lindsey, M. L., Jung, M., Hall, M. E., and DeLeon-Pennell, K. Y. (2018). Proteomic analysis of the cardiac extracellular matrix: clinical research applications. *Expert Rev. Proteomics* 15, 105–112. doi: 10.1080/14789450.2018.1421947

Lindsey, S. E., Butcher, J. T., and Yalcin, H. C. (2014). Mechanical regulation of cardiac development. *Front. Physiol.* 5:318. doi: 10.3389/fphys.2014.00318

Liu, B., Lee, B. W., Nakanishi, K., Villasante, A., Williamson, R., Metz, J., et al. (2018). Cardiac recovery via extended cell-free delivery of extracellular vesicles secreted by cardiomyocytes derived from induced pluripotent stem cells. *Nat. Biomed. Eng.* 2, 293–303. doi: 10.1038/s41551-018-0229-7

Liu, Q., Jiang, C., Xu, J., Zhao, M. T., Van Bortle, K., Cheng, X., et al. (2017). Genome-wide temporal profiling of transcriptome and open chromatin of early cardiomyocyte differentiation derived from hiPSCs and hESCs. *Circ. Res.* 121, 376–391. doi: 10.1161/CIRCRESAHA.116.310456

Liu, T., Zhang, R., Guo, T., Ma, S., Han, D., Li, X. J., et al. (2015). Cardiotrophin-1 promotes cardiomyocyte differentiation from mouse induced pluripotent stem cells via JAK2/STAT3/Pim-1 signaling pathway. *J. Geriatr. Cardiol.* 12, 591–599. doi: 10.11909/j.issn.1671-5411.2015.06.002

Liu, W., and Foley, A. C. (2011). Signaling pathways in early cardiac development. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 3, 191–205. doi: 10.1002/wsbm.112

Lock, M. C., Tellam, R. L., Botting, K. J., Wang, K. C. W., Selvanayagam, J. B., Brooks, D. A., et al. (2018). The role of miRNA regulation in fetal cardiomyocytes, cardiac maturation and the risk of heart disease in adults. *J. Physiol.* 596, 5625–5640. doi: 10.1113/JP276072

Lockhart, M., Wirrig, E., Phelps, A., and Wessels, A. (2011). Extracellular matrix and heart development. *Birth Defects Res. A Clin. Mol. Teratol.* 91, 535–550. doi: 10.1002/bdra.20810

Lu, J., Kaestle, K., Huang, J., Liu, Q., Zhang, P., Gao, L., et al. (2017). Interactions of human embryonic stem cell-derived cardiovascular progenitor cells with immobilized extracellular matrix proteins. *J. Biomed. Mater. Res. Part A* 105, 1094–1104. doi: 10.1002/jbm.a.36005

Lu, T.-Y., Lin, B., Kim, J., Sullivan, M., Tobita, K., Salama, G., et al. (2013). Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells. *Nat. Commun.* 4:2307. doi: 10.1038/ncomms3307

Lundy, S. D., Zhu, W.-Z., Regnier, M., and Laflamme, M. A. (2013). Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. *Stem Cells Dev.* 22, 1991–2002. doi: 10.1089/scd.2012.0490

Lyu, L., Wang, H., Li, B., Qin, Q., Qi, L., Nagarkatti, M., et al. (2013). A critical role of cardiac fibroblast-derived exosomes in activating renin angiotensin system in cardiomyocytes. *J. Mol. Cell Cardiol.* 82, 34–44. doi: 10.1016/j.yjmcc.2015.10.022

Ma, X., Yu, C., Wang, P., Xu, W., Wan, X., Lai, C. S. E., et al. (2018). Rapid 3D bioprinting of decellularized extracellular matrix with regionally varied mechanical properties and biomimetic microarchitecture. *Biomaterials* 185, 310–321. doi: 10.1016/J.BIOMATERIALS.2018.09.026

Machiraju, P., and Greenway, S. C. (2019). Current methods for the maturation of induced pluripotent stem cell-derived cardiomyocytes. *World J. Stem Cells* 11, 33–43. doi: 10.4252/wjsc.v11.i1.33

Majkut, S., Dingal, P. C. D. P., and Discher, D. E. (2014). Stress Sensitivity and mechanotransduction during heart development. *Curr. Biol.* 24, R495–R501. doi: 10.1016/j.cub.2014.04.027

Martin, M. L., and Blaxall, B. C. (2012). Cardiac intercellular communication: are myocytes and fibroblasts fair-weather friends? *J. Cardiovasc. Transl. Res.* 5, 768–782. doi: 10.1007/s12265-012-9404-9405

Marvin, M. J., Di Rocco, G., Gardiner, A., Bush, S. M., and Lassar, A. B. (2001). Inhibition of Wnt activity induces heart formation from posterior mesoderm. *Genes Dev.* 15, 316–327. doi: 10.1101/gad.855501

Mayourian, J., Ceholski, D. K., Gorski, P. A., Mathiyalagan, P., Murphy, J. F., Salazar, S. I., et al. (2018). Exosomal microRNA-21-5p mediates mesenchymal stem cell paracrine effects on human cardiac tissue contractility. *Circ. Res.* 122, 933–944. doi: 10.1161/CIRCRESAHA.118.312420

Moghaddam, A. S., Afshari, J. T., Esmaeili, S.-A., Saburi, E., Joneidi, Z., and Momtazi-Borojeni, A. A. (2019). Cardioprotective microRNAs: lessons from stem cell-derived exosomal microRNAs to treat cardiovascular disease. *Atherosclerosis* 285, 1–9. doi: 10.1016/j.atherosclerosis.2019.03.016

Mummery, C., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., et al. (2003). Differentiation of human embryonic stem cells to cardiomyocytes. *Circulation* 107, 2733–2740. doi: 10.1161/01.CIR.0000068356.38592.68

Mummery, C. L., Ward, D., and Passier, R. (2007). Differentiation of human embryonic stem cells to cardiomyocytes by coculture with endoderm in serum-free medium. *Curr. Protoc. Stem Cell Biol.* 2:1F.2.1-1F.2.14. doi: 10.1002/9780470151808.sc01f02s2

Mummery, C. L., Zhang, J., Ng, E. S., Elliott, D. A., Elefanty, A. G., and Kamp, T. J. (2012). Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. *Circ. Res.* 111, 344–358. doi: 10.1161/CIRCRESAHA.110.227512

Murry, C. E., and Keller, G. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. *Cell* 132, 661–680. doi: 10.1016/j.cell.2008.02.008

Naba, A., Clauser, K. R., Ding, H., Whittaker, C. A., Carr, S. A., and Hynes, R. O. (2016). The extracellular matrix: tools and insights for the "omics" era. *Matrix Biol.* 49, 10–24. doi: 10.1016/j.matbio.2015.06.003

Naba, A., Clauser, K. R., Hoersch, S., Liu, H., Carr, S. A., and Hynes, R. O. (2012). The matrisome: in silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. *Mol. Cell. Proteomics* 11, M111.014647. doi: 10.1074/mcp.M111.014647

Ng, S. L. J., Narayanan, K., Gao, S., and Wan, A. C. A. (2011). Lineage restricted progenitors for the repopulation of decellularized heart. *Biomaterials* 32, 7571–7580. doi: 10.1016/j.biomaterials.2011.06.065

Nguyen, D. T., O'Hara, M., Graneli, C., Hicks, R., Miliotis, T., Nyström, A. C., et al. (2018). Humanizing miniature hearts through 4-flow cannulation perfusion decellularization and recellularization. *Sci. Rep.* 8, 1–10. doi: 10.1038/s41598-018-25883-x

Noor, N., Shapira, A., Edri, R., Gal, I., Wertheim, L., and Dvir, T. (2019). 3D printing of personalized thick and perfusable cardiac patches and hearts. *Adv. Sci.* 6:1900344. doi: 10.1002/advs.201900344

Nunes, S. S., Miklas, J. W., Liu, J., Aschar-Sobbi, R., Xiao, Y., Zhang, B., et al. (2013). Biowire: a platform for maturation of human pluripotent stem cell–derived cardiomyocytes. *Nat. Methods* 10, 781–787. doi: 10.1038/nmeth.2524

Oberwallner, B., Brodarac, A., Aniæ, P., Šariæ, T., Wassilew, K., Neef, K., et al. (2015). Human cardiac extracellular matrix supports myocardial lineage commitment of pluripotent stem cells. *Eur. J. Cardio-thoracic Surg.* 47, 416–425. doi: 10.1093/ejcts/ezu163

Ogasawara, T., Okano, S., Ichimura, H., Kadota, S., Tanaka, Y., Minami, I., et al. (2017). Impact of extracellular matrix on engraftment and maturation of pluripotent stem cell-derived cardiomyocytes in a rat myocardial infarct model. *Sci. Rep.* 7:8630. doi: 10.1038/s41598-017-09217-x

Ogle, B. M., Bursac, N., Domian, I., Huang, N. F., Menasché, P., Murry, C. E., et al. (2016). Distilling complexity to advance cardiac tissue engineering. *Sci. Transl. Med.* 8, s13–s342. doi: 10.1126/scitranslmed.aad2304

Ott, H. C., Matthiesen, T. S., Goh, S.-K., Black, L. D., Kren, S. M., Netoff, T. I., et al. (2008). Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat. Med.* 14, 213–221. doi: 10.1038/nm1684

Paige, S. L., Plonowska, K., Xu, A., and Wu, S. M. (2015). Molecular regulation of cardiomyocyte differentiation. *Circ. Res.* 116, 341–353. doi: 10.1161/CIRCRESAHA.116.302752

Paige, S. L., Thomas, S., Stoick-Cooper, C. L., Wang, H., Maves, L., Sandstrom, R., et al. (2012). A temporal chromatin signature in human embryonic stem cells identifies regulators of cardiac development. *Cell* 151, 221–232. doi: 10.1016/j.cell.2012.08.027

Paradis, A., Xiao, D., Zhou, J., and Zhang, L. (2014). Endothelin-1 promotes cardiomyocyte terminal differentiation in the developing heart via heightened DNA methylation. *Int. J. Med. Sci.* 11, 373–380. doi: 10.7150/ijms.7802

Pedrotty, D. M., Klinger, R. Y., Kirkton, R. D., and Bursac, N. (2009). Cardiac fibroblast paracrine factors alter impulse conduction and ion channel expression of neonatal rat cardiomyocytes. *Cardiovasc. Res.* 83, 688–697. doi: 10.1093/cvr/cvp164

Perea-Gil, I., Gálvez-Montón, C., Prat-Vidal, C., Jorba, I., Segú-Vergés, C., Roura, S., et al. (2018). Head-to-head comparison of two engineered cardiac grafts for myocardial repair: from scaffold characterization to pre-clinical testing. *Sci. Rep.* 8, 1–13. doi: 10.1038/s41598-018-25115-25112

Pereira, I. T., Spangenberg, L., Robert, A. W., Amorín, R., Stimamiglio, M. A., Naya, H., et al. (2018). Polysome profiling followed by RNA-seq of cardiac differentiation stages in hESCs. *Sci. Data* 5, 1–11. doi: 10.1038/sdata.2018.287

Pereira, I. T., Spangenberg, L., Robert, A. W., Amorín, R., Stimamiglio, M. A., Naya, H., et al. (2019). Cardiomyogenic differentiation is fine-tuned by differential mRNA association with polysomes. *BMC Genomics* 20:219. doi: 10.1186/s12864-019-5550-5553

Pinto, A. R., Ilinykh, A., Ivey, M. J., Kuwabara, J. T., D'antoni, M. L., Debuque, R., et al. (2016). Revisiting cardiac cellular composition. *Circ. Res.* 118, 400–409. doi: 10.1161/CIRCRESAHA.115.307778

Piryani, S. O., Jiao, Y., Kam, A. Y. F., Liu, Y., Vo-Dinh, T., Chen, B. J., et al. (2019). Endothelial cell-derived extracellular vesicles mitigate radiation-induced hematopoietic injury. *Int. J. Radiat. Oncol. Biol. Phys.* 104, 291–301. doi: 10.1016/j.ijrobp.2019.02.008

Poon, E., Keung, W., Liang, Y., Ramalingam, R., Yan, B., Zhang, S., et al. (2015). Proteomic analysis of human pluripotent stem cell-derived, fetal, and adult ventricular cardiomyocytes reveals pathways crucial for cardiac metabolism and maturation. *Circ. Cardiovasc. Genet.* 8, 427–436. doi: 10.1161/CIRCGENETICS.114.000918

Protze, S. I., Liu, J., Nussinovitch, U., Ohana, L., Backx, P. H., Gepstein, L., et al. (2016). Sinoatrial node cardiomyocytes derived from human pluripotent cells function as a biological pacemaker. *Nat. Biotechnol.* 35, 56–68. doi: 10.1038/nbt.3745

Pursani, V., Pethe, P., Bashir, M., Sampath, P., Tanavde, V., and Bhartiya, D. (2017). Genetic and epigenetic profiling reveals EZH2-mediated down regulation of OCT-4 involves NR2F2 during cardiac differentiation of human embryonic stem cells. *Sci. Rep.* 7, 1–14. doi: 10.1038/s41598-017-13442-13449

Ravenscroft, S. M., Pointon, A., Williams, A. W., Cross, M. J., and Sidaway, J. E. (2016). Cardiac non-myocyte cells show enhanced pharmacological function suggestive of contractile maturity in stem cell derived cardiomyocyte microtissues. *Toxicol. Sci.* 152, 99–112. doi: 10.1093/toxsci/kfw069

Ren, Y., Lee, M. Y., Schliffke, S., Paavola, J., Amos, P. J., Ge, X., et al. (2011). Small molecule Wnt inhibitors enhance the efficiency of BMP-4-directed cardiac differentiation of human pluripotent stem cells. *J. Mol. Cell. Cardiol.* 51, 280–287. doi: 10.1016/j.yjmcc.2011.04.012

Reus, T. L., Robert, A. W., Da Costa, M. B. A., de Aguiar, A. M., and Stimamiglio, M. A. (2016). Secretome from resident cardiac stromal cells stimulates proliferation, cardiomyogenesis and angiogenesis of progenitor cells. *Int. J. Cardiol.* 221, 396–403. doi: 10.1016/j.ijcard.2016.06.199

Ricard-Blum, S., and Salza, R. (2014). Matricryptins and matrikines: biologically active fragments of the extracellular matrix. *Exp. Dermatol.* 23, 457–463. doi: 10.1111/exd.12435

Rienks, M., and Papageorgiou, A.-P. (2016). Novel regulators of cardiac inflammation: matricellular proteins expand their repertoire. *J. Mol. Cell. Cardiol.* 91, 172–178. doi: 10.1016/j.yjmcc.2016.01.008

Rienks, M., Papageorgiou, A.-P., Frangogiannis, N. G., and Heymans, S. (2014). Myocardial extracellular matrix: an ever-changing and diverse entity. *Circ. Res.* 114, 872–888. doi: 10.1161/CIRCRESAHA.114.302533

Robert, A. W., Schittini, A. V., Marchini, F. K., Batista, M., Affonso Da Costa, M. B., Senegaglia, A. C., et al. (2017). Tissue-derived signals for mesenchymal stem cell stimulation: role of cardiac and umbilical cord microenvironments. *Cells Tissues Organs* 203, 173–182. doi: 10.1159/000450600

Robertson, C., Tran, D. D., and George, S. C. (2013). Concise review: maturation phases of human pluripotent stem cell-derived cardiomyocytes. *Stem Cells* 31, 829–837. doi: 10.1002/stem.1331

Ronaldson-Bouchard, K., Ma, S. P., Yeager, K., Chen, T., Song, L., Sirabella, D., et al. (2018). Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature* 556, 239–243. doi: 10.1038/s41586-018-0016-13

Rozario, T., and DeSimone, D. W. (2010). The extracellular matrix in development and morphogenesis: a dynamic view. *Dev. Biol.* 341, 126–140. doi: 10.1016/j.ydbio.2009.10.026

Rupert, C. E., and Coulombe, K. L. K. (2015). The roles of neuregulin-1 in cardiac development, homeostasis, and disease. *Biomark. Insights* 10, 1–9. doi: 10.4137/BMIMI.S20061

Rupert, C. E., and Coulombe, K. L. K. (2017). IGF1 and NRG1 enhance proliferation, metabolic maturity, and the force-frequency response in hESC-derived engineered cardiac tissues. *Stem Cells Int.* 2017, 1–13. doi: 10.1155/2017/7648409

Sa, S., Wong, L., and McCloskey, K. E. (2014). Combinatorial fibronectin and laminin signaling promote highly efficient cardiac differentiation of human embryonic stem cells. *Biores. Open Access* 3, 150–161. doi: 10.1089/biores.2014.0018

Samal, R., Sappa, P. K., Gesell Salazar, M., Wenzel, K., Reinke, Y., Völker, U., et al. (2018). Global secretome analysis of resident cardiac progenitor cells from wild-type and transgenic heart failure mice: why ambience matters. *J. Cell. Physiol.* 234, 10111–10122. doi: 10.1002/jcp.27677

Sánchez, P. L., Fernández-Santos, M. E., Costanza, S., Climent, A. M., Moscoso, I., Gonzalez-Nicolas, M. A., et al. (2015). Acellular human heart matrix: a critical step toward whole heart grafts. *Biomaterials* 61, 279–289. doi: 10.1016/j.biomaterials.2015.04.056

Sartiani, L., Bettiol, E., Stillitano, F., Mugelli, A., Cerbai, E., and Jaconi, M. E. (2007). Developmental changes in cardiomyocytes differentiated from human embryonic stem cells: a molecular and electrophysiological approach. *Stem Cells* 25, 1136–1144. doi: 10.1634/stemcells.2006-2466

Scarrit, M. E., Pashos, N. C., and Bunnell, B. A. (2015). A review of cellularization strategies for tissue engineering of whole organs. *Front. Bioeng. Biotechnol.* 3:43. doi: 10.3389/fbioe.2015.00043

Schenke-Layland, K., Nsair, A., Van Handel, B., Angelis, E., Gluck, J. M., Votteler, M., et al. (2011). Recapitulation of the embryonic cardiovascular progenitor cell niche. *Biomaterials* 32, 2748–2756. doi: 10.1016/j.biomaterials.2010.12.046

Schittini, A. V., Celedon, P. F., Stimamiglio, M. A., Krieger, M., Hansen, P., Da Costa, F. D. A., et al. (2010). Human cardiac explant-conditioned medium: soluble factors and cardiomyogenic effect on mesenchymal stem cells. *Exp. Biol. Med.* 235, 1015–1024. doi: 10.1258/ebm.2010.010003

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* 4, 7–25.

Schwan, J., Kwaczala, A. T., Ryan, T. J., Bartulos, O., Ren, Y., Sewanan, L. R., et al. (2016). Anisotropic engineered heart tissue made from laser-cut decellularized myocardium. *Sci. Rep.* 6, 1–12. doi: 10.1038/srep32068

Shadrin, I. Y., Allen, B. W., Qian, Y., Jackman, C. P., Carlson, A. L., Juhas, M. E., et al. (2017). Cardiopatch platform enables maturation and scale-up of human pluripotent stem cell-derived engineered heart tissues. *Nat. Commun.* 8:1825. doi: 10.1038/s41467-017-01946-x

Shah, A. M. (1996). Paracrine modulation of heart cell function by endothelial cells. *Cardiovasc. Res.* 31, 847–867. doi: 10.1016/0008-6363(96)00025-29

Sharma, S., Mishra, R., Bigham, G. E., Wehman, B., Khan, M. M., Xu, H., et al. (2017). A deep proteome analysis identifies the complete secretome as the functional unit of human cardiac progenitor cells. *Circ. Res.* 120, 816–834. doi: 10.1161/CIRCRESAHA.116.309782

Sharma, S., Mishra, R., Simpson, D., Wehman, B., Colletti, E. J., Deshmukh, S., et al. (2015). Cardiosphere-derived cells from pediatric end-stage heart failure patients have enhanced functional activity due to the heat shock response regulating the secretome. *Stem Cells* 33, 1213–1229. doi: 10.1002/stem.1937

Smith, A. S. T., Macadangdang, J., Leung, W., Laflamme, M. A., and Kim, D.-H. (2017). Human iPSC-derived cardiomyocytes and tissue engineering strategies for disease modeling and drug screening. *Biotechnol. Adv.* 35, 77–94. doi: 10.1016/J.BIOTECHADV.2016.12.002

Stik, G., Crequit, S., Petit, L., Durant, J., Charbord, P., Jaffredo, T., et al. (2017). Extracellular vesicles of stromal origin target and support hematopoietic stem and progenitor cells. *J Cell Biol.* 216, 2217–2230. doi: 10.1083/JCB.201601109

Stoppel, W. L., Kaplan, D. L., and Black, L. D. (2016). Electrical and mechanical stimulation of cardiac cells and tissue constructs. *Adv. Drug Deliv. Rev.* 96, 135–155. doi: 10.1016/j.addr.2015.07.009

Sun, C., and Kontaridis, M. I. (2018). Physiology of cardiac development: from genetics to signaling to therapeutic strategies. *Curr. Opin. Physiol.* 1, 123–139. doi: 10.1016/j.cophys.2017.09.002

Sylva, M., van den Hoff, M. J. B., and Moorman, A. F. M. (2014). Development of the human heart. *Am. J. Med. Genet. Part A* 164, 1347–1371. doi: 10.1002/ajmg.a.35896

Takeda, N., and Manabe, I. (2011). Cellular interplay between cardiomyocytes and nonmyocytes in cardiac remodeling. *Int. J. Inflam.* 2011, 1–13. doi: 10.4061/2011/535241

Tallquist, M. D., and Molkentin, J. D. (2017). Redefining the identity of cardiac fibroblasts. *Nat. Rev. Cardiol.* 14, 484–491. doi: 10.1038/nrcardio.2017.57

Talman, V., and Kivelä, R. (2018). Cardiomyocyte—endothelial cell interactions in cardiac remodeling and regeneration. *Front. Cardiovasc. Med.* 5:101. doi: 10.3389/fcvm.2018.00101

Tang-Quan, K. R., Mehta, N. A., Sampaio, L. C., and Taylor, D. A. (2018). "Whole cardiac tissue bioscaffolds," in *Advances in Experimental Medicine and Biology*, eds E. G. Schmuck, P. Hematti, and A. N. Raval (Cham: Springer International Publishing), 85–114. doi: 10.1007/978-3-319-97421-7_5

Thimm, T. N., Squirrell, J. M., Liu, Y., Eliceiri, K. W., and Ogle, B. M. (2015). Endogenous optical signals reveal changes of elastin and collagen organization during differentiation of mouse embryonic stem cells. *Tissue Eng. Part C Methods* 21, 995–1004. doi: 10.1089/ten.tec.2014.0699

Tirziu, D., Giordano, F. J., and Simons, M. (2010). Cell communications in the heart. *Circulation* 122, 928–937. doi: 10.1161/CIRCULATIONAHA.108.847731

Tohyama, S., Hattori, F., Sano, M., Hishiki, T., Nagahata, Y., Matsuura, T., et al. (2013). Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell Stem Cell* 12, 127–137. doi: 10.1016/j.stem.2012.09.013

Tompkins, J. D., Jung, M., Chen, C. Y., Lin, Z., Ye, J., Godatha, S., et al. (2016). Mapping human pluripotent-to-cardiomyocyte differentiation: methylomes, transcriptomes, and exon DNA methylation "memories. *EBioMedicine* 4, 74–85. doi: 10.1016/j.ebiom.2016.01.021

Torán, J. L., López, J. A., Gomes-Alves, P., Aguilar, S., Torroja, C., Trevisan-Herraz, M., et al. (2019). Definition of a cell surface signature for human cardiac progenitor cells after comprehensive comparative transcriptomic and proteomic characterization. *Sci. Rep.* 9, 1–16. doi: 10.1038/s41598-019-39571-x

Torella, D., Ellison, G. M., Méndez-Ferrer, S., Ibanez, B., and Nadal-Ginard, B. (2006). Resident human cardiac stem cells: role in cardiac cellular homeostasis and potential for myocardial regeneration. *Nat. Clin. Pract. Cardiovasc. Med.* 3, 8–13. doi: 10.1038/ncpcardio0409

Tulloch, N. L., Muskheli, V., Razumova, M. V., Korte, F. S., Regnier, M., Hauch, K. D., et al. (2011). Growth of engineered human myocardium with mechanical loading and vascular coculture. *Circ. Res.* 109, 47–59. doi: 10.1161/CIRCRESAHA.110.237206

Tyukavin, A. I., Belostotskaya, G. B., Golovanova, T. A., Galagudza, M. M., Zakharov, E. A., Burkova, N. V., et al. (2015). Stimulation of proliferation and differentiation of rat resident myocardial cells with apoptotic bodies of cardiomyocytes. *Bull. Exp. Biol. Med.* 159, 138–141. doi: 10.1007/s10517-015-2909-6

Ueno, S., Weidinger, G., Osugi, T., Kohn, A. D., Golob, J. L., Pabon, L., et al. (2007). Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9685–9690. doi: 10.1073/pnas.0702859104

Van Hoof, D., Dormeyer, W., Braam, S. R., Passier, R., Monshouwer-kloots, J., Oostwaard, D. W., et al. (2010). Identification of cell surface proteins for antibody-based selection of human embryonic stem cell-derived cardiomyocytes. *J Proteome Res.* 9, 1610–1618. doi: 10.1021/pr901138a

van Laake, L. W., van Donselaar, E. G., Monshouwer-Kloots, J., Schreurs, C., Passier, R., Humbel, B. M., et al. (2010). Extracellular matrix formation after transplantation of human embryonic stem cell-derived cardiomyocytes. *Cell. Mol. Life Sci.* 67, 277–290. doi: 10.1007/s00018-009-0179-z

Vidarsson, H., Hyllner, J., and Sartipy, P. (2010). Differentiation of human embryonic stem cells to cardiomyocytes for in vitro and in vivo applications. *Stem Cell Rev. Reports* 6, 108–120. doi: 10.1007/s12015-010-9113-x

Wagner, M., and Siddiqui, M. A. (2007). Signal transduction in early heart development (I): cardiogenic induction and heart tube formation. *Exp. Biol. Med.* 232, 852–865.

Wang, Y., Zhao, R., Liu, D., Deng, W., Xu, G., Liu, W., et al. (2018). Exosomes derived from miR-214-enriched bone marrow-derived mesenchymal stem cells regulate oxidative damage in cardiac stem cells by targeting CaMKII. *Oxid. Med. Cell. Longev.* 2018, 1–21. doi: 10.1155/2018/4971261

Wang, Z., Long, D. W., Huang, Y., Chen, W. C. W., Kim, K., and Wang, Y. (2019). Decellularized neonatal cardiac extracellular matrix prevents widespread ventricular remodeling in adult mammals after myocardial infarction. *Acta Biomater.* 87, 140–151. doi: 10.1016/j.actbio.2019.01.062

Wendt, S., Goetzenich, A., Goettsch, C., Stoppe, C., Bleilevens, C., Kraemer, S., et al. (2018). Evaluation of the cardioprotective potential of extracellular vesicles – a systematic review and meta-analysis. *Sci. Rep.* 8, 1–14. doi: 10.1038/s41598-018-33862-5

Weymann, A., Patil, N. P., Sabashnikov, A., Jungebluth, P., Korkmaz, S., Li, S., et al. (2014). Bioartificial heart: a human-sized porcine model – the way ahead. *PLoS One* 9:e111591. doi: 10.1371/journal.pone.0111591

White, M. C., Pang, L., and Yang, X. (2016). MicroRNA-mediated maturation of human pluripotent stem cell-derived cardiomyocytes: towards a better model

for cardiotoxicity? *Food Chem. Toxicol.* 98, 17–24. doi: 10.1016/j.fct.2016.05.025

Wolling, H., Konze, S. A., Höfer, A., Erdmann, J., Pich, A., Zweigerdt, R., et al. (2018). Quantitative secretomics reveals extrinsic signals involved in human pluripotent stem cell cardiomyogenesis. *Proteomics* 18, 1–12. doi: 10.1002/pmic.201800102

Xin, T., Greco, V., and Myung, P. (2016). Hardwiring stem cell communication through tissue structure. *Cell* 164, 1212–1225. doi: 10.1016/j.cell.2016.02.041

Xu, C., Police, S., Rao, N., and Carpenter, M. K. (2002). Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. *Circ. Res.* 91, 501–508. doi: 10.1161/01.RES.0000035254.80718.91

Xu, Q. X., Zweigerdt, R., Soo, S. Y., Ngoh, Z. X., Tham, S. C., Wang, S. T., et al. (2008). Highly enriched cardiomyocytes from human embryonic stem cells. *Cytotherapy* 10, 376–389. doi: 10.1080/14653240802105307

Xu, X. Q., Soo, S. Y., Sun, W., and Zweigerdt, R. (2009). Global expression profile of highly enriched cardiomyocytes derived from human embryonic stem cells. *Stem Cells* 27, 2163–2174. doi: 10.1002/stem.166

Yang, L., Soonpaa, M. H., Adler, E. D., Roepke, T. K., Kattman, S. J., Kennedy, M., et al. (2008). Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* 453, 524–528. doi: 10.1038/nature06894

Yang, X., Pabon, L., and Murry, C. E. (2014). Engineering adolescence: maturation of human pluripotent stem cell–derived cardiomyocytes. *Circ. Res.* 114, 511–523. doi: 10.1161/CIRCRESAHA.114.300558

Yap, L., Wang, J.-W., Moreno-Moral, A., Chong, L. Y., Sun, Y., Harmston, N., et al. (2019). In Vivo generation of post-infarct human cardiac muscle by laminin-promoted cardiovascular progenitors. *Cell Rep.* 26, 3231.e–3245.e. doi: 10.1016/j.celrep.2019.02.083

Yoshida, S., Miyagawa, S., Fukushima, S., Kawamura, T., Kashiyama, N., Ohashi, F., et al. (2018). Maturation of human induced pluripotent stem cell-derived cardiomyocytes by soluble factors from human mesenchymal stem cells. *Mol. Ther.* 26, 2681–2695. doi: 10.1016/j.ymthe.2018.08.012

Yu, C., Ma, X., Zhu, W., Wang, P., Miller, K. L., Stupin, J., et al. (2019). Scanningless and continuous 3D bioprinting of human tissues with decellularized extracellular matrix. *Biomaterials* 194, 1–13. doi: 10.1016/J.BIOMATERIALS.2018.12.009

Zhang, D., Shadrin, I. Y., Lam, J., Xian, H.-Q., Snodgrass, H. R., and Bursac, N. (2013). Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. *Biomaterials* 34, 5813–5820. doi: 10.1016/j.biomaterials.2013.04.026

Zhang, J., Klos, M., Wilson, G. F., Herman, A. M., Lian, X., Raval, K. K., et al. (2012). Extracellular matrix promotes highly efficient cardiac differentiation of human pluripotent stem cells: the matrix sandwich method. *Circ. Res.* 111, 1125–1136. doi: 10.1161/CIRCRESAHA.112.273144

Zhang, P., Su, J., and Mende, U. (2012). Cross talk between cardiac myocytes and fibroblasts: from multiscale investigative approaches to mechanisms and functional consequences. *Am. J. Physiol. Circ. Physiol.* 303, H1385–H1396. doi: 10.1152/ajpheart.01167.2011

Zhang, X., Guo, J. P., Chi, Y. L., Liu, Y. C., Zhang, C. S., Yang, X. Q., et al. (2012). Endothelin-induced differentiation of Nkx2.5 + cardiac progenitor cells into pacemaking cells. *Mol. Cell. Biochem.* 366, 309–318. doi: 10.1007/s11010-012-1309-8

Zhang, J., Wilson, G. F., Soerens, A. G., Koonce, C. H., Yu, J., Palecek, S. P., et al. (2009). Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ. Res.* 104, 30–41. doi: 10.1161/CIRCRESAHA.108.192237

Zhang, Q., Jiang, J., Han, P., Yuan, Q., Zhang, J., Zhang, X., et al. (2011). Direct differentiation of atrial and ventricular myocytes from human embryonic stem cells by alternating retinoid signals. *Cell Res.* 21, 579–587. doi: 10.1038/cr.2010.163

Zhang, W., Kong, C. W., Tong, M. H., Chooi, W. H., Huang, N., Li, R. A., et al. (2017). Maturation of human embryonic stem cell-derived cardiomyocytes (hESC-CMs) in 3D collagen matrix: effects of niche cell supplementation and mechanical stimulation. *Acta Biomater.* 49, 204–217. doi: 10.1016/j.actbio.2016.11.058

Zhang, Y., Sivakumaran, P., Newcomb, A. E., Hernandez, D., Harris, N., Khanabdali, R., et al. (2015). Cardiac repair with a novel population of mesenchymal stem cells resident in the human heart. *Stem Cells* 33, 3100–3113. doi: 10.1002/stem.2101

Zhu, R., Blazeski, A., Poon, E., Costa, K. D., Tung, L., and Boheler, K. R. (2014). Physical developmental cues for the maturation of human pluripotent stem cell-derived cardiomyocytes. *Stem Cell Res. Ther.* 5:117. doi: 10.1186/scrt507

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REVIEW
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A Revised Perspective of Skeletal Stem Cell Biology

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Bone-related maladies are a major health burden on modern society. Loss of skeletal integrity and regeneration capacity through aging, obesity, and disease follows from a detrimental shift in bone formation and resorption dynamics. Targeting tissue-resident adult stem cells offers a potentially innovative paradigm in the development of therapeutic strategies against organ dysfunction. While the essential role of skeletal stem cells (SSCs) for development, growth, and maintenance of the skeleton has been generally established, a common consensus on the exact identity and definition of a pure *bona fide* SSC population remains elusive. The controversies stem from conflicting results between different approaches and criteria for isolation, detection, and functional evaluation; along with the interchangeable usage of the terms SSC and "mesenchymal stromal/stem cell (MSC)". A great number of prospective bone-forming stem cell populations have been reported with various characteristic markers, often describing overlapping cell populations with widely unexplored heterogeneity, species specificity, and distribution at distinct skeletal sites, bone regions, and microenvironments, thereby creating confusion that may complicate future advances in the field. In this review, we examine the state-of-the-art knowledge of SSC biology and try to establish a common ground for the definition and terminology of specific bone-resident stem cells. We also discuss recent advances in the identification of highly purified SSCs, which will allow detailed interrogation of SSC diversity and regulation at the single-cell level.

Keywords: bone, skeletal stem cell, bone marrow/mesenchymal stromal/stem cell, stem cell niche, heterogeneity, regeneration, aging

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INTRODUCTION

The skeleton is a composite of diverse tissue types, including cells of osseous, adipogenic, cartilaginous, stromal/endothelial, and hematopoietic lineages. Beyond its central role in locomotion, it has also been established as an endocrine organ that is essential for mineral homeostasis and systemic health (Karsenty and Ferron, 2012). Bone is fundamental for structural support and movement, which is reflected in the observation that people suffering from impaired bone fitness inevitably experience a decline in quality of life and increased morbidity (Demontiero et al., 2012; Lee et al., 2014). There is a growing focus on skeletal health worldwide due to the rising number of elderly people, which has dramatically increased the burden of bone fractures and

skeletal disorders. One of the main metabolic diseases, which has expanded exponentially alongside that development, is osteoporosis, a condition resulting from an imbalance of the ability to form bone at the same speed as it is resorbed. Osteoporosis leads to increased fragility and occurrence of fractures (Pagnotti et al., 2019). There are over six million fractures per year in the United States alone, with the majority occurring in the older population. While in healthy individuals bones normally have the ability to heal without scarring, in up to ten percent of the cases fractures do not heal in an adequate time frame and develop to so-called nonunions, with limited treatment options (Ekegren et al., 2018). Along with osteoporosis, degenerative joint disease, or osteoarthritis, is a top cause of disability in the aged adult, which is characterized by deteriorated cartilage resulting in bone-to-bone rubbing creating increased stiffness, pain, and impaired movement. It is currently unclear why adult cartilage does not naturally regenerate like bone tissue. Importantly, osteoporosis and osteoarthritis are not only associated with aging but also sedentary lifestyle and metabolic health, underlining the systemic interrelation between bone health and physiological health (Chen et al., 2017). Therefore, advances in understanding of the stem and progenitor cells of bone and cartilage could have major ramifications for global health.

The cellular origins of bone and cartilage have been extensively studied. Like all connective tissues, the bone compartment contains cell populations with multipotent differentiation potential, generally referred to as "mesenchymal stromal/stem cells" (MSCs). Beside putative roles in controlling the bone marrow niche, these cells are thought to be the main source of bone formation during organ growth, maintenance, and repair. Given their beneficial properties, differentiation capacity, and expandability, strong efforts have been made to exploit those characteristics for cell-based regenerative therapies. The success of "MSC"-based approaches have been hampered by the high heterogeneity of the transplanted cell populations, mainly attributable to their varying tissue source but also to discrepancies in the techniques for detection, purification, and isolation of prospective stem cells (Galipeau and Sensebe, 2018). Research in mice is continually adding to the number of potential bone stem cell populations defined by cell surface proteins and genetic labeling, however, the majority of reports are limited by concerns of cell purity, lack information on the degree of overlap with previously reported cell populations, and most importantly are inappropriate for translation to the human setting (Bianco and Robey, 2015). A major drawback is that the identity and use of human bone marrow-derived stem cells have long been based on plastic culture adherence. Unfortunately, the relative technical ease of this technique and its use for isolating other morphologically similar fibroblastic cell types from various connective tissues coupled to deceptive *in vitro* differentiation regiments has helped fuel doubtful claims, offering "MSC cell therapies" for regenerative purposes, resulting in detrimental rather than beneficial outcomes (Sipp et al., 2018). First and foremost, there is no scientific rationale, or much less pre-clinical data, justifying the use of those cells from any tissue source for clinical application. Considering the extensive literature on bone-residing stem cells, there is a need for a more standardized functional characterization of potential cell types. Reported "MSCs," or rather multipotent bone marrow stromal cell (BMSC) populations, display a variety of differences including developmental occurrence (e.g., pre- vs. post-natal), localization, and differentiation potential, with the most striking differences being obvious between classical perisinusoidal and growth plate/periosteal bone-forming cells, which will be discussed in detail (Sacchetti et al., 2007; Tormin et al., 2011; Chan et al., 2015, 2018; Ambrosi et al., 2017). Accumulating evidence suggests that the terms "MSC"/BMSC and skeletal stem cell (SSC), which have been used interchangeably, are describing both distinct and overlapping stem cell population with different properties and functions.

In light of these observations, this review aims to collectively compare reported bone-residing stem cell populations in mice and humans; and to establish a common terminology in order to promote a better basis for the development of successful research strategies. We have focused on findings of the appendicular skeleton, as the majority of scientific reports are based on experiments using limb and hip bone tissues. This is likely assignable to the ready access of specimen for these skeletal sites in mice and humans. It remains to be shown if findings can be generalized to all bone compartments and future investigations will have to explore if embryonic origin, skeletal form, and cell composition affect the SSC source. Importantly, existing controversies in the field are due to laboratory-specific availability as well as preference of technology and genetic models for the identification of "MSCs"/SSCs. Establishing a common ground will have great importance for a better understanding of scientific data and more efficient paradigms of regenerative approaches.

DEFINING SKELETAL STEM CELLS

Stem cells are characterized by their ability to self-renew and to differentiate into multiple cell fates thereby contributing to tissue ontogeny, growth, and turnover for regeneration throughout life (Bianco and Robey, 2015). All cells of an organism are descendants of a zygote with unique totipotency, which is lost after the preimplantation stage of the blastocyst, with exception of germline stem cells (Evans and Kaufman, 1981; Martin, 1981). At that timepoint, defined multipotent, fate-restricted fetal stem cells (and then postnatal stem cells) emerge, orchestrating organ maturation and maintenance. It has to be stressed that despite some early controversial claims there is no evidence for the existence of stem cells with *in vivo* pluripotency in adult tissue (Jiang et al., 2002; Miyanishi et al., 2013). However, ground-breaking advancements in cellular reprograming have been able to generate induced pluripotent stem cells from diverse somatic cell origins *in vitro* (Takahashi and Yamanaka, 2006).

The concept of stem cells dates back as far as the middle of the 19th century, when Ernst Haeckel first coined the term "Stammzelle" (Dose, 1981), suggesting the origin of living cells as an evolutionary sequence. This theory was extended and experimentally addressed by contributions of pioneers including Arthur Pappenheim and Alexander Maximov, eventually leading

to the seminal finding of the existence of a hematopoietic stem cell (HSCs) by Till and McCulloch, as they described that single rare bone marrow cells could form multilineage myelo-erythroid colonies in the spleen of lethally irradiated mice (Till and McCulloch, 1961; Becker et al., 1963). This discovery provided the first definitive proof of the presence of a postnatal *bona fide* stem cell but did not yet enable the prospective isolation of phenotypically defined cells. With the development of more sophisticated technologies such as flow cytometry, a cell population considerably enriched for HSCs was later first described by Spangrude et al. (1988) building the foundation for today's concept of the hematopoietic lineage tree (Laurenti and Gottgens, 2018).

Recent efforts have led to the identification of multiple types of lineage-restricted postnatal stem cells with self-renewal and multipotent differentiation ability in many other organs. The study of stem cells has given important insights into mechanisms of tissue biology that provide great potential for the development of novel cell-based regenerative therapies. However, the exciting nature of stem cell concepts has also promoted the spread of dubious physicians and companies offering unproven stem cell-based therapies that lack peer-reviewed demonstrations of their efficacy as well as IRB (Internal Review Board)- and FDA (Federal Drug Administration)-type approvals, leading to adverse outcomes and harming the reputation of the stem cell research field (Sipp et al., 2018). One of the main causes is the incorrect use of the term "stem cell" for weakly defined cell populations comprised of heterogeneous cell types, i.e., more committed cell populations with varying differentiating potential and cells from other lineages. A striking example can be found in the field of bone marrow transplantations, which are often advertised as "hematopoietic stem cell transplants" but which in actuality are relatively unenriched mixtures of many cell types with only a very small number of stem cells. Some of the cell types that are co-transplanted include lymphocytes that could cause graft vs. host disease (GVHD), though in some cases they could also lead to helpful graft vs. tumor responses (Szyska and Na, 2016). Transplanting impure, undefined mixtures of cells not only hampers the predictability and reproducibility of treatments but may additionally lead to false assumptions by patients and disappointing results that could tarnish popular perceptions of stem cell research and stem cell-based medicine. It is, therefore, of great importance to re-establish solid foundations for stem cell research that focuses on highly purified, homogeneous populations and uses both, *in vitro* and *in vivo* assays, to identify true stem cells that display all the hallmark stem cell characteristics, i.e., quiescence, self-renewal, and multipotency at the single-cell level (Weissman, 2002; Bianco et al., 2008).

The Road to Identifying the Skeletal Stem Cell

Almost paralleling the description of the HSC, evidence for the existence of a second bone-resident stem cell appeared. This type of stem cell was distinct from HSCs as it was proposed to be the origin of non-hematopoietic stroma. Groundbreaking experiments by Tavassoli and Crosby (1968) in the 1960s reported that boneless marrow pieces have the capacity to reconstitute hematopoietic and adventitial structures, complete with bone, cartilage, and stromal tissue, providing a newly formed microenvironment for functional hematopoiesis. Friedenstein was one of the first to assign osteogenic potential to non-hematopoietic plastic-adherent cells with fibroblast colony-forming unit (CFU-F) capacity and multi-differentiation ability upon *in vivo* transplantation (Friedenstein et al., 1966; Friedenstein et al., 1974; Castro-Malaspina et al., 1980; Kuznetsov et al., 1997). It took another decade before Owen and Friedenstein (1988) proposed the concept of clonogenic, multipotent, self-renewing stromal cells *in vivo*, which were shortly after defined as "Mesenchymal Stem Cells" (Caplan, 1991; Pittenger et al., 1999). The International Society of Cellular Therapies (ISCT) later re-defined the same cells as "Mesenchymal Stromal Cells" since "stroma" (*Greek*: bed) better reflected the hematopoiesis-supportive function and heterogeneous mixture of cell types included in "MSCs" (Dominici et al., 2006). In contrast to HSCs, which were conclusively shown to be able to give rise to phenotypically defined cells reconstituting hematopoiesis under limiting dilution conditions in serial transplants, *in vivo* self-renewing capacity had not been convincingly proven for cells of mesenchymal origin in mice and humans for another substantial period of time (Sacchetti et al., 2007; Mendez-Ferrer et al., 2010). By developing a renal transplant assay for single skeletal progenitor cells, we could further test the *in vivo* self-renewal capabilities of bone-forming stem cells without prior CFU-F expansion (Chan et al., 2013). Perplexingly, and as a result of the relative ease of culturing plastic-adherent fibroblastic cells, non-skeletal "multipotent stromal cell" populations have incorrectly been defined by their ability to make bone, cartilage, fat, tendon, and muscle in the culture dish, sometimes even suggested to be pluripotent depending on the assay conducted (Via et al., 2012).

While mostly associated with bone and adipose tissue, "MSC"-like populations have been identified in other connective tissues as well as perinatal tissues such as the umbilical cord and the placenta. As a consequence, the denomination "MSC" was established as a universal term for adult tissue-resident stem cells with putative therapeutic promise and is up to today routinely used by many scientists to wrongly describe stem cell populations (da Silva Meirelles et al., 2006). The general use of the term "Mesenchymal Stem Cell" is flawed in many ways leading us to stress that its use should be avoided. The intrinsic properties of these cells are tightly correlated with their tissue source. They contain cells of divergent developmental origins and as mostly looked from the perspective of adult stem cells should not be named "mesenchymal" in the first place, since this term should be reserved for embryonic tissue. Above all, "MSCs" describe a highly heterogeneous cell population with seemingly multilineage differentiation capacity which actually results from the aggregate potential of a mixture of individual, distinct cell types. Over time, the promiscuous use of the "MSC" terminology resulted in a great number of publications with conflicting claims and it became necessary for the scientific community to address the inconsistencies. In an attempt to unify researchers and to rectify falsely assumed stem cell characteristics the ISCT published minimal criteria for the definition of "MSCs"

and proposed to call them "Multipotent Mesenchymal Stromal Cells," rather than "Mesenchymal Stem Cells" (Dominici et al., 2006). However, it should be noted that even "stromal" is an inaccurate generalization as it does not apply to fibroblasts of certain tissue sources, e.g., umbilical cord and amniotic fluid. Minimal criteria for human "MSCs" included plastic-adherence under standard culture conditions, which upon culture express CD105, CD73, CD90 and lack CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules, and exhibit tri-lineage differentiation potential into osteogenic, chondrogenic, and adipogenic fates *in vitro*. It needs to be stated that these cell surface markers are not specific for stem cells but rather confirm the fibroblastic identity of cultured cells. As a consequence, many laboratories are currently using widely differing marker profiles additionally stemming from differences in marker expression on freshly isolated cell populations. *In vitro* culture alters surface marker profiles and does not reflect the true biological activity of investigated cells, underscoring the need for the ISCT standards to be reassessed. Lastly, stemness of a homogeneous cell population is often not rigorously interrogated which in part could be attributed to the lack of clonal self-renewal as one defining criteria in the guidelines agreed on by the ISCT, in turn hampering success of clinical applications utilizing stromal cell populations (Robey, 2017). The FDA has published a study that describes the disagreement over molecular characteristics of "MSCs" (Mendicino et al., 2014), which is also supported by the report that identical surface protein profiles between stromal cell populations from different source tissues display differential transcriptional expression patterns (Sacchetti et al., 2016). Caplan, who originally coined "MSCs," recently suggested refraining from the use of the word "stem cells" considering the issue of cellular heterogeneity and the inconclusive reports that are prevalent in "MSC"-related literature (Caplan, 2017).

The recent identification of *bona fide* lineage-restricted SSCs within the skeletal compartment, which give rise to a hematopoiesis-like hierarchy of committed progenitors *in vivo,* supported the need to re-think the nomenclature of skeletal derived "mesenchymal" stem cell-like cells (Sacchetti et al., 2007; Chan et al., 2015, 2018). In light of these developments, the terms "Bone marrow stromal cells" (BMSCs) and "Skeletal stem cells" (SSCs) have been suggested (Bianco and Robey, 2015). Further adding to the panoply of previously characterized skeletal progenitors are recent experimental findings that imply the existence of more than one *bona fide* SSC population, which will be discussed in the following sections.

Getting a Grasp on SSC Terminology

An overwhelming number of researchers use the terms SSC and "MSC" interchangeably to describe bone-residing "stem" cell types, while actually reporting different or only partially overlapping skeletogenic populations without testing for basic stem cell characteristics, such as self-renewal. On the background of earlier mentioned discrepancies in the field, one should carefully distinguish SSCs as cells locally restricted to bone with the ability to reconstitute an environment for active hematopoiesis, as being able to self-renew on the clonal level, and to show multipotency within the same clone under *in vivo* conditions (Bianco et al., 2008). "MSCs" on the other hand, represent a heterogeneous cell mixture roughly complying with ISCT's minimal criteria that can be isolated from tissues such as muscle, fat, bone, and heart, thereby proposing equal suitability for regeneration across connective tissues. While these stromal cell populations may genuinely contain some tissue-specific stem cells capable of generating tissue fates of their native tissue origin (Sacchetti et al., 2016), it is unlikely that they are intrinsically pluripotent. For example, BMSCs are inherently osteochondrogenic, muscle "MSCs" are myogenic, and cord blood-derived cells display spontaneous chondrogenesis *in vivo*. Functionally, categorization of SSCs vs. "MSCs" has been attempted by stemness assays (*in vivo* vs. *in vitro*), mode of action (regeneration vs. cues), therapeutic potential (regeneration by living cells vs. paracrine modulation/anti-inflammatory signaling), and type of administration (transplantation for engraftment vs. infusion for embolization) (Bianco et al., 2013), albeit with limited success.

On the basis of immune-localization studies with stromal cell markers that are also associated with blood vessels in different tissues (Crisan et al., 2008; Sacchetti et al., 2016), some stromal cell populations have further been described to be pericytes (Sacchetti et al., 2007; Caplan, 2008). In contrast, SSCs are enriched in and around the avascular regions of the hypertrophic growth plates, though they have also been found in the periosteum as well as fracture calluses (Chan et al., 2015, 2018; Debnath et al., 2018; Duchamp de Lageneste et al., 2018). It is, therefore, possible that BMSCs are either comprised of more committed progenitor populations descending from SSCs settling in sinusoidal regions or contain a multipotent pericyte-like stem cell giving rise to more restricted progenitor cell types itself. While neither scenario can be excluded, a combination of both might be an additional alternative explanation but remains to be investigated. An argument supporting this theory is the fact that we and others have found SSCs to be restricted to give rise to bone, cartilage, and stroma, but not fat *in vivo* (Chan et al., 2013, 2015, 2018; Mizuhashi et al., 2018). The CD146-positive SSC population identified by Sacchetti et al. (2007) does not overlap with our described SSC, which is in compliance with its adipogenic potential and subendothelial localization. However, we have also shown that a distinct lineage of CD146-positive multilineage skeletal progenitors is derived from CD146-negative SSCs (Chan et al., 2018). Additionally to osteochondrogenic mouse SSCs, an adipogenic lineage with phenotypically defined subpopulations sharing a common "mesenchymal" stem cell-like origin has been reported, further supporting the potential existence of two distinct SSC populations (Chan et al., 2015; Ambrosi et al., 2017). The collaboration of multiple stem and progenitor cell types in orchestrating the highly complex cell network within the bone compartment seems more reasonable given the manifold niches existing in the skeleton. At this point, however, we cannot definitively rule out that divergent findings might be the result of different experimental techniques, a topic that will be discussed in depth in sections below. Should it turn out to be confirmed, it could pose a great opportunity to unite the scientific community in agreeing on a common nomenclature

which could accommodate the existence of two or more *bona fide* SSC populations in bone.

PROSPECTIVE ISOLATION OF SKELETAL STEM CELLS

Over time, stem cell hallmark criteria have been extended by single and combinatorial use of surface marker/genetic labeling, transplantation assays, and *in vivo* lineage tracing. This has led to the discovery of multiple SSC populations in the growth plate, the endosteal and perisinusoidal bone marrow space, or the perichondrium. Nevertheless, the description of these cell populations is often incomplete or redundant leaving contradictory results that hamper the advancement of the field. A potential explanation can be found in the complex and plastic nature of bone tissues and might be vested in technical limitations or an indication for the presence of an everchanging network of multiple subtypes of SSCs orchestrating skeletal homeostasis and repair.

Human Bones

Until the invention of flow cytometry by the Herzenbergs (Dangl and Lanier, 2013), which enables high-throughput, prospective isolation of antibody-labeled and fluorescence-tagged cell types, conclusions about putative SSC populations could only be drawn retrospectively leaving reasonable doubt about their true identity. Taking advantage of this technology, the CD45-negative non-hematopoietic nature of fibroblast colony-forming cells has been confirmed and copious markers, including CD105 (ENG), CD140a (PDGFRα), CD73 (NT5E), CD90 (THY1), STRO-1 (HSC70;HSPA8), CD271 (NGFR), and CD44 have been proposed to broadly label human SSC populations with variable CFU-F ability and differentiation to osteogenic, chondrogenic, adipogenic, and stromal lineages (Shi and Gronthos, 2003; Lange et al., 2005; Sorrentino et al., 2008; Pinho et al., 2013). CD271 (/CD140a$^{-/low}$) and STRO-1 turned out to most efficiently select for perivascular residing SSC-like cells that are also able to maintain human HSCs for extended time in culture but are less restricting than CD146 (MCAM), which allegedly identified the entire CFU-forming fraction (Simmons and Torok-Storb, 1991; Sacchetti et al., 2007; Li et al., 2014). Single cell-derived colonies of CD45^{-}CD146^{+} cells are sufficient to recreate hematopoiesis-supportive human ossicles in a mouse, representing an enriched SSC population with serially transplantability, multipotency, self-renewal, and localization along marrow sinusoids expressing endothelial and hematopoiesis supporting factors. However, a later paper by Tormin et al. (2011) reported at least similar CFU-F ability using CD271^{+}CD145$^{-/low}$ compared to CD271^{+} CD146^{+} cells. This is in line with our recently identified human SSC which falls into the CD146-negative fraction and underscores the necessity to enrich single marker labeled cell population with additional markers to derive homogeneous cell populations more depictive of the complex composition of skeletal tissue (Chan et al., 2018).

Assigning specific marker profiles to plated SSC populations is a major pitfall as *ex vivo* culturing alters the endogenous expression of surface markers. Cell epitope expression can be artificially skewed as seen with the loss of CD271 expression in cultured bone marrow cells in media containing basic Fibroblast Growth Factor (bFGF), as well as with CD146 which possesses a serum response element in its promoter and is enhanced in stromal cells under standard culture conditions and downregulated if exposed to a hypoxic environment (Tormin et al., 2011). Alike, CD44 is expressed by cultured "MSCs" only (Qian et al., 2012). Notably, simple variations in cell-detaching methods and culture media before flow cytometric analysis can alter cell surface antigen expression (Hagmann et al., 2013; Tsuji et al., 2017). The same thing might hold true for the digestion of freshly harvested tissue routinely conducted for FACS preparations which can have profound effects on the integrity of surface molecule expression and additionally is prone to loss of more fragile cell types (Autengruber et al., 2012). Altogether, this might under- or overestimate the abundance of cell populations as well as likely contaminate investigated candidate SSCs compromising conclusions drawn from experiments. It is, therefore, of the highest importance to confirm flow cytometric findings with *in situ* analysis of marker expression on tissue sections or by other methods.

In a meticulous endeavor, our laboratory was able to identify a highly purified *bona fide* SSC distinct from the reported CD146-positive SSC; and found in fetal and all adult stages throughout different skeletal sites but specifically enriched in the hypertrophic zones of the growth plate labeled by PDPN, CD73, CD164 and lacking expression of CD235, CD45, CD146, Tie2, and CD31 (Chan et al., 2018). We arrived at that surface marker panel by micro-dissecting seven regions of the long bones, looking for the enrichment of SSC markers found in the previously identified mouse SSC (Chan et al., 2015). Of note, in our analysis, CD146 was specifically enriched in diaphyseal regions abundant with vasculature separate from the growth plate – a site that is highly enriched with SSCs (Chan et al., 2018). We stringently tested *in vitro* and *in vivo* capacity of this candidate SSC population by serial transplantations, self-renewal, and lineage diversity to prove stemness. These human SSCs are activated by skeletal injuries, such as fractures, to locally increase proliferation and differentiation in order to facilitate regeneration. Importantly, we now have established a hierarchy with a SSC at the top giving rise to defined stromal subpopulations with osteogenic and/or chondrogenic fates; and the missing ability to give rise to fat. Although flow cytometric analyses identify phenotypic human SSCs at various skeletal compartments, future studies have to assess if cells from different bone types share functional properties with the investigated long bone-derived human SSCs. It is likely that bones with varying developmental origin do not share the same SSC and that transcriptional differences in SSCs from different skeletal sites determine skeletal shape and size.

Taken together, the distinct cell marker expression (CD146^{-} vs. CD146^{+}) of our and Sacchetti et al.'s (2007) SSC population, the divergent anatomical sites (epiphyseal vs. perivascular), and the disparity in lineage output (osteochondrogenic vs. osteochondroadipogenic) might be reconciled by our observation that CD146^{-} give rise to CD146^{+} multipotent

skeletal progenitors. It might also be possible that the two cell types mark specific subsets of SSCs. This is further supported by the notion that CD146-negative cells were reported to migrate from bone marrow sinusoids to an endosteal niche (Tormin et al., 2011), a site we did not specifically investigate, but that would make sense given the role of SSCs in injury response (**Figure 1**). Intriguingly, independent observations in mouse bones add evidence for at least two SSC forms and will be discussed hereafter. Future studies will have to directly experimentally address this idea and will help to get more insight into potential functional differences between these two cell types.

Mouse Bones

Mice provide an ideal model organism to research SSC biology. In contrast to humans, mice offer the accessibility of transgenic tools to specifically label and track cell populations beyond cell surface marker expression. Besides fluorescence reporter expression from an endogenous promoter of putative marker genes, the development of lineage tracing technology facilitated by the Cre-LoxP system has noticeably progressed the field, albeit with recognizable caveats (discussed later). Cre-LoxP recombination allows site-specific deletion, insertions, translocations, and inversions in the DNA of cells. This is facilitated by a single enzyme, called Cre-recombinase, which was originally derived from the bacteriophage P1 together with the LoxP site (Bouabe and Okkenhaug, 2013). Upon Cre-recombinase expression under the promoter of a target transgene, the enzyme acts on the second transgene containing the LoxP site. This leads to an irreversible gene sequence alteration which can be exploited in manifold ways, e.g., to continually turn on (fluorescence protein) or excise a gene (primary fluorescence protein or functional gene). Cre-reporter systems enable investigations in an unperturbed manner by either constitutive Cre-expression or time-point restricted induction through tamoxifen application in the modified CreERt version, being dependent on structural changes for nucleus translocation before eliciting recombinant properties.

Using this technology in combination with Rainbow-mice which carry an allele that through Cre-mediated recombination allows the stochastic expression of different fluorescence colors, we were able to show clonal expansion within the growth plate, conveying initial evidence for SSC activity in this region (Chan et al., 2015). This was preceded by reports of a purified cell population containing putative SSCs that started accumulating in the late 2000s when the Matsuzaki group first published a defined cell population with stem cell characteristics in mice. Establishing that stromal cells come from a distinct lineage compared to HSCs, the initial discovery found fibroblast activity within the PDGFRβ-positive cell fraction (Koide et al., 2007). They later narrowed down their findings to a perivascular population with *in vivo* self-renewal/multipotency to $CD45^{-}Ter119^{-}Sca1^{+}PDGFR\alpha^{+}$ expressing cells, which *in vitro* contains clones with osteogenic, chondrogenic, adipogenic, and even angiogenic and neural crest differentiation ability (Morikawa et al., 2009a,b). They also suggested that the double-negative $Sca1^{-}PDGFR\alpha^{-}$ cells were more restricted and committed osteoprogenitors. Many more surface proteins have been proposed to label multipotent clonogenic stromal cell types, partially mirroring reports in humans, e.g., CD271, CD90, CD51, CD44, CD146, CD106, and LepR with variations depending on the developmental stages investigated (Mabuchi et al., 2013; Isern et al., 2014; Zhou B.O. et al., 2014). Building on these studies and employing a surface marker configuration described for progenitor cell populations of adipose tissue (Rodeheffer et al., 2008), a bone-resident stem cell-like population that is $CD45^{-}CD31^{-}Sca1^{+}PDGFR\alpha^{+}CD24^{+}$ was isolated which shows high purity for clonal tri-lineage potential in *in vitro* studies and *in vivo* when transplanted in bulk (Ambrosi et al., 2017). In the bone marrow these cells give rise to committed adipogenic progenitors ($CD24^{-}$) that further make Zfp423-expressing pre-adipocytes before becoming mature fat cells. In agreement with the observations by Morikawa et al. (2009b), an alternate differentiation route creates osteochondrogenic-restricted $Sca1^{-}PDGFR\alpha^{-}$ cells. Our lab first described another subpopulation within the latter, defined by CD105 expression bringing along minimal properties for the generation of a hematopoietic niche through endochondral ossification and found in fetal, neonatal, and adult mouse bone (Chan et al., 2009, 2013). Using a renal capsule transplantation model, we unprecedently showed that single cells termed Bone Cartilage Stromal Progenitor (BCSPs; $CD45^{-}Ter119^{-}Tie2^{-}Thy1^{-}6C3^{-}CD51^{+}$) were *bona fide* stem cells *in vivo*, spawning stromal subsets crucial for niche functions (Chan et al., 2013). Hypothesizing that the skeletal lineage might follow an analogous differentiation tree as described for the hematopoietic compartment, we set out to delineate the SSC and its downstream stroma. We succeeded in showing that skeletogenesis proceeds through a developmental hierarchy of lineage-restricted progenitors with a *bona fide* SSC at the top ($CD45^{-}Ter119^{-}Tie2^{-}Thy1^{-}6C3^{-}CD51^{+}CD105^{-}CD200^{+}$) giving rise to the BCSP and seven defined and restricted stromal progenitor cell types, never producing fat (Chan et al., 2015; Gulati et al., 2018). In concordance with the different locations SSCs have been identified this raises the possibility that the perivascular adipocyte-producing multipotent stromal cells represent a distinct stem cell-like population, as also observed in experiments with human bone marrow cells. One major limitation of the multi-surface marker approach is the inability to investigate the exact niche composition and to trace cell fates through *in situ* labeling. Using transcriptomic data from highly purified cell populations representing all cell types of long bones might allow for the identification of a single specific marker for mouse SSCs which then can be used to generate transgenic mice for a more defined interrogation of their biological activity.

Concurrently, the generation and analysis of genetically modified mice have yielded enormous insights into skeletal biology. Using single fluorescence reporters, e.g., GFP, dtTomato, mCherry, YFP, etc., under the control of target gene expression, many cell populations have been described enriching for colony-forming fibroblasts, relating to widely overlapping cell populations termed SSCs, "MSCs," pericytes, or CXCL12-abundant reticular (CAR) cells depending on the publication. Furthermore, genetic manipulation of stromal populations *in situ* employing the Cre-system has allowed investigations of skeletal development, homeostasis, and regeneration through temporal lineage tracing and targeted ablation of specific cell types.

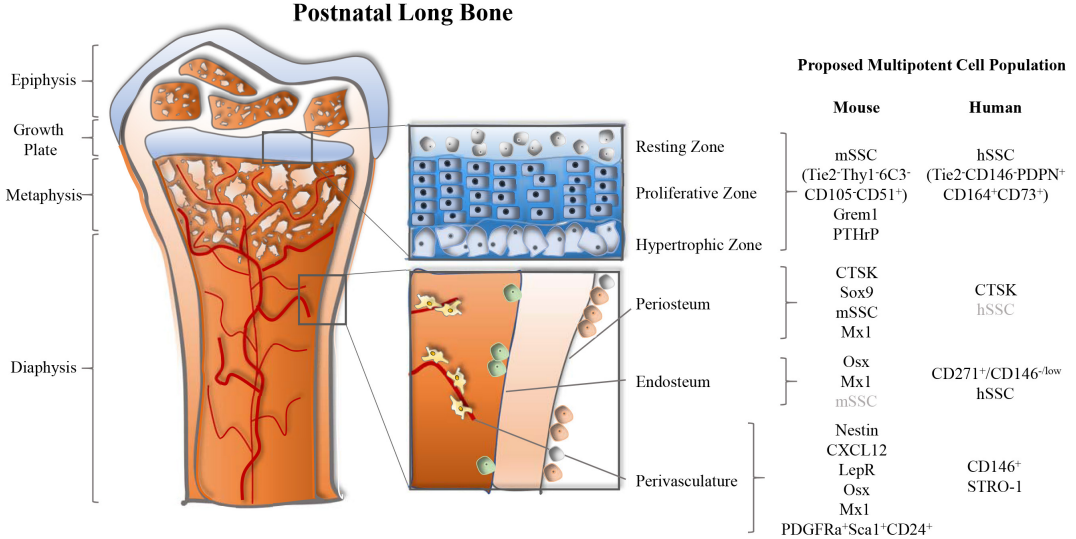

FIGURE 1 | Multiple anatomical sites of long bone-resident skeletal stem and progenitor cell populations in mice. Postnatal long bones of mice harbor skeletal stem and progenitor cells at the growth plate, periosteum, endosteum, and perivascular sites. Several markers to identify these cell populations have been proposed and are summarized by location.

Cre-mediated recombination by the transcription co-activator Paired-related homebox 1 (Prx1) is very effective in inclusive labeling of all clonogenic stromal cells as it incorporates but is not limited to PDGFRα/β- and Sca1-expressing cells (Logan et al., 2002; Omatsu et al., 2010; Ambrosi et al., 2017). It also includes CAR cells, a very crude collection of stromal progenitor cells (PDGFRα-positive cells are a subpopulation), since it also strongly labels endothelial and mature osteogenic cells (Sugiyama et al., 2006; Greenbaum et al., 2013), which all play crucial roles for normal homeostatic processes of the skeleton, as shown by aberrant differentiation and hematopoietic support when specifically ablated (Omatsu et al., 2010; Seike et al., 2018).

One of the first fluorescent labels to be used for mouse BMSCs, initially reported as a marker for neural stem and progenitor cells and also expressing PDGFRα, is Nestin (Mignone et al., 2004). In the marrow, Nestin-GFP marks perivascular, clonogenic, self-renewing cells, which can form mesenspheres that differentiate in the osteochondral lineages *in vivo* as well as to adipocytes *in vitro* (Mendez-Ferrer et al., 2010). A subsequent demonstration by the same group showed that Nestin-expressing cells could be divided into Nestin^{dim} and Nestin^{bright} cells, with the cells having the brightest signal being NG2-positive, more quiescent, and containing the highest CFU-F potential while the dimly labeled cells overlap with LepR (Kunisaki et al., 2013). Surprisingly, no Nestin or NG2 gene expression can be found in Prx1-Cre labeled cells, an observation that has been explained to potentially be due to incomplete tracing (Greenbaum et al., 2013). The stem cell-like nature of Nestin-GFP cells has also been implied by their descent from Col2-expressing cells, however, although Col2 marks progenitor cell types it is rather ubiquitously expressed which make such conclusions questionable (Maes et al., 2010; Ono et al., 2014a). Furthermore, the Nestin gene has been utilized for lineage tracing through Cre-recombination which revealed varying proportions of endothelial cells that were marked depending on the model used, leaving considerable doubt with regard to its use as a proper stem cell marker (Ding et al., 2012; Ono et al., 2014a). Similarly, the heterogeneous Mx-1-Cre line overlaps with Nestin-GFP, PDGFRα, Sca1, CD105 but also robustly labels more mature osteogenic populations (Osterix^{+}, Osteopontin^{+}, Osteocalcin^{+}) as well as hematopoietic cells (Park et al., 2012).

In part due to the vague labeling of stromal cells by Nestin, LepR-expressing cells (traced by Cre and CreERt) have emerged as the prevailing multipotent stromal cell marker over the last years (Ding et al., 2012; Zhou B.O. et al., 2014). In a series of elegant studies, the Morrison lab could show that LepR-expressing cells are the major source of osteogenic, chondrogenic, and adipogenic cells formed during adulthood and are involved in homeostasis, fracture healing, and hematopoiesis (Zhou B.O. et al., 2014; Yue et al., 2016). LepR-expression in the bone marrow overlaps with all known stromal cell markers to varying extents. If interpreting histological data of LepR-reporter mice bones, caution is advised as they also label substantial proportions of hematopoietic and endothelial cell types which can be excluded during FACS-purification. Just under 10% of LepR-labeled cells are CFU-F of which about the same percentage possesses *in vitro* clonogenic tri-lineage potential underlining the often wrongly used term SSC for these cells as they are not pure *bona fide* SSCs (Zhou B.O. et al., 2014). Recent single-cell RNA-sequencing reports of LepR-positive cells have suggested that this population contains at least four subpopulations with differing osteogenic and adipogenic commitments (Tikhonova et al., 2019). In accordance with that as well as our proposed skeletal lineage tree, David Scadden's group also using single-cell RNA-sequencing identified seventeen marrow stromal subsets characterized by fibroblastic, endothelial, pro-adipogenic, and

pro-osteochondrogenic phenotypes, of which all expressed LepR in at least some cells (Baryawno et al., 2019). Although these studies shed exciting new light on the abundant heterogeneity of single fluorescence-labeled cell populations, attention is warranted, as results are mostly descriptive, and assumptions are based on former knowledge. Future studies will have to provide evidence that stromal sub-populations identified by singe cell RNA-sequencing are following proposed differentiation trajectories *in vivo* and fulfill functions assigned based on gene expression.

LepR-expression can also be found in the mouse SSCs (mSSCs; plus, all downstream lineages) identified by us, which show a strong resemblance to BMP-antagonist Gremlin1-Cre traced cells (Worthley et al., 2015). Grem1^{+} cells predominantly reside in metaphyseal areas, show stem cell hallmark characteristics on the clonal level, and are non-adipogenic. However, neither Nestin-Cre nor LepR-Cre seem to substantially trace Grem1 cells, which on the one hand supports the notion of a concept with at least two skeletogenic stem cell populations; and on the other hand might be the result of the infidelity of these Cre-reporters, e.g., incomplete recombination, or the time of initiated tracing. Latest single-cell data is supportive of a stem cell model in which one SSC source is following a classic tri-lineage differentiation pathway that is complemented by a second origin for osteoblastic cells created from a "chondrogenic cluster" (Baryawno et al., 2019). The latter might entail the mSSC/Grem1^{+} cells as they are found within the boundaries of highly chondrocyte-abundant growth plate areas.

Along this chain of arguments, a recent publication describes parathyroid hormone-related protein (PTHrP) as a label for chondrocytes of the resting zone in the growth plate of long bones which descend from a PTHrP^{+} SSC. PTHrP indeed seems to enrich for mSSCs but remains heterogeneous for other more committed cell populations (Mizuhashi et al., 2018). These cells are shown to contribute to CAR cells and bone stroma (not fat) below the chondrogenic zones, even though only to a low degree. A potential explanation is the incomplete labeling of stem cells by the Cre-driver, also observed when trying to ablate PTHrP-expressing cells. Another study reported Glioma-associated oncogene 1 (Gli1) as a marker for early postnatal multipotent progenitor cells in the metaphyseal region of long bones (Shi et al., 2017). Shi et al. showed crucial functions for Gli1-cells in maintaining bone mass and the capacity to produce chondrocytes, osteocytes, and adipocytes *in vivo*. Experiments also confirmed a strong overlap with other BMSC markers such as PDGFRα/β and LepR. Combined with the observation of expression of markers usually seen in more mature cell types (i.e., Osx, Col1), this data implies that Gli1 enriches for multipotent stromal cells but does not uniquely mark a homogeneous SSC population. Future studies could more closely assess SSC content of Gli1-positive cells by clonal analysis, an approach that was not pursued in that work.

Aside from the association of SSCs at and around the growth plate, they are also abundant along the periosteum, where they can quickly get "activated" upon injury and facilitate proper fracture healing (Marecic et al., 2015). We discovered that these stem cells additionally express CD49f and display stronger proliferative and bone-forming capacity than their CD49f-negative counterparts. Colnot and colleagues confirmed the existence of a multipotent periosteal cell pool with high clonogenic as well as tri-lineage differentiation potential *in vitro* and *in vivo* that plays an important role in fracture regeneration (Duchamp de Lageneste et al., 2018). These cells were shown to be dependent on the expression of the extracellular matrix protein Periostin, as the loss of it impairs their number and function. A unique marker for SSCs of the periosteum has long remained elusive and could also not be provided by the latter study which relied on plastic adhering cells derived from micro-dissected periosteum and bone marrow cultured in media. Recently, however, Cathepsin K (CTSK), best known as an osteoclast marker, has been demonstrated to label a set of skeletal cells present on the periosteum (Debnath et al., 2018). Functional characterization of CTSK-Cre;mTmG reporter mice revealed general multipotent stromal cell characteristics and a role in fracture healing, but no involvement in hematopoiesis support. Looking ahead, the ability of periosteal SSCs (pSSCs) to be efficiently activated upon injury and their anatomical localization lining the bone surface makes them an attractive source and target for clinical treatments.

In sum, single-gene transgenic lineage tracing approaches have key limitations since many of the frequently used genes to trace SSC lineages, including PTHrP, Nestin, and LepR, are broadly expressed by multiple tissue types and are almost ubiquitously expressed within the skeletal lineages independent of developmental stages (**Table 1**). There is enough reason to believe that all here reviewed putative multipotent stromal populations are comprised of cell mixtures that gain multipotency from heterogeneity rather than being one defined cell type. Another major caveat of most mouse SSC markers is the inability to translate them to humans (**Figure 1**). This has also become obvious when we identified a human SSC, which exhibits a different marker profile than the mouse SSC population we described. Investigating *bona fide* SSCs is crucial since the study of impure cell populations will keep on hampering the discovery of promising therapeutic approaches.

SKELETAL STEM CELL DEVELOPMENT

Bone formation mainly occurs through two mechanisms: intramembranous and endochondral ossification, with the former describing direct osteogenesis from stromal cells, while the latter develops mineralized tissue through a cartilage template. As the primary form of bone formation in the appendicular skeleton, endochondral ossification is crucial for establishing the hematopoietic niche (Chan et al., 2009). Craniofacial bones arise from paraxial mesoderm and primitive neural crest following both, intramembranous and endochondral ossification routes. Excitingly, in a model of mandibular distraction, we could show that mSSCs can reverse to their early neural crest state in order to orchestrate directed regeneration (Ransom et al., 2018). Many proposed SSC markers of the cranium are distinct from the appendicular skeleton, which might be at least in part explained by their differential developmental

TABLE 1 | Summary of selected markers proposed to enrich for mouse skeletal stem cells and multipotent stromal cells.

Candidate marker	References	Reported overlap	Gene expression in mSSC lineage (Chan et al., 2015)				Self-renewal/ clonogenicity	Differentiation/lineage contribution	Reconstitution of niche
			Cell	Neonate	Young	Old			
Sox9	Akiyama et al., 2005; Mizoguchi et al., 2014; He et al., 2017	Ocn, Osx, Prx1, Runx2, LepR	SSC	Yes	Yes	Yes	*In vitro*: no data	*In vitro*: no data	Not assessed
			BCSP	Yes	Yes	Yes			
			Thy1+	Yes	Yes	No	*In vivo*: no data	*In vivo*: osteochondrogenic (no clonal data), adipogenesis not assessed	
			6C3+	Yes	Yes	Yes			
Grem1	Worthley et al., 2015	CD105, Pdgfrα (low), Sca1 (low), Osx, Acan	SSC	Yes	Yes	Yes	*In vitro*: low CFU-F (approx. 1%)	*In vitro*: 84% of clones osteochondrogenic	Not assessed; contribution to niche components
			BCSP	Yes	Yes	Yes			
			Thy1+	Yes	Yes	Yes	*In vivo*: at least long-term presence (> 12mo) of labeled cells	*In vivo*: clonally osteochondrogenic (% unknown)	CFU-F selected clones are serial transplantable to fracture site, give rise to osteochondrogenic fates
			6C3+	Yes	Yes	Yes			
Nestin	Mendez-Ferrer et al., 2010; Kunisaki et al., 2013; Isern et al., 2014; Ono et al., 2014a	NG2, Pdgfrα, αSma, Cxcl12, Scf, Angpt1, Vcam1, Opn, LepR, Sox10, CD90 (low)	SSC	No	No	No	*In vitro*: approx. 5% of clones form secondary mesenspheres	*In vitro*: clonally osteochondroadipogenic (<50%)	Yes, not quantified
			BCSP	No	No	No			
			Thy1+	Yes	No	No	*In vivo*: single culture-derived mesenspheres serial transplantable	*In vivo*: clonally osteochondroadipogenic (<40%)	
			6C3+	Yes	No	No			
LepR	Ding et al., 2012; Zhou B.O. et al., 2014	Pdgfrα, Pdgfrβ, CD51, CD105, Cxcl12, Scf, Dmp-1, Ocn, Sca1 (low)	SSC	No	Yes	Yes	*In vitro*: approx. 10%	*In vitro*: approx. 10% clonally osteochondroadipogenic	Yes, approx. 30% of clones upon subcutaneous transplantation
			BCSP	No	Yes	Yes			
			Thy1+	Yes	Yes	Yes	*In vivo*: not directly assessed	*In vivo*: clonally osteochondroadipogenic	
			6C3+	Yes	Yes	Yes			

(Continued)

TABLE 1 | Continued

Candidate marker	References	Reported overlap	Gene expression in mSSC lineage (Chan et al., 2015)				Self-renewal/clonogenicity	Differentiation/lineage contribution	Reconstitution of niche
			Cell	Neonate	Young	Old			
Osx	Maes et al., 2010; Park et al., 2012; Mizoguchi et al., 2014; Ono et al., 2014a,b	Vcam-1, αSma, Angpt1, Pdgfrα, Pdgfrβ, Vegf, Nestin, Sox9, Cxcl12, Ocn, Mx-1	SSC	Yes	Yes	Yes	*In vitro*: 0.5% of P5-labeled clones form secondary mesenspheres	*In vitro*: P5-labeled clonally osteochondrogenic (% unknown)	Not assessed; P5-labeled cells contribute to tissue remodeling upon fracture
			BCSP	Yes	Yes	Yes			
			Thy1+	No	No	Yes	*In vivo*: no data	*In vivo*: osteochondroadipogenic (no clonal data)	
			6C3+	No	Yes	Yes			
Cxcl12	Greenbaum et al., 2013	Pdgfrα, Sca1, Prx1, Osx	SSC	Yes	Yes	Yes	*In vitro*: no data	*In vitro*: osteoadipogenic as bulk (chondrogenic potential not assessed)	Not assessed
			BCSP	Yes	Yes	Yes			
			Thy1+	Yes	Yes	Yes	*In vivo*: no data	*In vivo*: no data	
			6C3+	Yes	Yes	Yes			
Prx1	Akiyama et al., 2005; Greenbaum et al., 2013; Zhou B.O. et al., 2014; Ambrosi et al., 2017	Pdgfrα, Sca1, LepR, Cxcl12	SSC	Yes	Yes	Yes	*In vitro*: as bulk	*In vitro*: osteochondroadipogenic as bulk	Not directly assessed
			BCSP	Yes	Yes	Yes			
			Thy1+	Yes	Yes	Yes	*In vivo*: as bulk	*In vivo*: contributes to all skeletal mesenchymal tissues (clonal % unknown)	
			6C3+	Yes	Yes	Yes			
Ctsk	Debnath et al., 2018	CD105 (low), Pdgfrα, CD51, Grem1, Nestin, Runx2, Ocn, Acan, Sox9	SSC	Yes	Yes	Yes	*In vitro*: as bulk, in mesensphere culture	*In vitro*: clonally uniformly osteochondroadipogenic	As bulk intramembranous bone formation without hematopoiesis support, endochondral upon injury
			BCSP	Yes	Yes	Yes			
			Thy1+	Yes	Yes	Yes	*In vivo*: upon bulk serial transplant into fat pad and under renal capsule	*In vivo*: osteogenic, chondrogenic upon fracture (bulk)	
			6C3+	Yes	Yes	Yes			

(Continued)

TABLE 1 | Continued

Candidate marker	References	Reported overlap	Gene expression in mSSC lineage (Chan et al., 2015)				Self-renewal/clonogenicity	Differentiation/lineage contribution	Reconstitution of niche
			Cell	Neonate	Young	Old			
Pthrp	Mizuhashi et al., 2018	CD105	SSC	Yes	Yes	Yes	*In vitro*: approx. 15%	*In vitro*: clonal analysis (low number), max. 50% osteochondrogenic, negligibly adipogenic	lineage tracing shows continuous contributions to stroma
			BCSP	Yes	Yes	Yes			
			Thy1+	Yes	Yes	Yes	*In vivo*: clonally shown (total percentage unclear)	*In vivo*: osteochondrogenic (not clonally shown)	
			6C3+	Yes	Yes	Yes			
Mx-1	Park et al., 2012	Pdgfrα, Sca1, CD105, Ocn, Osx, Opn, CD29, CD44, CD133	SSC	No	No	No	*In vitro*: approx. 5% of clones	*In vitro*: clonally osteochondroadipogenic (unknown %)	Only contribute to osteolineage in homeostasis and injury
			BCSP	No	No	No			
			Thy1+	No	No	No	*In vivo*: long-term labeling (6–10mo)	*In vivo*: osteogenic only	70% of transplants home to bone marrow and display long-term engraftment upon i.v. transplantation
			6C3	No	No	No			

Microarray gene expression data of markers as expressed in the mouse SSC lineage tree (marker gene expressed: green; marker gene not expressed: red), showing no restricted labeling to highly purified SSCs independent of age group (SSC: Skeletal Stem Cell; BCSP: Bone and Cartilage Stromal Progenitor Cell; Thy1+: Committed osteoprogenitor; 6C3+: hematopoiesis-supportive stroma). Information on stem cell properties as reported for each population targeted by the candidate marker is summarized.

origin. Axin2, for example, labels a stem cell pool throughout all stages of development and adulthood of mice which is virtually absent in long bones (Maruyama et al., 2016). These cells are also functionally distinct as they normally do not form chondrocytes and are less supportive of an ectopic hematopoietic niche when transplanted under the renal capsule. Together with Gli1-positive cells Axin2 is recognized as the best marker for cranial SSCs (Zhao et al., 2015). Gli1-expressing cells share characteristics with long bone BMSCs and are abundantly present along the whole craniofacial bone gaps, called sutures, while Axin2-positive stromal cells are restricted to the midline of these sutures. Both cell types are thought to contribute to growth and regeneration. These and other stromal cell markers of the cranium have been more comprehensively reviewed elsewhere (Doro et al., 2017).

