CLINICAL TRANSLATION AND COMMERCIALISATION OF ADVANCED THERAPY MEDICINAL PRODUCTS

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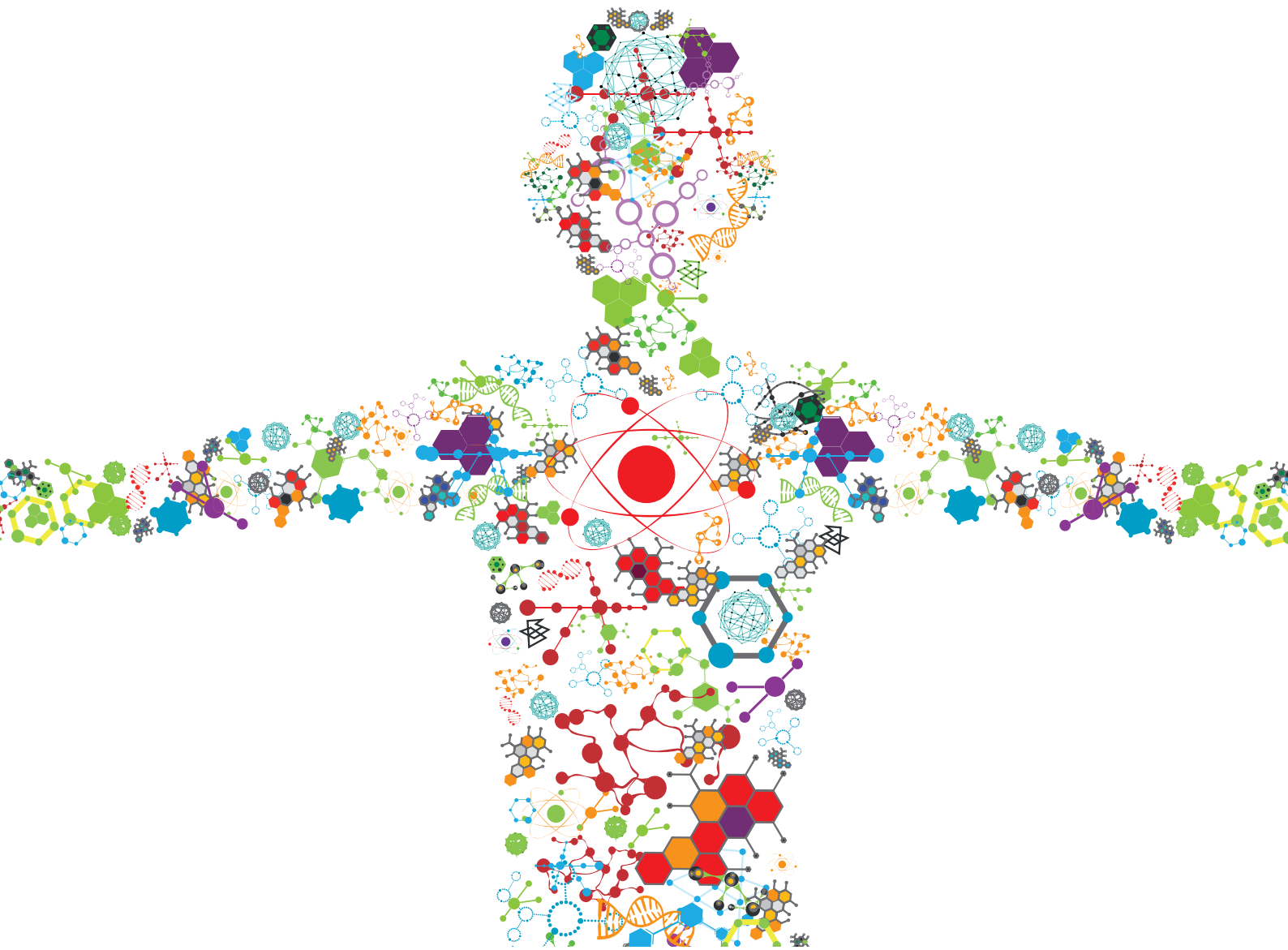

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CLINICAL TRANSLATION AND COMMERCIALISATION OF ADVANCED THERAPY MEDICINAL PRODUCTS

Topic Editors:
Tracy Tong Li Yu, M Ventures, Netherlands
Yves Bayon, Other, France
Alain A. Vertes, NxR Biotechnologies GmbH, Switzerland
Ivan Martin, University of Basel, Switzerland

Dr. Yves Bayon is a Senior Principal Scientist at Medtronic and Dr. Alain Vertes is affiliated with NxR Biotechnologies GmbH. All other Topic Editors declare no competing interests with regards to the Research Topic subject.

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EDITORIAL
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Editorial: Clinical Translation and Commercialisation of Advanced Therapy Medicinal Products

Ivan Martin[1,2], *Yves Bayon*[3], *Tracy T. L. Yu*[4*] and *Alain A. Vertès*[5]

[1] Department of Biomedicine, University Hospital Basel, University of Basel, Basel, Switzerland, [2] Department of Biomedical Engineering, University of Basel, Basel, Switzerland, [3] Medtronic – Sofradim Production, Trévoux, France, [4] M Ventures, Amsterdam, Netherlands, [5] NxR Biotechnologies GmbH, Basel, Switzerland

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Editorial on the Research Topic

Clinical Translation and Commercialisation of Advanced Therapy Medicinal Products

In the past 5 years, the number of ongoing clinical trials in advanced therapy medicinal products (ATMPs) worldwide has been increasing tremendously, at a staggering compounded annual growth rate (CAGR) of 14% (from 631 in 2015 to 1066 in 2019, source: Alliance for Regenerative Medicine). The field as a whole, though still emerging, has attracted on average $10 billion of financings every year, with the Gene & Gene-modified Cell Therapy category leading the way, accounting for more than half of the total financings toward all ATMPs. This funding injection among other factors has fuelled the growth in the number of companies created in this space, at a worldwide CAGR of 10.1% (2015-2019; source: Alliance for Regenerative Medicine). Geographically, Asia emerges as the fastest growing region in the number of companies enabled not only by foreign direct investments but also by national investments, followed by North America and Oceania (CAGR from 2015 to 2019: 12.6%, 11.2%, and 10%, respectively; source: Alliance for Regenerative Medicine). Remarkably, Asian countries, and notably Japan amongst the very first, have adapted their regulatory processes to implement faster these transformational therapies in areas of high unmet needs. Nonetheless, there still remains several critical hurdles before the full realization of the therapeutic, market, and economic potential of ATMPs, including: (1) remaining perceived technology risk; (2) still slow rate of overall technology adoption; (3) still limited market access complexified by business model challenges particularly when it comes to autologous products and exacerbated by previously unexplored pricing and reimbursement issues; and finally (4) still limited overall manufacturing capacity compared to the fast increasing demand from numerous clinical trials and from the market itself post novel ATMPs' product launch. On the other hand, there are key enabling factors including: investments; policies and notably advanced regulatory policies; Phase III data readouts and product approvals; as well as tailor-designed business ecosystems adapted to the intrinsic characteristics of cell and gene-based therapies. These four dimensions constitute important catalysts to accelerate the pace of the development of this sector. Currently, more than 25 ATMPs are available in the market such as Alofisel (2018), LUXTURNA® (2017), YESCARTA® (2017), KYMRIAH® (2017), and INVOSSA™ (2017).

When tackling this Research Topic, we aimed to provide diverse perspectives and learnings on the clinical translation and commercialization front of the ATMP field, as well as to feature several biotechnologically significant original research work. Ghamari et al. highlighted the current discrepancies in the marker characterization of placenta-derived amniotic cells with the aim of achieving better clinical translation and safer practices. Similarly, in an original research article,

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Edited and reviewed by:
Ranieri Cancedda,
Independent Researcher, Genoa, Italy

***Correspondence:**
Tracy T. L. Yu
tracy_tongyu@outlook.com

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Asnaghi et al. exemplified the importance of biomarker signatures for the quality of engineered nasal chondrocyte-derived cartilage, where they proposed gene expression patterns and generalized linear models could be used to define the molecular signatures of identity, purity, and potency of regenerative cellular therapies. In addition, in another original research article, Rothweiler et al. proposed a novel predictive MSC chondrogenesis marker, the ratio of TGFβ-RI/TGFβ-RII, which could be tuned by siRNA knockdown of TGFβ-RII and subsequently recover the chondrogenic differentiation ability of non-responsive MSCs.

As alternatives to autologous/allogenic cell therapy approaches, cell-derived extracellular vesicles, and acellular xenografts could bring unique opportunities, at the same time associated with distinct challenges. In a review article, Maumus et al. brought forward some of the key aspects in production, regulation, and clinical translation, while He et al. presented a pre-clinical study of acellular xenograft from whole porcine meniscus as a potential substitute to partially replace irreparable damaged meniscus.

Looking at ATMP applications by tissue types, Sallent et al. reviewed and discussed the clinical translation and regulatory aspects of bone grafts by identifying the patterns of successful clinical translations, which are related to the understanding of the mechanism of action of the various device components and their compliance with regulatory frameworks. In another review, Magrelli et al. analyzed the ophthalmology field as an example of the health economics of ATMPs in comparison with conventional therapies, coming to the interesting conclusion that these two therapeutic approaches are actually economically comparable.

Needless to say, it is impossible to have a complete Research Topic on ATMPs without considering cell manufacturing and its delivery logistics. The former was featured in a mini review article by Doulgkeroglou et al., in which they proposed that it is critical to consider automation, monitoring, digital tools and standardization of cell product manufacturing at the early phase of the product development cycle. The latter was exemplified by a cell therapy logistic tool innovation that has the potential to offer a novel portable solution for live cell transportation by Willbrand et al..

A highlight of this Research Topic is from the remarkable translation of an allogenic cell product—primary dermal progenitor fibroblasts (FE002-SK2 cell type). In this original research article, Laurent et al. reported a success story and learnings ranging from the mechanistic characterization, bioprocessing up-scaling, and all the way to cross-continental technology and material transfer.

The field of ATMPs is likely at or nearing its inflection point, thus announcing tremendous changes to come and a further acceleration in discoveries, development, and translation to the bed-side, which will profoundly transform healthcare in disease areas and indications that have all but vexed the pharmaceutical and biotechnological industry to this date. Transformational changes are desperately needed in numerous indications with high unmet need, such as heart diseases, neurodegenerative diseases, fibroses of the liver or kidney, solid tumours, and not to forget tissue and organ replacement, where conventional therapeutic modalities have failed to this date to provide a suitable solution. However, there are multifarious challenges that must be overcome to reach clinical fruition for a large number of patients. We trust that this Research Topic highlights relevant readings to capture the current status and the potential for the development of ATMPs.

The previous decade was that of reaching a generally recognized proof of concept for cell and gene-base therapies. This coming decade is that of implementing in a large number of indications the promises of the novel therapies. Such promises are best exemplified by Provenge, Glybera, KYMRIAH®, and Yescarta®. These products all constitute critical precursor products regardless of their past, present, or future commercial successes. The first approvals of cell and gene therapies in key jurisdictions have paved the way for a transformation of healthcare as radical as the one triggered by the technology of monoclonal antibodies. Successive innovations, market access, and advanced manufacturing will increasingly play a central role as the first technology wave matures. They should enable, in a domino effect, the emergence of the next generation ATMPs, such as: (1) living drugs that are engineered to a higher degree of complexity to permit better therapeutic function in terms of both safety and efficacy, (2) novel cell types (e.g., macrophages) that harness unspoiled yet fundamental biological properties, and (3) novel combination therapies to exploit synergies between conventional and advanced therapies to deliver greatly optimized treatment algorithms to patients with life-threatening diseases.

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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REVIEW
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The Bottlenecks in Translating Placenta-Derived Amniotic Epithelial and Mesenchymal Stromal Cells Into the Clinic: Current Discrepancies in Marker Reports

Seyyed-Hadi Ghamari[1†], *Mohsen Abbasi-Kangevari*[2†], *Tahereh Tayebi*[1], *Soheyl Bahrami*[3] *and Hassan Niknejad*[1*]

[1] *Department of Pharmacology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran,* [2] *Student Research Committee, Social Determinants of Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran,* [3] *Ludwig Boltzmann Institute for Experimental and Clinical Traumatology in AUVA Research Center, Vienna, Austria*

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Edited by:
Ivan Martin,
University of Basel, Switzerland

Reviewed by:
Ornella Parolini,
Catholic University of the Sacred Heart, Italy
Anna Lange-Consiglio,
University of Milan, Italy

***Correspondence:**
Hassan Niknejad
niknejad@sbmu.ac.ir

[†] *These authors share first authorship*

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Placenta-derived amniotic cells have prominent features for application in regenerative medicine. However, there are still discrepancies in the characterization of human amniotic epithelial and mesenchymal stromal cells. It seems crucial that the characterization of human amniotic membrane cells be investigated to determine whether there are currently discrepancies in their characterization reports. In addition, possible causes for the witnessed discrepancies need to be addressed toward paving the way for further clinical application and safer practices. The objective of this review is to investigate the marker characterization as well as the potential causes of the discrepancies in the previous reports on placenta-derived amniotic epithelial and mesenchymal stromal cells. The current discrepancies could be potentially due to reasons including passage number and epithelial to mesenchymal transition (EMT), cell heterogeneity, isolation protocols and cross-contamination, the region of cell isolation on placental disk, measuring methods, and gestational age.

Keywords: amniotic epithelial cells, cell heterogeneity, cross-contamination, epithelial to mesenchymal transition, isolation protocol, mesenchymal stromal cells, passage number, simultaneous isolation

INTRODUCTION

Human amniotic membrane has increasingly attracted the attention of basic and clinical scientists in recent years as a promising source of cells for regenerative medicine. It is a thin avascular membrane which forms a fluid-filled sac enclosing the fetus, which consists of an epithelial layer, a basal lamina, and an avascular mesenchymal layer which includes a compact layer, a fibroblast layer and a spongy layer (Gupta et al., 2015). The epithelial layer consists of flat, cuboidal and columnar epithelial cells which are in contact with amniotic fluid. Attached to the epithelial layer is the basal lamina composed of collagen, fibronectin, and laminin (Hilmy et al., 2018). The mesenchymal layer is connected to the basal lamina and includes fibroblast-like mesenchymal stromal cells, and a defined population of HLA-DR-expressing cells with macrophage-monocyte phenotypic (Magatti et al., 2008). A spongy layer consisting of loosely arranged collagen fibers separates the mesenchymal layer and the chorion. The number of human amniotic epithelial cells (hAECs) is

four to eight times greater than human amniotic mesenchymal stromal cells (hAMSCs), depending on the gestational age (Ochsenbein-Kolble et al., 2003).

Extensive research has focused on placenta-derived amniotic membrane as a potential cost-effective unlimited source of cells which could be obtained less invasively compared to procedures like bone marrow biopsy. With parental consent, the placenta could be obtained from elective caesarian sections which have lower risk of microbial contamination compared with vaginal births (Adds et al., 2001). In addition, obtaining amniotic membrane does not require any embryos to be destroyed and is associated with limited ethical considerations compared with the use of embryonic stem cells (Parolini et al., 2008). Human amniotic cells could be used rather safely due to lack of tumorigenicity (Liu et al., 2018; Abbasi-Kangevari et al., 2019). Furthermore, they do not express MHC class II surface markers and have low immunogenicity (Peric et al., 2018). Therefore, they could be used as an allogenic transplant which highlights their potential application among the elderly who do not possess sufficient pools of stem cells for autograft transplantation (Ahmed et al., 2017). Although characteristics of the cells could be affected according to the culture condition such as the components of culture medium, hAECs can sufficiently be expanded under certain culture condition and maintain their reproducible biologic characteristics including expressing major pluripotent genes as well as embryonic stem cell specific surface markers in the subculturing process (Evron et al., 2011). Like many stem cells, human amniotic cells could also be cryopreserved which makes them suitable for banking due to low costs in terms of expense, time, and human resources (Murphy et al., 2014; Yazdanpanah et al., 2015). Moreover, they have ease of isolation and self-renewal capacities which make them a promising option for applications in regenerative medicine. Human amniotic cells have the potential to differentiate into all the three germ layers including endoderm, mesoderm, and ectoderm; i.e., hepatocytes, pancreatic cells (Wei et al., 2003), cardiomyogenic (Miki, 2011), chondrogenic, osteogenic, adipogenic (Shu et al., 2011; Topoluk et al., 2017; Ghasemzadeh et al., 2018), and neurogenic cell lines (Portmann-Lanz et al., 2006).

There is a rapidly growing body of literature on clinical trials which investigate the potential application of hAECs and hAMSCs in the clinic, considering their immunomodulatory features (Yamahara et al., 2019), wound healing promotion (Prakoeswa et al., 2018), prevention and treatment of pulmonary disorders (Moodley et al., 2010; Baker et al., 2019), treatment of premature infants with bronchopulmonary dysplasia (Lim et al., 2018). In addition, they are being investigated in recruiting or non-recruiting clinical trials in Asherman's Syndrome (The Second Affiliated Hospital of Chongqing Medical University and Shanghai iCELL Biotechnology Co., Ltd, Shanghai, China, 2017) and Graft-vs. -Host Disease (PUPS and Shanghai iCELL Biotechnology Co., Ltd, Shanghai, China, 2018).

An international workshop was held in 2008 which focused on the structure of amnion and discussed isolation, characterization, and differentiation protocols for hAECs, and hAMSC, as well as the immunomodulatory properties, *in vitro* and *in vivo* preclinical studies, and cell banking strategies for these cell populations (Parolini et al., 2008). However, there are still discrepancies in the recent reports on the characterization of human amniotic epithelial and mesenchymal stromal cells. Possible causes for the witnessed discrepancies among the characterization reports need to be addressed toward paving the way for further clinical application and safer practices. The objective of this review is to investigate the marker characterization as well as the potential causes of the discrepancies in the previous reports on placenta-derived amniotic epithelial cells and mesenchymal stromal cells.

DISCREPANCIES IN CHARACTERIZATION OF HUMAN AMNIOTIC CELLS

Human amniotic cells including hAECs and hAMSCs are derived from the epiblast and hypoblast layers of amnion after 8 days of fertilization, respectively. These cells form a heterogeneous population of pluripotent, multipotent, progenitor, and mature cells (Miki and Strom, 2006; Rennie et al., 2012) which are characterized by the presence of embryonic stem cell and pluripotency markers. Moreover, the expression of epithelial, mesenchymal, and Human Leukocyte Antigens (HLAs) varies among hAECs and hAMSCs. As the amniotic membrane is adjacent to the chorion, the isolated cells of amnion origin need to be negative for hematopoietic markers to rule out hematopoietic cell contamination. In addition, hAECs and hAMSCs express various lineage-associated markers, which represents their potential to differentiate to several cell lineages as progenitor cells. Characterization markers of hAECs and hAMSCs are presented in the following categories (**Figure 1**, **Table 1**).

Embryonic Stem Cell, Self-Renewal, and Pluripotency Markers

Although the specific phenotypic features of hAECs and hAMSCs including plastic-adherence, microscopic shape of the cells and the potential to form colony-forming units is of value in cell characterization, their identification essentially relies on characterization using markers of embryonic stem cell, self-renewal, and pluripotency which remains challenging. The expression of specific surface markers of undifferentiated embryonic stem cells including Tumor Rejection Antigen (TRA) 1-60, 1-81, Stage Specific Embryonic Antigens (SSEA)-3, SSEA-4, Octamer-Binding Transcription Factor 4 (OCT-4), Nanog, SOX-2, Kruppel-like factor 4 (KLF-4), REX-1, CFC-1, Developmental Pluripotency Associated 3 (DPPA-3), Prominin 1 (PROM-1), Paired Box Protein 6 (PAX-6), Forkhead box D3 (FOXD3), Growth differentiation factor-3 (GDF3), TFE3, and c-MYC has been studied among hAECs and hAMSCs. It has been shown that SSEA-3 and SSEA-4 are present on 9% and 44% of hAECs, respectively (Miki et al., 2005). Another study also detected SSEA-3 and SSEA-4 on hAECs, but in a different quantity: 40% and 97%, respectively (Zhou et al., 2013). While almost 10% of hAECs express TRA1-60 and TRA1-81 on their surface (Miki et al., 2005), a study suggested that TRA1-60 could be a

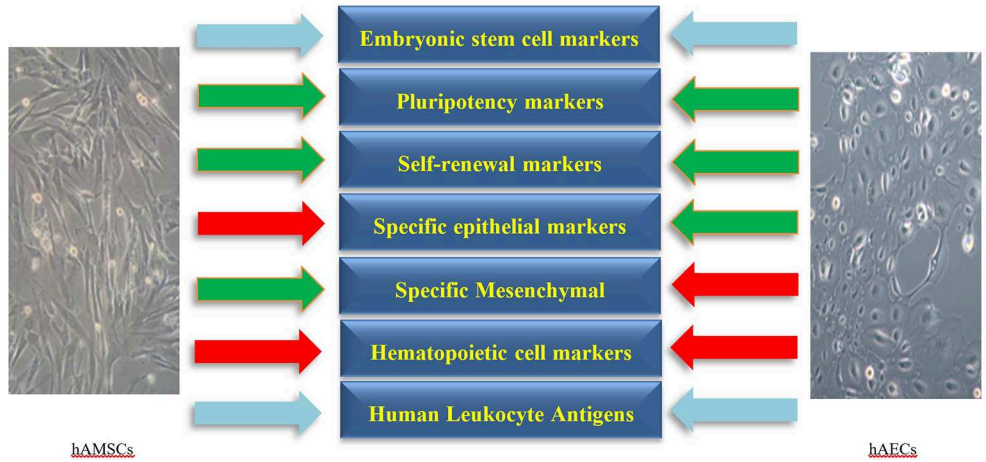

FIGURE 1 | The main negative (red arrows) and positive (green arrows) markers on human amniotic epithelial (hAECs) and mesenchymal stromal cells (hAMSCs). The expression of Human Leukocyte Antigens and embryonic stem cell markers on hAECs and hAMSCs is variable (sky-blue arrows). Specific markers for each category are presented in more details in **Table 1**.

ubiquitous marker for isolating stem cells from heterogeneous amnion epithelial cells (Koike et al., 2014). A study suggested that hAECs, but not hAMSCs, express TRA1-60, TRA1-81, SSEA-3, and SSEA-4 (Zhou et al., 2013). Consistently, it has been reported that low or no protein levels of TRA1-60 and TRA1-80 were detected on hAMSCs (Roubelakis et al., 2012; Koike et al., 2014). A study reported that only a small percentage of hAMSCs expressed SSEA-4 at P0, which decreased during P2 (Magatti et al., 2016). In contrast, it has been reported that SSEA-4 was expressed among 43% of hAMSCs (Koike et al., 2014). There are also other studies that reported the expression of SSEA-3 and SSEA-4 among hAMSCs (Roubelakis et al., 2012; Samsonraj et al., 2017).

The expression of pluripotency markers including Oct3/4, Nanog and KLF-4 is higher among hAMSCs than other sources of mesenchymal stem cells (Koike et al., 2014). In a study, reverse transcriptase-PCR analysis exhibited transcripts of Oct-3/4 among both hAMSCs and hAECs; however, immunocytochemistry confirmed translation into Oct-3/4 protein by a sub-population of hAECs, but not among hAMSCs (Bilic et al., 2008). There are studies which reported pluripotency of hAMSCs with a high expression of pluripotency-specific genes including Nanog and OCT-4 among other pluripotency genes (Miki et al., 2005; Ge et al., 2012). It has been reported that the expression of pluripotency markers including SOX-2, Nanog, KLF-4, and c-MYC decreased during the culture of mesenchymal stromal cell (Chen et al., 2015).

It has been reported that hAECs express molecular markers that are known to be essential for self-renewal and pluripotency including OCT-4, Nanog, SOX-2, KLF-4 and REX-1 at first culture (P_0) and also during passages (Miki et al., 2005; Garcia-Castro et al., 2015). Term freshly isolated (P_0) hAECs express mRNA of OCT-4, SOX-2, CFC-1, Nanog, DPPA-3, PROM-1, and PAX-6, while the mRNA of the pluripotency markers FOXD3 and growth differentiation factor 3 (GDF3) were not detected among hAECs population (Ilancheran et al., 2007). A study quantified the expression of pluripotency surface and molecular markers in the first culture of hAECs and through the passages by quantitative real-time PCR and immunostaining. They reported that 10%, 17%, and 52% of hAECs were positive for OCT4, SOX2, Nanog at P_0, respectively. Therefore, it could be suggested that at least 10% of the isolated epithelial cells population from human amniotic membrane have pluripotency features which makes it an appropriate source for cells. The percentage of hAECs that expressed OCT-4, SOX-2, and Nanog did not change significantly during passages one to four (P_{1-4}). Furthermore, they measured the presence of E-cadherin (CD324) which was expressed by human pluripotent stem cells and demonstrated that almost 100% of hAECs were positive for E-cadherin. Protein expression of KLF-4 and transcription factor binding to IGHM enhancer 3 (TfE3) was high in P_0 culture of hAECs and increased significantly during the second passage (P_2) (Garcia-Castro et al., 2015). Therefore, although both hAECs and hAMSCs express embryonic stem cell, self-renewal, and pluripotency markers, the level of marker expression remains variable.

Epithelial Cell Markers

Studies indicate that hAECs express epithelial specific markers including pan-cytokeratin (CK) 1, 2, 3, 4, 5, 6, 7, 8, 10, 13, 14, 15, 16, 19, Carbohydrate Antigen (CA) 125, Mucin (MUC) 16, EpCAM (CD326), and E-Cadherin (Nanbu et al., 1989; Diaz-Prado et al., 2011; Pratama et al., 2011; Caruso et al., 2012). It has been reported that more than 98% of hAECs express E-Cadherin and CD73 (Centurione et al., 2018). Amniotic epithelial cells showed high expression of integrin and increasing expression of CK3 and CK19 during serial passages; however, the expression of CK1 and CK14 decreased during serial passages which suggests that hAECs may be differentiated during passages (Fatimah et al., 2010). Several studies reported negative expression of epithelial cell markers by hAMSCs (Koike et al., 2014; Si et al.,

TABLE 1 | Characterization of human amniotic membrane-derived cells.