Genetic lineage tracing in mice has allowed an unperturbed look into the developmental paths of SSCs. In the early embryo, bone-forming cells originate from the adjacent perichondrium of the cartilage anlage and migrate into the bone marrow cavity where they transiently undergo osteogenesis before disappearing in early postnatal life (Maes et al., 2010; Mizoguchi et al., 2014; Ono et al., 2014b). These cells have been tracked through Osterix-CreERt and a Col1(3.2kb)-CreERt lines. While Osx-expressing cells are enriched for cell populations with high CFU-F shortly after birth, they show a limited capacity to contribute to mature bone, cartilage, and fat lineages during adulthood. It has been implied that perinatal Osx-positive cells are a source of $LepR^{+}$ cells which are the main origin of bone formed in adult mice (Liu Y. et al., 2013; Mizoguchi et al., 2014; Ono et al., 2014a).

Initial cartilage templates for bone formation are derived from Sox9-expressing progenitors, which are essential for skeletal growth and if traced from early embryonic stages mark the entity of osteogenic, chondrogenic, adipogenic, and stromal cell types including CAR cells of the adult bone marrow (Akiyama et al., 2005). They have also been assigned important roles as pSSCs during fracture healing (He et al., 2017). CD105 is an early marker of skeletal lineage commitment and can be detected as early as E13 (Chan et al., 2013). E14.5 limbs digested to single-cell dilution can re-create an ectopic bone marrow niche when transplanted under the renal capsule of adult mice, inferring that a SSC is present before blood vessel network formation (Chan et al., 2009). In support, E13.5 Grem1-Cre traces almost the entire embryonic mesenchyme and primary spongosia, which in combination with the metaphyseal anatomical localization of adulthood Grem1-expressing cells further strengthens the fact that a putative stem cell population has an inherent chondrogenic phenotype. This also fits with a theory in favor of the claim that the hypertrophic chondrogenic zone contains non-apoptotic chondrocytes acting as stem cells and when marked by Col10-Cre or Col10-CreERt can be followed to differentiated osteoblasts (Yang et al., 2014; Zhou X. et al., 2014; Mizuhashi et al., 2018). Lastly, Newton et al. (2019) recently reported the formation of a distinct stem cell niche in the early postnatal growth plate with a hierarchical structure important for tissue renewal, backing the presence of non-perivascular SSCs at this specific site.

The establishment of a blood vessel network in the newly formed bone marrow cavity from around E14.5 allows the formation of perivascular niches which are essential for the establishment of hematopoiesis. HSCs are present as early as E17.5 in long bones of mice and co-emerge with 6C3-supportive stroma and LepR-expressing cells, underscoring the necessity of the existence of a proper niche (Chan et al., 2013; Zhou B.O. et al., 2014). Furthermore, LepR-Cre targeted cells contribute to all osteogenic, chondrogenic, and adipogenic lineages in the adult skeleton (Ding et al., 2012; Zhou B.O. et al., 2014). They, however, very likely do not label a unique SSC population, due to their ubiquitous expression, enforcing the need for more specific SSC tracers.

In summary, Grem1-positive and mSSC cells are the first stem cell populations to appear during skeletal development and can be found throughout adulthood with contributions to osteogenic and chondrogenic cells for homeostasis and fracture repair. Perisinusoidal multipotent cell types arise during late embryogenesis and are a source for bone, cartilage, marrow fat. The exact proportions of the output to skeletal tissues and functional differences of these two cell types warrant further investigations in the future.

FUNCTIONAL CHARACTERISTICS OF THE SKELETAL STEM CELL LINEAGE

Seminal findings of clonogenic fibroblasts in the bone stroma have early on described the capacity of these cells to form an ectopic bone marrow compartment complete with host-derived hematopoietic tissue. This entails the interaction of complex three-dimensional architecture with controlled expression of niche factors guiding cell fates. SSCs and bone "mesenchymal stromal cells" give rise to various stroma populations with distinct contributions to the tissue, i.e., bone formation, chondrocyte replenishment, bone marrow adipocytes accrual, and hematopoiesis-support, which all play their part in orchestrating skeletal organ function. The interaction of skeletal lineage cells with the blood-forming compartment has since been shown to be of uttermost importance. Aberrant changes in those tightly regulated networks result in stem cell lineage-based impairments which are potentially a cause for many bone-related diseases. Skewing SSC fates into desired lineages might hold great potential for translational approaches beyond skeletal tissue maintenance and repair; and toward treatments for hematological malignancies. Current therapeutic strategies involving "mesenchymal stromal cells" are mostly unsuccessful or moderate in effect as they largely rely on their immune-modulatory effects. Working with defined and controllable stem cell populations will pave the way to new, more effective approaches.

Hematopoietic Niche Regulation

The conception of a stem cell niche, as envisioned by Schofield, set the stage for investigations into the regulation of HSCs by other cell types (Schofield, 1978; Kfoury and Scadden, 2015). Osteoprogenitor and osteoblastic cells were the first cells thought to have an important function in controlling hematopoiesis (Taichman et al., 2001). Later, transplantation experiments revealed that hematopoietic stem and progenitor cells (HSPCs)

homed to perivascular and endosteal sites where they self-renewed and differentiated (Kiel et al., 2005; Sipkins et al., 2005; Lo Celso et al., 2009), an observation in close alignment with the primary localization of multipotent stromal cells. Cre-transgene mediated ablation of the osteoblastic compartment or expressed regulatory genes thereof, e.g., Scf, Cxcl12, subsequently confirmed that the osteogenic lineage did not markedly interact with hematopoiesis (Kiel et al., 2007). In contrast, the significance of more immature cell types, in particular labeled by Prx1, LepR, or Nestin, could be shown by targeted depletion of HSPC maintenance genes from the same (Mendez-Ferrer et al., 2010; Greenbaum et al., 2013; Zhou B.O. et al., 2014). Endothelial cell subsets are equally important for hematopoiesis which has been reported to be through Notch signaling as well as their permeability regulating reactive oxygen species exposure on HSPCs which affects features such as migration, survival, differentiation, and long-term repopulation capability (Itkin et al., 2016; Kusumbe et al., 2016). On top of that, the endothelium mediates important niche functions by directly acting on stromal cells involved with HSC control (Kusumbe et al., 2014). Lastly, bone marrow adipose tissue controls niche behavior in a context-dependent manner. Marrow adipocyte accumulation during obesity and aging impair hematopoiesis (Naveiras et al., 2009; Ambrosi et al., 2017), while adipocyte-derived SCF has been implied to promote regeneration after irradiation damage (Zhou et al., 2017).

Hematopoietic stem and progenitor cells and downstream lineages express many cognate receptors for factors produced by the stromal cell compartment but produce cytokines allowing two-way communication alike (Chan et al., 2015). Reverse regulation of HSCs to stromal cells is an intriguing idea and would give credit to the complexity of the niche model, a concept that needs to be looked at more intensively. Interestingly, metabolic sensing might be mostly restricted to the stromal compartment, since HSPCs express no common receptors involved in this process, e.g., LepR, PTH1R. Overall, this supports a model in which the skeletal niche tightly controls HSC maintenance and output.

It has to be noted that most experiments studying the interaction of blood stem cells with the stromal cell compartment are conducted with cytotoxic pre-conditioning, e.g., irradiation, and the use of unspecific Cre-driver transgenes. This most definitely affects experimental readouts, supported by divergent results for studies under homeostatic conditions and after transplant. The development of more cell type-restrictive transgenic mice and novel methods for niche interrogations are constantly adding to this active field of research and will shed more light on the regulation of HSCs by specific cell populations.

Factors Impacting Lineage Fate

Diverse signals can direct lineage fates of multipotent BMSCs, which has consequences for the hematopoietic niche and is equally important for the balance of bone formation and resorption. Metabolic and age-related changes can favor adipogenic over osteochondrogenic differentiation paths in multipotent stromal cells, thereby impeding bone homeostasis (Yue et al., 2016; Ambrosi and Schulz, 2017; Tencerova et al., 2019). It remains to be shown if "our" *bona fide* SSCs, which under homeostatic and injury conditions do not contribute to the adipocytic lineage, are also able to acquire adipogenic fates during obesity, aging, or disease. The intercommunication between bone and other tissues has been established and is to a large extent mediated through stem cells (Idelevich and Baron, 2018). For instance, gut microbiota can control circulating IGF1 levels to induce bone formation (Yan et al., 2016). Moreover, a central signaling axis from the brain might be governing activity and lineage decisions of SSCs opening up a whole new research area (Herber et al., 2019).

Cartilage degeneration causing arthritis is one of the major health burdens of our society. It is unclear why articular cartilage at bone surfaces fails to be repaired. Currently, it is thought to be because of the avascular and structural nature of cartilage. Alternatively, it could be implied that SSCs lose their intrinsically defaulted chondrogenic fate displayed during growth and are skewed toward osteogenic and fibroblastic phenotypes. Strategies to target SSCs for stimulated cartilage output hold great promise for future treatments of this disease. Notably, we were able to show that BMP2 alone promotes expansion to an osteogenic fate of SSCs, while an additional VEGF-inhibitor application results in abundant cartilage formation (Chan et al., 2015). SSC lineages are main sources of these two factors accentuating that they are in part regulating their own niche by paracrine signaling, which can be modulated according to prevalent cues from the surroundings. Simultaneously, extrinsic alterations interfering with the niche guide aberrant lineage output. This is exemplified by the observation that cells labeled by PTHrP-CreERt directly differentiate into Col1a1(2.3kb)-GFP^{+} osteoblasts upon micro-perforation injury (Mizuhashi et al., 2018), losing their controlled fate upon niche interruption. Single multipotent BMSCs are reliant on a supportive niche in the form of feeder cells for engraftment and the ability to efficiently proliferate and differentiate in *in vitro* assays, respectively, underlining the necessity of a supportive niche (Chan et al., 2013; Ambrosi et al., 2017). Altogether these findings substantiate the notion that microenvironmental crosstalk and integrity is essential for proper stem cell function.

Age-related decline of tissue function is associated with bone loss and increased fracture risk, promoted by an osteoporotic status (Burge et al., 2007). Several reasons for stem cell aging have been described, i.e., telomere attrition, genomic instability, epigenetic alterations, loss of proteostasis, cellular exhaustion/senescence, and mitochondrial dysfunction/oxidative stress (Lopez-Otin et al., 2013). *In vivo* findings on the mechanism of compartmental aging of skeletal stromal cells is limited (Ganguly et al., 2017). Recent work has suggested that inflammation drives intrinsic adaptations in LepR-positive stromal cells with advancing age that decrease numbers and display loss of osteogenic capacity leading to impaired regeneration (Yukata et al., 2014; Josephson et al., 2019). Similarly, perivascular SSCs lose their osteochondrogenic differentiation ability while maintaining adipocyte formation *in vitro* and preferentially generate adipogenic cell types *in vivo* (Ambrosi et al., 2017). Using advanced stem cell characterization techniques, we could also demonstrate that metabolic disease

such as diabetes mellitus represses expression of Indian Hedgehog (IHH) in SSCs, vested on high exposure of circulating tumor necrosis factor-alpha (TNFα), impairing bone repair. Pharmacological re-exposure of SSCs to exogenous IHH could rescue diabetic bone healing (Tevlin et al., 2017).

Furthermore, compositional changes of the bone stroma during aging might disrupt the hematopoietic niche causing the pronounced skewing to myeloid fates and mediating expansion of the HSPC pool. A consequence of the niche dysregulated in this manner is the increased production of bone-resorbing osteoclasts and simultaneous decrease of osteochondrogenic tissue formation, additionally corroborating loss of skeletal mass. RANKL and MCSF are essential and sufficient to drive osteoclast maturation, survival, and differentiation from the monocytic lineage (Cenci et al., 2000). While mature osteocytes have long been thought to be the main source of pro-resorbing cytokines (Nakashima et al., 2011), other findings favor the importance of more immature stromal cell populations as a source for osteoclastic factors (Cao et al., 2005; Tikhonova et al., 2019). Recently, it was demonstrated that RANKL reverse signaling from osteoclasts to osteoblasts can also stimulate bone formation (Ikebuchi et al., 2018). Follow-up investigations will have to unravel the implications of this mechanism as well as the exact role of stromal cell-derived signals for age-related processes. Finally, the possibility of a switch of skeletal lineages to an abundantly fibroblastic phenotype accompanying modifications of the extracellular matrix accelerating aging symptoms has prevailed to be an understudied direction of research. Targeting stem cell-sensed inflammation, reciprocal signaling with the hematopoietic niche, and cellular senescence comprise promising interventions for beneficial effects on bone health (Baker et al., 2011; Campisi, 2013).

CAVEATS OF SKELETAL STEM CELL BIOLOGY

The field of SSC research has made fundamental progress over the last 60 years driven by technological advancements and the evolving potential of stem cell-based therapeutics. Results describing prospective SSC populations and mechanistic backgrounds of their function and regulation have been pleiotropic. Although we have learned important lessons from basic science, we are far from effective translational strategies. Reasons can be found in the examination of incompletely purified stem cell populations, discordant use of definitions and terminology as well as overinterpretation and conclusions drawn from flawed experimental models.

A major caveat of BMSC populations is their varying heterogeneity in terms of the actual percentage of cells with *bona fide* stem cell characteristics which in this new research era we are now able to address with single-cell genomics. It also will be compelling to see if highly purified cell populations such as the mSSC can undergo "clonal skeletogenesis", meaning that single cells acquire a fitness advantage over others, may it be through genetic mutations or local stimuli. This will be particularly interesting from the perspective of skeletal maintenance, aging, and disease, comparable to what has been shown for HSCs (Jaiswal et al., 2014, 2017). Through the recent demonstration of mouse and human SSCs we are now in the position to interrogate inter-species and inter-bone site differences of SSCs, providing us with a platform for the delineation of clues on the intrinsic determinants of skeletal size and shape. We envision this to be harnessed for novel therapeutic strategies.

Pitfalls of Skeletal Stem Cell Characteristics Assessment

The matter of heterogeneity warrants a re-examination of results by Owen and Friedenstein; that is the varying differentiation potential of CFU-F cells derived from marrow aspirates and their depiction as a non-homogeneous cell mixture manipulatable for multiple directions of differentiation. For a long time, the lack of well-defined functional assays similar to what is available for HSCs allowed only retrospective analysis of stem cell characteristics. The nature of plastic adherence cultures has strongly limited the analysis to a few primitive cells, ignoring the fact that quiescent stem cells might not readily attach in these assays. The underestimation and inaccurate characterization of stem cell-like cells become obvious when comparing cultures of the same cell type with or without feeder layer. Single feeder layer-supported candidate SSCs show higher survival and tri-lineage potential *in vitro* (Ambrosi et al., 2017). Two-dimensional culture limitations are partially overcome by mesensphere cultures which nevertheless still have to deal with the exposure to random factors compared to their endogenous niche. Fetal calf serum is an essential additive for stromal cell culture, however, is of unknown composition (e.g., hormones, growth factors, steroids, etc.) depending on lot number, which very likely affects properties of cultured cells and makes it hard to compare or reproduce results between laboratories. The ISCT has agreed on a marker panel expression for multipotent cultured stromal cell populations, which might hold true for the *in vitro* setting but is not transferable to the physiological setting, as often done; and more importantly characterizes fibroblastic instead of stem cell characteristics (Dominici et al., 2006). Cell surface epitope expression is prone to change depending on isolation method, culture conditions, and cell-detachment methods (discussed earlier).

Functional analyses are usually conducted on a selected number of highly proliferative clones which are passaged and treated with artificial cues for acquiring putative cell fates *in vitro*. There is also no common agreement on how to interpret the maturation stage of stem and progenitor cells by *in vitro* differentiation assays. While robust differentiation toward induced fates is mostly understood as multipotency originating from a stem cell, some laboratories have argued that populations with less pronounced differentiation phenotypes might be indicative of a more immature hierarchical state (Merrick et al., 2019).

Flow cytometry allows the prospective isolation of putative SSC populations that can be investigated for stem cell properties by direct transplantation into an *in vivo* setting without prior culture. Preparation of FACS samples entails the use of

mechanical and enzymatic digestion, which also brings about risks for cell loss and changes of cell epitopes. Transcriptomic comparisons of bulk tissue versus sorted viable populations using deconvolution techniques such as CIBERSORT can help to identify potential cell types that may be only inefficiently isolated by FACS (Newman et al., 2015). Yet, it can be assumed that isolation of cells by surface marker expression is superior to selective and artificially skewed adherence cultures. It is also important to control if antibody panels for distinct cell types are inclusive of the same cells when working with treatment, disease, or aged cohort samples. The same applies when examining stromal cells from different skeletal sites. In this context, reporting of cell type abundance is highly ambiguous, since cellular composition, preparation method, and presentation of analysis often vary.

Taken together, the scientific community should refrain from defining "stem cells" based on their *in vitro* behavior. Rigorous *in vivo* testing of prospective cell populations will give a better understanding of true SSCs. Proof for the translatability of findings from mice to humans should always be aimed for, specifically, since many stem cell markers are not conserved between the two species, a prominent example being the cell surface configuration of mouse and human SSCs (Chan et al., 2015, 2018). The development of novel and more sophisticated techniques are needed to enable interrogations of SSCs *in situ* across species.

Limitations of Transgenic Mouse Models

One means of looking at stem cell dynamics without perturbations is with the help of transgenic mice. Unfortunately, the use of gene promoters is inherently limited and biased (Mendez-Ferrer et al., 2015). No single genetic marker can target a pure skeletal or stromal stem cell population to date. Fluorescent reporter protein presence under control of a target gene can be misleading and not reflective of the actual expression. For instance, minimal thresholds of transcriptional activity for fluorescence protein expression and detection are varying depending on gene construct and reporter protein used. Immature stromal cells also tend to express high levels of genes necessary for differentiation, which not necessarily means that they have already committed to a lineage which would be represented by the investigated marker. Lastly, fluorescent protein retention after gene expression has ceased also gives a false readout.

The gold standard for lineage tracing is the Cre-LoxP system. Although very efficient for a broad majority of tissues it comes with major limitations for bone tissue (Elefteriou and Yang, 2011). Potential causes have been implied to result from the chromosomal location of floxed alleles, the distance between LoxP sites, and cell-specific Cre-activity (Liu J. et al., 2013). A number of Cre-lines targeting common stromal cell markers (PDGFRα, Prx1, Osx) have been demonstrated to only incompletely recombine in the bone marrow (Krueger et al., 2014; Mizoguchi et al., 2014; Zhou B.O. et al., 2014; Ambrosi et al., 2017). To the opposite, some Cre-reporters show fluctuating levels of leakage in cells not expressing the transgene. Different models for the same reporter gene have yielded divergent results. Reporter mice for the osteogenic transcription factor Osterix, for example, label only committed osteoblastic cells in an earlier model while also recombining in stromal cell types in newer inducible versions (Madisen et al., 2010; Maes et al., 2010; Liu Y. et al., 2013). Evidence that Cre-expression alone can affect skeletal maturation has also been reported in an Osx-Cre model, necessitating a rigorous assessment of each transgenic line (Wang et al., 2015).

Investigations of functional effects mediated by Cre-drivers often ignore quantitative and qualitative off-target effects. In the context of constitutive Cre-expression, compensatory effects for the lack of a deleted target gene have to be considered, especially during development. Cre-driven gene deletions are also often rather inefficient, reflected in the moderate phenotypes contrary to the expectation. Timepoint-specific deletions by inducible Cre-ERt models are many times hard to interpret as they require tamoxifen administration which has been shown to have side effects, even more pronounced when applied long-term. This has been partially overcome with the development of Cre-ERt2 models, which require up to ten times lower amounts of tamoxifen administration. Possible side issues should also be accounted for when using other activators of genetic modifications such as doxycycline, tetracycline, and diphtheria toxin. Drawbacks of single markers used to characterize stem cell populations are obvious. Prominent examples of Cre-lines misinterpreted as selective multipotent stromal cell markers include Mx-1, Nestin, NG2, and LepR (Ulyanova et al., 2007; Park et al., 2012; Mizoguchi et al., 2014; Zhou B.O. et al., 2014). Aside the broad spectrum of stromal cell types they select for, they additionally span hematopoietic and endothelial cell populations. When using these Cre-models for directed deletion of genes in bone populations it is also important to consider functional readouts of indirect origin, in these cases of neural origin, especially on the note that brain-derived factors affect bone physiology.

Thus, data has to be interpreted with all these potential pitfalls in mind. Appropriate controls for background signal determination are important. Transgene reporter expressions should always be validated in multiple ways, e.g., FACS, qPCR, or histology. In general, it is advisable to combine fluorescence protein expression with additional antibodies against surface proteins. This is allowing the investigation of more purified cell types, which couldn't be looked at without fluorescence-tagging of non-surface markers. Overall, comprehensive approaches combining rigorous validation of designated cells and functional testing of stem cell hallmarks in *in vivo* assays are necessary. This will also help to lead the way to the identification and development of more specific SSC Cre-models.

CONCLUSION AND FUTURE DIRECTIONS

The field of SSC research holds enormous promise for a paradigm shift in the treatment of bone-related diseases and the understanding of pathogenetic mechanisms. In order to accomplish meaningful translational results, the research

community will be better served if there are updated guidelines for the definition and nomenclature of SSCs; in the spirit of the ISCT publication (Dominici et al., 2006). However, it is necessary to amend the ISCT guidelines as they continue to build on the initial problematic definition of "MSCs" (Caplan, 1991). Constituting a revised definition will have to be based on the latest technological advancements that have granted a better understanding of the function and regulation of single stem and progenitor cell types. Integrating the state-of-the-art knowledge and available resources of bone stem cell biology to us, we have come to suggest minimal criteria and terms for defined *bona fide* stem cells of the skeleton.

Proposed Bona Fide SSC Criteria

In vitro methods to derive true tissue-specific stem cells, expand patient-derived stem cells, and protocols to differentiate them into functional tissues are highly important topics in stem cell research and bioengineering. However, *in vitro* cell culture experiments have limited value in assessing *in vivo* cell behavior and should not exclusively be considered as evidence in the determination of stem cell activity. This has been extensively shown for bone marrow-derived stromal cells, which display differentiation capacity beyond mesodermal fates *in vitro* but are much more restricted *in vivo* (Bianco et al., 2006). SSCs should be able to self-renew, give rise to progeny of more restricted cell fate, and differentiate at least into osteoblasts/osteocytes and chondrocytes, all on the clonal level *in vivo* without prior *in vitro* culture (Bianco et al., 2008). Self-renewal should be considered maintaining the original phenotype while simultaneously giving rise to cell populations and stroma of more mature states which together are able to reconstitute an entire stem cell compartment *in vivo* able to recruit active hematopoiesis; and not be confused with sustained growth and differentiation capacity in culture.

These properties should be observed upon transplantation of freshly isolated cells into the endogenous environment but can also be assessed when transplanted into ectopic sites, e.g., renal capsule, adipose tissue, subcutaneous; to determine their intrinsic skeletogenic potential. It remains to be investigated, however, if SSCs might suffer from exhaustion much faster than HSCs because the inherent tissue turnover is manifolds slower. In contrast to HSCs, which can home to their bone marrow niches after intravenous injections, SSCs have to be transplanted into the desired tissue and, more importantly, need stromal cell support in order to engraft at the single-cell level (Chan et al., 2013). Functionally, as also exemplified by HSC approaches, proof of substantial contributions to skeletal tissue during homeostasis and/or injury should be provided for candidate cell populations, may it be through lineage tracing or long-term tracking of donor cells after transplantation.

Based on the possibility that multiple stem cell types exist within the bone and the observation that SSCs defined by us and others do not make adipocytes, tri-lineage differentiation capacity should not be a prerequisite for stem cells. Likewise, at this point, we are not able to define common markers for all bone-resident stem cells, except that they are most likely of non-hematopoietic ($CD45^-$) and non-endothelial ($CD31^-$) origin. A major challenge will be to discern the existence of SSCs at multiple anatomical sites throughout development, adulthood, and repair. The constant dynamic adaptations of skeletal tissue will require a careful characterization of all potential stem cell niches. Currently, it seems likely that discrete microenvironments of the same bone harbor distinct stem cell populations with similar properties but dedicated physiological functions depending on the localization.

SSC Terminology From Here on Onward

While considering the difficulties in altering a widely engrained nomenclature and acknowledging the improbability that the general usage of "MSC" will be discarded any time soon (Caplan, 2017), we would still like to propose a couple of specifications to address some of the more confusing and negative connotations of the term. The name "mesenchymal stem cell" is chronically used in an inappropriate fashion to describe a cell population with stem cell features as a whole but high heterogeneity on the single-cell level and, therefore, should be abandoned. *Bona fide* SSCs can be considered bone-resident stromal stem cells. Similarly, any other connective tissue contains a comparable, but specific stem cell type. If minimal criteria of stem cell state have not been properly investigated, cell populations from these tissues should be referred to as "multipotent stromal cells," indicative of mixed stromal cell types of the corresponding source tissue. "SSC" should be reserved for the highly selective and homogeneous cell population responsible for bone organ development, growth, and regeneration/repair only. Contingent upon the anatomical microenvironment these cells reside in, SSCs could be further specified, e.g., growth plate SSCs (gpSSC), periosteal SSCs (pSSCs), etc.

Connective tissues harbor different amounts of stem cell types depending on the lineages required for proper organ function. The terminology of stem cells should be reflective of the tissue origin and contributions, as successfully implemented with SSCs. Evidence for a second distinct perivascular stem cell in long bones, which seems to be predominantly involved in hematopoietic maintenance and niche support, and which is more representative of classic "MSCs" when assayed *in vitro* leads us to propose the classification of a perivascular SSC (pvSSC), based on its localization and properties. Future experiments will have to shed more light on the characteristics and functions of subpopulations of SSCs and will have to further evolve terminology applied to these cell types. Eventually the scientific community should establish a timely and accurate cell atlas for reporting new skeletal lineages within the context of previously discovered lineages in real-time.

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REFERENCES

Akiyama, H., Kim, J.-E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., et al. (2005). Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14665–14670. doi: 10.1073/pnas.0504750102

Ambrosi, T. H., and Schulz, T. J. (2017). The emerging role of bone marrow adipose tissue in bone health and dysfunction. *J. Mol. Med.* 95, 1291–1301. doi: 10.1007/s00109-017-1604-7

Ambrosi, T. H., Scialdone, A., Graja, A., Gohlke, S., Jank, A. M., Bocian, C., et al. (2017). Adipocyte accumulation in the bone marrow during obesity and aging impairs stem cell-based hematopoietic and bone regeneration. *Cell Stem Cell.* 20, 771–784.e6. doi: 10.1016/j.stem.2017.02.009

Autengruber, A., Gereke, M., Hansen, G., Hennig, C., and Bruder, D. (2012). Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. *Eur. J. Microbiol. Immunol.* 2, 112–120. doi: 10.1556/EuJMI.2.2012.2.3

Baker, D. J., Wijshake, T., Tchkonia, T., Lebrasseur, N. K., Childs, B. G., Van De Sluis, B., et al. (2011). Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 479, 232–236. doi: 10.1038/nature10600

Baryawno, N., Przybylski, D., Kowalczyk, M. S., Kfoury, Y., Severe, N., Gustafsson, K., et al. (2019). A cellular taxonomy of the bone marrow stroma in homeostasis and leukemia. *Cell* 177, 1915–1932.e16. doi: 10.1016/j.cell.2019.04.040

Becker, A. J., Mc, C. E., and Till, J. E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* 197, 452–454. doi: 10.1038/197452a0

Bianco, P., Cao, X., Frenette, P. S., Mao, J. J., Robey, P. G., Simmons, P. J., et al. (2013). The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat. Med.* 19, 35–42. doi: 10.1038/nm.3028

Bianco, P., Kuznetsov, S. A., Riminucci, M., and Gehron Robey, P. (2006). Postnatal skeletal stem cells. *Methods Enzymol.* 419, 117–148. doi: 10.1016/s0076-6879(06)19006-0

Bianco, P., and Robey, P. G. (2015). Skeletal stem cells. *Development* 142, 1023–1027. doi: 10.1242/dev.102210

Bianco, P., Robey, P. G., and Simmons, P. J. (2008). Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2, 313–319. doi: 10.1016/j.stem.2008.03.002

Bouabe, H., and Okkenhaug, K. (2013). Gene targeting in mice: a review. *Methods Mol. Biol.* 1064, 315–336. doi: 10.1007/978-1-62703-601-6_23

Burge, R., Dawson-Hughes, B., Solomon, D. H., Wong, J. B., King, A., and Tosteson, A. (2007). Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. *J. Bone Miner. Res.* 22, 465–475. doi: 10.1359/jbmr.061113

Campisi, J. (2013). Aging, cellular senescence, and cancer. *Annu. Rev. Physiol.* 75, 685–705. doi: 10.1146/annurev-physiol-030212-183653

Cao, J. J., Wronski, T. J., Iwaniec, U., Phleger, L., Kurimoto, P., Boudignon, B., et al. (2005). Aging increases stromal/osteoblastic cell-induced osteoclastogenesis and alters the osteoclast precursor pool in the mouse. *J. Bone Miner. Res.* 20, 1659–1668. doi: 10.1359/jbmr.050503

Caplan, A. I. (1991). Mesenchymal stem cells. *J. Orthop. Res.* 9, 641–650.

Caplan, A. I. (2008). All MSCs are pericytes? *Cell Stem Cell* 3, 229–230. doi: 10.1016/j.stem.2008.08.008

Caplan, A. I. (2017). Mesenchymal stem cells: time to change the name! *Stem Cells Transl. Med.* 6, 1445–1451. doi: 10.1002/sctm.17-0051

Castro-Malaspina, H., Gay, R. E., Resnick, G., Kapoor, N., Meyers, P., Chiarieri, D., et al. (1980). Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood* 56, 289–301.

Cenci, S., Weitzmann, M. N., Gentile, M. A., Aisa, M. C., and Pacifici, R. (2000). M-CSF neutralization and egr-1 deficiency prevent ovariectomy-induced bone loss. *J. Clin. Invest.* 105, 1279–1287. doi: 10.1172/jci8672

Chan, C. K., Chen, C. C., Luppen, C. A., Kim, J. B., Deboer, A. T., Wei, K., et al. (2009). Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature* 457, 490–494. doi: 10.1038/nature07547

Chan, C. K., Lindau, P., Jiang, W., Chen, J. Y., Zhang, L. F., Chen, C. C., et al. (2013). Clonal precursor of bone, cartilage, and hematopoietic niche stromal cells. *Proc. Natl. Acad. Sci. U.S.A.* 110, 12643–12648. doi: 10.1073/pnas.1310212110

Chan, C. K., Seo, E. Y., Chen, J. Y., Lo, D., Mcardle, A., Sinha, R., et al. (2015). Identification and specification of the mouse skeletal stem cell. *Cell* 160, 285–298. doi: 10.1016/j.cell.2014.12.002

Chan, C. K. F., Gulati, G. S., Sinha, R., Tompkins, J. V., Lopez, M., Carter, A. C., et al. (2018). Identification of the human skeletal stem cell. *Cell* 175:e21.

Chen, D., Shen, J., Zhao, W., Wang, T., Han, L., Hamilton, J. L., et al. (2017). Osteoarthritis: toward a comprehensive understanding of pathological mechanism. *Bone Res.* 5:16044. doi: 10.1038/boneres.2016.44

Crisan, M., Yap, S., Casteilla, L., Chen, C. W., Corselli, M., Park, T. S., et al. (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 3, 301–313. doi: 10.1016/j.stem.2008.07.003

da Silva Meirelles, L., Chagastelles, P. C., and Nardi, N. B. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J. Cell Sci.* 119, 2204–2213. doi: 10.1242/jcs.02932

Dangl, J. L., and Lanier, L. L. (2013). Founding father of FACS: Professor Leonard A. *Herzenberg (*1931-2013). *Proc. Natl. Acad. Sci. U.S.A.* 110, 20848–20849. doi: 10.1073/pnas.1321731111

Debnath, S., Yallowitz, A. R., Mccormick, J., Lalani, S., Zhang, T., Xu, R., et al. (2018). Discovery of a periosteal stem cell mediating intramembranous bone formation. *Nature* 562, 133–139. doi: 10.1038/s41586-018-0554-8

Demontiero, O., Vidal, C., and Duque, G. (2012). Aging and bone loss: new insights for the clinician. *Ther. Adv. Musculoskelet Dis.* 4, 61–76. doi: 10.1177/1759720X11430858

Ding, L., Saunders, T. L., Enikolopov, G., and Morrison, S. J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481, 457–462. doi: 10.1038/nature10783

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., et al. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. *Cytotherapy* 8, 315–317. doi: 10.1080/14653240600855905

Doro, D. H., Grigoriadis, A. E., and Liu, K. J. (2017). Calvarial suture-derived stem cells and their contribution to cranial bone repair. *Front. Physiol.* 8:956. doi: 10.3389/fphys.2017.00956

Dose, K. (1981). Ernst Haeckel's concept of an evolutionary origin of life. *Biosystems* 13, 253–258. doi: 10.1016/0303-2647(81)90005-8

Duchamp de Lageneste, O., Julien, A., Abou-Khalil, R., Frangi, G., Carvalho, C., Gagnard, N., et al. (2018). Periosteum contains skeletal stem cells with high bone regenerative potential controlled by Periostin. *Nat. Commun.* 9:733. doi: 10.1038/s41467-018-03124-z

Ekegren, C. L., Edwards, E. R., De Steiger, R., and Gabbe, B. J. (2018). Incidence, costs and predictors of non-union, delayed union and mal-union following long bone fracture. *Int. J. Environ. Res. Public Health* 15:E2845. doi: 10.3390/ijerph15122845

Elefteriou, F., and Yang, X. (2011). Genetic mouse models for bone studies–strengths and limitations. *Bone* 49, 1242–1254. doi: 10.1016/j.bone.2011.08.021

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154–156. doi: 10.1038/292154a0

Friedenstein, A. J., Deriglasova, U. F., Kulagina, N. N., Panasuk, A. F., Rudakowa, S. F., Luria, E. A., et al. (1974). Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp. Hematol.* 2, 83–92.

Friedenstein, A. J., Piatetzky, S. I. I., and Petrakova, K. V. (1966). Osteogenesis in transplants of bone marrow cells. *J. Embryol. Exp. Morphol.* 16, 381–390.

Galipeau, J., and Sensebe, L. (2018). Mesenchymal stromal cells: clinical challenges and therapeutic opportunities. *Cell Stem Cell* 22, 824–833. doi: 10.1016/j.stem.2018.05.004

Ganguly, P., El-Jawhari, J. J., Giannoudis, P. V., Burska, A. N., Ponchel, F., and Jones, E. A. (2017). Age-related changes in bone marrow mesenchymal stromal cells: a potential impact on osteoporosis and osteoarthritis development. *Cell Transplant* 26, 1520–1529. doi: 10.1177/0963689717721201

Greenbaum, A., Hsu, Y. M., Day, R. B., Schuettpelz, L. G., Christopher, M. J., Borgerding, J. N., et al. (2013). CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* 495, 227–230. doi: 10.1038/nature11926

Gulati, G. S., Murphy, M. P., Marecic, O., Lopez, M., Brewer, R. E., Koepke, L. S., et al. (2018). Isolation and functional assessment of mouse skeletal stem cell lineage. *Nat. Protoc.* 13, 1294–1309. doi: 10.1038/nprot.2018.041

Hagmann, S., Moradi, B., Frank, S., Dreher, T., Kammerer, P. W., Richter, W., et al. (2013). Different culture media affect growth characteristics, surface marker distribution and chondrogenic differentiation of human bone marrow-derived mesenchymal stromal cells. *BMC Musculoskelet Disord.* 14:223. doi: 10.1186/1471-2474-14-223

He, X., Bougioukli, S., Ortega, B., Arevalo, E., Lieberman, J. R., and Mcmahon, A. P. (2017). Sox9 positive periosteal cells in fracture repair of the adult mammalian long bone. *Bone* 103, 12–19. doi: 10.1016/j.bone.2017.06.008

Herber, C. B., Krause, W. C., Wang, L., Bayrer, J. R., Li, A., Schmitz, M., et al. (2019). Estrogen signaling in arcuate Kiss1 neurons suppresses a sex-dependent female circuit promoting dense strong bones. *Nat. Commun.* 10:163. doi: 10.1038/s41467-018-08046-4

Idelevich, A., and Baron, R. (2018). Brain to bone: what is the contribution of the brain to skeletal homeostasis? *Bone* 115, 31–42. doi: 10.1016/j.bone.2018.05.018

Ikebuchi, Y., Aoki, S., Honma, M., Hayashi, M., Sugamori, Y., Khan, M., et al. (2018). Coupling of bone resorption and formation by RANKL reverse signalling. *Nature* 561, 195–200. doi: 10.1038/s41586-018-0482-7

Isern, J., Garcia-Garcia, A., Martin, A. M., Arranz, L., Martin-Perez, D., Torroja, C., et al. (2014). The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. *eLife* 3:e03696. doi: 10.7554/eLife.03696

Itkin, T., Gur-Cohen, S., Spencer, J. A., Schajnovitz, A., Ramasamy, S. K., Kusumbe, A. P., et al. (2016). Distinct bone marrow blood vessels differentially regulate haematopoiesis. *Nature* 532, 323–328. doi: 10.1038/nature17624

Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P. V., Mar, B. G., et al. (2014). Age-related clonal hematopoiesis associated with adverse outcomes. *N. Engl. J. Med.* 371, 2488–2498. doi: 10.1056/NEJMoa1408617

Jaiswal, S., Natarajan, P., Silver, A. J., Gibson, C. J., Bick, A. G., Shvartz, E., et al. (2017). Clonal Hematopoiesis and risk of atherosclerotic cardiovascular disease. *N. Engl. J. Med.* 377, 111–121. doi: 10.1056/NEJMoa1701719

Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., et al. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418:6893.

Josephson, A. M., Bradaschia-Correa, V., Lee, S., Leclerc, K., Patel, K. S., Muinos Lopez, E., et al. (2019). Age-related inflammation triggers skeletal stem/progenitor cell dysfunction. *Proc. Natl. Acad. Sci. U.S.A.* 116, 6995–7004. doi: 10.1073/pnas.1810692116

Karsenty, G., and Ferron, M. (2012). The contribution of bone to whole-organism physiology. *Nature* 481, 314–320. doi: 10.1038/nature10763

Kfoury, Y., and Scadden, D. T. (2015). Mesenchymal Cell Contributions to the Stem Cell Niche. *Cell Stem Cell* 16, 239–253. doi: 10.1016/j.stem.2015.02.019

Kiel, M. J., Radice, G. L., and Morrison, S. J. (2007). Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. *Cell Stem Cell* 1, 204–217. doi: 10.1016/j.stem.2007.06.001

Kiel, M. J., Yilmaz, O. H., Iwashita, T., Yilmaz, O. H., Terhorst, C., and Morrison, S. J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121, 1109–1121. doi: 10.1016/j.cell.2005.05.026

Koide, Y., Morikawa, S., Mabuchi, Y., Muguruma, Y., Hiratsu, E., Hasegawa, K., et al. (2007). Two distinct stem cell lineages in murine bone marrow. *Stem Cells* 25, 1213–1221. doi: 10.1634/stemcells.2006-0325

Krueger, K. C., Costa, M. J., Du, H., and Feldman, B. J. (2014). Characterization of Cre recombinase activity for *in vivo* targeting of adipocyte precursor cells. *Stem Cell Rep.* 3, 1147–1158. doi: 10.1016/j.stemcr.2014.10.009

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., et al. (2013). Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* 502, 637–643. doi: 10.1038/nature12612

Kusumbe, A. P., Ramasamy, S. K., and Adams, R. H. (2014). Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature* 507, 323–328. doi: 10.1038/nature13145

Kusumbe, A. P., Ramasamy, S. K., Itkin, T., Mae, M. A., Langen, U. H., Betsholtz, C., et al. (2016). Age-dependent modulation of vascular niches for haematopoietic stem cells. *Nature* 532, 380–384. doi: 10.1038/nature17638

Kuznetsov, S. A., Krebsbach, P. H., Satomura, K., Kerr, J., Riminucci, M., Benayahu, D., et al. (1997). Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation *in vivo*. *J. Bone Miner. Res.* 12, 1335–1347. doi: 10.1359/jbmr.1997.12.9.1335

Lange, C., Schroeder, J., Stute, N., Lioznov, M. V., and Zander, A. R. (2005). High-potential human mesenchymal stem cells. *Stem Cells Dev.* 14, 70–80. doi: 10.1089/scd.2005.14.70

Laurenti, E., and Gottgens, B. (2018). From haematopoietic stem cells to complex differentiation landscapes. *Nature* 553, 418–426. doi: 10.1038/nature25022

Lee, Y. K., Lee, Y. J., Ha, Y. C., and Koo, K. H. (2014). Five-year relative survival of patients with osteoporotic hip fracture. *J. Clin. Endocrinol. Metab.* 99, 97–100. doi: 10.1210/jc.2013-2352

Li, H., Ghazanfari, R., Zacharaki, D., Ditzel, N., Isern, J., Ekblom, M., et al. (2014). Low/negative expression of PDGFR-alpha identifies the candidate primary mesenchymal stromal cells in adult human bone marrow. *Stem Cell Rep.* 3, 965–974. doi: 10.1016/j.stemcr.2014.09.018

Liu, J., Willet, S. G., Bankaitis, E. D., Xu, Y., Wright, C. V., and Gu, G. (2013). Non-parallel recombination limits Cre-LoxP-based reporters as precise indicators of conditional genetic manipulation. *Genesis* 51, 436–442. doi: 10.1002/dvg.22384

Liu, Y., Strecker, S., Wang, L., Kronenberg, M. S., Wang, W., Rowe, D. W., et al. (2013). Osterix-cre labeled progenitor cells contribute to the formation and maintenance of the bone marrow stroma. *PLoS One* 8:e71318. doi: 10.1371/journal.pone.0071318

Lo Celso, C., Fleming, H. E., Wu, J. W., Zhao, C. X., Miake-Lye, S., Fujisaki, J., et al. (2009). Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* 457, 92–96. doi: 10.1038/nature07434

Logan, M., Martin, J. F., Nagy, A., Lobe, C., Olson, E. N., and Tabin, C. J. (2002). Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. *Genesis* 33, 77–80. doi: 10.1002/gene.10092

Lopez-Otin, C., Blasco, M. A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. *Cell* 153, 1194–1217. doi: 10.1016/j.cell.2013.05.039

Mabuchi, Y., Houlihan, D. D., Akazawa, C., Okano, H., and Matsuzaki, Y. (2013). Prospective isolation of murine and human bone marrow mesenchymal stem cells based on surface markers. *Stem Cells Int.* 2013, 507301. doi: 10.1155/2013/507301

Madisen, L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A., Gu, H., et al. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat. Neurosci.* 13, 133–140. doi: 10.1038/nn.2467

Maes, C., Kobayashi, T., Selig, M. K., Torrekens, S., Roth, S. I., Mackem, S., et al. (2010). Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Dev. Cell* 19, 329–344. doi: 10.1016/j.devcel.2010.07.010

Marecic, O., Tevlin, R., Mcardle, A., Seo, E. Y., Wearda, T., Duldulao, C., et al. (2015). Identification and characterization of an injury-induced skeletal progenitor. *Proc. Natl. Acad. Sci. U.S.A.* 112, 9920–9925. doi: 10.1073/pnas.1513066112

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78, 7634–7638. doi: 10.1073/pnas.78.12.7634

Maruyama, T., Jeong, J., Sheu, T. J., and Hsu, W. (2016). Stem cells of the suture mesenchyme in craniofacial bone development, repair and regeneration. *Nat. Commun.* 7:10526. doi: 10.1038/ncomms10526

Mendez-Ferrer, S., Michurina, T. V., Ferraro, F., Mazloom, A. R., Macarthur, B. D., Lira, S. A., et al. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466, 829–834. doi: 10.1038/nature09262

Mendez-Ferrer, S., Scadden, D. T., and Sanchez-Aguilera, A. (2015). Bone marrow stem cells: current and emerging concepts. *Ann. N. Y. Acad. Sci.* 1335, 32–44. doi: 10.1111/nyas.12641

Mendicino, M., Bailey, A. M., Wonnacott, K., Puri, R. K., and Bauer, S. R. (2014). MSC-based product characterization for clinical trials: an FDA perspective. *Cell Stem Cell* 14, 141–145. doi: 10.1016/j.stem.2014.01.013

Merrick, D., Sakers, A., Irgebay, Z., Okada, C., Calvert, C., Morley, M. P., et al. (2019). Identification of a mesenchymal progenitor cell hierarchy in adipose tissue. *Science* 364:eaav2501. doi: 10.1126/science.aav2501

Mignone, J. L., Kukekov, V., Chiang, A. S., Steindler, D., and Enikolopov, G. (2004). Neural stem and progenitor cells in nestin-GFP transgenic mice. *J. Comp. Neurol.* 469, 311–324. doi: 10.1002/cne.10964

Miyanishi, M., Mori, Y., Seita, J., Chen, J. Y., Karten, S., Chan, C. K., et al. (2013). Do pluripotent stem cells exist in adult mice as very small embryonic stem cells? *Stem Cell Rep.* 1, 198–208. doi: 10.1016/j.stemcr.2013.07.001

Mizoguchi, T., Pinho, S., Ahmed, J., Kunisaki, Y., Hanoun, M., Mendelson, A., et al. (2014). Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Dev. Cell* 29, 340–349. doi: 10.1016/j.devcel.2014.03.013

Mizuhashi, K., Ono, W., Matsushita, Y., Sakagami, N., Takahashi, A., Saunders, T. L., et al. (2018). Resting zone of the growth plate houses a unique class of skeletal stem cells. *Nature* 563, 254–258. doi: 10.1038/s41586-018-0662-5

Morikawa, S., Mabuchi, Y., Kubota, Y., Nagai, Y., Niibe, K., Hiratsu, E., et al. (2009a). Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J. Exp. Med.* 206, 2483–2496. doi: 10.1084/jem.20091046

Morikawa, S., Mabuchi, Y., Niibe, K., Suzuki, S., Nagoshi, N., Sunabori, T., et al. (2009b). Development of mesenchymal stem cells partially originate from the neural crest. *Biochem. Biophys. Res. Commun.* 379, 1114–1119. doi: 10.1016/j.bbrc.2009.01.031

Nakashima, T., Hayashi, M., Fukunaga, T., Kurata, K., Oh-Hora, M., Feng, J. Q., et al. (2011). Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat. Med.* 17, 1231–1234. doi: 10.1038/nm.2452

Naveiras, O., Nardi, V., Wenzel, P. L., Hauschka, P. V., Fahey, F., and Daley, G. Q. (2009). Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* 460, 259–263. doi: 10.1038/nature08099

Newman, A. M., Liu, C. L., Green, M. R., Gentles, A. J., Feng, W., Xu, Y., et al. (2015). Robust enumeration of cell subsets from tissue expression profiles. *Nat. Methods* 12, 453–457. doi: 10.1038/nmeth.3337

Newton, P. T., Li, L., Zhou, B., Schweingruber, C., Hovorakova, M., Xie, M., et al. (2019). A radical switch in clonality reveals a stem cell niche in the epiphyseal growth plate. *Nature* 567, 234–238. doi: 10.1038/s41586-019-0989-6

Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., et al. (2010). The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* 33, 387–399. doi: 10.1016/j.immuni.2010.08.017r

Ono, N., Ono, W., Mizoguchi, T., Nagasawa, T., Frenette, P. S., and Kronenberg, H. M. (2014a). Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. *Dev. Cell* 29, 330–339. doi: 10.1016/j.devcel.2014.03.014

Ono, N., Ono, W., Nagasawa, T., and Kronenberg, H. M. (2014b). A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. *Nat. Cell Biol.* 16, 1157–1167. doi: 10.1038/ncb3067

Owen, M., and Friedenstein, A. J. (1988). Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp.* 136, 42–60.

Pagnotti, G. M., Styner, M., Uzer, G., Patel, V. S., Wright, L. E., Ness, K. K., et al. (2019). Combating osteoporosis and obesity with exercise: leveraging cell mechanosensitivity. *Nat. Rev. Endocrinol.* 15, 339–355. doi: 10.1038/s41574-019-0170-1

Park, D., Spencer, J. A., Koh, B. I., Kobayashi, T., Fujisaki, J., Clemens, T. L., et al. (2012). Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. *Cell Stem Cell* 10, 259–272. doi: 10.1016/j.stem.2012.02.003

Pinho, S., Lacombe, J., Hanoun, M., Mizoguchi, T., Bruns, I., Kunisaki, Y., et al. (2013). PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J. Exp. Med.* 210, 1351–1367. doi: 10.1084/jem.20122252

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., et al. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–147. doi: 10.1126/science.284.5411.143

Qian, H., Le Blanc, K., and Sigvardsson, M. (2012). Primary mesenchymal stem and progenitor cells from bone marrow lack expression of CD44 protein. *J. Biol. Chem.* 287, 25795–25807. doi: 10.1074/jbc.M112.339622

Ransom, R. C., Carter, A. C., Salhotra, A., Leavitt, T., Marecic, O., Murphy, M. P., et al. (2018). Mechanoresponsive stem cells acquire neural crest fate in jaw regeneration. *Nature* 563, 514–521. doi: 10.1038/s41586-018-0650-9

Robey, P. (2017). "Mesenchymal stem cells": fact or fiction, and implications in their therapeutic use. *F1000Res* 6:F1000FacultyRev–524. doi: 10.12688/f1000research.10955.1

Rodeheffer, M. S., Birsoy, K., and Friedman, J. M. (2008). Identification of white adipocyte progenitor cells *in vivo*. *Cell* 135, 240–249. doi: 10.1016/j.cell.2008.09.036

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., et al. (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 131, 324–336. doi: 10.1016/j.cell.2007.08.025

Sacchetti, B., Funari, A., Remoli, C., Giannicola, G., Kogler, G., Liedtke, S., et al. (2016). No Identical "Mesenchymal Stem Cells" at different times and sites: human committed progenitors of distinct origin and differentiation potential are incorporated as adventitial cells in microvessels. *Stem Cell Rep.* 6, 897–913. doi: 10.1016/j.stemcr.2016.05.011

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* 4, 7–25.

Seike, M., Omatsu, Y., Watanabe, H., Kondoh, G., and Nagasawa, T. (2018). Stem cell niche-specific Ebf3 maintains the bone marrow cavity. *Genes Dev.* 32, 359–372. doi: 10.1101/gad.311068.117

Shi, S., and Gronthos, S. (2003). Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J. Bone Miner. Res.* 18, 696–704. doi: 10.1359/jbmr.2003.18.4.696

Shi, Y., He, G., Lee, W. C., McKenzie, J. A., Silva, M. J., and Long, F. (2017). Gli1 identifies osteogenic progenitors for bone formation and fracture repair. *Nat. Commun.* 8:2043. doi: 10.1038/s41467-017-02171-2

Simmons, P. J., and Torok-Storb, B. (1991). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* 78, 55–62.

Sipkins, D. A., Wei, X., Wu, J. W., Runnels, J. M., Cote, D., Means, T. K., et al. (2005). *In vivo* imaging of specialized bone marrow endothelial microdomains for tumour engraftment. *Nature* 435, 969–973. doi: 10.1038/nature03703

Sipp, D., Robey, P. G., and Turner, L. (2018). Clear up this stem-cell mess. *Nature* 561, 455–457. doi: 10.1038/d41586-018-06756-9

Sorrentino, A., Ferracin, M., Castelli, G., Biffoni, M., Tomaselli, G., Baiocchi, M., et al. (2008). Isolation and characterization of CD146+ multipotent mesenchymal stromal cells. *Exp. Hematol.* 36, 1035–1046. doi: 10.1016/j.exphem.2008.03.004

Spangrude, G. J., Heimfeld, S., and Weissman, I. L. (1988). Purification and characterization of mouse hematopoietic stem cells. *Science* 241, 58–62. doi: 10.1126/science.2898810

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 25, 977–988. doi: 10.1016/j.immuni.2006.10.016

Szyska, M., and Na, I. K. (2016). Bone marrow GvHD after allogeneic hematopoietic stem cell transplantation. *Front. Immunol.* 7:118. doi: 10.3389/fimmu.2016.00118

Taichman, R., Reilly, M., Verma, R., Ehrenman, K., and Emerson, S. (2001). Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors. *Br. J. Haematol.* 112, 438–448. doi: 10.1046/j.1365-2141.2001.02568.x

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. doi: 10.1016/j.cell.2006.07.024

Tavassoli, M., and Crosby, W. H. (1968). Transplantation of marrow to extramedullary sites. *Science* 161, 54–56. doi: 10.1126/science.161.3836.54

Tencerova, M., Frost, M., Figeac, F., Nielsen, T. K., Ali, D., Lauterlein, J. L., et al. (2019). Obesity-Associated hypermetabolism and accelerated senescence of bone marrow stromal stem cells suggest a potential mechanism for bone fragility. *Cell Rep.* 27, 2050–2062. doi: 10.1016/j.celrep.2019.04.066

Tevlin, R., Seo, E. Y., Marecic, O., Mcardle, A., Tong, X., Zimdahl, B., et al. (2017). Pharmacological rescue of diabetic skeletal stem cell niches. *Sci. Trans. Med.* 9:eaag2809. doi: 10.1126/scitranslmed.aag2809

Tikhonova, A. N., Dolgalev, I., Hu, H., Sivaraj, K. K., Hoxha, E., Cuesta-Dominguez, A., et al. (2019). The bone marrow microenvironment at single-cell resolution. *Nature* 569, 222–228. doi: 10.1038/s41586-019-1104-8

Till, J. E., and McCulloch, E. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat. Res.* 14, 213–222.

Tormin, A., Li, O., Brune, J. C., Walsh, S., Schutz, B., Ehinger, M., et al. (2011). CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with *in situ* localization. *Blood* 117, 5067–5077. doi: 10.1182/blood-2010-08-304287

Tsuji, K., Ojima, M., Otabe, K., Horie, M., Koga, H., Sekiya, I., et al. (2017). Effects of different cell-detaching methods on the viability and cell surface antigen expression of synovial mesenchymal stem cells. *Cell Transplant* 26, 1089–1102. doi: 10.3727/096368917X694831

Ulyanova, T., Priestley, G. V., Nakamoto, B., Jiang, Y., and Papayannopoulou, T. (2007). VCAM-1 ablation in nonhematopoietic cells in MxCre+ VCAM-1f/f mice is variable and dictates their phenotype. *Exp. Hematol.* 35, 565–571. doi: 10.1016/j.exphem.2007.01.031

Via, A. G., Frizziero, A., and Oliva, F. (2012). Biological properties of mesenchymal Stem Cells from different sources. *Muscles Ligaments Tendons J.* 2, 154–162.

Wang, L., Mishina, Y., and Liu, F. (2015). Osterix-Cre transgene causes craniofacial bone development defect. *Calcif. Tissue Int.* 96, 129–137. doi: 10.1007/s00223-014-9945-5

Weissman, I. L. (2002). The road ended up at stem cells. *Immunol. Rev.* 185, 159–174. doi: 10.1034/j.1600-065x.2002.18514.x

Worthley, D. L., Churchill, M., Compton, J. T., Tailor, Y., Rao, M., Si, Y., et al. (2015). Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. *Cell* 160, 269–284. doi: 10.1016/j.cell.2014.11.042

Yan, J., Herzog, J. W., Tsang, K., Brennan, C. A., Bower, M. A., Garrett, W. S., et al. (2016). Gut microbiota induce IGF-1 and promote bone formation and growth. *Proc. Natl. Acad. Sci. U.S.A.* 113, E7554–E7563.

Yang, L., Tsang, K. Y., Tang, H. C., Chan, D., and Cheah, K. S. (2014). Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12097–12102. doi: 10.1073/pnas.1302703111

Yue, R., Zhou, B. O., Shimada, I. S., Zhao, Z., and Morrison, S. J. (2016). Leptin receptor promotes adipogenesis and reduces osteogenesis by regulating mesenchymal stromal cells in adult bone marrow. *Cell Stem Cell* 18, 1–15. doi: 10.1016/j.stem.2016.02.015

Yukata, K., Xie, C., Li, T. F., Takahata, M., Hoak, D., Kondabolu, S., et al. (2014). Aging periosteal progenitor cells have reduced regenerative responsiveness to bone injury and to the anabolic actions of PTH 1-34 treatment. *Bone* 62, 79–89. doi: 10.1016/j.bone.2014.02.002

Zhao, H., Feng, J., Hi, T. V., Grimes, W., Urata, M., and Chai, Y. (2015). The suture provides a niche for mesenchymal stem cells of craniofacial bones. *Nat. Cell Biol.* 17, 386–396. doi: 10.1038/ncb3139

Zhou, B. O., Yu, H., Yue, R., Zhao, Z., Rios, J. J., Naveiras, O., et al. (2017). Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. *Nat. Cell Biol.* 8, 891–903. doi: 10.1038/ncb3570

Zhou, B. O., Yue, R., Murphy, M. M., Peyer, J. G., and Morrison, S. J. (2014). Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell* 15, 154–168. doi: 10.1016/j.stem.2014.06.008

Zhou, X., Von Der Mark, K., Henry, S., Norton, W., Adams, H., and De Crombrugghe, B. (2014). Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice. *PLoS Genet.* 10:e1004820. doi: 10.1371/journal.pgen.1004820

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REVIEW
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Understanding the Cellular and Molecular Mechanisms That Control Early Cell Fate Decisions During Appendicular Skeletogenesis

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The formation of the vertebrate skeleton is orchestrated in time and space by a number of gene regulatory networks that specify and position all skeletal tissues. During embryonic development, bones have two distinct origins: bone tissue differentiates directly from mesenchymal progenitors, whereas most long bones arise from cartilaginous templates through a process known as endochondral ossification. Before endochondral bone development takes place, chondrocytes form a cartilage analgen that will be sequentially segmented to form joints; thus, in the cartilage template, either the cartilage maturation programme or the joint formation programme is activated. Once the cartilage differentiation programme starts, the growth plate begins to form. In contrast, when the joint formation programme is activated, a capsule begins to form that contains special articular cartilage and synovium to generate a functional joint. In this review, we will discuss the mechanisms controlling the earliest molecular events that regulate cell fate during skeletogenesis in long bones. We will explore the initial processes that lead to the recruitment of mesenchymal stem/progenitor cells, the commitment of chondrocyte lineages, and the formation of skeletal elements during morphogenesis. Thereafter, we will review the process of joint specification and joint morphogenesis. We will discuss the links between transcription factor activity, cell–cell interactions, cell–extracellular matrix interactions, growth factor signalling, and other molecular interactions that control mesenchymal stem/progenitor cell fate during embryonic skeletogenesis.

Keywords: skeletal stem cells, chondrogenesis, endochondral bone development, limb development, joint development

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INTRODUCTION

The formation of the vertebrate skeletal system is a paradigmatic model process for the study of differentiation, patterning, and morphogenesis during embryogenesis. The appearance of an endoskeleton during evolution has favoured the radiation of many forms of tetrapods that have adapted to many ecological niches (Vogel et al., 1996; Ohuchi et al., 1997; Shubin et al., 1997). An intricate network of genetic regulatory programmes that coordinate three-dimensional organization, differentiation, patterning, and morphogenesis hierarchically regulates skeleton formation during embryogenesis. During skeletogenesis, the sequences of events that occur during

bone and cartilage development are temporally and spatially regulated to not only control the patterning of the early skeleton but also ensure the correct shapes of all skeletal elements. At the same time as patterning, precursor cells are committed to the cartilage lineage under precise spatial and temporal control of cell differentiation. Once chondrogenesis starts in the early stages of embryogenesis, cartilage forms and is gradually replaced by bone; however, it persists on articular surfaces and forms the sole skeletal support system for the larynx, trachea, bronchi, and other structures. Cartilage is usually an avascular tissue, except in areas where blood vessels pass through other tissues and in regions where endochondral ossification occurs (Mackie et al., 2008). According to its nature and visibility, cartilage can be subdivided into three varieties: 1) hyaline, 2) elastic, and 3) fibrous, with hyaline cartilage being the most widely distributed type. As a tissue, cartilage has multiple embryological origins, including the cranial neural crest (CNC) (Chai et al., 2000), dorsal paraxial mesoderm, and lateral plate mesoderm (LPM) (Hall, 1981; Takahashi et al., 2001). CNC cells migrate from the dorsal neural tube into the branchial arches and other regions of the developing face and head, where they give rise to bones and cartilage (Hall, 1981; Takahashi et al., 2001). The ribs and vertebrae originate from somites, which are formed by segmentation of the paraxial mesoderm. The somites de-epithelize ventrally to give rise to the sclerotome. Mesenchymal cells from the sclerotome receive signals from the notochord to form vertebrae and intervertebral discs, while cells from the lateral sclerotome migrate out to form the ribs (Chal and Pourquié, 2017). Finally, cells from the LPM participate in the establishment of the appendicular skeleton. LPM cells proliferate and migrate from the flanks of the embryo and form the limb buds (Vogel et al., 1996; Ohuchi et al., 1997; Jin et al., 2018). As development progresses, these initially undifferentiated cells interpret signals according to their positions and differentiate into a variety of tissues that compose the adult limb while simultaneously shaping each limb into its final form.

The aim of this review is to explore the earliest molecular processes that control cell fate decisions during appendicular skeletogenesis. Specifically, we will focus on the cellular and molecular mechanisms that govern the early fate decisions of mesenchymal stem/progenitor cells that give rise to the appendicular skeleton, using limb development as a model. Here, the term stem/progenitor cells will be used despite there is no evidence that mesenchymal cells are either stem or progenitor cells. Stem cells are considered to have the capacity to reproduce themselves. By asymmetric division, these cells can generate progeny with the ability to acquire distinct cell fates and to differentiate into functional cell types. Stem cells are present in many tissues for long durations and are regulated by the microenvironment, the stem cell niche. In contrast, progenitor cells arise from asymmetric division of stem cells and differentiate into distinct functional cell types. When progenitor cells proliferate to amplify their populations, they are also called transient amplifying cells (Slack, 2018). First, we will review the initial process that leads to the recruitment of mesenchymal stem/progenitor cells to the chondrocyte and osteoblast lineage and to the formation of skeletal elements located in the core of the limb and the subsequent process of endochondral ossification in long skeletal elements. We will also explore the early steps of joint specification.

A Brief Overview of Limb Development

Tetrapod limbs are adapted to different habitats. Despite their diversity, limbs have three anatomical regions that can be distinguished: the stylopod in the proximal region, which gives

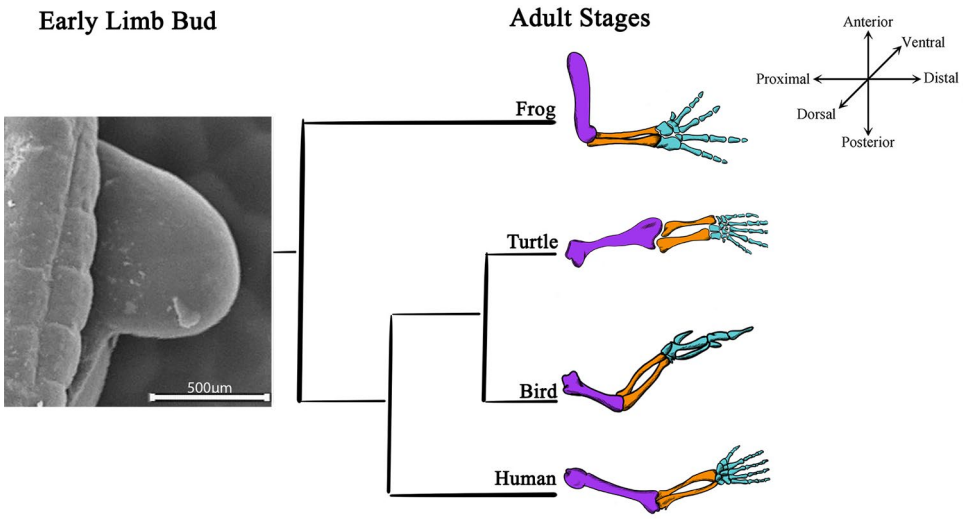

FIGURE 1 | Limb bud development and adult limb structures are conserved among tetrapods. A scanning electron microscopy image of a 22HH chick forelimb shows limb bud, the structure where all the skeletal elements arise during development. Schematic representations of adult skeletal structures of forelimbs from distinct tetrapods (frog, turtle, bird, and human) are shown. The most proximal skeletal element, the stylopod, is characterized for a single skeletal element (purple); two middle elements represent the zeugopod (orange), and the distal region highly segmented represents the autopod (blue).

rise to the humerus of the forelimb and the femur of the hindlimb; the zeugopod, which corresponds to the two middle elements, the radius/ulna of the forelimb or the tibia/fibula of the hindlimb; and the autopod, which corresponds to the highly segmented distal elements, developing into the metacarpal/carpal/finger bones of the forelimb and the metatarsal/tarsal/toe bones of the hindlimb (**Figure 1**; Towers and Tickle, 2009; Tickle, 2015).

The beginning of limb development is evidenced by the emergence of a limb bud (Tickle, 2015). Embryonic limb buds are formed by mesenchymal cells covered with ectoderm. The mesenchyme gives rise to the skeleton and ligaments, while the ectoderm gives rise to the skin and its derivates. However, although a homogeneous mesenchymal cell population is initially observed, cell populations that secrete molecular signals to establish limb spatial patterns are localized in certain limb bud zones (Shimizu et al., 2007; Zeller et al., 2009; Tickle, 2015) i.e., the arrangement of limb structures and the formation of a functional limb originates from the spatiotemporal organization of cells. The establishment of spatial patterns is closely related to morphogenesis, the process by which an organism develops its final shape. The three-dimensional organization of the limb depends on molecular interactions between three different signalling centres that govern limb shape and patterning in the proximo-distal (PD), antero-posterior (AP), and dorso-ventral (DV) axes (Towers and Tickle, 2009; Tickle, 2015). The outgrowth of the limb bud is coordinated by the apical ectodermal ridge (AER), positioned at the distal part of the limb at the boundary between the dorsal and ventral ectoderm (**Figure 2**; Saunders, 1948; Laufer et al., 1997; Rodriguez-Esteban et al., 1997). This structure releases signals to the undifferentiated region beneath it, promoting proliferation and maintaining the undifferentiated state of mesenchymal stem/precursor cells. Once mesenchymal stem/progenitor cells leave the region under the influence of the AER, they acquire spatiotemporal cues that allow them to commit to different cell lineages (Tabin and Wolpert, 2007). On the other hand, the AP polarity of the limb is directed by the zone of polarizing activity situated in the posterior region of the early limb (Saunders and Gasseling, 1968; Riddle et al., 1993). Finally, the dorsal ectoderm controls the DV polarity of the limb (**Figure 2**; Yang and Niswander, 1995, Parr and McMahon, 1995). Mesodermal cells from all axes interpret the molecular signals from the three signalling centres, giving rise to skeletal elements, muscles, tendons, and ligaments.

In all vertebrates, the formation of the appendicular skeleton originates from a cartilage template initiated in the core of the limb mesenchyme (Shum et al., 2003). The development of appendicular skeletal elements requires the proliferation and migration of mesenchymal cells from the LPM to the limb bud (Shimizu et al., 2007). Mesenchymal cells at the periphery of the limb bud are maintained in an undifferentiated state by signals released from the ectoderm (ten Berge et al., 2008). However, in the centre of the limb bud, chondrogenic signals induce the aggregation of mesenchymal stem/progenitor cells, giving rise to condensations that will later form the cartilage templates. The production of cartilage anlagen of specific sizes, shapes, and positions allows the precise patterning of limb skeletal elements. Except in the joints, the cartilage tissue is eventually replaced by bone in a process called endochondral ossification.

Maintenance of the Undifferentiated State of Limb Mesenchyme Prior to Skeletal Specification

Multiple cell types form an adult limb, but cartilage, bone, perichondrium, dermis, ligaments, and tendons originate from the limb mesenchymal stem/progenitor cells. Preceding lineage commitment, mesenchymal stem/progenitor cells are maintained in an undifferentiated state due to the influence of the AER and ectodermal signals. When they abandon the area of influence of these signals, begin the cellular commitment to give rise to different cellular lineages.

The Importance of the Establishment of the AER-FGF Signalling

The undifferentiated zone under the AER is approximately 200 μm in size and is located in the distal region of the limb bud (**Figure 3**; Summerbell et al., 1973; Dudley et al., 2002). Pioneering work by Saunders (Saunders, 1948) and later work by Summerbell (Summerbell, 1974) demonstrated the role of the AER in the outgrowth and formation of the skeletal elements of the embryonic limb. Using chicken embryos, they showed that

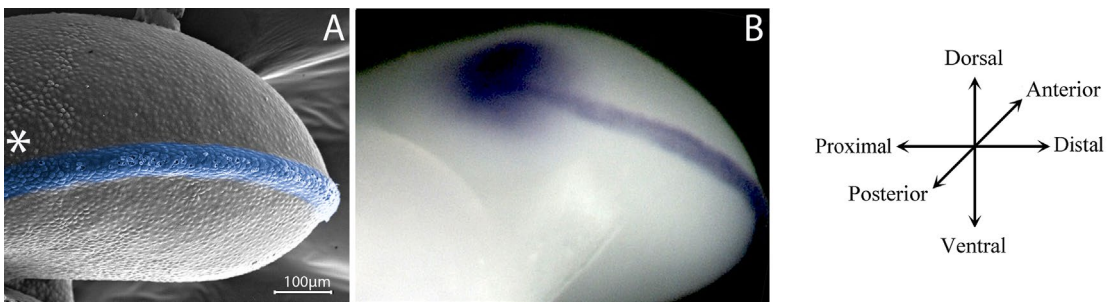

FIGURE 2 | Signalling centres controls limb development. **(A)** A posterior view of a scanning electron microscopy of a 22HH chicken hindlimb bud showing in blue the apical ectodermal ridge (AER) between the dorsal and ventral ectoderm. The localization of the zone of polarizing activity (ZPA) is showed with an asterisk. **(B)** In situ hybridization in a 22HH chicken hindlimb showing the expression of Shh in the ZPA and Fgf8 in the AER, signalling centres of the limb bud.

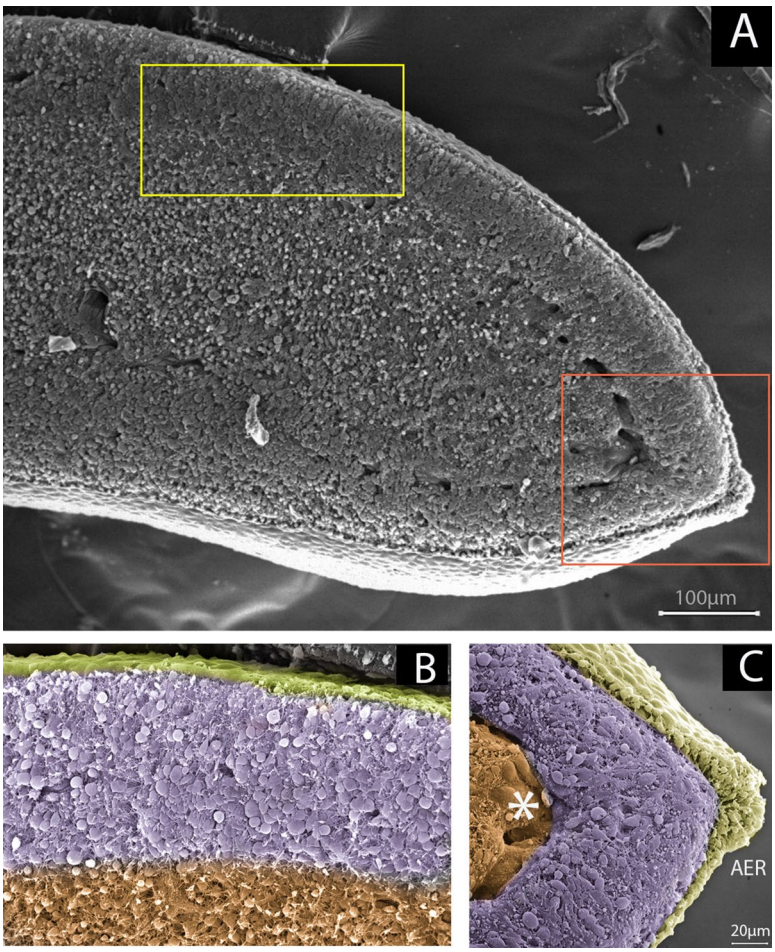

FIGURE 3 | Undifferentiated zones beneath limb ectoderm as reservoirs of stem/progenitor cells. **(A)** Scanning electron microscopy of a sagittal section of a 23HH chicken forelimb showing the undifferentiated zone. Yellow and orange squares-marked regions represents the image showed in B and C respectively. **(B)** Magnification of yellow boxed area. The undifferentiated zone, in purple, is under the dorsal ectoderm (yellow) influence. The region where mesenchymal cells are committed to chondrogenic/tenogenic lineage is showed in orange. **(C)** Magnification of orange boxed area. The undifferentiated zone (purple) underlies the Apical Ectodermal Ridge (AER) and dorsal and ventral ectoderm (yellow). Notice that the marginal vein, indicated with an asterisk, delimits the undifferentiated zone and the committed zone (orange).

AER removal at progressive stages of limb development caused a progressive loss of distal elements of the limb (Saunders, 1948; Summerbell, 1974). In the chicken embryo, the AER becomes distinguishable at Hamburger–Hamilton stage 18 (Hamburger and Hamilton, 1992) (18HH) during development, when distal ectodermal cells acquire columnar shapes distinguishing them from the cuboidal ectoderm. At stage 20HH, the AER becomes a pseudostratified epithelium that is maintained until the 23–24HH stage (Todt and Fallon, 1984). On the other hand, AER initiation in the mouse forelimb starts at approximately embryonic day 9 (E9). It is known that Wnt3a/β-catenin signalling in chicken and Wnt3/β-catenin signalling in mouse is required to maintain the AER after its initiation and maturation (Barrow et al., 2003; Kengaku et al., 1998). In 2002, it was demonstrated that both fibroblast growth factor (FGF) 8 and 4 signalling from the AER play instructive roles and are essential for the survival of mesodermal cells, maintaining the viability of the underlying AER mesodermal cells to ensure sufficient availability of progenitors for normal formation of the skeletal elements (Sun et al., 2002). However, *Fgf9* and *Fgf17* are also expressed in the AER and contribute to limb development. Interestingly, an *Fgf8* knockout (KO) mutant has been found to show more severe limb defects than individual and compound *Fgf4/Fgf9/Fgf17* mutants. This result suggests that the presence of *Fgf8* is sufficient for normal limb development. An explanation for the diverse range of phenotypes obtained with various *Fgf* KOs is that the AER-FGFs (*Fgf8, Fgf4, Fgf9*, and *Fgf17*) are functionally equivalent but differ in the extent to which they contribute to the AER-FGF signal; thus, changes in the different components produce different changes in the total AER-FGF signal (Mariani et al., 2008). On the other hand, Lu et al. (2008) through genetic ablation of FGFR2 function found evidence suggesting that FGFR2 promotes the survival of AER cells and interacts with Wnt/β-catenin signalling during AER maintenance. Additionally, using *Hoxa13* as an early autopod progenitor cell marker, the authors found that premature AER loss in mutant

limb buds may delay the generation of autopod progenitors, in turn preventing the progenitors from reaching the threshold number required to form a normal (Lu et al., 2008). However, mesenchymal expression of *Fgfr2* (Coumoul et al., 2005) and *Fgfr1* (Li et al., 2005; Verheyden et al., 2005) is also necessary for skeletal progenitor cells to respond to AER signals.

Molecular Control in the Maintenance of an Undifferentiated State of Mesodermal Cells

In the first stages, all mesenchyme in the limb bud is composed of undifferentiated cells. While the limb grows, an undifferentiated distal zone is always maintained (**Figure 3**). The region from which digital rays later extend and where joints are sequentially formed also features an undifferentiated zone known as the digital crescent (DC) (Montero et al., 2008) or phalanx-forming region (PFR) (Suzuki et al., 2008), which is positive for pSMAD1/5/8 and pSMAD2. The maintenance of mesenchymal stem/progenitor cells during development in an undifferentiated, proliferative, and viable state is highly regulated by ectodermal signals.

It is known that combinations of Wnt and FGF signals from the limb ectoderm, specifically FGF8 and WNT3A signals, have different effects on the mesenchymal stem/progenitor cells of the undifferentiated region than either signal alone. Mesenchymal cells are maintained in a multipotent and proliferative state by the synergistic action of both growth factors, but they retain the ability to undergo chondrogenesis. In the absence of both signals, mesenchymal stem/progenitor cells exit the cell cycle and begin chondrogenic differentiation. Continuous exposure to Wnt induces *Nbl1* expression, which maintains proliferation and re-specifies the cells towards soft connective tissue lineages (ten Berge et al., 2008). Additionally, *N-Myc* plays a significant role in the expansion of undifferentiated mesenchymal cells, which gives rise to chondrocyte and osteoblast progenitors, while *c-Myc* participates in the proliferative expansion of osteoblast progenitors (Zhou et al., 2011). Furthermore, FGF and WNT family members secreted from the ectoderm promote *N-Myc* expression in the mouse limb and consequent proliferation of the underlying mesenchyme lineages (ten Berge et al., 2008). Thus, newly generated undifferentiated cells cannot reach the most central part of the limb bud and are maintained in their undifferentiated state. When undifferentiated mesenchymal cells are far enough from the AER signals, they exit the cell cycle and commit to becoming *Sox9*-positive chondro-osteoprogenitors (Akiyama et al., 2005). In addition, in the chick embryo has been demonstrated that the dorsal ectoderm releases a probable chondrogenesis inhibitory factor (WNT6), which restricts the formation of cartilage towards the central region of the limb. If the dorsal limb ectoderm is removed, chondrogenesis differentiation extends to the mesenchyme under the dorsal ectoderm, probably at the expense of tendon formation (Geetha-Loganathan et al., 2010).

Thus, in the appendicular skeleton, the commitment of mesenchymal cells to the chondrogenic and tenogenic lineages occurs when cells in the undifferentiated zone reach the differentiation front and leave the influence of the AER and ectodermal signals (Tabin and Wolpert, 2007). The details of the molecular basis of the maintenance and cell fate regulation of mesenchymal stem/progenitor cells in the limb have been elucidated. Kumar and Lassar demonstrated in mouse embryonic limb that Wnt signals irreversibly block the induction of chondrogenesis through CpG methylation and H3K27me3 modification in the *Sox9* promoter. In contrast, the FGF signal blocks the recruitment of the *de novo* methyltransferase DNMT3A to the *Sox9* promoter, maintaining competence for eventual *Sox9* gene expression by blocking Wnt signals from inducing stable CpG methylation at the *Sox9* locus. Thus, FGF signalling controls whether *Sox9* expression is irreversibly or reversibly silenced by Wnt signals in limb bud undifferentiated mesenchymal cells (Kumar and Lassar, 2014). Furthermore, the arginine methyltransferase PMRT5 present in the DC/PFR is considered to promote the survival and proliferation of mesenchymal stem/progenitor cells, thus maintaining the undifferentiated pool of progenitor cells and limiting cell differentiation in the distal tips of digital rays (Norrie et al., 2016). Moreover, the Notch pathway is active in mesenchymal stem/progenitor cells and inhibits their differentiation *via* upregulation of the transcription factor *Twist1* (Tian et al., 2015).

On the other hand, in chicks and mice, hypoxic environments and hypoxia-inducible factor 1 alpha (*Hif1a*) upregulation participate in the fate determination that initiates the Sox9-positive chondrocyte lineage and represses the *Runx2*-positive osteoblast lineage and tenogenic differentiation (Salim et al., 2004; Amarilio et al., 2007; Lorda-Diez et al., 2013). Furthermore, the initial low-level *c-Myc* expression in newly *Sox9*-positive cells has been proposed to promote the proliferation of these cells but maintain their multipotent progenitor character (Zhou et al., 2011). Ultimately, limb mesenchymal cells are definitively committed to cartilage or soft connective tissue lineages at the chick embryo 24HH stage (Searls and Janners, 1969).

Coordinated Regionalization and Differentiation of Limb Mesodermal Cells

It is known that limb patterning and cell differentiation occur in an orchestrated manner (Dudley et al., 2002; Sun et al., 2002). A detailed fate map for the limb bud of the chicken at stage 19HH demonstrates that only the distal undifferentiated mesenchyme is maintained in a non-regionalized condition and that each limb structure is likely to be regionalized in the proximal-to-distal direction (Sato et al., 2007). Additionally, using KO mutant mice lacking different combinations of *Fgf4*, *Fgf8*, *Fgf9*, and *Fgf17*, it was demonstrated that AER-FGFs function as initial distal signals during the early stages of limb development, playing instructive roles in specifying distal domains for PD patterning and repressing *Meis1* expression (Mariani et al., 2008). Thus, the co-existence of two parallel mechanisms during limb PD patterning has been demonstrated: one based on MEIS, retinoic acid (RA), and FGF signalling and a second based on chromatin regulation that delays *Hoxa13* activation to provide the time needed for the accumulation of sufficient precursors to ensure proper limb formation (Rosello-Diez et al., 2014). Hence, cells in the undifferentiated zone are constantly under the

influence of FGF signalling. Once undifferentiated cells cross the differentiation front, aside from committing to a chondrogenic lineage, they become sequentially specified as stylopod, zeugopod, and autopod cells due to increased RA signalling (Rosello-Diez et al., 2014). On that basis, undifferentiated cells interpret signals that lead them to differentiate while they acquire their identities from a specific segment of the limb.

Are Early Limb Mesodermal Cells Stem Cells?

The existence of a distal undifferentiated zone in the limb as a source of stem/progenitor cells has been well established. It has been clearly demonstrated *in vitro* and *in vivo* that mesenchymal cells at early stages of development are capable of differentiating into different limb lineages (Ahrens et al., 1977; Akiyama et al., 2005; Marin-Llera and Chimal-Monroy, 2018), Even extra-limb lineages belonging to ectodermal and endodermal germ layers have been obtained *in vitro* (Jiao et al., 2012). However, it remains unclear whether the mesenchyme is composed of a collection of distinct types of stem/progenitor cells with restricted differentiation potential or of a group of equipotential cells capable of giving rise to the same cell lineages. Using a library of retroviral vectors to trace the cellular fate and location of the progeny of individually marked single cells during limb development, Pearse II and collaborators demonstrated that subsets of multipotent cells that can give rise to between two and five different lineages (muscle connective tissue, tendon, dermis, perichondrium, and cartilage) are present in the limb mesenchyme. Their results showed that at the 19HH stage, cells are not specified to any lineage, and at least a subset of progenitor cells can generate five mature tissue types. Additionally, a progressive restriction of multipotency was observed through development. In that work, the authors concluded that cells in the early limb bud are not committed to individual fates, nor do they appear to be restricted to any particular PD segment along the length of the limb bud (Pearse et al., 2007). Although the multipotency of undifferentiated limb cells is clear, the signature of each type of subpopulation has not been determined, nor has it been determined whether the cells are multipotent stem cells. Detection of specific signatures of cell surface molecules, such as mesenchymal stromal cell-associated markers (MSCams), can enable the identification and study of distinct limb subpopulations at different developmental stages. In this sense, it has been demonstrated that few limb cells express different MSCams (Marin-Llera and Chimal-Monroy, 2018). Gretel Nusspaumer et al. traced the ontogeny and relationships of distinct mesenchymal stromal cell populations previously reported in the mouse limb and found that the PDGFRa^{pos}/CD51 cell population is the largest population of progenitors, containing mouse skeletal stem cell (mSSC) (CD200^{pos}, CD51^{pos}, CD90^{neg}, CD105^{neg}, and 6C3^{neg}) and PαS subpopulations. Additionally, in the PαS population (PDGFRa^{pos}, Sca1^{pos}, CD45^{neg}, and TER119^{neg}), four subpopulations can be distinguished based on their potential to differentiate into cartilage (Nusspaumer et al., 2017). The use of mesenchymal stromal cell (MSC) surface markers enables the identification, isolation, and characterization of different types of cells in the limb; however, the expression of these molecules alone does not indicate stemness. In particular, when expression has been measured after culture, it has been demonstrated that cells from a variety of tissues acquire surface signatures or modify their surface signatures *in vitro* (Qian et al., 2012; Lei et al., 2013; Guimaraes-Camboa et al., 2017; Marin-Llera and Chimal-Monroy, 2018).

Despite the existing knowledge of the origins, maintenance, and cell fate regulation of stem/progenitor cells in the limb, their self-renewal capacity and the timing of cell fate decisions by undifferentiated cells in the context of the developing limb is still unclear.

Cartilage Commitment and Differentiation

Some of the important characteristics of skeletal development are that all skeletal elements are confined to specific locations with specific shapes and are present in defined numbers. The particular organization of the skeleton depends on its particular locomotor function for each species. However, the initial steps that drive chondrogenesis are conserved in many species of vertebrates.

Stem/precursor cells are committed to the chondrocyte lineage in the core of the limb. Ectoderm tissue releases signals that block cartilage differentiation in the margin of the early limb bud, restricting cartilage formation to the core of the limb mesenchyme (ten Berge et al., 2008; Cooper et al., 2011). Once chondrogenic signals are received and/or inhibitory signals are removed, mesenchymal cells begin to aggregate and differentiate into early chondrocytes, building the cartilage anlagen, proliferating and producing characteristic extracellular matrix (ECM; **Figure 4A**; Hall and Miyake, 1995). During this process, Wnt and FGF signals from the ectoderm control the expression of Sox9 through epigenetic modification (Kumar and Lassar, 2014).

First Steps of Chondrocyte and Osteoblast Commitment

As mentioned above, AER-FGF signals determine whether mesenchymal cells are responsive to chondrogenic signals by regulating epigenetic modifications in the *Sox9* promoter (Kumar and Lassar, 2014). However, once *Sox9* is expressed, there is evidence that functional and physical interactions occur between Sox9 and β-catenin during cartilage development. If an interaction occurs between the Sox9 C-terminus and β-catenin, the transcriptional activity of the β-catenin/Tcf-Lef complex is inhibited (Akiyama et al., 2004; Topol et al., 2009). Alternatively, translocation of the Sox9 and β-catenin complex into the nucleus is necessary and sufficient to induce β-catenin degradation (Topol et al., 2009).

The cellular recruitment of mesenchymal cells towards the chondrogenic lineage occurs in the undifferentiated zone beneath the AER (**Figure 4B**). The formation of precartilage condensations in the core of the limb depends on cell–cell and cell–ECM interactions. N-Cadherin and N-CAM are two cell adhesion molecules expressed during precartilage condensation formation. Blocking the function of these molecules prevents cell aggregation and subsequent cartilage differentiation in micromass cultures prepared from chicken limb precartilage

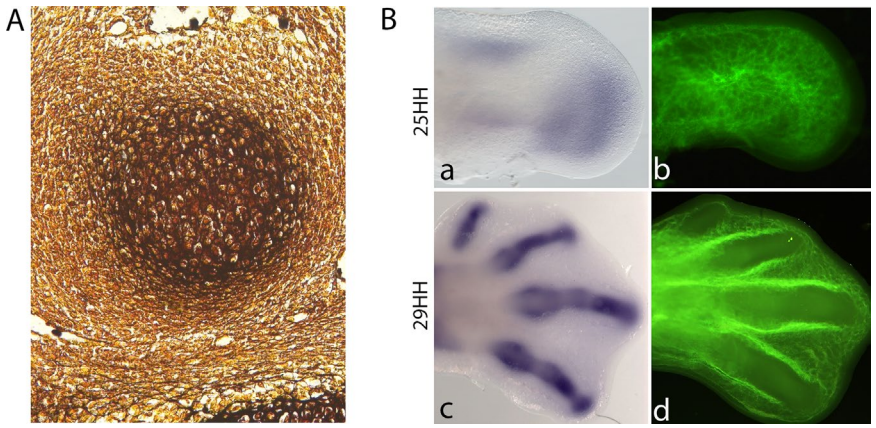

FIGURE 4 | Chondrogenesis starts when the limb mesenchymal cells leave the influence of the AER-FGFs signalling. **(A)**. Histological section of a silver-stained 28-HH chicken digit showing a cartilage condensation. **(B)**. In situ hybridization for Sox9 (a,c) and blood vessel cell-tracker staining (b,d) in chicken hindlimbs at 25HH and 29HH stages. Noticed that cartilage differentiation is accompanied by vascular regression. Avascular zones can be observed at the later stage in the digits.

cells (Chuong et al., 1993; Widelitz et al., 1993; Oberlender and Tuan, 1994a, Oberlender and Tuan, 1994b). However, no limb phenotype has been reported in N-CAM knockout mice (Cremer et al., 1994). Regarding cell–ECM interactions, fibronectin and tenascin are ECM proteins expressed during the formation of precartilage condensations (Hall and Miyake, 1995). Similarly, blocking the function of fibronectin with a specific antibody for exon IIIA of fibronectin (Gehris et al., 1997) or with an antibody directed against the 29 kDa amino-terminal heparin-binding domain of fibronectin and the oligopeptide gly-arg-gly impedes the formation of pre-cartilage condensations (Frenz et al., 1989). Interestingly, blocking the function of β1 integrins inhibits cell aggregation and thus impedes cartilage differentiation, at least *in vitro* (Shakibaei, 1998).

However, the condensing mesenchyme in the limb bud shows a bi-potential character, as it is able to differentiate into both chondrogenic and osteogenic lineages. As mentioned above, the chondrogenic lineage is characterized by the expression of Sox9, whereas the osteogenic lineage is characterized by the expression of Runx2. The activation of Wnt/β-catenin signalling is involved in controlling the decision to become osteoblasts or chondrocytes in non-committed cells in the undifferentiated region (Hill et al., 2005). Conditional deletion of β-catenin in the limb mesenchyme results in ectopic cartilage differentiation at the expense of osteoblast differentiation (Akiyama et al., 2005; Day et al., 2005; Hill et al., 2005). Similarly, inhibition of Runx2 results in the inhibition of osteogenesis but not chondrogenesis. Thus, during direct ossification processes, such as intramembranous ossification, Wnt/β-catenin signalling is active; *Sox9* expression is downregulated; and *Runx2* expression is activated. In contrast to Runx2, deletion of the *Sox9* gene before chondrocyte commitment results in the complete inhibition of skeletal element formation (Akiyama et al., 2002). Thus, during the formation of long skeletal bones, Wnt/β-catenin signalling is inhibited such that *Sox9* expression is upregulated. In this context, condensing pre-cartilage cells exit the cell cycle and express Hif1a, and a hypoxic environment is created. At the same time, hypoxia inhibits osteogenic and tenogenic differentiation, but the chondrogenic lineage avoids this inhibition because of the upregulation of *Sox9* by Hif1α (Salim et al., 2004).

Chondrogenesis During Digit Formation

During digit formation in chicken embryos, chondrogenic differentiation begins when the TGFβ/activin signalling pathway directs the pool of mesenchymal precursor cells in the DC/PFR region to a cartilage fate by inducing *Sox9* and *Bmpr1b* expression (Montero et al., 2008). *Activin/Tgfβ* expression is restricted to the earliest chondrogenic condensations of the growing tips of digits until the last phalanx is formed but is not observed in cellular condensations of proximal skeletal elements. In contrast, *Activin* βB transcripts are observed only in the initial stages of digit formation. The expression of activin receptor II, *ActRIIb*, is intense in the precursor cells surrounding the distal tip of the digit. In addition, *Tgfβ2* expression is observed at stage 29 during the formation of growing digits (Merino et al., 1998). In contrast to chick embryos, single mouse mutants for *Tgfβ1, Tgfβ2,* and *Tgfβ3* do not exhibit digit abnormalities that suggest any participation of these genes in digit development. Nevertheless, severe syndactyly is observed in double knockout animals for *Tgfβ2* and *Tgfβ3* (Shull et al., 1992; Kaartinen et al., 1995; Dunker et al., 2002). Moreover, Activin- or Follistatin-deficient mice show no digit phenotype (Matzuk et al., 1995a; Matzuk et al., 1995b; Matzuk et al., 1995c).

During digit development, it has been shown that non-canonical Wnt signalling through Wnt5a occurs at higher levels than Wnt/β-catenin signalling in the distal tips of mouse limb buds. In *Wnt5a*$^{-/-}$ animals, chondrocyte differentiation is constrained because the levels of β-catenin are elevated, inhibiting *Sox9* expression; however, inhibition of β-catenin partially rescues chondrocyte differentiation (Topol et al., 2003). These results suggest that Wnt5a signalling promotes cartilage differentiation by inhibiting the antagonistic effect

of Wnt/β-catenin signalling (Topol et al., 2003). On the other hand, the antichondrogenic *Sox* trio (Sox4, Sox11, and Sox12) cooperates to stabilize the β-catenin protein and replace Sox9 in the Sox9-β-catenin complex (Bhattaram et al., 2014). The antichondrogenic Sox trio also inhibits the activity of GSK3β, preventing the formation of degradation complexes. Interestingly, Wnt antagonists such as *Frzb1* are expressed during chondrogenesis in skeletal elements (Wada et al., 1999), and misexpression of Frzb1 in the developing limbs of chick embryos maintains chondrocytes at the initial stages of differentiation; they do not begin chondrocyte maturation. Thus, the skeletal elements are short with no endochondral ossification or joint fusion (Enomoto-Iwamoto et al., 2002), suggesting that these antagonists protect cartilage from the inhibitory effects of Wnt/β-catenin signalling on *Sox9* expression. However, in Frzb-knockout mice, no defects in early skeletogenesis are observed (Lories et al., 2007).

It has been shown that TGFβ and activin can induce extra digit formation during development (Ganan et al., 1996). The experimental model of ectopic digit formation induced by activin/TGFβ in interdigital tissue in chicken embryos is an excellent chondrogenesis model with which to elucidate the early molecular cascade that leads to cartilage differentiation *in vivo* (Chimal-Monroy et al., 2003). This model shows that the expression of *Sox9* is induced immediately after TGFβ treatment and that the expression of *Smad6* and *Bambi* [bone morphogenetic protein (BMP) antagonists] is also inhibited, favouring the receptivity of precartilage cells to BMP/SMAD1/5/8 signalling. BMP signalling plays an important role in prechondrogenic condensation and chondrogenic differentiation during digit formation (Pizette and Niswander, 2000; Barna and Niswander, 2007). Although, it is thought that the onset of chondrogenesis depends on BMP signalling, double mutant mice for *Bmp2* and *Bmp4* display skeletal malformations in the stylopod and the zeugopod, and in this region, one skeletal element is missing, whereas in the autopod, the size of the skeletal elements is reduced, and the two posterior-most digits are absent. Thus, although some skeletal elements are lost in the absence of Bmp2 and Bmp4, chondrogenic differentiation continues normally in the other condensations that are formed. The skeletal defects present in double mutant mice for *Bmp2* and *Bmp7* are less severe than those observed in double mutant mice for *Bmp2* and *Bmp4*. However, skeletal differentiation is normal (Bandyopadhyay et al., 2006).

Treatment of digit primordia in the DC/PFR region with different BMP proteins promotes expansion of cartilage at the digit tips in the chick limb (Macias et al., 1997; Merino et al., 1999a). In contrast, treatment with BMP antagonists such as Noggin and Gremlin results in digit truncation (Merino et al., 1999b). These results suggest that BMP plays an important role in the control of digit formation at two levels, first promoting cartilage condensation and second promoting chondrogenic differentiation. Nevertheless in the experimental model of ectopic digit formation, TGFβ but not BMP treatment induces the molecular cascade of chondrogenesis, and *Bmpr1b* is induced after 6 h of TGFβ treatment. BMP treatment induces cell death in the interdigital tissue, while treatment with BMP antagonists inhibits cell death (Chimal-Monroy et al., 2003). However, if BMP protein is implanted after 6 h of TGFβ treatment, coinciding with the time of *Bmpr1b* expression, BMP enhances chondrogenesis, promoting cartilage condensation and *Sox9* expression (Chimal-Monroy et al., 2003). Thus, it is possible that during digit development, BMP may act as a permissive signal. In addition, it is known that BMP signalling promotes the compaction of prechondrogenic cells during cell condensation (Barna and Niswander, 2007). This compaction promotes cohesive cell behaviour in mesenchymal cells to delineate the boundaries and sizes of cartilage elements. During the formation of digit primordia, the expression of the Iroquois (*Irx*) genes *Irx1* and *Irx2* is detectable at the boundaries of skeletal condensations and non-cartilage tissue (Diaz-Hernandez et al., 2013). It has been suggested that this expression may reflect the range of diffusion of TGFβ at growing digits to promote chondrogenesis, and IRX proteins might allow the formation of the prospective perichondrium by repressing the expression of genes involved in the cell death process (Diaz-Hernandez et al., 2013). A similar function has been suggested for *SoxC* genes, which may specify the fates of perichondrium and joint cells through a β-catenin-independent mechanism (Bhattaram et al., 2014).

The Earliest Molecular Steps of Chondrogenic Specification

Recently, on the basis of the molecular cascade for chondrogenesis induced in the experimental model of ectopic digit formation, it was established that three consecutive periods precede cartilage formation (Lorda-Diez et al., 2011). The first corresponds to the first three hours and is a pre-condensation stage characterized by the upregulated expression of *Sox9, Scleraxis, Ephrin A5,* and *Bigh3*, which are important regulators of cell adhesion. The functions of these molecules, together with cell morphology controlled by the actin cytoskeleton, are very important for chondrogenesis, as demonstrated by the fact that co-treatment with cytochalasin D inhibits chondrogenesis induced by TGFβ. Interestingly, new early molecular markers, the matricellular proteins CCN1 and CCN2, have been discovered. They are expressed very early at the same time as *Sox9*, but they are unable to induce chondrogenesis; instead, they induce the expression of *Tenascin-C*. On this basis, the commitment of stem/precursor cells to the chondrocyte lineage occurs in this period. Thus we suggest that it should be named the commitment stage, as the master gene *Sox9* is expressed as early as 30 min, triggering gene regulatory networks that control the correct positioning of all skeletal elements in time and space. The second period is named the condensation stage, which corresponds to the period in which committed cells initiate the process of mesenchymal condensation. In this phase, the expression of cell–cell adhesion (*N-Cam*) and cell–ECM (*Tenascin C, alpha 5 integrin*) genes is increased, as is that of *Activin* βA and other transcription factors. The third period, named the precartilage period, is characterized by the upregulation of genes expressed in the previous period in addition to other genes, such as ECM genes and transcription factors (**Figure 4A**). In particular, the expression of *Sox9* is

several times higher in this stage than in the previous periods. It is possible that this increase allows the commencement of molecular network control to regulate the expression of genes involved in the establishment of the cartilage phenotype (Lorda-Diez et al., 2011).

Altogether, *Sox9* is very important for the control of cell fate decisions, i.e., the commitment of mesenchymal stem/precursor cells to the cartilage lineage. As mentioned above, cartilage differentiation continues after *Sox9* is expressed in mesenchymal cells (**Figure 4B**), promoting cell aggregation and the expression of cartilage-specific proteins such as type II collagen, aggrecan, and sulphated proteoglycans. Additionally, in the absence of *Sox9* or after deletion of its expression in prechondrogenic mesenchyme, cartilage differentiation is inhibited, preventing the formation of skeletal elements. Massive cell death rather than cartilage differentiation is observed in *Sox9* mutant mice, suggesting that undifferentiated mesenchymal cells are committed to differentiation or to cell death depending on their positions inside the limb (Akiyama et al., 2002). Misexpression of *Sox9* induces extra digit formation in developing limb buds and attenuates hypodactyly caused by a *Hoxa13* mutation (Akiyama et al., 2007). *Sox9* expression in mesenchymal progenitors precedes chondrogenesis and increases at the onset of *Sox5* and *Sox6* expression in early chondrocytes (Lefebvre, 2019).

In summary, the programme of cartilage differentiation starts once *Sox9* is expressed and then cell aggregations are established to preconfigure skeletal elements; these aggregations give rise to skeletal elements shaped by proliferating chondrocytes. The early chondrocytes proliferate and secrete ECM composed mainly of collagen type II, aggrecan, and link protein. During the early stages of precursor mesenchymal cell condensation, vascular regression occurs in the mesenchyme of developing limbs (**Figure 4B**; Hallmann et al., 1987). This vascular regression induces reductions in oxygen levels. Under such hypoxic microenvironments, *Hif1a* is expressed. *Hif1a* expression is considered an important factor promoting the expression of *Sox9* and other factors that enable chondrocytes to adapt to hypoxic conditions (Amarilio et al., 2007). Furthermore, expression of Sox9 in precartilage condensations also regulates angiogenic patterning (Eshkar-Oren et al., 2009). The expression of the chondrogenic SOX trio genes (*Sox9, Sox5,* and *Sox6*) controls cell cycle progression in the proliferating chondrocytes of the central regions of cartilage elements (Akiyama et al., 2002). Thus, the expression of *Sox9*, *Sox5,* and *Sox6* are implicated in the maintenance of the cartilage phenotype. In contrast, Dy et al. demonstrated that Sox*9* is also required to activate cartilage hypertrophy (Dy et al., 2012).

The orchestrated regulation of the molecular network that controls the differentiation of cartilage leads to the establishment of embryonic skeletal elements, subsequently initiating the maturation process leading to cartilage hypertrophy and thus to elongation of the skeletal elements.

Establishment of the Synovial Joints

Functional joints require the coordinated expression of many molecules that participate in the joint commitment and differentiation of cartilage, bone, meniscus, and other tissues. During normal limb development, the process of joint formation occurs concomitantly with the formation of the cartilage anlagen. Diarthrodial or synovial joints, such as the phalange, knee, and elbow joints, result from segmentation of continuous skeletal elements into individualized skeletal structures.

The first evidence of joint development is the formation of the interzone (IZ), a specific region of higher cell density that forms where chondrocytes flatten and become fibroblastic tissue (**Figure 5**). During the establishment of the IZ, differentiation of the articular cartilage occurs, and separation of the two skeletal elements later occurs by a process named cavitation, thereby forming the joint capsule. It has been demonstrated that the most important process for joint establishment is the formation of the IZ. Holder (1977) and later Rux et al. (2019) found that joint formation is inhibited after removal of the IZ, resulting in a lack of segmented skeletal elements.

The formation of the IZ begins at the site of the future joint, establishing the boundary between two prospective individual skeletal elements. The flattened, fibroblastic-like cells of the IZ evolve into three cellular layers: one central intermediate lamina with low cell density flanked by two areas of high cell density. The two areas of high density differentiate into articular cartilage covering the articulating surfaces at either end of the future joint. In the case of the future knee, the central layer gives rise to the internal elements of the joint: the synovial tissue, menisci, and joint ligaments. The joint capsule arises from the mesenchymal sheath surrounding the entire IZ (**Figure 5**; Rux et al., 2019). In addition, many studies have been demonstrated that movement and mechanical forces are important to joint development and its maintenance (Drachman and Sokoloff, 1966; Kahn et al., 2009; Felsenthal and Zelzer, 2017).

Molecular Scenario to Trigger Joint Development

Each mature skeletal element is organized with a diaphysis in the central portion and an epiphysis at each end. The epiphysis is the region resulting from joint formation. The newly formed individual embryonic skeletal element continues to grow; remodelling of the cartilaginous template into bone occurs in the diaphysis, in which proliferating chondrocytes become pre-hypertrophic and then hypertrophic, initiating the process of endochondral ossification.

Thus, a pivotal process in joint induction is the signalling of proliferating chondrocytes to begin the programme of joint formation while stopping the programme of cartilage differentiation, preventing endochondral ossification (Garciadiego-Cazares et al., 2004). At the molecular level, cells of joint IZs begin to express different members of the Wnt gene family, such as Wnt4, Wnt9a, and Wnt16 (Guo et al., 2004). Signalling by Wnt9a and Wnt16 involves the activation of β-catenin. *Wnt9a* and β-catenin misexpression trigger a molecular cascade of joint marker expression at chondrogenic sites but may not induce true ectopic joints (Hartmann and Tabin, 2001; Guo et al., 2004). In mice lacking β-catenin in the entire limb mesenchyme the onset of *Gdf5* expression and other joint markers is not affected. Yet, no true joints are formed as *type II collagen* expression is not down-regulated (Spater

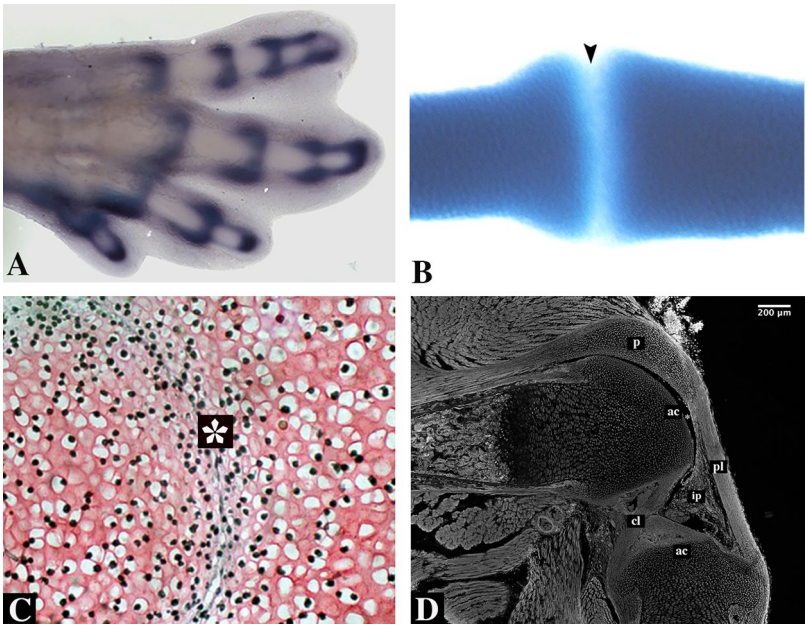

FIGURE 5 | Synovial joint formation. **(A)** 31HH chick hindlimb showing an in situ hybridization for Gdf5. **(B)** Alcian blue staining of a developing digit of a turtle embryo. Notice lack of staining in the interphalangeal zone (arrowhead). **(C)** An early developing joint stained with Safranin O of a E13.5 mouse embryo showing the interzone (asterisk). Subpopulations from this area give rise to the different tissues of the joint. **(D)** A sagittal section of a knee-joint of a newborn mouse stained with phalloidin showing the different tissues of the joint; articular cartilage (ac), cruciate ligaments (cl), cells that will give rise to the infrapatellar pad (ip), patella (p), and patellar ligament (pl). Joint cavity is indicated with an asterisk.

et al., 2006a). Similarly, joint fusion defects are observed upon deletion of β-catenin in *type II collagen* expressing cells (Guo et al., 2004). Furthermore, joint initiation is not affected by the single or combined loss of two of the Wnt ligands, Wnt9a and Wnt4, expressed in the joint interzone, yet, their loss leads to ectopic chondrocyte differentiation in joint structures (Spater et al., 2006b). Together, these observations suggest that Wnt/β-catenin signalling is not necessary for the initiation of the joints but for their subsequent maintenance. Kan and Tabin (2013) demonstrated that c-Jun is expressed in prospective joints in a similar manner as Wnt9a and Gdf5. Interestingly, the active form of c-Jun is observed during joint development, and its expression and activity coincide with Wnt9a expression in the IZ region. In addition, c-Jun binds directly to the Wnt9a promoter, activating its expression. In mice lacking c-Jun in the mesenchyme of the early limb bud, mainly interphalangeal joint initiation and successive programmes of joint differentiation are inhibited, although other joints are also affected. The formation of many synovial joints in the appendicular skeleton is inhibited because the joint IZ is disrupted; it has also been found that c-Jun is involved in postnatal joint maturation. Inhibition of the expression of Wnt9a/Wnt16 results in continuous skeletal elements that never form an IZ. However, it is important to establish that although Wnt/β-catenin signalling occurs very early in the joint IZ, it is not necessary for early joint formation; moreover, it is regulated downstream by c-Jun. Therefore, it is possible to speculate the existence of a process of de-differentiation of proliferating chondrocytes that, if controlled by Wnt/β-catenin, is subordinate to IZ specification. In addition, there is no evidence regarding whether these de-differentiated cells are joint stem cells. Although c-Jun induces commitment according to the molecular cascade of joint formation, it is possible that some proliferating chondrocytes are converted into joint stem cells through c-Jun activation (Kan and Tabin, 2013).

While Wnt9a commits the joint IZ, Sox9 expression continues in proliferating chondrocytes, but matrillin-1 expression is inhibited (Hyde et al., 2007). Therefore, it is possible that Wnt/β-catenin signalling, once activated by c-Jun, regulates the continued differentiation of chondrocytes to prevent endochondral ossification promoting joint formation. It was mentioned above that Sox9 physically interacts with β-catenin during cartilage development. Thus, it is probable that during joint IZ formation, the ratio of Sox9 to β-catenin may lead to the induction of the molecular cascade to prevent chondrocytes from undergoing endochondral ossification. However, the effects of c-Jun regarding joint formation are restricted to interphalangeal joint initiation. Similar results have been found in the mice lacking the TGF-β type II receptor gene (TGFβ; Spagnoli et al., 2007).

The molecular cascade of joint formation indicates that members of the BMP family, such as Gdf5, Gdf6, Bmp7, and Bmp2 (Francis-West et al., 1999a; Merino et al., 1999a; Francis-West et al., 1999b; Storm and Kingsley, 1999) are expressed in the IZ. Overexpression of BMPs expands the cartilage differentiation zone and inhibits joint formation (Duprez et al., 1996; Brunet et al., 1998; Storm and Kingsley, 1999), while, loss of noggin affects all joints (Brunet et al., 1998; Tylzanowski et al., 2006). In contrast, single mutations in humans or single or double KO of

Gdf5 or Gdf6 in mice results in joint fusion (Settle et al., 2003). However, despite the specific expression of BMP family members in developing joints, these molecules are not involved in the induction of the IZ or in the formation of ectopic joints. On this basis, many studies have focused on determining the progeny of IZ cells. Using the reporter mouse line Gdf5-Cre;R26R-LacZ to map the fates of these cells and other studies have determined that the joint IZ is formed by Sox9+ and Matrillin-1, Gdf5+ cells and that they are present in the articular cartilage, meniscus, and synovial lining. These results might suggest that Gdf5+-descendent cells from the IZ become different progenitor cells that will differentiate into the tissues that compose the mature joint, such as the articular cartilage, meniscus, synovial lining, and joint capsule (Koyama et al., 2008).

However, a recent study (Shwartz et al., 2016) suggests a distinct model in which there is a constant inflow of new GDF5+ cells that are recruited into different joint cell lineages, demonstrating that cells from various joint tissues originate from GDF5+ interzone cells. In the knee, GDF5+ cells are observed in the epiphysis, articular cartilage, meniscus, and intra-articular ligaments, and they are recruited at different developmental stages of joint formation. The elbow and metacarpophalangeal joints show a similar trend, but they differ in the time in which GDF5+ cells contribute to specific tissues. Thus, joint development involves constant recruitment of new Gdf5-positive cells derived from Sox9+ chondroprogenitors (Sox9+/GDF5-), and they must presumably express *Sox5* and *Sox6*, since the deletion of both genes in chondrocytes results in a joint morphogenesis block, but if *Sox5* and *Sox6* inactivation occurs in GDF5+ cells, it results in milder joint defects and normal growth plates (Dy et al., 2010). In addition, it has been found that Tgfbr2+ cells are present within specific niches throughout embryonic life to early adulthood. They express markers of joint progenitors such as Gfg5 and Noggin (Li et al., 2013). However, it is unknown if they represent cells that originate from or give rise to Sox9+ Gdf5- chondroprogenitors.

On the other hand, it has been proposed in a previous study that whether proliferating chondrocytes enter the joint formation, or the endochondral ossification programme depends on the presence or absence of α5β1 integrin (Garciadiego-Cazares et al., 2004). In that work, it was shown that cell–ECM interactions are important during joint formation and cartilage differentiation. The study demonstrated that inhibition of α5β1 integrin signalling was necessary for joint formation. Inhibition of the function of α5 or β1 integrin with specific neutralizing antibodies or arginine-glycine-aspartic acid (RGD)-blocking peptides in forelimb organ cultures resulted in the formation of an ectopic joint. This was a consequence of inhibition of endochondral ossification. During the formation of ectopic joints, the molecular cascade of joint formation is triggered by the disruption of proliferating chondrocyte–ECM interactions mediated by α5β1 integrin. Wnt9a, Gdf5, chordin, autotaxin, type I collagen, and CD44 are expressed in ectopic joints, whereas Indian hedgehog (IHH) and type II collagen are downregulated. As mentioned above, BMP signalling potentiates chondrogenesis and inhibits joint formation, but after inhibition of α5β1 integrin, BMP signalling improves joint formation (Garciadiego-Cazares et al., 2004). These data strongly suggest that once the molecular cascade of joint formation is initiated, BMP signalling contributes to joint formation. As mentioned by Attila Aszodi and his group (Docheva et al., 2014), "although this hypothesis is clearly attractive, genetic experiments with conditional knockout mice did not support a mechanistic role α5β1-mediated matrix attachments in joint morphogenesis". However, the β1 integrin allele was floxed under the Col2a1-cre or Prx1-cre promoter in transgenic mice. Thus, an inducible β1 integrin or α5 integrin allele under the Col2a1-cre promoter may give a better response.

In conclusion, the formation of the IZ is an excellent model with which to study the mechanisms that control the appearance of joint stem/progenitor cells during development. Until now, it has remained unknown if the IZ appears and then the joint stem/progenitor cells migrate or dedifferentiate from other sources or if the stem/progenitor joint cells determine IZ formation. It is possible that new microenvironments for IZ formation are created during the establishment of the joint IZ within a source/reservoir of joint stem/progenitor cells that will ultimately form the distinct tissues of mature joints, such as the articular cartilage, meniscus, synovial lining, and joint capsule.

Stem Cell Renewal and Cell Differentiation During Endochondral Ossification

Endochondral ossification programme initiates in the centres of the skeletal elements. This process is characterized by the replacement of cartilaginous templates with bone. In the peripheries of the skeletal elements, flattened cells differentiate into perichondrial cells. Proliferative chondrocytes differentiate into permanent articular cartilage at the end of the skeletal elements giving rise to zone I chondrocytes that are round or slow-proliferative. In the centres of long bones are the proliferating chondrocytes or Zone II chondrocytes, which exhibit flat morphology and are organized in parallel columns. At the centres of skeletal elements, proliferating chondrocytes undergo differentiation to form first prehypertrophic and then hypertrophic chondrocytes. Surrounding the newly formed skeletal elements, there is a thin layer of mesenchymal cells that will form the perichondrium; when ossification begins, this layer will form the periosteum (**Figure 6**). During this process, environmental conditions change to support vascular invasion, and periosteal cells invade the zone of hypertrophic cells to begin bone formation (Kozhemyakina et al., 2015). The same process of endochondral ossification is recapitulated during the formation of growth plates in infants. These structures are formed through secondary ossification, so they are localized between the primary ossification centre and the newly formed ossification centre (**Figure 6**). Notably, the proliferative activity of resting chondrocytes in the growth plates of bones during embryonic and foetal development is higher than that during puberty. Then, proliferating chondrocytes become prehypertrophic and undergo hypertrophy and mineralization. This coordinated process enables longitudinal growth in both embryonic and postnatal skeletal elements (Karsenty and Wagner, 2002; Kozhemyakina et al., 2015).

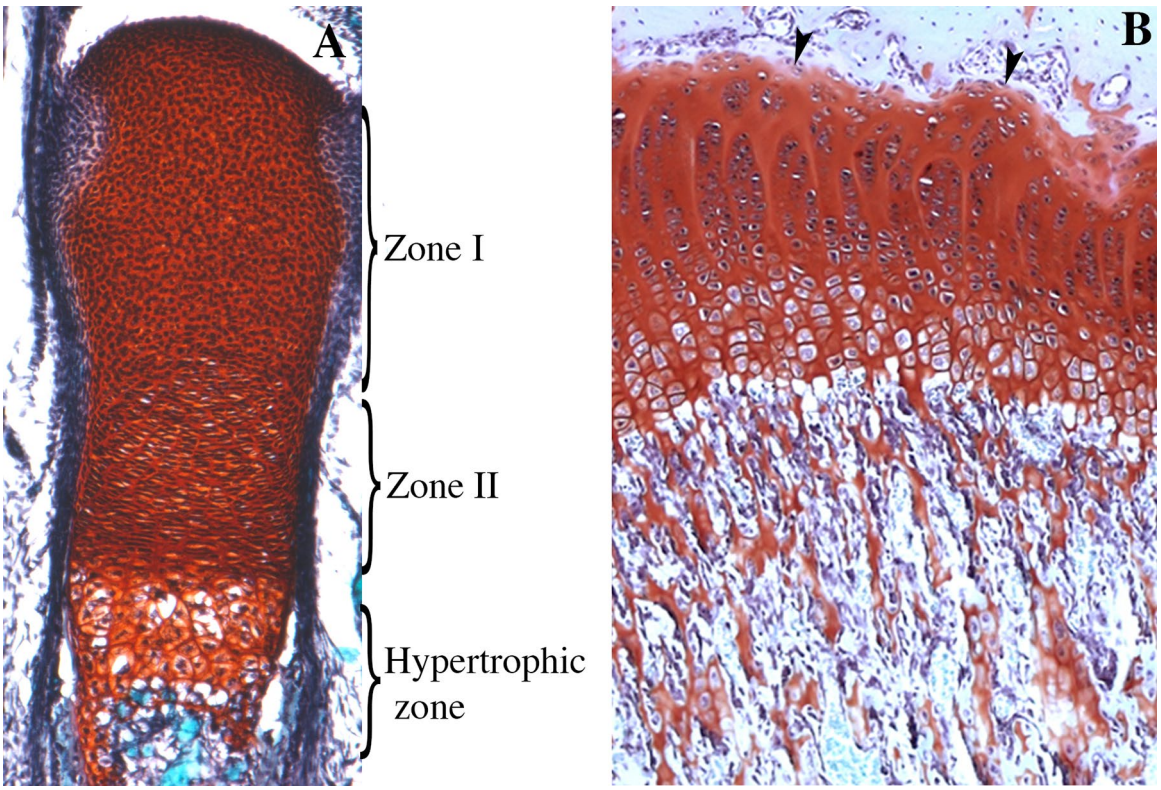

FIGURE 6 | Skeletal stem cells during endochondral ossification. **(A)** Longitudinal section of an E18.5 mouse tibia stained with Safranin O. Zone I, zone II, and the zone of hypertrophic chondrocytes are represented. Zone I corresponds to the entire epiphysis and contains differentiated chondrocytes that in this stage are all proliferative and originates the articular cartilage. Zone II corresponds to cells, originated from zone I, organized in parallel columns. **(B)** Growth plate of 6-weeks-old rat. In the juvenile stage, chondrocytes from the resting zone (arrowheads) behave as stem cells giving rise to different clones disposed in a column, that contributes to the longitudinal growth of the skeletal elements. In foetal, neonatal, and juvenile stages columns extend up to the primary spongiosa (ps).

Early Endochondral Ossification: From Proliferative to Hypertrophic Chondrocytes

A pivotal point of control for the growth of skeletal elements is the transition of resting chondrocytes into proliferating chondrocytes followed by the differentiation of these cells into hypertrophic chondrocytes. The mechanism by which signalling molecules control cell proliferation and differentiation involves key intracellular regulatory factors, including cell cycle regulators and transcription factors. Proliferating chondrocytes exit the cell cycle through the activity of the cyclin-dependent kinase (CDK) inhibitors p21, $p57^{Kip2}$, p107, and p130 (Rossi et al., 2002; MacLean et al., 2004; Goldring and Marcu, 2009). Similar to the case during the onset of cartilage differentiation, the proliferating chondrocytes still express Sox9, which together with Sox-5 and Sox-6 maintain the cartilage in a proliferative stage (Ikeda et al., 2004; Kim et al., 2011). This transcription factor regulates the expression of ECM cartilage genes such as *collagens (types 2, 9,* and *11*) and proteoglycans (*aggrecan*) (Bi et al., 1999). Hypertrophic differentiation depends on *Runx*-2, which directs *collagen 10a1* (*Col10a1*) and matrix metalloproteinase 13 (*Mmp13*) expression (Takeda et al., 2001). It is well known that during this process, Runx-2 inhibits Sox-9 function, and *Col2a1* and *aggrecan* are consequently downregulated (Zhou et al., 2006). In contrast, conditional *Sox9* or Sox trio (*Sox9, Sox5*, and *Sox6*) overexpression delays or inhibits hypertrophic differentiation (Ikeda et al., 2004; Kim et al., 2011), respectively.

With regard to the transition from chondrocyte proliferation to differentiation, two signalling molecules, parathyroid-related protein (PthrP) and IHH, interact in a negative feedback loop to regulate the onset of hypertrophic differentiation (Vortkamp et al., 1996). *PthrP* is expressed in the perichondrium, while its receptor, *Ppr*, is expressed in proliferative chondrocytes (Amizuka et al., 1994; Karaplis et al., 1994) and promotes chondrocyte proliferation (Lanske et al., 1996). IHH is expressed in prehypertrophic chondrocytes and binds to its receptor Patched, which is also expressed in chondrocytes and perichondral cells (Tavella et al., 2004). Activation of the IHH pathway induces *PthrP* expression in perichondral cells, promoting the proliferation of chondrocytes (Vortkamp et al., 1996). Cartilage hypertrophic differentiation begins when the proliferating chondrocytes are sufficiently far from the source of PTHrP. The cells stop proliferating and, then the site at which chondrocytes differentiate into hypertrophic cartilage is established. Then, IHH is expressed together with type X collagen, which is characteristic of hypertrophic cartilage. However, IHH is only expressed in early hypertrophic chondrocytes. In this process, other signalling molecules are involved, such as Wnt, FGF, and BMP (Kozhemyakina et al., 2015).

The Growth Plate Formation

As the first ossification centre enlarges, the secondary ossification centre is established in the epiphyses (proximal or distal) of long bones. Here, chondrocytes stop proliferation and undergo hypertrophy, attracting blood vessel invasion. Between primary and secondary ossification centres, growth chondrocytes continue to proliferate, forming a distinct plate of cells known as the growth plate. At the top of the growth plate, the proliferation rate of round chondrocytes slows, and these cells are then referred to as resting chondrocytes (Kronenberg, 2003). In humans, secondary ossification centres form during late embryogenesis, while in the mouse they are formed during early postnatal development P7–P15. In these structures, cell differentiation is active and is responsible for the growth of long bones, while in the articular cartilage, the rates of proliferation and differentiation are severely reduced (Kozhemyakina et al., 2015).

During foetal and neonatal endochondral ossification, resting chondrocytes with rounded morphology behave like stem cells, while proliferating chondrocytes behave like transient amplifying cells. Research using clonal genetic tracing with multicolour reporters or *Pthrp-mCherry* knock-in reporter alleles has demonstrated that cells of the resting zone give rise to flat chondrocytes of the proliferating zone and to hypertrophic chondrocytes by clonogenic division (Mizuhashi et al., 2018; Newton et al., 2019). The findings suggest that the cells of the resting zone give rise to all the cells clonogenically. However, the cells in the resting zone become depleted, and bone growth in the first ossification centre does not continue. Then, the supply of proliferating and hypertrophic chondrocytes ceases. Once the secondary ossification centre begins to form, the growth plate is established. Here, among resting chondrocytes, some *Pthrp-mCherry* cells behave like stem cells. They acquire the capacity for self-renewal, resulting in the formation of entire monoclonal columns of chondrocytes from the resting zone to the hypertrophic zone. In these niches, the skeletal stem cells begin to express stem cell markers and to undergo symmetric cell division, thus renewing the population of stem cells (Mizuhashi et al., 2018; Newton et al., 2019).

The control of both self-renewal and cell differentiation of skeletal stem cells is important for maintaining the pool of these cells. These processes are associated with determination of stem cell proliferation and stem cell identity. Hedgehog signalling and Pthrp signalling are both involved in promoting self-renewal and cell proliferation. *Pthrp-mCherry* cells are less proliferative than proliferating chondrocytes and are present in the growth plate in the CD45neg cell population (Chan et al., 2018; Mizuhashi et al., 2018). In the growth plate, activation of the IHH pathway induces *PthrP* expression in resting cells. Inhibition of hedgehog signalling reduces the clonal size of chondrocytes in the resting region but does not cause premature hypertrophy of the cartilage. This suggests that IHH controls the proliferation of skeletal stem cells (Newton et al., 2019). On the other hand, it has been reported that activation of mTORC1 (Newton et al., 2018) controls the organization of the resting zone without affecting cell proliferation. Activation of this signalling pathway regulates stem cell renewal because it controls the transition of skeletal stem cell division from asymmetric to symmetric.

Concluding Remarks

Our understanding of skeletal development has grown considerably in recent years. From the perspective of developmental biology, we have deep knowledge of the molecular mechanisms that control the shaping and positioning of cartilage and bone primordia during embryonic development. The use of genetic and molecular approaches together with experimental manipulation of embryos *in vivo* has favoured exploration of the requirements for particular genes at early stages during the commitment of mesenchymal cells. Such approaches have also allowed researchers to elucidate how committed cells are organized in space and time to produce three-dimensional skeletal organization and to drive morphogenesis and patterning. This knowledge is of great interest in disciplines such as the study of skeletal malformations and particularly in newly emerging fields such as regenerative medicine. However, this progress presents new challenges, such as how to use this information to rebuild damaged tissues. The rapid expansion of stem cell research has allowed us to understand how stem cells acquire different cell fates during development and how these cells can contribute to the formation of damaged tissues or regenerate them *in vitro*. Knowledge of stem cells provides indications/clues about how to activate the endogenous programme of cell differentiation to a correct fate and the creation of organoids in non-regenerative tissues. On the other hand, progress in the design of appropriate materials for controlling cell fate behaviour and or mimicking the ECM has generated advances in regenerative medicine but has not necessarily achieved the recapitulation of *in vivo* development. This is especially important in transplantation because the response of the newly delivered cells to their new environment is usually unknown. Complications with currently used materials, the control of factor release kinetics, and the vascularization and innervation of the tissue to allow graft survival and function persist. To promote the tissue regeneration of a whole limb (or any other limb tissue) *via* regenerative medicine, it will first be necessary to obtain a deeper understanding of the cellular mechanisms that regulate the microenvironment, the expression of morphogens, and the activation of signalling pathways in appropriate sites and cells, without ignoring how and what types of signals regulate the early cell fate decisions of the stem/progenitor limb cells. Thus, to repair or regenerate a tissue or structure in a whole limb, the translation of basic research discoveries to clinical applications must be considered.

Concerning developmental biology, knowledge regarding stem/progenitor cells and their interactions with their microenvironment *in vivo* is pivotal to understanding how mesenchymal cells sequentially undergo developmental commitment during skeletogenesis, transforming from mesenchymal cells to cartilage/bone cells; from cartilage cells to joint cells; from joint cells to articular cartilage, meniscus,

synovial lining, and joint capsule cells; and from cartilage cells to bone cells during endochondral ossification. A main question associated with the study of skeletal development is whether all skeletal stem cells originate from undifferentiated mesenchymal cells at early stages of limb development. If not, where do these stem cells come from, or how are they locally induced? Are the joint IZ regions reservoirs of stem cells or progenitor cells? Is IZ formation a consequence of local induction of joint stem cells? Further studies are needed to find new skeletal stem cell niches for the various developing tissues during skeletogenesis.

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JCM-L and JC-M conceived and wrote the paper. DG-C wrote the paper.

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REFERENCES

Ahrens, P. B., Solursh, M., and Reiter, R. S. (1977). Stage-related capacity for limb chondrogenesis in cell culture. *Dev. Biol.* 60 (1), 69–82. doi: 10.1016/0012-1606(77)90110-5

Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A., and de Crombrugghe, B. (2002). The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev.* 16 (21), 2813–2828. doi: 10.1101/gad.1017802

Akiyama, H., Kim, J. E., Nakashima, K., Balmes, G., Iwai, N., Deng, J. M., et al. (2005). Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc. Natl. Acad. Sci. U. S. A.* 102 (41), 14665–14670. doi: 10.1073/pnas.0504750102

Akiyama, H., Lyons, J. P., Mori-Akiyama, Y., Yang, X., Zhang, R., Zhang, Z., et al. (2004). Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev.* 18 (9), 1072–1087. doi: 10.1101/gad.1171104

Akiyama, H., Stadler, H. S., Martin, J. F., Ishii, T. M., Beachy, P. A., Nakamura, T., et al. (2007). Misexpression of Sox9 in mouse limb bud mesenchyme induces polydactyly and rescues hypodactyly mice. *Matrix Biol.* 26 (4), 224–233. doi: 10.1016/j.matbio.2006.12.002

Amarilio, R., Viukov, S. V., Sharir, A., Eshkar-Oren, I., Johnson, R. S., and Zelzer, E. (2007). HIF1alpha regulation of Sox9 is necessary to maintain differentiation of hypoxic prechondrogenic cells during early skeletogenesis. *Development* 134 (21), 3917–3928. doi: 10.1242/dev.008441

Amizuka, N., Warshawsky, H., Henderson, J. E., Goltzman, D., and Karaplis, A. C. (1994). Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *J. Cell Biol.* 126 (6), 1611–1623. doi: 10.1083/jcb.126.6.1611

Bandyopadhyay, A., Tsuji, K., Cox, K., Harfe, B. D., Rosen, V., and Tabin, C. J. (2006). Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet.* 2 (12), e216. doi: 10.1371/journal.pgen.0020216

Barna, M., and Niswander, L. (2007). Visualization of cartilage formation: insight into cellular properties of skeletal progenitors and chondrodysplasia syndromes. *Dev. Cell* 12 (6), 931–941. doi: 10.1016/j.devcel.2007.04.016

Barrow, J. R., Thomas, K. R., Boussadia-Zahui, O., Moore, R., Kemler, R., Capecchi, M. R., et al. (2003). Ectodermal Wnt3/beta-catenin signaling is required for the establishment and maintenance of the apical ectodermal ridge. *Genes Dev.* 17 (3), 394–409. doi: 10.1101/gad.1044903

Bhattaram, P., Penzo-Mendez, A., Kato, K., Bandyopadhyay, K., Gadi, A., Taketo, M. M., et al. (2014). SOXC proteins amplify canonical WNT signaling to secure nonchondrocytic fates in skeletogenesis. *J. Cell Biol.* 207 (5), 657–671. doi: 10.1083/jcb.201405098

Bi, W., Deng, J. M., Zhang, Z., Behringer, R. R., and de Crombrugghe, B. (1999). Sox9 is required for cartilage formation. *Nat. Genet.* 22 (1), 85–89. doi: 10.1038/8792

Brunet, L. J., McMahon, J. A., McMahon, A. P., and Harland, R. M. (1998). Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* 280 (5368), 1455–1457. doi: 10.1126/science.280.5368.1455

Chai, Y., Jiang, X., Ito, Y., Bringas, P., Jr., Han, J., Rowitch, D. H., et al. (2000). Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 127 (8), 1671–1679.

Chal, J., and Pourquié, O. (2017). Making muscle: skeletal myogenesis *in vivo* and *in vitro. Development* 144, 2104–2122. doi: 10.1242/dev.151035

Chan, C. K. F., Gulati, G. S., Sinha, R., Tompkins, J. V., Lopez, M., Carter, A. C., et al. (2018). Identification of the human skeletal stem cell. *Cell* 175 (1), 43–56 e21. doi: 10.1016/j.cell.2018.07.029

Chimal-Monroy, J., Rodriguez-Leon, J., Montero, J. A., Ganan, Y., Macias, D., Merino, R., et al. (2003). Analysis of the molecular cascade responsible for mesodermal limb chondrogenesis: Sox genes and BMP signaling. *Dev. Biol.* 257 (2), 292–301. doi: 10.1016/S0012-1606(03)00066-6

Chuong, C. M., Widelitz, R. B., Jiang, T. X., Abbott, U. K., Lee, Y. S., and Chen, H. M. (1993). Roles of adhesion molecules NCAM and tenascin in limb skeletogenesis: analysis with antibody perturbation, exogenous gene expression, talpid mutants and activin stimulation. *Prog. Clin. Biol. Res.* 383B, 465–474.

Cooper, K. L., Hu, J. K., ten Berge, D., Fernandez-Teran, M., Ros, M. A., and Tabin, C. J. (2011). Initiation of proximal-distal patterning in the vertebrate limb by signals and growth. *Science* 332 (6033), 1083–1086. doi: 10.1126/science.1199499

Coumoul, X., Shukla, V., Li, C., Wang, R. H., and Deng, C. X. (2005). Conditional knockdown of Fgfr2 in mice using Cre-LoxP induced RNA interference. *Nucleic Acids Res.* 33 (11), e102. doi: 10.1093/nar/gni100

Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., et al. (1994). Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature* 367 (6462), 455–459. doi: 10.1038/367455a0

Day, T. F., Guo, X., Garrett-Beal, L., and Yang, Y. (2005). Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev. Cell* 8 (5), 739–750. doi: 10.1016/j.devcel.2005.03.016

Diaz-Hernandez, M. E., Bustamante, M., Galvan-Hernandez, C. I., and Chimal-Monroy, J. (2013). Irx1 and Irx2 are coordinately expressed and regulated by retinoic acid, TGFbeta and FGF signaling during chick hindlimb development. *PLoS One* 8 (3), e58549. doi: 10.1371/journal.pone.0058549

Docheva, D., Popov, C., Alberton, P., and Aszodi, A. (2014). Integrin signaling in skeletal development and function. *Birth Defects Res. C Embryo Today* 102 (1), 13–36. doi: 10.1002/bdrc.21059

Drachman, D. B., and Sokoloff, L. (1966). The role of movement in embryonic joint development. *Dev. Biol.* 14, 401–420. doi: 10.1016/0012-1606(66)90022-4

Dudley, A. T., Ros, M. A., and Tabin, C. J. (2002). A re-examination of proximodistal patterning during vertebrate limb development. *Nature* 418 (6897), 539–544. doi: 10.1038/nature00945

Dunker, N., Schmitt, K., and Krieglstein, K. (2002). TGF-beta is required for programmed cell death in interdigital webs of the developing mouse limb. *Mech. Dev.* 113 (2), 111–120. doi: 10.1016/S0925-4773(02)00015-1

Duprez, D., Bell, E. J., Richardson, M. K., Archer, C. W., Wolpert, L., Brickell, P. M., et al. (1996). Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mech. Dev.* 57 (2), 145–157. doi: 10.1016/0925-4773(96)00540-0

Dy, P., Smits, P., Silvester, A., Penzo-Mendez, A., Dumitriu, B., Han, Y., et al. (2010). Synovial joint morphogenesis requires the chondrogenic action of Sox5 and Sox6 in growth plate and articular cartilage. *Dev. Biol.* 341 (2), 346–359. doi: 10.1016/j.ydbio.2010.02.024

Dy, P., Wang, W., Bhattaram, P., Wang, Q., Wang, L., Ballock, R. T., et al. (2012). Sox9 directs hypertrophic maturation and blocks osteoblast differentiation of growth plate chondrocytes. *Dev. Cell* 22 (3), 597–609. doi: 10.1016/j.devcel.2011.12.024

Enomoto-Iwamoto, M., Kitagaki, J., Koyama, E., Tamamura, Y., Wu, C., Kanatani, N., et al. (2002). The Wnt antagonist Frzb-1 regulates chondrocyte maturation and long bone development during limb skeletogenesis. *Dev. Biol.* 251 (1), 142–156. doi: 10.1006/dbio.2002.0802

Eshkar-Oren, I., Viukov, S. V., Salameh, S., Krief, S., Oh, C. D., Akiyama, H., et al. (2009). The forming limb skeleton serves as a signaling center for limb vasculature patterning *via* regulation of Vegf. *Development* 136 (8), 1263–1272. doi: 10.1242/dev.034199

Felsenthal, N., and Zelzer, E. (2017). Mechanical regulation of musculoskeletal system development. *Development* 144, 4271–4283. doi: 10.1242/dev.151266

Francis-West, P. H., Abdelfattah, A., Chen, P., Allen, C., Parish, J., Ladher, R., et al. (1999a). Mechanisms of GDF-5 action during skeletal development. *Development* 126 (6), 1305–1315.

Francis-West, P. H., Parish, J., Lee, K., and Archer, C. W. (1999b). BMP/GDF-signalling interactions during synovial joint development. *Cell Tissue Res.* 296 (1), 111–119. doi: 10.1007/s004410051272

Frenz, D. A., Akiyama, S. K., Paulsen, D. F., and Newman, S. A. (1989). Latex beads as probes of cell surface-extracellular matrix interactions during chondrogenesis: evidence for a role for amino-terminal heparin-binding domain of fibronectin. *Dev. Biol.* 136 (1), 87–96. doi: 10.1016/0012-1606(89)90132-2

Ganan, Y., Macias, D., Duterque-Coquillaud, M., Ros, M. A., and Hurle, J. M. (1996). Role of TGF beta s and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. *Development* 122 (8), 2349–2357.

Garciadiego-Cazares, D., Rosales, C., Katoh, M., and Chimal-Monroy, J. (2004). Coordination of chondrocyte differentiation and joint formation by alpha5beta1 integrin in the developing appendicular skeleton. *Development* 131 (19), 4735–4742. doi: 10.1242/dev.01345

Geetha-Loganathan, P., Nimmagadda, S., Christ, B., Huang, R., and Scaal, M. (2010). Ectodermal Wnt6 is an early negative regulator of limb chondrogenesis in the chicken embryo. *BMC Dev. Biol.* 10, 32. doi: 10.1186/1471-213X-10-32

Gehris, A. L., Stringa, E., Spina, J., Desmond, M. E., Tuan, R. S., and Bennett, V. D. (1997). The region encoded by the alternatively spliced exon IIIA in mesenchymal fibronectin appears essential for chondrogenesis at the level of cellular condensation. *Dev. Biol.* 190 (2), 191–205. doi: 10.1006/dbio.1997.8693

Goldring, M. B., and Marcu, K. B. (2009). Cartilage homeostasis in health and rheumatic diseases. *Arthritis Res. Ther.* 11 (3), 224. doi: 10.1186/ar2592

Guimaraes-Camboa, N., Cattaneo, P., Sun, Y., Moore-Morris, T., Gu, Y., Dalton, N. D., et al. (2017). Pericytes of multiple organs do not behave as mesenchymal stem cells *in vivo*.. *Cell Stem Cell* 20 (3), 345–359 e345. doi: 10.1016/j.stem.2016.12.006

Guo, X., Day, T. F., Jiang, X., Garrett-Beal, L., Topol, L., and Yang, Y. (2004). Wnt/beta-catenin signaling is sufficient and necessary for synovial joint formation. *Genes Dev.* 18 (19), 2404–2417. doi: 10.1101/gad.1230704

Hall, B. K. (1981). The induction of neural crest-derived cartilage and bone by embryonic epithelia: an analysis of the mode of action of an epithelial-mesenchymal interaction. *J. Embryol. Exp. Morphol.* 64, 305–320.

Hall, B. K., and Miyake, T. (1995). Divide, accumulate, differentiate: cell condensation in skeletal development revisited. *Int. J. Dev. Biol.* 39 (6), 881–893.

Hallmann, R., Feinberg, R. N., Latker, C. H., Sasse, J., and Risau, W. (1987). Regression of blood vessels precedes cartilage differentiation during chick limb development. *Differentiation* 34 (2), 98–105. doi: 10.1111/j.1432-0436.1987.tb00055.x

Hamburger, V., and Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo. 1951. *Dev. Dyn.* 195 (4), 231–272. doi: 10.1002/aja.1001950404

Hartmann, C., and Tabin, C. J. (2001). Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. *Cell* 104 (3), 341–351. doi: 10.1016/S0092-8674(01)00222-7

Hill, T. P., Spater, D., Taketo, M. M., Birchmeier, W., and Hartmann, C. (2005). Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev. Cell* 8 (5), 727–738. doi: 10.1016/j.devcel.2005.02.013

Holder, N. (1977). An experimental investigation into the early development of the chick elbow joint. *J. Embryol. Exp. Morphol.* 39, 115–127.

Hyde, G., Dover, S., Aszodi, A., Wallis, G. A., and Boot-Handford, R. P. (2007). Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms. *Dev. Biol.* 304 (2), 825–833. doi: 10.1016/j.ydbio.2007.01.026

Ikeda, T., Kamekura, S., Mabuchi, A., Kou, I., Seki, S., Takato, T., et al. (2004). The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. *Arthritis Rheum.* 50 (11), 3561–3573. doi: 10.1002/art.20611

Jiao, F., Wang, J., Dong, Z. L., Wu, M. J., Zhao, T. B., Li, D. D., et al. (2012). Human mesenchymal stem cells derived from limb bud can differentiate into all three embryonic germ layers lineages. *Cell Reprogram.* 14 (4), 324–333. doi: 10.1089/cell.2012.0004

Jin, L., Wu, J., Bellusci, S., and Zhang, J. S. (2018). Fibroblast growth factor 10 and vertebrate limb development. *Front. Genet.* 9, 705. doi: 10.3389/fgene.2018.00705

Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., et al. (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat. Genet.* 11 (4), 415–421. doi: 10.1038/ng1295-415

Kahn, J., Shwartz, Y., Blitz, E., Krief, S., Sharir, A., Breitel, D. A., et al., , et al. (2009). Muscle contraction is necessary to maintain joint progenitor cell fate. *Dev. Cell* 16, 734–743. doi: 10.1016/j.devcel.2009.04.013

Kan, A., and Tabin, C. J. (2013). c-Jun is required for the specification of joint cell fates. *Genes Dev.* 27 (5), 514–524. doi: 10.1101/gad.209239.112

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M., et al. (1994). Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev.* 8 (3), 277–289. doi: 10.1101/gad.8.3.277

Karsenty, G., and Wagner, E. F. (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell* 2 (4), 389–406. doi: 10.1016/S1534-5807(02)00157-0

Kengaku, M., Capdevila, J., Rodriguez-Esteban, C., De La Pena, J., Johnson, R. L., Belmonte, J. C., et al. (1998). Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science* 280 (5367), 1274–1277. doi: 10.1126/science.280.5367.1274

Kim, Y., Murao, H., Yamamoto, K., Deng, J. M., Behringer, R. R., Nakamura, T., et al. (2011). Generation of transgenic mice for conditional overexpression of Sox9. *J Bone Miner. Metab.* 29 (1), 123–129. doi: 10.1007/s00774-010-0206-z

Koyama, E., Shibukawa, Y., Nagayama, M., Sugito, H., Young, B., Yuasa, T., et al. (2008). A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. *Dev. Biol.* 316 (1), 62–73. doi: 10.1016/j.ydbio.2008.01.012

Kozhemyakina, E., Lassar, A. B., and Zelzer, E. (2015). A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. *Development* 142 (5), 817–831. doi: 10.1242/dev.105536

Kronenberg, H. M. (2003). Developmental regulation of the growth plate. *Nature* 423 (6937), 332–336. doi: 10.1038/nature01657

Kumar, D., and Lassar, A. B. (2014). Fibroblast growth factor maintains chondrogenic potential of limb bud mesenchymal cells by modulating DNMT3A recruitment. *Cell Rep.* 8 (5), 1419–1431. doi: 10.1016/j.celrep.2014.07.038

Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., et al. (1996). PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 273 (5275), 663–666. doi: 10.1126/science.273.5275.663

Laufer, E., Dahn, R., Orozco, O. E., Yeo, C. Y., Pisenti, J., Henrique, D., et al. (1997). Expression of Radical fringe in limb-bud ectoderm regulates apical ectodermal ridge formation. *Nature* 386 (6623), 366–373. doi: 10.1038/386366a0

Lefebvre, V. (2019). Roles and regulation of SOX transcription factors in skeletogenesis. *Curr. Top. Dev. Biol.* 133, 171–193. doi: 10.1016/bs.ctdb.2019.01.007

Lei, H., Yu, B., Huang, Z., Yang, X., Liu, Z., Mao, X., et al. (2013). Comparative analysis of mesenchymal stem cells from adult mouse adipose, muscle, and fetal muscle. *Mol. Biol. Rep.* 40 (2), 885–892. doi: 10.1007/s11033-012-2129-3

Li, C., Xu, X., Nelson, D. K., Williams, T., Kuehn, M. R., and Deng, C. X. (2005). FGFR1 function at the earliest stages of mouse limb development plays an indispensable role in subsequent autopod morphogenesis. *Development* 132 (21), 4755–4764. doi: 10.1242/dev.02065

Li, T., Longobardi, L., Myers, T. J., Temple, J. D., Chandler, R. L., Ozkan, H., et al. (2013). Joint TGF-beta type II receptor-expressing cells: ontogeny and characterization as joint progenitors. *Stem Cells Dev.* 22 (9), 1342–1359. doi: 10.1089/scd.2012.0207

Lorda-Diez, C. I., Montero, J. A., Diaz-Mendoza, M. J., Garcia-Porrero, J. A., and Hurle, J. M. (2011). Defining the earliest transcriptional steps of chondrogenic progenitor specification during the formation of the digits in the embryonic limb. *PLoS One* 6 (9), e24546. doi: 10.1371/journal.pone.0024546

Lorda-Diez, C. I., Montero, J. A., Diaz-Mendoza, M. J., Garcia-Porrero, J. A., and Hurle, J. M. (2013). betaig-h3 potentiates the profibrogenic effect of TGFbeta signaling on connective tissue progenitor cells through the negative regulation of master chondrogenic genes. *Tissue Eng Part A* 19 (3-4), 448–457. doi: 10.1089/ten.tea.2012.0188

Lories, R. J., Peeters, J., Bakker, A., Tylzanowski, P., Derese, I., Schrooten, J., et al. (2007). Articular cartilage and biomechanical properties of the long bones in Frzb-knockout mice. *Arthritis Rheum.* 56 (12), 4095–4103. doi: 10.1002/art.23137

Lu, P., Yu, Y., Perdue, Y., and Werb, Z. (2008). The apical ectodermal ridge is a timer for generating distal limb progenitors. *Development* 135 (8), 1395–1405. doi: 10.1242/dev.018945

Macias, D., Ganan, Y., Sampath, T. K., Piedra, M. E., Ros, M. A., and Hurle, J. M. (1997). Role of BMP-2 and OP-1 (BMP-7) in programmed cell death and skeletogenesis during chick limb development. *Development* 124 (6), 1109–1117.

Mackie, E. J., Ahmed, Y. A., Tatarczuch, L., Chen, K. S., and Mirams, M. (2008). Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int. J. Biochem. Cell Biol.* 40 (1), 46–62. doi: 10.1016/j.biocel.2007.06.009

MacLean, H. E., Guo, J., Knight, M. C., Zhang, P., Cobrinik, D., and Kronenberg, H. M. (2004). The cyclin-dependent kinase inhibitor p57(Kip2) mediates proliferative actions of PTHrP in chondrocytes. *J. Clin. Invest.* 113 (9), 1334–1343. doi: 10.1172/JCI21252

Mariani, F. V., Ahn, C. P., and Martin, G. R. (2008). Genetic evidence that FGFs have an instructive role in limb proximal-distal patterning. *Nature* 453 (7193), 401–405. doi: 10.1038/nature06876

Marin-Llera, J. C., and Chimal-Monroy, J. (2018). A small population of resident limb bud mesenchymal cells express few MSC-associated markers, but the expression of these markers is increased immediately after cell culture. *Cell Biol. Int.* 42 (5), 570–579. doi: 10.1002/cbin.10933

Matzuk, M. M., Kumar, T. R., and Bradley, A. (1995a). Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature* 374 (6520), 356–360. doi: 10.1038/374356a0

Matzuk, M. M., Kumar, T. R., Vassalli, A., Bickenbach, J. R., Roop, D. R., Jaenisch, R., et al. (1995b). Functional analysis of activins during mammalian development. *Nature* 374 (6520), 354–356. doi: 10.1038/374354a0

Matzuk, M. M., Lu, N., Vogel, H., Sellheyer, K., Roop, D. R., and Bradley, A. (1995c). Multiple defects and perinatal death in mice deficient in follistatin. *Nature* 374 (6520), 360–363. doi: 10.1038/374360a0

Merino, R., Ganan, Y., Macias, D., Economides, A. N., Sampath, K. T., and Hurle, J. M. (1998). Morphogenesis of digits in the avian limb is controlled by FGFs, TGFbetas, and noggin through BMP signaling. *Dev. Biol.* 200 (1), 35–45. doi: 10.1006/dbio.1998.8946

Merino, R., Macias, D., Ganan, Y., Economides, A. N., Wang, X., Wu, Q., et al. (1999a). Expression and function of Gdf-5 during digit skeletogenesis in the embryonic chick leg bud. *Dev. Biol.* 206 (1), 33–45. doi: 10.1006/dbio.1998.9129

Merino, R., Rodriguez-Leon, J., Macias, D., Ganan, Y., Economides, A. N., and Hurle, J. M. (1999b). The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. *Development* 126 (23), 5515–5522.

Mizuhashi, K., Ono, W., Matsushita, Y., Sakagami, N., Takahashi, A., Saunders, T. L., et al. (2018). Resting zone of the growth plate houses a unique class of skeletal stem cells. *Nature* 563 (7730), 254–258. doi: 10.1038/s41586-018-0662-5

Montero, J. A., Lorda-Diez, C. I., Ganan, Y., Macias, D., and Hurle, J. M. (2008). Activin/TGFbeta and BMP crosstalk determines digit chondrogenesis. *Dev. Biol.* 321 (2), 343–356. doi: 10.1016/j.ydbio.2008.06.022

Newton, P. T., Li, L., Zhou, B., Schweingruber, C., Hovorakova, M., Xie, M., et al. (2019). A radical switch in clonality reveals a stem cell niche in the epiphyseal growth plate. *Nature* 567 (7747), 234–238. doi: 10.1038/s41586-019-0989-6

Newton, P. T., Xie, M., Medvedeva, E. V., Savendahl, L., and Chagin, A. S. (2018). Activation of mTORC1 in chondrocytes does not affect proliferation or differentiation but causes the resting zone of the growth plate to become disordered. *Bone Rep.* 8, 64–71. doi: 10.1016/j.bonr.2018.02.006

Norrie, J. L., Li, Q., Co, S., Huang, B. L., Ding, D., Uy, J. C., et al. (2016). PRMT5 is essential for the maintenance of chondrogenic progenitor cells in the limb bud. *Development* 143 (24), 4608–4619. doi: 10.1242/dev.140715

Nusspaumer, G., Jaiswal, S., Barbero, A., Reinhardt, R., Ishay Ronen, D., Haumer, A., et al. (2017). Ontogenic identification and analysis of mesenchymal stromal cell populations during mouse limb and long bone development. *Stem Cell Reports* 9 (4), 1124–1138. doi: 10.1016/j.stemcr.2017.08.007

Oberlender, S. A., and Tuan, R. S. (1994a). Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. *Development* 120 (1), 177–187.

Oberlender, S. A., and Tuan, R. S. (1994b). Spatiotemporal profile of N-cadherin expression in the developing limb mesenchyme. *Cell Adhes. Commun.* 2 (6), 521–537. doi: 10.3109/15419069409014216

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., et al. (1997). The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* 124 (11), 2235–2244.

Parr, B. A., and McMahon, A. P. (1995). Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature* 374 (6520), 350–353. doi: 10.1038/374350a0

Pearse, R. V., Scherz, P. J., Campbell, J. K., and Tabin, C. J. (2007). A cellular lineage analysis of the chick limb bud. *Dev. Biol.* 310 (2), 388–400. doi: 10.1016/j.ydbio.2007.08.002

Pizette, S., and Niswander, L. (2000). BMPs are required at two steps of limb chondrogenesis: formation of prechondrogenic condensations and their differentiation into chondrocytes. *Dev. Biol.* 219 (2), 237–249. doi: 10.1006/dbio.2000.9610

Qian, H., Le Blanc, K., and Sigvardsson, M. (2012). Primary mesenchymal stem and progenitor cells from bone marrow lack expression of CD44 protein. *J. Biol. Chem.* 287 (31), 25795–25807. doi: 10.1074/jbc.M112.339622

Riddle, R. D., Johnson, R. L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75 (7), 1401–1416. doi: 10.1016/0092-8674(93)90626-2

Rodriguez-Esteban, C., Schwabe, J. W., De La Pena, J., Foys, B., Eshelman, B., and Belmonte, J. C. (1997). Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. *Nature* 386 (6623), 360–366. doi: 10.1038/386360a0

Rosello-Diez, A., Arques, C. G., Delgado, I., Giovinazzo, G., and Torres, M. (2014). Diffusible signals and epigenetic timing cooperate in late proximo-distal limb patterning. *Development* 141 (7), 1534–1543. doi: 10.1242/dev.106831

Rossi, F., MacLean, H. E., Yuan, W., Francis, R. O., Semenova, E., Lin, C. S., et al. (2002). p107 and p130 Coordinately regulate proliferation, Cbfa1 expression, and hypertrophic differentiation during endochondral bone development. *Dev. Biol.* 247 (2), 271–285. doi: 10.1006/dbio.2002.0691

Rux, D., Decker, R. S., Koyama, E., and Pacifici, M. (2019). Joints in the appendicular skeleton: Developmental mechanisms and evolutionary influences. *Curr. Top. Dev. Biol.* 133, 119–151. doi: 10.1016/bs.ctdb.2018.11.002

Salim, A., Nacamuli, R. P., Morgan, E. F., Giaccia, A. J., and Longaker, M. T. (2004). Transient changes in oxygen tension inhibit osteogenic differentiation and Runx2 expression in osteoblasts. *J. Biol. Chem.* 279 (38), 40007–40016. doi: 10.1074/jbc.M403715200

Sato, K., Koizumi, Y., Takahashi, M., Kuroiwa, A., and Tamura, K. (2007). Specification of cell fate along the proximal-distal axis in the developing chick limb bud. *Development* 134 (7), 1397–1406. doi: 10.1242/dev.02822

Saunders, J.W., Jr. (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J. Exp. Zool.* 108 (3), 363–403. doi: 10.1002/jez.1401080304

Saunders, J. W., Jr., and Gasseling, M. T. (1968). "Ectodermal-mesodermal interactions in the origin of limb symmetry," in *Epithelial-Mesenchymal Interactions*. Eds. R. Fleischmajer and R. E. Billingham (Baltimore: Williams & Wilkins), 78–97.