	Human amniotic epithelial cells (hAECs)	Human amniotic mesenchymal cells (hAMCs)	References
Phenotype of cells	**Flat, cuboidal, and columnar epithelial cells**	**Plastic-adherent, spindle-shaped cells**	
Positive markers during passages			
Embryonic stem cell, self-renewal, and pluripotency markers	TRA1-60$^{\dagger\times}$, TRA1-81$^{\dagger\times}$, SSEA-3*, SSEA-4$^{*\times}$, OCT4$^{*\times}$, Nanog$^{*\times}$, SOX-2$^{*\times}$, SOX17$^{*\times}$, KLF-4$^{*\times}$, c-MYC$^{*\times}$, REX-1, CFC-1, DPPA-3, PROM-1, PAX-6, FOXD3, GDF3, TfE3	TRA1-60$^{\dagger\times}$, TRA1-81$^{\dagger\times}$, OCT3, OCT4$^{*\times}$, Nanog$^{*\times}$, SOX-2$^{*\times}$, SOX17$^{*\times}$, KLF-4$^{*\times}$, c-MYC$^{*\times}$	Miki et al., 2005; Ilancheran et al., 2007; Bilic et al., 2008; Ge et al., 2012; Roubelakis et al., 2012; Zhou et al., 2013; Koike et al., 2014; Chen et al., 2015; Garcia-Castro et al., 2015; Samsonraj et al., 2017
Epithelial cell markers	CK-1, CK-2, CK-3, CK-4, CK-5, CK-6, CK-7, CK-8, CK-10, CK-13, CK-14, CK-15, CK-16, CK-19, CA-125, MUC-16, CD326$^{\times*}$ (EpCAM), CD324* (E-Cadherin), CD73	CD324*, CD326$^{\times*}$ (EpCAM)	Nanbu et al., 1989; Diaz-Prado et al., 2011; Pratama et al., 2011; Caruso et al., 2012; Paracchini et al., 2012; Centurione et al., 2018
Mesenchymal cell markers	CD73*, CD271*, CD24†*, CD90†*, CD133*†, CD44$^{\dagger\times}$	CD73*, CD271*, CD24*, CD90*, CD133*, CD105$^{\times}$	Soncini et al., 2007; Murphy et al., 2010; Roubelakis et al., 2012; Iaffaldano et al., 2013; Sivasubramaniyan et al., 2013; Koike et al., 2014; Spitzhorn et al., 2017; Schmelzer et al., 2019
Human leukocyte antigens	HLA-A*, HLA-B* HLA-C*, HLA-DR*	HLA-A*, HLA-B*, HLA-C*	Bilic et al., 2008; Fatimah et al., 2010; Koike et al., 2014; Magatti et al., 2015, 2016; Pogozhykh et al., 2015
Specific cell lineage and functional markers	Apo-D, A2B5*, MMP-1, PDGF Receptor ß (CD140b), Musashi-1, Nestin*, Vimentin$^{*\times}$, PSA-NCAM, β-tubulin-III, Catecholamine, Norepinephrine, Dopamine, DOPAC, Choline acetyltransferase (ChAT), Acetylcholine, GATA-4*, Hepatocyte nuclear factor-3ß, AFP$^{\times}$, Albumin*, Glucose-sensing molecule (GLUT-2), Insulin, RCI*, Neurofilament proteins*, MAP2 kinase*, Microtubule-associated protein 2* (MAP2), Glial fibrillary acidic protein*, CNPase*, Myelin basic protein*, Galactocerebroside*, Atrial myosin light chain- 2* (MLC-2A), Ventricular myosin light chain- 2* (MLC-2V), Nkx 2.5*, α-actinin, collagen type II*, Osteocalcin*, Osteopontin*, ALP*, Type I collagen	CD133, Nestin*, Albumin*, α-fetoprotein$^{\times}$ (α-FP), Cytokeratin 18 (CK18), α1-Antitrypsin (α1-AT), Hepatocyte nuclear factor 4α (HNF4α), PDX-1, RCI*, A2B5*, Neurofilament proteins*, MAP2 kinase*, Microtubule-associated protein 2* (MAP2), Glial fibrillary acidic protein*, CNPase*, Myelin basic protein*, Galactocerebroside*, Atrial myosin light chain- 2* (MLC-2A), Ventricular myosin light chain- 2* (MLC-2V), GATA-4*, Nkx 2.5*, α-actinin, collagen type II*, Osteocalcin*, Osteopontin*, ALP*, type I collagen*	Sakuragawa et al., 1996, 2000, 2004; Elwan and Sakuragawa, 1997; Kakishita et al., 2000; Takahashi et al., 2001, 2002; Wei et al., 2003; Portmann-Lanz et al., 2006; Kim et al., 2007; Gomez Dominguez, 2008; Kong et al., 2008; Parolini et al., 2008; Tamagawa et al., 2008; Manuelpillai et al., 2011; Miki, 2011; Niknejad et al., 2012; Alcaraz et al., 2013; Fatimah et al., 2013; Koike et al., 2014; Garcia-Lopez et al., 2015; Sarvandi et al., 2015; Bollini et al., 2018; Centurione et al., 2018; Liu et al., 2018; Maymo et al., 2018
Other markers	CD1b,CD9*, CD10, CD13†, CD24, CD26, CD29*,CD31, CD34, CD46, CD49a, CD49b, CD49c, CD49d, CD49e*, CD49f, CD55, CD58, CD59, CD63, CD77, CD81,CD83, CD91, CD95, CD98, CD104, CD109, CD117$^{\times*}$, CD 133, CD142, CD144,CD146, CD147, CD151, CD164,CD166*, CD227, ABCG2/BCRP	CD9*, CD13*, CD27†, CD29*, CD31, CD49e*, CD54, CD166*, CD117$^{*\times}$, CD349, Vimentin$^{*\times}$, STRO-1, BMP-4	Gomez Dominguez, 2008; Fatimah et al., 2010; Murphy et al., 2010; Pratama et al., 2011; Zhou et al., 2013; Koike et al., 2014; Pozzobon et al., 2014; Magatti et al., 2015
Negative markers during passages	CD11,CD14*, CD31*, CD62, CD349, HLA-A2, VWF	CD3, CD14*, CD34, CD45, CD324 (E-cadherin), HLA-DR$^{\times}$, HLA-DP, HLA-DQ	Miki et al., 2005; Portmann-Lanz et al., 2006; Ilancheran et al., 2007; Wolbank et al., 2007; Bilic et al., 2008; Murphy et al., 2010; Pratama et al., 2011; Magatti et al., 2012, 2016; Koike et al., 2014; Alikarami et al., 2015; Si et al., 2015; Phermthai et al., 2017; Samsonraj et al., 2017

**Expressed on both hAECs and hAMSCs.*
† Low expression.
$^{\times}$ Discrepancy witnessed.

2015; Phermthai et al., 2017; Magatti et al., 2018); however, a study reported low level of expression of E-Cadherin (Iaffaldano et al., 2013). Another study on hAMSCs reported that CK19 was strongly positive at passage 0 and 1 and decreased to zero level at passage 6 (Gomez Dominguez, 2008). E-Cadherin has been used as a marker to prove epithelial contamination in the characterization of isolated hAMSCs (Mariotti et al., 2008).

Mesenchymal Cell Markers

There are studies which reported that hAMSCs expressed mesenchymal markers including vimentin, CD73, CD90, and CD105. Vimentin remained strongly positive at passage 0, 1 and 6 among hAMSCs (Roubelakis et al., 2012; Koike et al., 2014; Spitzhorn et al., 2017). The expression of CD73, CD90, and CD105 increased during passages and more than 95% of

hAMSCs expressed CD73, CD90, and CD105 from P2 to P4 (Samsonraj et al., 2017). A recent study confirmed that more than 90% of hAMSCs were positive for CD90 and CD73; however, they reported that CD105 was expressed only by 4% of hAMSCs (Schmelzer et al., 2019). Mesenchymal cell-related markers including CD24, CD133 and CD271 were positive on hAMSCs; however, the expression of CD271 remained controversial. A study identified mesenchymal stromal cells via CD271, while another study reported that only bone marrow-derived mesenchymal stromal cells among all types of mesenchymal stromal cells expressed CD271 (Soncini et al., 2007; Sivasubramaniyan et al., 2013). In addition, a study reported a lack of expression of CD271 on hAMSCs (Iaffaldano et al., 2013), while another study indicated that 50% of hAMSCs expressed CD271 (Koike et al., 2014).

Moreover, hAECs express mesenchymal stromal cell and mesenchymal cell-related antigens. Almost 69% and 38% of hAECs express CD73 and CD271, respectively (Zhou et al., 2013; Koike et al., 2014). The expression of CD24, CD90, and CD133 by hAECs was also observed (Fatimah et al., 2010; Zhou et al., 2013; Koike et al., 2014); however, <1% of hAECs expressed CD44 (Zhou et al., 2013; Koike et al., 2014). The expression of mesenchymal markers including vimentin and CD140-B increased on hAECs during passages, which is possibly suggestive of the epithelial-mesenchymal transition potential of hAECs (Miki and Strom, 2006; Pratama et al., 2011).

Hematopoietic Cell Markers

Almost none of the hAECs and hAMSCs express hematopoietic markers including CD14, CD34, and CD45 (Murphy et al., 2010; Koike et al., 2014; Si et al., 2015; Phermthai et al., 2017; Samsonraj et al., 2017). However, a study detected CD14, CD34 and CD45 on 10%, 3% and 17% of hAMSCs, respectively. There might be small colonies of CD14 and CD45-positive mesenchymal cells at P0, which could be attributed to human amniotic mesenchymal tissue cell contamination (Magatti et al., 2012, 2015, 2016).

Human Leukocyte Antigens

Despite hAECs and hAMSCs possess different morphology and marker expression, they have the same potential of modulating immunoreactions (Wolbank et al., 2007). The immunologic profiles of hAECs and hAMSCs showed that they both expressed very low levels of HLA A, B and C immediately after isolation (P0); however, the level of these antigens on hAECs increased significantly by P2 (Fatimah et al., 2010; Pogozhykh et al., 2015). Furthermore, a study reported that freshly isolated hAECs may express some type-I-MHC antigens including HLA-A, HLA-B or HLA-C as evaluated by a pan antibody against HLA-ABC (Magatti et al., 2015). It has been observed that hAECs displayed negligible expression of type II MHC including HLA-DR, DP and DQ (Fatimah et al., 2010), while the expression of HLA-DR on hAMSCs remained controversial. Although some studies reported negative expression of HLA-DR by hAMSCs, a study indicated that 14% of hAMSCs were HLA-DR-positive (Bilic et al., 2008; Koike et al., 2014; Magatti et al., 2016). Magatti et al. reported small groups of HLA-DR-positive hAMSCs at P0, as well. However, the level of HLA-DR deceased during P2 compared to P0 (Magatti et al., 2016).

It is worth mentioning that the low-level expression of type I HLA and the lack of expression of type II HLA markers alongside the expression of immune privileging HLA-G and co-stimulatory molecules including CD40, CD40 ligand, CD80 (B7-1), and CD86 (B7-2) on hAECs and hAMSCs demonstrates their potential immunomodulatory value in transplantation, which may enable them to be applied across the major histocompatibility barrier (Lefebvre et al., 2000; Chang et al., 2006; Banas et al., 2008; Parolini et al., 2008; Lim et al., 2013; Peric et al., 2018). In addition, hAECs and hAMSCs neither express the Programmed Death-1 (PD-1), an inhibitory receptor that is generally expressed on activated T and B cells, nor Programmed Death Ligand 1 and 2 (PDL-1/2) (Okazaki and Honjo, 2007; Wu et al., 2014).

Specific Cell Lineage Markers

Amniotic membrane cells include a heterogeneous population of stromal cells and precursor cells. These cells are clonogenic and their primary cultures could differentiate into specific cells of three germ lineages which express various markers of ectodermal, endodermal, and mesodermal cells (Miki et al., 2005).

Since hAECs and hAMSCs express specific markers of ectodermal cells derived neuronal, oligodendrocytes, and glial cells, they have shown potential for treating central nervous system disorders as well as having the capacity to produce and secrete neurotransmitters. In 2008, Sakuragawa et al. reported for the first time that these cells expressed high levels of neural cells specific antigens including RCI, A2B5, vimentin, Neurofilament proteins, microtubule-associated protein (MAP) 2, and MAP2 kinase. It has been reported that hAECs had a high expression level of neural specific markers including Musashi-1, Nestin, vimentin, PSA-NCAM, and β-tubulin-III (Kong et al., 2008). There are studies which reported the production of neurotransmitters related proteins in hAECs, including catecholamine, norepinephrine, dopamine, 3,4-Dihydroxyphenylacetic acid (DOPAC), choline acetyltransferase (ChAT) and acetylcholine (Elwan and Sakuragawa, 1997; Sakuragawa et al., 1997; Kakishita et al., 2000). They also showed high expression of glial cells specific markers including glial fibrillary acidic protein, CNPase, myelin basic protein, and Galactocerebroside (Sakuragawa et al., 2004; Portmann-Lanz et al., 2006; Kim et al., 2007; Tamagawa et al., 2008). In addition, it has been reported that fibroblast markers including matrix metallopeptidase 1 (MMP-1) and ApoD were expressed in freshly isolated hAECs; however, the expression level was low in comparison to adult fibroblasts (Koike et al., 2014). PDGF Receptor ß (CD140b) expression on freshly isolated hAECs (P0) alongside vimentin expression is negative; however, 90% of hAECs are positive for CD140b at passage 6 (P6) (Parolini et al., 2008; Miki, 2011; Alcaraz et al., 2013; Centurione et al., 2018). Hepatic markers including GATA-4 and hepatocyte nuclear factor-3ß were detected in amnion-derived epithelial cells by RT-PCR (Wei et al., 2003; Bollini et al., 2018). The expression of the hepatic markers and proteins including alpha fetoprotein (AFP) and albumin were high on both mRNA and

protein levels among freshly isolated hAECs (Takahashi et al., 2001, 2002; Liu et al., 2018; Maymo et al., 2018). Amniotic epithelial cells strongly expressed glucose-sensing molecule GLUT-2 on mRNA levels which is characteristic for beta cells of pancreas and hepatocytes (Wei et al., 2003; Garcia-Lopez et al., 2015). Furthermore, albumin synthesis and excretion by hAECs have been detected by immunostaining and enzyme-linked immunoassay (Sakuragawa et al., 2000). It has been shown that undifferentiated hAMSCs expressed genes associated with hepatocytes and pancreatic cells including albumin, AFP, CK18, α1-Antitrypsin (α1-AT), hepatocyte nuclear factor 4α (HNF4α) and the pancreatic lineage-associate marker PDX-1 (Manuelpillai et al., 2011; Sarvandi et al., 2015).

Although hAMSCs originate from avascular stromal layer of the amniotic membrane, they express endothelial and angiogenic markers including von Willebrand Factor (vWF), platelet/endothelial cell adhesion molecule (PECAM-1/CD31), vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor-2 (VEGFR-2), Fibroblast Growth Factor (FGF) and angiopoietin-1 (Fatimah et al., 2013). It has been reported PECAM-1, bFGF, eNOS, VEGF, VEGFR-2, and vWF expressions decreased during passage of hAMECs; however, angiopoietin-1 expression was increased. On the other hand, endothelial markers including PECAM-1 (CD31), E-selectin (CD62e), and vWF were almost negative on hAECs (Gomez Dominguez, 2008).

It has been reported that hAECs and hAMSCs express cardiac-specific genes including atrial myosin light chain- 2 (MLC-2A), ventricular myosin light chain- 2 (MLC-2V), GATA-4, and Nkx 2.5 in media supplemented with ascorbic acid. In addition, it has been observed that they expressed α-actinin, a mature cardiomyocyte marker, which was detected by Immunohistochemical analysis (Miki et al., 2005). A summary of characterization markers of human amniotic membrane-derived cells is presented in **Table 1**.

CAUSES OF DISCREPANCIES IN REPORTS ON PHENOTYPE AND MARKERS

Extensive research has focused on the possible applications of amnion-derived cells in the clinic. However, the discrepancies in the reports on characterization of hAECs and hAMSCs need to be considered to further pave the way for their clinical utilization. There are various possible causes for the current discrepancies in reports on characterization markers of hAECs and hAMSCs, which are mentioned here (**Figure 2**).

Passage Number and Epithelial to Mesenchymal Transition

Several studies indicate that the number of passages influence the marker expression on hAMSCs and hAECs. Like mesenchymal stromal cells from other sources, hAMSCs do not express HLA-DR in earlier passages (Pittenger et al., 1999; Covas et al., 2003; In 't Anker et al., 2003; D'Ippolito et al., 2004; Gotherstrom et al., 2004; Lee et al., 2004; Portmann-Lanz et al., 2006; Koike et al., 2014). However, a study on amniotic membranes without culturing reported expression of HLA-DR on hAMSCs (Kubo et al., 2001). Another study reported the expression of MHC antigens in the early passages of hAMSCs which disappeared in later passages (Kim et al., 2007). A suggested explanation for the reported discrepancy among studies has been the multiple passages of the cells which diminished the expression of HLA-DR (Kim et al., 2007).

Along with alterations in surface marker expressions, hAECs' morphology gradually changes toward mesenchymal phenotype over several passages (Bilic et al., 2008) and transmission electron microscopic studies of hAMSCs suggested an epithelial–mesenchymal hybrid phenotype (Pasquinelli et al., 2007). These observations are interpreted as epithelial to mesenchymal transition (EMT) (Portmann-Lanz et al., 2006; Bilic et al., 2008; Pratama et al., 2011). EMT occurs in the natural process of placental development, where extravillous cytotrophoblasts transit to mesenchymal phenotype which allows them to migrate and infiltrate into the maternal decidua and vessels. It has been shown that hAECs are initially negative for the mesenchymal marker vimentin; however, they become vimentin positive during the EMT process (Alcaraz et al., 2013). Along with the rise in vimentin, fibronectin, and *N*-cadherin levels (Guarino et al., 2009; Tsuji et al., 2009), E-cadherin protein levels which are high at early passages become undetectable in later passages which confirms EMT among the amniotic cells (Alcaraz et al., 2013). Although some studies consider CD73 to be a mesenchymal-specific marker (Brown et al., 2019), others introduce it as an epithelial-specific marker (Centurione et al., 2018). A recent study has suggested that CD73 mechanistically promotes the expression of EMT-associated genes, which could shed more light on the witnessed discrepancy regarding the role of CD73 in characterization of hAMSCs and hAECs (Lupia et al., 2018).

Cell Heterogeneity

Cell heterogeneity could be another explanation for the current discrepancies in reports on characterization of hAECs and hAMSCs. Amniotic membrane cell populations are seemingly heterogeneous and thus may differ in their phenotypic and molecular properties (Roubelakis et al., 2012; Niknejad et al., 2016). A study reported that among mesenchymal cells isolated from chorion, placental decidua, and amniotic membrane, the highest heterogeneity could be from those isolated from amniotic membrane. Miki et al. suggested that there might be various lineage-committed multipotent cells in the population of hAECs (Miki, 2011). The heterogeneity of hAMSCs and hAECs has been confirmed by immuno-phenotypic and morphological analysis (Araujo et al., 2017). Various levels of expression of pluripotency and proliferation markers in hAECs including OCT-4, CD117, SOX-2, a-fetoprotein, CREB, and p- CREB; various proliferation capability; and osteogenic potential could indicate their heterogeneity. In addition, Sakuragawa et al. demonstrated that hAECs express phenotype of both neural and glial cells (Sakuragawa et al., 1996). Heterogeneity also applies to functional molecules as well as growth factors secreted by hAECs and hAMSCs. Several studies have shown that amniotic membrane cells are capable of expressing erythropoietin (Ogawa

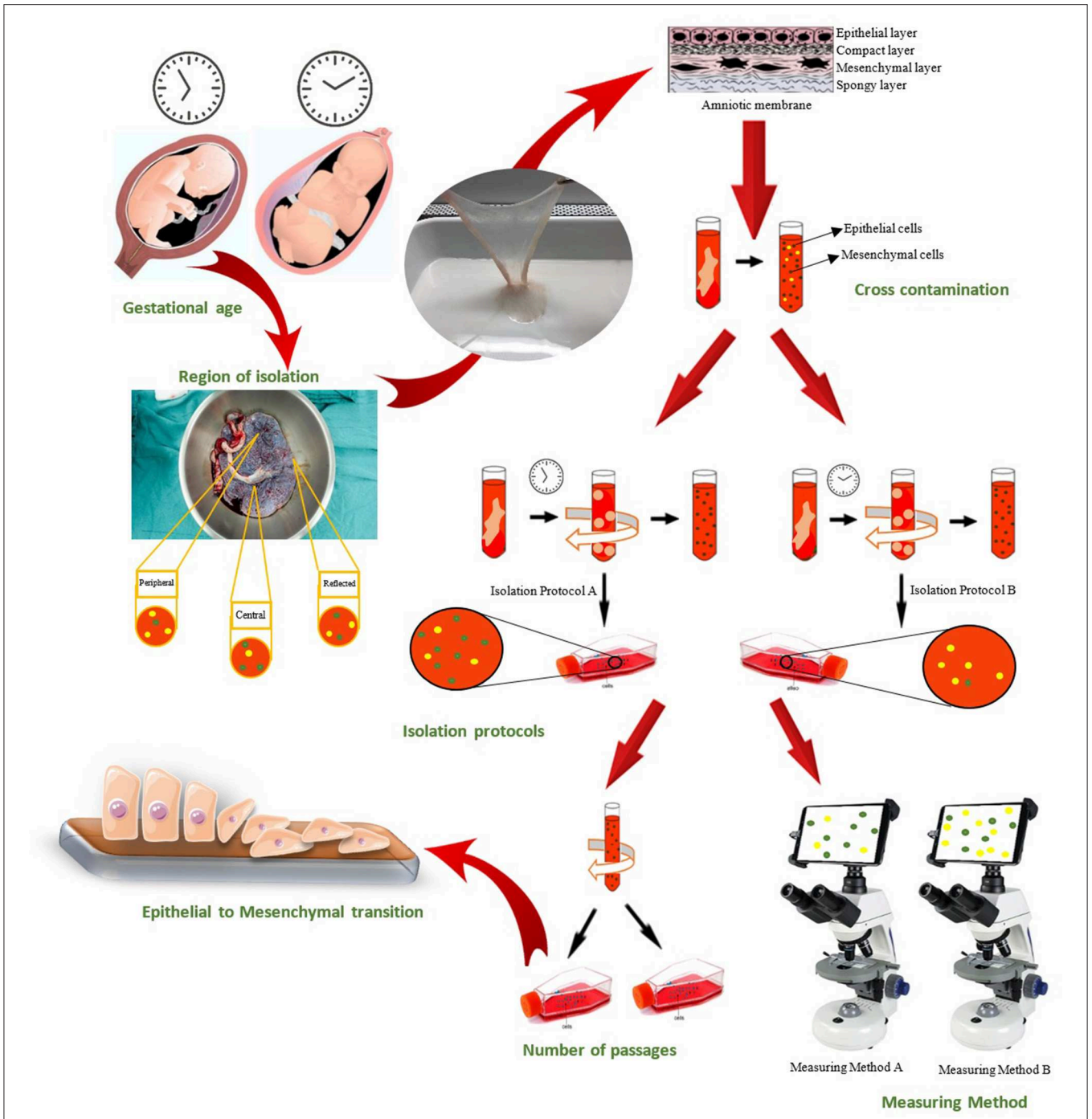

FIGURE 2 | Causes of witnessed discrepancies in characterization of human amniotic epithelial and mesenchymal stromal cells could be categorized in seven groups: Gestational age of placenta, Region of cell isolation on placenta, Cross-contamination of amniotic epithelial and mesenchymal stromal cell, Isolation protocol, Epithelial-to-mesenchymal transition of hAECs, Passage number of isolated cells, and Measuring methods that used for characterization.

et al., 2003), pulmonary surfactant (Lemke et al., 2017), dopamine (Niknejad et al., 2012), catecholamine (Sakuragawa et al., 1996) activin (Koyano et al., 2002), brain-derived neurotrophic factor, neurotrophin-3, and nerve growth factor (Uchida et al., 2000; Jin et al., 2015), all of which are involved in fetal early development.

Isolation Protocols and Cross-Contamination

Isolation of cells of human amniotic membrane could become challenging due to its histologic feature. hAECs and hAMSCs are located within layers adjacent to each other, which increases the

risk of simultaneous isolation and cross-contamination. Cross-contamination is defined as the contamination of hAECs with hAMSCs and vice versa rather than the pure population of the desired cells. Although the expression of CD117 in hAECs is almost always positive, it has been reported that hAMSCs are negative or weakly positive for CD117 (Bilic et al., 2008; Roubelakis et al., 2012; Magatti et al., 2016). While CD44 is a characteristic marker of mesenchymal stromal cells, a study reported that CD44 is a positive marker for the isolated hAECs (Roubelakis et al., 2012; Insausti et al., 2014). Furthermore, several studies for isolation of hAECs yielded cells that were positive for CD105, which is a defined mesenchymal stromal cells marker (Miki et al., 2010; Murphy et al., 2010, 2014; Tabatabaei et al., 2014). In a protocol for the isolation of hAECs more than 56 percent of cells were CD105 positive (Gramignoli et al., 2016). Simultaneous isolation of cells could be a possible cause for the witnessed discrepancy.

Several protocols have been proposed for isolation of hAECs and hAMSCs with a wide range of cells yielded, viability, and purity (Motedayyen et al., 2017; Araujo et al., 2018; Kitala et al., 2018). However, the protocol of isolation affects phenotype and function of the yielded cells. A study compared two protocols and reported that the isolated hAMSCs had some differences (Diaz-Prado et al., 2011). Soncini's protocol yielded hAMSCs by cutting amniotic membrane into small pieces; enzymatic digestion for 7 min by Dispase; resting period for 10 min; second enzymatic digestion by collagenase and DNase for 3 h, centrifuge at 200 g for 10 min; and culture (Soncini et al., 2007). Alviano's protocol is consisted of mincing; two enzymatic digestion of amniotic membrane by Trypsin/EDTA, collagenase and DNase for 15 and 5 min, respectively; centrifuge at 200 g for 10 min; and culture in DMEM with 20% FBS and 1% penicillin–streptomycin (P/E) (Alviano et al., 2007). The expression of CD117 marker was significantly higher in hAMSCs isolated by Soncini's protocol, which suggested that this protocol isolated more progenitor cells than Alviano's protocol. In addition, the expression of SSEA-4 and STRO-1 were higher among hAMSCs isolated from Soncini's protocol. The authors hypothesized that Soncini's protocol isolated cells in an earlier state of stemness (Diaz-Prado et al., 2011). Therefore, it seems that current protocols could potentially fuel simultaneous isolation and cross-contamination and hence are not yet eligible enough to ensure isolation of the desired cells.

Region of Cell Isolation on Placental Disk

Region at the amniotic membrane from which cells have been isolated may determine the characteristics of the cells (**Figure 2**). A study demonstrated that cells isolated from placental region had significantly higher mitochondrial activity while significantly fewer reactive oxygen species (ROS) (Banerjee et al., 2015). Centurione et al. isolated cells from four different regions according to their position relative to the umbilical cord. The first area, closer to umbilical cord, was named the central area; the second, in the middle, was considered the intermediate area; the third was named the peripheral area; and the fourth, the reflected area, corresponded to the chorion leave (Centurione et al., 2018). They reported that the peripheral and reflected areas had the highest levels of expression of OCT-4 and SOX-2. On the contrary, the expression of embryonic markers, SSEA-4 and TRA-1-60, was not different among the different areas which indicated homogeneity (Centurione et al., 2018).