Shwartz, Y., Viukov, S., Krief, S., and Zelzer, E. (2016). Joint development involves a continuous influx of Gdf5-positive cells. *Cell Rep.* 15 (12), 2577–2587. doi: 10.1016/j.celrep.2016.05.055

Searls, R. L., and Janners, M. Y. (1969). The stabilization of cartilage properties in the cartilage-forming mesenchyme of the embryonic chick limb. *J. Exp. Zool.* 170 (3), 365–375. doi: 10.1002/jez.1401700313

Settle, S. H., Jr., Rountree, R. B., Sinha, A., Thacker, A., Higgins, K., and Kingsley, D. M. (2003). Multiple joint and skeletal patterning defects caused by single and double mutations in the mouse Gdf6 and Gdf5 genes. *Dev. Biol.* 254 (1), 116–130. doi: 10.1016/S0012-1606(02)00022-2

Shakibaei, M. (1998). Inhibition of chondrogenesis by integrin antibody *in vitro*. *Exp. Cell Res.* 240 (1), 95–106. doi: 10.1006/excr.1998.3933

Shimizu, H., Yokoyama, S., and Asahara, H. (2007). Growth and differentiation of the developing limb bud from the perspective of chondrogenesis. *Dev. Growth Differ.* 49 (6), 449–454. doi: 10.1111/j.1440-169X.2007.00945.x

Shubin, N., Tabin, C., and Carroll, S. (1997). Fossils, genes and the evolution of animal limbs. *Nature* 388 (6643), 639–648. doi: 10.1038/41710

Shull, M. M., Ormsby, I., Kier, A. B., Pawlowski, S., Diebold, R. J., Yin, M., et al. (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359 (6397), 693–699. doi: 10.1038/359693a0

Shum, L., Coleman, C. M., Hatakeyama, Y., and Tuan, R. S. (2003). Morphogenesis and dysmorphogenesis of the appendicular skeleton. *Birth Defects Res. C Embryo Today* 69 (2), 102–122. doi: 10.1002/bdrc.10012

Slack, J. M. W. (2018). What is a stem cell? *Wiley Interdiscip Rev. Dev. Biol.*, 15:e323. doi: 10.1002/wdev.323

Spagnoli, A., O'Rear, L., Chandler, R. L., Granero-Molto, F., Mortlock, D. P., Gorska, A. E., et al. (2007). TGF-beta signaling is essential for joint morphogenesis. *J. Cell Biol.* 177 (6), 1105–1117. doi: 10.1083/jcb.200611031

Spater, D., Hill, T. P., Gruber, M., and Hartmann, C. (2006a). Role of canonical Wnt-signalling in joint formation. *Eur. Cell Mater.* 12, 71–80. doi: 10.22203/eCM.v012a09

Spater, D., Hill, T. P., O'Sullivan R, J., Gruber, M., Conner, D. A., and Hartmann, C. (2006b). Wnt9a signaling is required for joint integrity and regulation of Ihh during chondrogenesis. *Development* 133 (15), 3039–3049. doi: 10.1242/dev.02471

Storm, E. E., and Kingsley, D. M. (1999). GDF5 coordinates bone and joint formation during digit development. *Dev. Biol.* 209 (1), 11–27. doi: 10.1006/dbio.1999.9241

Summerbell, D. (1974). A quantitative analysis of the effect of excision of the AER from the chick limb-bud. *J. Embryol. Exp. Morphol.* 32 (3), 651–660.

Summerbell, D., Lewis, J. H., and Wolpert, L. (1973). Positional information in chick limb morphogenesis. *Nature* 244 (5417), 492–496. doi: 10.1038/244492a0

Sun, X., Mariani, F. V., and Martin, G. R. (2002). Functions of FGF signalling from the apical ectodermal ridge in limb development. *Nature* 418 (6897), 501–508. doi: 10.1038/nature00902

Suzuki, T., Hasso, S. M., and Fallon, J. F. (2008). Unique SMAD1/5/8 activity at the phalanx-forming region determines digit identity. *Proc. Natl. Acad. Sci. U. S. A.* 105 (11), 4185–4190. doi: 10.1073/pnas.0707899105

Tabin, C., and Wolpert, L. (2007). Rethinking the proximodistal axis of the vertebrate limb in the molecular era. *Genes Dev.* 21 (12), 1433–1442. doi: 10.1101/gad.1547407

Takahashi, K., Nuckolls, G. H., Takahashi, I., Nonaka, K., Nagata, M., Ikura, T., et al. (2001). Msx2 is a repressor of chondrogenic differentiation in migratory cranial neural crest cells. *Dev. Dyn.* 222 (2), 252–262. doi: 10.1002/dvdy.1185

Takeda, S., Bonnamy, J. P., Owen, M. J., Ducy, P., and Karsenty, G. (2001). Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes Dev.* 15 (4), 467–481. doi: 10.1101/gad.845101

Tavella, S., Biticchi, R., Schito, A., Minina, E., Di Martino, D., Pagano, A., et al. (2004). Targeted expression of SHH affects chondrocyte differentiation, growth plate organization, and Sox9 expression. *J. Bone Miner. Res.* 19 (10), 1678–1688. doi: 10.1359/JBMR.040706

ten Berge, D., Brugmann, S. A., Helms, J. A., and Nusse, R. (2008). Wnt and FGF signals interact to coordinate growth with cell fate specification during limb development. *Development* 135 (19), 3247–3257. doi: 10.1242/dev.023176

Tian, Y., Xu, Y., Fu, Q., Chang, M., Wang, Y., Shang, X., et al. (2015). Notch inhibits chondrogenic differentiation of mesenchymal progenitor cells by targeting Twist1. *Mol. Cell Endocrinol.* 403, 30–38. doi: 10.1016/j.mce.2015.01.015

Tickle, C. (2015). How the embryo makes a limb: determination, polarity and identity. *J. Anat.* 227 (4), 418–430. doi: 10.1111/joa.12361

Todt, W. L., and Fallon, J. F. (1984). Development of the apical ectodermal ridge in the chick wing bud. *J. Embryol. Exp. Morphol.* 80, 21–41.

Topol, L., Chen, W., Song, H., Day, T. F., and Yang, Y. (2009). Sox9 inhibits Wnt signaling by promoting beta-catenin phosphorylation in the nucleus. *J. Biol. Chem.* 284 (5), 3323–3333. doi: 10.1074/jbc.M808048200

Topol, L., Jiang, X., Choi, H., Garrett-Beal, L., Carolan, P. J., and Yang, Y. (2003). Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation. *J. Cell Biol.* 162 (5), 899–908. doi: 10.1083/jcb.200303158

Towers, M., and Tickle, C. (2009). Growing models of vertebrate limb development. *Development* 136 (2), 179–190. doi: 10.1242/dev.024158

Tylzanowski, P., Mebis, L., and Luyten, F. P. (2006). The Noggin null mouse phenotype is strain dependent and haploinsufficiency leads to skeletal defects. *Dev. Dyn.* 235 (6), 1599–1607. doi: 10.1002/dvdy.20782

Verheyden, J. M., Lewandoski, M., Deng, C., Harfe, B. D., and Sun, X. (2005). Conditional inactivation of Fgfr1 in mouse defines its role in limb bud establishment, outgrowth and digit patterning. *Development* 132 (19), 4235–4245. doi: 10.1242/dev.02001

Vogel, A., Rodriguez, C., and Izpisua-Belmonte, J. C. (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* 122 (6), 1737–1750.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M., and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* 273 (5275), 613–622. doi: 10.1126/science.273.5275.613

Wada, N., Kawakami, Y., Ladher, R., Francis-West, P. H., and Nohno, T. (1999). Involvement of Frzb-1 in mesenchymal condensation and cartilage differentiation in the chick limb bud. *Int. J. Dev. Biol.* 43 (6), 495–500.

Widelitz, R. B., Jiang, T. X., Murray, B. A., and Chuong, C. M. (1993). Adhesion molecules in skeletogenesis: II. Neural cell adhesion molecules mediate precartilaginous mesenchymal condensations and enhance chondrogenesis. *J. Cell Physiol.* 156 (2), 399–411. doi: 10.1002/jcp.1041560224

Yang, Y., and Niswander, L. (1995). Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* 80 (6), 939–947. doi: 10.1016/0092-8674(95)90297-X

Zeller, R., Lopez-Rios, J., and Zuniga, A. (2009). Vertebrate limb bud development: moving towards integrative analysis of organogenesis. *Nat. Rev. Genet.* 10 (12), 845–858. doi: 10.1038/nrg2681

Zhou, G., Zheng, Q. P., Engin, F., Munivez, E., Chen, Y. Q., Sebald, E., et al. (2006). Dominance of SOX9 function over RUNX2 during skeletogenesis. *Proceedings of the National Academy of Sciences of the United States of America* 103 (50), 19004–19009. doi: 10.1073/pnas.0605170103

Zhou, Z. Q., Shung, C. Y., Ota, S., Akiyama, H., Keene, D. R., and Hurlin, P. J. (2011). Sequential and coordinated actions of c-Myc and N-Myc control appendicular skeletal development. *PLoS One* 6 (4), e18795. doi: 10.1371/journal.pone.0018795

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REVIEW
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Clinical Translation of Mesenchymal Stromal Cell Therapy for Graft Versus Host Disease

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Graft versus host disease (GVHD) is a common condition in patients subjected to allogeneic hematopoietic stem cell transplantation (HSCT). The immune cells derived from the grafted stem cells attack recipient's tissues, including those from the skin, liver, eyes, mouth, lungs, gastrointestinal tract, neuromuscular system, and genitourinary tract, may lead to severe morbidity and mortality. Acute GVHD can occur within few weeks after the allogeneic cells have engrafted in the recipient while chronic GVHD may occur any time after transplant, typically within months. Although treatable by systemic corticosteroid administration, effective responses are not achieved for a significant proportion of patients, a condition associated with poor prognosis. The use of multipotent mesenchymal stromal cells (MSCs) as an alternative to treat steroid-refractory GVHD had improved last decade, but the results are still controversial. Some studies have shown improvement in the life quality of patients after MSCs treatment, while others have found no significant benefits. In addition to variations in trial design, discrepancies in protocols for MSCs isolation, characterization, and *ex vivo* manipulation, account for inconsistent clinical results. In this review, we discuss the immunomodulatory properties supporting the therapeutic use of MSCs in GVHD and contextualize the main clinical findings of recent trials using these cells. Critical parameters for the clinical translation of MSCs, including consistent production of MSCs according to Good Manufacturing Practices (GMPs) and informative potency assays for product quality control (QC), are addressed.

Keywords: mesenchymal stromal cells, immunomodulation, graft versus host disease, bone marrow, good manufacturing practices

INTRODUCTION

Allogeneic hematopoietic stem cell transplant (HSCT) is a treatment for high risk hematological and malignant diseases. Conditioning regimen, immunosuppressive strategies, supportive care and prophylaxis for infectious disease are improving, reducing mortality related to transplant (Appelbaum, 2001). However, graft versus host disease (GVHD) remains one of the most common complication with high rate of disability and mortality (Perkey and Maillard, 2018).

Graft versus host disease occurs when immunologically competent donor T lymphocytes recognize recipient's tissue as foreign resulting in damage in many organs systems including skin, liver, gastrointestinal tract, and lung. Clinical manifestations are classified as acute or chronic GVHD. In the past, all clinical manifestations of GVHD occurring before 100 days of transplant where classified as acute GVHD. When clinical manifestations occurred later on, after 100 days of transplant, it was considered chronic GVHD. In 2005, the National Institutes of Health (NIH) Consensus Conference determined new criteria of diagnosis and scoring and abolished the 100 days criterion. NIH Consensus considered acute and chronic GVHD as two distinguished symptoms without restriction of time (Filipovich et al., 2005; **Figure 1**).

Among all patients undergoing allogeneic HSCT 30–50% will have acute GVHD (grade 1–4) and 14% of patients exhibit chronic GVHD. The most important acute GVHD risk factor is human leukocyte antigen (HLA) mismatch (Petersdorf et al., 1995; Flomenberg et al., 2004). Transplants involving female donor/male recipient or unrelated donor are also associated with higher risk of developing late acute and chronic GVHD (Arora et al., 2016).

Pathophysiology of acute GVHD involves engraftment of immunocompetent cells in a host with mismatched antigens that is incapable to respond against graft cells, allowing donor lymphocytes activation to attack host tissue (Billingham, 1966). The damage to host tissues leads to production of proinflammatory cytokines, such as tumor necrosis factor (TNF) α, interleukin (IL) 1, 2, and 6, chemokines and increased expression of adhesion molecules, costimulatory molecules and major histocompatibility complex (MHC) antigens on the tissue (Jamil and Mineishi, 2015). Regulatory T Cells (Tregs) have been shown to downregulate the alloreactivity of T cells *in vitro* and *in vivo* and natural killer cells (NK cells) have been shown to modulate GVHD in a clinical trial reducing incidence of GVHD (Zeng et al., 1999; Cohen and Boyer, 2006).

The pathophysiology of chronic GVHD is more complex. All mechanisms reported in acute GVHD are relevant, however, other pathways are under investigation. The presence of auto and alloantibodies is described but is unclear whether these antibodies are involved in pathogenesis or are just markers of B cell dysregulation (Shimabukuro-Vornhagen et al., 2009). The presence of these auto antibodies is also described along with implication of Treg dysfunction in the development of chronic GVHD (Martin, 2008).

Acute and chronic GVHD are first treated by glucocorticoids. However, 50–60% of patients are resistant to glucocorticoids (Flowers and Martin, 2015; Mielcarek et al., 2015) and they have poor long-term prognosis with overall survival rate of only 5–30% (Zeiser and Blazar, 2017). Alternative treatments involve different immunosuppressive drugs like Calcineurin inhibitor, Antithymocyte globulin (ATG), Anti-interleukin 2 receptor antibodies, Anti-TNFα agents, Extracorporeal photopheresis (ECP), Mycophenolate mofetil (MMF), Sirolimus, and Pentostatin. None of them are fully effective and new therapeutic modalities for refractory GVHD are currently under investigation, including therapy with mesenchymal stromal cells (MSCs).

MESENCHYMAL STROMAL CELL IDENTITY

After their first description in bone marrow by Friedenstein et al. (1968), mesenchymal cells were later found to reside in almost all post-natal tissues, being recruited to sites of tissue injury. Although at variable quantities, mesenchymal stem cells are also isolated from cord blood (Erices et al., 2000), umbilical cord (Wang et al., 2004), amnion (Kaviani et al., 2001), placenta

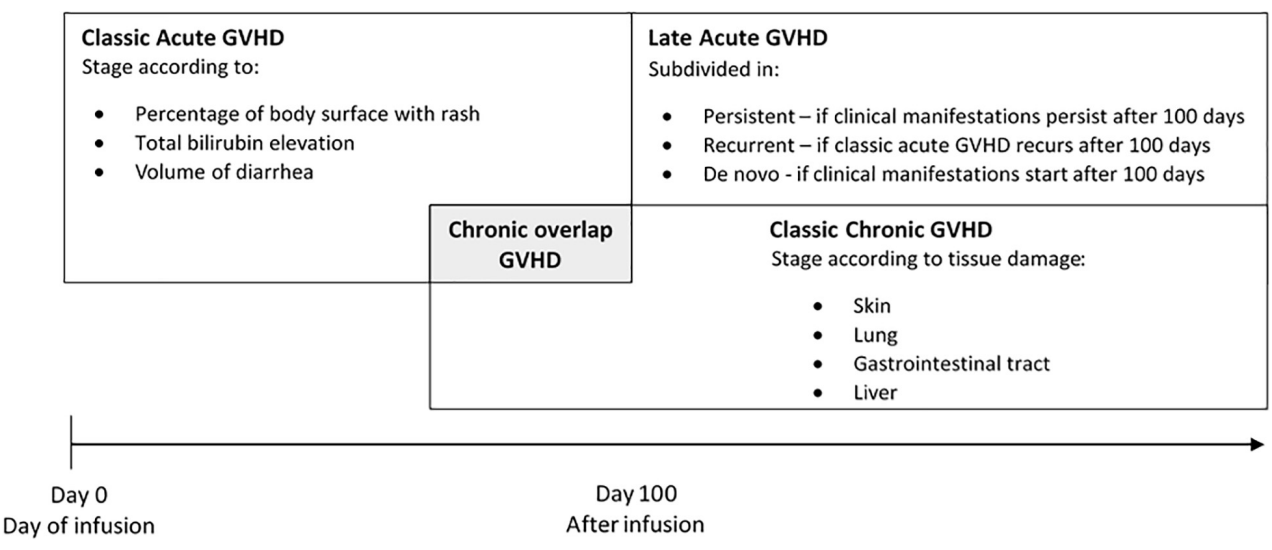

FIGURE 1 | Classification and stage of graft versus host disease (GVHD).

(Fauza, 2004), peripheral blood (Kassis et al., 2006), adipose tissue (Zuk et al., 2002), dental pulp (Gronthos et al., 2000), maternal milk (Patki et al., 2010), skin (Shih et al., 2005), and menstrual blood (Meng et al., 2007), among others. However, the great variability in the protocols for mesenchymal stem cell isolation and *ex vivo* expansion may sometimes result in cultures of cells with distinct properties.

In attempt to help standardize the growing research field with such mesenchymal cells, the International Society for Cellular Therapy suggested using the term "MSCs," due to the lack of uniform evidences for their stem cell activity (Horwitz et al., 2005). The same Society also proposed minimum criteria to characterize MSCs, namely culture plastic adherence, ability to differentiate *in vitro* into adipocytes, chondrocytes and osteocytes, and expression of specific membrane surface antigens (Dominici et al., 2006).

Although widely accepted, these criteria do not guarantee purity of MSC preparations since other cell types, such as fibroblasts, to some extent comply with these same requirements (Junker et al., 2010; Pereira et al., 2011). Heterogeneity in MSC products may lead to discrepant clinical outcomes. Indeed, in an experimental model of Parkinson's Disease, contamination of MSC preparations with fibroblasts abolished MSC-induced therapeutic effects and enhanced degeneration of dopaminergic neurons (Pereira et al., 2011). Therefore, defining clear threshold levels of critical cell parameters may improve MSC quality testing. Assessment of alternative membrane markers enriched in MSCs compared to other cell types, such as CD166 (Halfon et al., 2011), CD271 (Jones et al., 2002), or CD146 (Sacchetti et al., 2007) have also been proposed for MSC immunophenotyping.

Therefore, following strict criteria for MSC identity is essential for comparability and reproducibility studies. Nonetheless, it is also important to continuously revise these consensus criteria once knowledge is updated in the literature.

IMMUNOMODULATORY PROPERTIES OF MESENCHYMAL STROMAL CELLS

Mesenchymal stromal cells are highly metabolically active, secreting not only extracellular matrix molecules (Wight et al., 1986), but also a variety of cytokines (Horwitz and Dominici, 2008). Indeed, the paracrine effects of MSCs, such as those related to regulation of immune response, seem more relevant under certain physiopathological conditions than their multipotency. Some studies reported that MSCs are able to affect the activity of both, T and B cells. MSCs may suppress T cell proliferation, cytokine release, cytotoxicity, and Th1/Th2 balance (Puissant et al., 2005; Selmani et al., 2008). MSCs also affect B cells viability, antibodies secretion and the co-stimulatory production of molecules released by B cells (Corcione et al., 2006). Some studies have also reported MSC to be capable of inhibiting antigen maturation, activation and presentation by dendritic cells (Ramasamy et al., 2007), as well as inhibiting interleukin-2 (IL-2) production by NK cells (Spaggiari et al., 2006).

It is known that the immunomodulatory effects of MSCs depend on cell activation by stimulatory molecules in the microenvironment. The main factors leading to MSCs activation are IFN-gamma, TNF-α, and IL-1β (Krampera, 2011). The release and ligation of IFN-gamma to receptors in MSCs are key factors inducing immunomodulatory properties not only in T cells, but also in B and NK cells, which are not responsive to IFN-gamma by itself (Duffy et al., 2011; Franquesa et al., 2012).

The stimulation by TNF-α or IL-1β cause significant modification in MSCs phenotype, which include MHC class I expression and increase in ICAM-1 and VCAM-1 expression (Ren et al., 2010). The combinatory action of IFN-gamma and TNF-α increases IL-6, IL-8, HGF, PGE-2, and cyclo-oxigenase-2 (COX-2) expression in MSCs (Hemeda et al., 2010). IFN-gamma action alone results in induction of MHC class II, indoleamine 2,3-dioxygenase (IDO) and PD-L1 expression (Sheng et al., 2008). Up-regulation of IDO has been shown to have therapeutic potential in preventing GvHD. IDO activity leads to production of kynurenine, a tryptophan breakdown product capable of inducing apoptosis of T cells and suppression of inflammation (Jasperson et al., 2009). Programed death 1 (PD-1) and its ligand (PD-L1) are important players in GVHD, by delivering inhibitory signals avoiding immune mediated tissue damage (Blazar et al., 2003). IFN-gamma and TNF-α co-activation induce expression of chemokines such as CCR5, CCR10, CXCR3, CXCL9, and CXCL10, which are involved in chemotaxis and may inhibit the proliferation of effector cells in the immune system (Ren et al., 2008; English, 2013; Najar et al., 2016). The interplay between pro-inflammatory factors, production/activation of immunomodulatory molecules by MSCs, and ensuing consequences on immune system cells is illustrated in **Figure 2**.

In a recent study using C57BL/6 mice, it was found that 24 h post-injection of umbilical cord MSCs in the tail vein, most cells were dead and located in lungs and liver, with a huge presence of $Ly6C^{low}$ monocytes. *In vitro* assays showed that human monocytes were polarized from a $CD14^{++}/CD16^{-}$ to a $CD14^{++}/CD16^{+}/CD206^{+}$ phenotype after MSCs phagocytosis. These monocytes also expressed PDL-1 and IL-10, while TNF-α was reduced. These modified monocytes after MSCs phagocytosis induced Treg $Foxp3^{+}$ formation, indicating that monocytes play a key role in the MSCs immune modulatory response (de Witte et al., 2018). Some *in vitro* studies showed that MSCs stimulated monocytes to acquire an anti-inflammatory phenotype with IL-10 production (Melief et al., 2013; Deng et al., 2016). Gonçalves et al. (2017) demonstrated that particles derived from the plasma membrane of MSCs were able to induce pro-inflammatory monocytes to apoptosis, thereby modulating the immune response.

Apoptosis seems to be involved in different mechanisms leading to immunosuppression. Another study in a mouse model of GVHD reported that the immunosuppression effect of MSCs depends on the recipient's cytotoxic T cell activity. It was found that highly cytotoxic T cells in the recipient induces MSC apoptosis and that apoptotic MSCs are cleared by recipient's phagocytes. This process induces production of IDO by the phagocytes, thereby promoting immunosuppression (Galleu et al., 2017). In this study, the authors confirmed that the cytotoxic activity of GVHD patient's T cells against MSCs

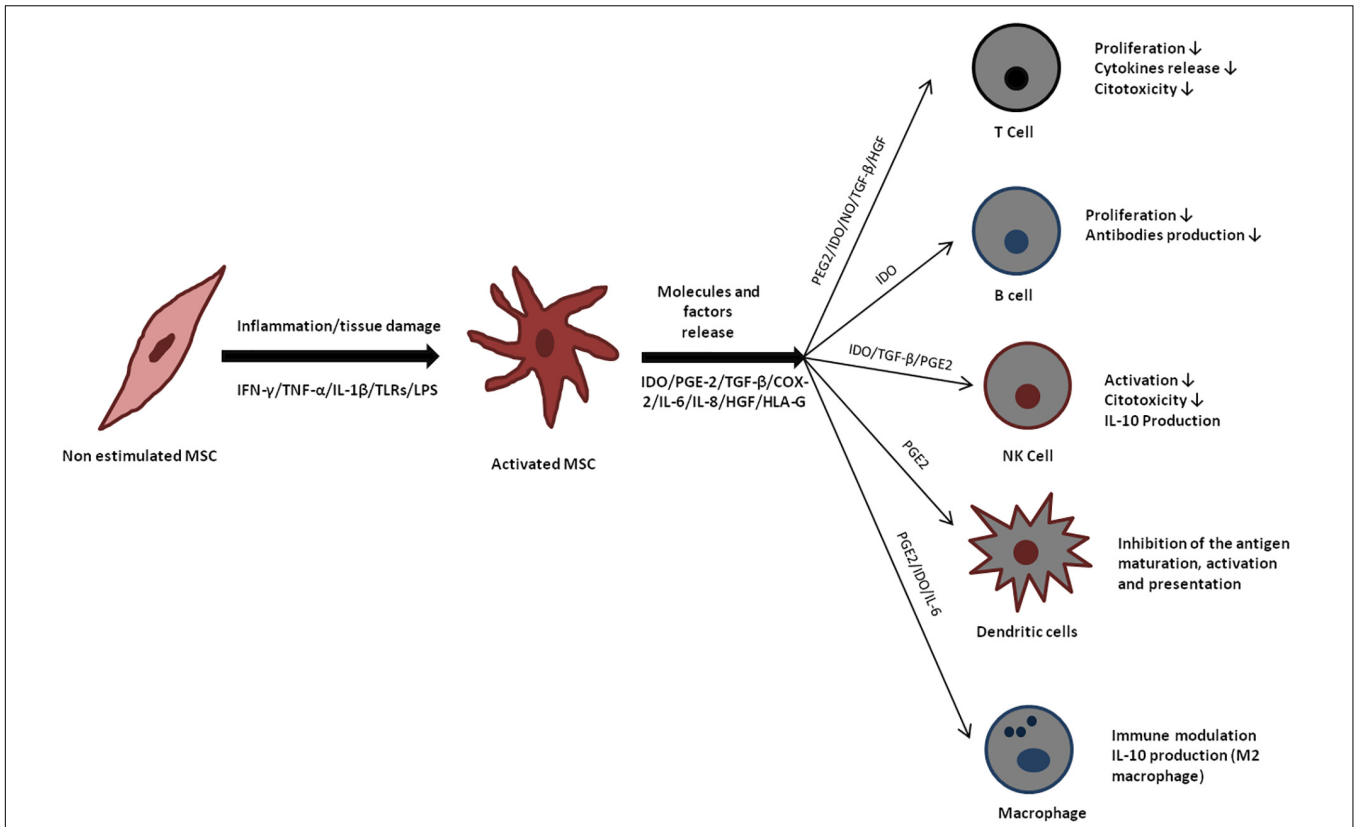

FIGURE 2 | Immunomodulatory effects of mesenchymal stromal cells (MSC). Mesenchymal stromal cells release several molecules that act directly in cells from the immune system, favoring an anti-inflammatory microenvironment.

was positively correlated with clinical response, which led them to propose the use of an *in vitro* cytotoxicity test against MSCs to help screening eligible GVHD patients to undergo treatment with MSCs.

As many signaling pathways involved in MSCs immunomodulatory effects are mediated by soluble factors, cell-free therapy strategies such as those with MSC-derived exosomes are also being considered. Exosomes can carry important active molecules such as cytokines, mRNAs and regulatory miRNAs (Yin et al., 2019). Exosomes released by bone marrow MSCs were reported to improve GVHD in mice by inhibiting CD4 T cells, reducing inflammatory cytokines and increasing IL-10 expressing Treg cells (Lai et al., 2018).

MESENCHYMAL STROMAL CELLS FOR GRAFT VERSUS HOST DISEASE

The better understanding of the MSCs immunomodulatory properties and of the GVHD pathophysiology has supported a rationale for potential benefits of a MSC-based therapy for this condition. Given that MSCs suppress proliferation of activated lymphocytes, reduce IFN-gamma production and upregulate activation markers (Klyushnenkova et al., 2005), in 2004, MSC was first used successfully to treat GVHD (Le Blanc et al., 2004). This first approach from a haploidentical third party donor showed that MSCs treatment could be safe and potentially effective. Since then, MSC has been studied to prove its efficacy, but the results are still controversial, probably as a consequence of variations in trial design.

SOURCE OF MSC

Studies evaluating MSC for GVHD normally use allogeneic MSCs as patients usually don't have clinical condition for this donation. In the beginning, HLA matched donors were selected, however, *in vitro* culture showed similar suppressive effects despite HLA matched (Le Blanc et al., 2003). This finding resulted in studies using third party donors without HLA matched without impact in clinical outcome (Le Blanc et al., 2008; Kebriaei et al., 2009). Third party donor cells have the advantage of prior cryopreservation, allowing cell product availability in just few days after a clinical indication of MSCs treatment.

Most GVHD studies use bone marrow aspirate as a source of MSCs production. Of 30 clinical studies assessed in the literature, 25 used bone marrow MSCs (18 of which for GVHD treatment and seven for GVHD prevention). Four studies using umbilical cord-derived MSC to prevent GVHD and one study using adipose-derived MSCs to treat GVHD have also been reported. These studies have compared MSCs treatment only

with conventional treatment or historical control. Thus far, no studies have compared the impact of different biological sources of MSC in clinical outcome. Variabilities in trial design is one main limitation for carrying out a meta-analysis to determine which biological source is better or if they are all equivalent in terms of adverse effects and therapeutic benefits (Rizk et al., 2016).

SAFETY OF MSC

Although malignant transformation of MSCs is a theoretical risk, a systematic review conducted by a Canadian group in 2012 found no significant association between MSC infusion and tumor formation. Malignancy were detected only in patients with ongoing malignancies or as recurrence events. No *de novo* malignancies have been reported, although the clinical follow-up in the examined trials were rather short, ranging from 3 to 60 months (Lalu et al., 2012).

Another meta-analysis of clinical trials enrolled seven studies with a total of 593 patients (334 treated with MSCs and 259 without MSC treatment). Infusion was safe and well tolerated in all studies (doses ranging from 0.1 to 10 million cells per kg) and there was no report of oncogenesis in the follow-up period. Trials using MSCs prophylactically had a median follow-up of 10–60 months. Follow-up of trials using MSCs as treatment varied from 2 to 23 months (Fisher et al., 2019).

As immunosuppressive treatment can reduce graft-versus-leukemia (GVL) effect, two studies reported relapse of malignant disease in short follow up (Kebriaei et al., 2009; Martin et al., 2010). However, there is insufficient evidence to determine this association with high risk of malignant relapse (RR 0.83, 95%, CI 0.37–1.84; participants 275; studies 2) (Fisher et al., 2019). Also in long term follow up, six trials reported this complication but there is insufficient evidence to associate this risk with MSC infusion (RR 1.08, 95%, CI 0.73–1.59; participants 323; studies 6).

Safety regarding the use of MSCs also involves evaluation of possible complications related to embolism in capillary-rich organs. Among the most common routes of MSCs delivery, including topical application, intramuscular or direct injection into organs, intravenous and intra-arterial infusion, the preferred route is the intravenous (Moll et al., 2019). Despite its simplicity, possible complications are related to embolism or thrombus formation. Indeed, Wu et al. (2017) reported that two patients with renal transplantation and chronic kidney disease presented thromboembolism after infusion of MSCs derived from umbilical cord.

A study by Cui et al. (2015) showed in a mouse model that cerebral blood flow was reduced when infusing high amounts of MSCs, which could lead to more severe embolism events. They also showed that not only the number of MSCs is important for this effect but also the speed of cell infusion. The authors demonstrated that longer infusion times were more related to embolism complications. However, given that the follow-up period of this study was limited to 3 days, the authors suggest that these complications could be a transient event.

In another experimental model, Gleeson et al. (2015) showed that, when infused in female pigs, bone marrow MSCs expressed active tissue factor, a key factor of the soluble coagulation cascade that supports thrombin generation and thrombus formation. To counteract this effect, the authors suggest the use of an antithrombotic therapy when MSCs are administered. Christy et al. (2017) also reported a procoagulant activity of MSCs; they showed that adipose-derived MSCs displayed a higher procoagulant activity than MSCs derived from bone marrow. The authors suggest that MSCs should be tested for coagulation activity and that patients should be monitored for these possible complications.

Altogether these studies indicate that patients in cell therapy protocols should be monitored for possible embolism or thrombus events related to MSCs infusion and, when applicable, antithrombotic therapies could be applied. Preclinical studies like these are of utmost importance to prevent translational failure. To this end, critical parameters such as the therapeutic window, delivery route, type of cells, immunogenicity, comorbidity, and concomitant treatment, should be considered in these studies.

EFFICACY OF MSC

Evidence of efficacy is difficult to determine since the clinical studies published so far are highly heterogeneous. Some studies use MSCs as prophylactic scheme, with infusion in pre-determined days. Six studies analyzed incidence of acute GVHD and other six studies evaluated chronic GVHD (**Tables 1**, **2**). Possible therapeutic benefit from MSC treatment seems to occur in chronic GVHD patients, although the quality of evidence is low as studies had different schemes of infusion (Fisher et al., 2019).

TABLE 1 | Prophylactic trials for acute GVHD.

Study	MSC		No MSC		Risk ratio
	aGVHD events	Total participants	aGVHD events	Total participants	
Ghavamzadeh et al., 2010	6	25	4	23	1.38 (0.45–4.28)
Kuzmina et al., 2012	4	39	8	38	0.49 (0.16–1.48)
Liu et al., 2011	16	27	16	28	1.04 (0.66–1.62)
Mareika et al., 2016	1	10	3	12	0.40 (0.05–3.27)
Ning et al., 2008	5	10	11	15	0.68 (0.34–1.36)
Wu et al., 2013	4	8	8	12	0.75 (0.34–1.67)

Adapted from Fisher et al. (2019).

TABLE 2 | Prophylactic trials for chronic GVHD.

Study	MSC		No MSC		Risk ratio
	cGVHD events	Total participants	cGVH events	Total participants	
Gao et al., 2016	17	62	30	62	0.57 (0.35–0.92)
Kuzmina et al., 2012	5	19	6	18	0.79 (0.29–2.14)
Liu et al., 2011	13	27	19	28	0.71 (0.44–1.13)
Mareika et al., 2016	1	10	4	12	0.30 (0.04–2.27)
Ning et al., 2008	4	10	5	15	1.20 (0.42–3.41)
Wu et al., 2013	1	8	5	12	0.30 (0.04–2.11)

Adapted from Fisher et al. (2019).

Only two controlled trials evaluated efficacy in acute GVHD treatment without difference in clinical manifestation in both groups (MSCs versus no MSCs). In one study with pediatric patients, complete and partial responses were reported in 58 and 17% patients, respectively (RR 2.0, 95%, CI 0.20–19.6 participants 28). One trial evaluated MSC for treatment of chronic GVHD in 40 patients. In this study, complete and partial responses were observed in 15 and one patients, respectively (RR 5.0, 95%, CI 0.75–33.21) (Fisher et al., 2019).

As for safety, the cell administration route is a variable potentially impacting therapeutic effects. MSC systemic delivery is one of the most common administration route in cell therapy since it does not require an invasive procedure but, on the other hand, it relies on a transendothelial cell migration process toward the lesion site, which could have a direct influence in the treatment efficacy Nitzsche et al. (2017). MSCs take a longer time to complete diapedesis than leukocytes (Teo et al., 2012) and this process is not improved by increased persistence of circulating MSC, affecting the amount of cells reaching the targeted organs (Schmidt et al., 2006).

Mesenchymal stromal cells should be able to exit circulation and migrate to tissues/organs in order to repair local lesions due to GvHD. This process initially involves a contact between the cells and the endothelium and, subsequently, transmigration toward the target tissue/organ. MSCs rolling depends on presence of platelets (Teo et al., 2015) and different ligands such as P-selectin (Rüster et al., 2006), glycoproteins and galectin-1 (Suila et al., 2014). MSCs also express a variety of integrins, which can be responsible for the rolling process (Nitzsche et al., 2017). After this first step, MSCs should adhere firmly to the endothelium, which is supported by the expression of various chemokines such as CCR2, CCR4, CCR7, CCR10, CXCR5, CXCR6, and CXCR4 (Andreas et al., 2014); immediately after adhesion, MSCs reorganize the cytoskeleton inducing a polarization before transmigration (Belema-Bedada et al., 2008). MSCs migration to the subendothelial space is mediated by integrins and metalloproteinases that breakdown the basal lamina (Cheng et al., 2012).

Enhancing migration and homing of MSCs to target tissue/organ could be achieved by genetic engineering these cells to increase the expression of chemokines, integrins or selectins (Nowakowski et al., 2013). This approach could help increasing the effectiveness of MSC in GvHD patients.

DOSE OF MSC

One of the earliest trials of MSC infusions in humans occurred in 1995 (Lazarus et al., 1995), in which patients with hematologic malignancies and in complete remission received a one-time infusion of autologous bone marrow MSC at doses of 1, 5, or 50×10^6 cells. Since this pioneer study, many others have been performed over the last decade establishing an excellent safety profile for both, autologous and allogeneic MSC infusions, over a range of cell doses and different schemes (**Table 3**).

For most clinical indications, human MSCs are transfused intravenously at doses typically in the one to two million cells per kilogram. For instance, Le Blanc et al. (2008) treated 55 acute GVHD patients with a median dose of 1.4×10^6 cells per kg. Almost a half of them received two doses and six patients, three to five doses. Complete responders had lower transplantation-related mortality 1 year after infusion than patients with partial or no response, as well as higher overall survival 2 years after hemopoietic stem cell transplantation (Le Blanc et al., 2008).

The Osiris trial Protocol 260 compared two different doses of MSCs for acute GVHD. Patients were randomized in two arms: high-dose (8×10^6 MSC/kg) or low dose (2×10^6 MSC/kg). Patients were stratified for dose levels between grades II and grades III/IV of acute GVHD. Standard steroid therapy using glucocorticosteroids and cyclosporine or tacrolimus, was continued at therapeutic dose levels. Seventy seven percent of patients had complete response and 16% partial response (RR 0.76 CI 0.51–1.14 for complete response, RR 11.69 CI 0.7–194.79) without difference in both arms (Kebriaei et al., 2009; Fisher et al., 2019).

For chronic GVHD patients, a team from Karolinska University Hospital, Stockholm, Sweden, reported a study with 11 patients who received six doses of 2×10^6 MSC/kg, at a 4–6-weeks interval. Patients who responded and tolerated the initial six dose regimen received one to three additional MSC doses. Two patients have discontinued all systemic immunosuppression and another two patients were free of steroids and tapering calcineurin inhibitors. The median follow-up time of this study was 29 months. Quality of life was evaluated using the FACT-BMT questionnaire, and responders showed a mean increase in FACT-BMT total score of 6.6 points, or 8%, compared to baseline values, at last follow-up (Von Bahr et al., 2015).

TABLE 3 | Dosing scheme of selected clinical studies testing MSCs for treatment of GVHD.

References	Acute or chronic	Source of MSCs	Dose	Scheme
von Bonin et al., 2009	aGVHD	Allogeneic	0.9×10^6/kg	Weekly
Jitschin et al., 2013	aGVHD	Allogeneic	2×10^6/kg	Single dose
Shipounova et al., 2014	aGVHD	Allogeneic	1.2×10^6/kg	Single dose
Maziarz et al., 2015	aGVHD	Allogeneic	$1–10 \times 10^6$/kg	Single dose or weekly
Kebriaei et al., 2009	aGVHD	Allogeneic	2 or 8×10^6/kg	Two infusions
Gao et al., 2016	cGVDH	Allogeneic	3×10^7/kg	Monthly (four times)
Kuçi et al., 2016	aGVHD	Allogeneic	$50–129 \times 10^6$	Not specified
Yin et al., 2014	aGVHD	Allogeneic	2×10^6/kg	Weekly (three times)
Bader et al., 2018	aGVHD	Allogeneic	$1–2 \times 10^6$/kg	Weekly (one to four times)
Salmenniemi et al., 2017	aGVHD or cGVHD	Allogeneic	2×10^6/kg	Once or biweekly (six times)
Dotoli et al., 2017	aGVHD	Allogeneic	2×10^6/kg	Weekly (three times)
von Dalowski et al., 2016	aGVHD	Allogeneic	0.99×10^6/kg	Two times
Kurtzberg et al., 2014	aGVHD	Allogeneic	2 × 10e6/kg	Biweekly (eight times)

A recent Cochrane review identified 12 studies and 13 ongoing trials involving adult or pediatric patients with GvHD (acute or cronic). In these studies, patients were treated with MSCs doses ranging from 10^5 to 10^7 cells/kg, but no differences in clinical outcome could be associated with MSC dose (Fisher et al., 2019).

PRODUCTION OF MSCs UNDER GMP CONDITIONS FOR CLINICAL USE

The MSCs anti-inflammatory properties as well as homing to sites of inflammation, immunomodulatory properties, and trophic influence on tissue repair, have made these cells very popular for clinical studies (Trounson and McDonald, 2015; de Witte et al., 2018). Up to February 2019, there were 936 registered clinical trials using MSCs with 181 recruiting status (**Figure 3**). Most of these MSCs clinical trials use allogeneic cells and these trials have the highest activity in United States, Europe, and China. Conditions more frequently indicated for MSC therapy include degenerative osteoarthritis, defect of articular cartilage, rheumatoid arthritis, GVHD, sickle cell disease, thalassemia, leukemia, nephrotic syndrome, liver cirrhosis, diabetes mellitus, lupus, Crohn's Disease, multiple sclerosis, amyotrophic lateral sclerosis, autism spectrum disorder, ischemic heart disease, among many others. Although there are a high number of ongoing clinical studies, only few MSC commercial products are approved by regulatory agencies (**Table 4**).

Currently, distinct strategies are used to produce human MSCs *ex vivo* for clinical purposes, as an alternative method for regenerative therapy in many diseases. In any case, regulatory issues related to the safety, efficacy and quality of MSC therapies must be considered while preparing a cell- or tissue-based product for clinical and commercial use. Quality assurance (QA) and quality control (QC) must be provided in any cell manipulation under good manufacturing practices (GMP) grade.

Thus far, there is no consensus on the production of MSCs regarding GMP system. The QC standards may be discussed individually on each project following the local regulatory agency authority. In Brazil, the regulatory agency (ANVISA – Agência Nacional de Vigilância Sanitária), establish specific criteria for advanced therapy products to assure the quality, safety and efficacy of cell therapy in the country (Resolutions: RDC 214/2018 and RDC 260/2018). Cell viability, cytogenetics, potency tests and sterility tests to verify contamination by mycoplasma, aerobic and anaerobic bacteria, fungus, and special microorganisms such as filamentous fungus, are routinely used to ensure the quality of the cell product. Main GMP issues addressed by regulatory agencies in different countries are comparable and can be assessed in **Supplementary Table S1**.

According to the Foundation for the Accreditation of Cell Therapy (FACT) and Brazil's ANVISA, cytogenetic testing is mandatory to release a cell product. Most manufacturing units perform karyotyping on the final cell product and the results are an important component of the release criteria. In case of any chromosomal abnormalities in the manipulated cells, the incoming samples may be karyotyped to ensure that the donor does not have any constitutive chromosomal abnormalities and that the alteration has probably originated in a cell clone during *ex vivo* expansion. A study by Nikitina and colleagues estimated that around 10% of MSC samples contain chromosomal aberrations after expansion, although the clinical consequences of such aberrations are unknown (Nikitina et al., 2018).

However, since the potential clinical impact of minor changes in karyotyping is difficult to evaluate, not all services consider the cytogenetic testing as a release criterion (Philippe et al., 2010; Tarte et al., 2010; Hanley et al., 2013).

There are many different variations in existing manufacturing protocols for MSC production that may influence the final characteristics of the cells. The type of media supplementation is a typical example. Fetal bovine serum (FBS) is the most common supplementation used. FBS concentration in media ranges from 2 to 20%, with 10% FBS being the most common concentration. This variability on FBS concentration may result in different amounts of growth factors to stimulate cell survival and proliferation (Carmen et al., 2012; Mendicino et al., 2014). Xeno-free media supplementation is also used, including human platelet lysate and human serum. Some cell culture media are fully

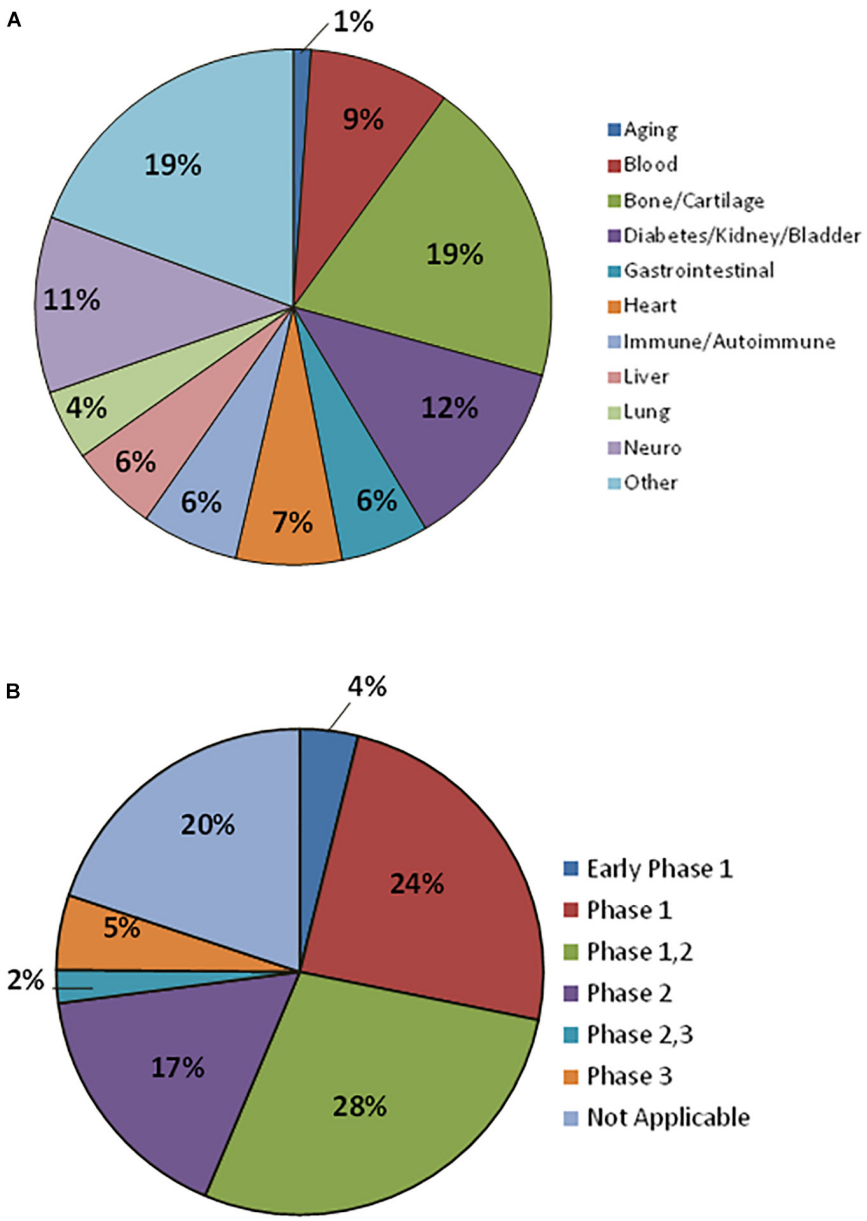

FIGURE 3 | Mesenchymal stromal cells in Clinical Trials. **(A)** Therapeutic indications being addressed with MSCs. **(B)** MSCs Clinical Trials classified by Clinical Phase. Data for 181 registered clinical trials with recruiting status.

defined and do not require extra supplementation. However, the lack of serum or platelet lysate could impair MSCs attachment to the surface and there is a need of a coating substrate that is derived from animal or human tissue. In our GMP unit, we have validated the use autologous serum (patient-specific) to expand MSC products for infusion in patients enrolled in official clinical protocols. Under such condition, typically, a 20 mL bone marrow aspirate yields 10 million MSCs after three cell culture passages.

Many efforts have been made to develop technologies to achieve production of adequate number of cells with high therapeutic quality. *Ex vivo* MSC expansion may be performed by conventional cell culture techniques or by using bioreactors. Considering autologous use, it is possible to produce lower cell quantity and the scale-out approach can be performed, using planar culture systems with multiple flasks in cell factories. For allogeneic use, MSCs can be expanded to a large number of cells in bioreactor systems (scale-up approach) and cryopreserved in cell banks for future use (Pittenger et al., 1999; dos Santos et al., 2013; Mizukami and Swiech, 2018). However, it is critical to determine cell viability, potency and sterility post-thawing to validate the cryopreservation method before routine implementation (Galipeau, 2013; Mendicino et al., 2014).

Monolayer culture is the traditional technique for MSC expansion; however, excessive manipulation may interfere in

TABLE 4 | Commercially approved MSCs-based products.

Medicinal product	Company	hMSC type	Clinical indication	Marketing authorization
Allostem	AlloSource	Allogeneic hASC	Bone regeneration	FDA (United States)
Cartistem	Medipost	Allogeneic UCB-MSC	Traumatic and degenerative osteoarthritis	SKFDA (Korea)
HeartiCellgram	FCB-Pharmicell	Autologous BM-MSC	Acute myocardial infarction	SKFDA (Korea)
Grafix	Osiris Therapeutics	Allogeneic BM-MSC	Soft tissue defects (acute and chronic wounds)	FDA (United States)
Prochymal	Mesoblast	Allogeneic BM-MSC	GVHD	Health Canada (Canada); Medsafe (New Zealand)
OsteoCel	NuVasive	Allogeneic BM-MSC	Spinal bone regeneration	FDA (United States)
OvationOD	Osiris Therapeutics	Allogeneic BM-MSC	Bone regeneration	FDA (United States)
Cartiform	Osiris Therapeutics	Allogeneic BM-MSC	Cartilage repair	FDA (United States)
Stravix	Osiris Therapeutics	Allogeneic BM-MSC	Wound repair	FDA (United States)
Cupistem	Anterogen	Autologous hASC	Crohn's fistula, reduce inflammation and regenerate damaged joint tissues	SKFDA (Korea)
TEMCELL	Mesoblast	Allogeneic BM-MSC	Acute radiation injury, Crohn's disease, GVHD, type I diabetes, and myocardial infarction	PMDA (Japan); Health Canada (Canada); Medsafe (New Zealand)
Trinity evolution	Orthofix	Allogeneic BM-MSC	Bone regeneration	FDA (United States)
Trinity elite	Orthofix	Allogeneic BM-MSC	Bone regeneration	FDA (United States)
QueenCell	Anterogen	Autologous hASC	Regeneration of subcutaneous adipose tissue	SKFDA (Korea)
Ossron	RMS	Autologous BM-MSC	Bone regeneration	SKFDA (Korea)
Alofisel	TiGenix/Takeda	Allogeneic hASC	Complex perianal fistulas	EMA (Denmark)

the functional properties of cells due to enzymatic treatments in many passages, higher contamination risk due to intense manipulation, prolonged culture to generate adequate amounts of cells, impairing cell physiology. Scale-up based cell expansion meets the criteria for GMP with quality standards, allowing monitoring of pH, temperature, carbon dioxide, and oxygen concentration over time, and maintenance of cells behavior (adherent or suspension cells). There are several bioreactors available for cell expansion such as stirred tank bioreactor, rocking bioreactor, hollow fiber bioreactor, and fixed bed bioreactor. The choice of bioreactor will depend on the aim to be achieved, i.e., final amount of cells required for infusion, type of cell (adherent or suspension growing cells), mid/long term use, etc (Jung et al., 2012; Clarke, 2013; Mizukami and Swiech, 2018).

After MSC expansion, cells are harvested by using an enzyme, usually trypsin. Since this enzyme is normally of porcine origin, alternative GMP recombinant enzymes are available and their use should be prioritized. Mechanical detachment of cells cultured under GMP facilities is also possible by using a cell scraper tool, although detachment must be done gently in order to avoid cell damage and death. While enzymatic MSC detachment can be adapted in expansion protocols using bioreactors, the same adaptation is harder to achieve for mechanical detachment techniques.

When high MSC doses and multiple cell infusions are required, which is typically the case of GVHD treatment, cryopreservation of previously expanded cells is an optimal solution to have high amounts of GMP-grade cells readily available for infusion. Nonetheless, cryopreservation is a critical step in MSC manipulation. Although either cryopreservation bags or regular cryotubes could be used for this purpose, the use of the latter is limited by the low volume of cell preparation that can be stored per vial (usually 1.5–2 mL). Cryopreservation media usually contain DMSO as cryoprotectant but it can cause some adverse effects when infused in patients. Typically, cells are stored using a ratio of 10% DMSO and 90% of serum. Thus, after thawing, some laboratories centrifuge and wash cells to remove DMSO. An alternative is to use cryoprotectants containing methylcellulose, sucrose, trehalose, glycerol, hydroxyethyl starch, and polyvinylpyrrolidone. Some companies already developed serum-free and xeno-free cryopreservation media (e.g., Cryostor – StemCell Technologies, Plasmalyte-A – Baxter and Synth-a-Freeze – Gibco) to circumvent toxicity. Importantly, validation of progressive freezing and thawing cycles is required to avoid significant loss of cell viability.

Despite the lack of consensus in critical steps of MSCs production under GMP conditions, different protocols are available to attain high yields of expanded MSCs. However, these *ex vivo* expanded MSC need to meet the quality standards required by regulatory agencies.

INFORMATIVE MSCs POTENCY ASSAYS FOR USE IN GVHD TREATMENT

Cellular products intended for clinical use must also pass functional evaluation, a key part of a GMP quality control program. Currently, there is no specific release criterion required by the authorities for testing MSC potency. The most used and accepted potency tests for MSCs evaluate their ability to differentiate into three different cell types (osteoblasts, adipocytes, and chondroblasts), to inhibit T-lymphocyte proliferation or to promote endothelial tube formation.

All these tests involve *in vitro* assays that can be easily adapted for routine screening of cell preparations. Nonetheless, adoption of different assays for extensive functional characterization of

cells is expensive and time consuming, which may delay the availability of freshly produced MSCs for infusion in patients at critical clinical conditions. Alternatively, a key test could be performed to evaluate a specific MSC property that is correlated with the aimed therapeutic effect. To this end, knowledge of the mechanism of cell action *in vivo* is of fundamental importance, which reinforces the relevance of basic stem cell biology studies.

For instance, exploration of MSC immunosuppressive potential is well established in immunological-based diseases such as diabetes, multiple sclerosis, and GVHD. For this type of application, assaying MSC immunomodulatory activity would better suit a potency test then assaying MSC multipotency. This could be achieved by co-culture assays with T cells to evaluate MSC effects on T cell proliferation and/or by cytokine release by MSC (Wuchter et al., 2015; Mizukami and Swiech, 2018).

Di Nicola et al. (2002) showed that MSCs were able to suppress T-cell proliferation when these cells were added to mixed lymphocyte reactions. They also demonstrated that MSCs were able to inhibit both $CD4^+$ and $CD8^+$ cells. This study suggested that cell-cell contact between MSC and effector cells was not necessary to inhibit T-lymphocyte proliferation (Di Nicola et al., 2002). However, cell-cell contact was important for T-cell arrest in G0 phase of the cell cycle (Glennie et al., 2005). MSC do not seem to induce T-cell apoptosis *in vitro* (Krampera et al., 2006).

The immuno-suppressive activity of MSCs may also be determined after exposure to IFN-gamma. In this assay, presence of primed MSC could be indicated by expression of MHC class I, MHC II, PDL-1, or other modulatory molecules. Release of certain chemokine receptors such as CXCR3, CXCR4, CXCR5, and CCR7 could also be assayed to indicate immunomodulatory active MSC (Krampera et al., 2013). As previously addressed, potency tests based on the cytotoxicity of recipient's cells toward donor MSCs is another example of informative test associated with clinical response of GVHD patients (Galleu et al., 2017). Incorporation of such assays in the routine QA tests is feasible, since it would only require basic flow cytometry and ELISA platforms, or similar alternatives.

Specific MSC properties involved in their expected therapeutic effect may be affected by different factors, including cryopreservation (Weise et al., 2014). Thus, proper potency assays are also valuable to assess stability of cell therapy products at different storage conditions over time. Donor age is another factor that influences MSC activity. Stolzing et al. (2008) analyzed expression of cell surface markers, oxidative cell damage and senescence in MSCs derived from adults and children. They have found a reduction in CFU-F (colony forming unit-fibroblast) generation and in the subset of $CD45^{low}/D7fib^{+ve}/LNGF^{+ve}$ cells in samples derived from adults compared with samples derived from children. MSCs obtained from elderly people also showed increased levels of ROS, p21, and p53 proteins. The authors suggested that active MSCs derived from bone marrow decrease in number with age and that these cells are not as potent as the ones isolated from younger patients. Such aging effect on MSC properties should be considered when defining inclusion criteria for MSC donors in cell therapy protocols.

CONCLUDING REMARKS

Evidences in the literature support safety of MSCs treatment for GVHD, whereas efficacy of this type of cell therapy still needs further clarification. Efficacy of MSC-based treatment is more evident for chronic GVHD patients. Double-blind randomized controlled trials with steroid-refractory GVHD patients should be designed to better address MSCs efficacy. Well-defined inclusion/exclusion criteria for patient accrual and a standardized protocol for GMP production of MSCs should facilitate multicenter studies and acquisition of faster results for clinical outcome assessment. Regarding MSCs production, uniformity in donor age and automated *ex vivo* expansion of cells should help minimize product variability. Also, the choice of potency test is critical in evaluating suitability of the final product, since the desirable therapeutic effect may differ among clinical trials. In the case of GVHD, potency tests addressing MSCs immunomodulatory activity are key factors for obtaining high quality MSCs for therapeutic purposes. Allogeneic MSCs transplantation seem highly appropriate for GVHD treatment, due to the poor clinical condition of patients for tissue donation and lack of necessity of HLA match. In this scenario, the clinical use of cryopreserved third party MSC products offers the additional advantage of faster cell product availability, compared with autologous transplantation. Additional tests to address the cytotoxicity of recipient's cells toward allogeneic MSCs should help refining the selection of eligible patients. Distribution of MSC products stored in cryobanks to different hospitals is also feasible, allowing potential therapeutic benefits for a greater number of patients in need.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Andreas, K., Sittinger, M., and Ringe, J. (2014). Toward in situ tissue engineering: chemokine-guided stem cell recruitment. *Trends Biotechnol.* 32, 483–492. doi: 10.1016/j.tibtech.2014.06.008

Appelbaum, F. R. (2001). Hematopoietic cell transplantation as immunotherapy. *Nature* 411, 385–389.

Arora, M., Cutler, C. S., Jagasia, M. H., Pidala, J., Chai, X., Martin, P. J., et al. (2016). Late acute and chronic graft-versus-host disease after allogeneic hematopoietic cell transplantation. *Biol. Blood Marrow Transpl.* 22, 449–455. doi: 10.1016/j.bbmt.2015.10.018

Bader, P., Kuçi, Z., Bakhtiar, S., Basu, O., Bug, G., and Dennis, M. (2018). Effective treatment of steroid and therapy-refractory acute graft-versus-host disease with a novel mesenchymal stromal cell product (MSC-FFM). *Bone Marrow Transplant.* 53, 852–862. doi: 10.1038/s41409-018-0102-z

Belema-Bedada, F., Uchida, S., Martire, A., Kostin, S., and Braun, T. (2008). Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2. *Cell Stem Cell.* 5, 566–575. doi: 10.1016/j.stem.2008.03.003

Billingham, R. E. (1966). The biology of graft-versus-host reactions. *Harvey Lect.* 62, 21–78.

Blazar, B. R., Carreno, B. M., Panoskaltsis-Mortari, A., Carter, L., Iwai, Y., Yagita, H., et al. (2003). Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. *J. Immunol.* 171, 1272–1277. doi: 10.4049/jimmunol.171.3.1272

Carmen, J., Burger, S. R., McCaman, M., and Rowley, J. A. (2012). Regen. *Medcine.* 7, 85–100. doi: 10.2217/rme.11.105

Cheng, H., Reddy, A., Sage, A., Lu, J., Garfinkel, A., Tintut, Y., et al. (2012). Focal high cell density generates a gradient of patterns in self-organizing vascular mesenchymal cells. *J. Vasc. Res.* 49, 441–446. doi: 10.1159/000339568

Christy, B. A., Herzig, M. C., Montgomery, R. K., Delavan, C., Bynum, J. A., Reddoch, K. M., et al. (2017). Procoagulant activity of human mesenchymal stem cells. *J. Trauma Acute Care. Surg.* 83(1 Suppl. 1), S164–S169. doi: 10.1097/TA.0000000000001485

Clarke, D. (2013). Single-use technologies in cell therapy. *Bioprocess. Intern.* 11(Supp. 1).

Cohen, J. L., and Boyer, O. (2006). The role of CD4+ CD25 hi regulatory T cells in the physiopathogeny of graft-versus-host disease. *Curr. Opin. Immunol.* 18, 580–585.

Corcione, A., Benvenuto, F., Ferretti, E., Giunti, D., Cappiello, V., Cazzanti, F., et al. (2006). Human mesenchymal stem cells modulate B-cell functions. *Blood* 107, 367–372.

Cui, L. L., Kerkelä, E., Bakreen, A., Nitzsche, F., Andrzejewska, A., Nowakowski, A., et al. (2015). The cerebral embolism evoked by intra-arterial delivery of allogeneic bone marrow mesenchymal stem cells in rats is related to cell dose and infusion velocity. *Stem Cell Res. Ther.* 27:11. doi: 10.1186/scrt544

de Witte, S. F. H., Luk, F., Sierra Parraga, J. M., Gargesha, M., Merino, A., Korevaar, S. S., et al. (2018). I*mmunomodulation* by therapeutic mesenchymal stromal Cells (MSC) is triggered through phagocytosis of msc by monocytic cells. *Stem Cells* 36, 602–615. doi: 10.1002/stem.2779

Deng, Y., Zhang, Y., Ye, L., Zhang, T., Cheng, J., Chen, G., et al. (2016). Umbilical cord-derived mesenchymal stem cells instruct monocytes towards an IL10-producing phenotype by secreting IL6 and HGF. *Sci. Rep.* 6:37566. doi: 10.1038/srep37566

Di Nicola, M., Carlo-Stella, C., Magni, M., Milanesi, M., Longoni, P. D., Matteucci, P., et al. (2002). Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 99, 3838–3843.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., et al. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. the international society for cellular therapy position statement. *Cytotherapy* 8, 315–317.

dos Santos, F. F., Andrade, P. Z., da Silva, C. L., and Cabral, J. M. S. (2013). Bioreactor design for clinical-grade expansion of stem cells,". *Biotechnol. J.* 8, 644–654.

Dotoli, G. M., De Santis, G. C., Orellana, M. D., de Lima Prata, K., Caruso, S. R., Fernandes, T. R., et al. (2017). Mesenchymal stromal cell infusion to treat steroid-refractory acute GvHD III/IV after hematopoietic stem cell transplantation. *Bone Marrow Transplant.* 52, 859–862. doi: 10.1038/bmt.2017.35

Duffy, M. M., Ritter, T., Ceredig, R., and Griffin, M. D. (2011). Mesenchymal stem cell effects on T-cell effector pathways. *Stem Cell Res Ther.* 2:34. doi: 10.1186/scrt75

English, K. (2013). Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol. Cell Biol.* 91, 19–26. doi: 10.1038/icb.2012.56

Erices, A., Conget, P., and Minguell, J. J. (2000). Mesenchymal progenitor cells in human umbilical cord blood. *Br. J. Haematol.* 109, 235–242.

Fauza, D. (2004). Amniotic fluid and placental stem cells. *Best Pract. Res. Clin. Obstet. Gynaecol.* 18, 877–891.

Filipovich, A. H., Weisdorf, D., Pavletic, S., Socie, G., Wingard, J. R., Lee, S. J., et al. (2005). National institutes of health consensus development project on criteria for clinical trials in chronic graft-versus-host disease: i. diagnosis and staging working group report. *Biol. Blood Marrow Transpl.* 11, 945–956.

Fisher, S. A., Cutler, A., Doree, C., Brunskill, S. J., Stanworth, S. J., Navarrete, C., et al. (2019). Mesenchymal stromal cells as treatment or prophylaxis for acute or chronic graft-versus-host disease in haematopoietic stem cell transplant (HSCT) recipients with a haematological condition. *Cochrane Database Syst. Rev.* 30:CD009768.

Flomenberg, N., Baxter-Lowe, L. A., Confer, D., Fernandez-Vina, M., Filipovich, A., Horowitz, M., et al. (2004). Impact of HLA class I and class II high-resolution matching on outcomes of unrelated donor bone marrow transplantation: HLA-C mismatching is associated with a strong adverse effect on transplantation outcome. *Blood* 104, 1923–1930.

Flowers, M. E. D., and Martin, P. J. (2015). How we treat chronic graft-versus-host disease. *Blood* 125, 606–615. doi: 10.1182/blood-2014-08-551994

Franquesa, M., Hoogduijn, M. J., Bestard, O., and Grinyó, J. M. (2012). Immunomodulatory effect of mesenchymal stem cells on B cells. *Front. Immunol.* 3:212. doi: 10.3389/fimmu.2012.00212

Friedenstein, A. J., Petrakova, K. V., Kurolesova, A. I., and Frolova, G. P. (1968). Heterotopic of bone marrow. *Anal. Precursor Cells Osteogenic Hematopoietic Tissues. Transpl.* 6, 230–247.

Galipeau, J. (2013). The mesenchymal stromal cells dilemma–does a negative phase III trial of random donor mesenchymal stromal cells in steroid-resistant graft-versus-host disease represent a death knell or a bump in the road? *Cytotherapy* 15, 2–8. doi: 10.1016/j.jcyt.2012.10.002

Galleu, A., Riffo-Vasquez, Y., Trento, C., Lomas, C., Dolcetti, L., Cheung, T. S., et al. (2017). Apoptosis in mesenchymal stromal cells induces in vivo recipient-mediated immunomodulation. *Sci. Transl. Med.* 15:eaam7828. doi: 10.1126/scitranslmed.aam7828

Gao, L., Zhang, Y., Hu, B., Liu, J., Kong, P., Lou, S., et al. (2016). Phase II multicenter, randomized, double-blind controlled study of efficacy and safety of umbilical cord-derived mesenchymal stromal cells in the prophylaxis of chronic graft-versus-host disease after HLA-haploidentical stem-cell transplantation. *J. Clin. Oncol.* 34, 2843–2850. doi: 10.1200/JCO.2015.65.3642

Ghavamzadeh, A., Alimoghaddam, K., Hamidieh, A. A., Karimi, A., Bashtar, M., and Shamshiri, A. R. (2010). Co-transplantation of HLA-matched related donors culture-expanded mesenchymal stromal cells and hematopoietic stem cells in thalassemia major patients. *Biol. Blood Marrow Trans.* 16(2 Suppl. 2), S214. doi: 10.1016/j.bbmt.2013.12.564

Gleeson, B. M., Martin, K., Ali, M. T., Kumar, A. H., Pillai, M. G., Kumar, S. P., et al. (2015). Bone marrow-derived mesenchymal stem cells have innate procoagulant activity and cause microvascular obstruction following intracoronary delivery: amelioration by antithrombin therapy. *Stem Cells* 33, 2726–2737. doi: 10.1002/stem.2050

Glennie, S., Soeiro, I., Dyson, P. J., Lam, E. W., and Dazzi, F. (2005). Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 105, 2821–2827.

Gonçalves, F. D. C., Luk, F., Korevaar, S. S., Bouzid, R., Paz, A. H., López-Iglesias, C., et al. (2017). Membrane particles generated from mesenchymal stromal cells modulate immune responses by selective targeting of pro-inflammatory monocytes. *Sci. Rep.* 7:12100. doi: 10.1038/s41598-017-12121-z

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 97, 13625–11630.

Halfon, S., Abramov, N., Grinblat, B., and Ginis, I. (2011). Markers distinguishing mesenchymal stem cells from fibroblasts are downregulated with passaging. *Stem Cells Dev.* 20, 53–66. doi: 10.1089/scd.2010.0040

Hanley, P. J., Mei, Z., Cabreira-Hansen, M. G., Klis, M., Li, W., Zhao, Y., et al. (2013). Manufacturing mesenchymal stromal cells for phase I clinical trials. *Cytotherapy* 1, 416–422.

Hemeda, H., Jakob, M., Ludwig, A. K., Giebel, B., Lang, S., and Brandau, S. (2010). Interferon-gamma and tumor necrosis factor-alpha differentially affect cytokine expression and migration properties of mesenchymal stem cells. *Stem Cells Dev.* 19, 693–706. doi: 10.1089/scd.2009.0365

Horwitz, E. M., and Dominici, M. (2008). How do mesenchymal stromal cells exert their therapeutic benefit? *Cytotherapy* 10, 771–774. doi: 10.1080/14653240802618085

Horwitz, E. M., Le Blanc, K., Dominici, M., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., et al. (2005). International society for cellular therapy. clarification of the nomenclature for MSC: the international society for cellular therapy position statement. *Cytotherapy* 7, 393–395.

Jamil, M. O., and Mineishi, S. (2015). State-of-the-art acute and chronic GVHD treatment. *Int. J. Hematol.* 101, 452–466. doi: 10.1007/s12185-015-1785-1

Jasperson, L. K., Bucher, C., Panoskaltsis-Mortari, A., Mellor, A. L., Munn, D. H., and Blazar, B. R. (2009). Inducing the tryptophan catabolic pathway, indoleamine 2,3-dioxygenase (IDO), for suppression of graft-versus-host disease (GVHD) lethality. *Blood* 114, 5062–5070. doi: 10.1182/blood-2009-06-227587

Jitschin, R., Mougiakakos, D., Von Bahr, L., Völkl, S., Moll, G., Ringden, O., et al. (2013). Alterations in the cellular immune compartment of patients treated with third-party mesenchymal stromal cells following allogeneic hematopoietic stem cell transplantation. *Stem Cells* 31, 1715–1725. doi: 10.1002/stem.1386

Jones, E. A., Kinsey, S. E., English, A., Jones, R. A., Straszynski, L., Meredith, D. M., et al. (2002). Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arth. Rheum.* 46, 3349–3360.

Jung, S., Panchalingam, K. M., Wuerth, R. D., Rosenberg, L., and Behie, L. A. (2012). Large-scale production of human mesenchymal stem cells for clinical applications. *Biotechnol. Appl. Biochem.* 59, 106–120. doi: 10.1002/bab.1006

Junker, J. P., Sommar, P., Skog, M., Johnson, H., and Kratz, G. (2010). Adipogenic, chondrogenic and osteogenic differentiation of clonally derived human dermal fibroblasts. *Cells Tissues Organs.* 191, 105–118. doi: 10.1159/000232157

Kassis, I., Zangi, L., Rivkin, R., Levdansky, L., Samuel, S., Marx, G., et al. (2006). Isolation of mesenchymal stem cells from G-CSF-mobilized human peripheral blood using fibrin microbeads. *Bone Marrow Transpl.* 37, 967–976.

Kaviani, A., Perry, T. E., Dzakovic, A., Jennings, R. W., Ziegler, M. M., and Fauza, D. O. (2001). The amniotic fluid as a source of cells for fetal tissue engineering. *J. Pediatr. Surg.* 36, 1662–1665.

Kebriaei, P., Isola, L., Bahceci, E., Holland, K., Rowley, S., McGuirk, J., et al. (2009). Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. *Biol. Blood Marrow Transpl.* 15, 804–811. doi: 10.1016/j.bbmt.2008.03.012

Klyushnenkova, E., Mosca, J. D., Zernetkina, V., Majumdar, M. K., Beggs, K. J., Simonetti, D. W., et al. (2005). T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance and suppression. *J. Biomed. Sci.* 12, 47–57.

Krampera, M. (2011). Mesenchymal stromal cell 'licensing': a multistep process. *Leukemia* 25, 1408–1414. doi: 10.1038/leu.2011.108

Krampera, M., Cosmi, L., Angeli, R., Pasini, A., Liotta, F., Andreini, A., et al. (2006). Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 24, 386–398.

Krampera, M., Galipeau, J., Shi, Y., Tarte, K., and Sensebe, L. (2013). MSC committee of the international society for cellular therapy (ISCT). immunological characterization of multipotent mesenchymal stromal cells–the international society for cellular therapy (ISCT) working proposal. *Cytotherapy* 15, 1054–1061. doi: 10.1016/j.jcyt.2013.02.010

Kuçi, Z., Bönig, H., Kreyenberg, H., Bunos, M., Jauch, A., Janssen, J. W., et al. (2016). Mesenchymal stromal cells from pooled mononuclear cells of multiple bone marrow donors as rescue therapy in pediatric severe steroid-refractory graft-versus-host disease: a multicenter survey. *Haematologica* 101, 985–994. doi: 10.3324/haematol.2015.140368

Kurtzberg, J., Prockop, S., Teira, P., Bittencourt, H., Lewis, V., Chan, K. W., et al. (2014). Allogeneic human mesenchymal stem cell therapy (remestemcel-L, Prochymal) as a rescue agent for severe refractory acute graft-versus-host disease in pediatric patients. *Biol Blood Marrow Transplant.* 20, 229–235. doi: 10.1016/j.bbmt.2013.11.001

Kuzmina, L. A., Petinati, N. A., Parovichnikova, E. N., Lubimova, L. S., Gribanova, E. O., and Gaponova, T. V. (2012). Multipotent mesenchymal stromal cells for the prophylaxis of acute graft-versus-host disease-a phase II study. *Stem Cells Int.* 2012:968213. doi: 10.1155/2012/968213

Lai, P., Chen, X., Guo, L., Wang, Y., Liu, X., Liu, Y., et al. (2018). A potent immunomodulatory role of exosomes derived from mesenchymal stromal cells in preventing cGVHD. *J. Hematol. Oncol.* 11:135. doi: 10.1186/s13045-018-0680-7

Lalu, M. M., McIntyre, L., Pugliese, C., Fergusson, D., Winston, B. W., Marshall, J. C., et al. (2012). Canadian critical care trials group. safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS One* 7:e47559. doi: 10.1371/journal.pone.0047559

Lazarus, H. M., Haynesworth, S. E., Gerson, S. L., Rosenthal, N. S., and Caplan, A. I. (1995). Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. *Bone Marrow Transpl.* 16, 557–564.

Le Blanc, K., Frassoni, F., Ball, L., Locatelli, F., Roelofs, H., Lewis, I., et al. (2008). Developmental committee of the european group for blood and marrow transplantation. mesenchymal stem cells for treatment of steroid-resitant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 371, 1579–1586. doi: 10.1016/S0140-6736(08)60690-X

Le Blanc, K., Rasmusson, I., Sundberg, B., Götherström, C., Hassan, M., Uzunel, M., et al. (2004). Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 363, 1439–1441.

Le Blanc, K., Tammik, L., Sundberg, B., Haynesworth, S. E., and Ringdén, O. (2003). Mesenchymal stem cells inhibit and stimulate mixed lymphocute cultures and mitogenic responses independently of major histocompatibility complex. *Scand. J. Immunol.* 57, 11–20.

Liu, K., Chen, Y., Zeng, Y., Xu, L., Liu, D., Chen, H., et al. (2011). Coinfusion of mesenchymal stromal cells facilitates platelet recovery without increasing leukemia recurrence in haploidentical hematopoietic stem cell transplantation: a randomized, controlled clinical study. *Stem Cells Dev.* 20, 1679–1685. doi: 10.1089/scd.2010.0447

Mareika, I., Shman, T., Isailina, Y., Minakovskaya, N., and Aleinikova, O. (2016). Influence of mesenchymal stem cells co-transplantation on post-transplant period after allogeneic hematopoietic cell transplantation in children with acute leukemia. *Bone Marrow Transplant.* 51(Suppl. 1S), S118–S119.

Martin, P. J. (2008). Biology of chronic graft-versus-host disease: implications for a future therapeutic approach. *Keio J. Med.* 57, 177–183.

Martin, P. J., Uberti, J. P., Soiffer, R. J., Klingermann, H., Waller, E. K., Daly, A. S., et al. (2010). Prochymal improves response rates inpatients with steroid-refractory acute graft-versus-host disease involving liver and gut: results of a randomizes, placebo-controlled, multicenter phase III trial in GVHD. *Bone Marrow Transpl.* 45(Suppl. 2):S17.

Maziarz, R. T., Devos, T., Bachier, C. R., Goldstein, S. C., Leis, J. F., Devine, S. M., et al. (2015). Single and multiple dose multistem (multipotent adult progenitor cell) therapy prophylaxis of acute graft-versus-host disease in myeloablative allogeneic hematopoietic cell transplantation: a phase 1 trial. *Biol Blood Marrow Transplant.* 21, 720–728. doi: 10.1016/j.bbmt.2014.12.025

Melief, S. M., Geutskens, S. B., Fibbe, W. E., and Roelofs, H. (2013). Multipotent stromal cells skew monocytes towards an anti-inflammatory interleukin-10-producing phenotype by production of interleukin-6. *Haematologica* 98, 888–895. doi: 10.3324/haematol.2012.078055

Mendicino, M., Bailey, A. M., Wonnacott, K., Puri, R. K., and Bauer, S. R. (2014). MSC-based product characterization for clinical trials: an FDA perspective. *Cell Stem Cell* 14, 141–145. doi: 10.1016/j.stem.2014.01.013

Meng, X., Ichim, T. E., Zhong, J., Rogers, A., Yin, Z., Jackson, J., et al. (2007). Endometrial regenerative cells: a novel stem cell population. *J. Transl. Med.* 5:57.

Mielcarek, M., Furlong, T., Storer, B. E., Green, M. L., McDonald, G. B., Carpenter, P. A., et al. (2015). Effectiveness and safety of lower dose prednisone for initial treatment of acute graft-versus-host disease a randomized controlled trial. *Haematologica* 100, 842–848. doi: 10.3324/haematol.2014.118471

Mizukami, A., and Swiech, K. (2018). Mesenchymal stromal cells: from discovery to manufacturing and commercialization. *Stem Cells Int.* 2018:13. doi: 10.1155/2018/4083921

Moll, G., Ankrum, J. A., Kamhieh-Milz, J., Bieback, K., Ringdén, O., Volk, H. D., et al. (2019). Intravascular mesenchymal stromal/stem cell therapy product diversification: time for new clinical guidelines. *Trends Mol. Med.* 25, 149–163. doi: 10.1016/j.molmed.2018.12.006

Najar, M., Raicevic, G., Fayyad-Kazan, H., Bron, D., Toungouz, M., and Lagneaux, L. (2016). Mesenchymal stromal cells and immunomodulation: a gathering of regulatory immune cells. *Cytotherapy* 18, 160–171. doi: 10.1016/j.jcyt.2015.10.011

Nikitina, V., Astrelina, T., Nugis, V., Ostashkin, A., Karaseva, T., Dobrovolskaya, E., et al. (2018). Clonal chromosomal and genomic instability during human multipotent mesenchymal stromal cells long-term culture. *PLoS One* 13:e0192445. doi: 10.1371/journal.pone.0192445

Ning, H., Yang, F., Jiang, M., Hu, L., Feng, K., Zhang, J., et al. (2008). The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. *Leukemia* 22, 593–599. doi: 10.1038/sj.leu.2405090

Nitzsche, F., Müller, C., Lukomska, B., Jolkkonen, J., Deten, A., and Boltze, J. (2017). Concise review: msc adhesion cascade-insights into homing and transendothelial migration. *Stem Cells* 35, 1446–1460. doi: 10.1002/stem.2614

Nowakowski, A., Andrzejewska, A., Janowski, M., Walczak, P., and Lukomska, B. (2013). Genetic engineering of stem cells for enhanced therapy. *Acta. Neurobiol. Exp.* 73, 1–18.

Patki, S., Kadam, S., Chandra, V., and Bhonde, R. (2010). Human breast milk is a rich source of multipotent mesenchymal stem cells. *Hum. Cell* 23, 35–40. doi: 10.1111/j.1749-0774.2010.00083.x

Pereira, M. C., Secco, M., Suzuki, D. E., Janjoppi, L., Rodini, C. O., Torres, L. B., et al. (2011). Contamination of mesenchymal stem-cells with fibroblasts accelerates neurodegeneration in an experimental model of Parkinson's disease. *Stem Cell Rev. Rep.* 7, 1006–1017. doi: 10.1007/s12015-011-9256-4

Perkey, E., and Maillard, I. (2018). New insights into graft-versus-host disease and graft rejection. *Annu. Rev. Pathol. Mec. Dis.* 13, 219–245. doi: 10.1146/annurev-pathol-020117-043720

Petersdorf, E. W., Longton, G. M., Anasetti, C., Martin, P. J., Mickelson, E. M., Smith, A. G., et al. (1995). The significance of HLA-DRB1 matching on clinical outcome after HLA-A, B, DR identical unrelated donor marrow transplantation. *Blood* 86, 1606–1613.

Philippe, B., Luc, S., Valérie, P. B., Jérôme, R., Alessandra, B. R., and Louis, C. (2010). Culture and use of mesenchymal stromal cells in phase I and II. *Stem Cells Int.* 2010:503593. doi: 10.4061/2010/503593

Pittenger, M. F., Mackay, A. M., and Beck, S. C. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–147.

Puissant, B., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., et al. (2005). Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. *Br. J. Haematol.* 129, 118–129.

Ramasamy, R., Fazekasova, H., Lam, E. W., Soeiro, I., Lombardi, G., and Dazzi, F. (2007). Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. *Transplantation* 83, 71–76.

Ren, G., Zhang, L., Zhao, X., Xu, G., Zhang, Y., Roberts, A. I., et al. (2008). Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2, 141–150. doi: 10.1016/j.stem.2007.11.014

Ren, G., Zhao, X., Zhang, L., Zhang, J., L'Huillier, A., Ling, W., et al. (2010). Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. *J. Immunol.* 184, 2321–2328. doi: 10.4049/jimmunol.0902023

Rizk, M., Monaghan, M., Shorr, R., Kekre, N., Bredeson, C. N., and Allan, D. S. (2016). Heterogeneity in studies of mesenchymal stromal cells to treat or prevent graft-versus-host disease: a scoping review of the evidence. *Biol. Blood Marrow Transpl.* 22, 1416–1423. doi: 10.1016/j.bbmt.2016.04.010

Rüster, B., Göttig, S., Ludwig, R. J., Bistrian, R., Müller, S., Seifried, E., et al. (2006). Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 108, 3938–3944.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., et al. (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 131, 324–336.

Salmenniemi, U., Itälä-Remes, M., Nystedt, J., Putkonen, M., Niittyvuopio, R., Vettenranta, K., et al. (2017). Good responses but high TRM in adult patients after MSC therapy for GvHD. *Bone Marrow Transplant.* 52, 606–608. doi: 10.1038/bmt.2016.317

Schmidt, A., Ladage, D., Steingen, C., Brixius, K., Schinköthe, T., Klinz, F. J., et al. (2006). Mesenchymal stem cells transmigrate over the endothelial barrier. *Eur. J. Cell Biol.* 85, 1179–1188.

Selmani, Z., Naji, A., Zidi, I., Favier, B., Gaiffe, E., Obert, L., et al. (2008). Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. *Stem Cells* 26, 212–222.

Sheng, H., Wang, Y., Jin, Y., Zhang, Q., Zhang, Y., Wang, L., et al. (2008). A critical role of IFNgamma in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. *Cell Res.* 18, 846–857. doi: 10.1038/cr.2008.80

Shih, D. T., Lee, D. C., Chen, S. C., Tsai, R. Y., Huang, C. T., Tsai, C. C., et al. (2005). Isolation and characterization of neurogenic mesenchymal stem cells in human scalp tissue. *Stem Cells* 23, 1012–1020.

Shimabukuro-Vornhagen, A., Hallek, M. J., Storb, R. F., and von Bergwelt-Baildon, M. S. (2009). The role of B cells in the pathogenesis of graft-versus-host disease. *Blood* 114, 4919–4927. doi: 10.1182/blood-2008-10-161638

Shipounova, I. N., Petinati, N. A., Bigildeev, A. E., Zezina, E. A., Drize, N. I., Kuzmina, L. A., et al. (2014). Analysis of results of acute graft-versus-host disease prophylaxis with donor multipotent mesenchymal stromal cells in patients with hemoblastoses after allogeneic bone marrow transplantation. *Biochemistry* 79, 1363–1370. doi: 10.1134/S0006297914120104

Spaggiari, G. M., Capobianco, A., Becchetti, S., Mingari, M. C., and Moretta, L. (2006). Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* 107, 1484–1490.

Stolzing, A., Jones, E., McGonagle, D., and Scutt, A. (2008). Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech. Ageing Dev.* 129, 163–173. doi: 10.1016/j.mad.2007.12.002

Suila, H., Hirvonen, T., Kotovuori, A., Ritamo, I., Kerkelä, E., Anderson, H., et al. (2014). Human umbilical cord blood-derived mesenchymal stromal cells display a novel interaction between P-selectin and galectin-1. *Scand. J. Immunol.* 80, 12–21. doi: 10.1111/sji.12179

Tarte, K., Gaillard, J., Lataillade, J. J., Fouillard, L., Becker, M., Mossafa, H., et al. (2010). Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. *Blood* 115, 1549–1553. doi: 10.1182/blood-2009-05-219907

Teo, G. S., Ankrum, J. A., Martinelli, R., Boetto, S. E., Simms, K., Sciuto, T. E., et al. (2012). Mesenchymal stem cells transmigrate between and directly through tumor necrosis factor-α-activated endothelial cells via both leukocyte-like and novel mechanisms. *Stem Cells* 30, 2472–2486.

Teo, G. S., Yang, Z., Carman, C. V., Karp, J. M., and Lin, C. P. (2015). Intravital imaging of mesenchymal stem cell trafficking and association with platelets and neutrophils. *Stem Cells* 33, 265–277. doi: 10.1002/stem.1848

Trounson, A., and McDonald, C. (2015). Stem cell therapies in clinical trials: progress and challenges. *Cell Stem Cell* 17:2015. doi: 10.1016/j.stem.2015.06.007

Von Bahr, L., Davies, L., Afram, G., Modin, C., Garming-Legert, K., Hägglund, H., et al. (2015). Treatment of chronic GvHD with mesenchymal stromal cells induces durable responses. a phase II study. *Blood* 126:3138.

von Bonin, M., Stölzel, F., Goedecke, A., Richter, K., Wuschek, N., Hölig, K., et al. (2009). Treatment of refractory acute GVHD with third-party MSC expanded in platelet lysate-containing medium. *Bone Marrow Transplant.* 43, 245–251. doi: 10.1038/bmt.2008.316

von Dalowski, F., Kramer, M., Wermke, M., Wehner, R., Röllig, C., Alakel, N., et al. (2016). Mesenchymal stromal Cells for treatment of acute steroid-refractory graft versus host disease: clinical responses and long-term outcome. *Stem Cells* 34, 357–366. doi: 10.1002/stem.2224

Wang, H. S., Hung, S. C., Peng, S. T., Huang, C. C., Wei, H. M., Guo, Y. J., et al. (2004). Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells* 22, 1330–1337.

Weise, G., Lorenz, M., Pösel, C., Maria Riegelsberger, U., Störbeck, V., Kamprad, M., et al. (2014). Transplantation of cryopreserved human umbilical cord blood

mononuclear cells does not induce sustained recovery after experimental stroke in spontaneously hypertensive rats. *J. Cereb. Blood Flow Metab.* 34:e1-9.

Wight, T. N., Kinsella, M. G., Keating, A., and Singer, J. W. (1986). Proteoglycans in human long-term bone marrow cultures: biochemical and ultrastructural analyses. *Blood* 67, 1333–1343.

Wu, K. H., Tsai, C., Wu, H. P., Sieber, M., Peng, C. T., and Chao, Y. H. (2013). Human application of ex vivo expanded umbilical cord-derived mesenchymal stem cells: enhance hematopoiesis after cord blood transplantation. *Cell Transplant.* 22, 2041–2051. doi: 10.3727/096368912X663533

Wu, Z., Zhang, S., Zhou, L., Cai, J., Tan, J., Gao, X., et al. (2017). Thromboembolism induced by umbilical cord mesenchymal stem cell infusion: a report of two cases and literature review. *Transplant Process.* 49, 1656–1658. doi: 10.1016/j.transproceed.2017.03.078

Wuchter, P., Bieback, K., and Schrezenmeier, H. (2015). Standardization of good manufacturing practice-compliant production of bone marrow-derived human mesenchymal stromal cells for immunotherapeutic applications. *Cytotherapy* 17, 128–139. doi: 10.1016/j.jcyt.2014.04.002

Yin, F., Battiwalla, M., Ito, S., Feng, X., Chinian, F., Melenhorst, J. J., et al. (2014). Bone marrow mesenchymal stromal cells to treat tissue damage in allogeneic stem cell transplant recipients: correlation of biological markers with clinical responses. *Stem Cells* 32, 1278–1288. doi: 10.1002/stem.1638

Yin, K., Wang, S., and Zhao, R. C. (2019). Exosomes from mesenchymal stem/stromal cells: a new therapeutic paradigm. *Biomark Res.* 7:8. doi: 10.1186/s40364-019-0159-x

Zeiser, R., and Blazar, B. R. (2017). Pathophysiology of chronic graft-versus-host disease and therapeutic targets. *N. Engl. J. Med.* 377, 2565–2579. doi: 10.1056/nejmra1703472

Zeng, D., Gazit, G., Dejbakhsh-Jones, S., Balk, S. P., Snapper, S., Taniguchi, M., et al. (1999). Heterogeneity of NK1.1+ T cells in the bone marrow: divergence from the thymus. *J. Immunol.* 163, 5338–5345.

Zuk, P. A., Zhu, M., Ashjian, P., De Ugarte, D. A., Huang, J. I., Mizuno, H., et al. (2002). Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* 13, 4279–4295.

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REVIEW
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Coding Cell Identity of Human Skeletal Muscle Progenitor Cells Using Cell Surface Markers: Current Status and Remaining Challenges for Characterization and Isolation

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Skeletal muscle progenitor cells (SMPCs), also called myogenic progenitors, have been studied extensively in recent years because of their promising therapeutic potential to preserve and recover skeletal muscle mass and function in patients with cachexia, sarcopenia, and neuromuscular diseases. SMPCs can be utilized to investigate the mechanisms of natural and pathological myogenesis via *in vitro* modeling and *in vivo* experimentation. While various types of SMPCs are currently available from several sources, human pluripotent stem cells (PSCs) offer an efficient and cost-effective method to derive SMPCs. As human PSC-derived cells often display varying heterogeneity in cell types, cell enrichment using cell surface markers remains a critical step in current procedures to establish a pure population of SMPCs. Here we summarize the cell surface markers currently being used to detect human SMPCs, describing their potential application for characterizing, identifying and isolating human PSC-derived SMPCs. To date, several positive and negative markers have been used to enrich human SMPCs from differentiated PSCs by cell sorting. A careful analysis of current findings can broaden our understanding and reveal potential uses for these surface markers with SMPCs.

Keywords: skeletal muscle, skeletal muscle progenitor cells, cell surface markers, human pluripotent stem cells, human induced pluripotent stem cells, muscular dystrophy, neuromuscular diseases

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INTRODUCTION

The most pronounced symptom of neuromuscular disorders is loss of skeletal muscle mass and strength, which causes functional decline and loss of independence in patients (Morrison, 2016; Mary et al., 2018). However, muscular deterioration is not always indicative of disease. Age-related progressive muscle atrophy and reduction of muscle function in healthy older adults is known as sarcopenia (Santilli et al., 2014; Ogawa et al., 2016; Marzetti et al., 2017). Although both a decrease in myocyte number (hypoplasia) and size (atrophy) are consistently observed in sarcopenic skeletal muscle (Deschenes, 2004; Narici and Maffulli, 2010), the precise biological mechanisms driving the precipitous decline in muscle mass and function are not well-understood. Current treatment options for these muscle conditions are only palliative, and to date there is no effective cure for any type of muscle wasting.

Stem cells offer the potential to become a realistic means to suppress the aging process in humans. To treat muscle wasting, stem cell-based therapy is the most attractive approach, as demonstrated in numerous pre-clinical studies and several clinical trials (Wilschut et al., 2012; Bajek et al., 2015; Ryder et al., 2017; Tompkins et al., 2017). For full muscle repair and regeneration, the formation of contractile muscle units is required. The obvious candidates for cell therapy are skeletal muscle progenitor cells (SMPCs, also known as myogenic progenitors). These cells can differentiate into skeletal myocytes with high efficiency and terminally achieve the formation of contractile muscle units, which is required for muscle repair and regeneration in the implanted muscle. There is a range of available cell sources to propagate SMPCs in culture, including from fetal muscle, adult muscle, and non-muscle somatic tissues. Furthermore, recent advances in stem cell technology allow us to use human pluripotent stem cells (PSCs) as a virtually indefinite new cell source for SMPC preparation. Human PSCs, which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), can overcome problems associated with expansion to large cell numbers for clinical use. Therefore, human PSC-derived SMPCs hold great promise for cell-based therapy to achieve muscle regeneration.

Not limited for use in cell-based regenerative therapy, human PSC-derived SMPCs are also available for *in vitro* modeling to study normal and pathological mechanisms in human skeletal muscle. As there is a large void between pre-clinical work carried out in rodent models and translating these therapies to humans, utilizing human PSC-derived SMPCs to study muscle wasting would help bridge the gap in knowledge. While *in vitro* culture systems have limitations and cannot completely recapitulate the complex *in vivo* milieu, they have powerful experimental advantages that enable us to study inaccessible human cell types in a controlled setting. Through *in vitro* drug screening using human PSC-derived SMPCs, we can possibly identify new mechanisms and molecules that have the capacity to prevent muscle wasting and atrophy during normal aging or disease processes.

This review catalogs the current findings on cell surface markers to identify human SMPCs. Here we focus on surface markers that have been reported in human PSC-derived SMPCs and compare their expression in other systems. Specific cell markers and/or cell surface proteins can be used for isolation, identification, and characterization of viable SMPCs. A better understanding of how SMPC markers are regulated *in vitro* and *in vivo* can help resolve enduring questions and challenges such as (1) the origins of SMPCs; (2) signaling mechanisms that drive lineage progression; (3) optimal isolation techniques; (4) selective enrichment of populations with clinical relevance, either for *in vitro* modeling and/or *ex vivo* therapy; and (5) potential genetic manipulations and/or pharmaceutical interventions to correct deteriorating muscle phenotypes. Similarities or differences in SMPC surface marker expression might be indicative of their stemness, myogenic differentiation propensity, and lineage potential to assume non-myogenic fates.

SKELETAL MUSCLE DEVELOPMENT AND SMPCS

There are various types of progenitor cells that have the ability to differentiate into skeletal myocytes. These cells include muscle satellite cells, muscle-derived stem cells (MDSCs), side population (SP) cells, mesoangioblasts and pericytes (reviewed in Hosoyama et al., 2014). Different sources have been used to propagate SMPCs in culture, including fetal muscle, adult muscle, non-muscle somatic tissues, and pluripotent stem cells (PSCs).

Skeletal muscle satellite cells are a type of adult SMPC localized beneath the basal lamina of adult muscle fibers. Regeneration of postnatal and adult muscles relies on satellite cells (Mauro, 1961; Starkey et al., 2011; Pallafacchina et al., 2013; Xu et al., 2015). These cells are mitotically quiescent in adult muscles. When the muscle is stimulated by stress or trauma, satellite cells are activated to divide, giving rise to daughter satellite cells to replenish the quiescent satellite cell pool and/or to undergo terminal differentiation for muscle repair (Bischoff and Heintz, 1994; Morgan and Partridge, 2003; Kuang et al., 2007; Le Grand et al., 2009; Xu et al., 2015). Both quiescent and activated satellite cells express Pax7 (Seale et al., 2000), whereas Myf5 is only expressed in activated satellite cells (Crist et al., 2012; Xu et al., 2015). With the expression of a muscle determinant factor MyoD, satellite cells are committed to become myoblasts, or myogenic precursor cells, which then terminally differentiate into multinucleated myotubes (Tapscott et al., 1988; Bischoff and Heintz, 1994; Seale et al., 2000; Morgan and Partridge, 2003; Kuang et al., 2007; Le Grand et al., 2009; Crist et al., 2012).

Muscle-derived stem cells (MDSCs) can be isolated from adult muscle biopsies by a combination of enzyme digestion and serial plating to collagen-coated culture plates, as these cells are less adhesive compared to other cell types in skeletal muscle (Vella et al., 2011). MDSCs are biologically, biochemically and genetically distinct from satellite cells (Qu-Petersen et al., 2002; Alessandri et al., 2004; Deasy et al., 2005; Usas et al., 2011). Human MDSCs are positive for CD105, CD133, vimentin and desmin, but negative for CD31, CD34, CD45, FLK-1/KDR, von Willebrand factor, VE-cadherins, and BCL2 (Alessandri et al., 2004). On the other hand, murine MDSCs have been known to express Sca-1 and CD34 (Cao et al., 2003; Deasy et al., 2005). Human MDSCs induced for myogenic differentiation in culture express striated-muscle actin, smooth-muscle actin, and desmin (Alessandri et al., 2004). Intramuscular transplant of human MDSCs was able to reverse muscle atrophy and promote phrenic nerve regeneration in pre-injured and immunocompetent alpha-sarcoglycan null mice (Lavasani et al., 2014). MDSCs can self-renew and differentiate into various cell types including non-muscle cells such as astrocytes, neurons, osteoblasts, chondrocytes, adipocytes, and cardiomyocytes (Deasy et al., 2001; Alessandri et al., 2004; Usas et al., 2011; Tchao et al., 2013). As demonstrated in a mouse model of acute hindlimb injury, multipotent MDSCs can be especially useful when muscle degeneration affects the myotendinous junction and the muscle-associated tendon. This is because MDSCs can potentially

differentiate into fibroblasts or tenocytes to reconstitute the wasted muscle-tendon units (Hashimoto et al., 2016).

Side population (SP) cells are named after their ability to efflux the DNA-binding dye Hoechst 33342 and form a side population on Fluorescence Activated Cell Sorting (FACS) analysis (Goodell et al., 1996). This cell type was first identified in murine bone marrow as a subset of hematopoietic stem cells (HSCs; Goodell et al., 1996). Murine bone marrow SP cells were found to participate in muscle regeneration (Gussoni et al., 1999). Later, an isolation protocol was developed to enrich for murine skeletal muscle SP cells (Gussoni et al., 1999). These murine skeletal muscle SP cells were also able to replenish the satellite cell pool, which can contribute to muscle regeneration following systemic delivery (Gussoni et al., 1999; Asakura et al., 2002; Bachrach et al., 2006). While significant information is available for murine SP cells, studies in human skeletal muscle SP cells are relatively limited due to their scarcity, heterogeneity and difficulty to expand *in vitro*. It has been shown that human fetal muscle SP cells, which express CD146, Myf5, and Pax7, are highly myogenic and expandable *in vitro* but retain limited engraftment potential *in vivo* (Lapan et al., 2012).

Mesoangioblasts and pericytes are adult progenitor cells associated with vasculature running through the muscle tissue with a significant capacity to regenerate skeletal muscle. Mesoangioblasts are a type of mesodermal stem cell located in the walls of the embryonic aorta and the vessels of postnatal tissues (Tonlorenzi et al., 2007). As mesoangioblasts possess strong myogenic potential, systemic delivery of these cells into the blood stream enables them to reach and integrate into the target site of pathological muscles (Sampaolesi et al., 2003; Cossu et al., 2015). Intramuscular transplantation of muscle-derived $CD133^+$ mesoangioblasts into a mouse cryoinjury model re-populated the satellite cell niche and was able to activate a regenerative response after subsequent re-injury (Meng et al., 2014). Interestingly, mesoangioblasts are known to express angiogenic cell markers such as CD34, Sca-1, and Fetal Liver Kinase 1 (De Angelis et al., 1999). Pericytes are embedded within the basement membrane of micro-vasculatures in adult skeletal muscles (Armulik et al., 2011; Cappellari and Cossu, 2013). These cells express Myf5 and Tissue-Nonspecific Alkaline Phosphatase (Dellavalle et al., 2007; Cappellari and Cossu, 2013).

A number of recent studies acknowledge that human PSCs, which include ESCs and iPSCs, can serve as a promising source for SMPC preparation. PSCs have the potential to differentiate into any cell lineage (Thomson, 1998; Takahashi et al., 2007). As iPSCs can be prepared using the somatic cells of a patient, they are a powerful tool for disease modeling, gene correction and drug screening in culture, and are a robust cell source for immuno-privileged clinical applications. In the last decade, SMPCs have been derived from PSCs via either transgene-based methods (i.e., overexpression of myogenic genes) or transgene-free approaches (i.e., supplementation of myogenic growth factors and/or signaling molecules in defined culture) (reviewed in Jiwlawat et al., 2018). Most protocols aim to generate satellite cell-like populations because satellite cells are considered *bona fide* skeletal muscle stem cells with both stem-like properties and myogenic activities. When deriving SMPCs from PSCs, especially via transgene-free approaches, the resulting cell populations commonly display high heterogeneity. Therefore, characterization and purification of PSC-derived SMPCs is crucial to promote culture expansion efficiency and increase engraftment rate following transplantation (Kim et al., 2017). Currently available methods for SMPC isolation often require fixation and intracellular staining, which prevents further examination of behavior and functionality after sorting. To preserve the viability of enriched transgene-free SMPCs, isolation should be based exclusively on unique surface markers that do not disrupt or compromise the integrity of the cells. Moreover, profiling by cell surface markers would also be valuable to identify how PSC-derived SMPCs resemble SMPC types such as mesoangioblasts, SP cells, and satellite cells.

CELL SURFACE MARKERS TO DEFINE HUMAN PSC-DERIVED SMPCS

Different molecular signatures are displayed in the SMPCs derived from various cell sources. Therefore, a profile of cell surface proteins can be used to define a specific cell type. A cell isolation procedure can use either positive selection or negative selection. Positive selection isolates the target cell type from the entire population, whereas negative selection depletes all other cell types of the population with only the target cells remaining. A combined use of different surface markers has worked successfully to enrich human PSC-derived SMPCs and deplete undesirable cell types by cell sorting. Although these individual works support the feasibility of SMPC sorting using multiple markers, such complicated procedures would critically dampen enthusiasm for potential therapeutic applications of PSC-derived SMPCs. In this section, we will introduce cell surface markers that can positively or negatively identify SMPCs derived from human ESCs and iPSCs (**Table 1**); however, this is not an exhaustive list. We will also summarize the expression of these markers on SMPCs from the other tissue sources. For consistency, SMPCs in this review are defined by: (1) ability to self-renew prior to differentiation induction and upon differentiation induction; (2) ability to form myofibers; and (3) expression of at least one protein marker that has been commonly seen in skeletal muscle progenitors, myoblasts, and myocytes. All cells and tissues mentioned onwards are sourced from humans, unless specified otherwise.

Positive Surface Markers for Human SMPCs

CD10

CD10 is commonly recognized as a cancer marker but is also used to identify specific populations of SMPCs (Maguer-Satta et al., 2011). This marker represents a type of endopeptidase protein also known as neprilysin, membrane metallo-endopeptidase, neutral endopeptidase, or common acute lymphoblastic leukemia antigen (Maguer-Satta et al., 2011). CD10 is also involved in hematopoiesis and B cell-innate immunology (Maguer-Satta et al., 2011). In a recent study using transgene-free PSCs and Pax7/Myf5 reporter ESCs, $CD10^+/CD24^-$ cells represented

TABLE 1 | Common markers to identify SMPCs from human PSCs and muscles.

		PSCs				Adult muscle		Fetal muscle		Other sources (see NOTE)
		iPSCs		ESCs						
		Presence	References	Presence	References	Presence	References	Presence	References	
Positive markers	CD10	+	Wu et al., 2018	+	Wu et al., 2018	+	Crisan et al., 2008; Lecourt et al., 2010; Wu et al., 2018	+	Crisan et al., 2008	
	CD13	+	Darabi et al., 2012; Tedesco et al., 2012	+	Darabi et al., 2012	+	Morosetti et al., 2006; Dellavalle et al., 2007; Crisan et al., 2008; Lecourt et al., 2010; Pisani et al., 2010b	+	Crisan et al., 2008; Castiglioni et al., 2014	
	CD29	+	Awaya et al., 2012; Darabi et al., 2012; Abujarour et al., 2014; Magli et al., 2017; Sakai-Takemura et al., 2018	+	Awaya et al., 2012; Darabi et al., 2012; Magli et al., 2017	+	Lecourt et al., 2010; Woodard et al., 2014; Charville et al., 2015; Xu et al., 2015; Garcia et al., 2018*; Lorant et al., 2018	+	Castiglioni et al., 2014	
	CD44	+	Darabi et al., 2012; Tedesco et al., 2012; Abujarour et al., 2014	+	Darabi et al., 2012	+	Morosetti et al., 2006; Dellavalle et al., 2007; Lecourt et al., 2010; Pisani et al., 2010b; Lorant et al., 2018	+	Crisan et al., 2008; Castiglioni et al., 2014	
	CD54	+	Magli et al., 2017	+	Magli et al., 2017					
	CD56	+/–	(+): Awaya et al., 2012; Darabi et al., 2012; Abujarour et al., 2014; Choi et al., 2016; Uezumi et al., 2016; Hicks et al., 2017; Sakai-Takemura et al., 2018 (–): Darabi et al., 2012; Awaya et al., 2012; Tedesco et al., 2012; Uezumi et al., 2016; Sakai-Takemura et al., 2018	+/low	(+): Barberi et al., 2007; Awaya et al., 2012; Darabi et al., 2012; Albini et al., 2013; Hwang et al., 2014, Goudenege et al., 2012; Hicks et al., 2017; Rao et al., 2018 (low): Caron et al., 2016	+/–	(+): Sinanan et al., 2004; Dellavalle et al., 2007; Zheng et al., 2007; Crisan et al., 2008; Lindström and Thornell, 2009; Negroni et al., 2009; Lecourt et al., 2010; Pisani et al., 2010a; Okada et al., 2012; Zheng et al., 2012; Agley et al., 2013; Bareja et al., 2014; Marg et al., 2014; Woodard et al., 2014; Xu et al., 2015; Alexander et al., 2016; Franzin et al., 2016; Uezumi et al., 2016; Garcia et al., 2018*; Lorant et al., 2018 (–): Proksch et al., 2009	+	Castiglioni et al., 2014; Hicks et al., 2017	Placenta (Park et al., 2011)
	CD63	+	Darabi et al., 2012	+	Darabi et al., 2012	+	Dellavalle et al., 2007			
	CD73	+	Awaya et al., 2012	+	Barberi et al., 2005, 2007; Awaya et al., 2012; Goudenege et al., 2012	+	Crisan et al., 2008; Lecourt et al., 2010; Woodard et al., 2014; Uezumi et al., 2016; Lorant et al., 2018			
	CD82	+	Uezumi et al., 2016; Sakai-Takemura et al., 2018			+	Alexander et al., 2016; Uezumi et al., 2016; Lorant et al., 2018	+	Alexander et al., 2016	

(Continued)

TABLE 1 | Continued

	PSCs				Adult muscle		Fetal muscle		Other sources (see NOTE)
	iPSCs		ESCs						
	Presence	References	Presence	References	Presence	References	Presence	References	
CD90	+	Darabi et al., 2012	+	Darabi et al., 2012	+/–	(+): Morosetti et al., 2006; Dellavalle et al., 2007; Zheng et al., 2007; Crisan et al., 2008; Lecourt et al., 2010; Pisani et al., 2010b; Woodard et al., 2014; Uezumi et al., 2016; Lorant et al., 2018 (–): Proksch et al., 2009	+	Crisan et al., 2008; Castiglioni et al., 2014	
CD105	+/–	(+): Awaya et al., 2012; Darabi et al., 2012 (–): Sakai-Takemura et al., 2018	+	Awaya et al., 2012; Darabi et al., 2012	+/–	Lecourt et al., 2010; Pisani et al., 2010b; Woodard et al., 2014; Uezumi et al., 2016; Lorant et al., 2018 (–): Alessandri et al., 2004	+	Crisan et al., 2008	Wharton's jelly (Conconi et al., 2006)
CD146	+/low	(+): Darabi et al., 2012; Tedesco et al., 2012 (Low): Sakai-Takemura et al., 2018	+	Darabi et al., 2012	+/low	(+): Cerletti et al., 2006; Morosetti et al., 2006; Dellavalle et al., 2007; Crisan et al., 2008; Lecourt et al., 2010; Pisani et al., 2010b; Okada et al., 2012; Zheng et al., 2012 (Low): Lorant et al., 2018	+	Cerletti et al., 2006; Lapan et al., 2012; Alexander et al., 2016;	Placenta (Park et al., 2011)
CD166	+	Awaya et al., 2012	+	Awaya et al., 2012; Darabi et al., 2012	+	Lecourt et al., 2010			
CD184	+/–	(+): Borchin et al., 2013 (–): Awaya et al., 2012; Darabi et al., 2012	+/–	Awaya et al., 2012	+	Bareja et al., 2014; Marg et al., 2014; Garcia et al., 2018*	+	Castiglioni et al., 2014	
CD271	+	Hicks et al., 2017; Sakai-Takemura et al., 2018	+	(+): Borchin et al., 2013 (–): Awaya et al., 2012; Darabi et al., 2012	+	Sakai-Takemura et al., 2018*	+	Alexander et al., 2016; Hicks et al., 2017	
CD318	+/–	(+): Sakai-Takemura et al., 2018 (–): Uezumi et al., 2016			+	Uezumi et al., 2016; Lorant et al., 2018; Sakai-Takemura et al., 2018			
CD362	+	Magli et al., 2017							
ErbB3	+	Hicks et al., 2017; Sakai-Takemura et al., 2018	+	Hicks et al., 2017			+	Alexander et al., 2016; Hicks et al., 2017	
c-Met	+/–	(+): Borchin et al., 2013 (–): Sakai-Takemura et al., 2018	+	Borchin et al., 2013					
HLA-ABC	+	Darabi et al., 2012	+	Darabi et al., 2012					
ITGA7	+	Darabi et al., 2012	+	Darabi et al., 2012	+	Castiglioni et al., 2014			

(Continued)

TABLE 1 | Continued

		PSCs				Adult muscle		Fetal muscle		Other sources (see NOTE)
		iPSCs		ESCs						
		Presence	References	Presence	References	Presence	References	Presence	References	
Negative marker	CD15					–	Lecourt et al., 2010; Pisani et al., 2010a; Agley et al., 2013			
	CD24	–	Wu et al., 2018	–		–	Wu et al., 2018			
	CD31	–	Darabi et al., 2012; Tedesco et al., 2012	–	Hicks et al., 2017	–	Alessandri et al., 2004; Dellavalle et al., 2007; Bareja et al., 2014; Charville et al., 2015; Xu et al., 2015; Garcia et al., 2018*; Lorant et al., 2018	–	Cerletti et al., 2006; Castiglioni et al., 2014	
	CD33	–	Darabi et al., 2012	–	Borchin et al., 2013					
	CD34	–	Awaya et al., 2012; Darabi et al., 2012	+/–	(+): Hwang et al., 2014 (–): Awaya et al., 2012; Darabi et al., 2012	+/–	(+): Proksch et al., 2009; Pisani et al., 2010a; Okada et al., 2012; Marg et al., 2014; Sidney et al., 2014 (–): Alessandri et al., 2004; Morosetti et al., 2006; Dellavalle et al., 2007; Zheng et al., 2007, 2012; Crisan et al., 2008; Negroni et al., 2009; Lecourt et al., 2010; Pisani et al., 2010b; Bareja et al., 2014; Charville et al., 2015; Franzin et al., 2016; Garcia et al., 2018*; Lorant et al., 2018	–	Crisan et al., 2008; Castiglioni et al., 2014	Placenta (Park et al., 2011) Umbilical cord blood (Koponen et al., 2007; Nunes et al., 2007)
	CD45	–	Darabi et al., 2012; Tedesco et al., 2012	–	Darabi et al., 2012	–	Alessandri et al., 2004; Morosetti et al., 2006; Dellavalle et al., 2007; Crisan et al., 2008; Proksch et al., 2009; Lecourt et al., 2010; Okada et al., 2012; Zheng et al., 2012; Bareja et al., 2014; Charville et al., 2015; Xu et al., 2015; Garcia et al., 2018*; Lorant et al., 2018	–	Crisan et al., 2008; Castiglioni et al., 2014, Franzin et al., 2016	Placenta (Park et al., 2011) Umbilical cord blood (Nunes et al., 2007)
	CD57	–	Borchin et al., 2013; Choi et al., 2016; Hicks et al., 2017; Sakai-Takemura et al., 2018	–	Darabi et al., 2012					

(Continued)

TABLE 1 | Continued

	PSCs				Adult muscle		Fetal muscle		Other sources (see NOTE)
	iPSCs		ESCs						
	Presence	References	Presence	References	Presence	References	Presence	References	
CD106	–	Awaya et al., 2012; Darabi et al., 2012	–	Awaya et al., 2012; Darabi et al., 2012	+/–	(+): Sinanan et al., 2004; Lecourt et al., 2010 (–): Morosetti et al., 2006; Dellavalle et al., 2007; Crisan et al., 2008; Pisani et al., 2010b	–	Crisan et al., 2008	
CD108	–	Sakai-Takemura et al., 2018			+	Sakai-Takemura et al., 2018			
HLA-DR	–	Darabi et al., 2012	–	Darabi et al., 2012	–	Dellavalle et al., 2007			
VEGFR2	–	Darabi et al., 2012; Tedesco et al., 2012	–	Darabi et al., 2012	–	Lorant et al., 2018			

*+, expression detected (>40% or not specified); low, expression detected but low (20–40%); –, expression not detected or very low (<20%). The Other sources column lists the sources where expression of "positive markers" was detected and expression of "negative markers" was not detected. *Studies were done using postnatal muscle (Crisan et al., 2008); midterm placenta, fetal and adult muscle, fetal skin, fetal and adult pancreas, fetal bone marrow, fetal and adult adipose tissue (Franzin et al., 2016); fetal tissues, including skeletal muscle, myocardium, skin, pancreas, brain, bone marrow.*

the SMPC population (Wu et al., 2018). However, CD10 expression does not always correspond to SMPCs. A study using adult human muscle showed that ∼80% of $Pax7^+$ cells expressed CD10, suggesting that not all adult satellite cells express CD10 (Wu et al., 2018). Human primary myoblasts also uniformly express CD10 (∼97%) (Wu et al., 2018). When a $CD146^+/CD34^-/CD45^-/CD56^-$ cell subset was isolated from multiple human organs, co-expression of CD10 was identified in the isolated cells (Crisan et al., 2008).

CD13

CD13 is also known as aminopeptidase N, alanyl aminopeptidase or lamina-associated polypeptide 1. This marker was initially found to regulate adhesion and differentiation of muscle satellite cells following ischemic injury in mice (Rahman et al., 2014). CD13 plays important roles for the migration of cancer and endothelial cells (Kehlen et al., 2003; Fukasawa et al., 2006). In the human brain, CD13 is expressed on capillary pericytes (Smyth et al., 2018). Although CD13 has not been studied much in the context of human myogenic cells, one study reported that human adult pericytes were almost homogeneously positive (∼97%) for CD13 (Dellavalle et al., 2007). Positive CD13 expression has also been detected in SMPCs derived from human PSCs (Darabi et al., 2012; Tedesco et al., 2012), adult muscle (Morosetti et al., 2006; Dellavalle et al., 2007; Lecourt et al., 2010; Pisani et al., 2010b), and fetal muscle (Castiglioni et al., 2014). Similar to CD10, co-expression of CD13 was observed in the $CD146^+/CD34^-/CD45^-/CD56^-$ cell subset isolated from multiple human organs (Crisan et al., 2008).

CD29

CD29, or integrin beta-1, is a well-established marker widely utilized to isolate or identify human myogenic cells. CD29 expression has been confirmed in PSC-derived SMPCs (Awaya et al., 2012; Darabi et al., 2012; Abujarour et al., 2014; Magli et al., 2017; Sakai-Takemura et al., 2018), fetal muscle (Castiglioni et al., 2014), postnatal muscle (Garcia et al., 2018), and adult muscle (Lecourt et al., 2010; Woodard et al., 2014; Charville et al., 2015; Xu et al., 2015; Lorant et al., 2018). An isoform of CD29, integrin beta-1D, has been shown to be severely reduced in patients with limb girdle muscular dystrophy type 2C (Anastasi et al., 2004) or sensitive-motor polyneuropathy (Anastasi et al., 2008). These results imply that normal levels of CD29 may be required for maintenance of healthy skeletal muscle.

In healthy adult skeletal muscle, all $Pax7^+$ satellite cells displayed co-expression of CD56 and CD29, although a population of $CD56^+/CD29^+$ cells without Pax7 expression was still identified (Xu et al., 2015). Compared to an unselected cell population, $CD56^+/CD29^+$ cells exhibited a higher level of myofiber regeneration following cell transplantation into the pre-injured hindlimb muscle (tibialis anterior muscle) of immunodeficient NOD scid gamma (NSG) mice. In contrast, $CD56^-/CD29^+$ cells showed insufficient or non-existent myofiber regeneration (Xu et al., 2015). This study indicates that CD29 should be used in combination with other markers to define human SMPCs, as not only satellite cells but the myofiber and some non-muscle cells within skeletal muscle

tissue also expressed CD29 (Xu et al., 2015). In a different study, when $CD31^-/CD34^-/CD45^-/CD29^+$/ epidermal growth factor receptor $(EGFR)^+$ cells were isolated from human adult skeletal muscle, these cells expressed a series of myogenic cell markers such as Pax7, Pax3, Myf5, MyoD, Myogenin, and Myocyte Enhancer Factor 2C. Further, the isolated cells can differentiate into myotubes *in vitro* and when transplanted into NSG mice (Charville et al., 2015). In contrast, the remaining fraction (i.e., unselected cells) did not give rise to any $Pax7^+$ cells and did not exhibit myogenic activity in culture, further supporting the notion that CD29 can indeed efficiently enrich myogenic cells when used in combination with other markers (Charville et al., 2015).

A study using an inducible gene expression system demonstrated that the expression of CD29/Integrin alpha-9 dimer (α9β1) was upregulated in human PSCs following Pax7 induction (Magli et al., 2017). In this study, Pax7-overexpressing cells were tagged with a green fluorescent protein (GFP) reporter protein using a Pax7 promotor. When $\alpha9\beta1^+/CD54^+$ cells were isolated from Pax7-overexpressing PSCs, ∼95% of cells were CD362-positive, and nearly 100% of GFP^+ cells were triple positive ($\alpha9\beta1^+/CD54^+/CD362^+$). The triple positive cells demonstrated robust regenerative capacity *in vivo*, as human-specific satellite cells and myofibers were confirmed in the muscle of NSG mice at 10 months post-transplantation (Magli et al., 2017).

CD44

CD44 is also known as homing cell adhesion molecule, P-glycoprotein 1, lymphocyte homing receptor, extracellular matrix receptor III or HUTCH-1. The expression of CD44 is commonly seen in mesenchymal stem cells (MSCs) (Ramos et al., 2016), tenocytes (Scutt et al., 2008; Lui, 2015; Stolk et al., 2017), HSCs, and cancer stem cells (Thapa and Wilson, 2016; Senbanjo and Chellaiah, 2017). CD44 has also been detected on SMPCs from different cell sources, including PSCs (Darabi et al., 2012; Tedesco et al., 2012; Abujarour et al., 2014), adult muscle (Morosetti et al., 2006; Dellavalle et al., 2007; Lecourt et al., 2010; Pisani et al., 2010b; Lorant et al., 2018), and fetal muscle (Crisan et al., 2008; Castiglioni et al., 2014). Although CD44 expression in SMPCs was already verified in a number of previous works, this marker has not been tested to isolate or characterize human SMPCs by FACS. Interestingly, CD44 interacts with hyaluronan and activates EGFR signaling (Thapa and Wilson, 2016), which suggests that CD44 may play a significant role in the process of myogenesis through the EGFR-mediated pathway (Leroy et al., 2013). As an additional note, CD44 expression was identified in the endomysium of adult skeletal muscle (Lecourt et al., 2010).

CD54

CD54 (intercellular adhesion molecule 1, ICAM-1) is a glycoprotein typically expressed on endothelial cells and leukocytes (Zadeh et al., 2000; Long, 2011). CD54 expression is absent in healthy human muscle but detected in patients with inflammatory myopathies (Bartoccioni et al., 1994; De Bleecker and Engel, 1994; Marino et al., 2003). Treatment with various cytokines can induce CD54 expression in cultured human skeletal muscle cells (Goebels et al., 1992; Michaelis et al., 1993; Bartoccioni et al., 1994; Marino et al., 2001). One study reported that CD54 was upregulated by inducible Pax7 overexpression in human PSCs (Magli et al., 2017). Chromatin immunoprecipitation sequencing (ChIP-Seq) data revealed that Pax7 binds the 5′ region of the CD54 gene, suggesting that Pax7 directly regulates CD54 gene expression. Using the PSCs with inducible Pax 7 expression, $CD54^+/CD362^+$/integrin $\alpha9\beta1^+$ cells were able to repopulate the satellite cell pool and generate new muscle fibers in an NSG mouse model at 10 months post-transplantation. In the same study, the potential of CD54 as a sole marker was also evaluated for SMPC isolation. When using the PSCs with a GFP reporter gene driven by a Pax7 promotor, GFP^+ cells were highly enriched by CD54-based selection. Notably, the isolated cells exhibited robust muscle differentiation *in vivo* without any signs of teratoma formation even after 12 months of transplantation in NSG mice (Magli et al., 2017).

CD56

CD56, or neural cell adhesion molecule (NCAM), has been utilized for isolation and identification of human myogenic cells in an extensive list of publications. CD56 expression is observed on SMPCs derived from PSCs (Barberi et al., 2007; Awaya et al., 2012; Darabi et al., 2012; Goudenege et al., 2012; Albini et al., 2013; Abujarour et al., 2014; Hwang et al., 2014; Caron et al., 2016; Choi et al., 2016; Uezumi et al., 2016; Hicks et al., 2017; Rao et al., 2018; Sakai-Takemura et al., 2018) postnatal muscle (Garcia et al., 2018), adult muscle (Sinanan et al., 2004; Dellavalle et al., 2007; Zheng et al., 2007, 2012; Crisan et al., 2008; Lindström and Thornell, 2009; Negroni et al., 2009; Proksch et al., 2009; Lecourt et al., 2010; Pisani et al., 2010a; Okada et al., 2012; Agley et al., 2013; Bareja et al., 2014; Marg et al., 2014; Woodard et al., 2014; Xu et al., 2015; Alexander et al., 2016; Franzin et al., 2016; Uezumi et al., 2016; Lorant et al., 2018), fetal muscle (Castiglioni et al., 2014; Hicks et al., 2017), and placenta (Park et al., 2011).

It has been deduced previously that $CD56^+/Pax7^-$ cells in adult muscle could represent activated satellite cells or myoblasts (Lindström and Thornell, 2009). Alongside other markers, CD56 expression has been used to enrich SMPCs, in which the isolated populations were able to form myotubes at high efficiency *in vitro* and/or contribute to muscle regeneration *in vivo* (Sinanan et al., 2004; Barberi et al., 2007; Dellavalle et al., 2007; Zheng et al., 2007, 2012; Crisan et al., 2008; Lecourt et al., 2010; Pisani et al., 2010a; Okada et al., 2012; Bareja et al., 2014; Castiglioni et al., 2014; Woodard et al., 2014; Xu et al., 2015; Alexander et al., 2016; Choi et al., 2016; Uezumi et al., 2016; Hicks et al., 2017; Lorant et al., 2018). CD56 expression was also used to identify and confirm SMPC fractions in the purified cells using other surface markers (Negroni et al., 2009; Proksch et al., 2009). Genetic analysis revealed that when compared to whole populations, both $CD56^+$ cultured fetal myocytes and human PSC-derived SMPCs highly expressed genes associated with myogenesis, embryonic development and cell migration (Hicks et al., 2017).

However, several other studies indicate that $CD56^-$ cells may also contain SMPCs. A population of $CD146^+/CD34^-/CD45^-/CD56^-$ cells were isolated from multiple human organs (Crisan et al., 2008) and placenta

(Crisan et al., 2008; Park et al., 2011). The isolated cells, specifically named "perivascular cells" in these studies, were able to form multinucleated Myosin heavy chain (MyHC)$^+$/desmin$^+$/dystrophin$^+$ myotubes *in vitro* as well as human spectrin$^+$/human dystrophin$^+$ myofibers and new blood vessels *in vivo*. After myogenic differentiation was induced in culture, these CD146$^+$/CD34$^-$/CD45$^-$/CD56$^-$ cells showed upregulated CD56 expression (Park et al., 2011). These cells were also able to differentiate into myotubes, chondrocytes, adipocytes and osteocytes upon appropriate inductions in culture (Crisan et al., 2008). In another report, CD146$^+$/CD45$^-$/CD56$^-$/UEA-1R$^-$ cells could generate a high number of human spectrin$^+$ myofibers in an immunodeficient mouse model (Severe Combined Immunodeficiency, SCID; Zheng et al., 2012). Termed "endothelial cells" in the specific study, a CD45$^-$/CD56$^-$/CD34$^+$/CD144$^+$ population derived from adult muscle formed MyHC$^+$ myotubes *in vitro* and a low number of human spectrin$^+$ myofibers *in vivo* (Okada et al., 2012). More than half of these "endothelial cells" eventually expressed CD56 after expansion in culture (Okada et al., 2012).

The absence of CD56 expression may indicate diminished myogenic potential and increased adipogenic potential. In separate reports, CD56$^-$/TE-7$^+$, CD56$^-$/CD15$^+$, CD56$^-$/CD34$^+$, and CD56$^-$/CD34$^-$ cells derived from adult muscle were differentiated into adipoblasts but not myotubes (Zheng et al., 2007; Lecourt et al., 2010; Pisani et al., 2010a,b). When adult muscle-derived CD56$^-$/CD29$^+$ SMPCs were transplanted into NSG mice, human cell-derived myofibers were detected in only 1 out of 7 recipients, probably reflecting contamination with the CD56$^+$/CD29$^+$ fraction or a very limited myogenic potential of CD56$^-$/CD29$^+$ cells (Xu et al., 2015).

Based on these findings, CD56 expression seems to be identified in cells with a high capacity to differentiate into muscle, but if used alone may not work to enrich human SMPCs as a highly purified population.

CD73

Expression of CD73 (or ecto-5′-nucleotidase) has been detected in growth plates, articular cartilage, and hypertrophic chondrocytes (Coutu et al., 2017). CD73 is considered a marker of tenocytes (Lui, 2015; Stolk et al., 2017), MSCs (Ramos et al., 2016), vascular smooth muscle cells (Tamajusuku et al., 2006; Yang et al., 2015), and cancer cells (Gao et al., 2014). Although CD73 has not been frequently used for SMPC identification, its expression was found almost homogenously on SMPCs derived from several sources, including human PSCs (Awaya et al., 2012), MyoD-overexpressing human ESCs (Goudenege et al., 2012), and adult muscle (Woodard et al., 2014). These SMPCs expressed CD73, even though CD73 was not initially used for cell isolation (Crisan et al., 2008; Lecourt et al., 2010; Uezumi et al., 2016; Lorant et al., 2018). It has been reported that myogenic cells resided in the CD73$^+$ fraction of ESC-derived MSCs, and that these cells were able to differentiate into myotubes, adipocytes, chondrocytes and osteoblasts *in vitro* (Barberi et al., 2005, 2007).

CD82

CD82, also known as Tespan-27, is a member of the tetraspanin protein family primarily identified as a metastasis suppressor (Tonoli and Barrett, 2005). Recently, CD82 has become a popular marker to use in identifying SMPCs, as this cell surface protein is expressed on SMPCs derived from human PSCs (Uezumi et al., 2016; Sakai-Takemura et al., 2018), adult muscle (Alexander et al., 2016; Uezumi et al., 2016; Lorant et al., 2018) and fetal muscle (Alexander et al., 2016). In healthy adult muscle, CD82 was detected on ∼97% of Pax7$^+$ or M-cadherin$^+$ satellite cells and a small number of interstitial cells (Uezumi et al., 2016). CD82 expression is also maintained in activated and differentiating myogenic cells (Alexander et al., 2016). This marker can be used alone to enrich SMPCs with high regenerative capacity post-transplantation (Uezumi et al., 2016). When this marker was used alongside CD146 and CD56 on separate occasions, increased myogenic activity was observed in the CD82$^+$ fractions both *in vitro* and *in vivo* (Alexander et al., 2016; Uezumi et al., 2016).

Recent studies support the idea that CD82 may play significant roles during the process of myogenesis. Overexpression of CD82 molecules enhanced muscle differentiation in primary myoblasts (Alexander et al., 2016), whereas its downregulation decreased their proliferation and differentiation (Alexander et al., 2016; Uezumi et al., 2016). In CD56$^+$/CD82$^+$ myogenic cells purified from human muscle, knockdown of CD82 increased the transcription of MyoD1 and Myogenin and led to premature differentiation even under culture conditions ideal for cell growth (Uezumi et al., 2016). Interestingly, a p38 inhibitor could suppress upregulation of MyoD1 and Myogenin induced by CD82 knockdown. These results implicate that CD82 likely regulates the balance between differentiation and self-renewal of SMPCs via a p38-mediated signaling pathway (Uezumi et al., 2016). CD82 forms a protein complex with integrin α7β1 and α-sarcoglycan, both of which have been linked to muscle disorders (Alexander et al., 2016). The muscles from patients with Duchenne muscular dystrophy (DMD) commonly showed a reduced expression of CD82, which suggests that this molecule may play essential roles of SMPC function in the process of muscle degeneration (Alexander et al., 2016).

CD90

CD90/Thy1 is a glycophosphatidylinositol-anchored glycoprotein frequently used as an MSC marker (Ramos et al., 2016). CD90 is also expressed on tenocytes (Scutt et al., 2008; Lui, 2015; Stolk et al., 2017), endothelial cells, HSCs, and in developing nervous tissues (Wetzel et al., 2004). A number of studies reported that CD90 expression was detected in SMPCs derived from human PSCs (Darabi et al., 2012), adult muscle (Morosetti et al., 2006; Dellavalle et al., 2007; Zheng et al., 2007; Proksch et al., 2009; Lecourt et al., 2010; Pisani et al., 2010b; Woodard et al., 2014; Uezumi et al., 2016; Lorant et al., 2018), and fetal muscle (Crisan et al., 2008; Castiglioni et al., 2014). In human adult muscle, CD90$^+$ cells were also identified in the endomysium and adventitia of venous blood vessels (Lecourt et al., 2010). When culture-expanded satellite cells were sorted into CD34$^+$/CD90$^-$, CD34$^-$/CD90$^-$, and CD34$^-$/CD90$^+$

fractions, all three fractions expressed myogenic markers (Myf-5, MyoD, and Myogenin) as well as endothelial markers (CD31 and von Willebrand factor) (Proksch et al., 2009). Although CD90 expression was confirmed throughout various types of SMPCs, it remains inconclusive whether CD90 alone could represent the entirety of the SMPC population.

CD105

CD105 (endoglin, Src Homology 2) is a well-acknowledged marker of endothelial cells. This molecule plays a crucial role in angiogenesis and tumor growth (Fonsatti et al., 2010). Not limited to endothelial cells, the expression of CD105 is also identified in various types of SMPCs. This includes SMPCs derived from PSCs (Awaya et al., 2012; Darabi et al., 2012), adult muscle (Alessandri et al., 2004; Lecourt et al., 2010; Pisani et al., 2010b; Woodard et al., 2014; Uezumi et al., 2016; Lorant et al., 2018), and fetal muscle (Crisan et al., 2008), although the level of CD105 expression ranged widely from rare to ubiquitous in different types of progenitor cells. In adult muscle, CD105 was expressed in the sinusoidal endothelium and endomysium (Lecourt et al., 2010; Coutu et al., 2017). When $CD105^+/CD31^-/KDR^-$ cells were isolated from Wharton's jelly (a gelatinous substance within the umbilical cord) and implanted into the muscle of Lewis male rats, the grafted cells were positive with sarcomeric tropomyosin, a protein that regulates muscle contraction, at 2 weeks post-transplantation (Conconi et al., 2006). These results indicate that the grafted $CD105^+$ cells could generate functional muscle fibers. Further, these $CD105^+/CD31^-/KDR^-$ cells were able to differentiate into myotubes, adipocytes and osteoblasts *in vitro*. In other studies, CD105 was also found on human tenocytes (Lui, 2015; Stolk et al., 2017). As such, CD105 may likely be indicative of mesodermal progenitor cells with multilineage potential.

CD146

CD146 (or melanoma cell adhesion molecule, MCAM) is well-studied for its important role in development, cell migration, immunology, angiogenesis, cancer progression, and myogenesis (Wang and Yan, 2013). In adult muscle, CD146 expression has been observed in the endomysium (Lecourt et al., 2010). In fetal skeletal muscle, $CD146^+$ cells were found adjacent to myofibers and within blood vessels. These $CD146^+$ cells were also positive for M-Cadherin, Pax7, and MyoD (Cerletti et al., 2006).

CD146 expression has been detected on SMPCs derived from PSCs (Darabi et al., 2012; Tedesco et al., 2012; Sakai-Takemura et al., 2018), adult muscle (Cerletti et al., 2006; Morosetti et al., 2006; Dellavalle et al., 2007; Crisan et al., 2008; Lecourt et al., 2010; Pisani et al., 2010b; Okada et al., 2012; Zheng et al., 2012; Lorant et al., 2018), fetal muscle (Cerletti et al., 2006; Crisan et al., 2008; Lapan et al., 2012; Alexander et al., 2016), and placenta (Park et al., 2011). It has been reported that the $CD146^+$ fraction of SMPCs derived from adult muscle, fetal muscle and placenta contained cells with higher myogenic potential (Cerletti et al., 2006; Crisan et al., 2008; Park et al., 2011; Zheng et al., 2012; Alexander et al., 2016).

When both SP and main population (MP) cells were selected from human fetal muscle cells by scatter gating following FACS analysis, the CD146-based selection could enrich a population of myogenic cells. However, freshly isolated cells remained heterogenous: only ∼50% of $CD146^+$ SP and MP cells expressed Pax7, Myf5, or MyoD (Lapan et al., 2012). If SP and MP cells were sorted based on CD146 expression and then maintained in culture conditions that favored muscle differentiation, only $CD146^+$ cell populations showed myotube formation. Interestingly, the highest number of myotube-positive cells was observed in the $CD146^+$ SP fraction. Furthermore, $CD146^-$ cell fractions subsequently acquired CD146 expression during expansion in culture but did not acquire myogenic potential (Lapan et al., 2012). These observations suggest that myogenic lineage enrichment based on CD146 may only be useful for freshly isolated cells. When transplanted into NOD mice, $CD146^+$ SP cells exhibited high muscle regenerative capacity and gave rise to new human muscle fibers, whereas $CD146^+$ total cells (i.e., a population containing both MP and SP cells) and $CD146^+$ MP cells showed low engraftment rate (Lapan et al., 2012).

CD184

CD184, or commonly recognized as C-X-C chemokine receptor type 4 (CXCR-4), is a molecule with potent chemotactic activity for lymphocytes. It is crucial for homing hematopoietic stem cells to their adult marrow (Villa et al., 2012). CD184 is widely expressed on blood cells, endothelial cells, and neural cells (Walenkamp et al., 2017). CD184 expression is also confirmed on PSC-derived SMPCs (Borchin et al., 2013) as well as in adult muscle (Bareja et al., 2014; Marg et al., 2014; Garcia et al., 2018) and fetal muscle (Castiglioni et al., 2014).

As CD184 is also expressed in neural cells (Kos et al., 1999) and definitive endoderm cells (Teo et al., 2012), a combination of CD184 and other cell markers would be necessary to eliminate these non-myogenic cell types in PSC-derived cell populations. For instance, one study used the depletion of $CD57^+$ cells and c-Met^- cells to sufficiently exclude neural cells among the $CD184^+$ cell population (Borchin et al., 2013). In this study, $CD57^-$/acetylcholine receptor $(AChR)^-$ cells were purified from differentiated PSCs and then sorted into c-$Met^+/CD184^+$, c-$Met^+/CD184^-$, c-$Met^-/CD184^+$, and c-$Met^-/CD184^-$ fractions. Immunocytochemical analysis immediately after sorting revealed that both c-$Met^+/CD184^+$ and c-$Met^+/CD184^-$ fractions contained highly pure SMPCs. In contrast, c-$Met^-/CD184^+$ fraction contained both SMPCs and $SOX1^+$ neural cells, and c-$Met^-/CD184^-$ fraction did not have any SMPCs. Although CD184 alone could not enrich SMPCs, CD184 expression may be indicative of myogenic lineage progression. Interestingly, both c-$Met^+/CD184^+$ and c-$Met^+/CD184^-$ fractions expressed early myogenic genes Six4 and Pax3 (paralogue of Pax7) at day 23 of post-myogenic differentiation of PSCs (Borchin et al., 2013). Both c-$Met^+/CD184^+$ and c-$Met^+/CD184^-$ cells gradually acquired Pax7 expression by day 25, and almost all these cells exhibited co-expression of Pax3 and Pax7 by day 35. At this point, the number of Pax7-positive cells was higher in c-$Met^+/CD184^+$ cells compared to c-$Met^+/CD184^-$ cells, while 97–98% of cells still retained Pax3 expression in both preparations. As Pax7 has

been considered a later-stage marker of myogenic progenitors compared to Pax3, a specific population in c-Met$^+$/CD184$^+$ cells might have proceeded farther along the differentiation process. On the other hand, c-Met$^+$/CD184$^-$ cells might represent a more primitive SMPC population than c-Met$^+$/CD184$^+$ cells. Furthermore, gene-expression analysis confirmed the presence of Pax3 and Pax7 mRNA transcripts together with LBX1 in both c-Met$^+$/CD184$^+$ and c-Met$^+$/CD184$^-$ sorted populations. These *in vitro* results seem to be consistent with the situations observed during *in vivo* muscle development: within the hypaxial domain of the embryonic dermomyotome, the delamination of Pax3$^+$/LBX1$^+$ migratory muscle precursors is dependent on c-Met expression (Bladt et al., 1995; Dietrich et al., 1999), whereas CD184 is essential for the subsequent survival and distribution of precursors at the site of migration (Vasyutina et al., 2005; Buckingham, 2006).

CD271

CD271, also known as p75 neurotrophin receptor or low-affinity nerve growth factor receptor (p75NGFR), has recently been recognized as a positive marker to identify SMPCs and MSCs (Álvarez-Viejo et al., 2015; Sakai-Takemura et al., 2018). This surface marker was first reported as a candidate SMPC marker in a study when CD271 expression was detected in culture-expanded postnatal myoblasts but not in fibroblasts (Sakai-Takemura et al., 2018). When terminally differentiated, myotubes were exclusively formed in the CD271$^+$ fraction of iPSC-derived SMPCs although some CD271$^+$ cells remained non-myogenic (Sakai-Takemura et al., 2018). The progenitor cells sorted with a combination of CD271$^+$ and other markers (CD57$^-$/CD108$^-$/ErbB3$^+$) were able to generate myofibers with high efficiency *in vitro* and *in vivo* (Sakai-Takemura et al., 2018). The other group prepared CD271$^+$ SMPCs from DMD patient-derived iPSCs (DMD iPSCs) that had been genetically corrected by CRISPR/Cas9 technology. These CD271$^+$ SMPCs exhibited superior myotube-forming potential; even higher than the cells in the CD56$^+$, CD146$^+$, or CD184$^+$ fractions (Hicks et al., 2017). In a different study, SMPCs derived from four human PSC lines (ESCs, wild type iPSCs, DMD iPSCs, and genetically corrected DMD iPSCs) commonly expressed CD271 and ErbB3. These CD271$^+$/ErbB3$^+$ cells displayed significantly higher expression of myogenic genes and increased efficiency of skeletal muscle differentiation (Hicks et al., 2017). When using genetically corrected DMD iPSC-derived CD271$^+$ cells, the cells showed better engraftment in the muscle of dystrophin-deficient NSG mice compared to CD56$^+$ cells (Hicks et al., 2017).

At early developmental stages before any other myogenic surface markers are presented on muscle progenitors, CD271/ErbB3-based isolation can be used to enrich Pax7$^+$ cells and Myf5$^+$ cells from human fetal muscles (Hicks et al., 2017). At 8 weeks of gestation, a CD271$^+$/ErbB3$^+$ subpopulation emerged in the muscle with expression of myogenic transcription factors. At 11 weeks, the CD271$^+$/ErbB3$^+$ subpopulation began to co-express some cell surface markers such as CD56, CD82, and CD146. These cells then started losing CD271 expression at 16 weeks of gestation. When CD271$^+$/ErbB3$^+$ cells were isolated at this time point, plated down, and differentiated in culture, the cells could form myotubes with nearly 100% efficiency in cell fusion (Hicks et al., 2017). Such changes of CD271 expression have been characterized during transitions from early to late waves of human fetal myogenesis, which also correlates to the development of primary limb myofibers or maturation of secondary fetal myofibers (Hicks et al., 2017). CD271/ErbB3 expression can be used to distinguish premature SMPCs from more committed MyoD$^+$ myocytes (Hicks et al., 2017). Together, these data supports the idea that CD271 can be used as a positive selection marker to enrich human PSC-derived SMPCs.

However, we should bear in mind that CD271 expression may not always correlate to a capacity of muscle differentiation in human PSC-derived SMPCs. A recent study, which had used a double reporter ESC line driven by Pax7 and Myf5 promoter genes, revealed that CD271$^+$/ErbB3$^+$, CD271$^+$/ErbB3$^-$, and CD271$^-$/ErbB3$^+$ fractions contained similar proportions of Pax7$^+$ and Myf5$^+$ cells (Wu et al., 2018). When CD271$^+$/ErbB3$^+$, CD271$^+$/ErbB3$^-$, and CD271$^-$/ErbB3$^+$ fractions were sorted from Pax7/Myf5 dual reporter ESCs and terminally differentiated *in vitro*, all three fractions exhibited similar proportions of MyHC$^+$ cells and displayed similar myotube formation efficiency (Wu et al., 2018).

ErbB3

As mentioned in the previous section, ErbB3 has also been used as a surface marker to efficiently purify human PSC-derived SMPCs (Hicks et al., 2017; Sakai-Takemura et al., 2018). ErbB3, also known as human epidermal growth factor receptor 3, is widely expressed in a variety of organs during human development, including skin, bone, muscle, nervous system, lungs, and intestines (Coussens et al., 1985). ErbB3 is also expressed in human fetal CD146$^+$ cells which demonstrate high myogenic capacity (Alexander et al., 2016). When iPSC-derived SMPCs were sorted solely based on ErbB3 expression, myotubes were exclusively formed in the enriched ErbB3$^+$ population following terminal differentiation in culture (Sakai-Takemura et al., 2018). ErbB3-based cell sorting could enrich myogenic cells much more efficiently compared to the isolation using a combination of CD56 and CD82 expression (Sakai-Takemura et al., 2018). In a different study, isolated iPSC-derived SMPCs with CD57$^-$/CD108$^-$/CD271$^+$/ErbB3$^+$ or CD271$^+$/ErbB3$^+$ were able to differentiate into myotubes with high efficiency in culture and *in vivo* (Sakai-Takemura et al., 2018). In a similar study using genetically corrected DMD iPSC-derived SMPCs, ErbB3$^+$ cells displayed high potential for myotube formation compared to the cells solely sorted by CD56, CD146, CD184, or CD271 expression (Hicks et al., 2017). When ErbB3$^+$ cells were prepared from four PSC lines (ESCs, wild type iPSCs, DMD iPSCs, and genetically corrected DMD iPSCs), all ErbB3$^+$ cells from the four PSC lines were positive with CD271. These ErbB3$^+$/CD271$^+$ SMPC lines exhibited higher capacity for muscle differentiation than double-negative fractions (Hicks et al., 2017). When iPSC-derived SMPCs were transplanted into the limb muscle of dystrophin-deficient NSG mice, the implanted cells promoted significant engraftment and restored dystrophin expression in the grafted area (Hicks et al., 2017).

As we already introduced in the section on CD271, CD271/ErbB3 expression can be used to enrich $Pax7^+$ and $Myf5^+$ cells from fetal muscles at the first and second trimesters of human development (Alexander et al., 2016). A $CD271^+/ErbB3^+$ subpopulation began to emerge in fetal muscle at 8 weeks of gestation and gradually increased the expression of myogenic genes (Hicks et al., 2017). If $CD271^+/ErbB3^+$ cells were isolated at 16 weeks of gestation, the cells could differentiate into myotubes with a fusion efficiency close to 100% when plated down and differentiated in culture (Hicks et al., 2017). The changes in $CD271^+/ErbB3^+$ expression possibly mark the progression of human fetal myogenesis.

Similar to CD271, ErbB3 expression is not always indicative of higher myogenic potential in human PSC-derived SMPCs. In another study, ESC-derived cells were sorted to three fractions based on CD271 and ErbB3 expression ($CD271^+/ErbB3^+$, $CD271^+/ErbB3^-$, and $CD271^-/ErbB3^+$) and then myogenic differentiation was induced. Among the three fractions, there was no difference in the number of $Pax7^+$ cells and $Myf5^+$ cells (Wu et al., 2018). When ESC-derived Pax7/Myf5 double reporter cells were sorted into three fractions and the cells in these fractions were plated for myotube differentiation, neither the efficiency of myotube formation nor the percentage of $MyHC^+$ cells were different when compared between three fractions (Wu et al., 2018).

CD318

CD318, also known as CUB domain-containing protein 1 (CDCP1) or transmembrane and associated with Src kinases (TRASK), is present on epithelial cells (Spassov et al., 2009), hematopoietic cells (Conze et al., 2003), MSCs (Buhring et al., 2004), and tumor cells (Uekita and Sakai, 2011). Some recent studies reported CD318 expression in myogenic cells. In satellite cells, cytoplasmic expression of CD318 was detected in ∼75% of total cells (Uezumi et al., 2016). $Pax7^+/MyoD1^+$ adult muscle-derived SMPCs grown in hypoxic conditions were reportedly only found in the $CD318^+$ fraction (Uezumi et al., 2016). In mice, satellite cells and myofibers positive with human cell specific markers were observed in recipients of $CD318^+$ adult muscle-derived cells, but rarely in recipients of $CD318^-$ cells (Uezumi et al., 2016). In contrast, it remains controversial whether CD318 can specifically identify SMPC pools in differentiated human iPSCs. To date, both the presence (Sakai-Takemura et al., 2018) and absence (Uezumi et al., 2016) of CD318 expression have been reported in two different studies. Such controversial results in CD318 expression may be caused by differences in culture conditions like oxygen concentration, as several studies demonstrated that CD318 expression was changed by hypoxia (Razorenova et al., 2011; Emerling et al., 2013; Cao et al., 2015). Since commercially available myoblasts and iPSC-derived cells cultured in normoxic conditions showed diminished or undetectable CD318 expression, CD318-based sorting failed to enrich myogenic cells in iPSC-derived SMPCs (Uezumi et al., 2016).

Negative Surface Markers for Human SMPCs

The negative markers listed in **Table 1** are typically absent on human SMPCs but mainly present on non-myogenic cell types. As the markers on their own are usually not indicative of SMPC properties, the cells positive with these markers should be depleted during SMPC isolation. In other words, a majority of SMPCs reside in the negative fraction of these markers. In this section, several representatives of negative surface markers are introduced.

CD15

CD15 (3-fucosyl-N-acetyl-lactosamine, Lewis X or stage-specific embryonic antigen 1) has been used to separate myogenic and adipogenic cells. $CD15^+$ cells can be found outside adult muscle fibers (Lecourt et al., 2010); more specifically, in the interstitial position between the basal lamina of adjacent myofibers (Pisani et al., 2010a). Based on recent observations, CD15 seems to be negative in a majority of SMPCs because CD15 is predominantly detectable in the adipogenic cells with relatively less myogenic capacity. Cell sorting by CD56 and CD15 expression ($CD56^+/CD15^-$, $CD56^-/CD15^+$, and $CD56^+/CD15^+$) could distinguish three cell types: myogenic cells without adipogenic capabilities, non-myogenic cells with adipogenic capabilities, and myogenic cells with adipogenic capabilities, respectively (Lecourt et al., 2010; Pisani et al., 2010a; Agley et al., 2013). $CD56^+/CD15^+$ cells were still able to retain muscle differentiation capacity even under conditions that strongly favor adipogenesis (Agley et al., 2013).

CD24

Known as a sialoglycoprotein and a cell adhesion molecule, CD24 is commonly expressed on the cellular membrane of B lymphocytes and neutrophils (Elghetany and Patel, 2002; Tan et al., 2016), as well as on neural progenitors (Poncet et al., 1996; Fang et al., 2017; Gilliam et al., 2017). Depletion of CD24-expressing cells can enrich human PSC-derived SMPCs when used in combination with positive selection of $CD10^+$ cells. When $CD10^+/CD24^-$ cells were enriched from a variety of PSC lines (Pax7/Myf5 double reporter ESCs, two reporter-free ESC lines, and one reporter-free iPSC line), the isolated cells showed a high level of myotube formation following terminal differentiation (Wu et al., 2018). CD24 expression was not detectable in primary myoblasts (Wu et al., 2018). Interestingly, specific deletion of Myogenin in adult SMPCs resulted in a 10-fold downregulation of CD24 (Meadows et al., 2008). At 16 and 19 weeks of human gestation, a specific cell population with strong CD24 expression was identified in the mesenchymal areas adjacent to developing muscles and intramuscular nerves (Figarella-Branger et al., 1993). In adult muscles, CD24 expression was identified in some unmyelinated nerve fibers and interstitial elongated cells near the neuromuscular junctions (Figarella-Branger et al., 1993). CD24-positive signals were also detected in regenerating muscle fibers following segmental necrosis (Figarella-Branger et al., 1993). These *in situ* locations of CD24 expression implicate that CD24 is possibly involved in the development, maintenance,

and regeneration of muscle innervation. In mice, CD24 is expressed by myofiber synaptic nuclei and plays a role in synaptic transmission (Jevsek et al., 2006).

CD31

CD31, specifically named platelet endothelial cell adhesion molecule (PECAM-1), is a member of the immunoglobulin superfamily. CD31 is primarily used to detect endothelial cells (Lertkiatmongkol et al., 2016). For SMPC purification, this surface marker has been used as a negative selector. In a number of different studies, a depletion step of $CD31^+$ cells has been applied to isolate SMPCs from adult muscle (Bareja et al., 2014; Xu et al., 2015; Garcia et al., 2018) and fetal muscle (Cerletti et al., 2006; Castiglioni et al., 2014). In adult muscle, CD31 expression was observed in the endomysium and intimae part of arteries (Lecourt et al., 2010). In a previous study using fetal muscle, both $CD146^+/CD31^+$ and $CD146^+/CD31^-$ cells were found adjacent to myofibers, and some $CD146^+/CD31^+$ were clearly located within blood vessels (Cerletti et al., 2006). When these cells were isolated from the tissues and cultured, both $CD146^+/CD31^+$ and $CD146^+/CD31^-$ cells were positive with myogenic markers such as Pax7, MyoD, and desmin. Notably, both cell types were able to differentiate into $MyHC^+$ myotubes. These results suggest that CD31 expression itself would not correspond to the ability of muscle differentiation in isolated cells (Cerletti et al., 2006).

CD34

Although CD34 has been considered as a marker of satellite cells in mice, it does not mark satellite cells in human muscle (Péault et al., 2007). CD34 has been used in human muscle satellite cells for negative selection (Sidney et al., 2014). This surface protein has often been utilized for human SMPC isolation as a negative selection marker along with a combination with other surface markers (Zheng et al., 2007; Crisan et al., 2008; Proksch et al., 2009; Pisani et al., 2010b; Okada et al., 2012; Bareja et al., 2014; Castiglioni et al., 2014; Garcia et al., 2018). CD34 is a transmembrane phosphoglycoprotein and widely accepted as a marker of hematopoietic lineage (Sidney et al., 2014). However, CD34 expression is also detected on various types of non-hematopoietic cells such as MSCs (Huss, 2000; Lin et al., 2012), interstitial cells (Popescu et al., 2007; Rasmussen et al., 2007; Zheng et al., 2007; Yu et al., 2012), epithelial progenitors (Blanpain et al., 2004), vascular endothelial progenitors (Fina et al., 1990), and corneal keratocytes (Poole et al., 1993; Polisetty et al., 2008). Although several publications reported CD34 expression in muscle satellite cells (Sidney et al., 2014) and other myogenic cells (Zheng et al., 2007; Proksch et al., 2009; Pisani et al., 2010a; Okada et al., 2012), it remains controversial whether this marker itself is commonly detectable in different types of SMPCs from various sources. Nonexistent or low levels of CD34 expression were detected in human SMPCs derived from PSCs (Awaya et al., 2012; Darabi et al., 2012; Hwang et al., 2014), adult muscle (Alessandri et al., 2004; Morosetti et al., 2006; Dellavalle et al., 2007; Negroni et al., 2009; Lecourt et al., 2010; Zheng et al., 2012; Charville et al., 2015; Franzin et al., 2016; Lorant et al., 2018), fetal muscle (Castiglioni et al., 2014), umbilical cord blood (Koponen et al., 2007; Nunes et al., 2007), and placenta (Park et al., 2011). In skeletal muscle, CD34 expression represents an endothelial nature due to the detection of $CD34^+$ cells in the endomysium and interstitial spaces (Hollemann et al., 2008; Lecourt et al., 2010; Pisani et al., 2010b). CD34 expression has also been detected in the blood vessels within skeletal muscle, particularly the tunica adventitia in veins and the tunica media in arteries (Lecourt et al., 2010).

Evidence in previous studies suggests that both $CD34^+$ and $CD34^-$ cells were present in SMPC pools, but the $CD34^-$ cell population seemed to be more homogeneous and committed to the myogenic lineage compared to $CD34^+$ cells. For instance, some groups of SMPCs have been confirmed as $CD34^-$ populations, which include proliferative and activated satellite cells ($CD34^-/CD56^+/Myf5^+$ cells), and a minority of pericytes and mesoangioblasts with adipogenic potential ($CD34^-/CD56^-$ cells) (Péault et al., 2007). In contrast, $CD34^+$ cells might contain several cell populations originated from the interstitial compartment in skeletal muscle, such as myoendothelial cells, endothelial cells, and SP cells. Interstitial $CD34^+$ cells may have been derived from resident endothelial cells (Vailhe et al., 2001) or have invaded skeletal muscle directly via circulation (Asahara and Kawamoto, 2004). Typically, $CD34^-$ cells show consistent myogenic potential across different preparations of SMPCs, whereas myogenic potential in $CD34^+$ cells tends to be inconsistent and shows variation in different preparations of SMPCs.

A series of studies using adult muscle-derived MDSCs also supports the efficiency of CD34-based isolation to enrich homogenous myogenic cells in the negative fraction. Regardless of muscle type and culture period, $CD34^-$ MDSCs displayed high expression of myogenic markers (Myf5, Pax7, MyoD, myogenin, desmin, and muscle creatine kinase) (Pisani et al., 2010b). These $CD34^-$ MDSCs consistently expressed CD56 and showed myogenic potential following differentiation (Pisani et al., 2010b). In contrast, $CD34^+$ MDSCs barely expressed these myogenic markers and exhibited inconsistent myogenic potential across different preparations of MDSCs (Pisani et al., 2010b). When MDSCs were sorted into $CD34^-/CD90^+$, $CD34^-/CD90^-$ and $CD34^+/CD90^-$ fractions, $CD34^+/CD90^-$ cells showed the lowest expression of Myf5, MyoD, and Myogenin (Proksch et al., 2009). Consistently, in a different study using $CD133^+$ myoblasts isolated from adult muscle, $CD133^+/CD34^-$ cells exhibited higher CD56 expression in culture compared to $CD133^+/CD34^+$ cells (Negroni et al., 2009). In another study, $CD34^-/CD56^+/CD144^-$ cells also retained a high level of CD56 even after expansion in culture, whereas these cells also maintained a myogenic and endothelial cell phenotype (Okada et al., 2012). Interestingly, $CD34^-$ MDSCs did not show adipogenic differentiation both *in vitro* and *in vivo*, whereas $CD34^+$ cells were able to generate adipocytes both in culture and following transplantation (Pisani et al., 2010b). $CD34^+$ MDSCs may retain cellular characteristics as multipotent stem cells, which possibly implies their limited commitment to myogenic lineage.

Although $CD34^+$ cells do not specifically represent SMPC pools, these cells may have supporting roles for the integration of SMPCs when transplanted with $CD34^+$ SMPCs. In one

study, three cell populations ($CD56^+/CD34^+/CD144^+$, $CD56^-/CD34^+/CD144^+$, and $CD56^+/CD34^-/CD144^-$) were isolated from human adult muscle as myoendothelial cells, endothelial cells, and myogenic cells, respectively. When their ability of cell survival and muscle regeneration was compared by intramuscular implantation into SCID mice, $CD56^+/CD34^+/CD144^+$ myoendothelial cells demonstrated the most significant results (Zheng et al., 2007). In a different study, $CD133^+/CD34^+$ myoblasts isolated from human adult muscle showed better regeneration capacity compared to $CD133^+/CD34^-$ cells following transplantation into the tibialis anterior muscles of immunodeficient mice (Negroni et al., 2009).

CD45

CD45, also known as leukocyte common antigen or protein tyrosine phosphatase receptor type C, is a hematopoietic marker (Kaplan et al., 1990) widely used as a negative marker for SMPCs. In a number of studies, $CD45^+$ cell depletion has already been used to enrich human SMPCs derived from adult muscle and fetal muscle (Zheng et al., 2007, 2012; Crisan et al., 2008; Lecourt et al., 2010; Okada et al., 2012; Bareja et al., 2014; Castiglioni et al., 2014; Xu et al., 2015; Garcia et al., 2018). In other studies, the absence of CD45 was used to define SMPC pools from the cell preparation derived from PSCs, adult muscle, placenta, or umbilical cord blood (Alessandri et al., 2004; Morosetti et al., 2006; Dellavalle et al., 2007; Nunes et al., 2007; Zheng et al., 2007; Proksch et al., 2009; Park et al., 2011; Awaya et al., 2012; Darabi et al., 2012; Tedesco et al., 2012; Castiglioni et al., 2014; Charville et al., 2015; Lorant et al., 2018).

CD57

CD57 is also known as beta-1,3-glucuronyltransferase 1 (B3GAT1), human natural killer 1 (HNK-1), or LEU-7. CD57 is a neuroectodermal marker expressed on natural killer cells and T-lymphocytes (Kared et al., 2016). Although knowledge about CD57 expression on SMPCs is relatively limited at this moment, a few studies reported that CD57 was useful to deplete neural cells during the purification of PSC-derived SMPCs (Borchin et al., 2013; Choi et al., 2016; Sakai-Takemura et al., 2018). When CD57 alone was used for sorting PSC-derived cells, only negative cells could differentiate into myotubes (Sakai-Takemura et al., 2018). Compared to $CD56^-$ and $CD57^+/CD56^+$ cells, $CD57^-/CD56^+$ cells showed significantly increased expression of MyoD1, Myogenin, and MyHC. $CD57^-/CD56^+$ cells also showed higher expression of Myogenin and Pax7 than human fetal skeletal muscle and undifferentiated human ESCs (Choi et al., 2016). In a different study, $CD57^-/CD56^+$ PSC-derived SMPCs showed higher expression of Pax7, Myf5, and MyHC than unsorted cells in culture. However, there was no difference in engraftment rate between $CD57^-/CD56^+$ cells and unsorted cells when intramuscularly transplanted into mdx-NSG mice (Hicks et al., 2017).

CD106

CD106 (vascular cell adhesion molecule 1) has been known to show expression in MSCs, activated endothelial cells, and macrophages (Yang et al., 2013). Although murine satellite cells express CD106 (Liu et al., 2015; Maesner et al., 2016), it seems that CD106 expression in human SMPCs remains controversial. Some studies showed that the expression of this marker was positive in adult muscle-derived SMPC populations (Sinanan et al., 2004; Lecourt et al., 2010) while other studies reported an absence of CD106 expression in different types of SMPCs derived from PSCs, adult muscle and fetal muscle (Morosetti et al., 2006; Dellavalle et al., 2007; Crisan et al., 2008; Pisani et al., 2010b; Awaya et al., 2012; Darabi et al., 2012). Further studies would be required to determine the expression of CD106 on human SMPCs.