Measuring Methods

Sensitivities of the methods employed to detect markers of hAECs and hAMSCs could also give rise to some discrepancies. A study used and compared immunocytochemistry and flow cytometry for SSEA-4 detection and reported that both hAECs and hAMSCs were positive for SSEA-4 in 100% of tested amnion samples as detected by flowcytometry. However, immunocytochemistry confirmed the expression of SSEA-4 on hAECs as well as hAMSCs only in 40% of samples. The authors hypothesized the higher sensitivity of flowcytometry vs. immunocytochemistry to be a possible explanation for this discrepancy (Bilic et al., 2008). Therefore, sensitivity and specificity of measuring methods should be considered not only upon their application but also while comparing the results of various studies.

The marker expression on gene and protein level should also be considered. A study reported that although Oct-3/4 transcripts were detected hAECs and hAMSCs, its protein was found only in hAECs by immunocytochemistry (Bilic et al., 2008). Therefore, it seems crucial that the level of expression, gene or protein, of a marker need to be considered while reporting comparing the results of studies.

Gestational Age

Human amniotic membrane is only easily obtainable after childbirth; therefore, there is limited information concerning the phenotypic and functional differences between cells isolated from amniotic membranes preterm and term cesarean sections. Gestational age is thought to have an effect on the expression of pluripotency markers of hAECs including Nanog, SOX2, TRA1-60, and TRA1-81 which have higher expression on hAECs isolated from preterm (17–19 weeks) than term cesarean sections (>37 weeks) (Izumi et al., 2009; Barboni et al., 2014). Although there is limited evidence on the effect of gestational age on the markers expressed on human amniotic cells, there are studies which investigated its effects among zoonotic samples. A study conducted on ovine amniotic epithelial cells reported that cells of amniotic membranes isolated at early stages of pregnancy expressed higher basal and sustained levels of telomerase reverse transcriptase (TERT), SOX2 and Nanog even after *in-vitro* adipogenic differentiation (Barboni et al., 2014). A study reported that there was no expression of TERT mRNA in hAECs isolated form term placenta which could be explained by a progressive switch off during pregnancy (Miki et al., 2005). In addition, telomerase activity in murine amniotic epithelia cells isolated from mid stage amniotic membrane was higher compared to that of the late stages (Nakajima et al., 2001). However, mRNA expression of OCT4 in human was not affected by gestational age (Izumi et al., 2009).

NEW INSIGHTS AND FUTURE DIRECTIONS

Herein, we reviewed and compared various studies to shed light on the existing discrepancies in characterization of human placenta-derived amniotic epithelial and mesenchymal stromal cells, which could be potentially due to reasons including epithelial to mesenchymal transition, cell heterogeneity, passage number, cross-contamination, region of cell isolation on placental disk, isolation protocols, measuring methods, and gestational age. The potential causes of discrepancies need further consideration prior to the application of these cells in the clinic. As an early step toward overcoming the challenges, some suggestions which could be of potential use in practice are discussed here.

Epithelial to mesenchymal transition could affect the function and marker characterization of the cells both in basic and clinical research. Although some factors involved in EMT have previously been described, including TGF-β (Alcaraz et al., 2013), TNF-α, and matrix metalloproteinases (Janzen et al., 2017), the whole mechanisms of EMT remain unclear to date. EMT needs to be considered for the subcultures aimed to prepare hAECs for research and clinical use. Some studies employed different methods to avoid EMT. A study used xenobiotic-free medium for the culture of hAECs to eliminate the potential effects of growth factors (Pratama et al., 2011). Although the use of EMT-inhibitors could be of value in minimizing the risk of EMT, their potential adverse effects on the cells need to be investigated for safe clinical use. The application of hAECs in their first passage culture for primary cell therapy could keep the occurrence of EMT to a minimum among cells. Nevertheless, the use of the cells in earlier passages, despite being seemingly useful in minimizing EMT, could adversely affect heterogeneity which is another concern involved in the witnessed discrepancies. Heterogeneity decreases during the culture of both hAECs and hAMSCs. In a study, hAECs downregulated dopaminergic markers after seven days of culture, probably through the dedifferentiation process, which resulted in reduced cell heterogeneity (Niknejad et al., 2012). Therefore, defining certain standards of the controlled sub-culture could eliminate heterogeneity as well as EMT.

Simultaneous isolation and cross-contamination of two cell types are among causes of discrepancies which have also been reported in tissues with similar histology to amniotic membrane including cornea with the possibility of simultaneous isolation of endothelial cells with stromal keratocytes, and skin with the possibility of simultaneous isolation of keratinocyte and fibroblasts. Therefore, it is assumed that the methods used to solve the problems in those tissues could be of value in amniotic membrane. A study used antifibroblast magnetic microbeads to deplete the majority of the contaminating corneal fibroblasts (Peh et al., 2012). The skin explant technique, physical agitation with magnetic stirring, density gradient centrifugation, gravity-assisted cell sorting based on a passive filtration of keratinocytes resulted in the propagation of a highly enriched keratinocyte population (Dragunova et al., 2012; Mahabal et al., 2016). It could be suggested that more innovative isolation techniques are required to isolate hAECs form one side of the amniotic membrane and hAMSCs from the other side in a separate manner.

The functional variety of cells isolated from different regions can be considered for further specific clinical use. The cytoplasm of hAECs isolated from the peripheral area contained the highest level of lipid granules. Centurione et al. suggested that this area could be the most capable of immune modulatory effects (Centurione et al., 2018) since the granules have been associated with prostaglandin E secretion by amniotic membrane (Kang et al., 2012; Park et al., 2016). Moreover, hAECs in the central area expressed higher levels of α-fetoprotein compared to other regions. Consequently, an enriched population of cells isolated from this region has the potential to be applied in hepatic differentiation (Centurione et al., 2018). The region of isolation needs to be determined based on the target clinical features of desired cells. Notably, not all regions of the amniotic membrane are suitable for clinical use. The zone of altered morphology, located near the lower uterine pole and cervix, is associated with apoptosis of cells and degradation of basement membrane by matrix metalloproteinases which results in structural weakness and marked disruption of the connective tissue layers and marked reduction of the thickness and cellularity of the amniotic membrane (Peirovi et al., 2012).

Gestational age, another cause for discrepancy, is a clinical term used by obstetricians that is timed from the first day of the last menstrual period in weeks and days. This clinical age differs by approximately 2 weeks from the time of fertilization used by embryologist in basic research. Therefore, the difference in the actual age of the membrane due to various definitions of gestational age could give rise to discrepancy. Therefore, researchers need to make sure that they have the same definition of GA and use it in a united way to avoid discrepancy. It is of importance to notice that preterm amniotic membrane could not be clinically used due to ethical and practical considerations. The amniotic membranes which could be used in the clinic need to be obtained from elective caesarian sections, which are referred to those caesarian sections that are not associated with any medical or surgical indications and have been conducted as per mother's request (Diema Konlan et al., 2019). Normal uncomplicated pregnancies usually reach term and thus preterm placenta of normal pregnancies are not accessible for clinical use. Preterm labors are usually associated with underling diseases or conditions and thus the amniotic membranes obtained from these births are not the clinicians first choice due to possible defects. Noteworthy, epithelial and mesenchymal stromal cells derived from preterm animal placenta is appropriate for mechanistical investigations and research use.

Considering the witnessed discrepancies in the characterization of hAECs and hAMSCs markers and their potential causes along with the promising results of clinical applications of these cells, more research is needed to address the sensitivity and specificity of markers in their characterization as well as determining the most suitable isolation marker(s) for hAMSCs and hAECs characterization. Until optimal approaches for overcoming the potential undesirable effects of above-mentioned causes of discrepancies are achieved, it is suggested that the passage number of cells mentioned in the study, isolation

protocols, region of isolation, and gestational age be stated in the articles and any future products with clinical applications.

AUTHOR CONTRIBUTIONS

S-HG, MA-K, and HN: conceptualization. SH-G, MA-K, HN, and TT: writing—original draft. HN and SB: writing—review, editing, and supervision. HN: resources.

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Biomarker Signatures of Quality for Engineering Nasal Chondrocyte-Derived Cartilage

M. Adelaide Asnaghi[1†], Laura Power[2†], Andrea Barbero[1], Martin Haug[3], Ruth Köppl[4], David Wendt[1] and Ivan Martin[1,2]*

[1] Department of Biomedicine, University Hospital Basel, University of Basel, Basel, Switzerland, [2] Department of Biomedical Engineering, University of Basel, Basel, Switzerland, [3] Department of Surgery, University Hospital Basel, Basel, Switzerland, [4] Otorhinolaryngology, Head and Neck Surgery, University Hospital Basel, Basel, Switzerland

The definition of quality controls for cell therapy and engineered product manufacturing processes is critical for safe, effective, and standardized clinical implementation. Using the example context of cartilage grafts engineered from autologous nasal chondrocytes, currently used for articular cartilage repair in a phase II clinical trial, we outlined how gene expression patterns and generalized linear models can be introduced to define molecular signatures of identity, purity, and potency. We first verified that cells from the biopsied nasal cartilage can be contaminated by cells from a neighboring tissue, namely perichondrial cells, and discovered that they cannot deposit cartilaginous matrix. Differential analysis of gene expression enabled the definition of identity markers for the two cell populations, which were predictive of purity in mixed cultures. Specific patterns of expression of the same genes were significantly correlated with cell potency, defined as the capacity to generate tissues with histological and biochemical features of hyaline cartilage. The outlined approach can now be considered for implementation in a good manufacturing practice setting, and offers a paradigm for other regenerative cellular therapies.

Keywords: regenerative medicine, engineered cartilage, perichondrium, identity/purity, potency, quality controls, advanced therapy medicinal product, good manufacturing practice

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***Correspondence:**
Ivan Martin
ivan.martin@usb.ch

[†] These authors have contributed equally to this work

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INTRODUCTION

Large cartilage defects in adults have limited capacity to regenerate, and state-of-the art regenerative medicine therapies do not induce reproducible or stable results (Kon et al., 2009; Teo et al., 2019). We previously demonstrated the safety and feasibility of autologous nasal chondrocyte-derived engineered cartilage for the treatment of focal traumatic lesions in the knee in a phase I clinical trial (Mumme et al., 2016), and a phase II clinical trial is ongoing to investigate efficacy. Briefly, autologous nasal chondrocytes are expanded *in vitro* before seeding onto a collagen I/III scaffold and cultured in chondrogenic conditions to produce a mature, hyaline-like cartilage graft that is then implanted into the knee cartilage defect of the same patient.

The starting material for this approach is a biopsy from the native nasal septum cartilage, which, like articular cartilage, is a hyaline cartilage (Al Dayeh and Herring, 2014) composed predominantly of water, type II collagen, glycosaminoglycan (GAG) containing proteoglycans, and the one cell type, chondrocytes (Buckwalter and Mankin, 1998). Mucoperichondrium is the tissue that overlays nasal cartilage; it consists of several layers, including mucosa, lamina propria, and perichondrium, the tissue directly adjacent to the cartilage that is tightly attached and cannot be easily distinguished

(Aksoy et al., 2012). Currently, nasal septal cartilage and mucoperichondrium are separated by pulling them apart with forceps and are identified based on their physical characteristics. Due to donor-related and operator-related variability, the resulting biopsy may not be completely pure after cleaning, with some overlaying tissue remaining attached to the cartilage.

When working with intrinsically variable donor-derived human materials, such as tissues and cells, establishing the quality and consistency of the starting material is key to ensuring reproducibly high quality engineered products, not least to avoid the consequences of cell misidentification (Editorial, 2009; Hyun-woo, 2019). This prompts for the development of identity and purity assays (Carmen et al., 2012), which can be based on various characteristics of the cells, such as gene or protein expression. Gene expression markers have been proposed for articular cartilage cell identity and purity assays (Mollenhauer and Gaissmaier, 2010; Bravery et al., 2013; Committee for Medicinal Products for Human Use [CHMP], 2017; Diaz-Romero et al., 2017). However, until now, there are no known biomarkers that can distinguish the cell types found in nasal cartilage biopsies. Moreover, the impact of possibly contaminating cells on nasal chondrocyte-based engineered cartilage has not been investigated.

Engineered products treat diseases or damage through repairing, replacing, or regenerating tissues or organs (Detela and Lodge, 2019). A potency assay must be developed based on the mode of action of the tissue engineered product (Committee for Medicinal Products for Human Use [CHMP], 2008)–in our case, the filling of cartilage defects with healthy, hyaline-like tissue–which is ideally correlated to the efficacy, leading to consistent quality of the tissue engineered product and good clinical outcome (Bravery et al., 2013). Gene expression markers have been investigated for human articular chondrocytes (Dell'Accio et al., 2001; Rapko et al., 2010), but not for cells from the nasal septum.

In this study, we first investigate which cell types are potentially contaminating in human nasal septum cartilage biopsies and their impact on the quality of engineered cartilage. We then investigated whether gene expression analysis could discriminate the contaminant cells found in nasal septal biopsies for the development of a characterization panel for identity and purity quality controls. To assess potency, we compared the gene expression of nasal septum biopsy-derived cells to their ability to produce cartilaginous tissue. Finally, we propose how these purity and potency assays could be implemented in a good manufacturing practice (GMP) compliant process for the translation of our regenerative therapy product.

MATERIALS AND METHODS

Cell Isolation and Expansion

Human nasal septum biopsies were collected from 17 donors (9 female, 8 male, mean age 46 years, range 16–84 years) undergoing reconstructive surgery after informed consent and in accordance with the local ethical commission (EKBB; Ref.# 78/07). Two samples derived from patients enrolled in the Nose2Knee clinical trials (ClinicalTrials.gov, number NCT01605201 and number NCT02673905).

For four donors, the biopsy was dissected to give a pure nasal cartilage sample (NC) and a pure perichondrium sample (PC).

Nasal chondrocytes were isolated from NC by enzymatic digestion as previously described (Jakob et al., 2003) with 0.15% collagenase II (Worthington) for 22 h at 37°C. After digestion, NCs were plated in tissue culture flasks at a density of 1×10^4 cells/cm^2 and cultured in medium consisting of *complete medium* [Dulbecco's Modified Eagle's Medium (DMEM)] containing 4.5 mg/mL D-glucose and 0.1 mM non-essential amino acids, 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.29 mg/mL L-glutamine (all from Invitrogen). Complete medium was supplemented with 1 ng/mL transforming growth factor beta-1 (TGF-β1) and 5 ng/mL fibroblast growth factor-2 (FGF-2) (both from R&D Systems) at 37°C and 5% CO_2 in a humidified incubator (Thermo Scientific Heraeus) as previously described (Jakob et al., 2003). When approaching 80% confluence, cells were detached using 0.05% trypsin-EDTA (Invitrogen) and re-plated.

Perichondrium tissue samples were cut in small pieces and put on the bottom of plastic culture dishes to isolate adherent cells that migrated out of the tissue for 1 week in complete medium. Cells were then detached using 0.05% trypsin-EDTA and further cultured until confluence in the same conditions as nasal chondrocytes.

Specific ratios of NC to PC cells were combined at passage two to generate mixed populations of *known* purity of 100, 90, 80, 70, 60, and 0% NC.

For all other biopsies, in case perichondrium was present, half was dissected, removing all perichondrium to obtain a NC, while the overlaying perichondrium remained intact on the other half (labeled as NC + PC samples, containing variable numbers of NC and PC cells). Cells were isolated from each sample by enzymatic digestion and expanded in complete medium supplemented with TGF-β1 and FGF-2 up to two passages as described above for nasal chondrocytes.

Proliferation Rate

Proliferation rates were calculated as the ratio of $\log_2 (N/N_0)$ to T, where N_0 and N are the numbers of cells respectively at the beginning and at the end of the expansion phase, $\log_2 (N/N_0)$ is the number of cell doublings, and T is the time required for the expansion.

Chondrogenic Redifferentiation

Micromass Pellets

Cells expanded until passage two were redifferentiated by culturing as 3D micromass pellets, as previously described (Asnaghi et al., 2018). 3D micromass pellets were formed by centrifuging 5×10^5 cells at $300 \times g$ in 1.5 mL conical tubes (Sarstedt) and cultured for 2 weeks in chondrogenic serum-free medium consisting of DMEM containing 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.29 g/mL L-glutamine, 1.25 mg/mL human serum albumin (CSL Behring), and 100 nM

dexamethasone (Sigma, Switzerland), supplemented with 10 ng/mL TGF-β1 (R&D), ITS + 1 (10 μg/mL insulin, 5.5 μg/mL transferrin, 5 ng/mL selenium; Gibco), 100 μM ascorbic acid 2-phosphate (Sigma), and 4.7 μg/mL linoleic acid (Sigma). Culture medium was changed twice weekly.

Engineered Cartilage on Chondro-Gide

Passage two cells were seeded on collagen type I/III membranes (Chondro-Gide; Geistlich Pharma AG) at a density of 4.17 million cells per cm^2. The resulting constructs were cultured for 2 weeks in chondrogenic medium consisting of complete medium supplemented with 10 μg/mL insulin (Novo Nordisk), and 0.1 mM ascorbic acid 2-phosphate (Sigma) at 37°C and 5% CO_2 with media changes twice/week.

The described protocols match the ones used in the context of the clinical trial, where GMP-grade reagents and autologous serum instead of FBS are used. Grafts for clinical use are produced at the GMP facility at the University Hospital Basel according to standard operating procedures under a quality management system, as described in Mumme et al. (2016).

Histology and Immunohistochemistry

Samples were fixed overnight in 4% formalin and embedded in paraffin. Sections 5 μm in thickness were stained with safranin O for GAGs and hematoxylin as a nuclear counterstaining as described elsewhere (Grogan et al., 2006). Immunohistochemistry against collagen type I (No. 0863170, MP Biomedicals, 1:5000) and collagen type II (No. 0863171, MP Biomedicals, 1:1000) was performed using the Vectastain ABC Kit (Vector Labs) with hematoxylin counterstaining as in standard protocols (Scotti et al., 2010). Incubation of tissues with only the secondary antibody were used as negative controls.

Histological scoring via the modified Bern score (MBS) was performed on safranin O-stained histological images as previously described (Lehoczky et al., 2019), as adapted from Grogan et al. (2006). Briefly, the MBS has two rating parameters that each receive a score between 0 and 3. First, the intensity of safranin-O staining (0 = no stain; 1 = weak staining; 2 = moderately even staining; 3 = even dark stain), and second, the morphology of the cells (0 = condensed/necrotic/pycnotic bodies; 1 = spindle/fibrous; 2 = mixed spindle/fibrous with rounded chondrogenic morphology; 3 = majority rounded/chondrogenic). The two values are summed together resulting in a maximum possible MBS of 6.

qPCR

We chose the gene expression markers to investigate based on a literature search. Interested in both purity and potency assays, we focused on matrix associated genes considering the two cell types potentially present in our starting material derive from tissues with structurally different ECM. The gene expression ratios of collagen II to I and aggrecan to versican are well-known chondrogenic markers (Martin et al., 2001). HAPLN1 has been found in most types of cartilage (Spicer et al., 2003), including in bovine nasal cartilage (Baker and Caterson, 1978). Versican protein expression has been found in perichondrium from other cartilage tissue sources (Shibata et al., 2001) and nestin has been shown to be expressed in embryonic perichondrium (Ono et al., 2014). MFAP5 is found in elastic as well as non-elastic extracellular matrixes (Halper and Kjaer, 2014) and has been used as a negative marker for chondrogenic cells from articular cartilage (Rapko et al., 2010).

Total RNA was extracted from expanded cells at both P1 and P2, 3D micromass pellets, and engineered cartilage grafts with the Quick RNA Miniprep Plus Kit (Zymo Research) and quantitative gene expression analysis was performed as previously described (Martin et al., 2001). Reverse transcription into cDNA was done from 3 μg of RNA by using 500 μg/mL random hexamers (Promega, Switzerland) and 0.5 μL of 200 UI/mL SuperScript III reverse transcriptase (Invitrogen). Assay on demand was used with TaqMan Gene Expression Master Mix to amplify type I collagen (Col I, Hs00164004), type II collagen (Col II, Hs00264051), aggrecan (Agg, Hs00153936_m1), Versican (Ver, Hs00171642_m1), link protein 1 (HAPLN1, Hs00157103_m1), MFAP5 (MFAP5, Hs00185803_m1), nestin (Nes, Hs00707120_s1), and GAPDH (GAPDH, Hs00233992_m1) (all from Applied Biosystems). The threshold cycle (C_T) value of the reference gene, GAPDH, was subtracted from the C_T value of the gene of interest to derive ΔC_T values. All displayed gene expression levels are, and statistical analyses were performed on, the ΔC_T values. GAPDH was found to be a stable reference gene for both nasal chondrocytes and perichondrial cells with a mean ΔC_T value of 18.1 (standard deviation of 0.68) at passage 2 and 22.6 (standard deviation of 0.80) for pelleted cells across both cell types.

Biochemical Quantification of GAG and DNA

Samples of engineered cartilage and micromass pellets were digested with proteinase K (1 mg/mL proteinase K in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide, and 10 mg/mL pepstatin A) for 16 h at 56°C. The GAG content was determined as previously described (Barbosa et al., 2003). Briefly, samples were incubated with 1 mL of dimethylmethylene blue assay (DMMB; Sigma-Aldrich 341088) solution (16 mg/L dimethylmethylene blue, 6 mM sodium formate, 200 mM GuHCL, pH 3.0) on a shaker at room temperature for 30 min. Precipitated DMMB-GAG complexes were centrifuged and supernatants were discarded. Complexes were dissolved in decomplexion solution (4 M GuHCL, 50 mM Na-acetate, 10% propan-1-ol, pH 6.8) at 60°C, absorption was measured at 656 nm and GAG concentrations were calculated using a standard curve prepared with purified bovine chondroitin sulfate. DNA content was measured using the CyQuant Cell Proliferation Assay Kit (Invitrogen) according to the instructions of the manufacturer.

Modeling

The generalized linear modeling (glm) function in R was used to build all the models. A logistic regression model was used to predict purity, where the response is a continuous probability between 0 (pure perichondrium) and 1 (pure cartilage) with samples from four donors and 48 independent experiments of known purities. For the logistic regression models, the

McFadden pseudo R^2 values were calculated with the pscl R package (Jackman, 2017) and the Hosmer–Lemeshow analysis was performed with the ResourceSelection R package (Lele et al., 2019). For the potency assay predicting GAG production, a gamma GLM with a log link was used to model quantified amounts of GAG (measured in μg). The MBS of chondrogenic pellets was modeled by first dividing the value by six, the maximum possible score, then training a multiple logistic regression model; the predicted responses were then multiplied by six. Samples from nine donors in 28 independent experiments were used to train the MBS potency assay and 25 independent experiments were used to train the GAG potency model and for gene selection. For all three assays, stepwise selection (Agostini et al., 2015) was performed in both directions; collagen II and I, aggrecan, versican, HAPLN1, and MFAP5 were tested and the model with the lowest Akaike information criterion (AIC) was chosen. Samples from five donors in 12 independent experiments were used to test the potency models. Residual plots were used to verify all the models. The correlation between the predicted and actual purity, GAG, and MBS values were calculated with the square of the Pearson correlation coefficient. The final equations of the potency models were rebuilt with both the training and test data together.

Statistical Analysis

All calculations were performed using standard functions, unless otherwise stated, in R (R Core Team, 2019). Statistical significance is defined as $p < 0.05$. Statistical significance for comparing two means was calculated using paired or unpaired *t*-tests and normality was checked with the Shapiro–Wilk test. To test multiple comparisons, a linear model was fitted, then the glht function of the multcomp R package (Hothorn et al., 2008) was used to test all the contrasts; *p*-values were corrected for multiple testing using the single-step Bonferroni method. Correlation plots using Spearman correlation coefficients (ρ) were created with the corrplot R package (Wei and Simko, 2017). Data are presented as mean and standard deviation of independent experiments with cells from at least 4 different donors. For each analysis at least 2 replicate micromass pellets were used per condition. Symbols used are: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, and $p < 0.1$.

RESULTS

Native Nasal Septum Biopsy Characterization

In the context of ongoing clinical trials, the nasal septum biopsy is harvested along the subperichondrial axis, so that most of the perichondrium remains in place in the patient's nose, not only an efficient risk-control measure, but also important for the stability and healing of the donor site. More heterogeneous samples are obtained from plastic surgeries unrelated to clinical trials, which include mixed cartilage and perichondrium. Safranin O staining of nasal septum specimens indicated the presence of tissues with distinct characteristics, i.e., GAG-rich cartilage with round chondrocytes residing in lacunae and adjacent GAG-negative perichondrium containing cells with fibroblast-like morphology, comparable to previous findings (Bairati et al., 1996). Immunohistochemical analysis showed more collagen II in the cartilage and more collagen I in the perichondrium, confirming previously reported results (Popko et al., 2007). The border between the two tissues is not clearly defined in our samples, as in previous reports (Bairati et al., 1996; **Figure 1A**).

The separation of the cartilage and overlaying tissue is done by pulling them apart with forceps; however, the efficiency of this technique is unknown. Histological analysis after physical separation of cartilage and perichondrium revealed that the resulting biopsy may have small amounts of safranin O-negative tissue on the cartilage after separation (**Figure 1B**). This safranin O-negative region includes cambium, which is hypothesized to be the source of cells with tissue forming capacity (Upton and Glowacki, 1981; Van Osch et al., 2000), and sometimes perichondrium that is difficult to remove (Hellingman et al., 2011). Deeper cleaning of the starting biopsy (e.g., via scraping or cutting with a scalpel) is not a suitable option, since we observed reduced cell yield and slightly lower chondrogenic capacity in preliminary experiments, supporting the theory that this superficial region contains more potent cells.

Characterization of Perichondrial Cells

The samples we classify as NC and PC are the tissues after separation using the aforementioned technique. Visually, under macroscopic observation during expansion in cell culture dishes, NC and PC cells are not distinguishable, both having the same characteristic fibroblastic-like cell morphology. The proliferation rates of the two cell types were measured and found to be about equal (**Figure 2A**). To compare the chondrogenic capacity of NC and PC cells, we engineered pellets and found that NCs could reproducibly produce GAG and collagen II while PCs could not and predominantly produced type I collagen, as seen by histological analyses and biochemical quantification (**Figures 2B,C**).

Identity Assay

We sought to distinguish the cells from these two tissues based on their gene expression profiles. NC cells expressed significantly higher levels of type II collagen and relative ratios of collagen II:I, aggrecan:versican and, at passage two, HAPLN1:MFAP5; whereas PC cells expressed significantly higher levels of versican, MFAP5, and nestin (**Figure 3**). Expanded cells were then cultured as 3D micromass pellets in chondrogenic conditions for two more weeks. NC cells from engineered pellets expressed significantly more collagen II and higher ratios of collagen II:I, aggrecan:versican, and HAPLN1:MFAP5, and PC cells expressed significantly higher levels of versican and MFAP5 (**Supplementary Figure S1**). In summary, these results demonstrate that nasal chondrocytes and perichondrial cells have statistically significant differential expression of cartilage-related genes both during expansion and after pellet culture.