CD108

CD108, or semaphorin 7A, is a glycophosphatidylinositol-linked glycoprotein. When CD108 was used as a sole marker for sorting iPSC-derived SMPCs, $CD108^-$ cells showed significantly higher levels of myotube formation *in vitro* compared to the unsorted cells. Isolated $CD57^-/CD108^-/CD271^+/ErbB3^+$ cells from iPSC-derived SMPCs were able to form myotubes with high efficiency in culture and when transplanted into the tibialis anterior muscles of immunodeficient dystrophin-deficient NSG-mdx^{4Cv} mice (Sakai-Takemura et al., 2018). In adult skeletal muscle, CD108 was expressed on both fibroblasts and myoblasts; this discrepancy is not well-understood (Sakai-Takemura et al., 2018).

Combinations of Surface Markers Used for SMPC Enrichment

To date, cell sorting by several combinations of cell surface markers has worked successfully to enrich human PSC-derived SMPCs and to deplete undesirable cell types (**Table 2**). Although these individual works supported the feasibility of SMPC isolation using different combinations of surface markers, one common challenge is that the procedure becomes complicated with multiple sorting steps.

Which Surface Markers Would Be Promising for Human SMPC Isolation?

Based on our comprehensive search of the literature as described above, we summarize the promising surface markers that have already been used to enrich human PSC-derived SMPCs (**Table 3**; the rows of these markers are highlighted in gray). We include possible markers that have confirmed expression in human PSC-derived SMPCs but have not been tested yet for sorting human PSC-derived SMPCs (**Table 3**; the rows of these markers remain white). We also made note of whether they were used alone or in combination with other markers, and in which PSC lines the markers have been tested. Although this is not a definitive list, we consider the bolded markers in **Table 3** to be highly promising for the enrichment of human PSC-derived SMPCs: CD10, CD54, CD56, CD82, CD271, ErbB3, and c-Met for positive selection; and CD24, CD57 for negative selection. Although it is difficult to propose the best combination of these markers at this moment, several combinations have successfully worked as described in the previous studies. They include $CD10^+/CD24^-$ (Wu et al., 2018), $CD57^-/CD108^-/CD271^+/ErbB3^+$ (Sakai-Takemura et al., 2018), $CD56^+/CD82^+$ (Uezumi et al., 2016),

TABLE 2 | Combinations of markers used to isolate human PSC-derived SMPCs and findings about their enrichment efficiency.

Isolated fractions	PSCs	*In vitro* analysis	*In vivo* analysis	References
$CD10^+/CD24^-$	Pax7/Myf5 dual reporter ESCs, and three transgene-free PSC lines	In all four cell lines, only $CD10^+/CD24^-$ cells exhibited myotube formation. $CD10^+/CD24^+$ $CD10^-/CD24^-$, and $CD10^-/CD24^+$ fractions contained non-myogenic cells and did not exhibit myotube formation	$CD10^+/CD24^-$ cells were sorted from Pax7/Myf5 dual reporter ESCs and transplanted into non-irradiated, cardiotoxin-damaged hindlimb (tibialis anterior, TA) muscles of immunodeficient dystrophin-deficient NSG-mdx^{4Cv} mice (2.5×10^5 cells/10 μL/muscle). At 4 weeks post-transplantation, $CD10^+/CD24^-$ cells formed maximum of 50–60 $dystrophin^+$ and lamin A/C^+ fibers, where 12 ± 1.9% out of all lamin A/C^+ cells were $Pax7^+$. $CD10^+/CD24^-$ cells isolated from transgene-free PSCs were also able to form human myofibers in NSG-mdx^{4Cv} mice	Wu et al., 2018
$CD57^-/CD108^-/CD271^+$/ $ErbB3^+$	iPSCs	At 48 h after serial sorting, most cells were $MyoD^+$, 30-40% were $Pax7^+$. The cells formed $Myogenin^+$ multinucleated myotubes. Pax7 was expressed in mononuclear cells between myotubes. When co-cultured with human adult myoblasts, the efficiency of myotube fusion was higher in the sorted cells compared to the unsorted ones	When the sorted cells were implanted into the TA muscles of NSG-mdx^{4Cv} mice, 12–13 myofibers with lamin A/C^+ and $Spectrin^+$ were found on transverse muscle sections at 3 weeks of post-transplantation. 1×10^5 sorted cells formed more human lamin A/C^+, $spectrin^+$ and $dystrophin^+$ myofibers compared to 1×10^6 unsorted cells	Sakai-Takemura et al., 2018
$CD54^+/\ \alpha9\beta1^+/\ CD362^+$	PSCs with Pax7 overexpression by doxycycline-based induction	100% of cells were $Pax7^+$. The isolated progenitors did not show expression of pluripotent stem cell markers (Oct3/4, Sox2 and Nanog) and were able to form multinucleated $MyHC^+$ myotubes following terminal differentiation	Sorted cells were transplanted into cardiotoxin-injured TA muscles of NSG mice (5×10^5 cells/10 μL/muscle). At 8 weeks and 10 months post-transplantation, ∼50 and ∼40 $dystrophin^+$/lamin A/C^+ myofibers were identified in each muscle section, respectively. Cell transplantation into NSG-mdx^{4Cv} mice showed similar results. Lamin A/C^+ cells, which also expressed M-cadherin or Pax7, were detected in satellite cell position	Magli et al., 2017
$CD56^+/CD82^+$	iPSCs	When compared to double-negative cells, Pax7 and MyoD expression was found exclusively in $CD56^+/CD82^+$ cells. When compared to $CD56^-/CD82^+$, $CD56^+/CD82^-$ and double-negative cells, $MyHC^+$ myotube formation was actively promoted in $CD56^+/CD82^+$ cells		Uezumi et al., 2016
$CD57^-/CD56^+$	PSCs	98% of single $CD57^-/CD56^+$ cells showed higher expression of Myogenin and Pax7 than human fetal skeletal muscle and undifferentiated ESCs. $CD57^-/CD56^+$ cells also expressed MyoD1, Myogenin and MyHC significantly higher than $CD56^-$ cells and $CD57^+/CD56^+$ cells. Gene expression analysis revealed that key markers and transcription factors of skeletal muscle structure development were enriched in the $CD57^-/CD56^+$ cell fractions. $CD57^-/CD56^+$ cells could be expanded up to the hundreds of millions of cells and were easily cryo-preserved without losing myotube-forming competence	The $CD57^-/CD56^+$ cells were prepared from genetically corrected DMD iPSC-derived SMPCs and then transplanted into irradiated cardiotoxin-injured TA muscles of NSG-mdx^{4Cv} mice and immunodeficient NOD-$Rag1^{null}IL2r\gamma^{null}$ (NRG) mice (2×10^6 cells/muscle). ∼100 and ∼380 $laminin^+$/lamin A/C^+ myofibers per muscle section were detected in NSG-mdx^{4Cv} mice and NRG mice, respectively	Choi et al., 2016
	PSCs	$CD57^-/CD56^+$ cells were positive with Pax7 and Myf5, specifically with ∼1.7-fold more compared to unsorted preparations	Sorted cells were transplanted into cardiotoxin-injured TA muscles of NSG-mdx^{4Cv} mice (2×10^6 cells/10 μL/muscle). $CD57^-/CD56^+$ cells did not show improved engraftment rate compared to unsorted cells. Significant differences were not found in $spectrin^+$/lamin A/C^+ and $dystrophin^+$ myofibers generated by both groups of cells	Hicks et al., 2017

(Continued)

TABLE 2 | Continued

Isolated fractions	PSCs	*In vitro* analysis	*In vivo* analysis	References
$CD271^+/ErbB3^+$	PSCs	$CD271^+/ErbB3^+$ expressed Pax7 and Myf5 20-fold more than double-negative cells. $CD271^+/ErbB3^+$ cells showed significantly higher myotube formation compared to $CD271^+/ErbB3^-$, $CD271^-/ErbB3^+$ and double-negative cells	DMD iPSC were genetically corrected by CRIPSR/Cas9 method and prepared SMPCs. $CD271^+$ cells and $ErbB3^+$ cells were sorted from these SMPCs and transplanted into cardiotoxin-injured TA muscles of NSG-mdx^{4Cv} mice (2×10^6 cells/10 μL/muscle). Both cell types showed significantly higher engraftment rate than $CD56^+$ and unsorted cells, as determined by the number of myofibers with human lamin A/C^+/$spectrin^+$/$dystrophin^+$. Engraftment of $ErbB3^+$ cells could restore dystrophin expression up to the levels observed in the muscles implanted freshly isolated human fetal myocytes	Hicks et al., 2017
	Pax7/Myf5 dual reporter ESCs, and three transgene-free PSC lines	At Day 5 and Day 15 in myogenic differentiation medium, $CD271^+/ErbB3^+$, $CD271^+/ErbB3^-$, and $CD271^-/ErbB3^+$ cells were evaluated with reporter protein expression (tdTomato driven by Pax7 promotor and EGFP by Myf5 promotor). At both timepoints, EGFP-positive myogenic cells were equally distributed in three fractions. When these fractions were plated and subjected to terminal differentiation, all of three fractions from Day 5 could not differentiate into myotubes. In contrast, all three fractions from Day 15 contained mixed populations of $MyHC^+$ myotubes and $MyHC^-$ non-myogenic cells	$CD271^+/ErbB3^+$ cells were isolated from Pax7/Myf5 dual reporter ESCs and transplanted into non-irradiated, cardiotoxin-damaged TA muscles of NSG-mdx^{4Cv} mice (2.5×10^5 cells/10 μL/muscle). At 4 weeks post-transplantation, $CD271^+/ErbB3^+$ cells were able to form maximum of 10–15 $dystrophin^+$ and lamin A/C^+ myofibers per section, but no $Pax7^+$ cells were detected	Wu et al., 2018
$CD73^+/CD56^+$	ESCs	60-80% of $CD73^+/CD56$ cells were $MyoD^+$. Cells adopted a bipolar cell morphology at 24 h in culture; 46 ± 3% and 7 ± 3% of total cells were $MyoG^+$ and $Pax7^+$, respectively. Upon terminal differentiation, the expression of Myogenin, desmin, actin and MyHC were confirmed. These differentiated myotubes were capable of spontaneous twitching in culture	Before transplantation, $CD73^+/CD56^+$-sorted cells were transduced with a lentiviral vector carrying a triple reporter construct expressing herpes simplex virus thymidine kinase, enhanced GFP, and luciferase. The cells were then transplanted into the cardiotoxin-injured TA muscles of immunodeficient SCID/Beige mice (5×10^5 cells/muscle). Stable bioluminescence signals were confirmed even after 6 months of cell transplantation. Immunohistochemical analyses of implanted muscles demonstrated that an average of 7% of muscle fibers was positive with human laminin	Barberi et al., 2007
$CD57^-/AChR^-/CD184^+$ /c-Met^+	PSCs	Cell sorting was performed at three time points (Day 23, 25, and 35) following directed myogenic differentiation of PSCs. At Day 23, sorted cells expressed Six4 and Pax3. At Day 25, sorted cells expressed Pax7. At Day 35, sorted cells were 98% $Pax3^+$ and 96% $Pax7^+$. The cells sorted at Day 35 demonstrated progressive terminal muscle differentiation when plated down, as shown by expression of Myf5, Myogenin, and MyHC. After 3 days of terminal differentiation, all cells expressed MYF5 and few retained Pax7 expression. After 9 days, myotubes were formed while most cells were $Myogenin^+$ and $MyHC^+$		Borchin et al., 2013

TABLE 3 | Surface markers and their potential to isolate human PSC-derived SMPCs.

Marker	+/– selection	Strength	Weakness	Expression level in SMPCs from other sources
CD10	+	• Enriched SMPC population from transgene-free PSCs and Pax7/Myf5 reporter ESCs when used in combination with $CD24^-$ selection (Wu et al., 2018)	• Efficiency as a sole marker is unknown • Efficiency when used with markers other than $CD24^-$ is unknown	• Detected on ~97% human primary myoblasts (Wu et al., 2018) • Detected on ~80% $Pax7^+$ cells derived from adult muscle (Wu et al., 2018)
CD11b	-	• Exclusion of $CD11b^+$ cells efficiently depleted hematopoietic cells among SMPC population derived from fetal muscle, when used in combination with $GlyA^-$ /$CD45^-$ selection (Castiglioni et al., 2014), and from adult muscle when used in combination with $CD45^-$ selection (Bareja et al., 2014)	• May be redundant as a negative marker, as the expression on human PSC-derived SMPCs was rarely reported • $CD11b^+$ population from CRISPR/Cas9 corrected DMD iPSC-derived SMPC was able to form myotube in culture (Hicks et al., 2017)	
CD13	+	• Expression was detected on 40-64% human Pax7-overexpressing PSC-derived SMPCs (Darabi et al., 2012) and iPSC-derived mesoangioblast-like stem/progenitor cells (Tedesco et al., 2012)	• Never used to isolate human PSC-derived SMPCs, efficiency is unknown whether as a sole marker or in combination	• Detected on ~97% human adult pericytes (Dellavalle et al., 2007) • Detected on 38.4% mononucleated cells derived from fetal muscle (Castiglioni et al., 2014)
CD15	-	• Presence or absence of expression could distinguish $CD56^+$ adult muscle-derived myogenic cells with or without adipogenic capabilities, respectively (Lecourt et al., 2010; Pisani et al., 2010a; Agley et al., 2013)	• May be redundant as a negative marker, as CD15 expression on human PSC-derived SMPCs has not been confirmed yet	
CD24	–	• Enriched SMPC population from transgene-free PSCs and Pax7/Myf5 reporter ESCs when used in combination with $CD10^+$ selection (Wu et al., 2018)	• Efficiency as a sole marker is unknown • Efficiency when used with markers other than $CD10^+$ is unknown	• Not detected on human primary myoblasts (Wu et al., 2018)
CD29	+	• CD29/Integrin alpha-9 dimer (α9β1) efficiently isolated SMPC population from Pax7-overexpressing PSCs (Magli et al., 2017) • Expression confirmed by >70% cells of PSC-derived SMPC populations (Awaya et al., 2012; Darabi et al., 2012; Abujarour et al., 2014; Magli et al., 2017; Sakai-Takemura et al., 2018) • Enriched SMPC population from adult muscle when used in combination with $CD31^-$/$CD34^-$/$CD45^-$/$EGFR^+$ selection (Charville et al., 2015), and with $CD56^+$/$CD31^-$/$CD45^-$ selection (Xu et al., 2015), as well as from fetal muscle when used in combination with $CD56^+$/$CD184^+$/$CD31^-$/$CD34^-$/$CD45^-$ selection (Garcia et al., 2018)	• Never used alone (as opposed to α9β1 dimer) to isolate human PSC-derived SMPCs; efficiency is unknown whether as a sole marker or in combination • A population of $CD56^+$/$CD29^+$ cells without Pax7 expression was identified in healthy adult human skeletal muscle, implying that CD29 expression is not exclusive to satellite cells (Xu et al., 2015)	• Detected on 90.4% mononucleated cells derived from fetal muscle (Castiglioni et al., 2014) • Co-expression with CD56 was detected on 100% $Pax7^+$ satellite cells in healthy adult skeletal muscle (Xu et al., 2015) • Detected on SMPCs derived from postnatal muscle (Garcia et al., 2018) and adult muscle (Lecourt et al., 2010; Woodard et al., 2014; Charville et al., 2015; Xu et al., 2015; Lorant et al., 2018)
CD31	-	• Exclusion of $CD31^+$ cells in a series of sequential isolation steps efficiently depleted endothelial cells among SMPC population derived from adult muscle (Bareja et al., 2014; Xu et al., 2015; Garcia et al., 2018) and fetal muscle (Cerletti et al., 2006; Castiglioni et al., 2014)	• May be redundant as a negative marker for PSC-derived SMPCs as expression was not detected on human Pax7-overexpressing PSC-derived SMPCs (Darabi et al., 2012) and iPSC-derived mesoangioblast-like stem/progenitor cells (Tedesco et al., 2012) • CD31 expression itself does not correspond to SMPC properties, as both $CD146^+$/$CD31^+$ and $CD146^+$/$CD31^-$ cells isolated from human fetal muscle and cultured were able to differentiate into myotubes (Cerletti et al., 2006)	• Expression on SMPCs derived from adult muscle had been reported to be both detectable (Hollemann et al., 2008; Lecourt et al., 2010; Bareja et al., 2014; Xu et al., 2015) and undetectable (Alessandri et al., 2004; Dellavalle et al., 2007; Charville et al., 2015; Lorant et al., 2018)

(Continued)

TABLE 3 | Continued

Marker	+/– selection	Strength	Weakness	Expression level in SMPCs from other sources
CD34	-	• $CD34^-$ MDSCs displayed high expression of myogenic markers and exhibited consistent myogenic potential regardless of preparation and culture period (Pisani et al., 2010b) • $CD34^-$ MDSCs retained higher expression of CD56 in culture when sorted along with both $CD90^+$ and $CD90^-$ selections (Proksch et al., 2009), $CD133^+$ selection (Negroni et al., 2009), as well as $CD56^+/CD144^-$ selection (Okada et al., 2012)	• May be redundant as a negative marker for PSC-derived SMPCs as expression was reported to be non-existent or low on human PSC-derived SMPCs (Awaya et al., 2012; Darabi et al., 2012; Hwang et al., 2014)	• Some groups of SMPCs have been confirmed as $CD34^-$ populations: proliferative satellite cells ($CD34^-/CD56^+/Myf5^+$ cells), and a minority of pericytes and mesoangioblasts with adipogenic potential ($CD34^-/CD56^-$ cells) (Péault et al., 2007). • Expression reported to be in varied levels on SMPCs from different sources (see section CD34)
CD45	-	• Exclusion of $CD45^+$ cells in a series of sequential isolation steps efficiently depleted hematopoietic cells among SMPC population derived from adult muscle (Zheng et al., 2007, 2012; Lecourt et al., 2010; Okada et al., 2012; Bareja et al., 2014; Xu et al., 2015) and fetal muscle (Crisan et al., 2008; Castiglioni et al., 2014; Garcia et al., 2018)	• May be redundant as a negative marker for PSC-derived SMPCs as expression was not detected on human Pax7-overexpressing PSC-derived SMPCs (Darabi et al., 2012) and iPSC-derived mesoangioblast-like stem/progenitor cells (Tedesco et al., 2012)	• Not detected on SMPC populations derived from adult muscle, placenta, and umbilical cord blood (see section CD45)
CD54	+	• Enriched SMPC population from Pax7-overexpressing PSCs when used alongside with $CD362^+$/integrin $\alpha9\beta1^+$ selection (Magli et al., 2017) • Able to act as a sole marker to isolate SMPC population from Pax7-overexpressing PSCs (Magli et al., 2017)	• Efficiency to isolate transgene-free PSC-derived SMPCs is unknown	• Expression on cultured human skeletal muscle cells can be induced with various cytokine treatments (Goebels et al., 1992; Michaelis et al., 1993; Bartoccioni et al., 1994; Marino et al., 2001)
CD56	+	• Enriched SMPCs from transgene-free ESC-derived MSCs when used alongside $CD73^+$ selection (Barberi et al., 2007) • Enriched transgene-free iPSC-derived SMPCs when used alongside $CD82^+$ selection (Uezumi et al., 2016) • Enriched transgene-free PSC-derived SMPCs when used alongside $CD57^-$ selection (Hicks et al., 2017) • Enriched SMPC population derived from fetal muscle (Crisan et al., 2008; Castiglioni et al., 2014) and adult muscle (Dellavalle et al., 2007; Zheng et al., 2007, 2012; Lecourt et al., 2010; Pisani et al., 2010a; Okada et al., 2012; Bareja et al., 2014; Woodard et al., 2014; Xu et al., 2015; Alexander et al., 2016; Choi et al., 2016) alongside other markers • Able to act as a sole marker to isolate SMPC population derived from adult muscle (Sinanan et al., 2004; Lecourt et al., 2010; Pisani et al., 2010a; Lorant et al., 2018)	• Efficiency to isolate transgene-based PSC-derived SMPCs is unknown • Efficiency as a sole marker to isolate PSC-derived SMPCs is unknown • May not improve SMPC purity by much as almost all cells in some PSC-derived SMPC populations express CD56: 98-100% of human Pax7-overexpressing PSC-derived SMPCs (Darabi et al., 2012), 95.3% of $Pax3^+$ or $Pax7^+$ cells from ESC-derived SMPCs (Awaya et al., 2012), 75% MyoD-overexpressing ESCs (Goudenege et al., 2012) • SMPCs may also reside in $CD56^-$ population, as evidenced by several $CD56^-$ populations that were able to form myotubes both *in vitro* and *in vivo*: a population of "endothelial cells" ($CD45^-/CD56^-/CD34^+/CD144^+$ selection) isolated from adult muscle (Okada et al., 2012), a population of "perivascular stem cells" ($CD146^+/CD45^-/CD56^-$/UEA-$1R^-$ selection) derived from adult muscle (Zheng et al., 2012), and a population of "perivascular cells" ($CD146^+/CD34^-/CD45^-/CD56^-$ selection) isolated from multiple human organs and placenta (Crisan et al., 2008; Park et al., 2011); the latter two populations showed upregulated CD56 expression after expression in culture	• Detected on >80% satellite cells derived from adult muscle (Negroni et al., 2009) • Detected on 83% "slowly adhering cell" fraction of culture derived from adult muscle (Okada et al., 2012) • Detected on 77.1% $CD45^-$ cultured cells derived from adult muscle (Zheng et al., 2012) • Detected on 69-99% SMPCs derived from adult muscle (Lorant et al., 2018) • Detected on SMPCs derived from PSCs, postnatal muscle, adult muscle, fetal muscle, and placenta (see section CD56)

(Continued)

TABLE 3 | Continued

Marker	+/– selection	Strength	Weakness	Expression level in SMPCs from other sources
CD57	–	• Exclusion of CD57$^+$ cells in a series of sequential isolation steps efficiently depleted neural cells among transgene-free PSC-derived SMPC populations (Borchin et al., 2013; Hicks et al., 2017; Sakai-Takemura et al., 2018) and Mesogenin1/eGFP reporter human ESC-derived SMPC population (Choi et al., 2016) • Able to enrich transgene-free iPSC-derived SMPCs when used as a sole marker in a single-step negative selection (Sakai-Takemura et al., 2018)	• May be redundant as a negative marker as expression on human PSC-derived SMPCs was rarely reported • May not improve SMPC purity by much if expression level is low in PSC-derived SMPC populations	
CD73	+	• The expression was confirmed in 99.4% Pax3$^+$ or Pax7$^+$ cells from ESC-derived SMPCs (Awaya et al., 2012), 96.8% MyoD-overexpressing ESCs (Goudenege et al., 2012) and 66% SMPCs derived from adult muscle (Woodard et al., 2014)	• Never used to isolate SMPCs from any source, efficiency is unknown whether as a sole marker or in combination	• Detected on SMPCs derived from fetal muscle (Crisan et al., 2008) and adult muscle (Lecourt et al., 2010; Uezumi et al., 2016; Lorant et al., 2018)
CD82	+	• Enriched transgene-free iPSC-derived SMPCs (Uezumi et al., 2016) and SMPCs derived from adult muscle (Alexander et al., 2016) when used alongside CD56+ selection • Enriched SMPCs derived from fetal muscle when used alongside CD146$^+$ selection (Alexander et al., 2016) • Can be used alone to enrich SMPCs derived from adult muscle (Uezumi et al., 2016)	• Efficiency to isolate transgene-based PSC-derived SMPCs is unknown • Efficiency as a sole marker to isolate PSC-derived SMPCs is unknown • Fairly new marker, expression level in PSC-derived SMPCs have not been reported much, therefore hard to gauge redundancy of isolation based on this marker	• Detected on ∼97% Pax7$^+$ or M-cadherin$^+$ satellite cells (Uezumi et al., 2016)
CD108	–	• Enriched transgene-free iPSC-derived SMPCs as a sole marker and when used alongside CD57$^-$/CD271$^+$/ErbB3$^+$ selection (Sakai-Takemura et al., 2018)	• Efficiency to isolate transgene-based PSC-derived SMPCs is unknown • Efficiency as a sole marker or when used with markers other than CD57$^-$/CD271$^+$/ErbB3$^+$ is unknown • Expression level in PSC-derived SMPCs is rarely reported, therefore hard to gauge redundancy of isolation based on this marker	• Detected on both fibroblasts and myoblasts in adult skeletal muscle (Sakai-Takemura et al., 2018)
CD146	+	• Expression confirmed in PSC-derived SMPCs (Darabi et al., 2012; Tedesco et al., 2012; Sakai-Takemura et al., 2018) • Enriched SMPC population derived from fetal muscle (Cerletti et al., 2006; Crisan et al., 2008; Lapan et al., 2012; Alexander et al., 2016), adult muscle (Zheng et al., 2012) and placenta (Park et al., 2011)	• Efficiency to isolate PSC-derived SMPCs is unknown • May not improve SMPC purity by much if very highly expressed in PSC-derived SMPC populations; for example, 100% of human Pax7-overexpressing PSC-derived SMPCs were CD146$^+$ (Darabi et al., 2012)	• Detected on 41.11% culture-expanded pericytes derived from adult muscle (Dellavalle et al., 2007) • Detected on 66.9% CD45$^-$ cultured cells derived from adult muscle (Zheng et al., 2012) • Detected on 35-80% mononucleated cells derived from fetal muscle (Lapan et al., 2012)
CD184	+	• Enriched highly pure SMPCs from transgene-free PSC-derived populations when used in combination with CD57$^-$/AChR$^-$/c-Met$^+$ selection (Borchin et al., 2013)	• Does not exclusively select SMPCs. Also expressed on neural cells, therefore most likely only reliable when used alongside c-Met: highly pure SMPCs were found to reside in c-Met$^+$/CD184$^-$ population; c-Met$^-$/CD184$^+$ fraction contained both SMPCs and neural cells; c-Met$^-$/CD184$^-$ fraction did not have any SMPCs (Borchin et al., 2013) • Efficiency when used with markers other than c-Met is unknown	• Detected on 63.2% mononucleated cells derived from fetal muscle (Castiglioni et al., 2014) • Detected on SMPCs derived from adult muscle (Bareja et al., 2014; Marg et al., 2014; Garcia et al., 2018)

(Continued)

TABLE 3 | Continued

Marker	+/– selection	Strength	Weakness	Expression level in SMPCs from other sources
CD271	+	• Enriched transgene-free iPSC-derived SMPC population when used as a sole marker and when used in combination with $CD57^-/CD108^-/ErbB3^+$ selection (Sakai-Takemura et al., 2018) • Resulted in the most efficient SMPC enrichment from CRISPR/Cas9 corrected DMD iPSC-derived SMPC population when used as a sole marker, compared to CD11b, CD56, CD146, CD184, c-Met, ITGA7, ErbB3 used as sole markers (Hicks et al., 2017) • Enriched transgene-free and transgene-based PSC-derived SMPC populations when used alongside $ErbB3^+$ selection (Hicks et al., 2017)	• May not be able to isolate SMPCs in all human PSC-derived SMPC populations; failed to enrich for SMPCs in a Pax7/Myf5 reporter ESC population when used alongside $ErbB3^+$ or $ErbB3^-$ selection (Wu et al., 2018) • Fairly new marker; expression level in PSC-derived SMPCs has not been reported much, therefore hard to gauge redundancy of isolation based on this marker	• Detected on cultured-expanded postnatal myoblasts (Sakai-Takemura et al., 2018) • Detected in a subpopulation co-expressing $CD271^+$ and myogenic transcription factors in the muscle at 8 weeks of gestation; these cells then started losing CD271 expression at 16 weeks of gestation
CD318	+	• Expression reported in 43.1% of transgene-free iPSC-derived SMPCs (Sakai-Takemura et al., 2018)	• Failed as a sole marker to enrich transgene-free iPSC-derived SMPCs due to undetectable expression level (Uezumi et al., 2016) • Efficiency to isolate PSC-derived SMPCs in combination with other markers is unknown	• Cytoplasmic expression was detected in ∼75% of satellite cells (Uezumi et al., 2016) • Only the CD318+ fraction contained $Pax7^+/MyoD1^+$ adult muscle-derived SMPCs grown in hypoxic conditions (Alexander et al., 2016)
CD362	+	• Enriched SMPC population from Pax7-overexpressing PSCs when used alongside with $CD54^+$/integrin $\alpha9\beta1^+$ selection (Magli et al., 2017) • Detected on ∼95% $CD54^+$/integrin $\alpha9\beta1^+$ proliferating SMPCs	• Efficiency to isolate transgene-free PSC-derived SMPCs is unknown • Efficiency as a sole marker or when used with markers other than $CD54^+$/integrin $\alpha9\beta1^+$ is unknown • Expression level in PSC-derived SMPCs is rarely reported, therefore hard to gauge redundancy of isolation based on this marker	
c-Met	+	• Enriched highly pure SMPCs and depleted neural cells and other non-myogenic cell types from transgene-free PSC-derived populations when used in combination with $CD57^-/AChR^-$ selection (Borchin et al., 2013)	• May be redundant if not expressed on human PSC-derived SMPCs; expressed only in 2.4% cells of an iPSC-derived SMPC population (Sakai-Takemura et al., 2018); failed to act as a sole marker to enrich CRISPR/Cas9 corrected DMD iPSC-derived SMPCs (Hicks et al., 2017) • Efficiency as a sole marker is unknown	
ErbB3	+	• When used as a sole marker yielded better SMPC enrichment from transgene-free iPSC-derived SMPC population than $CD56^+/CD82^+$ selection (Sakai-Takemura et al., 2018) • Enriched transgene-free iPSC-derived SMPC population when used in combination with $CD57^-/CD108^-/ErbB3^+$ selection (Sakai-Takemura et al., 2018) • Resulted in the second most efficient SMPC enrichment from CRISPR/Cas9 corrected DMD iPSC-derived SMPC population when used as a sole marker, compared to CD11b, CD56, CD146, CD184, CD271, c-Met, ITGA7 used as sole markers (Hicks et al., 2017)	• May not be able to isolate SMPCs in all human PSC-derived SMPC populations; failed to enrich for SMPCs in a Pax7/Myf5 reporter ESC population when used alongside $CD271^+$ or $CD271^-$ selection (Wu et al., 2018) • Fairly new marker; expression level in PSC-derived SMPCs have not been reported much, therefore hard to gauge redundancy of isolation based on this marker	• Detected on $CD146^+$ SMPCs derived from fetal muscle (Alexander et al., 2016) • Detected in a subpopulation co-expressing $ErbB3^+$ and myogenic transcription factors in the muscle at 8 weeks of gestation; these cells at 16 weeks of gestation exhibited close to 100% myotube-forming capability if plated down

Markers with gray background have already been used to isolate human PSC-derived SMPCs. The markers with white background are the ones that have not been tested for sorting of PSC-derived SMPC but are potentially useful for SMPC isolation. Based on our literature search, we propose that the bolded markers would be highly promising for SMPC isolation from human pluripotent sources.

$CD271^+/ErbB3^+$ (Hicks et al., 2017), $CD57^-/CD56^+$ (Choi et al., 2016; Hicks et al., 2017), and $CD57^-/AChR^-/CD184^+/c\text{-}Met^+$ (Borchin et al., 2013; **Table 2**). To further improve the efficiency of SMPC enrichment, additional studies would be necessary to test different combinations.

REMAINING CHALLENGES TO ISOLATE HUMAN SMPCS FROM PLURIPOTENT STEM CELLS

The current knowledge of cell surface markers supports our ability to isolate and characterize PSC-derived SMPCs. While a number of positive and negative markers are potentially available to define SMPC pools, it may still not be easy to prepare a pure population of SMPCs from PSC-derived cells.

In recent years, several culture protocols have been developed to derive skeletal myocytes from human PSCs. Transgene-based SMPC derivation uses the overexpression of myogenic genes, such as PAX7 (Darabi et al., 2012; Skoglund et al., 2014) and MYOD1 (Tanaka et al., 2013; Abujarour et al., 2014; Yasuno et al., 2014; Maffioletti et al., 2015). Although transgene-based approaches yield SMPCs with high efficiency, the resulting cells may not fully reflect the natural endogenous processes of SMPC proliferation, differentiation, and maturation, because such overexpression requires genetic modification (reviewed in Jiwlawat et al., 2018). Similarly, although fluorophore-labeled SMPCs warrant convenient isolation, the insertion of transgene constructs that link fluorophore reporter genes to myogenic genes present the same concerns associated with transgene-based approaches. As such, transgene-free methods may be more suitable to prepare SMPCs for clinical applications, because these methods only use defined culture conditions supplemented with factors that encourage myogenic lineage commitment. However, the efficiency of SMPC derivation is relatively lower in the existing transgene-free methods compared to transgene-based approaches. Further, transgene-free SMPC derivation gives rise to a heterogeneous cell population with an embryonic or perinatal phenotype which contains myocytes of various stages and other cell types (Jiwlawat et al., 2017). As such, to obtain SMPC populations with high quality and quantity, cell sorting using cell surface markers would be required. A better understanding of SMPC surface markers is crucial to improve the purity of resulting cell populations.

With the use of specific antibodies targeting only cells expressing a particular surface marker, positive selection yields a higher purity of the desired population. However, positively selected cells may retain antibodies and other labeling agents, which may interfere with downstream culture and assays. Negatively selected cells would not carry such concern, but it is less efficient in terms of purity to deplete all undesired cells by only relying on negative selection. Positive selection and negative selection can be used to purify a cell population sequentially through several cycles of the procedure.

When designing a strategy of SMPC enrichment, we should consider the expression level of each surface marker in unsorted populations. Intuitively, CD29 and CD56 are well-established surface markers that have been used for SMPC enrichment in various cell lines. However, if an SMPC surface marker is already very highly expressed in pre-sorted populations (>90%), it may be redundant to enrich using this surface marker, as the purity may not improve by much. The same applies to negative SMPC surface markers that are already minimally expressed in unsorted populations. A surface marker barely expressed in the unsorted population may play an important biological role but would not be useful as a target for live cell isolation. Similarly, if a surface marker is also highly expressed on non-SMPC cell types, it would no longer serve to distinguish SMPC identity from the background cell populations.

We also need to carefully compare the rate of improvement in muscle differentiation efficiency between the unsorted and sorted populations. When quantifying the success rate of SMPC enrichment, the *in vivo* capacity of muscle regeneration should be taken into consideration alongside the ability of the cells to differentiate into muscle *in vitro*. Forming new muscle fibers, repopulating the satellite cell niche, and vascularization of the affected area are all crucial to establish the structural integrity and functionality of the regenerated muscle tissue. It is possible that unsorted SMPC populations may perform better after transplantation, as these more heterogenous unsorted populations could contain cells that play a supporting role for muscle repair and regeneration. "Enriched" SMPC populations may show an increased rate of myotube formation *in vitro* where conditions are strictly controlled. However, these cells may engraft more poorly than unsorted populations due to the absence of non-SMPC support cells. Therefore, the purpose of SMPC enrichment should be considered when selecting surface markers, as the resulting cell population most ideal for *in vitro* modeling may not also be the most ideal for transplantation studies.

Our literature search further verifies that a surface marker profile cannot be assumed to be similar for all SMPC lines. A surface marker could enrich SMPCs from one cell line very well only to be rendered useless in another cell line. For example, CD271 and ErbB3 efficiently enriched SMPCs derived via both transgene-free and transgene-based methods from several PSC lines (Hicks et al., 2017; Sakai-Takemura et al., 2018). However in a different paper, when a combination of CD271 and Erb3 was used for an ESC line (Pax7/Myf5 reporter ESC), the similar efficiency of SMPC enrichment could not be replicated (Wu et al., 2018). Thus, more comprehensive isolation strategies that account for expression-level variability are warranted. The variance in antibody binding affinity from different suppliers should also be carefully considered when designing an antibody-based isolation strategy as well as in data interpretation.

When differentiating skeletal myocytes from human PSCs, there has been inconsistency between studies with regards to evaluating differentiation efficiency and myocyte maturity (reviewed in Jiwlawat et al., 2018). It would be greatly beneficial in the field to establish standards for these evaluations in order to make better comparisons across protocols. To achieve this goal, cell characterization using specific surface markers against PSC-derived SMPCs would be crucial. Another challenge in the field is that cultured skeletal myocytes often retain an embryonic

or perinatal phenotype. While better establishment of culture technique would be required to obtain SMPCs and myocytes with sufficient quality, it would also be worthwhile to define stage-specific markers to distinguish embryonic, perinatal, and adult phenotypes of SMPCs and myoblasts.

To date, a majority of SMPC surface markers were profiled based on the knowledge from satellite cells, resident SMPCs located between the basal lamina and sarcolemma of adult muscle fibers. Even though these possible markers can be used to sort SMPCs into subpopulations, it remains uncertain whether these subpopulations are homogeneous in their function and lineage commitment. Moreover, given the fact that satellite cells from different *in situ* locations within the same donor can have distinct molecular signatures (Harel et al., 2009; Sambasivan et al., 2009), these markers should not be utilized alone to unequivocally identify SMPCs, especially "*de novo* SMPCs" derived from PSCs. Additionally, contradictory reports on marker expression on SMPCs imply that different culture conditions may lead to the heterogeneity of SMPCs. Different culture media and durations would influence the expression of specific markers in timepoint- and sample-dependent manners. Ultimately, it would be more helpful if a sole marker was available to specifically enrich only SMPCs. For other stem cell types such as HSCs and MSCs, cell surface markers are already well-characterized and defined for identification. In contrast, our knowledge in the field of myogenic progenitors is still developing. To advance, additional studies are needed to discover specific markers that can be used alone to detect a pure population of SMPCs.

Lastly, another concern is that throughout the reports there is inconsistency on how to evaluate the myogenic potential in sorted cell pools. Some studies examined commitment to the myogenic lineage based on a pooled expression of common muscle cell markers, whereas several other studies only identified the expression of one marker. When comparing *in vivo* regenerative capacity using intramuscular transplantation, the results may also vary depending on the animal models used, how muscle injury was induced, the number of cells transplanted, the duration between transplantation and analysis, and the position in muscle used for section preparation and analysis. Furthermore, calculation of myotube formation efficiency may differ based on the usage of immunohistochemical staining, the counting of positive cells in a field of view, the number of nuclei per myotube, and the percentage of nuclei within myotubes. Since the ability to generate fully matured myotubes is pivotal for transplanted SMPCs to have clinical significance, a comprehensive analysis of the anatomical features, physiological functionality and fiber type expressed should be performed to gauge the state of myotube maturity.

CONCLUDING REMARKS

Recent studies have offered valuable knowledge regarding cell surface markers to identify SMPCs. While promising positive and negative markers have been identified using different SMPC types, it remains challenging to use them for sorting human PSC-derived SMPCs in efficient ways. Furthermore, there is still a need to standardize methods of quantifying SMPC properties in order to facilitate comparisons between surface marker expressions. This would allow for precise utilization of SMPC surface markers to enhance the enrichment and differentiation of SMPCs. Having control over the composition of SMPC populations could lead to new state of the art uses for disease modeling, drug testing, and therapeutic development.

AUTHOR CONTRIBUTIONS

S-RT designed the outline of manuscript, performed manuscript search, and primarily prepared the manuscript. SR and EL provided critical suggestions about the contents and edited the manuscript. MS proposed conception and design of the manuscript and wrote the manuscript. All authors approved the final version of the manuscript.

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REFERENCES

Abujarour, R., Bennett, M., Valamehr, B., Lee, T. T., Robinson, M., Robbins, D., et al. (2014). Myogenic differentiation of muscular dystrophy-specific induced pluripotent stem cells for use in drug discovery. *Stem Cells Transl. Med.* 3, 149–160. doi: 10.5966/sctm.2013-0095

Agley, C. C., Rowlerson, A. M., Velloso, C. P., Lazarus, N. R., and Harridge, S. D. R. (2013). Human skeletal muscle fibroblasts, but not myogenic cells, readily undergo adipogenic differentiation. *J. Cell Sci.* 126, 5610–5625. doi: 10.1242/jcs.132563

Albini, S., Coutinho, P., Malecova, B., Giordani, L., Savchenko, A., Forcales, S., et al. (2013). Epigenetic reprogramming of human ES cells into skeletal muscle cells and generation of contractile myospheres. *Cell Rep.* 3, 661–670. doi: 10.1016/j.celrep.2013.02.012

Alessandri, G., Pagano, S., Bez, A., Benetti, A., Pozzi, S., Iannolo, G., et al. (2004). Isolation and culture of human muscle-derived stem cells able to differentiate into myogenic and neurogenic cell lineages. *Lancet* 364, 1872–1883. doi: 10.1016/S0140-6736(04)17443-6

Alexander, M. S., Rozkalne, A., Colletta, A., Spinazzola, J. M., Johnson, S., Rahimov, F., et al. (2016). CD82 is a marker for prospective isolation of human muscle satellite cells and is linked to muscular dystrophies. *Cell Stem Cell* 19, 800–807. doi: 10.1016/j.stem.2016.08.006

Álvarez-Viejo, M., Menéndez-Menéndez, Y., and Otero-Hernández, J. (2015). CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. *World J. Stem Cells* 7, 470–476. doi: 10.4252/wjsc.v7.i2.470

Anastasi, G., Cutroneo, G., Santoro, G., Arco, A., Rizzo, G., Bramanti, P., et al. (2008). Costameric proteins in human skeletal muscle during muscular inactivity. *J. Anat.* 213, 284–295. doi: 10.1111/j.1469-7580.2008.00921.x

Anastasi, G., Cutroneo, G., Trimarchi, F., Santoro, G., Bruschetta, D., Bramanti, P., et al. (2004). Evaluation of sarcoglycans, vinculin-talin-integrin system and filamin2 in alpha- and gamma-sarcoglycanopathy: an immunohistochemical study. *Int. J. Mol. Med.* 14, 989–999. doi: 10.3892/ijmm.14.6.989

Armulik, A., Genove, G., and Betsholtz, C. (2011). Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev. Cell* 21, 193–215. doi: 10.1016/j.devcel.2011.07.001

Asahara,. T., and Kawamoto, A. (2004). Endothelial progenitor cells for postnatal vasculogenesis. *Am. J. Physiol. Cell Physiol.* 287, C572–C579. doi: 10.1152/ajpcell.00330.2003

Asakura, A., Seale, P., Girgis-Gabardo, A., and Rudnicki, M. A. (2002). Myogenic specification of side population cells in skeletal muscle. *J. Cell Biol.* 159, 123–134. doi: 10.1083/jcb.200202092

Awaya, T., Kato, T., Mizuno, Y., Chang, H., Niwa, A., Umeda, K., et al. (2012). Selective development of myogenic mesenchymal cells from human embryonic and induced pluripotent stem cells. *PLoS ONE* 7:e51638. doi: 10.1371/journal.pone.0051638

Bachrach, E., Perez, A. L., Choi, Y., Illigens, B. M. W., Jun, S. J., Del Nido, P., et al. (2006). Muscle engraftment of myogenic progenitor cells following intraarterial transplantation. *Muscle Nerve* 34, 44–52. doi: 10.1002/mus.20560

Bajek, A., Porowinska, D., Kloskowski, T., Brzoska, E., Ciemerych, M. A., and Drewa, T. (2015). Cell therapy in Duchenne muscular dystrophy treatment: clinical trials overview. *Crit. Rev. Eukaryot. Gene Expr.* 25, 1–11. doi: 10.1615/CritRevEukaryotGeneExpr.2015011074

Barberi, T., Bradbury, M., Dincer, Z., Panagiotakos, G., Socci, N. D., and Studer, L. (2007). Derivation of engraftable skeletal myoblasts from human embryonic stem cells. *Nat. Med.* 13, 642–648. doi: 10.1038/nm1533

Barberi, T., Willis, L. M., Socci, N. D., and Studer, L. (2005). Derivation of multipotent mesenchymal precursors from human embryonic stem cells. *PLoS Med.* 2:e161. doi: 10.1371/journal.pmed.0020161

Bareja, A., Holt, J. A., Luo, G., Chang, C., Lin, J., Hinken, A. C., et al. (2014). Human and mouse skeletal muscle stem cells: convergent and divergent mechanisms of myogenesis. *PLoS ONE* 9:e90398. doi: 10.1371/journal.pone.0090398

Bartoccioni, E., Gallucci, S., Scuderi, F., Ricci, E., Servidei, S., Broccolini, A., et al. (1994). MHC class I, MHC class II and intercellular adhesion molecule-1 (ICAM-1) expression in inflammatory myopathies. *Clin. Exp. Immunol.* 95, 166–172. doi: 10.1111/j.1365-2249.1994.tb06032.x

Bischoff, R., and Heintz, C. (1994). Enhancement of skeletal muscle regeneration. *Develop. Dyn.* 201, 41–54. doi: 10.1002/aja.1002010105

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. (1995). Essential role for the c-Met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* 376, 768–771. doi: 10.1038/376768a0

Blanpain, C., Lowry, W. E., Geoghegan, A., Polak, L., and Fuchs, E. (2004). Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 118, 635–648. doi: 10.1016/j.cell.2004.08.012

Borchin, B., Chen, J., and Barberi, T. (2013). Derivation and FACS-mediated purification of PAX3+/PAX7+ skeletal muscle precursors from human pluripotent stem cells. *Stem Cell Reports* 1, 620 – 631. doi: 10.1016/j.stemcr.2013.10.007

Buckingham, M. (2006). Myogenic progenitor cells and skeletal myogenesis in vertebrates. *Curr. Opin. Genet. Dev.* 16, 525–532. doi: 10.1016/j.gde.2006.08.008

Buhring, H. J., Kuci, S., Conze, T., Rathke, G., Bartolovic, K., Grunebach, F., et al. (2004). CDCP1 identifies a broad spectrum of normal and malignant stem/progenitor cell subsets of hematopoietic and nonhematopoietic origin. *Stem Cells* 22, 334–343. doi: 10.1634/stemcells.22-3-334

Cao, B., Zheng, B., Jankowski, R. J., Kimura, S., Ikezawa, M., Deasy, B., et al. (2003). Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. *Nat. Cell Biol.* 5:640–646. doi: 10.1038/ncb1008

Cao, M., Gao, J., Zhou, H., Huang, J., You, A., Guo, Z., et al. (2015). HIF-2alpha regulates CDCP1 to promote PKCdelta-mediated migration in hepatocellular carcinoma. *Tumour Biol.* 37, 1651–1662. doi: 10.1007/s13277-015-3527-7

Cappellari, O., and Cossu, G. (2013). Pericytes in development and pathology of skeletal muscle. *Circ. Res.* 113:341–347. doi: 10.1161/CIRCRESAHA.113.300203

Caron, L., Kher, D., Lee, K. L., McKernan, R., Dumevska, B., Hidalgo, A., et al. (2016). A human pluripotent stem cell model of facioscapulohumeral muscular dystrophy-affected skeletal muscles. *Stem Cells Transl. Med.* 5, 1145–1161. doi: 10.5966/sctm.2015-0224

Castiglioni, A., Hettmer, S., Lynes, M. D., Rao, T. N., Tchessalova, D., Sinha, I., et al. (2014). Isolation of progenitors that exhibit myogenic/osteogenic bipotency *in vitro* by fluorescence-activated cell sorting from human fetal muscle. *Stem Cell Rep.* 2, 92–106. doi: 10.1016/j.stemcr.2013.12.006

Cerletti, M., Molloy, M. J., Tomczak, K. K., Yoon, S., Ramoni, M. F., Kho, A. T., et al. (2006). Melanoma cell adhesion molecule is a novel marker for human fetal myogenic cells and affects myoblast fusion. *J. Cell Sci.* 119, 3117–3127. doi: 10.1242/jcs.03056

Charville, G. W., Cheung, T. H., Yoo, B., Santos, P. J., Lee, G. K., Shrager, J. B., et al. (2015). *Ex vivo* expansion and *in vivo* self-renewal of human muscle stem cells. *Stem Cell Rep.* 5, 621–632. doi: 10.1016/j.stemcr.2015.08.004

Choi, I. Y., Lim, H., Estrellas, K., Mula, J., Cohen, T. V., Zhang, Y., et al. (2016). Concordant but varied phenotypes among Duchenne Muscular Dystrophy patient-specific myoblasts derived using a human iPSC-based model. *Cell Rep.* 15, 2301–2312. doi: 10.1016/j.celrep.2016.05.016

Conconi, M. T., Burra, P., Di Liddo, R., Calore, C., Turetta, M., Bellini, S., et al. (2006). CD105(+) cells from Wharton's jelly show *in vitro* and *in vivo* myogenic differentiative potential. *Int. J. Mol. Med.* 118, 1089–1096. doi: 10.3892/ijmm.18.6.1089

Conze, T., Lammers, R., Kuci, S., Scheri-Mostageer, M., Schweifer, N., Kanz, L., et al. (2003). CDCP1 is a novel marker for hematopoietic stem cells. *Ann. N. Y. Acad. Sci.* 996, 222–226. doi: 10.1111/j.1749-6632.2003.tb03249.x

Cossu, G., Previtali, S. C., Napolitano, S., Cicalese, M. P., Tedesco, F. S., Nicastro, F., et al. (2015). Intra-arterial transplantation of HLA-matched donor mesoangioblasts in Duchenne muscular dystrophy. *EMBO Mol. Med.* 7, 1513–1528. doi: 10.15252/emmm.201505636

Coussens, L., Yang-Feng, T. L., Liao, Y. C., Chen, E., Gray, A., McGrath, J., et al. (1985). Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* 230, 1132–1139. doi: 10.1126/science.2999974

Coutu, D. L., Kokkaliaris, K. D., Kunz, L., and Schroeder, T. (2017). Three-dimensional map of nonhematopoietic bone and bone-marrow cells and molecules. *Nat. Biotechnol.* 35, 1202–1210. doi: 10.1038/nbt.4006

Crisan, M., Yap, S., Casteilla, L., Chen, C. W., Corselli, M., Park, T. S., et al. (2008). A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 3, 301–313. doi: 10.1016/j.stem.2008.07.003

Crist, C. G., Montarras, D., and Buckingham, M. (2012). Muscle satellite cells are primed for myogenesis but maintain quiescence with sequestration of Myf5 mRNA targeted by microRNA-31 in mRNP granules. *Cell Stem Cell* 11, 118–126. doi: 10.1016/j.stem.2012.03.011

Darabi, R., Arpke, R. W., Irion, S., Dimos, J. T., Grskovic, M., Kyba, M., et al. (2012). Human ES- and iPS-derived myogenic progenitors restore dystrophin and improve contractility upon transplantation in dystrophic mice. *Cell Stem Cell* 10, 610–619. doi: 10.1016/j.stem.2012.02.015

De Angelis, L., Berghella, L., Coletta, M., Lattanzi, L., Zanchi, M., Gabriella, M., et al. (1999). Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. *J. Cell. Biol.* 147, 869–878. doi: 10.1083/jcb.147.4.869

De Bleecker, J. L., and Engel, A. G. (1994). Expression of cell adhesion molecules in inflammatory myopathies and Duchenne dystrophy. *J. Neuropathol. Exp. Neurol.* 53, 369–376. doi: 10.1097/00005072-199407000-00008

Deasy, B. M., Gharaibeh, B. M., Pollett, J. B., Jones, M. M., Lucas, M. A., Kanda, Y., et al. (2005). Long-term self-renewal of postnatal muscle-derived stem cells. *Mol. Biol. Cell* 16, 3323–3333. doi: 10.1091/mbc.e05-02-0169

Deasy, B. M., Jankowski, R. J., and Huard, J. (2001). Muscle-derived stem cells: characterization and potential for cell-mediated therapy. *Blood Cells Mol. Dis.* 27, 924–933. doi: 10.1006/bcmd.2001.0463

Dellavalle, A., Sampaolesi, M., Tonlorenzi, R., Tagliafico, E., Sacchetti, B., Perani, L., et al. (2007). Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. *Nat. Cell Biol.* 9, 255–267. doi: 10.1038/ncb1542

Deschenes, M. R. (2004). Effects of aging on muscle fibre type and size. *Sports Med.* 34, 809–824. doi: 10.2165/00007256-200434120-00002

Dietrich, S., Abou-Rebyeh, F., Brohmann, H., Bladt, F., Sonnenberg-Riethmacher, E., Yamaai, T., et al. (1999). The role of SF/HGF and c-Met in the development of skeletal muscle. *Development* 126, 1621–1629.

Elghetany, M. T., and Patel, J. (2002). Assessment of CD24 expression on bone marrow neutrophilic granulocytes: CD24 is a marker for the myelocytic stage of development. *Am. J. Hematol.* 71, 348–349. doi: 10.1002/ajh.10176

Emerling, B. M., Benes, C. H., Poulogiannis, G., Bell, E. L., Courtney, K., Liu, H., et al. (2013). Identification of CDCP1 as a hypoxia-inducible factor 2alpha (HIF-2alpha) target gene that is associated with survival in clear cell renal cell carcinoma patients. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3483–3488. doi: 10.1073/pnas.1222435110

Fang, X., Zheng, P., Tang, J., and Liu, Y. (2017). CD24: from A to Z. *Cell. Mol. Immunol.* 7, 100–103. doi: 10.1038/cmi.2009.119

Figarella-Branger, D., Moreau, H., Pellissier, J. F., Bianco, N., and Rougon, G. (1993). CD24, a signal-transducing molecule expressed on human B lymphocytes, is a marker for human regenerating muscle. *Acta Neuropathol.* 86, 275–284. doi: 10.1007/BF00304142

Fina, L., Molgaard, H. V., Robertson, D., Bradley, N. J., Monaghan, P., Delia, D., et al. (1990). Expression of the CD34 gene in vascular endothelial cells. *Blood* 75, 2417–2426. doi: 10.1182/blood.V75.12.2417.2417

Fonsatti, E., Nicolay, H. J., Altomonte, M., Covre, A., and Maio, M. (2010). Targeting cancer vasculature via endoglin/CD105: a novel antibody-based diagnostic and therapeutic strategy in solid tumours. *Cardiovasc. Res.* 86, 12–19. doi: 10.1093/cvr/cvp332

Franzin, C., Piccoli, M., Urbani, L., Biz, C., Gamba, P., De Coppi, P., et al. (2016). Isolation and expansion of muscle precursor cells from human skeletal muscle biopsies. *Methods Mol. Biol.* 1516, 195–204. doi: 10.1007/7651_2016_321

Fukasawa, K., Fujii, H., Saitoh, Y., Koizumi, K., Aozuka, Y., Sekine, K., et al. (2006). Aminopeptidase N (APN/CD13) is selectively expressed in vascular endothelial cells and plays multiple roles in angiogenesis. *Cancer Lett.* 243, 135–143. doi: 10.1016/j.canlet.2005.11.051

Gao, Z., Dong, K., and Zhang, H. (2014). The roles of CD73 in cancer. *Biomed Res. Int.* 2014:460654. doi: 10.1155/2014/460654

Garcia, S. M., Tamaki, S., Lee, S., Wong, A., Jose, A., Dreux, J., et al. (2018). High-yield purification, preservation, and serial transplantation of human satellite cells. *Stem Cell Rep.* 10, 1160–1174. doi: 10.1016/j.stemcr.2018.01.022

Gilliam, D. T., Menon, V., Bretz, N. P., and Pruszak, J. (2017). The CD24 surface antigen in neural development and disease. *Neurobiology* 99, 133–144. doi: 10.1016/j.nbd.2016.12.011

Goebels, N., Michaelis, D., Wekerle, H., and Hohlfeld, R. (1992). Human myoblasts as antigen-presenting cells. *The Journal of Immunology* 149, 661–667.

Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., and Mulligan, R. C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J. Exp. Med.* 183, 1797–1806. doi: 10.1084/jem.183.4.1797

Goudenege, S., Lebel, C., Huot, N. B., Dufour, C., Fujii, I., Gekas, J., et al. (2012). Myoblasts derived from normal hESCs and dystrophic hiPSCs efficiently fuse with existing muscle fibers following transplantation. *Mol. Ther.* 20, 2153–2167. doi: 10.1038/mt.2012.188

Gussoni, E., Soneoka, Y., Strickland, C. D., Buzney, E. A., Khan, M. K., Flint, A. F., et al. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 401, 390–394. doi: 10.1038/43919

Harel, I., Nathan, E., Tirosh-Finkel, L., Zigdon, H., Guimarães-Camboa, N., Evans, S. M., et al. (2009). Distinct origins and genetic programs of head muscle satellite cells. *Dev. Cell* 16, 822–832. doi: 10.1016/j.devcel.2009.05.007

Hashimoto, H., Tamaki, T., Hirata, M., Uchiyama, Y., Sato, M., and Mochida, J. (2016). Reconstitution of the complete rupture in musculotendinous junction using skeletal muscle-derived multipotent stem cell sheet-pellets as a "bio-bond". *PeerJ* 4:e2231. doi: 10.7717/peerj.2231

Hicks, M. R., Hiserodt, J., Paras, K., Fujiwara, W., Eskin, A., Jan, M., et al. (2017). ERBB3 and NGFR mark a distinct skeletal muscle progenitor cell in human development and hPSCs. *Nat. Cell Biol.* 20, 46–57. doi: 10.1038/s41556-017-0010-2

Hollemann, D., Budka, H., Löscher, W. N., Yanagida, G., Fischer, M. B., and Wanschitz, J. V. (2008). Endothelial and myogenic differentiation of hematopoietic progenitor cells in inflammatory myopathies. *J. Neuropathol. Exp. Neurol.* 67, 711–719. doi: 10.1097/NEN.0b013e31817d8064

Hosoyama, T., McGivern, J. V., Van Dyke, J. M., Ebert, A. D., and Suzuki, M. (2014). Derivation of myogenic progenitors directly from human pluripotent stem cells using a sphere-based culture. *Stem Cells Transl. Med.* 3, 564–574. doi: 10.5966/sctm.2013-0143

Huss, R. (2000). Isolation of primary and immortalized CD34-hematopoietic and mesenchymal stem cells from various sources. *Stem Cells* 18, 1–9. doi: 10.1634/stemcells.18-1-1

Hwang, Y., Suk, S., Shih, Y. V., Seo, T., Du, B., Xie, Y., et al. (2014). WNT3A promotes myogenesis of human embryonic stem cells and enhances *in vivo* engraftment. *Sci. Rep.* 4:5916. doi: 10.1038/srep05916

Jevsek, M., Jaworski, A., Polo-Parada, L., Kim, N., Fan, J., Landmesser, L. T., et al. (2006). CD24 is expressed by myofiber synaptic nuclei and regulates synaptic transmission. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6374–6379. doi: 10.1073/pnas.0601468103

Jiwlawat, N., Lynch, E., Jeffrey, J., Van Dyke, J. M., and Suzuki, M. (2018). Current progress and challenges for skeletal muscle differentiation from human pluripotent stem cells using transgene-free approaches. *Stem Cells Int.* 2018:6241681. doi: 10.1155/2018/6241681

Jiwlawat, S., Lynch, E., Glaser, J., Smit-Oistad, I., Jeffrey, J., Van Dyke, J. M., et al. (2017). Differentiation and sarcomere formation in skeletal myocytes directly prepared from human induced pluripotent stem cells using a sphere-based culture. *Differentiation* 96, 70–81. doi: 10.1016/j.diff.2017.07.004

Kaplan, R., Morse, B., Huebner, K., Croce, C., Howk, R., Ravera, M., et al. (1990). Cloning of three human tyrosine phosphatases reveals a multigene family of receptor-linked protein-tyrosine-phosphatases expressed in brain. *Proc. Natl. Acad. Sci. U.S.A.* 87, 7000–7004. doi: 10.1073/pnas.87.18.7000

Kared, H., Martelli, S., Ng, T. P., Pender, S. L., and Larbi, A. (2016). CD57 in human natural killer cells and T-lymphocytes. *Cancer Immunol. Immunother.* 65, 441–452. doi: 10.1007/s00262-016-1803-z

Kehlen, A., Lendeckel, U., Dralle, H., Langner, J., and Hoang-Vu, C. (2003). Biological significance of aminopeptidase N/CD13 in thyroid carcinomas. *Cancer Res.* 63, 8500–8506. Available online at: https://cancerres.aacrjournals.org/content/63/23/8500

Kim, J., Magli, A., Chan, S. S. K., Oliveira, V. K. P., Wu, J., Darabi, R., et al. (2017). Expansion and purification are critical for the therapeutic application of pluripotent stem cell-derived myogenic progenitors. *Stem Cell Rep.* 9, 12–22. doi: 10.1016/j.stemcr.2017.04.022

Koponen, J. K., Kekarainen, T., Heinonen, S. E., Laitinen, A., Nystedt, J., Laine, J., et al. (2007). Umbilical cord blood–derived progenitor cells enhance muscle regeneration in mouse hindlimb ischemia model. *Mol. Ther.* 15, 2172–2177. doi: 10.1038/sj.mt.6300302

Kos, L., Aronzon, A., Takayama, H., Maina, F., Ponzetto, C., Merlino, G., et al. (1999). Hepatocyte growth factor/scatter factor-MET signaling in neural crest-derived melanocyte development. *Pigment Cell Res.* 12, 13–21. doi: 10.1111/j.1600-0749.1999.tb00503.x

Kuang, S., Kuroda, K., Le Grand, F., and Rudnicki, M. A. (2007). Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell* 129, 999–1010. doi: 10.1016/j.cell.2007.03.044

Lapan, A. D., Rozkalne, A., and Gussoni, E. (2012). Human fetal skeletal muscle contains a myogenic side population that expresses the melanoma cell-adhesion molecule. *Hum. Mol. Genet.* 21, 3668–3680. doi: 10.1093/hmg/dds196

Lavasani, M., Thompson, S. D., Pollett, J. B., Usas, A., Lu, A., Stolz, D. B., et al. (2014). Human muscle-derived stem/progenitor cells promote functional murine peripheral nerve regeneration. *J. Clin. Investig.* 124, 1745–1756. doi: 10.1172/JCI44071

Le Grand, F., Jones, A. E., Seale, V., Scime, A., and Rudnicki, M. A. (2009). Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. *Cell Stem Cell* 4, 535–547. doi: 10.1016/j.stem.2009.03.013

Lecourt, S., Marolleaude, J. P., Fromigue, O., Vauchez, K., Andriamanalijaona, R., Ternaux, B., et al. (2010). Characterization of distinct mesenchymal-like cell populations from human skeletal muscle *in situ* and *in vitro*. *Exp. Cell Res.* 316, 2513–2526. doi: 10.1016/j.yexcr.2010.04.020

Leroy, M. C., Perroud, J., Darbellay, B., Bernheim, L., and Konig, S. (2013). Epidermal growth factor receptor down-regulation triggers human myoblast differentiation. *PLoS ONE* 8:e71770. doi: 10.1371/journal.pone.0071770

Lertkiatmongkol, P., Liao, D., Mei, H., Hu, Y., and Newman, P. J. (2016). Endothelial functions of PECAM-1 (CD31). *Curr. Opin. Hematol.* 23, 253–259. doi: 10.1097/MOH.0000000000000239

Lin, C. S., Ning, H., Lin, G., and Lue, T. F. (2012). Is CD34 truly a negative marker for mesenchymal stromal cells? *Cytotherapy* 14, 1159–1163. doi: 10.3109/14653249.2012.729817

Lindström, M., and Thornell, L. E. (2009). New multiple labelling method for improved satellite cell identification in human muscle: application to a cohort of power-lifters and sedentary men. *Histochem. Cell Biol.* 132, 141–157. doi: 10.1007/s00418-009-0606-0

Liu, L., Cheung, T. H., Charville, G. W., and Rando, T. A. (2015). Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting. *Nat. Protoc.* 10, 1612–1624. doi: 10.1038/nprot.2015.110

Long, E. O. (2011). Intercellular Adhesion Molecule 1 (ICAM-1): Getting a grip on leukocyte adhesion. *J. Immunol.* 186, 5021–5023. doi: 10.4049/jimmunol.1100646

Lorant, J., Saury, C., Schleder, C., Robriquet, F., Lieubeau, B., Negroni, E., et al. (2018). Skeletal muscle regenerative potential of human MuStem cells following transplantation into injured mice muscle. *Mol. Ther.* 26, 618–633. doi: 10.1016/j.ymthe.2017.10.013

Lui, P. P. (2015). Markers for the identification of tendon-derived stem cells *in vitro* and tendon stem cells *in situ* - update and future development. *Stem Cell Res. Ther.* 6:106. doi: 10.1186/s13287-015-0097-y

Maesner, C. C., Almada, A. E., and Wagers, A. J. (2016). Established cell surface markers efficiently isolate highly overlapping populations of skeletal muscle satellite cells by fluorescence-activated cell sorting. *Skelet. Muscle* 6:35. doi: 10.1186/s13395-016-0106-6

Maffioletti, S. M., Gerli, M. F., Ragazzi, M., Dastidar, S., Benedetti, S., Loperfido, M., et al. (2015). Efficient derivation and inducible differentiation of expandable skeletal myogenic cells from human ES and patient-specific iPS cells. *Nat. Protoc.* 10, 941–958. doi: 10.1038/nprot.2015.057

Magli, A., Incitti, T., Kiley, J., Swanson, S. A., Darabi, R., Rinaldi, F., et al. (2017). PAX7 Targets, CD54, Integrin α9β1 and SDC2, allow isolation of human ES/iPS cell-derived myogenic progenitors. *Cell Rep.* 19, 2867–2877. doi: 10.1016/j.celrep.2017.06.005

Maguer-Satta, V., Besançon, R., and Bachelard-Cascales, E. (2011). Concise review: neutral endopeptidase (CD10): a multifaceted environment actor in stem cells, physiological mechanisms, and cancer. *Stem Cells* 29, 389–396. doi: 10.1002/stem.592

Marg, A., Escobar, H., Gloy, S., Kufeld, M., Zacher, J., Spuler, A., et al. (2014). Human satellite cells have regenerative capacity and are genetically manipulable. *J. Clin. Investig.* 124, 4257–4265. doi: 10.1172/JCI63992

Marino, M., Scuderi, F., Mannella, F., and Bartoccioni, E. (2003). TGF-beta 1 and IL-10 modulate IL-1 beta-induced membrane and soluble ICAM-1 in human myoblasts. *J. Neuroimmunol.* 134, 151–157. doi: 10.1016/S0165-5728(02)00399-5

Marino, M., Scuderi, F., Mazzarelli, P., Mannella, F., Provenzano, C., and Bartoccioni, E. (2001). Constitutive and cytokine-induced expression of MHC and intercellular adhesion molecule-1 (ICAM-1) on human myoblasts. *J. Neuroimmunol.* 116, 94–101. doi: 10.1016/S0165-5728(01)00287-9

Mary, P., Servais, L., and Vialle, R. (2018). Neuromuscular diseases: Diagnosis and management. *Orthop. Traumatol. Surg. Res.* 104, S89–S95. doi: 10.1016/j.otsr.2017.04.019

Marzetti, E., Calvani, R., Tosato, M., Cesari, M., Di Bari, M., Cherubini, A., et al. (2017). Sarcopenia: an overview. *Aging Clin. Exp. Re.* 29, 11–17. doi: 10.1007/s40520-016-0704-5

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* 9:493–495. doi: 10.1083/jcb.9.2.493

Meadows, E., Cho, J. H., Flynn, J. M., and Klein, W. H. (2008). Myogenin regulates a distinct genetic program in adult muscle stem cells. *Dev. Biol.* 322, 406–414. doi: 10.1016/j.ydbio.2008.07.024

Meng, J., Chun, S., Asfahani, R., Lochmüller, H., Muntoni, F., and Morgan, J. (2014). Human skeletal muscle-derived CD133(+) cells form functional satellite cells after intramuscular transplantation in immunodeficient host mice. *Mol. Ther. J. Am. Soc. Gene Ther.* 22, 1008–1017. doi: 10.1038/mt.2014.26

Michaelis, D., Goebels, N., and Hohlfeld, R. (1993). Constitutive and cytokine-induced expression of human leukocyte antigens and cell adhesion molecules by human myotubes. *Am. J. Pathol.* 143, 1142–1149.

Morgan, J. E., and Partridge, T. A. (2003). Muscle satellite cells. *Int. J. Biochem. Cell Biol.* 35, 1151–1156. doi: 10.1016/S1357-2725(03)00042-6

Morosetti, R., Mirabella, M., Gliubizzi, C., Broccolini, A., De Angelis, L., Tagliafico, E., et al. (2006). MyoD expression restores defective myogenic differentiation of human mesoangioblasts from inclusion-body myositis muscle. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16995–17000. doi: 10.1073/pnas.0603386103

Morrison, B. M. (2016). Neuromuscular diseases. *Semin. Neurobiol.* 36, 409–418. doi: 10.1055/s-0036-1586263

Narici, M. V., and Maffulli, N. (2010). Sarcopenia: characteristics, mechanisms and functional significance. *Br. Med. Bull.* 95, 139–159. doi: 10.1093/bmb/ldq008

Negroni, E., Riederer, I., Chaouch, S., Melicchi, M., Razini, P., Di Santo, J., et al. (2009). *In vivo* myogenic potential of human CD133+ muscle-derived stem cells: a quantitative study. *Mol. Ther. J. Am. Soc. Gene Ther.* 17, 1771–1778. doi: 10.1038/mt.2009.167

Nunes, V. A., Cavacana, N., Canovas, M., Strauss, B. E., and Zatz, M. (2007). Stem cells from umbilical cord blood differentiate into myotubes and express dystrophin *in vitro* only after exposure to *in vivo* muscle environment. *Biol. Cell* 99, 185–196. doi: 10.1042/BC20060075

Ogawa, S., Yakabe, M., and Akishita, M. (2016). Age-related sarcopenia and its pathophysiological bases. *Inflamm. Regener.* 36:17. doi: 10.1186/s41232-016-0022-5

Okada, M., Payne, T. R., Drowley, L., Jankowski, R. J., Momoi, N., Beckman, S., et al. (2012). Human skeletal muscle cells with a slow adhesion rate after isolation and an enhanced stress resistance improve function of ischemic hearts. *Mol. Ther.* 20:138145. doi: 10.1038/mt.2011.229

Pallafacchina, G., Blaauw, B., and Schiaffino, S. (2013). Role of satellite cells in muscle growth and maintenance of muscle mass. *Nutr. Metab. Cardiovasc. Dis.* 23(Suppl. 1), S12–S18. doi: 10.1016/j.numecd.2012.02.002

Park, T. S., Gavina, M., Chen, C. W., Sun, B., Teng, P., Huard, J., et al. (2011). Placental perivascular cells for human muscle regeneration. *Stem Cells Dev.* 20, 451–463. doi: 10.1089/scd.2010.0354

Péault, B., Rudnicki, M., Torrente, Y., Cossu, G., Tremblay, J. P., Partridge, T., et al. (2007). Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. *Mol. Ther.* 15, 867–877. doi: 10.1038/mt.sj.6300145

Pisani, D. F., Clement, N., Loubat, A., Plaisant, M., Sacconi, S., Kurzenne, J., et al. (2010a). Hierarchization of myogenic and adipogenic progenitors within human skeletal muscle. *Stem Cells* 28, 2182–2194. doi: 10.1002/stem.537

Pisani, D. F., Dechesne, C. A., Sacconi, S., Delplace, S., Belmonte, N., Cochet, O., et al. (2010b). Isolation of a highly myogenic CD34-negative subset of human skeletal muscle cells free of adipogenic potential. *Stem Cells J.* 28, 753–764. doi: 10.1002/stem.317

Polisetty, N., Fatima, A., Madhira, S. L., Sangwan, V. S., and Vemuganti, G. K. (2008). Mesenchymal cells from limbal stroma of human eye. *Mol. Vis.* 14, 431–442. Available online at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2268845/

Poncet, C., Frances, V., Gristina, R., Scheiner, C., Pellissier, J. F., and Figarella-Branger, D. (1996). CD24, a glycosylphosphatidylinositol-anchored molecule is transiently expressed during the development of human central nervous system and is a marker of human neural cell lineage tumors. *Acta Neuropathol.* 91, 400–408. doi: 10.1007/s004010050442

Poole, C. A., Brookes, N. H., and Clover, G. M. (1993). Keratocyte networks visualised in the living cornea using vital dyes. *J. Cell Sci.* 106, 685–691.

Popescu, L. M., Ciontea, S. M., and Cretoiu, D. (2007). Interstitial Cajal-like cells in human uterus and fallopian tube. *Ann. N. Y. Acad. Sci.* 1101, 139–165. doi: 10.1196/annals.1389.022

Proksch, S., Bel, A., Puymirat, E., Pidial, L., Bellamy, V., Peyrard, S., et al. (2009). Does the human skeletal muscle harbor the murine equivalents of cardiac precursor cells? *Mol. Ther.* 17, 733–741. doi: 10.1038/mt.2009.6

Qu-Petersen, Z., Deasy, B., Jankowski, R., Ikezawa, M., Cummins, J., Pruchnic, R., et al. (2002). Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration. *J. Cell Biol.* 157, 851–864. doi: 10.1083/jcb.200108150

Rahman, M. M., Ghosh, M., Subramani, J., Fong, G. H., Carlson, M. E., and Shapiro, L. H. (2014). CD13 regulates anchorage and differentiation of the skeletal muscle satellite stem cell population in ischemic injury. *Stem Cells* 32, 1564–1577. doi: 10.1002/stem.1610

Ramos, L. T., Sánchez-Abarca, L. I., Muntión, S., Preciado, S., Puig, N., Lopez-Ruano, G., et al. (2016). MSC surface markers (CD44, CD73, and CD90) can identify human MSC-derived extracellular vesicles by conventional flow cytometry. *Cell Commun. Signal.* 14:2. doi: 10.1186/s12964-015-0124-8

Rao, L., Qian, Y., Khodabukus, A., Ribar, T., and Bursac, N. (2018). Engineering human pluripotent stem cells into a functional skeletal muscle tissue. *Nat. Commun.* 9:126. doi: 10.1038/s41467-017-02636-4

Rasmussen, H., Hansen, A., Smedts, F., Rumessen, J. J., and Horn, T. (2007). CD34-positive interstitial cells of the human detrusor. *APMIS* 115, 1260–1266. doi: 10.1111/j.1600-0643.2007.00759.x

Razorenova, O. V., Finger, E. C., Colavitti, R., Chernikova, S. B., Boiko, A. D., Chan, C. K., et al. (2011). VHL loss in renal cell carcinoma leads to up-regulation

of CUB domain-containing protein 1 to stimulate PKCdelta-driven migration. *Proc. Natl. Acad. Sci. U.S.A.* 108, 1931–1936. doi: 10.1073/pnas.1011777108

Ryder, S., Leadley, R. M., Armstrong, N., Westwood, M., de Kock, S., Butt, T., et al. (2017). The burden, epidemiology, costs and treatment for Duchenne muscular dystrophy: an evidence review. *Orphanet J. Rare Dis.* 12:79. doi: 10.1186/s13023-017-0631-3

Sakai-Takemura, F., Narita, A., Masuda, S., Wakamatsu, T., Watanabe, N., Nishiyama, T., et al. (2018). Premyogenic progenitors derived from human pluripotent stem cells expand in floating culture and differentiate into transplantable myogenic progenitors. *Sci. Rep.* 8:6555. doi: 10.1038/s41598-018-24959-y

Sambasivan, R., Gayraud-Morel, B., Dumas, G., Cimper, C., Paisant, S., Kelly, R. G., et al. (2009). Distinct regulatory cascades govern extraocular and pharyngeal arch muscle progenitor cell fates. *Dev. Cell* 16, 810–821. doi: 10.1016/j.devcel.2009.05.008

Sampaolesi, M., Torrente, Y., Innocenzi, A., Tonlorenzi, R., D'Antona, G., Pellegrino, M. A., et al. (2003). Cell therapy of alpha-sarcoglycan null dystrophic mice through intraarterial delivery of mesoangioblasts. *Science* 301, 487–492. doi: 10.1126/science.1082254

Santilli, V., Bernetti, A., Mangone, M., and Paoloni, M. (2014). Clinical definition of sarcopenia. *Clin. Cases Mineral Bone Metab.* 11, 177–180. doi: 10.11138/ccmbm/2014.11.3.177

Scutt, N., Rolf, C. G., and Scutt, A. (2008). Tissue specific characteristics of cells isolated from human and rat tendons and ligaments. *J. Orthop. Surg. Res.* 3:32. doi: 10.1186/1749-799X-3-32

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., and Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell* 102, 777–786. doi: 10.1016/S0092-8674(00)00066-0

Senbanjo, L. T., and Chellaiah, M. A. (2017). CD44: A multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells. *Front. Cell Develop. Biol.* 5:18. doi: 10.3389/fcell.2017.00018

Sidney, L. E., Branch, M. J., Dunphy, S. E., Dua, H. S., and Hopkinson, A. (2014). Concise review: evidence for CD34 as a common marker for diverse progenitors. *Stem Cells* 32, 1380–1389. doi: 10.1002/stem.1661

Sinanan, A. C. M., Hunt, N. P., and Lewis, M. P. (2004). Human adult craniofacial muscle-derived cells: neural-cell adhesion-molecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells. *Biotechnol. Appl. Biochem.* 40, 25–34. doi: 10.1042/BA20030185

Skoglund, G., Lainé, J., Darabi, R., Fournier, E., Perlingeiro, R., and Tabti, N. (2014). Physiological and ultrastructural features of human induced pluripotent and embryonic stem cell-derived skeletal myocytes *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 111, 8275–8280. doi: 10.1073/pnas.1322258111

Smyth, L. C. D., Rustenhoven, J., Scotter, E. L., Schweder, P., Faull, R. L. M., Park, T. I. H., et al. (2018). Markers for human brain pericytes and smooth muscle cells. *J. Chem. Neuroanat.* 92, 48–60. doi: 10.1016/j.jchemneu.2018.06.001

Spassov, D. S., Baehner, F. L., Wong, C. H., McDonough, S., and Moasser, M. M. (2009). The transmembrane src substrate Trask is an epithelial protein that signals during anchorage deprivation. *Am. J. Pathol.* 174, 1756–1765. doi: 10.2353/ajpath.2009.080890

Starkey, J. D., Yamamoto, M., Yamamoto, S., and Goldhamer, D. J. (2011). Skeletal muscle satellite cells are committed to myogenesis and do not spontaneously adopt nonmyogenic fates. *J. Histochem. Cytochem.* 59, 33–46. doi: 10.1369/jhc.2010.956995

Stolk, M., Klatte-Schulz, F., Schmock, A., Minkwitz, S., Wildemann, B., and Seifert, M. (2017). New insights into tenocyte-immune cell interplay in an *in vitro* model of inflammation. *Sci. Rep.* 7:9801. doi: 10.1038/s41598-017-09875-x

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872. doi: 10.1016/j.cell.2007.11.019

Tamajusuku, A. S., Carrillo-Sepúlveda, M. A., Braganhol, E., Wink, E. R., Sarkis, J. J.,Barreto-Chaves, M. L., et al. (2006). Activity and expression of ecto-5′-nucleotidase/CD73 are increased by thyroid hormones in vascular smooth muscle cells. *Mol. Cell. Biochem.* 289, 65–72. doi: 10.1007/s11010-006-9148-0

Tan, Y., Zhao, M., Xiang, B., Chang, C., and Lu, Q. (2016). CD24: from a hematopoietic differentiation antigen to a genetic risk factor for multiple autoimmune diseases. *Clin. Rev. Allergy Immunol.* 50, 70–83. doi: 10.1007/s12016-015-8470-2

Tanaka, A., Woltjen, K., Miyake, K., Hotta, A., Ikeya, M., Yamamoto, T., et al. (2013). Efficient and reproducible myogenic differentiation from human iPS cells: prospects for modeling Miyoshi Myopathy *in vitro*. *PLoS ONE* 8:e61540. doi: 10.1371/annotation/63972dc9-3a31-43d0-ad52-bc46fd948c03

Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P. F., Weintraub, H., and Lassar, A. B. (1988). MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science* 242, 405–411. doi: 10.1126/science.3175662

Tchao, J., Kim, J. J., Lin, B., Salama, G., Lo, C. W., Yang, L., et al. (2013). Engineered human muscle tissue from skeletal muscle derived stem cells and induced pluripotent stem cell derived cardiac cells. *Int. J. Tissue Eng.* 2013:198762. doi: 10.1155/2013/198762

Tedesco, F. S., Gerli, M. F. M., Perani, L., Benedetti, S., Ungaro, F., Cassano, M., et al. (2012). Transplantation of genetically corrected human iPSC-derived progenitors in mice with limb-girdle muscular dystrophy. *Sci. Transl. Med.* 4:140ra89. doi: 10.1126/scitranslmed.3003541

Teo, A. K., Ali, Y., Wong, K. Y., Chipperfield, H., Sadasivam, A., Poobalan, Y., et al. (2012). Activin and BMP4 synergistically promote formation of definitive endoderm in human embryonic stem cells. *Stem Cells* 30, 631–642. doi: 10.1002/stem.1022

Thapa, R., and Wilson, G. D. (2016). The importance of CD44 as a stem cell biomarker and therapeutic target in cancer. *Stem Cells Int.* 2016:2087204. doi: 10.1155/2016/2087204

Thomson, J. A. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147. doi: 10.1126/science.282.5391.1145

Tompkins, B. A., DiFede, D. L., Khan, A., Landin, A. M., Schulman, I. H., Pujol, M. V., et al. (2017). Allogeneic mesenchymal stem cells ameliorate aging frailty: a phase II randomized, double-blind, placebo-controlled clinical trial. *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* 72, 1513–1522. doi: 10.1093/gerona/glx137

Tonlorenzi, R., Dellavalle, A., Schnapp, E., Cossu, G., and Sampaolesi, M. (2007). Isolation and characterization of mesoangioblasts from mouse, dog, and human tissues. *Curr. Protocols Stem Cell Biol.* Chapter 2:Unit 2B.1. doi: 10.1002/9780470151808.sc02b01s3

Tonoli, H., and Barrett, J. C. (2005). CD82 metastasis suppressor gene: a potential target for new therapeutics? *Trends Mol. Med.* 11, 563–570. doi: 10.1016/j.molmed.2005.10.002

Uekita, T., and Sakai, R. (2011). Roles of CUB domain-containing protein 1 signaling in cancer invasion and metastasis. *Cancer Sci.* 102, 1943–1948. doi: 10.1111/j.1349-7006.2011.02052.x

Uezumi, A., Nakatani, M., Ikemoto-Uezumi, M., Yamamoto, N., Morita, M., Yamaguchi, A., et al. (2016). Cell-surface protein profiling identifies distinctive markers of progenitor cells in human skeletal muscle. *Stem Cell Rep.* 7, 263–278. doi: 10.1016/j.stemcr.2016.07.004

Usas, A., Maciulaitis, J., Maciulaitis, R., Jakuboniene, N., Milasius, A., and Huard, J. (2011). Skeletal muscle-derived stem cells: implications for cellmediated therapies. *Medicina* 47, 469–479. doi: 10.3390/medicina47090068

Vailhe, B., Vittet, D., and Feige, J. J. (2001). *In vitro* models of vasculogenesis and angiogenesis. *Lab. Invest.* 81, 439–452. doi: 10.1038/labinvest.3780252

Vasyutina, E., Stebler, J., Brand-Saberi, B., Schulz, S., Raz, E., and Birchmeier, C. (2005). CXCR4 and Gab1 cooperate to control the development of migrating muscle progenitor cells. *Genes Dev.* 19, 2187–2198. doi: 10.1101/gad.346205

Vella, J. B., Thompson, S. D., Bucsek, M. J., Song, M., and Huard, J. (2011). Murine and human myogenic cells identified by elevated aldehyde dehydrogenase activity: implications for muscle regeneration and repair. *PLoS ONE* 6:e29226. doi: 10.1371/journal.pone.0029226

Villa, C. H., Shore, T., Van Besein, K., and Cushing, M. (2012). Addition of plerixafor to mobilization regimens in autologus peripheral blood stem cell transplants does not affect the correlation of preharvest hematopoietic precursor cell enumeration with first harvest CD34+ stem cell yield. *Biol. Blood Marrow Transplant.* 18, 1867–1875. doi: 10.1016/j.bbmt.2012.07.002

Walenkamp, A. M. E., Lapa, C., Herrmann, K., and Wester, H. J. (2017). CXCR4 ligands: The next big hit? *J. Nuclear Med.* 58, 77S–82S. doi: 10.2967/jnumed.116.186874

Wang, Z., and Yan, X. (2013). CD146, a multi-functional molecule beyond adhesion. *Cancer Lett.* 330, 150–162. doi: 10.1016/j.canlet.2012.11.049

Wetzel, A., Chavakis, T., Preissner, K. T., Sticherling, M., Haustein, U., Anderegg, U., et al. (2004). Human Thy-1 (CD90) on activated endothelial cells is a

counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J. Immunol.* 172, 3850–3859. doi: 10.4049/jimmunol.172.6.3850

Wilschut, K. J., Ling, V. B., and Bernstein, H. S. (2012). Concise review: stem cell therapy for muscular dystrophies. *Stem Cells Transl. Med.* 1, 833–842. doi: 10.5966/sctm.2012-0071

Woodard, G. E., Ji, Y., Christopherson, G. T., Wolcott, K. M., Hall, D. J., Jackson, W. M., et al. (2014). Characterization of discrete subpopulations of progenitor cells in traumatic human extremity wounds. *PLoS ONE* 9:e114318. doi: 10.1371/journal.pone.0114318

Wu, J., Matthias, N., Lo, J., Ortiz-Vitali, J. L., Shieh, A. W., Wang, S. H., et al. (2018). A myogenic double-reporter human pluripotent stem cell line allows prospective isolation of skeletal muscle progenitors. *Cell Rep.* 25, 1966–1981.e4. doi: 10.1016/j.celrep.2018.10.067

Xu, X., Wilschut, K. J., Kouklis, G., Tian, H., Hesse, R., Garland, C., et al. (2015). Human satellite cell transplantation and regeneration from diverse skeletal muscles. *Stem Cell Rep.* 5, 419–434. doi: 10.1016/j.stemcr.2015.07.016

Yang, J., Jian, R., Yu, J., Zhi, X., Liao, X., Yu, J., et al. (2015). CD73 regulates vascular smooth muscle cell functions and facilitates atherosclerotic plaque formation. *IUBMB Life* 67, 853–860. doi: 10.1002/iub.1448

Yang, Z. X., Han, Z., Ji, Y. R., Wang, Y. W., Liang, L., Chi, Y., et al. (2013). CD106 identifies a subpopulation of mesenchymal stem cells with unique immunomodulatory properties. *PLoS ONE* 8:e59354. doi: 10.1371/journal.pone.0059354

Yasuno, T., Osafune, K., Sakurai, H., Asaka, I., Tanaka, A., Yamaguchi, S., et al. (2014). Functional analysis of iPSC-derived myocytes from a patient with carnitine palmitoyltransferase II deficiency. *Biochem. Biophys. Res. Commun.* 448, 175–181. doi: 10.1016/j.bbrc.2014.04.084

Yu, W., Zeidel, M. L., and Hill, W. G. (2012). Cellular expression profile for interstitial cells of cajal in bladder - a cell often misidentified as myocyte or myofibroblast. *PLoS ONE* 7:e48897. doi: 10.1371/journal.pone.0048897

Zadeh, M. S., Kolb, J., Geromin, D., D'Anna, R., Boulmerka, A., Marconi, A., et al. (2000). Regulation of ICAM-1/CD54 expression on human endothelial cells by hydrogen peroxide involves inducible NO synthase. *J. Leukoc. Biol.* 67, 327–334. doi: 10.1002/jlb.67.3.327

Zheng, B., Cao, B., Crisan, M., Sun, B., Li, G., Logar, A., et al. (2007). Prospective identification of myogenic endothelial cells in human skeletal muscle. *Nat. Biotechnol.* 25, 1025–1034. doi: 10.1038/nbt1334

Zheng, B., Chen, C. W., Li, G., Thompson, S. D., Poddar, M., Peault, B., et al. (2012). Isolation of myogenic stem cells from cultures of cryopreserved human skeletal muscle. *Cell Transplant.* 21, 1087–1093. doi: 10.3727/096368912X636876

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REVIEW
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Biology of Tendon Stem Cells and Tendon in Aging

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Both tendon injuries and tendinopathies, particularly rotator cuff tears, increase with tendon aging. Tendon stem cells play important roles in promoting tendon growth, maintenance, and repair. Aged tendons show a decline in regenerative potential coupled with a loss of stem cell function. Recent studies draw attention to aging primarily a disorder of stem cells. The micro-environment ("niche") where stem cells resided *in vivo* provides signals that direct them to metabolize, self-renew, differentiate, or remain quiescent. These signals include receptors and secreted soluble factors for cell-cell communication, extracellular matrix, oxidative stress, and vascularity. Both intrinsic cellular deficits and aged niche, coupled with age-associated systemic changes of hormonal and metabolic signals can inhibit or alter the functions of tendon stem cells, resulting in reduced fitness of these primitive cells and hence more frequent injuries and poor outcomes of tendon repair. This review aims to summarize the biological changes of aged tendons. The biological changes of tendon stem cells in aging are reviewed after a systematic search of the PubMed. Relevant factors of stem cell aging including cell-intrinsic factors, changes of microenvironment, and age-associated systemic changes of hormonal and metabolic signals are examined, with findings related to tendon stem cells highlighted when literature is available. Future research directions on the aging mechanisms of tendon stem cells are discussed. Better understanding of the molecular mechanisms underlying the functional decline of aged tendon stem cells would provide insight for the rational design of rejuvenating therapies.

Keywords: tendon-derived stem cells, tendon stem cells, stem cell niche, tendon aging, stem cell aging

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INTRODUCTION - BIOLOGICAL CHANGES OF AGED TENDONS

Aging is known to reduce the regenerative capacity and increase the susceptibility of tissues and organs, including tendons, to injuries. Both tendon injuries and tendinopathies, particularly rotator cuff tears, increase with tendon aging (Teunis et al., 2014; Albers et al., 2016). In a systematic review including 30 studies with 6,112 shoulders, the prevalence of rotator cuff abnormalities increased with age, from 9.7% in patients aged 20 years and younger to 62% in patients aged 80 years and above (Teunis et al., 2014). Lower extremity tendinopathy was also reported to be more prevalent among older patients (Albers et al., 2016).

Structural Changes

Aged tendons exhibited structural, compositional and biomechanical changes. Aged tendons appeared yellowish (mucoid degeneration) (Zhang et al., 2016). Collagen fibers were less organized in aged mouse patellar tendons (Dunkman et al., 2013). Ultrastructural analysis of distribution of fibril diameters indicated an increase of a subpopulation of large fibrils in aged mouse patellar tendons (Dunkman et al., 2013) and Achilles tendons (Gehwolf et al., 2016). There were inconsistent findings regarding the change of vascularity and blood flow in tendon during aging, depending on the species, tendon types, and definition of aging. While there was no age-associated change in vascularity in equine superficial digital flexor tendons (aged 2 to 23 years) (Gillis et al., 1997), aged rat patellar tendons (20-month-old) were reported to be highly vascular (Zhang et al., 2016). An early human study reported a reduction of vascularity in the supraspinatus tendons with age (aged 20–70 years) (Brewer, 1979). While there was no change in the rate of blood flow in the flexor tendons with age in a rabbit study (Landi et al., 1983), there was a significant decrease in blood flow in the intratendinous region of rotator cuff of elderly subjects compared with younger subjects (Funakoshi et al., 2010). Rudzki et al. (2008) reported a significant decrease in blood flow in intact supraspinatus tendon in asymptomatic subjects older than 40 years compared with younger subjects after exercise. Non-tendinous tissues such as fatty and cartilaginous tissues as well as calcification were reported in aged tendons in both animal models and human (Iagnocco et al., 2013; Zhang and Wang, 2015; Gehwolf et al., 2016; Wood and Brooks, 2016; Zaseck et al., 2016). These non-tendinous tissues compromise tendon structure and reduce tendon's biomechanical properties.

Compositional Changes

Tenocytes are the major cell types in tendons. The cell number decreased; tenocyte dedifferentiated and senescent; and their proliferation and metabolic activity reduced with aging (Ippolito et al., 1980; Josa and Kannus, 1997; Tsai et al., 2011; Dunkman et al., 2013; Yu et al., 2013).

The expression of extracellular matrix (ECM) genes and ECM remodeling gene were significantly reduced in aged Achilles tendons (Kostrominova and Brooks, 2013; Yu et al., 2013; Gehwolf et al., 2016; Wood and Brooks, 2016). The mRNA expression of collagen types I, III, and V, elastin and proteoglycan 4 (lubricin) in rat tendons decreased with age though immunostaining showed no apparent differences in protein levels of collagen type I and type V (Kostrominova and Brooks, 2013). The mRNA expression of collagen type I was reported to be similar in rat Achilles tenocytes in young (2 months) and aged (24 months) groups but the mRNA expression and enzymatic activities of MMP-2 and MMP-9 was significantly higher while the mRNA expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 were significantly lower in the aged group compared to the young group *in vitro* (Yu et al., 2013). The expressions of collagen type I and type III genes were reduced in aged mouse Achilles tendons (Gehwolf et al., 2016). The collagen content of aged mouse Achilles tendon was similar to their young counterpart (Gehwolf et al., 2016) but the level decreased in canine patellar tendon with aging (Haut et al., 1992). In a small clinical study involving 7 old men and 10 young men, the collagen concentration in the patellar tendon biopsies was lower in the old men compared to that in the young men with a similar physical activity level, supporting the reduction in collagen during the aging process (Couppe et al., 2009).

Proteoglycans are important for regulating collagen fibril assembly, fiber size, and fiber sliding as well as cellular functions. The age-associated changes of proteoglycans were inconclusive. Thorpe et al. (2016) reported no change in the mRNA expression of collagens and proteoglycans as well as protein and mRNA levels of matrix remodeling enzymes in equine superficial digital flexor tendons with age (3.3 ± 0.6 years versus 19.0 ± 1.7 years). There was also no difference in the mRNA expression of biglycan, decorin, fibromodulin, and lumican in the patellar tendons of aged mice (Dunkman et al., 2013). While there was no change in the levels of major matrix components with age, there was a reduction in protein levels of several less abundant small leucine-rich proteoglycans (fibromodulin, mimecan, asporin) in aged equine superficial digital flexor tendons (Peffers et al., 2014). One study reported significant lower total glycosaminoglycan, chondroitin sulphate, and dermatan sulphate in healthy human supraspinatus tendons with age, although there was no change in the relative proportion of different glycosaminoglycan types (Riley et al., 1994). The concentration of nonenzymatic cross-links was higher in the patellar tendon biopsies of aged men compared to that in the young men in a small scale clinical study (Couppe et al., 2009). The advanced glycation end-products (AGEs) adduct level in tibialis anterior tendons was also higher in aged compared to adult mice (Wood and Brooks, 2016).

Biomechanical Changes

The biomechanical properties of aged tendon were reported to be inferior in animal and human studies. The viscoelastic properties and mechanical strength of aged equine and mouse tendons were reported to be lower than those of young tendons (Dudhia et al., 2007; Dunkman et al., 2013; Zaseck et al., 2016). The mechanical properties (maximum stress and modulus) of aged rat Achilles tendon decreased with increasing age (Pardes et al., 2017). While aging did not alter tendon mechanical properties during homeostasis, it impaired tendon healing and hence biomechanical properties of flexor tendon in mice (Ackerman et al., 2017). Aged flexor tendons showed similar mechanical strength (maximum load to failure and ultimate tensile stress) but was significantly stiffer (higher Young's modulus and stiffness) compared to young tendons (Gehwolf et al., 2016). Aged human Achilles tendons were also stiffer compared to young tendons as shown by sonoelastography (Turan et al., 2015). Aged human patellar tendons had significantly lower elastic modulus and shear wave velocity compared to young tendons as indicated by shear wave elastography (Hsiao et al., 2015). In a systematic review of age-related changes of biomechanical properties of healthy Achilles tendon, its stiffness, and elastic modulus decreased in older compared to younger adults (Delabastita et al., 2018). The responses of human Achilles and patellar tendons to transverse strain was reduced by 2.5% for every 10 years of life (Dudhia et al., 2007). **Table 1** summarizes the biological changes of aging tendons.

TABLE 1 | Summary of biological changes of aging tendons.

• Yellowish color
• Enlargement of a subpopulation of collagen fibril size and change of fibril size distribution
• Disorganized collagen fibers
• Inconclusive findings on blood flow and vascularity
• Ectopic formation non-tendinous tissues including fat, cartilage and bone tissue
• Decreased cellularity
• Dedifferentiation and senescence of tenocytes
• Reduced proliferation and metabolic activity of tenocyte
• Inconclusive findings on change of collagen and proteoglycan content and composition
• Accumulation of advanced glycation end-products (AGEs)
• Inferior biomechanical properties particularly viscoelasticity

Consequently, the structure, composition, and biomechanical properties of aged tendons are compromised and aged tendons are prone to higher risk of tendon injuries, re-injuries, and tendinopathy as well as treatment failure (de Jonge et al., 2011; Teunis et al., 2014; Salini et al., 2015; Albers et al., 2016). The odds of a rotator cuff tear increased by 2.69-fold for every 10 years of age (Fehringer et al., 2008). After injury, aged tendons require very long time for rehabilitation. The current treatment modalities fail to completely restore tendon function. A summary of biological changes of aging tendons is showed in **Table 1**.

Tendon Aging as a Problem of Aging of Tendon Stem Cells and Their Niche

Recent studies draw attention to aging being primarily a disorder of stem cells with a decrease in the number and functional fitness of tissue-specific adult stem cells (Sharpless and DePinho, 2007; Fukada et al., 2014). Tendon stem cells have been recently identified in tendons of various species (Bi et al., 2007; Rui et al., 2010). Stem/progenitor cells isolated from tendon mid-substances of various species, called tendon-derived stem cells (TDSCs), showed standard mesenchymal stem cell (MSC) characteristics, with expression of typical MSC surface antigens, self-renewal, colony-forming, and tri-lineage differentiation potential (Rui et al., 2010; Lui and Chan, 2011; Lui, 2015). In addition, they are able to form tendon- and enthesis- like tissues when implanted *in vivo*. TDSC expressed higher Oct4 level, showed higher clonogenicity and proliferative, and differentiation capacity compared to BMSC (Tan et al., 2012). TDSCs expressed embryonic stem cell markers and are different from tenocytes which are terminally differentiated cells (Zhang and Wang, 2010a). Recent studies showed that the functions of TDSCs declined or skewed with age *in vitro*. An in-depth understanding of the molecular mechanisms controlling tendon stem cell aging is essential to inform the development of stem cell-based therapeutics that can slow, or even forestall age-associated degenerative changes as well as enhance repair of aged tendons.

Niche is a specialized dynamic micro-environment which regulates stem cells' functions and fate. Aging of cellular and acellular components of the niche can alter stem cell functions. Numerous studies showed that exposure of stem cells to a young systemic environment restored the regenerative capacity of stem cells in various tissues in aged mice (Conboy et al., 2005; Ruckh et al., 2012). Transplantation of aged spermatogonial stem cells to the testes of young male mice was sufficient to maintain stem cell functions (Sato et al., 2011). These findings underscore a major role of aging of the niche in the functional decline of stem cells and suggest opportunities of reversing tissue aging by targeting the niche.

Scope of Review and Search Strategy

This review aims to summarize the biological changes of tendon stem cells in aging after a systematic search of PubMed. Relevant factors of stem cell aging including cell-intrinsic factors, changes of microenvironment, and systemic changes of hormonal and metabolic signals are reviewed, with findings on tendon stem cells highlighted when literature is available. Future research directions on the aging mechanisms of tendon stem cells are discussed.

In this review, "TDSCs" refer to stem/progenitor cells isolated from tendon mid-substances *in vitro* while "tendon stem cells" refer to stem/progenitor cells residing *in situ* in tendon mid-substances. TDSCs are often used as *in vitro* model to understand the physiology of tendon stem cells residing *in situ* in tendon mid-substances. The properties of tendon stem cells may change due to cell isolation and *in vitro* culture conditions. "Tenocytes" refer to terminally differentiated cells in tendons.

For the studies on aged TDSCs, the PubMed database was searched with the keywords ((tendon[Title/Abstract]) AND (ageing[Title/Abstract] OR aging[Title/Abstract] OR age[Title/Abstract])) AND (stem cells[Title/Abstract] OR progenitor) on 25 Jan 2019 and updated on 22 Sep 2019 with no restriction in language and year of publication. The studies were selected after reviewing the titles and abstracts. Original studies investigating the biological changes of stem cells in tendons, either *in vitro* or *in situ*, in aging were included. A total of 74 articles was identified. Of these, 16 studies are eligible. Another two studies were identified by hand search. Therefore, a total of 18 studies were included in this review and all of them are on the biological changes of TDSCs of aged tendons. There were no studies on aging of tendon stem cells *in situ*. **Table 2** summaries the studies on the biological changes of TDSCs in aging.

CHARACTERISTICS OF AGED TDSCS

Phenotypes of Aged TDSCs

Young TDSCs were reported as spindle or cobble-stone shape in previous studies (Rui et al., 2010; Zhang and Wang, 2015). However, TDSCs isolated from aged human, mouse and rat tendons were reported to be heterogeneous, much larger and show a round and flattened appearance (Wu et al., 2015; Zhang and Wang, 2015). The stiffness of aged rat TDSCs was higher than that of young TDSCs (Wu et al., 2015; Kiderlen et al., 2019). The increase in size and irregular shape of aged TDSCs might be associated with the dense cytoskeleton organization, which could lead to an increase in both stiffness and viscosity (Wu et al., 2015; Kiderlen et al., 2019). Indeed, the actin stress fiber content was higher and the turnover of actin filaments was slower in TDSCs isolated from aged/degenerated human Achilles tendon compared to TDSCs isolated from young/healthy tendons

TABLE 2 | Summary of biological changes of tendon-derived stem cells (TDSCs) in aging.

Species	Major findings	References
Mice	Young TDSCs were cobblestone-shaped while the shape of aged TDSCs were heterogeneous. Aged TDSCs proliferated slower and expressed lower levels of stem cell makers (Oct-4, nucleostemin, Sca1 and SSEA-1). They showed reduced tri-lineage differentiation potential compared to young TDSCs. Moderate mechanical stretching (4%) increased while 8% stretching decreased the expression of nucleostemin in aged TDSCs. Moderate mechanical stretching also increased the expression of *Nanog* and tendon-related markers (*Col1* and *Tnmd*). 8% stretching increased the expression non-tenocyte-related genes (*Lpl*, *Sox9* and *Runx2*) while 4% stretching had minimal effects on these genes. Moderate treadmill running of aged mice increased the proliferation of aged TDSCs in culture, decreased lipid deposition, proteoglycan accumulation and calcification, and increased the number of nucleostemin-expressing TDSCs in tendons.	Zhang and Wang, 2015
Mouse	The expressions of tendon transcription factor (*Scx*, *Mkx*), ECM (*Col1a1*, *Col3a1*, *Dcn*, *Bgn*, *Fmod*) and ECM-remodeling (Lox, *Thbs1*, *Sparc*) genes were significantly reduced in aged tendon tissue and aged TDSCs isolated from the Achilles tendons. There was no significant difference in the fascicle diameters between young and healthy-aged tail tendons. Aged Achilles tendon was thicker but there was no difference in the fascicle diameters, cell density and total collagen content compared to their young counterparts. Aged Achilles tendon displayed a bimodal distribution, with a more pronounced separation of large and small diameter fibrils and less interfibrillar area, showing no change in fibril number when compared to young tendons whose fibril size was normally distributed. The average fibril diameter was larger for aged Achilles tendon compared to young tendons. The fibers were less well-oriented in aged tendon. Aged flexor tendons showed similar mechanical strength (maximum load to failure and ultimate tensile stress) but was significantly stiffer (higher Young's modulus and stiffness) compared to young tendons. There was an accumulation of lipid droplets in aged Achilles and tail tendons, with an increased expression of adipogenic markers (*Pparg* and *Cebpα*) and reduced expression of β-catenin (regulator of adipogenesis).	Gehwolf et al., 2016
Mouse	Aged TDSCs (18-month old) showed lower collagen contractility than cells obtained from younger animals (6-, 9-, and 12- months old).	Yin et al., 2019
Rat	Both aged and young TDSCs expressed nucleostemin, Oct-4 and SSEA-4. Aged TDSCs showed slower proliferation rate, frequency and cell cycle progression compared to young TDSCs. The expression of tendon related marker genes (*Scx*, *Tnmd*) was reduced while adipocyte differentiation and adipogenic markers increased in aged TDSCs. Cited2 was downregulated while CD44 was upregulated in aged TDSCs.	Zhou et al., 2010
Rat	Aged TDSCs showed lower cell proliferation, migration and tendon-related marker expression (*Tnc*, *Tnmd*, Scx) than young TDSCs. Medium of young TDSCs under hypoxic culture condition enhanced the migration, proliferation and mRNA expression of tendon-related markers (*Tnc*, *Tnmd* but not *Scx*) as well as reduced senescence of aged TDSCs.	Jiang et al., 2014
Rat	miR-135a, which directly binds to the 3'-untranslated region of ROCK1, was significantly downregulated in aged compared with young TDSCs. Overexpression of miR-135a in young TDSCs suppressed senescence, promoted proliferation, and induced migration and tenogenic differentiation, whereas suppression of miR-135a in aged TDSCs had the opposite effects. ROCK1 mediated the effects of miR-135a in TDSCs as shown by gain-of-function and loss-of-function studies.	Chen et al., 2015b
Rat	Aged TDSCs were large, flat and heterogeneous in morphologies while young TDSCs were uniformly elongated. Aged TDSCs showed higher stiffness compared to young TDSCs.	Wu et al., 2015
Rat	Moderate treadmill running accelerated tendon wound healing compared to the cage control. Moderate treadmill running also increased the number, proliferation, stem cell marker expression (Oct-4, Nanog) and tendon-related marker expression (collagen type I, collagen type III, tenomoduline) as well as downregulated the expression of non-tenocyte genes (*Pparg*, *Col2* and *Runx2*) of TDSCs.	Zhang et al., 2016
Rat	Young decellarized ECM (DECM) preserved stem cell properties of aged TDSCs. DECM from young TDSCs enhanced the proliferation, mRNA expression of tendon-related marker (*Scx*, *Tnmd*), protein expression stem cell marker protein expression (Oct-4, SSEA-1) of aged TDSCs. The senescence-associated β-galactosidase activity of aged TDSCs was also decreased by young DECM.	Jiang et al., 2018
Rat	FOXP1 mRNA and protein levels were markedly decreased in the aged TDSCs. Overexpression of FOXP1 attenuates TDSCs aging, as shown by reduced senescence-associated β-gal staining and expression of senescence marker, $p16^{INK4A}$. FOXP1 overexpression also restores the age-associated reduction of self-renewal, migration and differentiation of TDSCs. Conversely, knockdown of FOXP1 promoted senescence in young TDSCs. In addition, FOXP1 overexpression rescued decreased levels of E2F1, pRb and cyclin D1 in aged TDSCs.	Xu and Liu, 2018
Human	Aged tenocyte-like cells showed lower proliferation rate, colony forming ability, collagen type I secretion and osteogenic differentiation potential compared to their young counterparts. However, there were no significant difference in the surface expression of MSC markers (CD29, CD44, CD73, CD90 and CD105) as well as mRNA expression of ECM (collagen type I, collagen type III, osteocalcin, decorin), TGF-β1, TGF-β2, TGF-β3 between aged and young tenocyte-like cells.	Klatte-Schulz et al., 2012
Human	Aged/degenerated TDSCs exhibited deficits in self-renewal and colony-forming ability. They showed significant decline in proliferative activity and poor response to FGF2 and TGF-β1. However, their multipotency was retained. Aged/degenerated TDSCs showed earlier cellular senescence with upregulation of $p16^{INK4a.}$ Microarray and gene ontology analyses revealed that differential expression of genes regulating cell adhesion, migration and actin cytoskeleton. Aged/degenerated TDSCs showed slower migration, higher actin stress fiber content and slower turnover of actin filaments. Analyses of microarray candidates revealed dysregulated cell-matrix interactions with lower gene expression of collagen type I and collagen type I-binding integrins α1, α2 and α11 but higher gene expression of fibronectin and fibronectin-binding integrins αv, β3 and β5. The ROCK1 and ROCK 2 mRNA and protein levels as well as activities increased in aged/degenerated TDSCs. Inhibition of ROCK1 and ROCK2 activities in aged/degenerated TDSCs with ROCK inhibitor restored the cell area, F-actin content and dynamics as well as migration.	Kohler et al., 2013
Human	Aging affected the number of TDSCs isolated and colony-forming ability of TDSCs. However, tri-lineage differentiation potential of TDSCs was not affected by age.	Ruzzini et al., 2014
Human	Pin1 mRNA and protein expression levels were significantly decreased during prolonged *in vitro* culture of human TDSCs. Overexpression of Pin1 delayed the progression of cellular senescence, increased telomerase activity and decreased level of the senescence marker, $p16^{INK4a}$. Conversely, Pin1 siRNA transfection promoted senescence in TDSCs.	Chen et al., 2015a
Human	The expression of ephrin receptors EphA4, EphB2 and B4 and ligands EFNB1 were down regulated in aged TDSCs. Activation of EphA4- or EphB2-dependent reverse signaling could restore the migratory ability and normalize the actin turnover of aged TDSCs. However, only EphA4-Fc stimulation improved aged TDSCs' proliferation to levels comparable to young TDSCs.	Popov et al., 2016

(Continued)

TABLE 2 | Continued

Species	Major findings	References
Human	CITED2 mRNA and protein expression levels were significantly higher in young TDSCs than in old TDSCs. Old TDSCs displayed lower cell proliferation and higher senescence compared with young TDSCs. High level of CITED2 protein expression in young TDSCs correlated with the downregulation of SP1 and p21 and upregulation of MYC, suggesting the mechanism by which CITED2 upregulates TDSC proliferation. TGFβ2 downregulated the expression of *CITED2* gene and knockdown of CITED2 abolished the effect of TGFβ2 on TDSC proliferation and senescence.	Hu et al., 2017
Human	Tenogenic differentiation capacity of TDSCs decreased significantly with advancing age. Aged TDSCs showed higher β-galactosidase activity and p16 and concurrently lower collagen type I concentration and expression of tendon-related markers (*Scx*, *Tnmd*, *Bgn*, *Dcn*, *Col1*, and *Col3*). Overexpression of p16 significantly inhibited tendon-related marker expression in young TDSCs and the inhibitory effect was mediated by miRNA-217 and Egr1.	Han et al., 2017
Human	The cell stiffness and size of aged TDSCs were higher than young TDSCs. The aged TDSCs also showed a denser, well-structured actin cytoskeleton which correlated with the augmented cell stiffness. Treating aged TDSCs with ROCK-inhibitor, reversed these age-related changes, and has rejuvenating effect on cell morphology and stiffness.	Kiderlen et al., 2019

(Kohler et al., 2013). There was also an increase in focal adhesion kinase complex protein paxillin and profound rearrangement of actin cytoskeleton with an increase in actin in aged mouse Achilles TDSCs (Gehwolf et al., 2016).

Frequency, Proliferative, and Colony-Forming Ability of Aged TDSCs

Fewer TDSCs were isolated from aged tendons compared to young tendons (Zhou et al., 2010; Ruzzini et al., 2014). Aged TDSCs showed delayed cell cycle progression (Zhou et al., 2010), lower proliferation (Zhou et al., 2010; Klatte-Schulz et al., 2012; Kohler et al., 2013; Jiang et al., 2014; Zhang and Wang, 2015; Hu et al., 2017), lower proliferative response to fibroblast growth factor-2 (FGF-2), and transforming growth factor β 1 (TGF-β1) (Kohler et al., 2013) as well as lower colony-forming ability (Klatte-Schulz et al., 2012; Kohler et al., 2013) compared to their young counterparts.

Stem Cell Markers and ECM Expression and Migration Ability of Aged TDSCs

Aged TDSCs showed lower expression of stem cell markers (Oct-4, nucleostemin, Sca-1, and SSEA-1) (Zhang and Wang, 2015) and tenocyte-related markers (Zhou et al., 2010; Jiang et al., 2014) as well as lower collagen type I secretion (Klatte-Schulz et al., 2012; Kohler et al., 2013) and higher fibronectin protein expression (Kohler et al., 2013) compared to young TDSCs. However, Klatte-Schulz et al. (2012) reported no significant difference in the surface expression of MSC markers (CD29, CD44, CD73, CD90, and CD105) as well as mRNA expression of ECM proteins (collagen type I, collagen type III, osteocalcin, decorin), TGF-β1, TGF-β2, TGF-β3 between aged and young human tenocyte-like cells. Zhou et al. (2010) reported no difference in stem cell markers (nucleostemin, Oct-4, and SSEA-4) between young and aged rat TDSCs. The aged/degenerated TDSCs were deficient in cell adhesion, migration and actin dynamics (Kohler et al., 2013; Jiang et al., 2014). Aged mouse TDSCs showed lower collagen adhesion and contractility compared to their younger counterparts (Yin et al., 2019). This is consistent with change of transcriptome, especially in genes regulating cell adhesion, migration, and actin cytoskeleton, in aged human Achilles TDSCs (Kohler et al., 2013).

Differentiation Potential of Aged TDSCs

While two studies reported similar tri-lineage differentiation potential of young and aged human TDSCs (Kohler et al., 2013; Ruzzini et al., 2014), one study reported higher adipogenic differentiation potential of aged rat TDSCs (Zhou et al., 2010) and another study showed lower osteogenic differentiation of aged human tenocyte-like cells (Klatte-Schulz et al., 2012). Zhang and Wang (2015) reported reduced tri-lineage differentiation potential of aged TDSCs compared to their young counterparts. Han et al. (2017) showed that the tenogenic differentiation capacity, collagen type I concentration, and expression of tendon-related markers (*Scx*, *Tnmd*, *Dcn*, *Bgn*, *Col1*, and *Col3*) of human Achilles TDSCs decreased with advancing age. Treatment of human TDSCs with tarzarotene, a retinoic acid receptor (RAR) agonist, induced nuclear translation of scleraxis and inhibited spontaneous cell differentiation *via* histone methylation but not histone acetylation in RAR signaling, suggesting that histone methylation plays roles in the differentiation of tendon stem cells (Webb et al., 2016). Regulation of methylation status of aged TDSCs may help to restore the tenogenic differentiation potential of TDSCs and this requires further research.

INTRINSIC CELLULAR DEFICITS

Cellular Senescence

Cellular senescence is a state in which a cell no longer has the ability to proliferate (Collado et al., 2007; Hoare et al., 2010). Senescent cells are irreversibly arrested at the G1 phase of the cell cycle and do not respond to various external stimuli, but they remain metabolically active (Collado et al., 2007; Hoare et al., 2010). Cellular senescence can be triggered by a number of cellular stresses, including oxidative stress, telomere dysfunction, non-telomeric DNA damage, epigenetic derepression of the *INK4a/ARF* locus, and oncogenic activation (Collado et al., 2007). Senescent cells are characterized by shortened telomeres, increased activity of senescence-associated β-galactosidase (SA-β-gal), increased expression of $p16^{INK4a}$, p53, $p15^{INK4B}$, and $p21^{WAF1/Cip1}$, and histological changes (Collado et al., 2007; Hoare et al., 2010). Cellular senescence is a potent tumor suppression mechanism. It is seen in aged or damaged tissues, and is associated with reduced regenerative capacity with age. Similar to other tissue-specific stem

cells, aged TDSCs displayed cellular senescence (Kohler et al., 2013; Jiang et al., 2014; Han et al., 2017; Hu et al., 2017). The expression of $p21^{WAF1/Cip1}$ (Hu et al., 2017) and $p16^{INK4a}$ (Han et al., 2017) was higher in human aged/degenerated Achilles TDSCs. In another study, upregulation of *p16*^{INK4a} gene and protein expression was observed, but the gene expression of *p14*, *p21*, and *p53* were unchanged in age/degenerated human Achilles TDSCs (Kohler et al., 2013). Further studies are required to confirm this finding. $p16^{INK4a}$ is a critical effector of cellular senescence. Many studies showed that the increase in the expression of $p16^{INK4a}$ with aging was linked to the impairment of the number and/or function of adult stem cells (Janzen et al., 2006; Molofsky et al., 2006; Sousa-Victor et al., 2014) while abolishing $p16^{INK4a}$ function enhanced the regenerative potential of stem cells (Shibata et al., 2007; Sousa-Victor et al., 2014). Indeed, overexpression of $p16^{INK4a}$ inhibited tenogenic differentiation of young TDSCs (Han et al., 2017). Analysis of the mechanism revealed that the effect was mediated by miR-217 and its target EGR1 (Han et al., 2017). Silencing *p16* in aged satellite cells restored their quiescence and regenerative potential (Sousa-Victor et al., 2014). miRNA are epigenetic regulators of multiple biological activities including stem cell differentiation. Microarray analysis suggested that dysregulation of the Rho-associated protein kinase (ROCK) pathway might be a key player in TDSC aging (Kohler et al., 2013). The ROCK1 and ROCK 2 mRNA and protein levels as well as activities increased in aged/degenerated TDSCs (Kohler et al., 2013). Inhibition of ROCK1 and ROCK2 activities in aged/degenerated human TDSCs with ROCK inhibitor restored the cell area, F-actin content, and dynamics as well as migration (Kohler et al., 2013). This is consistent with the findings of another study which treatment with ROCK inhibitor restored the morphology and stiffness of aged human TDSCs (Kiderlen et al., 2019). miR-135a which targets ROCK1, was significantly downregulated in aged compared to young TDSCs (Chen et al., 2015b). Inhibition of miR-135a was reported to increase senescence of TDSCs (Chen et al., 2015b). Overexpression of miR-135a in young TDSCs suppressed senescence, promoted proliferation, and induced migration and tenogenic differentiation *in vitro* (Chen et al., 2015b), suggesting that miR-135a is a key regulator of ROCK1-induced cellular senescence. The reduced expression of *Tnmd* in aged TDSCs (Zhou et al., 2010; Han et al., 2017) may affect cellular senescence of TDSCs as knockdown of *Tnmd* in mouse tail TDSC augmented cellular senescence, reduced the gene expression of cyclin D1, and increased the gene expression of *p53* but has no effects on the gene expression of *p16* and *p21* (Alberton et al., 2015). However, it is important to note that the complete knockout of *Tnmd* was artificially created in the animal model which might be different from the reduced expression of Tnmd in aged TDSCs. miR124 suppressed collagen formation during tenogenic differentiation of human TDSCs *via* suppressing EGR1 expression while anti-miR124 promoted it, suggesting that miR124 might be a promising therapeutic target for TDSC and tendon aging (Wang et al., 2016).

Pin1 (peptidyl-prolyl cis-trans isomerase), an enzyme which regulates conformational changes of phosphoproteins, is an important enzyme necessary for healthy aging and prevention of age-related diseases. Deficiency of Pin1 in mice caused an early-aging phenotype (Driver and Lu, 2010). Overexpression of Pin1 was reported to delay the progression of cellular senescence, increase telomerase activity and decrease expression of $p16^{INK4A}$ while Pin1 knockdown promoted senescence of TDSCs *in vitro*, confirming that Pin1 is also an important regulator of tendon stem cell aging (Chen et al., 2015a). The Pin1-mediated anti-aging signaling mechanism might offer an attractive therapeutic target for protection against tendon aging.

Forkhead box P1 (FOXP1) plays a crucial role in tendon stem cell aging. FOXP1 directly repressed transcription of *p16INK4A* in BMSCs (Li et al., 2017). It controlled MSC fate and senescence during skeletal aging (Li et al., 2017). A recent study showed that the expression of FOXP1 mRNA and protein levels decreased markedly in aged TDSCs. Overexpression of FOXP1 attenuated TDSC aging with a decrease in SA-β-gal staining and $p16I^{NK4A}$ expression (Xu and Liu, 2018). It also rescued decreased levels of E2F1, pRb, and cyclin D1 and restored self-renewal, migration and differentiation in aged TDSCs (Xu and Liu, 2018). Conversely, depletion of FOXP1 by siRNA promoted senescence of young TDSCs.

CITED2 is a transcription factor implicated in the control of cell proliferation and cellular senescence. Aged human Achilles TDSCs were shown to display lower mRNA and protein expression of CITED2 as well as lower proliferation and higher cellular senescence compared to young TDSCs (Zhou et al., 2010; Hu et al., 2017). Moreover, TGF-β2 was reported to downregulate the expression of *CITED2* gene and knockdown of *CITED2* abolished the effect of TGF-β2–mediated TDSC senescence (Hu et al., 2017). The effects of TGF-β2 inhibitors and CITED2 activators in reversing the senescence of aged TDSCs and tendon aging await further studies.

Accumulation of Damaged DNA and Proteins

Aging is one of the major risk factors for DNA damage, protein mis-folding and accumulation. Age-dependent deficits of the autophagy-lysosomal and ubiquitin-proteasome systems, which remove the damaged proteins, were reported in recent studies (Rubinsztein et al., 2011; Tomaru et al., 2012). However, other studies argued that it was the increase in damage caused by metabolic stress, not the decrease in autophagy potential and proteasome activity, which overwhelmed the protective capacity of proteolytic systems in aged cells (Warr et al., 2013). Inhibition of autophagy has been shown to inhibit self-renewal and differentiation of stem cells, including TDSCs (Cheng et al., 2015; Chen et al., 2016; Sbrana et al., 2016). Tendon aging is dependent at least in part on the actions of mechanistic target of rapamycin (mTOR). Long-term dietary administration of rapamycin, an inhibitor of mTOR, was reported to attenuate aged-associated increase in stiffness; decrease in elasticity, cellularity, and progression of spontaneous tendon calcification; as well as enhance collagen turnover in aged mouse tendons (Wilkinson et al., 2012; Zaseck et al., 2016). Autophagy plays an important role in regulating the self-renewal capacity and stemness of human TDSCs through suppressing reactive oxygen species (ROS) production. Treatment of human TDSCs isolated from

supraspinatus tendon of adult donors (26–34 years old) with starvation or rapamycin prevented H2O2-induced loss of self-renewal capacity and stemness (Chen et al., 2016). The effects were mediated by increasing autophagy activity and suppressing ROS production in TDSCs (Chen et al., 2016). On the other hand, inhibition of autophagy reduced the protective effects of starvation and rapamycin on H2O2-treated cells (Chen et al., 2016).

AGED NICHE

Cell-Cell Communication

Stem cells interact with each other or niche cells *via* direct interaction of membrane proteins or secretion of soluble factors. The changes of membrane proteins or secretome of stem cells or niche cells during aging therefore could impact stem cell fate and functions. Ephrin are receptor tyrosine kinases for mediating short-distance cell-cell communication. Specifically, ephrins were reported to regulate the attachment, spreading, migration, differentiation, and age-associated senescence of stem cells (Arthur et al., 2011; Goichberg et al., 2013; Li and Johnson, 2013). Dysregulated cell-cell communication and hence proliferation, motility, and actin turnover of aged TDSCs were reported to be mediated by down-regulation of ephrin receptors EphA4, EphB2, and EphB4 and ligands EFNB1 (Popov et al., 2016). Interestingly, activation of EphA4- or EphB2- dependent reverse signaling in aged TDSCs restored migratory ability and normalized actin turnover while activation of EphA4 reverse signaling increased proliferation of aged TDSCs (Popov et al., 2016).

Extracellular Matrix

The ECM of tendon consists primarily of collagens (mainly collagen type I), proteoglycans, and glycoproteins. ECM functions to provide mechanical strength to tendon. In addition, it also functions to retain resident cells in place, localize and create gradients of instructive signals that guide stem cells in their processes of self-renewal and differentiation.

The alterations in the composition, topography and organization of ECM of aged tendons therefore can contribute to the loss of functions of tendon stem cells. While tendon-derived decellularized matrix promoted tendinous phenotype and inhibited osteogenesis of TDSCs in the presence of osteogenic induction conditions, bone-derived decellularized matrix induced osteogenic differentiation of TDSCs (Yin et al., 2013). The accumulation of AGEs with age likely plays a significant role in stem cell dysfunction during tendon aging. AGEs are proteins or lipids that become nonenzymatically glycated and oxidized after exposure to carbohydrates. Proteins are usually glycated through the lysine resides. Collagen, which is rich in lysine, is frequently glycated. Non-enzymatic glycation of collagen type I diminished collagen-proteoglycan binding and weakened cell adhesion and migration (Reigle et al., 2008). The formation of AGEs also resulted in the denaturation and cross-linking of collagen and the AGEs were nearly irreversible once formed. The mechanical and biological functions of long-lived proteins like collagen are therefore affected in the aging process. AGEs also increased the activity of transglutaminase, a cross-linking enzyme of ECM proteins, in tenocytes *in vitro*, further promoting collagen cross-linking (Rosenthal et al., 2009). The collagen cross-links limited fiber sliding, reduced viscoelasticity of tendon, and hence increased its stiffness and brittleness (Reddy, 2004; Gautieri et al., 2017), consistent with the increase in tendon stiffness with age (Andreassen et al., 1981; Haut et al., 1992). Matrix stiffness is known to regulate proliferation and lineage commitment of MSCs (Engler et al., 2006; Nam et al., 2011; Lee et al., 2014; Navaro et al., 2015). Matrix with lower stiffness promoted chondrogenic differentiation, whereas matrix with high stiffness promoted osteogenic differentiation of stem cells (Nam et al., 2011; Navaro et al., 2015). The increase in matrix stiffness as a result of age-induced collagen cross-links might underlie higher non-tenogenic differentiation potential of aged TDSCs. Bone morphogenetic protein-2 (BMP-2) stimulated non-tenogenic differentiation and promote proteoglycan deposition of TDSCs *in vitro* (Rui et al., 2013). A recent study showed that AGEs increased BMP-2 expression and accelerated progression of atherosclerotic calcification in diabetes (Wang et al., 2012). AGEs were also reported to increase the mRNA expression of BMP2, BMP4, and osteogenic markers of human yellow ligament cells isolated from cervical spine (Yokosuka et al., 2007). There has been no study about the effects of AGEs on TDSCs and is an area worthy of investigation.

The ECM composition can affect tendon stem cell fate. A deficiency in proteoglycans (biglycan and fibromodulin) resulted in altered differentiation of TDSCs towards the osteogenic linage *via* modulating the BMP signaling (Bi et al., 2007). Secreted protein acidic and rich in cysteine (Sparc), also known as osteonectin, is a collagen-binding matricellular glycoprotein involved in collagen fibril assembly and procollagen processing. It regulates cell-ECM interactions impacting upon cell signaling, adhesion, proliferation, migration, and survival. Sparc also regulates adipogenesis by modulating cell adhesiveness and cytoarchitecture. A recent study reported that the expression of Sparc decreased in aged mouse Achilles tendons (Gehwolf et al., 2016). The decrease in Sparc expression led to a more contracted phenotype, an altered actin cytoskeleton, and an elevated expression of the adipogenic marker genes *Pparg* and *Cebpa* with a concomitant increase in lipid deposits in both aged and Sparc -/- mouse tendons, a phenomenon that is commonly seen in elderly (Gehwolf et al., 2016).

The cytoskeleton of a cell and cell-matrix interactions affect stem cell migration, proliferation, and differentiation. Cell migration is strongly dependent on fibronectin, actin cytoskeletal organization, and turnover rate of actin filament. Integrins bind stem cells to different ECM components and regulate downstream signaling which are important for stem cell self-renewal and proliferation (Piwko-Czuchra et al., 2009; Ellis and Tanentzapf, 2010). Aged/degenerated TDSCs showed lower actin turnover, gene expression of collagen type I, and collagen type I-binding integrins $\alpha1$, $\alpha2$, and $\alpha11$ but higher gene expression of fibronectin, fibronectin-binding integrins αv, $\beta3$, and $\beta5$ compared to young/healthy TDSCs (Kohler et al., 2013). The changes therefore might cause poor cell adhesion, migration,

and actin dynamics of aged/degenerated TDSCs (Kohler et al., 2013). Integrin receptors and their ECM ligands function upstream of RhoA/ROCK signaling. Both the expression and activities of ROCK1 and ROCK2, which mediate RhoA-induced actin stress fiber formation *via* phosphorylation of myosin light chain (Schmitz et al., 2000), increased in aged/degenerated TDSCs (Kohler et al., 2013). Inhibition of ROCK1 and ROCK2 activities in aged/degenerated TDSCs with ROCK inhibitor Y-27632 restored the cell area, F-actin content and dynamics as well as migration ability, with results comparable to young/healthy TDSCs (Kohler et al., 2013).

CD44 is a cell-surface glycoprotein involved in cell adhesion and migration. As the expression of CD44 is implicated in poor tendon healing (Favata et al., 2006; Ansorge et al.,2009), the increased expression of CD44 in aged rat TDSCs might contribute to reduced tendon repair capacity with age (Zhou et al., 2010). Overexpression and knockdown experiments of CD44 in aged TDSCs would shed light on the aging mechanisms.

Collagen fibers are poorly organized in aged tendons (Gehwolf et al., 2016). This is partly due to chronic-inflammation and alteration in the production and activities of MMPs in aged tendons (Yu et al., 2013). The change of organization of collagen fibers from an intact, tightly-packed parallel orientation to a more random arrangement in aged tendons could affect the fate of resident tendon stem cells. Human fetal TDSCs seeded onto randomly-oriented fibrous scaffold underwent osteogenesis with lower levels of integrin α1, α5, and β1 subunits compared to cells seeded onto aligned fibers (Yin et al., 2010).

Locally Produced Biological Signals

Aging causes changes in the local production of biological signals at the stem cell niche which can profoundly impact tissue stem cell functions. Growth factors such as FGF, insulin growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), TGF-β1, platelet-derived growth factor (PDGF) and BMPs, as well as proteins in the Wnt/β-catenin, notch, and angiopoietin-1 signaling pathways are important modulators of stem cell functions. The Wnt/β-catenin and BMP signaling pathways enhanced non-tenogenic differentiation of TDSCs *in vitro* (Lui et al., 2013; Rui et al., 2013). Dysregulation of Wnt/β-catenin signaling pathway was observed in different aged stem cell and animal models (Brack et al., 2007; Liu et al., 2007; Gehwolf et al., 2016). However, there was no differences in mRNA expression of connective tissue growth factor (CTGF), TGF-β1, or stromal cell-derived factor 1 in tendons of old and young rats (Kostrominova and Brooks, 2013). The mRNA and protein expression of TGF-β1 also did not change with age in rat Achilles tenocytes (Yu et al., 2013). There was disruption of circadian control in aged mouse tendon (Yeung et al., 2014). One of the circadian genes, Gremlin-2 (Grem2) oscillated antiphase to BMP signaling and inhibited BMP-2 induced osteogenic differentiation of human tenocytes (Yeung et al., 2014). The study reported reduced Grem2 expression, dysregulated BMP signaling, and spontaneous calcification in aged mouse tendon, suggesting that inhibition of Grem2 might be involved in the calcification of aged tendons (Yeung et al., 2014). The growth factor profile of aged tendons and their effects on functions of tendon stem cells should be examined. Modulation of these growth factor signaling pathways might shed light on strategies to prevent tendon aging and promote healing of aged tendons after injury.

Inflamm-Aging

Aging is associated with reduced ability of the body to modulate inflammation, resulting in chronic low-grade inflammation termed "inflamm-aging" (Franceschi et al., 2000). Aged mice were reported to show higher serum levels of inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) compared to young mice (Wahl et al., 2010). There were greater inflammatory changes with higher IL-1βand IL-6 levels in flexor digitorum tendons of aged rats performing high repetition low force handle-pulling task (HRLF) compared to tendons in young rats performing HRLF and aged rats not performing HRLF (Kietrys et al., 2012). More macrophages and CTGF-immunoreactive fibroblasts were observed in the peritendon of supraspinatus tendons in aged rats after HRLF (Kietrys et al., 2012). The greater chronic inflammation in aged rat tendons after upper extremity overuse was associated with a decrease in forelimb agility and lack of improvement of success in reaching task (Kietrys et al., 2012). Dakin et al. (2012) measured the level of prostaglandin E2 (PGE2) in injured equine flexor tendons and found that the level of PGE2 increased while the level of formyl peptide receptor 2 (FRP2)/ALX, a receptor responsible for suppressing inflammatory response, decreased during injury with increasing age of the horses. Uninjured tendon explants isolated from younger (<10 years) but not older horses (≥10 years) treated with IL-1β responded by increasing FRP2/ALX receptor (Dakin et al., 2012), suggesting that aged tendons have lower capacity to resolve inflammation. de Gonzalo-Calvo et al. (2010) measured the oxidative stress and inflammatory cytokines of three volunteer groups, consisting of normoxic middle-aged people, normoxic aged people older than 75 years, and hypoxic aged people older than 75 years. The results showed that the antioxidant defenses of older people with hypoxia was lower. Serum TNF-α was significantly increased in the aged population while IL-6 was highly elevated in the hypoxic elderly population (de Gonzalo-Calvo et al., 2010). Another recent clinical study on 831 subjects showed that circulating mitochondrial DNA (mtDNA) increased with age and significantly contributed to the maintenance of chronic, low-grade inflammation observed in the elderly subjects with an increase in serum TNF- α, IL-6, RANTES, and IL-1ra (Pinti et al., 2014). Moreover, mtDNA values of siblings were directly correlated, suggesting a role for familiar/genetic background in controlling the levels of circulating mtDNA (Pinti et al., 2014). *In vitro* stimulation of monocytes with mtDNA resulted in an increased production of TNF-α, supporting that mtDNA could modulate the production of proinflammatory cytokines (Pinti et al., 2014). These findings supported that aged individuals exhibited reduced capacity to resolve inflammation and aging might scramble tendon stem cell functions and promote the development of chronic tendinopathy through these pathways. Indeed, high concentration of PGE2 was reported to reduce the proliferation and enhance non-tenocyte differentiation of TDSCs (Zhang and Wang, 2014). IL-1β irreversibly inhibited tenogenic and

adipogenic differentiation as well as reversibly inhibited chondrogenic and osteogenic differentiation of TDSCs isolated from injured mouse Achilles tendons (Zhang et al., 2015). IL-6 was reported to stimulate rat Achilles TDSCs' proliferation and entry into cell cycle but inhibited the expression of tendon-related markers (Chen et al., 2018). Recent studies showed that senescent cells exhibited a robust increase in mRNA expression and secreted numerous bioactive factors, including degradative enzymes, inflammatory cytokines, and growth factors, which might drive stem cell dysfunction in aged stem cells through paracrine signaling (Coppe et al., 2010; Campisi et al., 2011). Whether this mechanism also accounts for age-related degenerative disorders of tendons and the factors secreted by senescent cells during tendon aging needs further research. The damage of DNA of stem cells and niche cells were hypothesized to be major contributors to inflamm-aging and the effect was propagated and amplified by macrophages (Bonafe et al., 2012). More researches are needed to verify this hypothesis in aged tendon stem cells.

SYSTEMIC CHANGES OF HORMONAL AND METABOLIC SIGNALS

Given the remarkable ability of stem cells to sense and respond to the external stimuli, it is not surprising that the age-related systemic changes t can also impact stem cells and their niches. Chronic conditions such as hormone deficiency, diabetes mellitus (DM) and hypertension are common in elderly. The systemic changes of hormonal and metabolic signals as well as drug used in the treatment of these chronic conditions can alter niche through circulation, causing stem cells to lose or deviate from their original functions.

DM has been reported to increase the prevalence/incidence of tendinopathy (Ranger et al., 2016; Lui, 2017). A recent meta-analysis of clinical studies provide strong evidence that diabetes is associated with higher risk of tendinopathy (Ranger et al., 2016), despite that there were inconsistent findings regarding the effects of DM on structural and biomechanical properties of tendon in animal models (Lui, 2017). Potential mechanisms of DM in causing and exacerbating tendinopathy by modifying stem cell functions *via* AGE formation, hyperglycemia, insulin deficiency/resistance, and adipokine dysregulation have been discussed in another review and will not be repeated (Lui, 2017).

In women, estrogen can enhance tendon collagen synthesis. Estrogen deficiency was reported to negatively affect tendon metabolism and healing. Tenocytes isolated from ovariectomised rats were reported to show lower proliferation rate, collagen type I, aggrecan, elastin, fibronectin content in the supernatant, and migration rate but higher apoptosis, VEGF and MMP-13 compared to young tenocytes (Aydin et al., 2013; Torricelli et al., 2013). Collagen content was lower in Achilles tendons after ovariectomy compared to control rats (Ramos et al., 2012). The Achilles tendons from ovariectomised rabbits also showed lower gene expressions of proteoglycans aggrecan, biglycan, decorin, and versican compared to tendons from intact normal rabbits (Huisman et al., 2014). However, there was no changes in the biomechanical properties of patellar tendon of monkeys after ovariectomy (Wentorf et al., 2006). Age-associated estrogen deficiency therefore can alter matrix composition of tendons and may influence the functions of tendon stem cells. Whether TDSCs are sensitive to estrogen is a first step to understand the influence of estrogen deficiency in age-associated loss or dysfunction of tendon stem cells.

POTENTIAL FUTURE RESEARCH DIRECTIONS

Research on Treatments to Reverse the Intrinsic Cellular Deficits and Aged Niche of Tendon Stem Cells

As a new cell type identified in tendon, more studies are needed to fill the gaps of the aging mechanisms of tendon stem cells. Interventions that can combat protein and DNA damages, cellular senescence, oxidative stress, chronic inflammation; and inhibit AGE formation or promote its removal may rejuvenate aged tendon stem cells. These are attractive anti-aging options to explore. The growth factor and cytokine profile of aged tendons and their effects on functions of tendon stem cells should be examined. Notably, medium of young TDSCs under hypoxic culture condition was reported to enhance the migration, proliferation and mRNA expression of tendon-related markers as well as reduced senescence of aged TDSCs (Jiang et al., 2014). Exposure of aged rat patellar tendon TDSCs to young decellularized tendon ECM enhanced their proliferation, tendon-related marker expression (*Scx*, *Tnmd*), and stem cell marker expression (Oct-4, SSEA-1) as well as reduced SA-β-gal activity (Jiang et al., 2018).

Research on the Effects and Mechanisms of Common Aging Conditions on Tendon Stem Cells and Tendon

The effects of common aging conditions particularly diabetes, hypertension and hormonal deficiency and the drugs used on the functions of tendon stem cells and tendon should be examined. The sensitivity of TDSCs to estrogen has not been explored and should be examined. Similarly, studies on the effects of hyperglycemia, AGE, insulin deficiency/resistance, and adipokine dysregulation in diabetes on functions of tendon stem cells are needed.

Research on the Effects and Mechanisms of Exercise on Tendon Stem Cells and Tendon

TDSCs are sensitive to mechanical load. High uniaxial cyclic tensile loading (8%) induced osteogenic differentiation of TDSCs while low uniaxial cyclic tensile loading (4%) promoted tenogenic differentiation of TDSCs (Zhang and Wang, 2010b; Rui et al., 2011; Wang et al., 2018). Modulating the mechanical load experienced by tendon stem cells therefore may counteract the effect of aging on tendon. In this regard, moderate mechanical stretching (4%) of aged TDSCs *in vitro* significantly increased the protein expression of nucleostemin and mRNA expression of *Nanog*. It also increased the expression of tenocyte-related genes (*Col1* and *Tnmd*) without increasing

the expression of non-tenocyte-related genes (*Lpl*, *Sox9* and *Runx2*) of aged TDSCs (Zhang and Wang, 2015).

In an *in vivo* study, moderate treadmill running of aged mice increased the proliferation rate of aged TDSCs in culture, decreased lipid deposition, proteoglycan accumulation and calcification, and increased the expression of nucleostemin-expressing TDSCs in the patellar tendons (Zhang and Wang, 2015). Moderate exercises can also mitigate the deleterious effects of aging on the niche of tendon stem cells by improving tendon microcirculation, matrix composition, and organization as well as reducing AGE level and matrix stiffness (Couppe et al., 2014; Wood and Brooks, 2016). With training, Col1a1 and Mmp8 expression in aged mouse Achilles tendons were restored to levels similar to adult controls (Wood and Brooks, 2016). Moderate treadmill running prior to an injury augmented tendon wound healing in an aged rat model with reduced vascularity, lipid-like structures, and senescent cells as well as increased production of more collagen fibrils and better fibril organization (Zhang et al., 2016). The effect was likely mediated by the improvement of metabolism and functional fitness of aged tendon stem cells because aged TDSCs isolated from exercised rats showed higher stem cell marker expression, proliferation, colony-forming ability, and tenocyte-related gene expression as well as lower non-tenocyte-related gene expression compared to aged cells isolated from cage control rats (Zhang et al., 2016). The beneficial effect of exercise on aged tendons could also be due to systemic effects in addition to direct loading as tail tendons from aged and exercised rats assumed the elastic characteristics of their younger counterparts after exercise (Lacroix et al., 2013). Stem cells expressed different channels that could sense membrane tension (Soria et al., 2013). One study showed that the stretch-mediated activation channel, cystic fibrosis transmembrane conductance regulator (CFTR), was up-regulated in TDSCs during tenogenic differentiation under mechanical stretching (Liu et al., 2017). Further studies to confirm the beneficial effects, optimal loading protocol, and mechanosensing mechanisms of excise on aged tendon stem cells and tendon should be done.

CONCLUSION AND FUTURE PERSPECTIVES

In summary, the composition, structure, and mechanical properties of tendons deteriorate with aging and are driven at least in part by an age-associated decline in the metabolism and functional fitness of tendon stem cells. Unlike their young and healthy counterparts, aged TDSCs were shown to be larger and flatten. The number and stemness of TDSCs isolated from aged tendons were lower. They exhibited altered multi-lineage differentiation potential, deficiency in cell adhesion, and migration as well as early cellular senescence. The intrinsic cellular deficits, aged niche, and systemic hormonal and metabolic changes associated with aging can drive the aging process of stem cells, some of which have been demonstrated in TDSCs recently. There is no evidence about the factor(s)

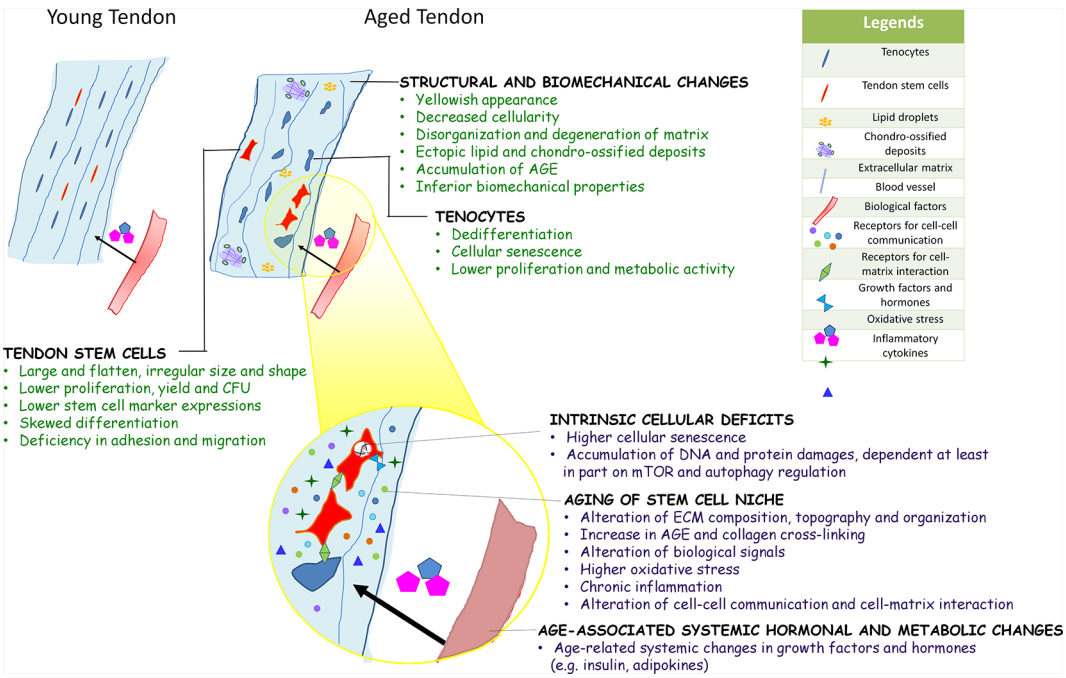

FIGURE 1 | Diagram summarizing the key biological characteristics of aged tendon, and potential factors influencing the fate and functions of TDSCs during tendon aging. Current evidences on aging of other adult stem cells and limited data on tendon-derived stem cells (TDSCs) suggested that aging of tendon stem cell likely occurs *via* intrinsic cellular deficits, aging of stem cell niche, and age-associated systemic hormonal and metabolic changes. These factors interact with each other to affect stem cell function at multiple levels.

triggering the aging of tendon stem cells. The authors speculate that they may work together in inducing functional deficits of tendon stem cells as aging is a continued process. **Figure 1** summarizes the key biological characteristics of aged tendon, and potential factors influencing the fate and functions of TDSCs during tendon aging. Uncovering the mechanisms underpinning the regenerative decline of metabolism and functions of aged tendon stem cells would have profound implications for the development of stem cell-based therapeutics to reset the aging clock of tendons.

AUTHOR CONTRIBUTIONS

PL conceived the idea, wrote, and proof-read the manuscript. CW wrote and proof-read the manuscript.

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REFERENCES

Ackerman, J. E., Bah, I., Jonason, J. H., Buckley, M. R., and Loiselle, A. E. (2017). Aging does not alter tendon mechanical properties during homeostasis, but does impair flexor tendon healing. *J. Orthop. Res.* 35 (12), 2716–2724. doi: 10.1002/jor.23580

Albers, I. S., Zwerver, J., Diercks, R. L., Dekker, J. H., and Van den Akker-Scheek, I. (2016). Incidence and prevalence of lower extremity tendinopathy in a Dutch general practice population: a cross-sectional study. *BMC Musculoskelet Disord.* 17, 16. doi: 10.1186/s12891-016-0885-2

Alberton, P., Dex, S., Popov, C., Shukunami, C., Schieker, M., and Docheva, D. (2015). Loss of tenomodulin results in reduced self-renewal and augmented senescence of tendon stem/progenitor cells. *Stem Cells Dev.* 24 (5), 597–609. doi: 10.1089/scd.20140314

Andreassen, T. T., Seyerhansen, K., and Bailey, A. J. (1981). Thermal-stability, mechanical-properties and reducible cross-links of rat tail tendon in experimental diabetes. *Biochim. Biophys. Acta* 677 (2), 313–317. doi: 10.1016/0304-4165(81)90101-X

Ansorge, H. L., Beredjiklian, P. K., and Soslowsky, L. J. (2009). CD44 deficiency improves healing tendon mechanics and increases matrix and cytokine expression in a mouse patellar tendon injury model. *J. Orthop. Res.* 27 (10), 1386–1391. doi: 10.1002/jor.20891

Arthur, A., Zannettino, A., Panagopoulos, R., Koblar, S. A., Sims, N. A., Stylianou, C., et al. (2011). EphB/ephrin-B interactions mediate human MSC attachment, migration and osteochondral differentiation. *Bone* 48 (3), 533–542. doi: 10.1016/j.bone.2010.10.180

Aydin, A., Kenar, H., Atmaca, H., Alici, T., Gacar, G., Muezzinoglu, U. S., et al. (2013). The short- and long- term effects of estrogen deficiency on apoptosis in musculoskeletal tissues: an experimental animal model study. *Arch. Iran Med.* 16 (5), 271–276. doi: 013165/AIM.006

Bi, Y., Ehirchiou, D., Kilts, T. M., Inkson, C. A., Embree, M. C., Sonoyama, W., et al. (2007). Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat. Med.* 13 (10), 1219–1227. doi: 10.1038/nm1630

Bonafe, M., Storci, G., and Franceschi, C. (2012). Inflamm-aging of the stem cell niche: breast cancer as a paradigmatic example: breakdown of the multi-shell cytokine network fuels cancer in aged people. *Bioessays* 34 (1), 40–49. doi: 10.1002/bies.201100104

Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., et al. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science* 317 (5839), 807–810. doi: 10.1126/science.1144090

Brewer, B. J. (1979). Aging of the rotator cuff. *Am. J. Sports Med.* 7 (2), 102–110. doi: 10.1177/036354657900700206

Campisi, J., Andersen, J. K., Kapahi, P., and Melov, S. (2011). Cellular senescence: a link between cancer and age-related degenerative disease? *Seminar Cancer Biol.* 21 (6), 354–359. doi: 10.1016/j.semcancer.2011.09.001

Chen, L., Liu, J., Tao, Z., Wang, G., Wang, Q., and Liu, X. (2015a). The role of Pin1 protein in aging of human tendon stem/progenitor cells. *Biochem. Biophys. Res. Commun.* 464 (2), 487–492. doi: 10.1016/j.bbrc.2015.06.163

Chen, L., Wang, G. D., Liu, J. P., Wang, H. S., Liu, X. M., Wang, Q., et al. (2015b). miR-135a modulates tendon stem/progenitor cell senescence *via* suppressing ROCK1. *Bone* 71, 210–216. doi: 10.1016/j.bone.2014.11.001

Chen, H., Ge, H. A., Wu, G. B., Cheng, B., Lu, Y., and Jiang, C. (2016). Autophagy prevents oxidative stress-induced loss of self-renewal capacity and stemness in human tendon stem cells by reducing ROS accumulation. *Cell Physiol. Biochem.* 39 (6), 2227–2238. doi: 10.1159/000447916

Chen, S., Deng, G., Li, K., Zheng, H., Wang, G., Yu, B., et al. (2018). Interleukin-6 promotes proliferation but inhibits tenogenic differentiation *via the* Janus Kinase/Signal transducers and activators of transcription 3 (JAK/STAT3) pathway in tendon-derived stem cells. *Med. Sci. Monit.* 24, 1567–1573. doi: 10.12659/MSM.908802

Cheng, Y., Wang, B., Zhou, H., Dang, S., Jin, M., Shi, Y., et al. (2015). Autophagy is required for the maintenance of liver progenitor cell functionality. *Cell Physiol. Biochem.* 36 (3), 1163–1174. doi: 10.1159/000430287

Collado, M., Blasco, M. A., and Serrano, M. (2007). Cellular senescence in cancer and aging. *Cell* 130 (2), 223–233. doi: 10.1016/j.cell.2007.07.003

Conboy, I. M., Conboy, M. J., Wagers, A. J., Girma, E. R., Weissman, I. L., and Rando, T. A. (2005). Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* 433 (7027), 760–764. doi: 10.1038/nature03260

Coppe, J. P., Desprez, P. Y., Krtolica, A., and Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu. Rev. Pathol.* 5, 99–118. doi: 10.1146/annurev-pathol-121808-102144

Couppe, C., Hansen, P., Kongsgaard, M., Kovanen, V., Suetta, C., Aagaard, P., et al. (2009). Mechanical properties and collagen cross-linking of the patellar tendon in old and young men. *J. Appl. Physiol.* (1985), 880–886. doi: 10.1152/japplphysiol.002912009

Couppe, C., Svensson, R. B., Grosset, J. F., Kovanen, V., Nielsen, R. H., Olsen, M. R., et al. (2014). Life-long endurance running is associated with reduced glycation and mechanical stress in connective tissue. *Age (Dordr)* 36 (4), 9665. doi: 10.1007/s11357-014-9665-9

Dakin, S. G., Dudhia, J., Werling, N. J., Werling, D., Abayasekara, D. R., and Smith, R. K. (2012). Inflamm-aging and arachidonic acid metabolite difference with stage of tendon disease. *PloS One* 7 (11), e48978. doi: 10.1371/journal.pone.0048978

de Gonzalo-Calvo, D., Neitzert, K., Fernandez, M., Vega-Naredo, I., Caballero, B., Garcia-Macia, M., et al. (2010). Differential inflammatory responses in aging and disease: TNF-alpha and IL-6 as possible biomarkers. *Free Radic. Biol. Med.* 49 (5), 733–737. doi: 10.1016/j.freeradbiomed.2010.05.019

de Jonge, S., van den Berg, C., de Vos, R. J., van der Heide, H. J., Weir, A., Verhaar, J. A., et al. (2011). Incidence of midportion Achilles tendinopathy in the general population. *Br. J. Sports Med.* 45 (13), 1026–1028. doi: 10.1136/bjsports-2011-090342

Delabastita, T., Bogaerts, S., and Vanwanseele, B. (2018). Age-related changes in Achilles tendon stiffness and impact of functional activities: a systematic review and meta-analysis. *J. Aging Phys. Act.* 27 (1), 116–127. doi: 10.1123/japa.2017-0359

Driver, J. A., and Lu, K. P. (2010). Pin1: a new genetic link between Alzheimer's disease, cancer and aging. *Curr. Aging Sci.* 3 (3), 158–165. doi: 10.2174/1874609811003030158

Dudhia, J., Scott, C. M., Draper, E. R., Heinegard, D., Pitsillides, A. A., and Smith, R. K. (2007). Aging enhances a mechanically-induced reduction in tendon strength by an active process involving matrix metalloproteinase activity. *Aging Cell.* 6 (4), 547–556. doi: 10.1111/j.1474-9726.2007.00307.x

Dunkman, A. A., Buckley, M. R., Mienaltowski, M. J., Adams, S. M., Thomas, S. J., Satchell, L., et al. (2013). Decorin expression is important for age-related

changes in tendon structure and mechanical properties. *Matrix Biol.* 32 (1), 3–13. doi: 10.1016/j.matbio.2012.11.005

Ellis, S. J., and Tanentzapf, G. (2010). Integrin-mediated adhesion and stem-cell-niche interactions. *Cell Tissue Res.* 339 (1), 121–130. doi: 10.1007/s00441-009-0828-4

Engler, A. J., Sen, S., Sweeney, H. L., and Sischer, D. E. (2006). Matrix elasticity directs stem cell lineage specification. *Cell* 126 (4), 677–689. doi: 10.1016/j.cell.2006.06.044

Favata, M., Beredjiklian, P. K., Zgonis, M. H., Beason, D. P., Crombleholme, T. M., Jawad, A. F., et al. (2006). Regenerative properties of fetal sheep tendon are not adversely affected by transplantation into an adult environment. *J. Orthop. Res.* 24 (11), 2124–2132. doi: 10.1002/jor.20271

Fehringer, E. V., Sun, J., VanOeveren, L. S., Keller, B. K., and Matsen, F. A. (2008). Full-thickness rotator cuff tear prevalence and correlation with function and co-morbidities in patients sixty-five years and older. *J. Shoulder Elbow Surg.* 17 (6), 881–885. doi: 10.1016/j.jse.2008.05.039

Franceschi, C., Bonafè, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., et al. (2000). Inflamm-aging: an evolutionary perspective on immunosenescence. *Ann. N. Y. Acad. Sci.* 908, 244–254. doi: 10.1111/j.1749-6632.2000.tb06651.x

Fukada, S., Ma, Y., and Uezumi, A. (2014). Adult stem cell and mesenchymal progenitor theories of aging. *Front. Cell. Dev. Biol.* 2, 10. doi: 10.3389/fcell.2014.00010

Funakoshi, T., Iwasaki, N., Kamishima, T., Nishida, M., Ito, Y., Kondo, M., et al. (2010). *In vivo* visualization of vascular patterns of rotator cuff tears using contrast-enhanced ultrasound. *Am. J. Sports Med.* 38 (12), 2464–2471. doi: 10.1177/0363546510375536

Gautieri, A., Passini, F. S., Silvan, U., Guizar-Sicairos, M., Carimati, G., Volpi, P., et al. (2017). Advanced glycation end-products: mechanics of aged collagen from molecule to tissue. *Matrix Biol.* 59, 95–108. doi: 10.1016/j.matbio.2016.09.001

Gehwolf, R., Wagner, A., Lehner, C., Bradshaw, A. D., Scharler, C., Niestrawska, J. A., et al. (2016). Pleiotropic roles of the matricellular protein Sparc in tendon maturation and ageing. *Sci. Rep.* 6, 32635. doi: 10.1038/srep32635

Gillis, C., Pool, R. R., Meagher, D. M., Stover, S. M., Reiser, K., and Willits, N. (1997). Effect of maturation and aging on the histomorphometric and biochemical characteristics of equine superficial digital flexor tendon. *Am. J. Vet. Res.* 58 (4), 425–430.

Goichberg, P., Kannappan, R., Cimini, M., Bai, Y., Sanada, F., Sorrentino, A., et al. (2013). Age-associated defects in EphA2 signaling impair the migration of human cardiac progenitor cells. *Circulation* 128 (20), 2211–2223. doi: 10.1161/CIRCULATIONAHA.113.004698

Han, W., Wang, B., Liu, J., and Chen, L. (2017). The p16/miR-217/EGR1 pathway modulates age-related tenogenic differentiation in tendon stem/progenitor cells. *Acta Biochim. Biophys. Sin. (Shanghai).* 49 (11), 1015–1021. doi: 10.1093/abbs/gmx104

Haut, R. C., Lancaster, R. L., and DeCamp, C. E. (1992). Mechanical properties of the canine patellar tendon: some correlations with age and the content of collagen. *J. Biomech.* 25 (2), 163–173. doi: 10.1016/0021-9290(92)90273-4

Hoare, M., Das, T., and Alexander, G. (2010). Ageing, telomeres, senescence, and liver injury. *J. Hepatol.* 53 (5), 950–961. doi: 10.1016/j.jhep.2010.06.009

Hsiao, M. Y., Chen, Y. C., Lin, C. Y., Chen, W. S., and Wang, T. G. (2015). Reduced patellar tendon elasticity with aging: *In vivo* assessment by shear wave elastography. *Ultrasound Med. Biol.* 41 (11), 2899–2905. doi: 10.1016/j.ultrasmedbio.2015.07.008

Hu, C., Zhang, Y., Tang, K., Luo, Y., Liu, Y., and Chen, W. (2017). Downregulation of CITED2 contributes to TGFβ-mediated senescence of tendon-derived stem cells. *Cell Tissue Res.* 368 (1), 93–104. doi: 10.1007/s00441-016-2552-1

Huisman, E. S., Andersson, G., Scott, A., Reno, C. R., Hart, D. A., and Thomton, G. M. (2014). Regional molecular and cellular differences in the female rabbit Achilles tendon complex: potential implications for understanding responses to loading. *J. Anat.* 224 (5), 528–547. doi: 10.1111/joa.12169

Iagnocco, A., Filippucci, E., Sakellariou, G., Ceccarelli, F., Di Geso, L., Carili, L., et al. (2013). Ultrasound imaging for the rheumatologist XLIV. Ultrasound of the shoulder in healthy individuals. *Clin. Exp. Rheumatol.* 31 (2), 165–171.

Ippolito, E., Natali, P. G., Postacchini, F., Accinni, L., and De Martino, C. (1980). Morphological, immunochemical and biochemical study of rabbit Achilles tendon at various ages. *JBJS* 62 (4), 583–598.