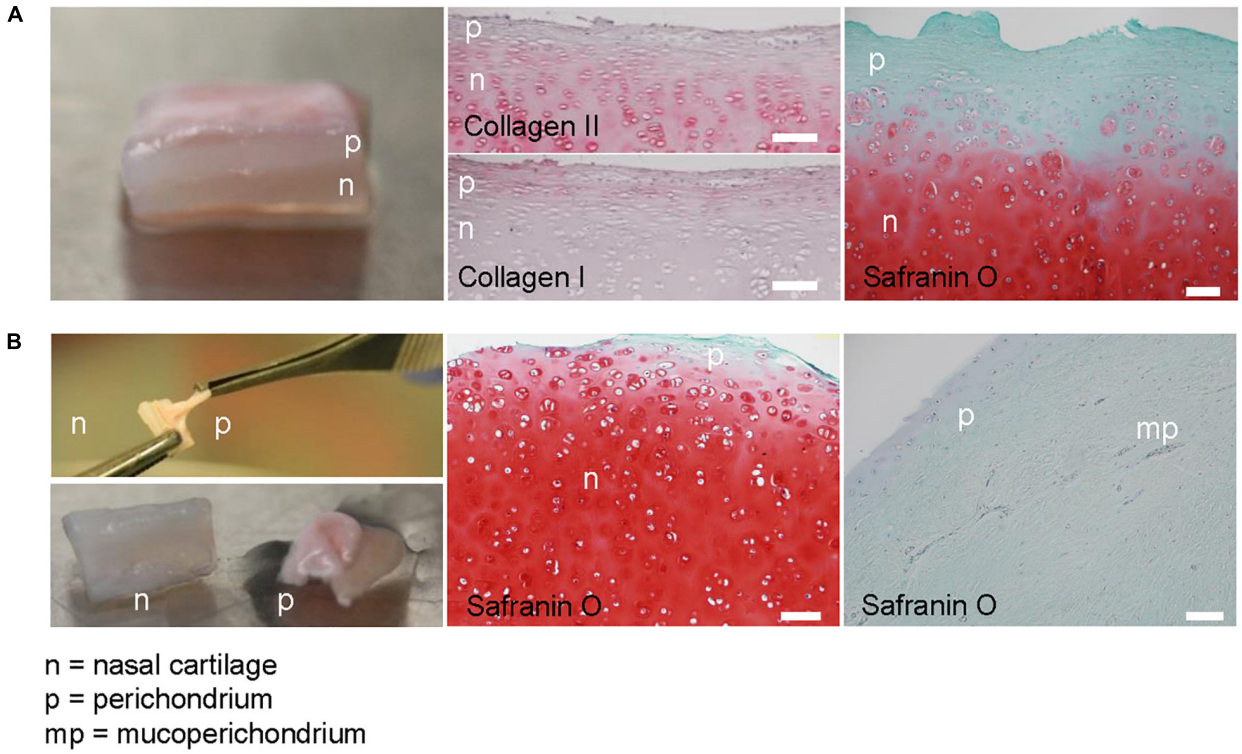


FIGURE 1 | Native nasal septal cartilage and overlaying tissue. **(A)** Photograph of native nasal cartilage with overlaying tissues. Safranin O-stained histological image, and collagen types I and II immunohistochemical images. **(B)** Photograph of the forceps separation technique and resulting separate nasal cartilage (n) and perichondrial tissue (p). Safranin O-stained histological images of separated nasal cartilage and perichondrial tissues. Scale bars are 100 μm.

Purity Assay

The only method currently available to assess the purity of the starting native cartilage biopsy is by manually counting the number of cells in each type of tissue in a histological image (**Supplementary Figure S2**). This method suffers from limitations due to histological artifacts, unclear distinction between tissue types, its semi-quantitative and destructive nature, and the fact that a histological section may not be representative of the whole tissue. Here we assessed if the purity of a *mixed* cell population could also be estimated based on gene expression analysis.

Spearman correlation coefficients (ρ) of the gene expression of cells at passage two that we combined at specific ratios of NC and PC cells revealed statistically significant trends across donors. Due to high donor-to-donor variability, the correlations between cell population purity and gene expression were higher per donor per gene than across donors. The highest correlation was found for the relative expression of aggrecan:versican (ρ = 0.69), where the ratio was higher in purer populations containing more NCs; per donor the correlations were even stronger (ρ = 0.61–0.98) (**Supplementary Figure S3A**).

In general, more significant differences in gene expression in individual genes and cell purity were seen at passage two compared to the pelleted cells' gene expression (**Supplementary Figure S3B**). Therefore, we focused on passage two for the subsequent purity model.

We performed multiple logistic regression to compare gene expression of collagen type I and II, aggrecan, versican, MFAP5, HAPL1, and nestin to the cell population purity. To gain insight into which genes were most important, stepwise selection (Agostini et al., 2015) was implemented and the model with the lowest AIC was chosen. Versican and collagen type II were found to be the factors most predictive of purity and significantly contributed to the model (p-value = 2.7e-3, p = 2.8e-3, respectively, and overall, the model was significant (Hosmer–Lemeshow p = 0.95 and McFadden pseudo R^2 = 0.53). The coefficient estimates from the model and the ΔCt values for versican and collagen II can be used to estimate the purity of a population of nasal cartilage-derived cells using Eq. 1, where inverse logit is exp (x) / [1 + exp (x)].

$$\text{Purity (NC\%)} = \text{inverse logit}\left[1.93 + (\text{Col2}) \times 0.64 - (\text{Ver}) \times 1.08\right] \quad (1)$$

The purity predicted by the model was plotted against the known purity and the resulting R^2 value of the observed and predicted values was 0.79 (**Figure 4**).

Potency Assay

We investigated whether predictive gene expression markers can be used to estimate the capacity of the cells to form engineered

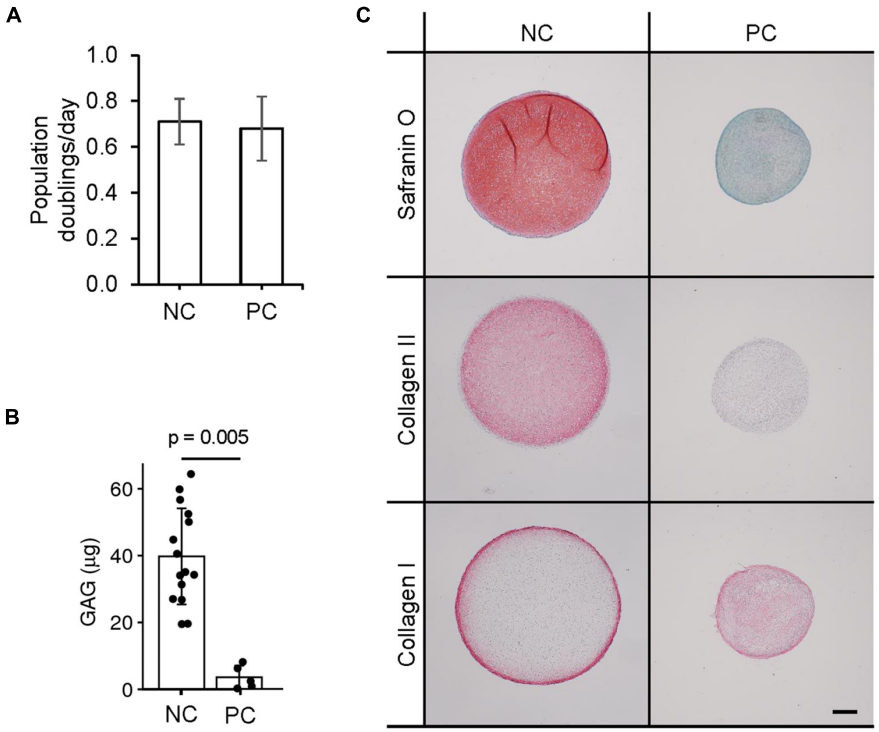


FIGURE 2 | Chondrogenic capacity of perichondrial cells. **(A)** Proliferation rates of nasal chondrocyte (NC) and perichondrial cells (PC). **(B)** Biochemical quantification of NC and PC chondrogenic pellets. *t*-test *p*-value displayed. **(C)** Safranin O staining and immunohistochemical staining of pellets engineered from nasal chondrocytes (NC) and perichondrial cells (PC). Scale bar is 200 μm.

cartilage. The final cartilage quality is currently assessed using the MBS, a semi-quantitative score of safranin O-stained histological images (Grogan et al., 2006; Lehoczky et al., 2019), and via GAG quantification (Thej and Kumar Gupta, 2019).

GAG content as well as the histological MBS score of the chondrogenic pellets were positively correlated to the cartilage identity gene expression markers and negatively correlated to the perichondrial identity markers (**Supplementary Figure S4**). The interrelationship of potency and purity is visualized in the top left corner of the correlation plot, which shows that purity (NC%), GAG, GAG/DNA, and MBS are highly correlated (**Figure 5**).

More significant gene expression trends were seen when analyzing the cells at passage two compared to after engineered pellet culture, so we developed a potency assay for this time point.

In order to develop a potency assay that could predict the amount of GAG in the final engineered cartilage based on the gene expression of the starting cell population, we trained a generalized linear model with a log-link and gamma distribution. The gamma distribution was selected because it only predicts positive values and because its distribution is flexible enough to fit many response shapes (Hardin and Hilbe, 2007). To select which gene expressions could best predict GAG produced by cells culture as pellets, stepwise selection was performed. Collagen II and MFAP5 were found to be the most significant and the model showed good results (training R^2 = 0.34 and testing R^2 = 0.78 of observed vs. predicted values; **Figure 6A**). The equation of the potency assay to predict the amount of GAG produced via the gene expression of passage two cells (Eq. 2), where the ΔCt values of the genes should be entered, was generated using both the test and training data together, to report the most accurate coefficient estimates possible.

$$\text{Potency}\,[\text{GAG}\ (\mu\text{g})] = \exp\left[2.55 + (\text{Col2}) \times 0.06 - (\text{MFAP5}) \times 0.14\right] \quad (2)$$

For a potency assay that can predict the histological score of the final engineered cartilage from passage two gene expression, a logistic regression model was trained. Stepwise selection found that the best model included only the MFAP5 gene, and showed good predictive ability in this dataset (training R^2 = 0.54 and testing R^2 = 0.64 of observed vs. predicted values; **Figure 6B**). The equation of the potency assay to predict the histological MBS score (Eq. 3), where the ΔCt value of the gene should be used, was generated with both the training and test data together. Again, the inverse logit is exp (x) / [1 + exp (x)], imposing upper and lower bounds on the model.

$$\text{Potency}\,(\text{histological score, MBS}) = 6 \times \text{inverse logit}\,[-0.84 - (\text{MFAP5}) \times 0.21] \quad (3)\ (4)$$

Implementation of In-Process Controls

Since isolated nasal septum-derived cell populations may include some perichondrial cells, we tested the impact of various amounts

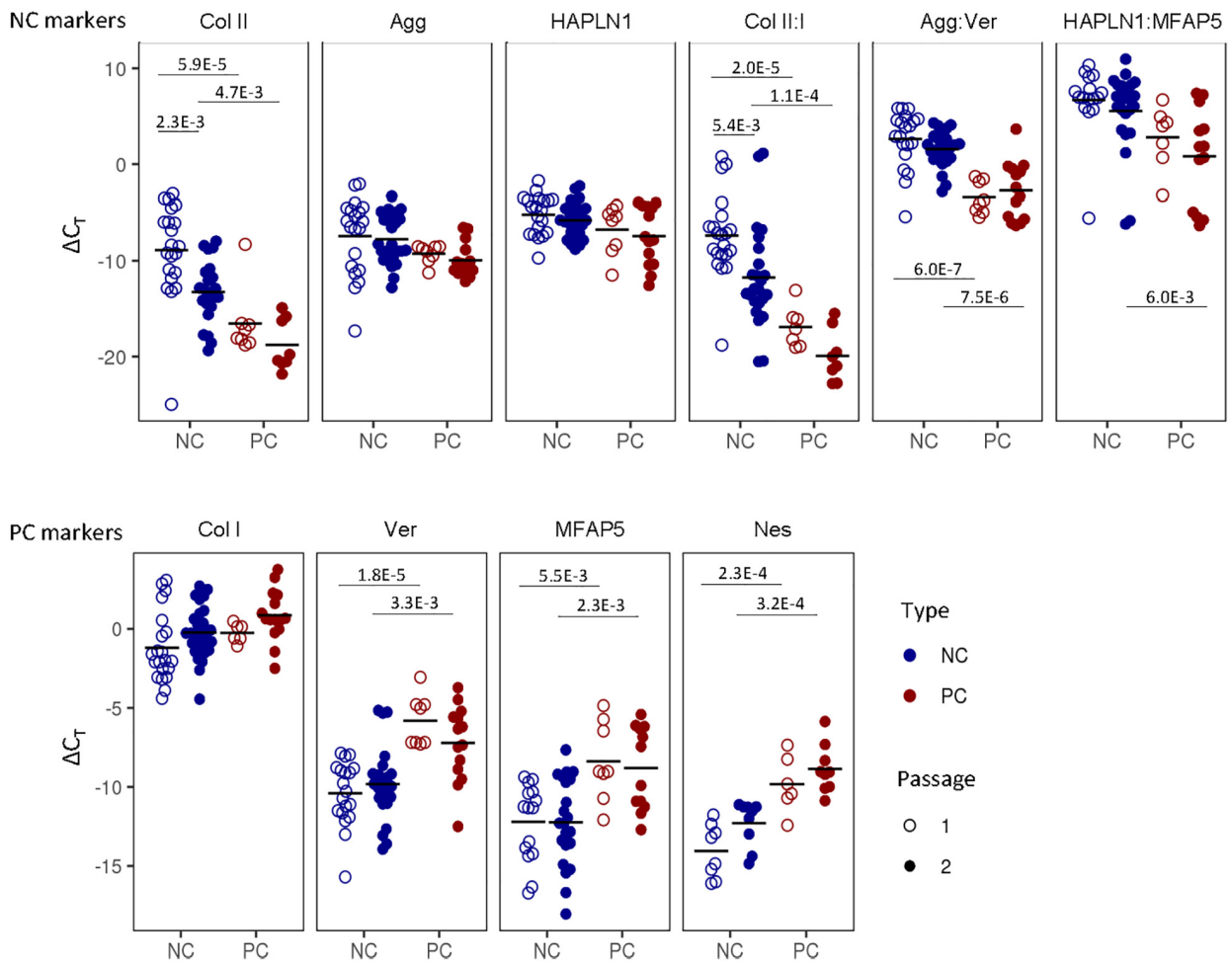
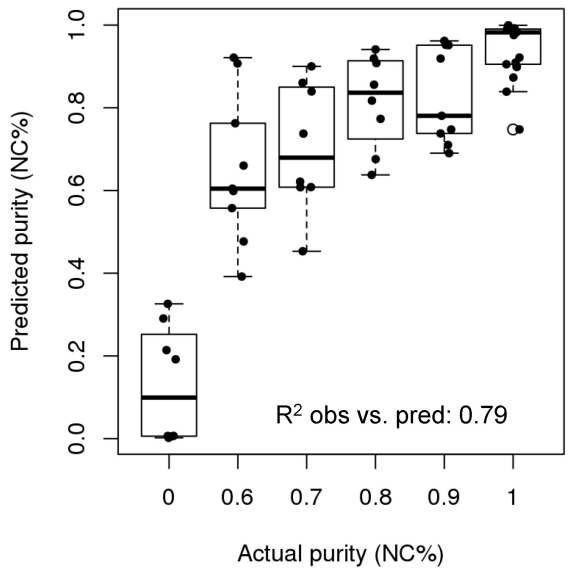


FIGURE 3 | Nasal chondrocyte and perichondrial cell identity. Gene expression comparisons between pure nasal chondrocyte (NC) and pure perichondrial cell (PC) populations at passage one and two. The fold change expression relative to GAPDH is displayed. Bonferroni multiple comparison corrected *p*-values are displayed.

of contaminating cells on final engineered cartilage quality. The chondrogenic capacity of contaminated cell populations was consistently lower than of pure cell populations, as observed across 15 donors, demonstrated by safranin O staining, GAG quantification, and immunohistochemical analysis of collagen types II and I (**Supplementary Figure S5**). We confirmed the negative effect of perichondrial cells on the engineered cartilage not only in pellet culture, but also when produced according to the clinical trial protocol where cells are seeded onto a collagen I/III scaffold (**Supplementary Figure S6**).

A threshold of acceptable purity needs to be set to guarantee the quality of the final product. Known quantities of NC and PC cells were mixed together and chondrogenic pellets were produced. Histological scoring was then used to set acceptable limits of PC cell contamination so that the quality of the final product would still meet the clinical trial release criteria (MBS $\geq$ 3). Due to donor-to-donor variability, potential cross-contamination from the mechanical tissue separation method, and considering the limitations of histological analysis, we show that some donors could still produce cartilage matrix of sufficient quality with up to 40% PC contamination, while the less potent donors could produce cartilage matrix with a PC contamination of up to 30% PC cells (**Figure 7A**).

Using the purity and potency assays we developed, the quality was estimated based on the gene expression of passage two cell populations for clinical trial samples and for heterogeneous biopsies collected from patients that underwent plastic surgeries with variable amounts of overlaying perichondrium. The predicted histological score results closely matched the actual values, and the quantified amounts of GAG could be estimated well, predicting if cells would produce high or low amounts of GAG (**Figure 7B**). The clinical trial starting materials were assessed to be pure. The potency assay predicted good chondrogenic capacity, confirmed by the high quality of the engineered cartilage produced in the clinical trial. The quality of the grafts also correlated to a good clinical outcome at the 24-month follow up examination, as demonstrated by a significant increase in KOOS scoring, where patients report their symptoms, pain levels, knee function, ability to do sport, and quality of life (Mumme et al., 2016). The more heterogeneous



	Beta est.	SE	p	Sig.
Intercept	1.93	1.37	1.6e-1	
Col II	0.64	0.21	2.7e-3	**
Ver	-1.08	0.36	2.8e-3	**

FIGURE 4 | Purity assay. Purity assay results. Multiple logistic regression model based on the expression of collagen II and versican to estimate cell purity (NC%).

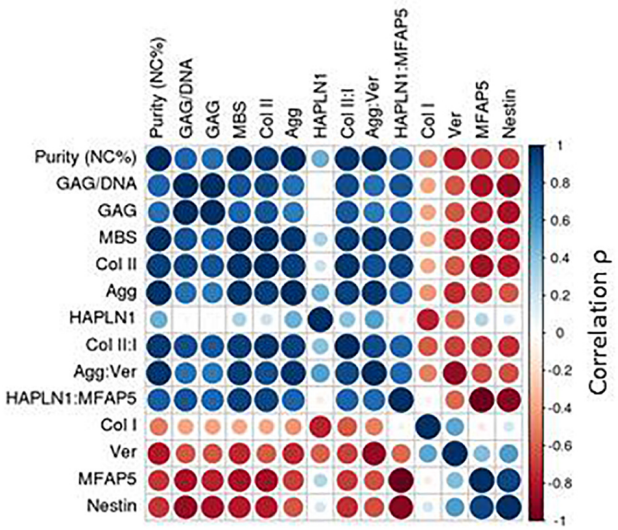


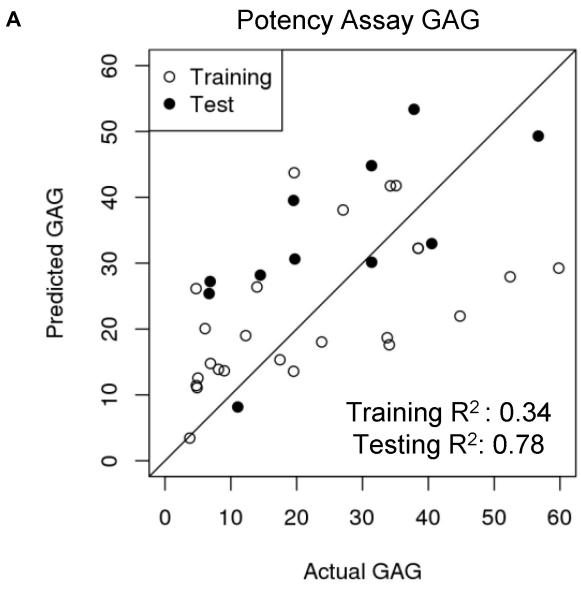
FIGURE 5 | Correlation plot with passage two gene expression. Spearman correlations (ρ) depicted for passage two gene expression.

cartilage samples from plastic surgery procedures had more variable results. The purity assay predicted the worst sample to have a purity of 20%, many samples to be 99% pure, and the mean purity of mixed samples to be 75% (**Figure 7C**). Consistent with the established purity threshold, cells that were predicted to be >70% pure were all able to produce cartilaginous tissues that passed the histological score release criteria. The sample with a predicted purity of 20%, on the other hand, produced a pellet that failed the release criteria (histological score = 2.3).

DISCUSSION

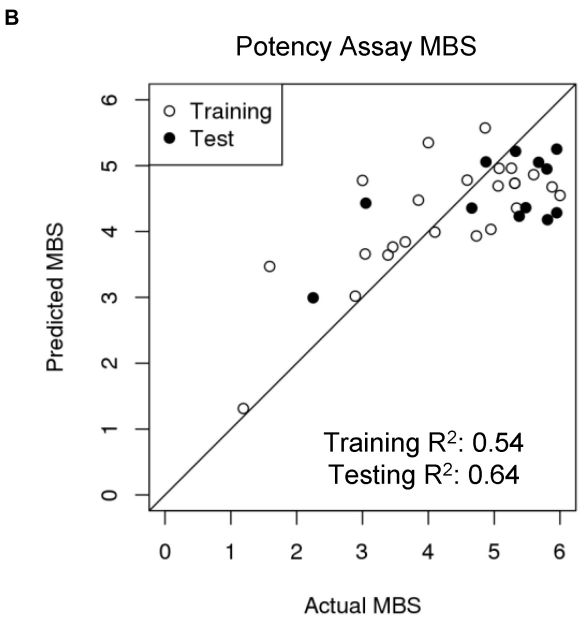
In this study, we established novel in-process controls to ensure the quality and standardization of nasal chondrocyte-based engineered cartilage grafts. Histological analysis revealed that nasal septal cartilage may be harvested with some adjacent tissue, and that there may still be fragments of perichondrial tissue overlaying the cartilage even after a trained operator further separates the tissues. Although some researchers claim that perichondrial cells from other cartilage sources have chondrogenic potential (Hellingman et al., 2011), we discovered that unlike chondrocytes, nasal septal perichondrial cells do not have the capacity to form GAG- and collagen type II-rich engineered tissues. We found that increasing amounts of perichondrium in the starting material profoundly decreases the quality of engineered cartilage, as seen by less GAG and collagen II production during chondrogenic culture. Therefore, minimal contamination of perichondrial cells must be ensured. The NC identity marker we found is collagen II, and the PC identity markers were, versican, MFAP5, and nestin. To quantitatively determine the percentage of contaminating cells in a population, we developed a model that correlates the expression of multiple gene expression markers to the purity of a cell population. Similarly, to predict the chondrogenic capacity of a cell population, we built models to estimate GAG production and the final histological MBS score in engineered cartilage. Finally, we discuss how such quality controls could be implemented during the production of cell or tissue therapies.

In practice, quantitative reverse-transcription polymerase chain reaction (qPCR) instrumentation is ubiquitous, so a gene expression-based quality control could be easily implemented. The cost of the quality control assay could be reduced by selecting a handful of genes for a standard qPCR analysis compared to transcriptomic analysis or single-cell RNA sequencing, and for a



Final model coefficient estimates

	Beta est.	SE	p	Sig.
Intercept	2.55	0.55	4.6E-05	***
Col II	0.06	0.03	8.8E-02	.
MFAP5	-0.14	0.03	1.2E-05	***



Final model coefficient estimates

	Beta est.	SE	p	Sig.
Intercept	-0.84	1.15	0.46	
MFAP5	-0.21	0.12	0.08	.

FIGURE 6 | Potency assay. **(A)** Generalized linear model with a gamma distribution and log-link to predict GAG. The estimated model coefficients, standard errors (SE), and significances are calculated with the training and test data combined. **(B)** Multiple logistic regression model to predict MBS. The estimated model coefficients, standard errors (SE), and significances are calculated with the training and test data combined.

routine test could be enough information to confirm cell identity (Maertzdorf et al., 2016).

To implement such gene expression-based quality controls, a suitable time point during the manufacturing process must be chosen. Biomarkers vary not only spatially within the tissue, but also temporally during monolayer expansion and after tissues are engineered (Tay et al., 2004; Späth et al., 2018; Detela and Lodge, 2019), and it also may be that the cells have more distinct gene expression profiles at certain time points than others (Tekari et al., 2014). From a practical perspective, an earlier quality control would save costs, because the quality of the cells could be established before an expensive production is undertaken. However, after 1 or 2 weeks of cell expansion, there are many more cells and an aliquot can be taken without depleting the whole cell population and the gene expression analysis of an aliquot of a cell suspension provides a broad readout of the total cellular material. Obtaining cells before they are embedded in the scaffold would allow to perform the analysis non-destructively. Interestingly, despite passage two corresponds to variable numbers of population doublings and thus to different degrees of cell de-differentiation, the biomarkers we investigated had the most distinct expression levels after the expansion phase.

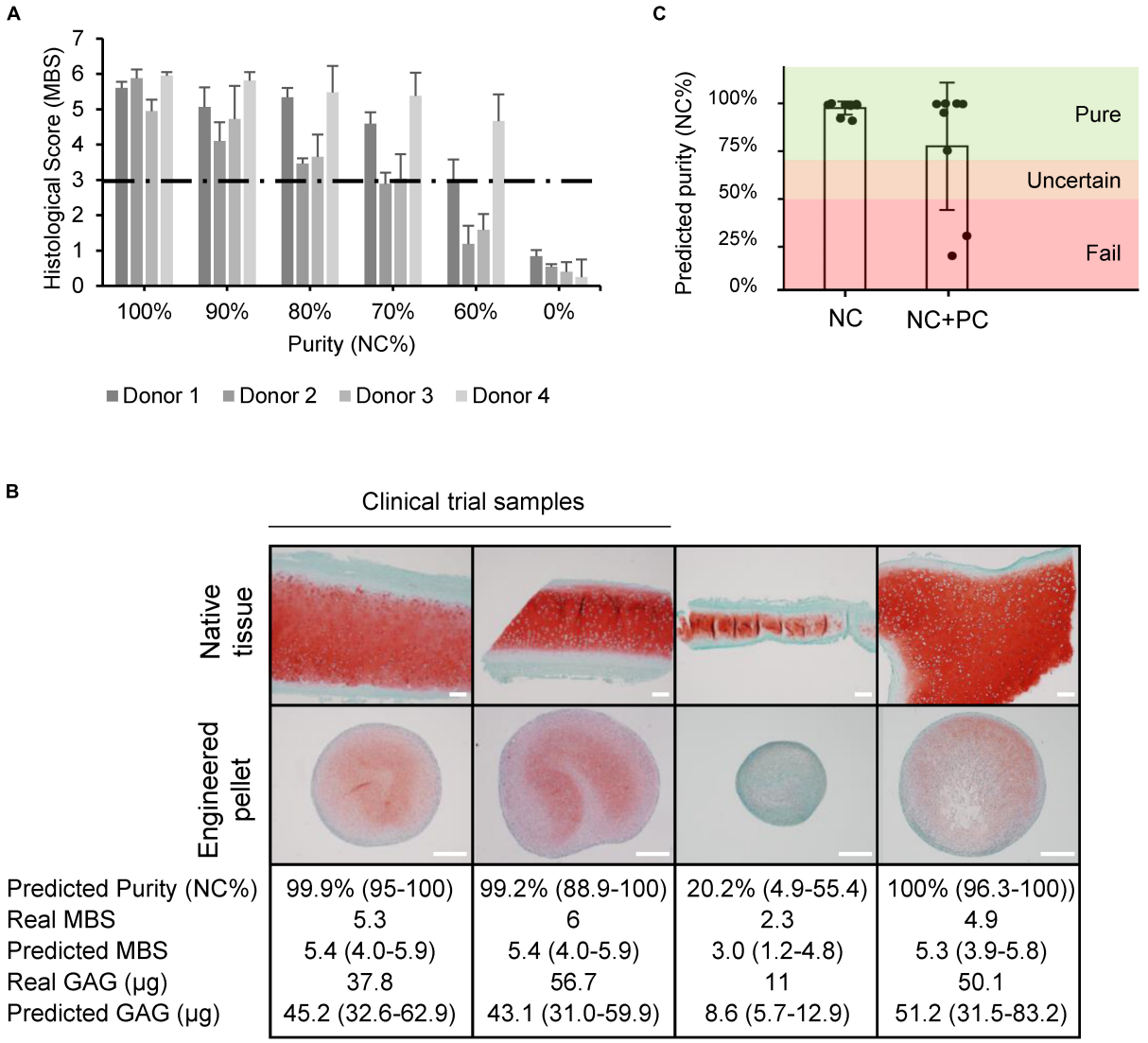


FIGURE 7 | Quality estimation and in-process control implementation. **(A)** Histological scores (Modified Bern Score) of engineered pellets derived from specific starting population purities. **(B)** Safranin O stained native and engineered cartilage harvested for a clinical trial study or for other purposes. The predicted purity and 95% CI, real MBS, predicted MBS and 95% CI, real amount of GAG, and predicted GAG with 95% CI. Two of the samples were produced from samples deriving from clinical trials. Scale bars are 200 µm. **(C)** Predicted purities of pure (NC) and mixed (NC + PC) biopsies.

Consequently, we propose that our gene expression-based assays should be implemented on expanded passage two cells.

Generalized linear models for the development of gene expression-based quality controls for regenerative medicine is a natural extension of their use in biomarker-based disease diagnosis (Faraway, 2006; Hosmer et al., 2013). Here we show how multiple logistic regression can be used to model purity percentages with the advantage of being able to provide biologically relevant estimates, i.e., between 0 and 100% (Zhao et al., 2001). When further screening the most significant genes that contributed to the purity model with stepwise selection (Ying et al., 2018; Liu et al., 2019), we found the combination of collagen II and versican expression to be predictive, a relatively uncommon gene pair compared to the often studied gene expression ratios of collagen II:I and aggrecan:versican. The selection appears reasonable, with one chondrocyte marker, collagen II, and one perichondrium marker, versican, used in the model and being inversely related to each other.

We showed how logistic regression can be used to estimate the histological score, a value bounded between 0 (worst) and 6 (best). Estimating GAG required modeling positive values only, therefore, we demonstrated how a generalized linear model with a gamma distribution and log-link could be implemented, similarly to other biomarker applications (García-Broncano et al., 2014; Schufreider et al., 2015). Stepwise selection was used again for the potency models, returning the combination of collagen II and MFAP5 for prediction of GAG, and MFAP5 alone for modeling histological MBS score. Increased MFAP5 expression

has been correlated with decreased chondrogenic potential in mesenchymal stem cells (Solchaga et al., 2010). MFAP-5 protein binds active TGFβ1, TGFβ2, and BMP2, sequestering these pro-chondrogenic factors in the matrix (Combs et al., 2013). Intracellular MFAP5 has been shown to bind and activate notch signaling (Miyamoto et al., 2006), which inhibits the regulator of cartilage formation, Sox9 (Hardingham et al., 2006). Notch has been found primarily in the perichondrium rather than the cartilage layer in mandibular condylar cartilage (Serrano et al., 2014), which, like nasal cartilage, is derived from cranial neural-crest cells (Chai et al., 2000). The predictive ability of these models are significant especially when considering that only ∼30–40% of the variance in protein abundance is explained by mRNA levels (Vogel and Marcotte, 2012). The selection of different genes for each potency assay may be due to the fact that they assess quality in slightly different ways; the histological score includes information not only of the GAG content, but also about the morphology of the cells.

We observed that all pellets that contained at least 70% NC cells pass the clinical trial release criteria, i.e., histological score ≥3, but more contamination could also lead to good results in some cases. To implement the purity assay, we propose a conservative three-category rating scale for the predicted purity (NC%), i.e., if cells are estimated to be more than 70% pure, they are labeled as *pure*, less than 50% pure, they are labeled as *fail*, otherwise the estimation is labeled as *uncertain*. We propose to introduce this uncertain region for the time being until further data can be collected and the estimates can be made more precise. In practice, we would recommend that starting cell populations labeled as pure or uncertain should continue in the production process, however, if the cells fail the purity test, the costly production should be halted. Cartilage engineered from cells of uncertain purity would nevertheless need to pass the release criteria (such as the histological score-based release criterial), ensuring the quality of the product.

The proposed models have been generated based on a limited number of genes. In future, it will be valuable to widen the panel of genes analyzed, based on other published studies (Dell'Accio et al., 2001) or more extensive unbiased transcriptomic analysis. The selected genes and coefficient estimates for the models will have to be updated as more data are obtained, and the in-process controls will have to be validated to meet GMP standards. Only then the models could be actually implemented as in-process control and release criteria, predicting if an engineered graft would pass or fail according to revised cut-off thresholds. Production would be stopped if the acceptance thresholds are not met. Moreover, the definition of a high quality graft may need to be revised as more long-term clinical outcome data are collected.

CONCLUSION

In conclusion, we have put forward gene expression-based assays for identity, purity, and potency to help ensure the safe and effective clinical use of nasal chondrocyte-derived engineered cartilage. More generally, we provide an example of the development and implementation of purity and potency assays based on relatively simple qPCR assays, stepwise selection of the most significant genes, and predictive *in silico* models. This approach could be relevant for the development of quality controls for other products in the emerging field of regenerative medicine, one of the biggest challenges for advanced therapy medicinal products to overcome for clinical translation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Human nasal septum biopsies were collected from 17 donors (9 female, 8 male, mean age 46 years, range 16–84 years) after informed consent and in accordance with the local ethical commission (EKBB; Ref.# 78/07). Two samples derived from patients enrolled in the Nose2Knee clinical trials (ClinicalTrials.gov, numbers NCT01605201 and NCT02673905).

AUTHOR CONTRIBUTIONS

MA conceived and designed the study. MA and LP performed the experiments, analyzed the data, and wrote the manuscript. LP created the models. AB contributed to the study design and revised the manuscript. MH and RK contributed to the sample preparation. DW contributed to manuscript revision. IM contributed to compiling the data and critically revised 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/fbioe.2020.00283/full#supplementary-material

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REVIEW
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Surgery Versus ATMPs: An Example From Ophthalmology

Federica M. Magrelli[1†], Alessia Merra[1†] and Graziella Pellegrini[1,2]*

[1] Holostem Terapie Avanzate S.r.l., Modena, Italy, [2] Stefano Ferrari Regenerative Medicine Center, University of Modena and Reggio Emilia, Modena, Italy

Advanced therapy medicinal products (ATMPs) are the new frontier of medicine. Advanced therapy medicinal products are set out to satisfy unmet medical needs and provide new innovative, cutting-edge therapies for serious or life-threatening diseases, thus providing new therapeutic options for people with few or no possibility of treatment. They are divided into four groups including gene therapy medicinal products, cell-based therapy medicinal products, tissue-engineered products, and combined ATMPs, which in Europe refer to products that incorporate one or more medical devices with any of the previously mentioned ATMPs as part of the advanced medicine product (AIFA, 2017; Ten Ham et al., 2018). Advanced therapy medicinal products can potentially have long-term benefits, thus bringing a long-lasting positive impact on patient health. Advanced therapy medicinal product therapies are often administered just once or twice, which gives patients the possibility to heal quickly compared to traditional therapies. They also provide a long-term saving opportunity, both in terms of costs of treatments and procedures that are no longer necessary and in terms of quality of life and productivity. The resolution of the patient's illness has a monetary impact on the patient, the patient's caretakers, and especially on the society (Alliance for Regenerative Medicine, 2019). The aim of this paper was to provide an overview on the use of ATMPs approved in Europe, with a focus on blindness and visual impairment and the related economic burden. In this case study, the effective cost of a blind patient in different European countries was compared after treatment with ATMPs or traditional therapies, focusing on visual impairment caused by corneal opacity. Our evaluation includes an overview of the global economic impact of the two types of therapies on the society. We estimated direct healthcare costs, direct non-healthcare costs, and labor productivity losses, to include costs on healthcare, services, patients, their families and for the society in general. We could conclude that the costs of the two therapeutic approaches are comparable.

Keywords: ATMP, surgery, ophthalmology, cost of treatment, public–private health care

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Ivan Martin,
University of Basel, Switzerland

Reviewed by:
Giovanni Migliaccio,
Consorzio per Valutazioni Biologiche e Farmacologiche, Italy
Mark W. Lowdell,
Royal Free London NHS Foundation Trust, United Kingdom

***Correspondence:**
Graziella Pellegrini
grzllpellegrini@gmail.com

† These authors have contributed equally to this work

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INTRODUCTION

Advanced therapy medicinal products (ATMPs) have unique attributes which differentiate them from standard pharmaceuticals and biologics. Indeed, ATMPs have curative potential as they address underlying genetic or cellular mechanisms of disease, which means that they can have a dramatic and long-lasting positive impact on health. They act through multiple mechanisms and on different cellular targets.

ATMPs are often administered just once or a handful of times within a short period. However, as they are typically paid as a one-time treatment, they have a high up-front cost. They are complex products and thus, have also difficult manufacturing processes, often requiring highly specialized manufacturing equipment, processes, and skills. Many cell-based gene therapies, for example CAR-T therapy used to treat some blood cancers, are individually manufactured for each patient: cells are collected from the patient's blood using a process called apheresis, they are modified and expanded in the laboratory, and then re-infused into the patient several hours later. These processes are usually carried out by trained individuals in specialized centers.

Overall, ATMPs can also have a positive impact on patient quality of life, caregivers and on the whole society. In fact, the use of ATMPs decreases hospitalization, avoids continuous drug administration, and reduces nursing. Notably, this approach speeds up the patient productivity, enabling quick return to work with no burden on the society.

Thus, ATMPs appear to have the extraordinary potential to offer durable, life-changing solutions for the society. These highly complex treatments rely on current surgical practice and could not prescind from it, but differ from traditional medicines, both in terms of how they are made/administered and by the type of benefits they may provide.

In particular, the 3.3% of total number of ATMP clinical trials worldwide are in the field of ophthalmology (Alliance for Regenerative Medicine, 2019). Globally, it is estimated that there are about 2.2 billion people with vision impairment or blindness and at least 1 billion people with a form of vision impairment that could have been prevented or has yet to be addressed (World Health Organization, 2019).

ATMPs APPROVED IN EUROPE

Advanced therapy medicinal products can be classified into three main types:

- **Gene therapy medicinal products:** consist of a vector or delivery formulation containing genes that lead to a therapeutic, prophylactic, or diagnostic effect. The genetic construct is engineered to express a specific transgene. The 'recombinant' genes are inserted into the body and by using such gene therapy constructs, *in vivo* genetic regulation or genetic modification of somatic cells can be achieved. A recombinant gene is a stretch of DNA that is created in the laboratory, bringing together DNA from different sources (European Medicines Agency, 2018).
- **Somatic-cell therapy medicinal products:** consist of cells or tissues that have been subjected to substantial manipulation, or that are not intended to be used for the same essential function(s) in the recipient body. They can be used to cure, diagnose, or prevent diseases.
- **Tissue-engineered products:** these contain engineered cells or tissues that have been modified so they can be administered with the aim of regenerating, repairing, or replacing human tissue.

In addition, there are ATMPs that consist of one of the first three categories combined with one or more medical devices as an integral part of the product, which are referred to as combined ATMPs (European Medicines Agency, 2017).

Up to June 2019, a total of 14 ATMPs have been granted marketing authorization in Europe: seven gene therapies, four cell therapies, and three tissue engineered products. However, four ATMPs have been withdrawn from the market because they did not obtain any reimbursement (**Table 1**; Ten Ham et al., 2018; Alliance for Regenerative Medicine, 2019).

Among the 10 approved ATMPs, two focus on eye diseases. More specifically, they have been developed to cure blindness or visual impairment. In 2014, the Committee for Advanced Therapies (CAT) recommended Holoclar®, the first ATMP ensuring a specific number of stem cells, for the treatment of moderate and severe Limbal Stem Cell Deficiency (LSCD). In February 2015, Holoclar® received conditional approval by the European Medicines Agency (EMA) for the use in the European Union (EU; European Medicines Agency, 2015).

The second was Luxturna®, approved in 2018 as the first gene therapy to restore vision in people with rare inherited retinal disease, caused by mutations in the *RPE65* gene. This therapy can be provided to patients with enough residual cells in the retina. Ten years of evidence of its safety were proposed and its use has been studied in patients with ages ranging between 4 and 44 years old.

However, being approved in November 2018, Luxturna® has not enough data available to assess the product's impact. It is still too early for a comparison of outcomes of patients treated with Luxturna® versus traditional therapies, after authorization (Luxturna, 2018).

Notably, the comparison of the social impacts of different types of therapies can be done when: (i) adequate time from approval is available to evaluate economic consequences, (ii) significant follow-up of patient outcomes is collected with the approved technique, and (iii) routine treatments are available as comparator of ATMP effects.

For example Strimvelis®, approved in 2016, has long term follow-up data, but no comparators for economic analysis are available.

As a consequence, we analyzed the use of the first CLET (Cultured limbal epithelial transplantation: Holoclar®) for the treatment of blindness and visual impairment. This product was launched in 2015, therefore, extensive information are available on more than 100 patient outcomes with several years of follow up. In addition, alternative approaches are available as comparators, as described below.

GLOBAL DATA: BLINDNESS AND VISUAL IMPAIRMENT

Eye health has profound and wide-spread implications in many aspects of life, health, sustainable development, and economy.

TABLE 1 | ATMPs approved in Europe (Alliance for Regenerative Medicine, 2019).

Drug name	Developer	Indication	Approval date (EU)	Status	Therapy
Chondrocelect®	TiGenix	To repair a cartilage defect of the knee	October 2009	X 01-2017	CT
Glybera®	uniQure	For lipoprotein lipase deficiency (LPLD)	October 2012	X 10-2017	GT
MACI®	Vericel	To repair a cartilage defect of the knee	June 2013	X 09-2014	T-B T
Provenge®	Dendreon	To treat advanced prostate cancer in men in whom chemotherapy is not yet clinically indicated	September 2013	X 05-2015	CT
Holoclar®	Holostem	In adult patients with moderate-to-severe limbal stem-cell deficiency caused by burns, including chemical burns to the eyes.	February 2015	✓	T-B T
Imlygic®	Amgen	For regionally or distantly metastatic unresectable melanoma	December 2015	✓	GT
Strimvelis®	GSK	Adenosine deaminase (ADA)−deficient severe combined immunodeficiency (SCID)	May 2016	✓	GT
Zalmoxis®	MolMed	Add-on treatment for HSCT of adult patients with high-risk haematological malignancies	August 2016	✓	CT
Spherox®	CO.DON	To repair a cartilage defect of the knee	July 2017	✓	T-B T
Alofisel®	TiGenix	To treat complex anal fistulas in adults with Crohn's disease	March 2018	✓	CT
Kymriah®	Novartis	Certain types of acute lymphoblastic leukemia in people up to 25 years old and in certain adult patients with large B-cell lymphoma	August 2018	✓	GT
Yescarta®	Gilead	CAR T therapy for adults living with certain types of non-Hodgkin lymphoma who have failed at least 2 other kinds of treatment.	August 2018	✓	GT
LUXTURNA®	Novartis	To treat an inherited retinal disease, indicated for children and adults with vision loss caused by mutations in both copies of the RPE65 gene and enough viable retinal cells	November 2018	✓	GT
Zynteglo®	BlueBird Bio	To treat a blood disorder known as beta thalassemia in patients 12 years and older who require regular blood transfusions	June 2019	✓	GT

GT, gene therapy; CT, cell therapy; T-B T, tissue based therapy; ✓, authorized; X, withdrawn.

Worldwide, visual impairment leads to a considerable economic burden for both affected and non-affected people. The estimated number of people with sight damage is more than 217 million, of which 47 million have severe damage and 170 million have moderate damage, while the number of people who are blind is estimated to be 36 million (**Table 2**; Bourne et al., 2017; The Lancet Global Health Commission, 2019).

Globally the major causes of visual impairment are uncorrected refractive errors (43%) and cataracts (33%). Other causes are glaucoma (2%); and age-related macular degeneration (AMD), trachoma, diabetic retinopathy, and corneal opacities (1%). Furthermore, a large proportion of cases (18%) have an undetermined cause.

Meanwhile, the main causes of blindness are cataracts (51%), glaucoma (8%), AMD (5%), corneal opacities (4%), uncorrected refractive errors and trachoma (3%), and diabetic retinopathy (1%), while 21% of cases have undetermined causes (**Figure 1**; Pascolini and Mariotti, 2012).

SOCIO-ECONOMIC IMPACT OF VISUAL IMPAIRMENT AND BLINDNESS: EXAMPLES FROM EUROPEAN COUNTRIES

Visual impairment and blindness have considerable socio-economic consequences attributable to the following:

- ***Direct healthcare costs*** incurred within the healthcare system by the government and/or other payers. These include, for example, general ophthalmic services, hospitalizations, treatment, expenditure associated with injurious falls due to visual impairment and blindness, and rehabilitation.
- ***Direct non-healthcare costs*** caused by the illness but not imputable to medical treatment: e.g., home improvements (e.g., ramps, door-opening devices, handlebars and tactile assistance systems), technical assistance such as sticks, guide dogs or computer interface, mobility, home care.
- ***Indirect costs*** include the economic impacts of this condition outside the healthcare system and on the wider society. These include:
  - *Reduced productivity* of the patients and their caregivers due to absenteeism, limited efficiency at work (presenteeism), part-time employment or loss of work,
  - *Informal cares* (family or social care),
  - *Social security costs* (invalidity pensions or accompanying allowance, financial support for income, residence, or benefits).
- ***Intangible costs*** reflecting the burden of the disease in terms of worsening of the patient's quality of life due to, for example, pain and other aspects such as the stress felt by the caregiver. Indeed, these can be tangible to some extent,

TABLE 2 | Global data on visual impairment and blindness in 2015 (Bourne et al., 2017).

	World population (million)	Blind (million)	Moderate-to-severe VI (million)	Mild VI (million)
TOTAL	7330	36	217	188.5
MEN	3700	15.87	97.76	87.11
WOMEN	3630	20.14	118.85	101.44

VI, visual impairment.

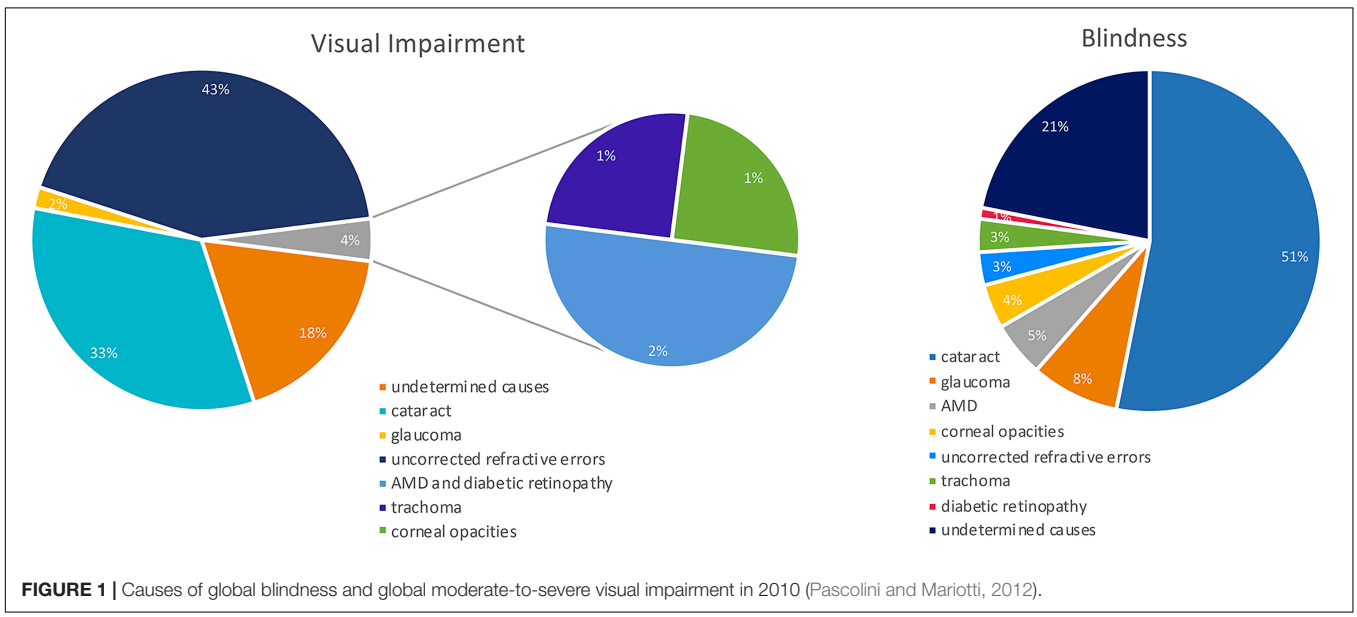

FIGURE 1 | Causes of global blindness and global moderate-to-severe visual impairment in 2010 (Pascolini and Mariotti, 2012).

such as in cases of costs related to depression, anxiety and further excess of morbidity. These can be traced back to other types of direct healthcare costs that can be easily calculated (Javitt et al., 2007; **Figure 2**).

Among direct non-healthcare costs, those dedicated to home improvements are not considered in a first analysis, but they have a consistent weight on total costs, as analyzed below.

Home adaptation-costs, reported from Italy, include kitchen and bathroom adjustments such as tactile assistance systems; cost

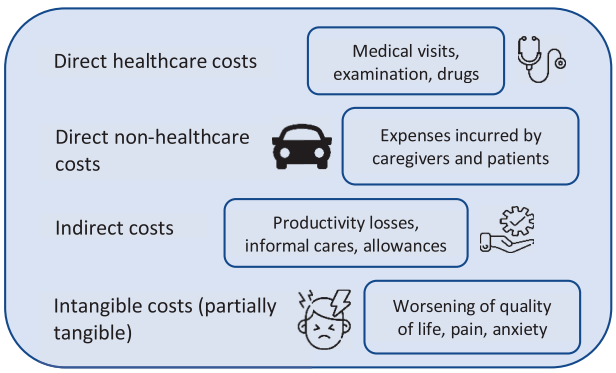

FIGURE 2 | Schematization of total costs for a blind and visually impaired patient.

€1,003.30 and €5,994.3, respectively. Similarly, in France, it is reported that the cost for a stair lift for a single patient is €6,000, while in Germany the cost of door-opening devices is €1,900 per unit (Lafuma et al., 2006).

Studies on cost of illness are a pivotal measure in healthcare, to assess the economic burden of a disease on the society. These studies support the quantification of the "hidden" costs of illness and so help to reveal the true disease-related charges. This is important because costs have a key role in public policy making and could help decision makers to prioritize medical costs, including research.

In fact, doing a cost-benefit analysis (CBA), the inclusion of marginal cost in the evaluation is emphasized, as it should not be a "on/off" decision, but rather a "more/less" decision.

An economic calculation may reveal, despite an apparent level of spending, that the additional (marginal) costs can change the final result of the cost evaluation (Zweifel and Telser, 2009).

Investments on ATMP that could cure patients with visual impairment, will lead to a healthier population, which in turn, could result in a more affordable medical budget for governments, a healthier tax-paying workforce, and lower productivity losses, improving the wellbeing and quality of life of patients and their caregivers.