Janzen, V., Forkert, R., Fleming, H. E., Saito, Y., Waring, M. T., Dombkowski, D. M., et al. (2006). Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* 443 (7110), 421–426. doi: 10.1038/nature05159

Jiang, D., Jiang, Z., Zhang, Y., Wang, S., Yang, S., Xu, B., et al. (2014). Effect of young extrinsic environment stimulated by hypoxia on the function of aged tendon stem cell. *Cell Biochem. Biophys.* 70 (2), 967–973. doi: 10.1007/s12013-014-0004-7

Jiang, D., Xu, B., and Gao, P. (2018). Effects of young extracellular matrix on the biological characteristics of aged tendon stem cells. *Adv. Clin. Exp. Med.* 27 (12), 625–1630. doi: 10.17219/acem/75503

Josa, L. G., and Kannus, P. (1997). *Human Tendons: Anatomy, Physiology and Pathology* (Champaign, IL: Human Kinetics Publishers).

Kiderlen, S., Polzer, C., Radler, J. O., Docheva, D., Clausen-Schaumann, H., and Sudhop, S. (2019). Age related changes in cell stiffness of tendon stem/progenitor cells an rejuvenating effect of ROCK-inhibition. *Biochem. Biophys. Res. Commun.* 509 (3), 839–844. doi: 10.1016/j.bbrc.2019.01.027

Kietrys, D. M., Barr-Gillespie, A. E., Amin, M., Wade, C. K., Popoff, S. N., and Barbe, M. F. (2012). Aging contributes to inflammation in upper extremity tendons and declines in forelimb agility in a rat model of upper extremity overuse. *PloS One* 7 (10), e46954. doi: 10.1371/journal.pone.0046954

Klatte-Schulz, F., Pauly, S., Scheibel, M., Greiner, S., Gerhardt, C., Schmidmaier, G., et al. (2012). Influence of age on the cell biological characteristics and the stimulation potential of male human tenocyte-like cells. *Eur. Cell Mater* 24, 74–89. doi: 10.22203/eCM.v024a06

Kohler, J., Popov, C., Klotz, B., Alberton, P., Prall, W. C., Haasters, F., et al. (2013). Uncovering the cellular and molecular changes in tendon stem/progenitor cells attributed to tendon aging and degeneration. *Aging Cell* 12 (6), 988–999. doi: 10.1111/acel.12124

Kostrominova, T., and Brooks, S. (2013). Age related changes in structure and extracellular matrix protein expression levels in rat tendons. *Age* 35 (6), 2203–2214. doi: 10.1007/s11357-013-9514-2

Lacroix, A. S., Duenwald-Kuehl, S. E., Brickson, S., Akins, T. L., Diffee, G., Aiken, J., et al. (2013). Effect of age and exercise on the viscoelastic properties of rat tail tendon. *Ann. Biomed. Eng.* 41 (6), 1120–1128. doi: 10.1007/s10439-013-0796-4

Landi, A., Elves, M., and Piaggi, W. (1983). The blood flow of rabbits' tendons. *Acta Orthop. Scand.* 54 (6), 832–835. doi: 10.3109/17453678308992917

Lee, J., Abdeen, A. A., Huang, T. H., and Kilian, K. A. (2014). Controlling cell geometry on substrates of variables stiffness can tune the degree of osteogenesis in human mesenchymal stem cells. *J. Mech. Behav. Biomed. Mater.* 38, 209–218. doi: 10.1016/j.jmbbm.2014.01.009

Li, J., and Johnson, S. E. (2013). Ephrin-A5 promotes bovine muscle progenitor cell migration before mitotic activation. *J. Anim. Sci.* 91 (3), 1086–1093. doi: 10.2527/jas.2012-5728

Li, H., Liu, P., Xu, S., Li, Y., Dekker, J. D., Li, B., et al. (2017). FOXP1 controls mesenchymal stem cell commitment and senescence during skeletal aging. *J. Clin. Invest.* 127 (4), 1241–1253. doi: 10.1172/JCI89511

Liu, H., Fergusson, M. M., Castilho, R. M., Liu, J., Cao, L., Chen, J., et al. (2007). Augmented Wnt signaling in a mammalian model of accelerated aging. *Science* 317 (5839), 803–806. doi: 10.1126/science.1143578

Liu, Y., Xu, J., Xu, L., Wu, T., Sun, Y., Lee, Y. W., et al. (2017). Cystic fibrosis transmembrane conductance regulator mediates tenogenic differentiation of tendon-derived stem cells and tendon repair: accelerating tendon injury healing by intervening in its downstream signaling. *FASEB J.* 31 (9), 3800–3815. doi: 10.1096/fj.201601181R

Lui, P. P., and Chan, K. M. (2011). Tendon-derived stem cells (TDSC): from basic science to potential roles in tendon pathology and tissue engineering applications. *Stem Cell Rev.* 7 (4), 883–897. doi: 10.1007/s12015-011-9276-0

Lui, P. P., Lee, Y. W., Wong, Y. M., Zhang, X., Dai, K., and Rolf, C. G. (2013). Expression of Wnt pathway mediators in metaplasic tissue in animal model and clinical samples of tendinopathy. *Rheumatol. (Oxford)* 52 (9), 1609–1618. doi: 10.1093/rheumatology/ket214

Lui, P. P. (2015). Markers for the identification of tendon-derived stem cells *in vitro* and tendon stem cells *in situ* – update and future development. *Stem Cell Res. Ther.* 6, 106. doi: 10.1186/s13287-015-0097-y

Lui, P. P. (2017). Tendinopathy in diabetes patients – epidemiology, pathogenesis and management. *Scand. J. Med. Sci. Sports* 27 (8), 776–787. doi: 10.1111/sms.12824

Molofsky, A. V., Slutsky, S. G., Joseph, N. M., He, S., Pardal, R., Krishnamurthy, J., et al. (2006). Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* 443 (7110), 448–452. doi: 10.1038/nature05091

Nam, J., Johnson, J., Lannutti, J. J., and Agarwal, S. (2011). Modulation of embryonic mesenchymal progenitor cell differentiation *via* control over pure mechanical modulus in electrospun nanofibers. *Acta Biomater.* 7 (4), 1516–1524. doi: 10.1016/j.actbio.2010.11.022

Navaro, Y., Bleich-Kimelman, N., Hazanov, L., Mironi-Harpaz, I., Shachaf, Y., Garty, S., et al. (2015). Matrix stiffness determines the fate of nucleus pulposus-derived stem cells. *Biomaterials* 49, 68–76. doi: 10.1016/j.biomaterials.2015.01.021

Pardes, A. M., Beach, Z. M., Raja, H., Rodriguez, A. B., Freedman, B. R., and Soslowsky, L. J. (2017). Aging leads to inferior Achilles tendon mechanics and altered ankle function in rodents. *J. Biomech.* 60, 30–38. doi: 10.1016/j.jbiomech.2017.06.008

Peffers, M. J., Thorpe, C. T., Collins, J. A., Eong, R., Wei, T. K. J., Screen, H. R. C., et al. (2014). Proteomic analysis reveals age-related changes in tendon matrix composition, with age- and injury- specific matrix fragmentation. *J. Biol. Chem.* 289 (37), 25867–25878. doi: 10.1074/jbc.M114.566554

Pinti, M., Cevenini, E., Nasi, M., De Biasi, S., Salvioli, S., Monti, D., et al. (2014). Circulating mitochondrial DNA increases with age and is a familiar trait: Implications for inflamm-aging. *Eur. J. Immunol.* 44 (5), 1552–1562. doi: 10.1002/eji.201343921

Piwko-Czuchra, A., Koegel, H., Meyer, H., Bauer, M., Werner, S., Brakebusch, C., et al. (2009). B1 integrin-mediated adhesion signaling is essential for epidermal progenitor cell expansion. *PloS One* 4 (5), e5488. doi: 10.1371/journal.pone.0005488

Popov, C., Kohler, J., and Docheva, D. (2016). Activation of EphA4 and EphB2 reverse signaling restores the age-associated reduction of self-renewal, migration, and actin turnover in human tendon stem/progenitor cells. *Front. Aging Neurosci.* 7, 246. doi: 10.3389/fnagi.2015.00246

Ramos, J. E., Al-Nakkash, L., Peterson, A., Gump, B. S., Janjulia, T., Moore, M. S., et al. (2012). The soy isoflavone genistein inhibits the reduction in Achilles tendon collagen content induced by ovariectomy in rats. *Scand. J. Med. Sci. Sports* 22 (5), e108–e114. doi: 10.1111/j.1600-0838.2012.01516.x

Ranger, T. A., Wong, A. M., Cook, J. L., and Gaida, J. E. (2016). Is there an association between tendinopathy and diabetes mellitus? *systematic Rev. meta-analysis. Br. J. Sports Med.* 50 (16), 982–989. doi: 10.1136/bjsports-2015-094735

Reddy, G. K. (2004). Cross-linking in collagen by nonenzymatic glycation increases the matrix stiffness in rabbit achilles tendon. *Exp. Diabetes Res.* 5 (2), 143–153. doi: 10.1080/15438600490277860

Reigle, K. L., Di Lullo, G., Turner, K. R., Last, J. A., Chervoneva, I., Birk, D. E., et al. (2008). Non-enzymatic glycation of type I collagen diminishes collagen-proteoglycan binding and weakens cell adhesion. *J. Cell Biochem.* 104 (5), 1684–1698. doi: 10.1002/jcb.21735

Riley, G. P., Harrall, R. L., Constnt, C. R., Chard, M. D., Cawston, T. E., and Hazleman, B. L. (1994). Glycosaminoglycans of human rotator cuff tendons: changes with age and in chronic rotator cuff tendinitis. *Ann. Rheumatol. Dis.* 53 (6), 367–376. doi: 10.1136/ard.53.6.367

Rosenthal, A. K., Gohr, C. M., Mitton, E., Monnier, V., and Burner, T. (2009). Advanced glycation end products increase transglutaminase activity in primary porcine tenocytes. *J. Investig. Med.* 57 (2), 460–466. doi: 10.2310/JIM.0b013e3181954ac6

Rubinsztein, D. C., Marino, G., and Kroemer, G. (2011). Autophagy and aging. *Cell* 146 (5), 682–695. doi: 10.1016/j.cell.2011.07.030

Ruckh, J. M., Zhao, J. W., Shadrach, J. L., van Wijngaarden, P., Rao, T. N., Wagers, A. J., et al. (2012). Rejuvenation of regeneration in the aging central nervous system. *Cell Stem Cell.* 10 (1), 96–103. doi: 10.1016/j.stem.2011.11.019

Rudzki, J. R., Adler, R. S., Warren, R. F., Kadrmas, W. R., Verma, N., Pearle, A. D., et al. (2008). Contrast-enhanced ultrasound characterization of the vascularity of the rotator cuff tendon: age- and activity-related changes in the intact asymptomatic rotator cuff. *J. Shoulder Elbow Surg.* 17 (1 suppl), 96S–100S. doi: 10.1016/j.jse.2007.07.004

Rui, Y. F., Lui, P. P., Li, G., Fu, S. C., Lee, Y. W., and Chan, K. M. (2010). Isolation and characterization of multipotent rat tendon-derived stem cells. *Tissue Eng. Part A.* 16 (5), 1549–1558. doi: 10.1089/ten.TEA.20090529

Rui, Y. F., Lui, P. P., Ni, M., Chan, L. S., Lee, Y. W., and Chan, K. M. (2011). Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells. *J. Orthop. Res.* 29 (3), 390–396. doi: 10.1002/jor.21218

Rui, Y. F., Lui, P. P., Wong, Y. M., Tan, Q., and Chan, K. M. (2013). BMP-2 stimulated non-tenogenic differentiation and promoted proteoglycan deposition of tendon-derived stem cells (TDSCs) *in vitro*. *J. Orthop. Res.* 31 (5), 746–753. doi: 10.1002/jor.22290

Ruzzini, L., Abbruzzese, F., Rainer, A., Longo, U., Trombetta, M., Maffulli, N., et al. (2014). Characterization of age-related changes of tendon stem cells from adult human tendons. *Knee Surg. Sports Traumatol. Arthrosc.* 22 (11), 2856–2866. doi: 10.1007/s00167-013-2457-4

Salini, V., Vanni, D., Pantalone, A., and Abate, M. (2015). Platelet rich plasma therapy in non-insertional achilles tendinopathy: the efficacy is reduced in 60-years old people compared to young and middle-age individuals. *Front. Aging Neurosci.* 7, 228. doi: 10.3389/fnagi.2015.00228

Sato, T., Katagiri, K., Yokonishi, T., Kubota, Y., Inoue, K., Ogonuki, N., et al. (2011). *In vitro* production of fertile sperm from murine spermatogonial stem cell lines. *Nat. Commun.* 2, 472. doi: 10.1038/ncomms1478

Sbrana, F. V., Cortini, M., Avnet, S., Perut, F., Columbaro, M., De Milito, A., et al. (2016). The role of autophagy in the maintenance of stemness and differentiation of mesenchymal stem cells. *Stem Cell Rev.* 12 (6), 621–633. doi: 10.1007/s12015-016-9690-4

Schmitz, A. A., Govek, E. E., Bottner, B., and Van Aelst, L. (2000). Rho GTPases: signaling, migration, and invasion. *Exp. Cell Res.* 261 (1), 1–12. doi: 10.1006/excr.20005049

Sharpless, N. E., and DePinho, R. A. (2007). How stem cells age and why this makes us grow old. *Nat. Rev. Mol. Cell. Biol.* 8 (9), 703–713. doi: 10.1038/nrm2241

Shibata, K. R., Aoyama, T., Shima, Y., Fukiage, K., Otsuka, S., Furu, M., et al. (2007). Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during *in vitro* expansion. *Stem Cells* 25 (9), 2371–2382. doi: 10.1634/stemcells.2007-0225

Soria, B., Navas, S., Hmadcha, A., and Hamill, O. P. (2013). Single mechanosensitive and Ca2+- sensitive channel currents recorded from mouse and human embryonic stem cells. *J. Membr. Biol.* 246 (3), 215–230. doi: 10.1007/s00232-012-9523-6

Sousa-Victor, P., Gutarra, S., Garcia-Prat, L., Rodriguez-Ubreva, J., Ortet, L., Ruiz-Bonilla, V., et al. (2014). Geriatric muscle stem cells switch reversible quiescence into senescence. *Nature* 506 (7488), 316–321. doi: 10.1038/nature13013

Tan, Q., Lui, P. P., Rui, Y. F., and Wong, Y. M. (2012). Comparison of potentials of stem cells isolated from tendon and bone marrow for musculoskeletal tissue engineering. *Tissue Eng. Part A.* 18 (7-8), 840–851. doi: 10.1089/ten.TEA.2011.0362

Teunis, T., Lubberts, B., Reilly, B. T., and Rin, D. (2014). A systematic review and pooled analysis of the prevalence of rotator cuff disease with increasing age. *J. Shoulder Elbow Surg.* 23 (12), 1913–1921. doi: 10.1016/j.jse.2014.08.001

Thorpe, C. T., McDermott, B. T., Goodship, A. E., Clegg, P. D., and Birch, H. L. (2016). Ageing does not result in a decline in cell synthetic activity in an injury prone tendon. *Scand. J. Med. Sci. Sports.* 26 (6), 684–693. doi: 10.1111/sms.12500

Tomaru, U., Takahashi, S., Ishizu, A., Miyatake, Y., Gohda, A., Suzuki, S., et al. (2012). Decreased proteasomal activity causes age-related phenotypes and promotes the development of metabolic abnormalities. *Am. J. Pathol.* 180 (3), 963–972. doi: 10.1016/j.ajpath.2011.11.012

Torricelli, P., Veronesi, F., Pagani, S., Maffulli, N., Masiero, S., Frizziero, A., et al. (2013). *In vitro* tenocyte metabolism in aging and oestrogen deficiency. *Age (Dordr)* 35 (6), 2125–2136. doi: 10.1007/s11357-012-9500-0

Tsai, W. C., Chang, H. N., Yu, T. Y., Chien, C. H., Fu, L. F., Liang, F. C., et al. (2011). Decreased proliferation of aging tenocytes is associated with down-regulation of cellular senescence-inhibited gene and up-regulation of p27. *J. Orthop. Res.* 29 (10), 1598–1603. doi: 10.1002/jor.21418

Turan, A., Teber, M. A., Yakut, Z. I., Unlu, H. A., and Hekimoglu, B. (2015). Sonoelastographic assessment of the age-related changes of the Achilles tendon. *Med. Ultrason.* 17 (1), 58–61. doi: 10.11152/mu.2013.2066.171.ayt

Wahl, E. C., Aronson, J., Liu, L., Fowlkes, J. L., Thrailkill, K. M., Bunn, R. C., et al. (2010). Restoration of regenerative osteoblastogenesis in aged mice:

modulation of TNF. *J. Bone Miner. Res.* 5 (1), 114–123. doi: 10.1359/jbmr.090708

Wang, Z., Jiang, Y., Liu, N., Ren, L., Zhu, Y., An, Y., et al. (2012). Advanced glycation end-product Nε-carboxymethyl-Lysine accelerates progression of artherosclerotic calcification in diabetes. *Atherosclerosis* 221 (2), 387–396. doi: 10.1016/j.atherosclerosis.2012.01.019

Wang, B., Guo, J., Feng, L., Suen, C. W., Fu, W. M., Zhang, J. F., et al. (2016). miR124 suppresses collagen formation of human tendon derived stem cells through targeting egr1. *Exp. Cell Res.* 347 (2), 360–366. doi: 10.1016/j.yexcr.2016.08.018

Wang, T., Thien, C., Wang, C., Ni, M., Gao, J., Wang, A., et al. (2018). 3D uniaxial mechanical stimulation induces tenogenic differentiation of tendon-derived stem cells through a PI3K/AKT signaling pathway. *FASEB J.* 32 (9), 4804–4814. doi: 10.1096/fj.201701384R

Warr, M. R., Binnewies, M., Flach, J., Reynaud, D., Garg, T., Malhotra, R., et al. (2013). FOXO3A directs a protective autophagy program in haematopoietic stem cells. *Nature* 494 (7437), 323–327. doi: 10.1038/nature11895

Webb, S., Gabrelow, C., Pierce, J., Gibb, E., and Elliott, J. (2016). Retinoic acid receptor signaling preserves tendon stem cell characteristics and prevents spontaneous differentiation *in vitro*. *Stem Cell Res Ther* 7, 45. doi: 10.1186/s13287-016-0306-3

Wentorf, F. A., Sudoh, K., Moses, C., Arendt, E. A., and Carlson, C. S. (2006). The effects of estrogen on material and mechanical properties of the intra- and extra- articular knee structures. *Am. J. Sports Med.* 34 (12), 1948–1952. doi: 10.1177/0363546506290060

Wilkinson, J. E., Burmeister, L., Brooks, S. V., Chan, C. C., Friedline, S., Harrison, D. E., et al. (2012). Rapamycin slows aging in mice. *Aging Cell* 11 (4), 675–682. doi: 10.1111/j.1474-9726.2012.00832.x

Wood, L. K., and Brooks, S. V. (2016). Ten weeks of treadmill running decreases stiffness and increases collagen turnover in tendons of old mice. *J. Orthop. Res.* 34 (2), 346–353. doi: 10.1002/jor.22824

Wu, H., Zhao, G., Zu, H., Wang, J. H., and Wang, Q. M. (2015). Aging-related viscoelasticity variation of tendon stem cells (TSCs) characterized by quartz thickness shear mode (TSM) resonators. *Sens. Actuators (Warrendale Pa).* 210, 369–380. doi: 10.1016%2Fj.snb.2014.12.117

Xu, H., and Liu, F. (2018). Downregulation of FOXP1 correlates with tendon stem/progenitor cells aging. *Biochem. Biophys. Res. Commun.* 504 (1), 96–102. doi: 10.1016/j.bbrc.2018.08.136

Yeung, C. Y., Gossan, N., Lu, Y., Hughes, A., Hensman, J. J., Bayer, M. L., et al. (2014). Gremlin-2 is a BMP antagonist that is regulated by the circadian clock. *Sci. Rep.* 4, 5183. doi: 10.1038/srep05183

Yin, Z., Chen, X., Chen, J. L., Shen, W. L., Hieu Nguyen, T. M., Gao, L., et al. (2010). The regulation of tendon stem cell differentiation by the alignment of nanofibers. *Biomaterials* 31 (8), 2163–2175. doi: 10.1016/j.biomaterials.2009.11.083

Yin, Z., Chen, X., Zhu, T., Hu, J. J., Song, H. X., Shen, W. L., et al. (2013). The effect of decellularized matrices on human tendon stem/progenitor cell differentiation and tendon repair. *Acta Biomater.* 9 (12), 9317–9329. doi: 10.1016/j.actbio.2013.07.022

Yin, H., Caceres, M. D., Yan, Z., Schieker, M., Nerlich, M., and Docheva, D. (2019). Tenomodulin regulates matrix remodeling of mouse tendon stem/progenitor cells in an *ex vivo* collagen I gel model. *Biochem. Biophys. Res. Commun.* 512 (4), 691–697. doi: 10.1016/j.bbrc.2019.03.063

Yokosuka, K., Park, J. S., Jimbo, K., Yoshida, T., Yamada, K., Sato, K., et al. (2007). Immunohistochemical demonstration of advanced glycation end products and the effects of advanced glycation end products in ossified ligament tissues *in vitro*. *Spine (Phila Pa* 1976) 32 (11), E337–E339. doi: 10.1097/01.brs.0000263417.17526.35

Yu, T. Y., Pang, J. H., Wu, K. P., Chen, M. J., Chen, C. H., and Tsai, W. C. (2013). Aging is associated with increased activities of matrix metalloproteinase-2 and -9 in tenocytes. *BMC Musculoskelet. Disord.* 14, 2. doi: 10.1186/1471-2474-14-2

Zaseck, L. W., Miller, R. A., and Brooks, S. V. (2016). Rapamycin attenuates age-associated changes in tibialis anterior tendon viscoelastic properties. *J. Gerontol. A Biol. Sci. Med. Sci.* 71 (7), 858–865. doi: 10.1093/gerona/glv307

Zhang, J., and Wang, J. H. (2010a). Characterization of differential properties of rabbit tendon stem cells and tenocytes. *BMC Musculoskelet Disord.* 11, 10. doi: 10.1186/1471-2474-11-10

Zhang, J., and Wang, J. H. (2010b). Mechanobiological response of tendon stem cells: implications of tendon homeostasis and pathogenesis of tendinopathy. *J. Orthop. Res.* 28 (5), 639–643. doi: 10.1002/jor.21046

Zhang, J., and Wang, J. H. (2014). Prostaglandin E2 (PGE2) exerts biphasic effects on human tendon stem cells. *PloS One* 9 (2), e87706. doi: 10.1371/journal.pone.0087706

Zhang, J., and Wang, J. H. (2015). Moderate exercise mitigates the detrimental effects of aging on tendon stem cells. *PloS One* 10 (6), e0130454. doi: 10.1371/journal.pone.0130454

Zhang, K., Asai, S., and Yu, B. (2015). Enomoto-Iwamoto M. IL-1 β irreversibly inhibits tenogenic differentiation and alters metabolism in injured tendon-derived progenitor cells *in vitro*. Biochem. *Biophys. Res. Commun.* 463 (4), 667–672. doi: 10.1016/j.bbrc.2015.05.122

Zhang, J., Yuan, T., and Wang, J. H. (2016). Moderate treadmill running exercise prior to tendon injury enhances wound healing in aging rats. *Oncotarget* 7 (8), 8498–8512. doi: 10.18632/oncotarget7381

Zhou, Z., Akinbiyi, T., Xu, L., Ramcharan, M., Leong, D. J., Ros, S. J., et al. (2010). Tendon-derived stem/progenitor cell aging: defective self-renewal and altered fate. *Aging Cell* 9 (5), 911–915. doi: 10.1111/j.1474-9726.2010.00598.x

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METHODS
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Methods for Accurate Assessment of Myofiber Maturity During Skeletal Muscle Regeneration

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Adult skeletal muscle has a remarkable ability to regenerate. Regeneration of mature muscle fibers is dependent on muscle stem cells called satellite cells. Although they are normally in a quiescent state, satellite cells are rapidly activated after injury, and subsequently proliferate and differentiate to make new muscle fibers. Myogenesis is a highly orchestrated biological process and has been extensively studied, and therefore many parameters that can precisely evaluate regenerating events have been established. However, in some cases, it is necessary to evaluate the completion of regeneration rather than ongoing regeneration. In this study, we establish methods for assessing the myofiber maturation during muscle regeneration. By carefully comparing expression patterns of several muscle regeneration-related genes, we found that expression of *Myozenin* (*Myoz1* and *Myoz3*), *Troponin I* (*Tnni2*), and *Dystrophin* (*Dmd*) is gradually increased as muscle regeneration proceeds. In contrast, commonly used regeneration markers such as *Myh3* and *Myh8* are transiently upregulated after muscle injury but their expression decreases as regeneration progresses. Intriguingly, upregulation of *Myoz1*, *Myoz3* and *Tnni2* cannot be achieved in cultured myotubes, indicating that these markers are excellent indicators to assess myofiber maturity. We also show that analyzing re-expression of Myoz1 and dystrophin in individual fiber during regeneration enables accurate assessment of myofiber maturity at the single-myofiber level. Together, our study provides valuable methods that are useful in evaluating muscle regeneration and the efficacy of therapeutic strategies for muscle diseases.

Keywords: skeletal muscle, muscle regeneration, muscle differentiation, satellite cells, muscle disease

INTRODUCTION

Skeletal muscle consists mainly of myofibers, which are large cylindrical cells with many nuclei. Myofibers are terminally differentiated post-mitotic cells; however, skeletal muscles possess a high ability to regenerate. Regeneration of mature myofibers is dependent on satellite cells. Satellite cells are mononucleated cells located between the plasma membrane of the myofiber and basal lamina.

They normally remain in a quiescent state, but are activated upon muscle injury, and then they proliferate and differentiate to regenerate myofibers. Genetically engineered mice in which satellite cells are ablated show a complete lack of regenerative response (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011), indicating that satellite cells are absolutely required for muscle regeneration and cannot be compensated by other cell types. Furthermore, single-satellite cell transplantation revealed that these cells indeed possess self-renewal potential, in addition to the ability to differentiate into myofibers (Sacco et al., 2008). Thus, satellite cells are considered as definitive adult muscle stem cells.

Adult myogenesis is a highly ordered process in which satellite cells proliferate, differentiate, and generate new myofibers. Myogenic regulatory factors (MRFs) are important regulators of myogenesis and their expression is tightly regulated. Quiescent satellite cells do not express detectable levels of MyoD but they begin to express high levels of MyoD upon activation (Zammit et al., 2004). Expression of MyoD is maintained during the proliferation phase and continues until the early differentiation phase (Zammit et al., 2004). Myogenin is not expressed in quiescent satellite cells and proliferating undifferentiated myoblasts, but its expression is significantly upregulated when cells begin to differentiate (Bentzinger et al., 2012). Therefore, MyoD and Myogenin are commonly used as activation and differentiation markers of myogenesis, respectively. Expression levels of MRF4 are highest of the MRFs in adult mature muscle and are considered to reflect muscle fiber maturity (Bentzinger et al., 2012; Zammit, 2017). Adult muscle regeneration recapitulates many aspects of embryonic myogenesis, including expression of embryonic- or perinatal-type myosin heavy chain (MyHC) (Sartore et al., 1982). Thus, expression of these embryonic-type contractile proteins is a hallmark of muscle regeneration and is often used to detect activity of regeneration.

Expression of MRFs or embryonic or perinatal MyHC is useful to examine regenerating events. However, the most important goal in tissue regeneration is that the normal condition is restored. From this perspective, expression of the above described regeneration markers reflects conditions where muscle is still abnormal. In certain cases, therefore, assessment of "normality" becomes more important than evaluating regenerating events, especially in studies that examine the efficacy of therapeutic methods for degenerative muscle diseases. If diseased muscle is successfully treated and restored to its healthy state, expression of regeneration markers should be downregulated. Based on this notion, some studies examined downregulation of embryonic MyHC to assess therapeutic efficacy (Guiraud et al., 2019). However, little is known about indicators that directly reflect normality of muscle tissue.

Although experimental muscle regeneration is a highly ordered process, it is not completely synchronized, and thus there is a regional difference in the progression of regeneration within a single muscle. In a regeneration model of grafted muscle, it was reported that a radial gradient of regeneration is formed, with more mature muscle at the periphery and less mature muscle toward the center in the regenerating grafted muscle (Carlson and Gutmann, 1975). Likewise, other muscle regeneration models, including cardiotoxin injury models, do not show completely uniform regeneration, with some regions showing accelerated regeneration while other regions are in a delayed phase of regeneration. Therefore, it is important to develop a reliable method for evaluating muscle regeneration accurately and quantitatively, taking spatial non-uniformity of regeneration into account.

In this study, we carefully examined several regeneration-related markers during muscle regeneration. These analyses revealed that expression of *Myozenin* (*Myoz1* and *Myoz3*), *Troponin I* (*Tnni2*), and *Dystrophin* (*Dmd*) correlates very well with the progression of regeneration. Their expression highly reflects myofiber maturity because high expression of these genes can only be achieved in muscle tissue *in vivo* and not in cultured myotubes *in vitro*. We also developed a method that can distinguish advanced regenerating areas from delayed regenerating areas within single muscle, which enables accurate and quantitative evaluation of muscle regeneration. Our study provides useful information for the studies of muscle regeneration and therapy for muscle diseases.

MATERIALS AND METHODS

Mice

C57BL/6 wild type mice were used to isolate satellite cells and to analyze muscle regeneration. DBA/2-mdx (D2-mdx) mice were provided from Central Institute for Experimental Animals in Japan. All animal experiments performed in this report were approved by the Animal Care and Use Committee of Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology.

Muscle Injury

Cardiotoxin (CTX, Sigma) was dissolved in sterile saline at a concentration of 10 μM. Tibialis anterior (TA) muscles of 2 to 3 month old mice were injected with 100 μl CTX. TA muscles were isolated at days 0, 3, 5, 7, and 14 of CTX injury, embedded in tragacanth gum, and frozen in liquid nitrogen-cooled isopentane.

Satellite Cell Isolation

Isolation of mouse satellite cells was reported previously (Uezumi et al., 2016). Hind limb muscles were collected, minced and digested with 0.2% type II collagenase (Worthington) for 60 min at 37°C using a magnetic stirrer. Digested muscles were passed through an 18-gauge needle several times and further digested for 30 min at 37°C. Digested samples were filtered through a 100-μm cell strainer, and then through a 40-μm cell strainer. Cells were resuspended in washing buffer and labeled with APC-eFluor 780-conjugated rat anti-mouse CD45 (1:250) (Invitrogen), PE/Cy7-conjugated rat anti-mouse CD31 (1:250) (Biolegend), biotin-conjugated SM/C-2.6 (1:250) (Fukada et al., 2004), and PE-conjugated

goat anti-mouse PDGFRα (15 μl/test) (R&D systems), followed by secondary staining with streptavidin-brilliant violet 421 (Biolegend) (1:250). $CD31^{-}CD45^{-}PDGFR\alpha^{-}SM/C\text{-}2.6^{+}$ cells were sorted and collected as satellite cells with FACSAria II (BD Biosciences).

Cell Culture

After cell sorting, satellite cells were seeded at a density of 1×10^4 cells/well on a 48-well cell culture plate coated with Matrigel (BD Biosciences) in growth medium (GM) consisting of DMEM supplemented with 20% FBS, 1% penicillin-streptomycin, and 2.5 ng/μl bFGF (Katayama Chemical), and cultured at 37°C in 5% CO_2 and 3% O_2. After 4 days of culture in GM, GM was changed to differentiation medium (DM) consisting of DMEM with 5% horse serum. Then cells were maintained at 37°C in 5% CO_2 and 20% O_2 for 3 days to induce myogenic differentiation.

RNA Extraction and Quantitative Reverse Transcription-PCR Analysis

Total RNA was extracted from cultured satellite cells and muscles using RNeasy Mini Kit (Qiagen) and miRNeasy Mini Kit (Qiagen), respectively. Pieces of muscle tissues collected from frozen TA muscles were crushed in QIAzol Lysis Reagent (Qiagen) using a Shakeman homogenizer (Bio Medical Science). Complementary DNA (cDNA) was synthesized using QuantiTect Transcription Kit (Qiagen). qRT-PCR was performed with SYBR Premix Ex Taq II (Takara) on a Takara Thermal Cycler Dice Real Time System (Takara) under the following cycling conditions: 94°C for 30 s followed by 40 cycles of amplification (94°C for 5 s, 60°C for 20 s, 72°C for 12 s) and dissociation curve analysis. For gene expression analysis in regenerating TA muscles and differentiating satellite cells, mRNA expression was normalized with *Cmas*. Relative mRNA expression was then calculated using the $2^{-\Delta\Delta}$ method. Specific primers used for qRT-PCR were listed in **Supplementary Table 1**. Primers for *Actb* were provided from QuantiTect Primer Assays Kit (Qiagen).

Immunohistochemistry

Frozen transverse sections were cut at the thickness of 8 μm and fixed for 5 min in ice-cooled acetone. After blocking with M.O.M.™ mouse IgG blocking reagent (Vector Laboratories), sections were incubated overnight at 4°C with primary antibodies diluted in M.O.M.™ diluent. After washing with PBS, sections were stained with secondary antibodies. Primary and secondary antibodies used were listed in **Supplementary Table 2**. Nuclei were counterstained with DAPI (Dojindo), and stained muscles were mounted with SlowFade Diamond anti-fade reagent (Invitrogen). Fluorescent signals were detected with confocal laser scanning microscope systems TCS-SP8 (Leica). The same sections were stained with hematoxylin and eosin (HE) after capturing fluorescent images. HE images were taken with microscope AXIO (Carl Zeiss) equipped with a digital camera, Axiocam ERc 5s (Carl Zeiss).

Quantitative Analysis of Mature Myofibers

Cross-sections were made by cutting at the mid-belly of TA muscle (at the position about 3 mm from proximal end of TA muscle). After immunostaining, fluorescent images of entire cross-sections were captured with fluorescent microscope system BZ-X710 (Keyence). Image recognition and quantification were performed by using the Hybrid Cell Count Application (Keyence). First, entire cross-sectional areas of TA muscle were measured. For quantification of Myoz1-positive area, Myoz1-stained area was recognized based on the intensity of Myoz1 staining by adjusting threshold. For quantification of dystrophin-positive area, dystrophin-stained sarcolemma was first recognized based on the intensity of dystrophin staining by adjusting threshold, and then dystrophin-positive fiber area was recognized by using inversion function. After recognition of Myoz1- and dystrophin-positive areas, the misrecognized small areas were excluded by adjusting lower limit in histogram function. Finally, errors in recognition step were corrected manually, and then Myoz1- and dystrophin-positive areas were measured. Myoz1- or dystrophin-positive area was divided by entire cross-sectional area to calculate percentage of area positive for each marker. Two side unpaired *t*-test was used to compare two groups.

Statistical Analysis

Statistical significance was evaluated using GraphPad Prism 8.0 (GraphPad Software). One-way analysis of variance (ANOVA) followed by Tukey's or Dunnett's test was used to compare more than two groups.

RESULTS

Optimum Internal Control Gene for Gene Expression Analysis During Muscle Regeneration

We first analyzed the expression of several internal control genes by qRT-PCR to determine the optimum control genes for the most accurate gene expression analysis during muscle regeneration. As shown in **Figure 1**, *Gapdh* and *Actb* (also called β-actin), commonly used control genes, were highly variable in their expression during muscle regeneration (**Figure 1**). Therefore, these genes are not suitable as internal control genes to normalize expression of target genes. One pioneering study on comprehensive gene expression analysis during muscle regeneration had previously pointed out this problem and identified two genes that are stably expressed across all time points during muscle regeneration (Zhao and Hoffman, 2004). Those two genes are *Cmas* (also called as CMP-N-acetylneuraminic acid synthase) and *Eif3c* (called as NIPI-like protein). We thus examined the expression of *Cmas* and found relatively stable expression of this gene during muscle regeneration (**Figure 1**). Therefore, we decided to use *Cmas* as an internal control gene for gene expression analysis during muscle regeneration.

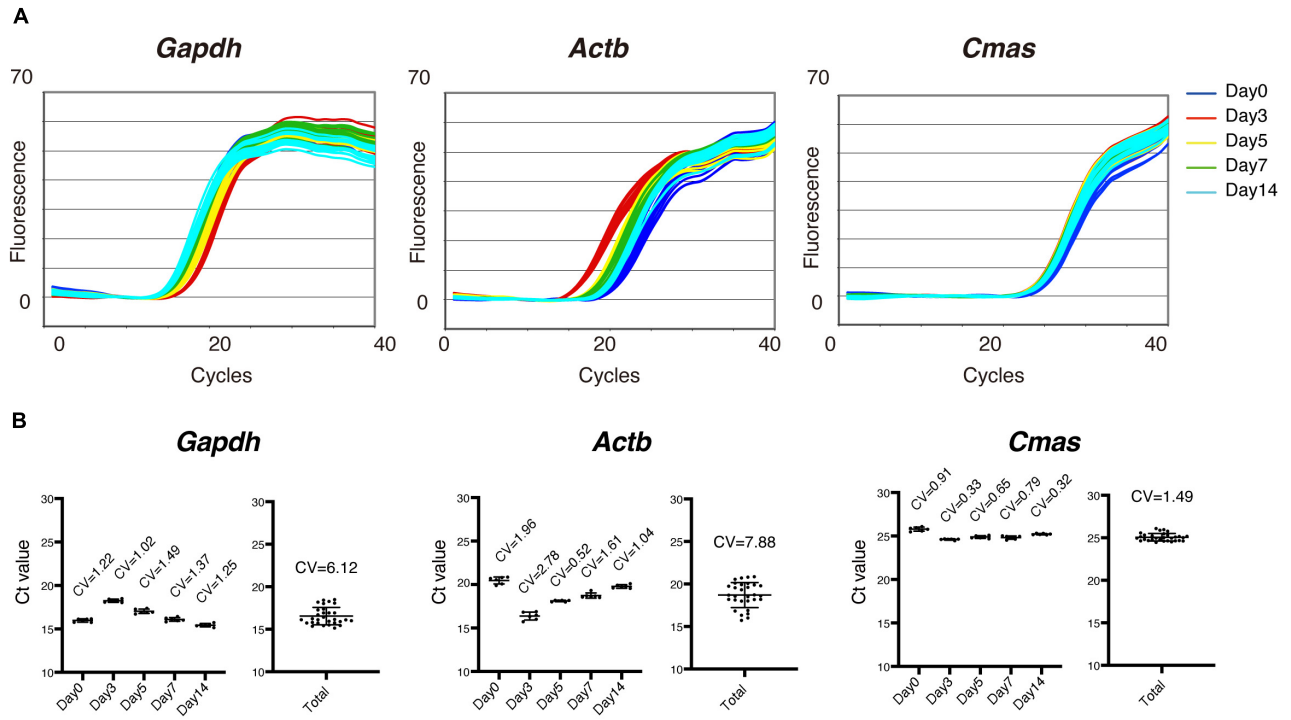

FIGURE 1 | Optimum internal control genes for gene expression analysis during muscle regeneration. **(A)** Amplification curves of quantitative reverse transcription-PCR (qRT-PCR) for *Gapdh*, β-*actin* (*Actb*), and *Cmas* using total RNA extracted from intact and regenerating tibialis anterior (TA) muscles 3, 5, 7, and 14 days after CTX injury. **(B)** Cycle threshold (Ct) values of indicated time points and total data for *Gapdh*, *Actb*, and *Cmas* are shown as mean ± SD of $n = 6$ mice at each time point. Coefficient of variation (CV) is shown in the graphs. Note that Ct value of *Cmas* showed smaller CV than that of *Gapdh* or *Actb*.

Gradual Upregulation of *Myoz1*, *Myoz3*, *Tnni2*, and *Dmd* Reflects the Myofiber Maturity During Regeneration

We next examined expression of several regeneration-related genes. As expected, expression of *MyoD* and *Myogenin* were highly induced upon muscle injury, and gradually downregulated thereafter (**Figure 2A**). We also observed similar dynamics in the expression of embryonic-type contractile genes. As shown in **Figure 2B**, expression of *Myh3* and *Myh8* was detected at day 3 of muscle injury, reached its peak at day 5, and then decreased to levels comparable to intact muscle. Thus, expression of above-described genes is transient during muscle regeneration and therefore does not reflect completion of regeneration accurately. Zhao et al. (2002) performed temporal gene expression profiling of muscle regeneration and showed that expression of muscle structural component genes is downregulated at early stages and then upregulated at late stages of muscle regeneration. Those include *Myozenin*, which encodes a Z-disk associated protein myozenin, and *Tnni2*, which encodes a fast skeletal type troponin I, a protein responsible for the calcium-dependent regulation of muscle contraction. Therefore, we examined expression of these muscle structural component genes. Expression of *Myoz1*, *Myoz3*, and *Tnni2* was sharply downregulated at day 3 of muscle injury, and then gradually upregulated as regeneration proceeded (**Figure 2C**), indicating that expression of these genes well reflects the extent of muscle regeneration. We also analyzed expression of *Dmd*, which encodes a dystrophin protein, and *Myh4*, which encodes a MyHC-IIb, a predominant type of MyHC expressed in TA muscle (Kammoun et al., 2014). Similar to *Myoz1*, *Myoz3* and *Tnni2*, expression of *Dmd* correlated well with the progression of regeneration (**Figure 2C**). Although *Myh4* showed a similar expression pattern, it reflected the extent of regeneration less accurately because there was no statistically significant difference in its expression levels between day 3 and day5, or day 5 and day 7 (**Figure 2C**). In contrast to these genes, expression of *Myoz2* and *Myf6* did not reflect muscle maturity (**Figure 2D**). These results clearly show that *Myoz1*, *Myoz3*, *Tnni2*, and *Dmd* are excellent markers for the assessment of myofiber maturity during muscle regeneration.

High Level Expression of *Myoz1*, *Myoz3*, and *Tnni2* Cannot Be Achieved in Cultured Myotubes

Results described above strongly suggest that expression of *Myoz1*, *Myoz3*, *Tnni2*, and *Dmd* correlates with myofiber maturity. It is well-known that cultured myotubes cannot mature into myofibers. To further confirm the relationship between expression of *Myoz1*, *Myoz3*, *Tnni2*, and *Dmd* and myofiber maturity, we examined the expression of these genes during myogenesis of cultured satellite cells. Satellite cells were FACS-sorted from hind limb muscles and cultured *in vitro* to obtain myotubes (**Figure 3A**). During the first 4 days of the growth

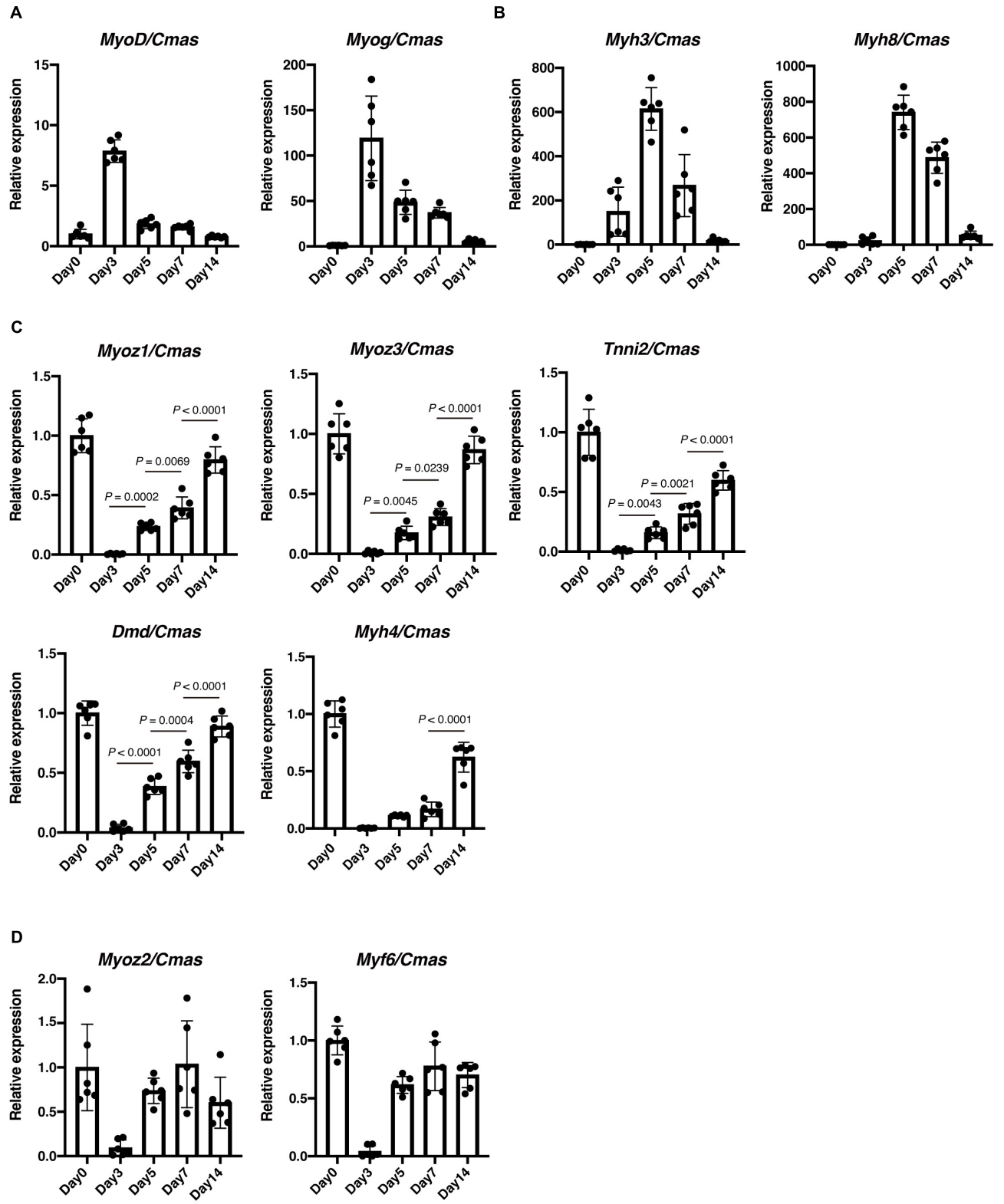

FIGURE 2 | Expression of *Myoz1*, *Myoz3*, *Tnni2*, and *Dmd* correlates with the progression of muscle regeneration. Expression of *MyoD* and *Myog* **(A)**, *Myh3* and *Myh8* **(B)**, *Myoz1*, *Myoz3*, *Tnni2*, *Dmd* and *Myh4* **(C)**, *Myoz2* and *Myf6* **(D)** during muscle regeneration was examined by qRT-PCR. Data are shown as relative value to intact muscle (Day 0) and represent mean ± SD of $n = 6$ mice at each time point. Data on *Myoz1*, *Myoz3*, *Tnni2*, *Dmd*, and *Myh4* from day 3 to day 14 were analyzed by ANOVA followed by Tukey's test to evaluate statistical difference.

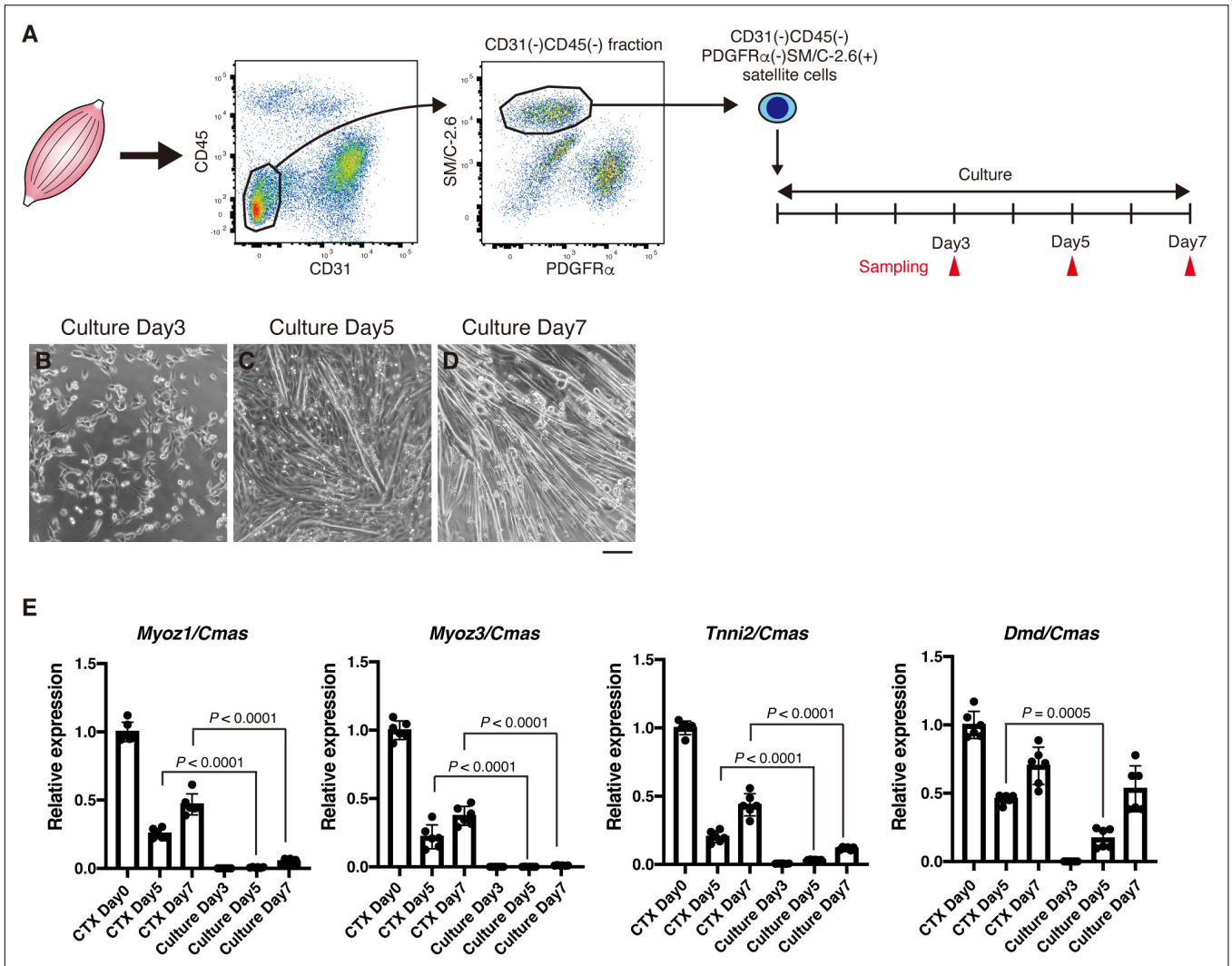

FIGURE 3 | *In vitro* cultured myotubes do not express high levels of *Myoz1*, *Myoz3*, and *Tnni2*. **(A)** Scheme of satellite cell isolation and culture. **(B–D)** Isolated satellite cells were cultured in GM for 4 days, then induced to differentiate into myotubes in DM. Representative images of cultured cells were taken at indicated time points. **(E)** Expression of *Myoz1*, *3*, *Tnni2*, and *Dmd* in intact (CTX Day 0), regenerating TA muscle (CTX Days 5 and 7) and cultured satellite cells was examined by qRT-PCR. Data are shown as relative value to intact TA muscle and represent the mean ± SD from independent experiments (n = 6). Data from regenerating TA muscle (CTX Days 5 and 7) and cultured cells were analyzed by ANOVA followed by Tukey's test to evaluate statistical difference. Scale bar: 100 μm **(B–D)**.

period, satellite cells became activated, and they proliferated extensively (**Figure 3B**). Upon induction of differentiation, they rapidly formed myotubes at day 5 of culture, and generated numerous myotubes by day 7 as they further differentiated (**Figures 3C,D**). In CTX muscle regeneration model, satellite cells proliferate extensively within 2 to 3 days of injury and begin to form regenerated myofibers approximately 5 days after injury, and regenerated myofibers mature afterward (Hawke and Garry, 2001). Thus, proliferation period and timing of differentiation of satellite cells are similar between *in vitro* myogenesis and *in vivo* regeneration model. As expected, undifferentiated myoblasts expressed very low levels of *Myoz1*, *Myoz3*, *Tnni2*, and *Dmd* similarly to CTX-injected muscle at day 3 (**Figures 2C**, **3E**). In later time points, expression levels of *Myoz1*, *Myoz3* and *Tnni2* remained quite low compared to levels in intact and regenerating (CTX day 5 and day 7) TA muscles *in vivo* (**Figure 3E**). Although expression of *Dmd* remained low levels until day 5 of culture, its expression in myotubes increased to the levels comparable to *in vivo* regenerating muscle at day 7 (**Figure 3E**). These data further reinforce the view that expression of *Myoz1*, *Myoz3*, and *Tnni2* well-reflects myofiber maturity that cannot be achieved in cultured myotubes.

Centrally Nucleated Fibers With Myoz1 and Dystrophin Expression Identify Areas of Advanced Regeneration

Evaluating *Myoz1*, *Myoz3*, *Tnni2*, and *Dmd* expression would be very useful for assessing the degree of muscle

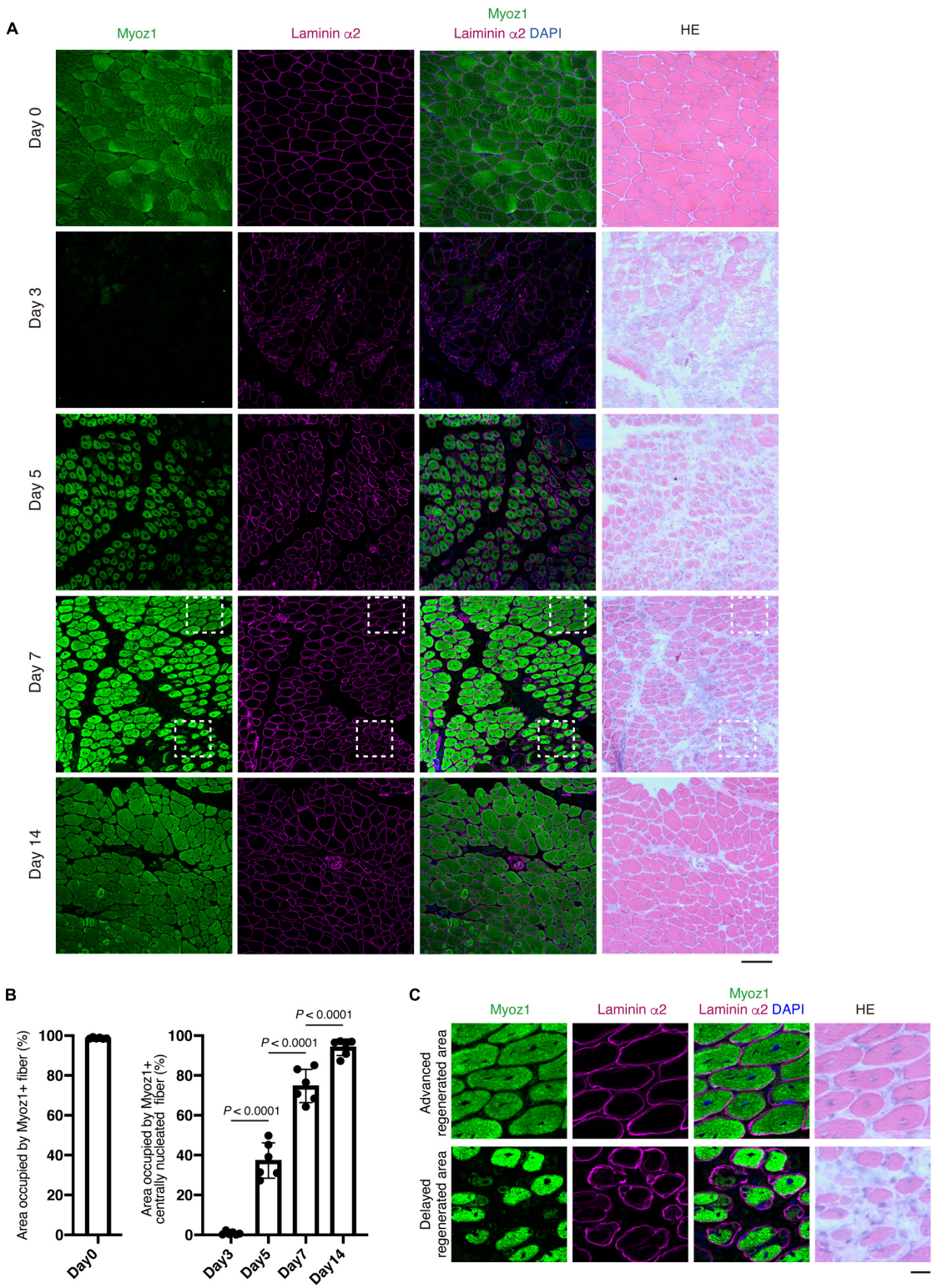

FIGURE 4 | Re-expression of Myoz1 protein is closely associated with the extent of myofiber regeneration. **(A)** TA muscle sections from the indicated time points were subjected to immunofluorescent staining for Myoz1 (green) and Laminin α2 (magenta) followed by HE staining. **(B)** Area occupied by Myoz1+ fibers in intact muscle (Day 0) and Myoz1+ centrally nucleated fibers in regenerating muscle was quantified. Data represent the mean ± SD of $n = 6$ mice at each time point. Data from regenerating muscle (Day 3 to Day 14) were analyzed by ANOVA followed by Tukey's test to evaluate statistical difference. **(C)** Magnified images of boxed areas in **(A)**. Upper panels show area with advanced regeneration and lower panels show area with delayed regeneration. Scale bars: 100 μm **(A)** and 20 μm **(C)**.

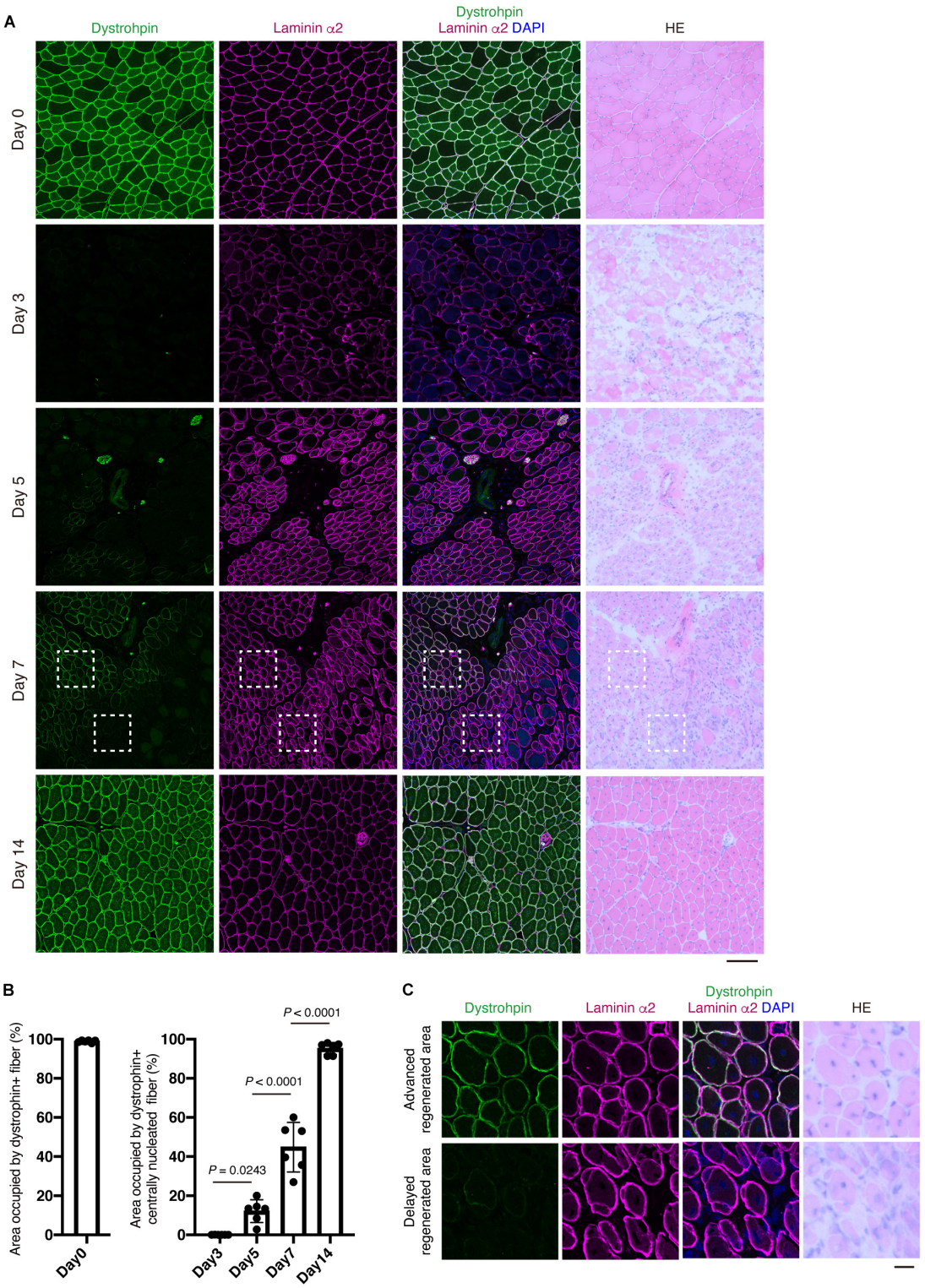

FIGURE 5 | Re-expression of dystrophin protein at the plasma membrane is closely associated the extent of myofiber regeneration. **(A)** TA muscle sections from the indicated time points were subjected to immunofluorescent staining for Dystrophin (green) and Laminin α2 (magenta) followed by HE staining. **(B)** Area occupied by Dystrophin+ fibers in intact muscle (Day 0) and Dystrophin+ centrally nucleated fibers in regenerating muscle was quantified. Data represent the mean ± SD of $n = 6$ mice at each time point. Data from regenerating muscle (Day 3 to Day 14) were analyzed by ANOVA followed by Tukey's test to evaluate statistical difference. **(C)** Magnified images of boxed areas in **(A)**. Upper panels show area with advanced regeneration and lower panels show area with delayed regeneration. Scale bars: 100 μm **(A)** and 20 μm **(C)**.

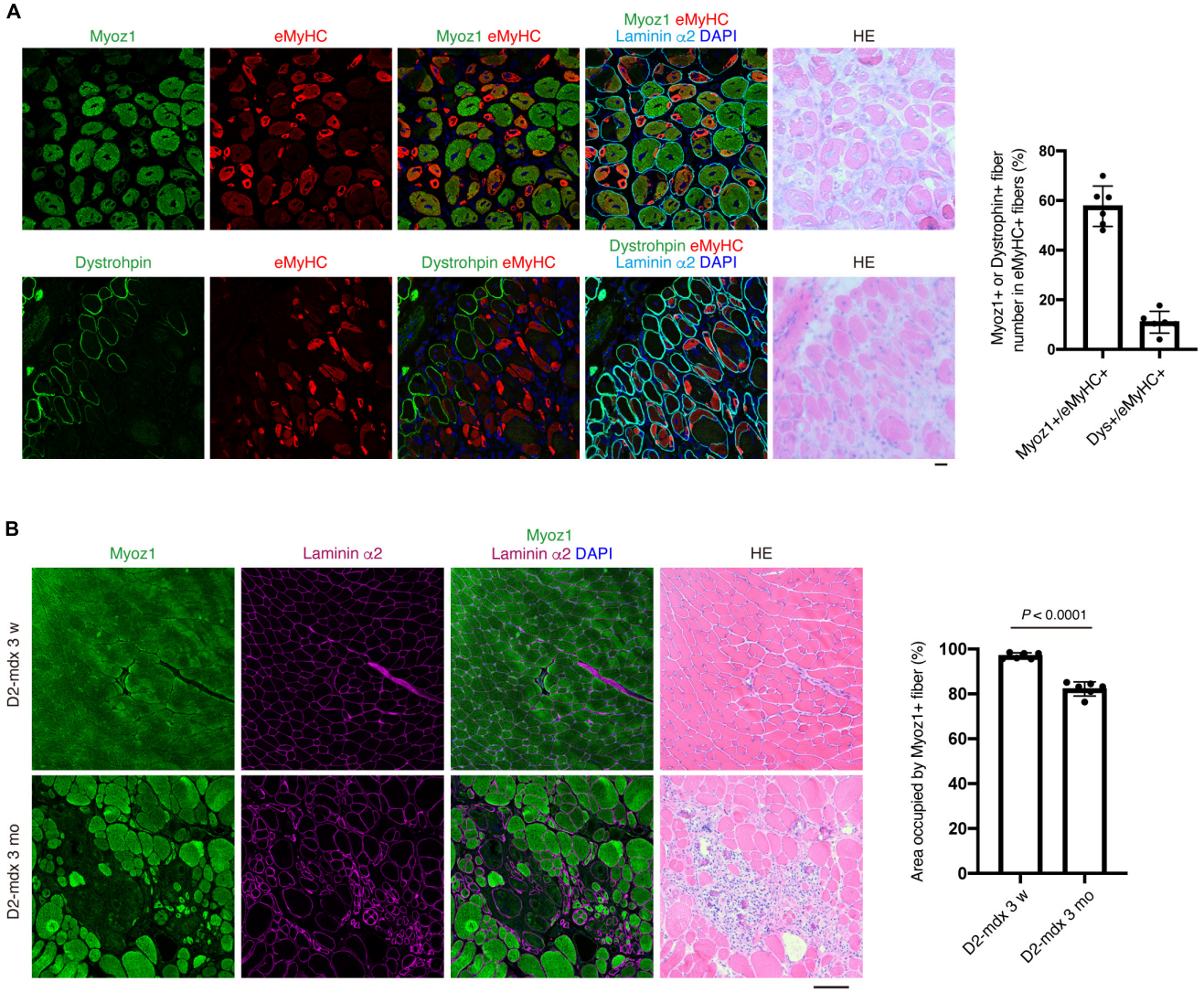

FIGURE 6 | Expression of Myoz1 and dystrophin protein is a good indicator of myofiber maturity at the single-fiber level. **(A)** TA muscle section from day 7 of CTX injection was subjected to immunofluorescent staining for Myoz1 or Dystrophin (green), eMyHC (red) and Laminin α2 (cyan) followed by HE staining. Graph shows percentage of Myoz1+ or Dystrophin+ fiber number in eMyHC+ fibers. Data represent the mean ± SD of $n = 6$ mice. **(B)** Quadriceps muscle section from D2-mdx mice at 3 weeks or 3 months of age was subjected to immunofluorescent staining for Myoz1 (green) and Laminin α2 (magenta) followed by HE staining. Area occupied by Myoz1+ fibers was quantified. Data represent the mean ± SD of $n = 6$ mice at each time point. Data was analyzed by two-sided unpaired *t*-test to evaluate statistical difference. Scale bars: 20 μm **(A)** and 100 μm **(B)**.

regeneration at the whole-tissue level. However, in some cases, it is necessary to assess muscle regeneration at the single-myofiber level because muscle regeneration is not a uniform process, with some regions showing advanced regeneration while other regions showing a delayed phase of regeneration. To overcome this problem, we developed a method that can accurately assess this spatial non-uniformity of regeneration. Centrally located nuclei are commonly used as an index of regenerated myofibers. However, central nuclei already exist in nascent myotubes, precluding its use as a reference index when assessing myofiber maturity. Among the markers whose expression correlate well with the progression of regeneration (*Myoz1*, *Myoz3*, *Tnni2*, and *Dmd*), we could obtain clear staining results for Myoz1 and dystrophin proteins. Because Myoz1 is a Z-disk associated protein, Myoz1-stained muscle showed sarcomere pattern, suggesting the specificity of the antibody used in this study (**Supplementary Figure 1**). We found that Myoz1 expression disappears upon muscle injury but gradually reappears as muscle regeneration proceeds (**Figures 4A,B**). Intriguingly, although well-differentiated large centrally nucleated fibers were strongly positive for Myoz1, small basophilic nascent myotubes were scantly positive or negative for Myoz1 even in contiguous areas of the same muscle

(**Figure 4C**). Dystrophin staining resulted in similar expression pattern, although dystrophin re-expression tended to be restricted to more mature myofibers (**Figure 5**). These results indicate that centrally nucleated myofiber with recovered Myoz1 or dystrophin expression is useful for assessing the spatial non-uniformity of muscle regeneration at single-myofiber level.

Re-expression of Myoz1 and Dystrophin Is a Good Indicator of Myofiber Maturity at the Single-Myofiber Level

To further understand the relationship between Myoz1 or dystrophin expression and myofiber maturity, we examined embryonic MyHC (eMyHC) expression, which is transiently upregulated in immature myofibers but downregulated as myofibers mature (d'Albis et al., 1988). Approximately 40 and 90% of eMyHC-positive immature myofibers were negative for Myoz1 and dystrophin, respectively (**Figure 6A**), indicating that re-expression of these markers occurs in myofibers with advanced maturation stage.

We also examined usefulness of these markers in evaluating disease progression of D2-mdx mice, a severe mouse model of Duchenne muscular dystrophy (DMD) (Fukada et al., 2010). Muscle of mdx background appears normal until approximately 3–4 weeks of age, but myofibers undergo massive degeneration afterward (DiMario et al., 1991). Because D2-mdx mice lack dystrophin expression, we examined expression of Myoz1 before and after disease onset. At 3 weeks of age, all myofibers of D2-mdx mice appeared normal and uniformly expressed Myoz1 (**Figure 6B**). After onset of symptoms, however, small immature myofibers located in degenerated area were scantly positive or negative for Myoz1, and total Myoz1-positive area was significantly decreased in ratio compared with pre-symptomatic stage (**Figure 6B**). These results indicate that expression of Myoz1 and dystrophin is a good indicator of myofiber maturity and useful for evaluating "normality" of myofiber in pathological conditions.

DISCUSSION

In this study, we describe methods for the assessment of myofiber maturity during skeletal muscle regeneration. Expression of *Myoz1*, *Myoz3*, *Tnni2*, and *Dmd* significantly correlates with progression of muscle regeneration, and therefore, these genes are quite useful to quantify and evaluate the extent of muscle regeneration at the whole-muscle tissue level. Meanwhile, re-expression of Myoz1 and dystrophin is an excellent indicator for the assessment of myofiber maturity at the single-fiber level.

Myozenin is specifically expressed in striated muscle and localized at Z-disks (Faulkner et al., 2000; Takada et al., 2001). Myozenin is reported to interact with other Z-disk proteins including a-actinin, filamin-C, telethonin and myotilin, and thought to be involved in the connection between the contractile apparatus and the sarcolemma (Faulkner et al., 2000; Frey et al., 2000; Takada et al., 2001; Frey and Olson, 2002; Gontier et al., 2005). In addition to its role as a structural protein, Myoz1 was shown to modulate calcineurin/NFAT activity (Frey et al., 2008), raising the possibility that Myozenin functions as a signaling molecule. In this study, we showed that expression of *Myoz1* and *Myoz3* is gradually upregulated as myofibers mature during muscle regeneration, and cultured myotubes do not express these genes at high levels. Therefore, it would be reasonable to assume that Myozenin plays some functional role in myofiber maturation.

We demonstrated that dystrophin begins to be re-expressed when myofibers mature in the late phase of muscle regeneration. Therefore, dystrophin re-expression closely reflects myofiber maturity. Although this method will provide a very powerful means of assessing muscle regeneration, there is a certain limitation. Because this evaluation method depends on dystrophin expression, it cannot be used in dystrophin-deficient conditions such as DMD. Evaluating Myoz1 expression offers an alternative method in such a situation as we showed in this study. However, definitive therapy for DMD is restoration of dystrophin expression. Therefore, dystrophin expression is commonly studied in research when evaluating the effects of DMD therapy. If the therapeutic strategy is based on endogenous gene expression machinery (such as exon skipping or gene editing therapy) and not on forced expression, restored dystrophin expression reflects not only proof of principle but also maturity of treated myofibers. Thus, our study provides additional rationale for examining dystrophin expression in DMD therapy research.

Methods described here are based on gene expression and histological analyses, but one of the most important functions of skeletal muscle is to contract for force generation. Therefore, measurement of contractile ability is one of the best ways to evaluate skeletal muscle property. However, evaluating contractile properties is accompanied by some technical difficulties. Our methods presented here are relatively stable and easy to perform. In addition, markers used in our methods seem to be functionally important because myozenin and troponin I are associated with contractile apparatus, and dystrophin is a causative gene for DMD. Thus, we believe that our methods provide convenient opportunity to assess myofiber maturity.

In conclusion, our study provides meaningful information that can be applied for accurate and quantitative assessment of muscle regeneration and effectiveness of therapy for muscle diseases.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Care and Use Committee of Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology.

AUTHOR CONTRIBUTIONS

YY performed qPCR, immunostaining and cell culture experiments, and analyzed the data. MI-U and KH induced muscle regeneration and retrieved samples. MI-U performed FACS experiments. AU and SF interpreted results and coordinated the project. AU conceived the whole project and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00267/full#supplementary-material

REFERENCES

Bentzinger, C. F., Wang, Y. X., and Rudnicki, M. A. (2012). Building muscle: molecular regulation of myogenesis. *Cold Spring Harb. Perspect. Biol.* 4:a008342. doi: 10.1101/cshperspect.a008342

Carlson, B. M., and Gutmann, E. (1975). Regneration in free grafts of normal and denervated muscles in the rat: morphology and histochemistry. *Anat. Rec.* 183, 47–62. doi: 10.1002/ar.1091830106

d'Albis, A., Couteaux, R., Janmot, C., Roulet, A., and Mira, J. C. (1988). Regeneration after cardiotoxin injury of innervated and denervated slow and fast muscles of mammals. myosin isoform analysis. *Eur. J. Biochem.* 174, 103–110. doi: 10.1111/j.1432-1033.1988.tb14068.x

DiMario, J. X., Uzman, A., and Strohman, R. C. (1991). Fiber regeneration is not persistent in dystrophic (MDX) mouse skeletal muscle. *Dev. Biol.* 148, 314–321. doi: 10.1016/0012-1606(91)90340-9

Faulkner, G., Pallavicini, A., Comelli, A., Salamon, M., Bortoletto, G., Ievolella, C., et al. (2000). FATZ, a filamin-, actinin-, and telethonin-binding protein of the Z-disc of skeletal muscle. *J. Biol. Chem.* 275, 41234–41242. doi: 10.1074/jbc.M007493200

Frey, N., Frank, D., Lippl, S., Kuhn, C., Kogler, H., Barrientos, T., et al. (2008). Calsarcin-2 deficiency increases exercise capacity in mice through calcineurin/NFAT activation. *J. Clin. Invest.* 118, 3598–3608. doi: 10.1172/jci36277

Frey, N., and Olson, E. N. (2002). Calsarcin-3, a novel skeletal muscle-specific member of the calsarcin family, interacts with multiple Z-disc proteins. *J. Biol. Chem.* 277, 13998–14004. doi: 10.1074/jbc.M200712200

Frey, N., Richardson, J. A., and Olson, E. N. (2000). Calsarcins, a novel family of sarcomeric calcineurin-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* 97, 14632–14637. doi: 10.1073/pnas.260501097

Fukada, S., Higuchi, S., Segawa, M., Koda, K., Yamamoto, Y., Tsujikawa, K., et al. (2004). Purification and cell-surface marker characterization of quiescent satellite cells from murine skeletal muscle by a novel monoclonal antibody. *Exp. Cell Res.* 296, 245–255. doi: 10.1016/j.yexcr.2004.02.018

Fukada, S., Morikawa, D., Yamamoto, Y., Yoshida, T., Sumie, N., Yamaguchi, M., et al. (2010). Genetic background affects properties of satellite cells and mdx phenotypes. *Am. J. Pathol.* 176, 2414–2424. doi: 10.2353/ajpath.2010.090887

Gontier, Y., Taivainen, A., Fontao, L., Sonnenberg, A., van der Flier, A., Carpen, O., et al. (2005). The Z-disc proteins myotilin and FATZ-1 interact with each other and are connected to the sarcolemma via muscle-specific filamins. *J. Cell Sci.* 118(Pt 16), 3739–3749. doi: 10.1242/jcs.02484

Guiraud, S., Edwards, B., Squire, S. E., Moir, L., Berg, A., Babbs, A., et al. (2019). Embryonic myosin is a regeneration marker to monitor utrophin-based therapies for DMD. *Hum. Mol. Genet.* 28, 307–319. doi: 10.1093/hmg/ddy353

Hawke, T. J., and Garry, D. J. (2001). Myogenic satellite cells: physiology to molecular biology. *J. Appl. Physiol. (1985)* 91, 534–551. doi: 10.1152/jappl.2001.91.2.534

Kammoun, M., Cassar-Malek, I., Meunier, B., and Picard, B. (2014). A simplified immunohistochemical classification of skeletal muscle fibres in mouse. *Eur. J. Histochem.* 58:2254. doi: 10.4081/ejh.2014.2254

Lepper, C., Partridge, T. A., and Fan, C. M. (2011). An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development* 138, 3639–3646. doi: 10.1242/dev.067595

Murphy, M. M., Lawson, J. A., Mathew, S. J., Hutcheson, D. A., and Kardon, G. (2011). Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development* 138, 3625–3637. doi: 10.1242/dev.064162

Sacco, A., Doyonnas, R., Kraft, P., Vitorovic, S., and Blau, H. M. (2008). Self-renewal and expansion of single transplanted muscle stem cells. *Nature* 456, 502–506. doi: 10.1038/nature07384

Sambasivan, R., Yao, R., Kissenpfennig, A., Van Wittenberghe, L., Paldi, A., Gayraud-Morel, B., et al. (2011). Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development* 138, 3647–3656. doi: 10.1242/dev.067587

Sartore, S., Gorza, L., and Schiaffino, S. (1982). Fetal myosin heavy chains in regenerating muscle. *Nature* 298, 294–296. doi: 10.1038/298294a0

Takada, F., Vander Woude, D. L., Tong, H. Q., Thompson, T. G., Watkins, S. C., Kunkel, L. M., et al. (2001). Myozenin: an alpha-actinin- and gamma-filamin-binding protein of skeletal muscle Z lines. *Proc. Natl. Acad. Sci. U.S.A.* 98, 1595–1600. doi: 10.1073/pnas.041609698

Uezumi, A., Kasai, T., and Tsuchida, K. (2016). Identification, isolation, and characterization of mesenchymal progenitors in Mouse and human skeletal muscle. *Methods Mol. Biol.* 1460, 241–253. doi: 10.1007/978-1-4939-3810-0_17

Zammit, P. S. (2017). Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis. *Semin. Cell Dev. Biol.* 72, 19–32. doi: 10.1016/j.semcdb.2017.11.011

Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A., and Beauchamp, J. R. (2004). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *J. Cell Biol.* 166, 347–357. doi: 10.1083/jcb.200312007

Zhao, P., and Hoffman, E. P. (2004). Embryonic myogenesis pathways in muscle regeneration. *Dev. Dyn.* 229, 380–392. doi: 10.1002/dvdy.10457

Zhao, P., Iezzi, S., Carver, E., Dressman, D., Gridley, T., Sartorelli, V., et al. (2002). Slug is a novel downstream target of MyoD. Temporal profiling in muscle regeneration. *J. Biol. Chem.* 277, 30091–30101. doi: 10.1074/jbc.M202668200

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