Here, we analyzed the annual costs of blindness and visual impairment reported in three western countries (from EU) in order to compare direct healthcare costs, direct non-healthcare

	Germany (€)	UK (€)	Netherlands (€)
Direct healthcare costs	**1,415.3**	**1,425.0**	**2,921.4**
Hospitalization	417.9	445.6	2,112.4
Other	997.4	979.5	809.0
Direct non-healthcare costs	**4,494.6**	**418.4**	**302.0**
Home care and Transports	13.2	168.7	263.3
Medical support (e.g. devices)	4,481.4	249.7	38.7
Indirect costs	**9,868.0**	**2,964.7**	**3,924.0**
Absenteeism at work (loss of productivity)	3,842.1	1,527.1	/
Informal care	6,026.0	1,437.6	/
Premature mortality/deadweight loss	/	232,3	/
Total cost	**15,778**	**4,808**	**7,147**

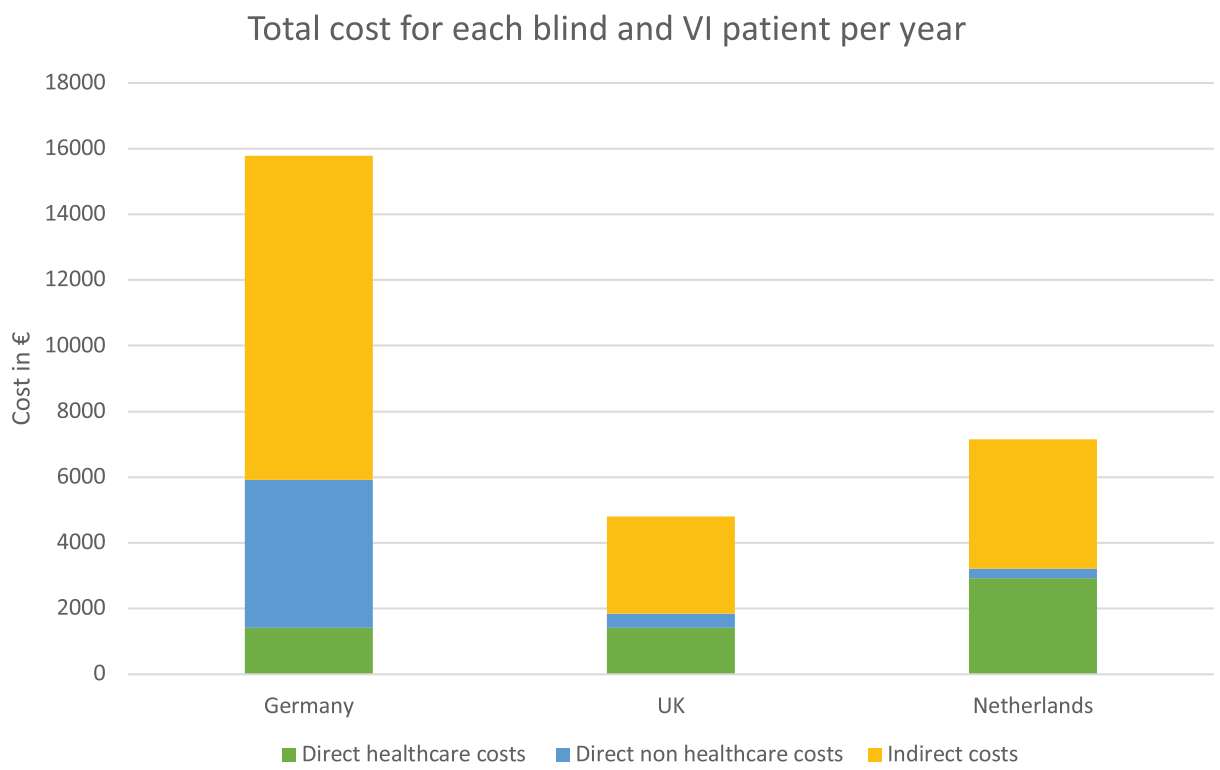

FIGURE 3 | In this table and figure are represented the direct healthcare costs, the direct non-healthcare costs and the indirect costs for some European Countries per unit (for each blind and visually impaired patient) (Pezzullo et al., 2018; Schakel et al., 2018; Chuvarayan et al., 2019). VI, visually impaired. Numerical data for UK have been converted from GBP to Euro.

costs, and indirect costs (**Figure 3**). Indeed, three studies were proposed by Netherland (Schakel et al., 2018), United Kingdom (Pezzullo et al., 2018), and Germany (Chuvarayan et al., 2019) for a cost-benefit analysis; this CBA was based on a previously made contingent valuation (CV), used to estimate economic values for all kinds of services. In the selected countries, the global cost for all visual impaired and blind individuals was divided by the number of the total patients in each Country, in the specific year

TABLE 3.1 | Summarizes an example of costs for different LSCD treatments.

LSCD Costs up to Surgery (€)	
CLAu	€ 21,893
Lr-CLAL	€ 65,479
KLAL	€ 77,393
SLET	€ 21,000*
BSC	€ 88,377
Holoclar	€ 93,907

*CLAu, limbal conjunctival autograft; lr-CLAL, conjunctival limbal allograft tissue from living relatives; KLAL, keratolimbal allograft; BSC, best supportive care, SLET, simple limbal epithelial transplantation. Data have been converted from GBP to Euro from NICE (2017a). *Data estimated by **Table 2** (Sangwan et al., 2012) that consider that SLET has the same cost as CLAu.*

TABLE 3.2 | Comparison between therapies – qualitative analysis.

	CLET (Holoclar® + surgery)	SLET
Costs to add to each surgery		
Hospitalization after surgery	1 day	Variable
Drugs/Medications needed		
Therapeutic contact lens	Not required	Yes
Amniotic membrane	Not required	Yes
Antibiotic eye drops	+	++
Steroid eye drops	+	++
Artificial tears	+	+
Outpatient appointments (first year)	At least 6 appointments	At least 9 appointments
Other costs to add to each therapy		
Home treatment	Self-medication	Medications At least 5 weeks up to complete re-epithelialization
Cost up to complete epithelialization	3–7 days	5–6 weeks
Invalidity/productivity loss	Days	Months
Pharmacovigilance on adverse event	Yes	Absent
Reproducibility of results	Highly standardized GMP setting	Not standardized setting
Proven inter-hospital consistency	Yes	No

This Table lists different treatments included in each therapy. The list of drugs and medications was taken from Basu et al. (2016) for SLET and AIFA (2019) for Holoclar. + used during the therapy, ++ used in higher amount than the comparator.

(Zweifel and Telser, 2009). In particular, results revealed that the direct healthcare costs of blind and visual impaired individuals represented just a small percentage of the total cost of this disability. Instead, the largest percentage of the costs, was due to productivity loss, social security costs, and informal support/care by caregivers. These results highlight the crucial role that indirect costs, usually not considered, play in the total cost of illness.

TABLE 3.3 | Comparison between therapies – quantitative analysis.

	CLET (Holoclar®)	SLET
Up-front cost of therapy	€ 93,907 (cost of surgery)	€ 21,000
Long-term healthcare costs		
Long-term stability**	23.4% failures up to 10 years (based on a proven follow-up)	24.8% failures up to 4 years (based on a proven 4 years follow-up) + 6 years hypothetical stability (best case) Or 6 years potential 100% failure (worst case)
Total potential cost of failures § in 10 years (follow-up)	€ 206,802	€ 220,943–€ 618,639
Total potential partial cost including surgery	€ 300,709	€ 241,943–€ 639,639

*In this table, the percentage of failure has been calculated from Rama et al. (2010) for Holoclar® and Basu et al. (2016) for SLET. **Failure rate and long-term stability were calculated on reported successful outcomes and proven 10 years follow-up for Holoclar® (Rama et al., 2010), and 4 years follow-up reported for SLET (Basu et al., 2016). The following 6 years (required to compare the data with ATMP) of SLET have been considered as a range: from a best case scenario as stable percentage of failure (24.8%) (Basu et al., 2016) to the worst case of late failure (100%). § The cost of failure was based on expenses for Best Supportive Care (BSC in **Table 3.1**).*

ECONOMIC BENEFIT OF ATMPs COMPARED TO TRADITIONAL THERAPIES: FOCUS ON TREATMENT OF LIMBAL STEM CELL DEFICIENCY

The aim of the treatment for LSCD is to restore the surface of the eye, achieve corneal clarity and improve visual acuity in monolateral or partial bilateral blindness. Current treatment practices usually start with supportive care treatments such as lubrication, autologous serum eye drops, antibiotics, anti-inflammatory drugs, and therapeutic soft or scleral contact lenses. Conservative surgery such as corneal scraping may also be offered before attempting limbal stem cell transplantation. The latter includes several types of invasive surgical options, the aim of which is to transplant stem cells to the affected eye. The surgical options differ in terms of where the cells come from and how they are transferred, specifically the following:

- Conjunctival limbal autograft (CLAu), in which stem cells are taken from the contralateral uninjured limbal tissue from the patient's healthy eye.
- Conjunctival limbal allograft (CLAL), in which stem cells are taken from a living, related donor or dead donor and transplanted into the diseased eye of the recipient.
- Keratolimbal allograft (KLAL), transplants the entire limbus from a dead donor using the corneoscleral carrier to deliver a large number of stem cells to the recipient.
- Simple limbal epithelial transplantation (SLET), reduces the tissue withdrawal of CLAu, but it can treat milder severity (superficial lesions) than CLET. In the SLET procedure,

ATMP treatment: LSCD symptoms pre and 12 months post-surgery		
	Pre-surgical assessment in %	12 months post-surgery in %
Any symptoms	38.5	11.5
Pain	6.7	0
Burning	28.8	6.7
Photophobia	33.7	7.7

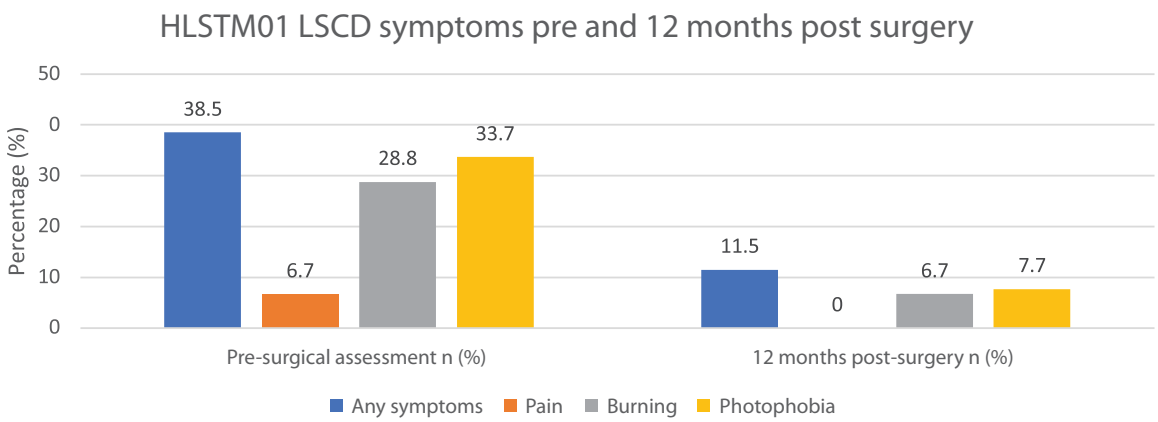

FIGURE 4 | Data from the first Holoclar clinical trial (HLSTM01) based on 97 patients (NICE, 2017a).

re-epithelialization is slower than in some of the other therapies (it takes about 5–6 weeks) (Sangwan et al., 2012; Swapna et al., 2019).

- Cultured limbal epithelial transplantation (CLET) procedure (Pellegrini et al., 1997) has some advantages compared to CLAu, for example it starts from a smaller amount of limbal tissue (1–2 mm^2), minimizing the risk of injury to the healthy eye. In addition, CLET does not require lifelong immunosuppression and in cases of failure, the treatment can be repeated multiple times.

Due to the evidence of its safety and efficacy, one specific CLET technique, under the name Holoclar®, has been conditionally approved in 2015 by the EMA as the first stem cell—based therapy (European Medicines Agency, 2015).

Some non ATMP-treatments can have disadvantages. For example, CLAu requires a large amount of donor tissue from the healthy eye (equivalent to around 40% of the available donor cornea). This increases the risk of damage to the donor eye and the treatment cannot be repeated in case of failure. The CLAu technique leads complete corneal epithelialization from day 18 up to several weeks after surgery (Kheirkhah et al., 2008).

In contrast, the use of the approved cultured stem cell therapy (Holoclar®) has several advantages, such as the absence of immunological rejection (autologous cells do not require immunosuppression), the use of a small limbal biopsy (1–2 mm^2), and the standardization of each preparation of the product made individually from the donor's cells for a single treatment. It is important to note that treatments can be repeated multiple times if both eyes need to be cured (European Medicines Agency, 2015; NICE, 2017a).

Thus, the cost of each traditional therapy could appear lower than the cost of an advanced therapy (see **Tables 3.1–3.3**), however, a more global evaluation of ATMPs leads to a different conclusion. Advanced therapy medicinal products can reduce hospital stay, medical evaluations, additional therapies, nursing costs, and finally, both direct non-healthcare and indirect costs. In fact, cost evaluation regarding cases of vision loss or blindness requires inputs to assist decision makers (e.g., surgeons, patients, payers), to calculate the cost effectiveness of different treatments as a whole and prioritize health expenditure for the society.

Procedure standardization, at the production and clinical application level, implies a clear definition of reproducibility on

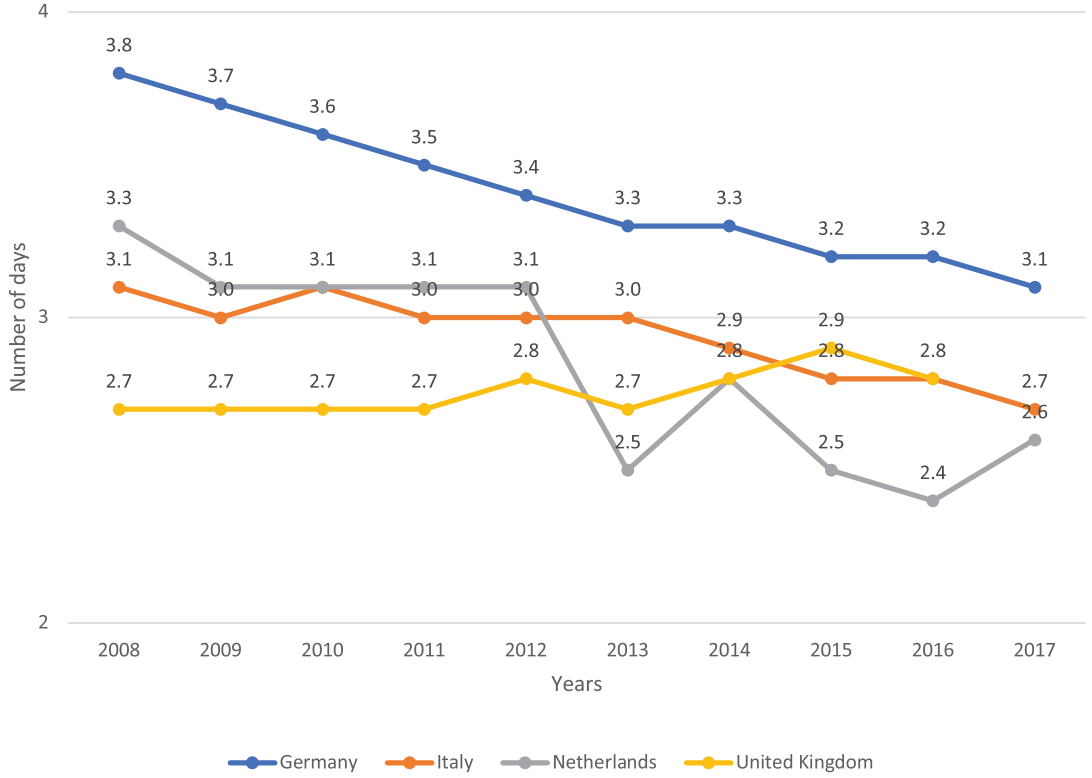

FIGURE 5 | In-patient average length of hospital stay (days) in different EU countries. This figure shows the number of days a single patient stays in hospital. It shows that the number of days in the hospital decreases with the years. These data refer to "diseases of the eye and adnexa" EUROSTAT category (Eurostat Database, 2016).

raw materials, production, clinical protocol, training of surgeons, long-term follow-up, and monitoring of adverse events, as requested by regulatory authorities.

Standardization has an impact on cost evaluation of the procedure. The absence of these guarantees results in highly variable results (potentially 0–100%), unknown long-term efficacy, and absence of pharmacovigilance on adverse events. Altogether, these missing points produce uncontrolled long-term increase/variability of costs (of the procedure) as calculated in **Table 3.3**.

Another advantage of the ATMP is that it uses new autologous healthy tissue grown in the laboratory that can be implanted onto the damaged cornea, healing the receiving eye in a few days with a rapid decrease in the patient's symptoms (**Figure 4**).

Results in **Figure 4** could be explained by the minimally invasive surgery required. Patients are usually able to go back home in one or a few days, compared to traditional surgeries requiring longer healing time.

Current clinical practices mainly rely on day surgeries, with a decrease on in-hospital patient average length of stay (days) in EU countries over the past few years, although the pace of diffusion has varied widely across countries.

Indeed, **Figure 5** shows that hospitalization has been reduced over the past 10 years, highlighting a clear tendency to decrease this cost for the society.

Therapies not following this trend, require an extension of the recovery and associated medical evaluations; they create cost-related problems and, above all, organizational problems to the entire healthcare system.

It is worth noting that patients with a less severe condition and an acceptable quality of life are less likely to suffer co-morbidities or adverse events requiring further, potentially expensive, therapies and support. Different studies have showed that visually impaired patients suffer from increased levels of depression, psychological stress, anxiety, and mental fatigue. It is estimated that depression occurs in patients with visual impairment more often (about 17%) than in patients with no vision damage (Van der Aa et al., 2016). Thus, depression is an additional cost for the society that could be avoided with timely patient treatment and effective sight recovery. For example, in Netherlands, psychological rehabilitation for each visually impaired patient costs about €432.6 per year (Schakel et al., 2018). This continuative cost, for both blind or visually impaired patients, for several years is a burden for the society and cannot be ignored.

CONCLUSION AND FUTURE PERSPECTIVES

A careful scientific and economic evaluation of the additional costs of each therapy should drive the selection of affordable medical solutions. As the number of ATMPs is increasing, the high prices associated with them have become the topic of many debates. Here, we have focused on sight recovery as it is a critical issue worldwide.

Beyond the value of ATMPs for healthcare and considering the total cost for a single patient, it is important to consider healthcare-related costs as well as non-healthcare and indirect costs to perform an appropriate evaluation. It is necessary to have a holistic view on expenses for the government, especially in the case of therapies with high up-front costs.

Hospitalization, home care, medical evaluations and adverse events are the most relevant costs for a blind or visual impaired patient. However, costs are not only related to the length of stay in hospital, but also to the hospital's logistic expenses, to the cost of physicians and nurses that take care of patients for months and to other expenditures. Such costs are widely reduced with the new innovative therapies.

Nevertheless, it is also important to consider the global experience of the patient and the possible correlated illnesses such as infections, pain and depression caused by prolonged and non-resolutive therapies. Patients often undergo long-term treatments with drugs in order to reduce symptoms but without any sight restoration. They live with pain and related depression and are not productive, representing often a burden for the society (NICE, 2017b).

Supportive care or surgical approaches can appear, at first, as cheaper choices but, afterward, they are often less effective than new therapies. These last ones potentially bring more significant benefits, especially in the long-term, not only for the success of the treatment but also for the caretakers, families, and for the society as a whole.

Therefore, the main aim of each therapy and treatment is patient's healthcare, its satisfaction and self-sufficiency, all in the frame of economic sustainability.

Finally, the costs of ATMPs include GMP production costs. The apparently high up-front costs of ATMPs are compensated by the high levels of therapy standardization and safety, ensuring a cost/benefit ratio. Concerning R&D, its related costs are compensated by the usefulness of R&D in assessing public policies and stimulating drug development and innovation.

Overall, our analysis highlights that, globally, there is not increase in the costs of ATMPs versus surgery, due to their guarantee of success and short duration.

AUTHOR CONTRIBUTIONS

FM and AM provided the acquisition. GP, FM, and AM analysis and interpretation of data for the work, drafted the work and revised it and approved for the publication of the content. All authors are accountable for all aspects of the work.

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frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
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Bringing Safe and Standardized Cell Therapies to Industrialized Processing for Burns and Wounds

Alexis Laurent[1,2†], *Poyin Lin*[3†], *Corinne Scaletta*[2], *Nathalie Hirt-Burri*[2], *Murielle Michetti*[2], *Anthony S. de Buys Roessingh*[4], *Wassim Raffoul*[5], *Bin-Ru She*[3‡] *and Lee Ann Applegate*[2,6,7*‡]

[1] Tec-Pharma SA, Bercher, Switzerland, [2] Regenerative Therapy Unit, Lausanne University Hospital, University of Lausanne, Epalinges, Switzerland, [3] Transwell Biotech Co. Ltd., Hsinchu, Taiwan, [4] Pediatric Surgery Service, Lausanne University Hospital, University of Lausanne, Lausanne, Switzerland, [5] Plastic, Reconstructive & Hand Surgery Service, Lausanne University Hospital, University of Lausanne, Lausanne, Switzerland, [6] Oxford Suzhou Center for Advanced Research, Science and Technology Co. Ltd., Oxford University, Suzhou, China, [7] Competence Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zurich, Switzerland

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Edited by:
Ivan Martin,
University of Basel, Switzerland

Reviewed by:
Sylvie Miot,
University Hospital of Basel, Switzerland
Pavel Makarevich,
Lomonosov Moscow State University, Russia

***Correspondence:**
Lee Ann Applegate
lee.laurent-applegate@chuv.ch

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share last authorship

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Cultured primary progenitor cell types are worthy therapeutic candidates for regenerative medicine. Clinical translation, industrial transposition, and commercial implementation of products based on such cell sources are mainly hindered by economic or technical barriers and stringent regulatory requirements. Applied research in allogenic cellular therapies in the Lausanne University Hospital focuses on cell source selection technique optimization. Use of fetal progenitor cell sources in Switzerland is regulated through Federal Transplantation Programs and associated Fetal Biobanks. Clinical applications of cultured primary progenitor dermal fibroblasts have been optimized since the 1990s as "Progenitor Biological Bandages" for pediatric burn patients and adults presenting chronic wounds. A single organ donation procured in 2009 enabled the establishment of a standardized cell source for clinical and industrial developments to date. Non-enzymatically isolated primary dermal progenitor fibroblasts (FE002-SK2 cell type) served for the establishment of a clinical-grade Parental Cell Bank, based on a patented method. Optimized bioprocessing methodology for the FE002-SK2 cell type has demonstrated that extensive and consistent progenitor cell banks can be established. *In vitro* mechanistic characterization and *in vivo* preclinical studies have confirmed potency, preliminary safety and efficacy of therapeutic progenitor cells. Most importantly, highly successful industrial transposition and up-scaling of biobanking enabled the establishment of tiered Master and Working Cell Banks using Good Manufacturing Practices. Successive and successful transfers of technology, know-how and materials to different countries around the world have been performed. Extensive developments based on the FE002-SK2 cell source have led to clinical trials for burns and wound dressing. Said trials were approved in Japan, Taiwan, USA and are continuing in Switzerland. The Swiss Fetal Transplantation Program and pioneer clinical experience in the Lausanne Burn Center over three decades constitute concrete indicators that primary progenitor dermal fibroblasts should be considered as therapeutic flagships in the domain of wound healing and for regenerative medicine in general. Indeed, one single

organ donation potentially enables millions of patients to benefit from high-quality, safe and effective regenerative therapies. This work presents a technical and translational overview of the described progenitor cell technology harnessed in Switzerland as cellular therapies for treatment of burns and wounds around the globe.

Keywords: cell therapy, clinical cell banking, progenitor cells, fibroblasts, GMP manufacturing, burns, chronic wounds

INTRODUCTION

Therapeutic developments driving regenerative medicine increasingly democratize cell-based therapies for treatment and prevention of wide arrays of acute and degenerative afflictions. Demographic shifts promoting higher incidence of chronic disease have prompted swift expansion of translational work pertaining to skin wound care, in particular to repair and restore function or supplement traditional therapeutic management (Vacanti and Langer, 1999; Marks and Gottlieb, 2018). The main indications of topical cell-based therapies remain set on complex, poorly-healing cutaneous affections. Corresponding clinical cases comprise ulcers, deep partial-thickness burn wounds or donor site wounds, which are highly demanding in classical wound care or necessitate skin grafting (Hernon et al., 2006; Li and Maitz, 2018). In such a context, wherein patient and practitioner expectations fall second highest only to regulatory requirements, cultured primary progenitor cells and derivatives have been demonstrably identified as worthy therapeutic candidates (Hebda and Dohar, 1999; Metcalfe and Ferguson, 2008; Larijani et al., 2015). Clinical-grade GMP-validated (Good Manufacturing Practices) allogenic progenitor cell sources trigger utmost interest. Indeed, pragmatic wielding of their tremendous therapeutic potential can minimize delays in medicinal product availability for the patient and provide maximal homogeneity, safety and stability of both biological starting materials and end-products (De Buys Roessingh et al., 2006; Applegate et al., 2009).

Multi- and inter-disciplinary professional approaches are instrumental in achieving implementation through clinical translation of allogenic cell-based therapies (Marks and Gottlieb, 2018). Market-approvals of Advanced Therapy Medicinal Products (ATMPs), although formidably tedious and complex to acquire, fall short of the challenge consisting in harnessing the optimal cell source (Applegate et al., 2009; Marks and Gottlieb, 2018). The latter must meet high quality standards, related to safety, stability and efficacy as a biological starting material intended for a therapy or product. Stringent requirements must be inherently met, comprising negligible probability of communicable disease transmission and prolonged maintenance of a defined and differentiated phenotype. Conserved proliferation characteristics throughout industrial production passages and relatively low technical limitations are equally important (Doyle and Griffiths, 1998).

Fetal progenitor dermal fibroblasts constitute worthy candidates in the search for the optimal cell source to be harnessed. Assuming that such cell types are properly isolated, expanded and preserved, progeny cells are reproducibly differentiated while maintaining low immunogenic properties, whereas the expansion and regenerative stimulation properties remain elevated (Quintin et al., 2007). Within consistent bioprocessing methodologies, these cells present low growth requirements (*in vitro* monolayers), are widely biocompatible with numerous natural and engineered scaffolds, are resistant to oxidative stress and are proven as effective trophic mediators of scarless wound healing (Shah et al., 1992; Cass et al., 1997; Doyle and Griffiths, 1998). Identity, purity, sterility, stability, safety and efficacy are furthermore most easily demonstrable when validating robust fetal progenitor cell banks (**Figure 1**) (Quintin et al., 2007).

An optimized process for cell source selection and primary cell isolation methodology, as described herein, results in highly consistent starting biological materials for therapeutic product development, such as the non-enzymatically isolated FE002-SK2 progenitor dermal fibroblast source and progeny (Swiss transplantation laws and approved Protocols from the Lausanne University Hospital Medical Ethics Committee) (Protocol #62/07: "Development of fetal cell banks for tissue engineering," August 2007; ECACC 12070301-FE002-SK2) (**Figure 1**) (Laurent-Applegate, 2012; Applegate et al., 2013). Such sources have been stringently optimized throughout tissue procurement, cellular isolation and whole-cell bioprocessing. Validated technical specifications for cell culture-expansion,

Abbreviations: ATMP, Advanced Therapy Medicinal Product; ATRI, Agricultural Technology Research Institute; BSA, Bovine Serum Albumin; CDEA, Cultured Dermal-Epidermal Autograft; CEA, Cultured Epithelial Autograft; cGMP, current Good Manufacturing Practices; CHUV, Centre Hospitalier Universitaire Vaudois; CMV, Cytomegalovirus; CPC, Cell Production Center; CQA, Critical Quality Attribute; DAPI, 4′,6-diamidino-2-phenylindole; DK-SFM, Defined Keratinocyte-Serum Free Medium; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethyl Sulfoxide; DPBS, Dulbecco's Phosphate-Buffered Saline; EBV, Epstein-Barr Virus; ECACC, European Collection of Authenticated Cell Cultures; ECM, Extra-Cellular Matrix; EDTA, Ethylenediaminetetraacetic acid; EOP, End Of Production; EOPCB, End Of Production Cell Bank; FBS, Fetal Bovine Serum; FDA, Food and Drug Administration; GLP, Good Laboratory Practices; GMP, Good Manufacturing Practices; HbsAg, Hepatitis B virus surface antigen; HBV, Hepatitis B Virus; hCMV, Human Cytomegalovirus; HCV, Hepatitis C Virus; HE, Hematoxylin and Eosin; HepB/C, Hepatitis B and C; HHV-6/7/8, Human Herpes Viruses types 6, 7 and 8; HIV-1/2, Human Immunodeficiency Viruses types 1 and 2; HTLV-1/2, Human T-cell Leukemia Viruses types 1 and 2; HUG, Hôpitaux Universitaires de Genève; IgG, Immunoglobulin G; IgM, Immunoglobulin M; ITRI, Industrial Technology Research Institute; K-SFM, Keratinocyte-Serum Free Medium; LOD, Limit Of Detection; MCB, Master Cell Bank; MMC, Mitomycin C; MOA, Mechanism Of Action; NIH, National Institutes of Health; PBB, Progenitor Biological Bandage; PBS, Phosphate-Buffered Saline; PCB, Parental Cell Bank; PMDA, Pharmaceuticals and Medical Devices Agency; Px, Passage number x; QC, Quality Control; Q-PCR, Quantitative Polymerase Chain Reaction; RNA, Ribonucleic Acid; SV40, Simian Virus 40; TFDA, Taiwan Food and Drug Administration; UTR, Regenerative Therapy Unit; WCB, Working Cell Bank.

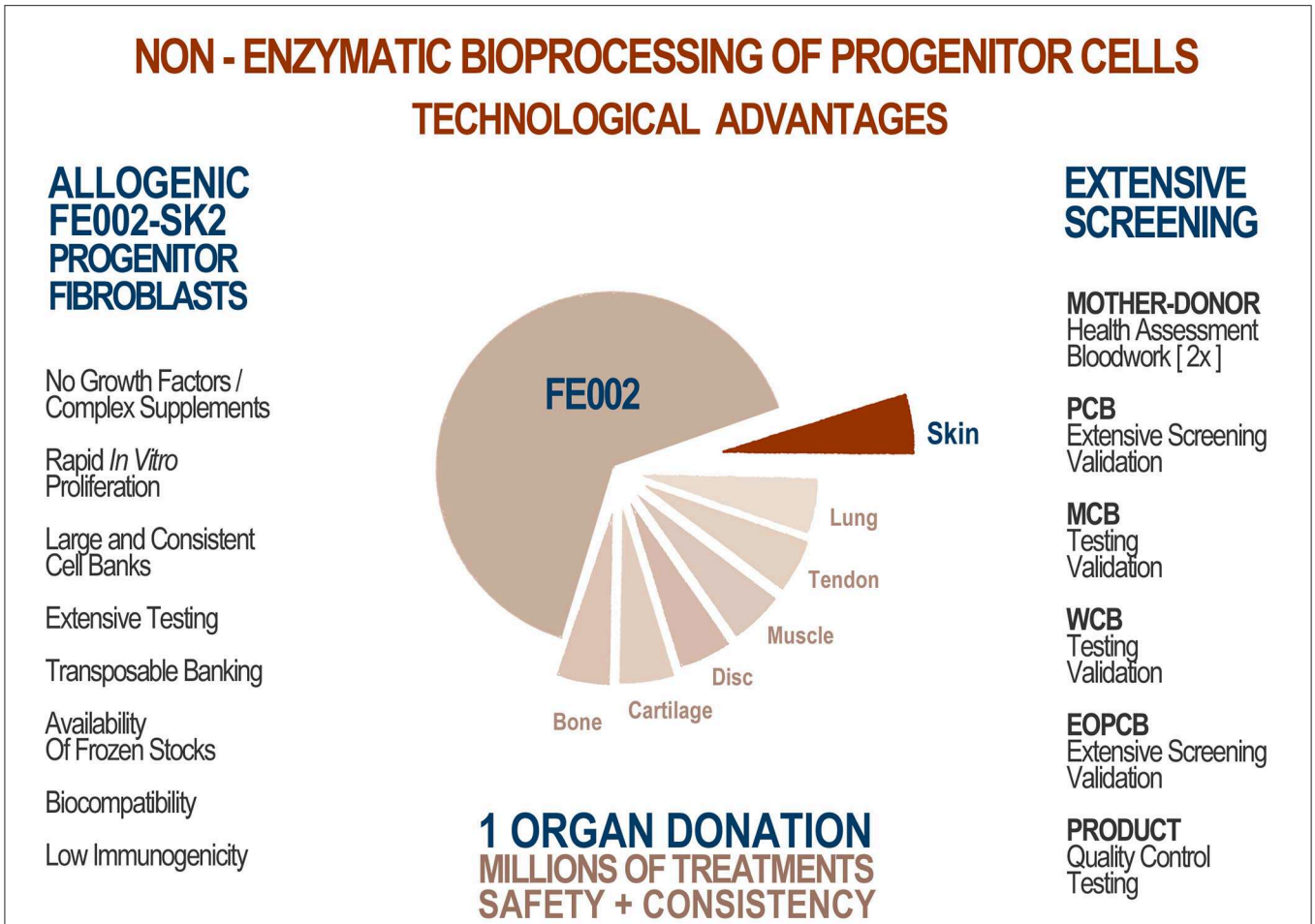

FIGURE 1 | Technological advantages of appropriate whole-cell bioprocessing for skin progenitors. From one single organ donation (FE002, 2009), various samples yielding differentiated tissue-specific progenitor cells were bioprocessed for isolation using the proprietary non-enzymatic method. Intrinsic cellular identity and characteristics were therein optimally maintained throughout the transposition to adherent monolayer culture. Inherent technical and clinical advantages are attributed to the specific choice of the cell source. Optimized and consistent biobanking allowed for establishment of extensive GMP cell banks. Thorough testing throughout manufacturing along with the consistency of the cell source guarantees optimal homogeneity and safety of the biological starting material for therapeutic products. Hundreds of millions of treatments can be produced based on the available stocks.

biobanking and extensive testing certify consistency and safety of the progeny cell banks (**Supplementary Figure 1**) (Quintin et al., 2007). To this day, FE002-SK2 fibroblasts and equivalents used in "Progenitor Biological Bandages" (PBB, skin progenitor fibroblasts on an equine collagen scaffold) have been applied clinically to treat severe burn patients in the Lausanne University Hospital (CHUV) with unique results (**Figure 2**) (Hohlfeld et al., 2005; Ramelet et al., 2009; De Buys Roessingh et al., 2015). Most importantly, successful up-scaling and industrial transposition of the novel progenitor cell technology have allowed significant translational research to advance both in Switzerland and in Asia. The fact that, withstanding the different regulatory and technical hurdles, a single organ donation (FE002) in Switzerland in 2009 was sufficient to furnish enough material to last 10 years to date for numerous research and clinical applications around the world is of utmost interest. When fully exploiting the potential of the cell banks under consideration, projected numbers of 9×12 cm PBBs *per* organ donation reach $>3.9 \times 10^{10}$, when using cells at stable defined passages (Passages 7 to 8, P7-8) within their *in vitro* life-span (**Figure 2**) (Ramelet et al., 2009). Recent regulatory shifts have at present led to the implementation of a Priority Project (Bru_PBB) in the CHUV, devising an internal randomized clinical trial to validate the continued clinical use of PBBs. Clinical batches of FE002-SK2 progenitor fibroblasts are manufactured for the CHUV Burn Center by its own GMP-certified (SwissMedic accreditation, 2015) Cell Production Center (CPC), a reference of technical expertise.

We present herein successful up-scaling and industrial transposition of original bioprocessing and biobanking protocols for the FE002-SK2 progenitor fibroblasts. Such processes are part of the historical description of the unique conjuncture and scientific progression that led to the establishment of the Swiss Fetal Transplantation Program. Original *in vitro* and animal *in vivo* data are presented herein, demonstrating

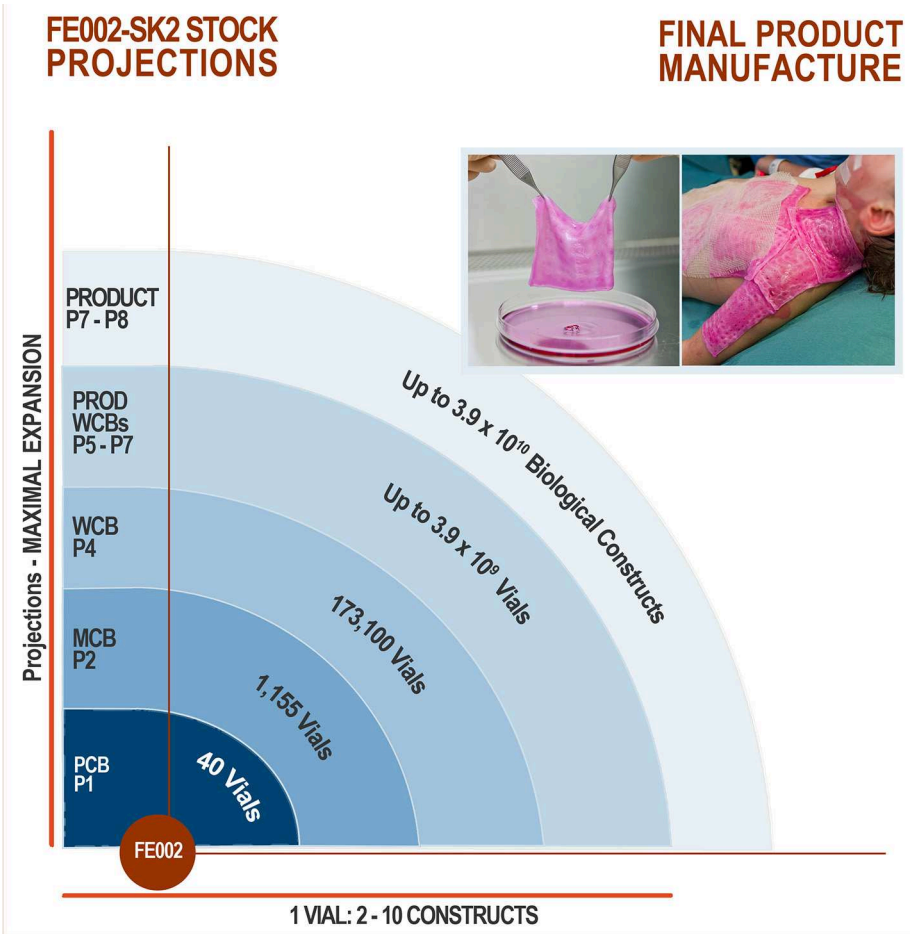

FIGURE 2 | FE002-SK2 stock projections and product manufacture. Assuming maximal expansion of all available cells, the theoretical number of final products is quantified in dozens of billions. Quality and safety testing are highly consumptive in materials throughout the GMP manufacturing process, but further optimization of bioprocessing and biobanking protocols would reasonably allow for such numbers of effective treatments to be produced, using one dedicated organ donation. To manufacture end-products, cryopreserved FE002-SK2 cells at specific passages are thawed and seeded on sterile bio-compatible scaffolds, which are applied to patients following defined clinical protocols.

safety and activity of the FE002-SK2 progenitor fibroblasts. These results strengthen the core technical concepts relative to bioprocessing and biobanking, as experimental assays studying cell-cell interactions, cell lysates and conditioned media were devised for determination of mechanisms of action. The ongoing clinical research around the world (clinical trial references: NCT02737748 and NCT03624023) focusing on the FE002-SK2 cell source will contribute synergistically to the vast local experience around such biological materials in Switzerland (De Buys Roessingh et al., 2015). The unique methodology adopted for the Swiss Fetal Transplantation Program development and the robustness of the FE002-SK2 clinical cell banks have already allowed for successful international GMP technology transfers to Europe and Asia. Through continued efforts directed at clinical translation, establishment of unified clinical protocols and product commercialization, quality and efficiency of patient care will in all probability be optimized worldwide with regard to all musculoskeletal tissues.

MATERIALS AND METHODS

Pilot Study and Establishment of FE002-SK2 MCBs and WCBs Within GMP Standards

After the FE002-SK2 Parental Cell Bank (PCB) was established from the FE002 organ donation in 2009 (**Supplementary Figure 2**), 8 PCB vials were sent frozen at −165°C to BioReliance, a GMP-certified production and storage facility (Merck Group, Glasgow, UK) for further testing (**Figure 3**). Initial tests were performed on quarantined vials in order to certify the admissibility of the PCB to GMP production. Stringent acceptance criteria had been defined and were also applied for the subsequent productions of tiered cell banks. The conforming assay results validated sterility of the materials, absence of bacteria, fungi, mycoplasma and viruses. Once the FE002-SK2 PCB vials were admitted for manufacturing processes, a crucial optimization phase was conducted, in

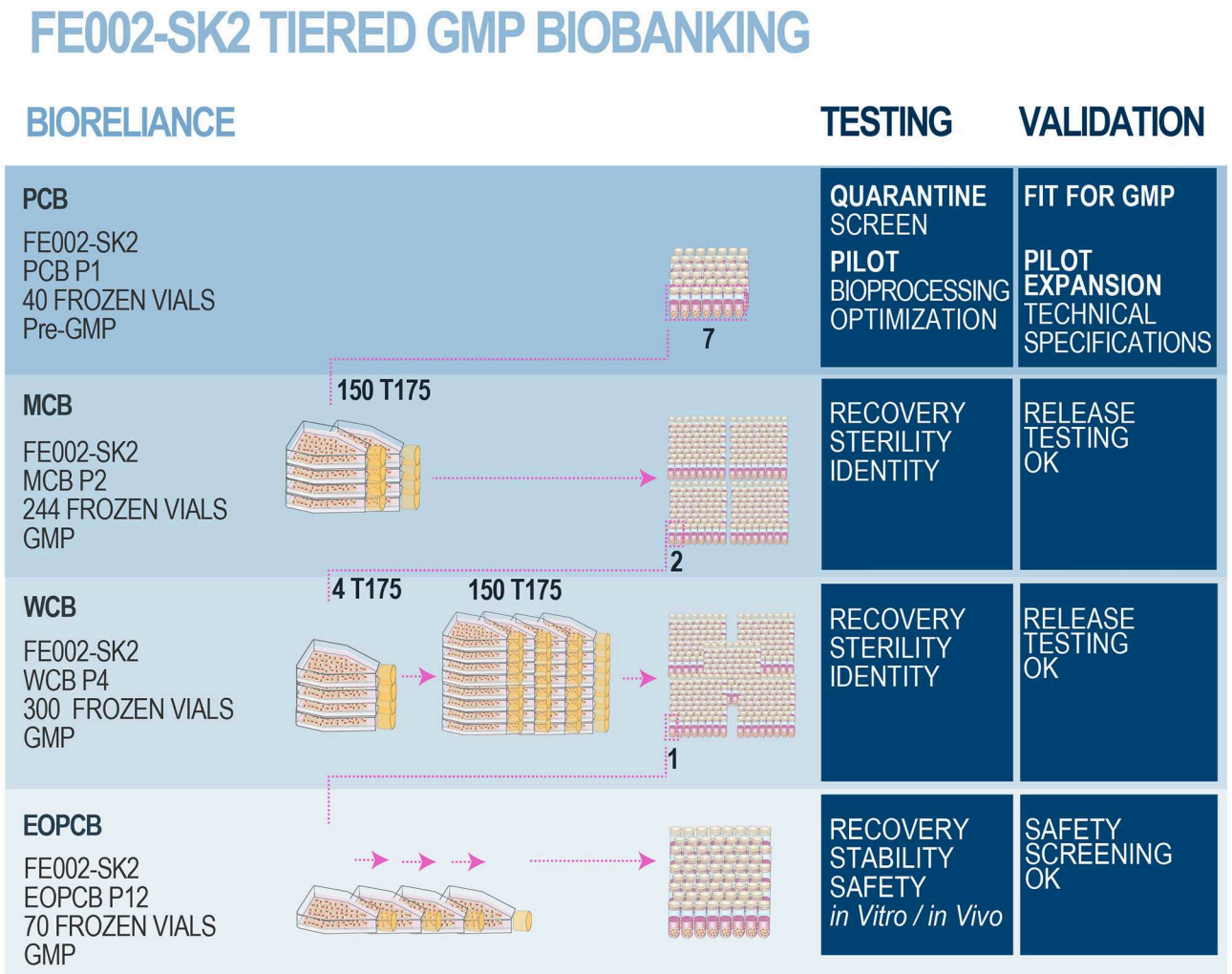

FIGURE 3 | FE002-SK2 GMP biobank establishment and validation. After the FE002-SK2 PCB was declared fit for GMP manufacturing, expansion campaigns were conducted for the establishment of extensive and consistent GMP FE002-SK2 Master (P2), Working (P4), and End of Production (P12) cell banks. All the progeny cells were stored in liquid nitrogen vapor phase. Several vials of each production batch were initiated for recovery studies and quality release testing. The EOPCB materials served for extensive safety and stability assays, both *in vitro* and *in vivo*. The complete battery of tests comprised (a) isoenzyme tests, (b) sterility tests, (c) mycoplasma detection, (d) karyology, (e) transmission electron microscopy (viruses, virus-like particles, mycoplasma, yeasts, fungi, bacteria), (f) *in vitro* adventitious viruses testing (picornavirus, orthomyxovirus, pramyxovirus, herpesvirus, adenovirus, reovirus, West Nile virus), (g) *in vivo* adventitious viruses testing (suckling mice, adult mice, guinea pigs, embryonated eggs), (h) Q-PCR (HepB/C, HIV-1/2, HTLV-1/2, HHV-6/7/8, EBV, hCMV, SV40, B19 parovirus) and (i) *in vivo* tumorigenicity testing in nude mice.

order to establish the technical specifications which would be used for the creation of progeny GMP cell banks. Optimized parameters comprised culture surface size, choice of various clinical-grade substrates (growth medium, supplements) and growth conditions such as cell seeding densities and culture periods (**Figure 3**).

Establishment of a FE002-SK2 GMP Master Cell Bank

Following validated processes and cGMP (current Good Manufacturing Practices) requirements (Commission Directive 2003/94/EC), a FE002-SK2 Master Cell Bank was manufactured and validated by BioReliance (**Figure 3**). A total of 7 vials from the FE002-SK2 PCB (**Supplementary Figure 2**) stored in liquid nitrogen vapor phase were used in a recovery procedure. The cells (characterized by a viability of 97% after recovery) served for the initiation of 150 × T175 cell culture flasks (175 cm^2, N°178883, Nunc®, USA) with a seeding density of 2.67×10^5 viable cells/flask or an approximate relative seeding density of 1.5×10^3 viable cells/cm^2. The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, N°04195943M, Invitrogen™, USA) containing 10% (v/v) FBS (Fetal Bovine Serum, N°10094, Invitrogen™, USA). The culture vessels were incubated at 37°C in a humidified atmosphere with 5% (v/v) CO_2. The culture medium was renewed every other day. The cultures were regularly photographically recorded. Cellular growth was reported as healthy (elongated fibroblast shape

observed) and within specifications. One T175 flask was lost to contamination during the final medium exchange phase. After the expansion reached the predefined limit parameters (100% confluency attained in 14 days), cells at Passage 2 were harvested (D-PBS, N°14190-94, Invitrogen™, USA; Trypsin-EDTA, N°25300-062, Invitrogen™, USA), counted, resuspended in a freezing solution (DMEM:FBS:DMSO−67.5%:27.5%:5.0%, DMSO N°D2438, Sigma-Aldrich®, USA) and conditioned in individual cryovials (244 vials each containing 1.0×10^7 viable cells/mL, 1.1 mL/vial, 1.8 mL capacity vials, N°368632, Nunc®, USA) to constitute the MCB. FE002-SK2 MCB vials were then frozen using a controlled-rate freezer and subsequently stored in liquid nitrogen vapor phase. Five days after the production of the MCB ended, MCB vials N°1, N°172, and N°243 were initiated to assess the recovery and cellular growth. Additional MCB vials were used in order to perform quality systems release testing. Testing of the FE002-SK2 MCB included sterility assays, mycoplasma and virus absence verification and an identity test. The MCB lot was liberated, as the samples qualified for cell viability and genetic identity, while being free of contamination by bacteria, fungi, mycoplasma, adventitious viruses, human specific viruses and bovine adventitious viruses. The total number of MCB vials produced was 244, while the number of vials released from GMP production after testing was 231.

Establishment of a FE002-SK2 GMP Working Cell Bank

Following validated processes and cGMP requirements, a FE002-SK2 Working Cell Bank was manufactured and validated by BioReliance (**Figure 3**). A total of 2 vials from the FE002-SK2 MCB stored in liquid nitrogen vapor phase were used in a recovery procedure (MCB vials N°90 and N°147). The cells (characterized by a viability of 98% after recovery) served for the initiation of 4 × T175 culture flasks (175 cm^2, Nunc®, USA) with a seeding density of 3.52×10^6 viable cells/flask or an approximate relative seeding density of 2×10^4 viable cells/cm^2. Cultures were processed in the same conditions as those of the MCB but without culture medium exchange steps. After the expansion reached the predefined limit parameters (100% confluency attained in 4 days), cells at Passage 3 were harvested as previously described, counted and used to initiate 150 × T175 culture flasks with a seeding density of 2.66×10^5 viable cells/flask or an approximate relative seeding density of 1.5×10^3 viable cells/cm^2. Cultures were processed in the same conditions as those of the MCB. Cellular growth was reported as healthy (elongated fibroblast shape observed) and within specifications. No deviations occurred. After the subsequent expansion and when cultures reached the predefined limit parameters (100% confluency attained in 12 days), cells at Passage 4 were harvested, counted and conditioned in individual cryovials (300 vials containing 1.0×10^7 viable cells/mL, 1.1 mL/vial) to constitute the WCB. FE002-SK2 WCB vials were then frozen as previously described and stored in liquid nitrogen vapor phase. One day after the production of the WCB ended, WCB vials N°2, N°140, and N°300 were initiated to assess cell recovery and growth. Additional FE002-SK2 WCB vials were used for the quality systems release testing. The WCB lot was liberated, as the samples qualified for cell viability and genetic identity, while being free of contamination by bacteria, fungi, mycoplasma, adventitious viruses, human specific viruses, and bovine adventitious viruses. The total number of WCB vials produced was 300, while the number of vials released from GMP production after testing was 287.

Establishment and Testing of an End of Production FE002-SK2 GMP Cell Bank

Following validated processes and cGMP requirements, a FE002-SK2 End of Production Cell Bank (EOPCB) was manufactured and validated by BioReliance (**Figure 3**). One vial from the FE002-SK2 WCB stored in liquid nitrogen vapor phase was used in a recovery procedure (WCB vial N°161). The cells (characterized by a viability of 98% after recovery) served for the initiation of 2 × T175 culture flasks (175 cm^2, Nunc®, USA) with a seeding density of 3.53×10^6 viable cells/flask. Cultures were processed in the same conditions as those of the MCB but without culture medium exchange steps. After the expansion (100% confluency attained in 4 days), cells at Passage 5 were harvested as previously described, counted and used to initiate 5 × T175 culture flasks with a seeding density of 3.45×10^6 viable cells/flask. The cells were allowed to expand and serial passaging was performed in the same way using 5 × T175 flasks until confluent cultures of FE002-SK2 cells were available at Passage 11. No medium exchange steps were required and average expansion times for individual expansions were of 4 days. The harvested cells at Passage 11 were then counted and split into 30 × T175 flasks with a seeding density of 3.00×10^5 viable cells/flask. Cultures were processed in the same conditions as those of the MCB and allowed to expand until 100% confluency was attained. Cellular growth was reported as healthy (elongated fibroblast shape observed) and within specifications. No deviations occurred. At the end of the final production expansion (100% confluency attained in 16 days), cells at Passage 12 were harvested, counted and conditioned in individual cryovials (70 vials containing 1.0×10^7 viable cells/mL, 1.1 mL/vial) to constitute the EOPCB. FE002-SK2 EOPCB vials were then frozen as previously described and stored in liquid nitrogen vapor phase. Two days after the production of the EOPCB ended, EOPCB vials N°4, N°37, and N°67 were initiated to assess cell recovery and growth. The material from EOPCB vial N°4 was lost due to contamination and the recovery study was complemented using EOPCB vial N°5. The total number of EOPCB vials produced was 70, while the number of vials released from GMP production after testing was 66.

Tests subsequently performed on cells initiated from the FE002-SK2 EOPCB (Passage 13 after recovery) comprised (a) identification of Caucasian human origin through isoenzyme testing, (b) sterility of culture conditions, (c) mycoplasma and retroviral reverse transcriptase activity tests, (d) transmission electron microscopy (TEM) imaging with a minimum of 200 cell profiles to detect the presence of pathogens (viruses, virus-like particles, mycoplasma, fungi, yeasts, and bacteria), (e) *in vitro* testing using three control cell lines to detect viral contaminants, (f) *in vivo* testing (inapparent viruses test in suckling mice, adult mice, guinea pigs and embryonated eggs), (g) *in vivo*

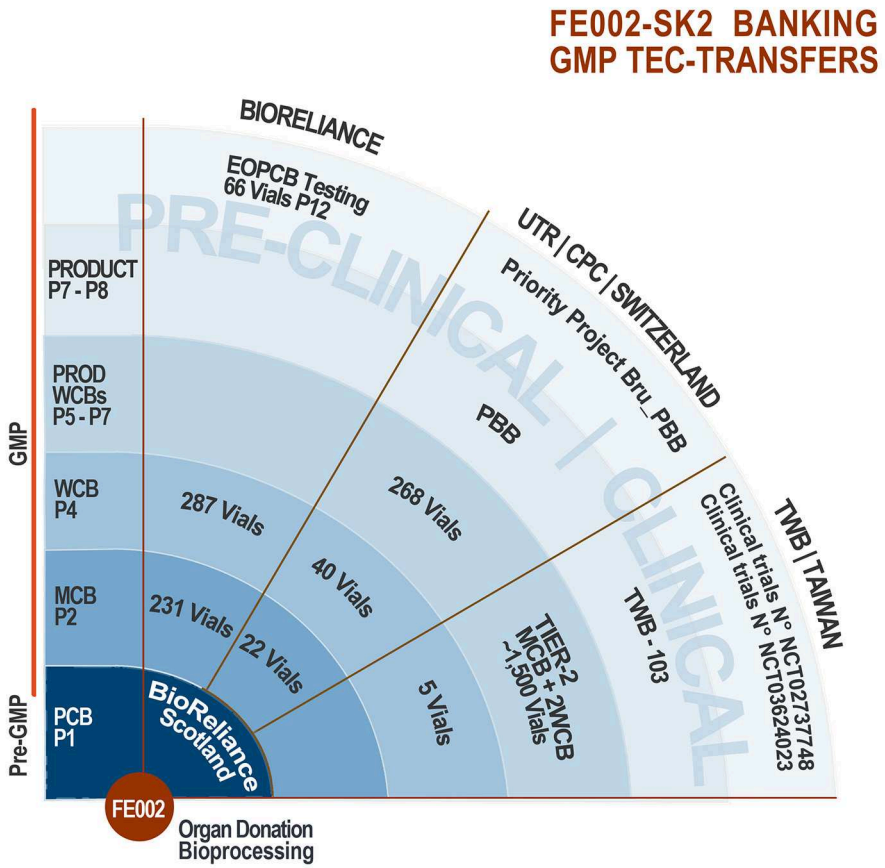

FIGURE 4 | FE002-SK2 tiered biobanking and GMP tec-transfers. The robust biobanking protocols devised for the FE002-SK2 progenitor fibroblasts allowed for successful technology transfers to BioReliance (Scotland), Transwell Biotech Co. Ltd. (Taiwan), and the CHUV CPC (Epalinges/Lausanne, Switzerland). Varying banking nomenclatures exist between the different groups, the pertinent passage numbers being those used by BioReliance. Quantities of vials listed exclude those consumed for recovery studies and release testing. Pre-clinical safety testing was performed on the EOPCB by BioReliance. Clinical applications comprise the ongoing use of PBBs in the CHUV Burn Center, the pending Priority Project Bru_PBB and both clinical trials sponsored in Asia by TWB.

tumorigenicity tests in order to determine the ability of the FE002-SK2 cell type to form tumors in mice using HeLa cells as positive controls, (h) Q-PCR for B19 parvovirus screening and (i) karyotyping (**Figure 3**).

Successive Technology Transfers of FE002-SK2 GMP Biobanking

Following the industrial up-scaling and transposition of FE002-SK2 banking to cGMP standards performed in collaboration with BioReliance, technology transfers were operated twice successfully to date (**Figure 4**). In an industrial setting, part of the FE002-SK2 cell source was made available to Transwell Biotech Co. Ltd. in Taiwan (TWB), at the ITRI (Industrial Technology and Research Institute) GMP facility in order to establish Tier-2 Working Cell Banks ("Tier-2 WCB"). Furthermore, cells from the Tier-2 WCBs were reinitiated, culture-expanded and cryopreserved as a component of the final TWB therapeutic product (FE002-SK2 progenitor fibroblasts renamed "TWB-102 cells" in a hydrogel scaffold) for preclinical research and clinical trials. Three co-Authors (BS, CS, LAA) oversaw the transmission of scientific and technical know-how in Taiwan. In a hospital/clinical setting, part of the FE002-SK2 cell source was donated to the CHUV Burn Center, represented by the state-of-the-art CPC for manufacturing purposes, for the continuation of burn patient care using Progenitor Biological Bandages. Manufacturing protocols were transposed to the CPC and subsequently internally optimized. Although FE002-SK2 banking nomenclatures vary between the different GMP production sites around the world, the same original stock of biological material (FE002-SK2 PCB) served as a source for the incremental development of all clinical-grade cell banks. Pre-GMP FE002 tissue donation bioprocessing and FE002-SK2 PCB establishment in the CHUV accredited laboratory under the Transplantation Program enabled the transition to full GMP banking, which was thereafter repeatedly and successfully transposed (**Figure 4**). This in turn allowed for the continuation of applied research and development related to the FE002-SK2 cell source of interest, both *in vitro* and *in vivo*, for which selected original data are presented hereafter.

In vitro Effects and *in vivo* Safety of FE002-SK2 Progenitor Fibroblasts

Transwell® Migration Assay of Primary Keratinocytes in Presence of FE002-SK2 Progenitor Fibroblasts

Sterile Transwell® inserts (6.5 mm diameter polycarbonate membranes with 8 μm pores, N°3422, Corning®, USA) and 24-well cell culture plates (N°353047, Corning®, USA) served as the experimental scaffold. In each assay repetition, triplicate wells were cultured in parallel for each study group. On Day 0 and for the test group, culture wells were seeded with 9×10^4 FE002-SK2 cells at P6 suspended in 0.5 mL of DMEM (N°11995, Gibco®, USA) supplemented with 10% FBS (N°10091, Gibco®, USA). Cell counting was performed using an ADAM-MC™ cell counter (NanoEnTek, South Korea). For the control group, the same culture medium without cells was added appropriately to additional wells. Cultures were incubated overnight at 37°C and under 5% CO_2. On Day 1, all media were removed and the culture wells were gently washed twice with DPBS (N°14190, Gibco®, USA), followed by the addition of 0.5 mL of K-SFM (Keratinocyte Serum-Free Medium, N°17005-042, ThermoFisher, USA) to each well. A sterile Transwell® was inserted into each well used in the assay. In the upper part of each Transwell®, 6×10^4 primary human foreskin keratinocytes (NHEK-Neo, N°00192906, Lonza, Switzerland, P3 or P4 at use) were then seeded, suspended in 0.3 mL of K-SFM. The culture vessels were transferred to a humidified incubator set at 37°C and 5% CO_2 for 15–18-h. On Day 2, after removal of the media from the Transwells®, the inserts were immersed in a 10% paraformaldehyde solution (N°16005, Sigma-Aldrich®, Germany) for 30 min. Thereafter, the Transwells® were immersed in a 1% crystal violet solution (N°V5265, Sigma-Aldrich®, USA) for 30 min for cell-staining. The inserts were then rinsed with sterile water and the keratinocytes which remained on the upper sides of the membranes were removed gently using cotton swabs. The keratinocytes which had migrated toward the lower sides of the membranes were further stained with DAPI (4′,6-diamidino-2-phenylindole, N°D8417, Sigma-Aldrich®, USA). Finally, after washing, the DAPI-stained cells on the membranes were visualized by fluorescence microscopy using excitation wavelengths 340–390 nm. Under 100X magnification, 5 photos were taken for each membrane including the upper, lower, left, right, and center fields. The photos were analyzed using ImageJ (NIH, USA). Cell counts (DAPI spots) on all 5 photos were accumulated. Mean total cell counts from the test groups were compared to those of the control groups.

Gap-Filling Assay Using Primary Keratinocytes in Presence of Media Conditioned by FE002-SK2 Progenitor Fibroblasts

The Culture-Insert 2 well in μ-Dish 35 mm (N°81176, ibidi®, Germany) served as an experimental scaffold. The device comprised a 35 mm dish with an insert disposed in its middle. The insert included 2 wells separated by a 0.5 mm wide divider. These wells were coated with collagen (rat tail collagen, N°354236, Corning®, USA, diluted with 0.02 N HCl to 50 μg/mL for coating) before use for the assay. To prepare FE002-SK2 cell conditioned media, the cells (P8) were grown in 6-well culture plates (N°140675, Nunc®, USA) in DMEM supplemented with 10% FBS (N°10091, Gibco®, USA) in a humidified incubator set at 37°C and 5% CO_2 until 70–100% confluency was attained. After media removal and two washes with DPBS, 2.5 mL of K-SFM were added to each well. The plates were re-incubated as mentioned hereabove. Conditioned media were collected 24-h later and processed using a 0.22 μm syringe filter (N°LGP033RB, Millipore, Germany) before direct use in assays or storage at −80°C until use. The sham medium was prepared following an identical process, except that no cells were present in culture. For the experimental assay, $2.25–4.20 \times 10^4$ primary human foreskin keratinocytes (N°881122-01-K, ATRI, Taiwan, P4 or P5 at use) suspended in fresh K-SFM were seeded in both wells of the ibidi® insert ($1.0–1.9 \times 10^5$ keratinocytes/cm^2). An additional 1 mL of fresh K-SFM was added to the dish area outside the insert and the device was incubated at 37°C with 5% CO_2. After 17-h of culture, 70 μL of test medium (conditioned medium or sham medium) were dispensed inside the wells to replace the K-SFM. The device was incubated at 37°C for another 6-h to allow the keratinocytes to respond to the test media. To initiate cell migration, the medium outside the insert was also replaced by test media and the insert was removed from the dish. At that moment, a 0.5×7.0 mm gap devoid of cells was created between areas of confluent keratinocytes. The assay plates were re-incubated, the keratinocytes being free to migrate. Representative imaging was performed at time points of 0, 3, 6, 9, 12, 15, and 18-h after initiation of migration. The resulting composed images were analyzed using ImageJ to integrate the area of cell migration.

Proliferation of Primary Keratinocytes in the Presence of FE002-SK2 Cell Extracts

Thawed suspensions of FE002-SK2 cells (P8, 7.5×10^6 cells/mL in a DMEM-based solution) were sequentially centrifuged at 20,600 × g for 10, 5, and 3 min, with the pellets being resuspended in the original medium after the first and second centrifugations. After the third centrifugation, supernatants were collected and defined as cell extracts. No cells were observed after cell extracts were put in culture (DMEM, 10% FBS). On Day 0, 1.92×10^4 keratinocytes (NHEK-Neo) suspended in 2 mL of DK-SFM (Defined Keratinocyte Serum-Free Medium, N°10785-012, Gibco®, USA) were seeded in each of the collagen-coated wells of 6-well culture plates. FE002-SK2 cell extracts were then added to the keratinocyte cultures in the amount of 0, 19.2, or 38.4 μL/well at the defined timepoints. Cultures were incubated at 37°C and under 5% CO_2. Cell extracts in appropriate doses were added once (on Day 0) or twice (on Day 0 and Day 2) over the course of the assay. The culture medium was changed on Day 2 (before adding the extracts) and on Day 5. On Day 7, the cells were detached (TrypLE™, N°12563, Gibco®, USA) and counted.

Proliferation of Primary Keratinocytes in the Presence of Mitomycin C-Treated FE002-SK2 Progenitor Fibroblasts

Mitomycin C (N°M4287, Sigma-Aldrich®, USA) was dissolved in DMSO (N°D2438, Sigma-Aldrich®, USA) to constitute a 1

mg/mL stock solution. FE002-SK2 cells at P10 were cultured in DMEM supplemented with 10% FBS in the same conditions as previously described until 90% confluency was attained. Mitomycin C stock solution was added to cultures by dilution in the growth medium, targeting a final concentration of 5 μg/mL. Cultures were re-incubated for 1-h. The supplemented medium was then removed and mitomycin C-treated FE002-SK2 cells were washed thrice with DPBS, trypsinized (Trypsin-EDTA, N°25300, Gibco®, USA), collected by centrifugation (7 min, 230 × g) and cryopreserved as previously described. After thawing, these cells attached to the culture vessels but did not proliferate. Both uncoated and collagen-coated 6-well culture plates (N°140675, Nunc®, USA) were used as scaffolds to assess the proliferation of primary keratinocytes (NHEK-Neo) in the presence of mitomycin C-treated progenitor fibroblasts (MMC-FE002-SK2). For the experimental assays, 1.92×10^4 keratinocytes suspended in DK-SFM were seeded in each well of the 6-well culture plates (2,000 cells/cm^2) and cultured as previously described for several hours until fully attached. Then, MMC-FE002-SK2 cells were thawed, washed, suspended in DK-SFM and seeded in the same wells at 2.90×10^4 cells/well (3,000 cells/cm^2) and the cultures were maintained for another 6 days. The media were renewed twice during this period. Wells containing only keratinocytes or only MMC-FE002-SK2 cells served as controls. Cell proliferation was monitored and photographically recorded. Proliferating cells in the co-cultures were verified as keratinocytes based on immunostaining using anti-cytokeratin-14 (CK-14) antibodies. Briefly, cultures in the 6-well plates were washed with DPBS and fixed with 4% paraformaldehyde. After cold DPBS wash and permeabilization of cells with 0.1% Triton™ X-100 (N°X100, Sigma-Aldrich®, USA), the wells were blocked with 1% bovine serum albumin (BSA, N°A7030, Sigma-Aldrich®, USA) at room temperature for 30 min. Then, 200X-diluted anti-CK-14 mouse antibodies (N°ab7800, Abcam, UK) were added to the wells and plates were incubated for 1-h at room temperature. The wells were then washed 3 times with DPBS. 1,000X-diluted Alexa Fluor 488 goat anti-mouse (H+L) antibodies (N°A-28175, ThermoFisher, USA) were added and plates were incubated at room temperature for 1-h. After 3 successive DPBS washes, wells were observed and recorded using appropriate fluorescence microscopy (excitation wavelengths filter 471–495 nm).

Proliferation of Primary Keratinocytes in Co-culture With FE002-SK2 Progenitor Fibroblasts and Biomarker Analysis of Conditioned Media

Cryopreserved keratinocyte suspensions (NHEK-Neo, P3) were thawed, diluted with DK-SFM, centrifuged and cells were resuspended in fresh DK-SFM. For the experimental assays, 1.92×10^4 keratinocytes suspended in DK-SFM were seeded in each well of collagen-coated 6-well culture plates (N°140675, Nunc®, USA, 2,000 cells/cm^2) and cultured for several hours as previously described until fully attached. Cryopreserved FE002-SK2 cells (P8, in non-DMSO cryopreservation solution) were thawed and diluted with DK-SFM before seeding at 4.80×10^4 cells/well (5,000 cells/cm^2) or 5.76×10^4 cells/well (6,000 cells/cm^2) on the same 6-well plates and cultures were maintained. Additional wells were prepared appropriately for controls. All wells were then supplemented with additional DK-SFM to the total volume of 2 mL. On Day 3 and Day 5, the media in all wells were replaced with 2 mL of fresh DK-SFM. On Day 6, the media (which had been conditioned for 24-h) were collected before the cells were washed, detached and counted. Conditioned media were collected from the cultures of FE002-SK2 cells (P9) and keratinocytes (P4) for biomarker analysis. These samples, along with the media collected from co-cultures on Day 6, were centrifuged at 805 × g for 5 min at 4°C. The supernatants were collected and kept at −80°C before being transported to RayBiotech Life (USA) for analysis by "Human 200 Biomarker Testing". There were 200 protein factors analyzed, including growth factors, cytokines, cell adhesion factors, ECM proteins and enzymatic modulators. Each factor was quantified using a reference curve.

GLP Porcine Study Using FE002-SK2 Progenitor Fibroblasts for Split-Thickness Wounds

The purpose of *in vivo* testing was to primarily evaluate safety of application of FE002-SK2 progenitor fibroblasts in treating split-thickness wounds (excision wound) on a porcine model. The primary goals were to evaluate whether a defined cell-based product caused adverse effects on wounds or peri-wound tissues, hindered wound healing, or caused adverse effects on general health of animals. The study was carried out, after proper ethical considerations were validated at the Agricultural Technology Research Institute in Taiwan, on 5 male domestic pigs (Landrace cross). Four test articles were investigated (sham control [A], hydrogel scaffold alone [B] and hydrogel scaffolds with low [C] and high doses [D] of progenitor fibroblasts). Four wounds were created on the dorsum of each pig. Using a dermatome (N°8821-01, Zimmer®, USA), square wounds (5.1 ± 1.0 cm sides, 0.45 ± 0.15 mm deep) were created on areas which were 4 cm distant from the spine. Two wounds were created on each dorsum side and were separated from each other by 5 cm. Every pig received four kinds of test articles (A-D), with one wound receiving one kind of test article (A, B, C, or D). There were 5 wounds for each group of test articles ($n = 5$ in each group). Each wound was treated with the respective test article on Days 0 (wound creation date), 3, 6, and 9. The wounds were then covered with 3M™ Tegaderm™ (3M, USA, waterproof and gas-permeable film gauze, 6 × 7 cm). Measurements of wound area and photographic recording were carried out on Days 0, 3, 6, 9, 13, and 14. Blood samples were drawn on Days 0, 3, and 14 and processed for hematology and biochemistry examinations. On Day 14, the skin samples on the original wound and peri-wound areas were excised (7.6 ± 1.0 cm sides, 0.60 ± 0.15 mm deep) and soaked in normal saline to rinse off blood. A half of the biopsy was soak-fixed in 10% (v/v) neutral formalin solution, while the other half was stored at −80°C. Tissue sections (6 μm) were stained using hematoxylin and eosin (HE) and histologically examined. Animals were sacrificed immediately after skin biopsies were harvested.

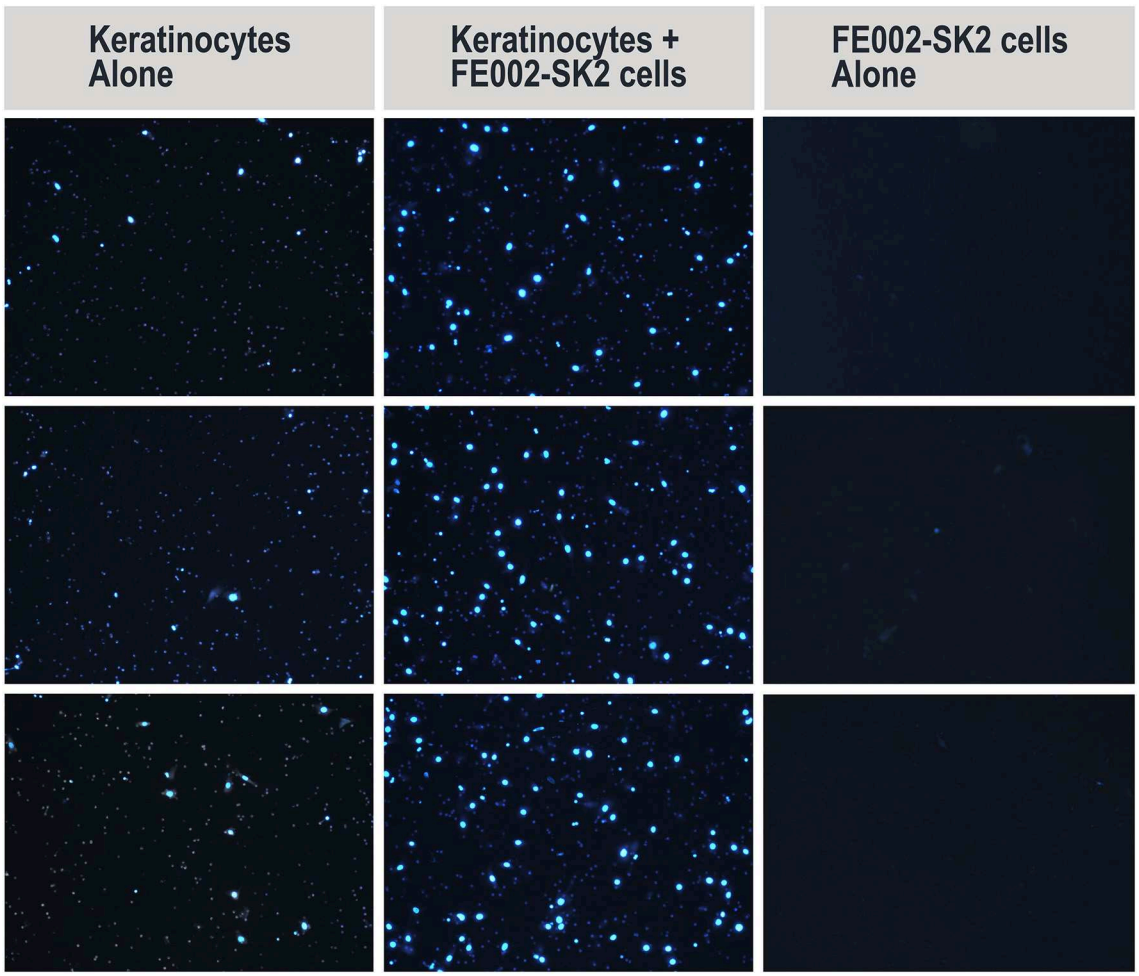

FIGURE 5 | Representative imaging of migrated primary human keratinocytes (DAPI staining) in Transwell® migration assay (left and middle columns). Representative imaging includes middle fields of 3 wells/group. Contamination of Transwells® by FE002-SK2 progenitor fibroblasts was negligible (right column). Mean cell counts are listed in **Table 1**.

RESULTS

Pilot Study and Establishment of FE002-SK2 MCBs and WCBs Within GMP Standards

Pre-GMP FE002 tissue donation bioprocessing and FE002-SK2 PCB establishment had been performed in the CHUV accredited laboratory under the Swiss Fetal Transplantation Program. FE002-SK2 progenitor fibroblasts had been extensively characterized in the same manner as the previous cell sources used in anterior successive Transplantation Programs (De Buys Roessingh et al., 2006; Quintin et al., 2007; Applegate et al., 2009; Laurent-Applegate, 2012). Extensive optimization steps, additional screening and close collaboration allowed for transposition of the biological materials and banking procedures to cGMP standards in BioReliance (**Figures 3**, **4**). Large and consistent GMP MCB (244 vials, cells at Passage 2), WCB (300 vials, cells at Passage 4) and EOPCB (70 vials, cells at Passage 12) were manufactured and qualified, based on a unified source and serving for further international research and clinical developments (**Figure 4**). Most importantly, in view of clinical applications, the FE002-SK2 progeny cell banks were extensively tested, and results fell within predefined validated specifications for all assays. Release testing was performed for FE002-SK2 Master and Working Cell Banks, while extensive *in vitro* and *in vivo* safety testing assays were performed on the EOPCB (**Figure 3**).

Screening tests that were accomplished on the FE002-SK2 EOPCB (cells at Passage 13 after recovery) confirmed the identification of Caucasian human origin through isoenzyme testing. Sterility of culture conditions was also confirmed. Mycoplasma and retroviral reverse transcriptase activity tests were negative. Transmission electron microscopy (TEM) imaging with a minimum of 200 cell profiles to detect the presence of pathogens (viruses, virus-like particles, mycoplasma, fungi, yeasts and bacteria) was accomplished and all test-results

TABLE 1 | Average counts of migrated primary keratinocytes in Transwell® migration assay, in the absence and presence of FE002-SK2 progenitor fibroblasts in the culture wells.

	A: Keratinocytes alone	B: Keratinocytes under influence of FE002-SK2 progenitor fibroblasts	Mean relative increase factor
Exp 1	88 ± 25 (28.0%)	238 ± 30 (12.8%)	2.71
Exp 2	152 ± 18 (11.7%)	409 ± 16 (3.9%)	2.70
Exp 3	113 ± 19 (16.8%)	299 ± 33 (10.9%)	2.64

Keratinocytes migrated downward through Transwell® membranes. Mean counts are presented for triplicates and three experimental repetitions (n = 3), with associated standard deviations and coefficients of variation [%]. Mean counts represent the sums of migrated keratinocytes in 5 integrated fields of each well. Cells were counted based on DAPI staining. Mean relative increase factors of migrated population counts are provided in the last column.

were documented as negative (data not shown). No contaminants were detected using all other assays including (a) *in vitro* testing using three control cell lines to detect viral contaminants, (b) *in vivo* testing (inapparent viruses test in suckling mice, adult mice, guinea pigs, and embryonated eggs) (100% survival was obtained for all the tests) and (c) Q-PCR for B19 parvovirus screening. *In vivo* tumorigenicity testing was performed in order to determine the ability of the FE002-SK2 cell type to form tumors in mice using HeLa cells as positive controls. Results indicated that no tumorigenicity was evident. Karyotyping was also accomplished to assess cell stability throughout passages and 50 metaphases were examined. No polyploidy was observed and no chromosome aberrations were noted. For each cell passage from Passage 5 to Passage 12, the viability from the recovery studies ranged from 98 to 100% with mean doubling times ranging from 37.5 to 71.6-h (P6-37.5 h; P7-46.9 h; P8-52.2 h; P9-47.5 h; P10-51.1 h; P11-71.6 h; P12-65.1 h) (data from FE002-SK2 EOPCB establishment). Furthermore, no contamination was found during all the processing except for one vial in one recovery study and one culture flask in a medium exchange procedure.

FE002-SK2 GMP Tec-Transfers and Product Development

Based on the results obtained during the successive GMP campaigns in BioReliance, the FE002-SK2 banking technology was transposed to TWB in Taiwan and to the CHUV Burn Center, represented for manufacturing purposes by the CPC (**Figure 4**). Continued development and parallel optimization steps were carried out between BioReliance and the UTR (Regenerative Therapy Unit, Applegate group) in the CHUV, to devise the most rational and efficient use of the biological materials for therapeutic application. Following sub-licensing of the technology to TWB in Taiwan, extensive product development (TWB-103 product) led to two registered clinical trials for donor site complications ("TWB-103 for Adult Patients with Split-Thickness Skin Graft Donor Site Wounds," NCT02737748, Japan and Taiwan) and for diabetic foot ulcers ("TWB-103 for Treating Lower Limb Ulcers on Patients With DM," NCT03624023, Taiwan). Following the long submission process, regulatory approval was therefore successfully obtained in both Taiwan (Taiwan Food and Drug Administration-TFDA) and Japan (Pharmaceuticals and Medical Devices Agency-PMDA) with associated documentation of Investigational Medicinal Product Dossiers and Investigator Brochures along with Clinical Trial documentation for monitoring. Part of the FE002-SK2 cell source was donated to the CHUV Burn Center, to continue clinical applications of Progenitor Biological Bandages (on-going in-house clinical practice and experience of 30 years) within a Priority Project (Bru_PBB).

In vitro Mechanism of Action Study and *in vivo* Preclinical Safety Evaluation of FE002-SK2 Progenitor Fibroblasts

FE002-SK2 Progenitor Fibroblasts Facilitate Keratinocyte Migration

Keratinocyte migration is critical for wound healing (Spiekstra et al., 2007). To test whether FE002-SK2 cells could promote keratinocyte migration, primary human keratinocytes from mixed donors were seeded on the upper side of Transwell® membranes in 24-well culture plates. It was found that the presence of FE002-SK2 fibroblasts in the culture wells increased the number of migrated keratinocytes (**Figure 5**, representative imaging, DAPI staining and **Table 1**). The relative increase factors for migrated populations of keratinocytes under the influence of FE002-SK2 progenitor fibroblasts ranged from 1.8 to 9.3 among 15 consecutive assays (mean value of 4.4 ± SD 2.4). It was found that the passage number and source of keratinocytes affected the baseline migration rate, which resulted in considerable inter-assay variation. However, the intra-assay coefficient of variation between triplicate wells was usually within 20%. The results of a series of three assays are presented in **Table 1**. Contamination of Transwells® by FE002-SK2 fibroblasts was negligible.

FE002-SK2 Cell-Conditioned Medium Facilitates Keratinocyte Migration

Wound-healing is typically modeled by closing of epithelial gaps developed *in vitro* (Pastar et al., 2014). The ability of FE002-SK2 conditioned medium to facilitate epithelial cell migration was tested using primary keratinocytes in gap-filling assays. The results showed that the rate of gap-filling was higher in the group using FE002-SK2 conditioned media than in the group using sham media (**Figure 6**). Adding conditioned medium promoted faster closing of the epithelial gaps, when considering appropriate timepoints and under specific experimental conditions. In three considered experimental repetitions, a significant difference in migration area covered by the keratinocytes was observed after 6–9-h of incubation. In this assay, before the initiation of migration (i.e., the time when the divider between two patches of confluent keratinocytes is removed) the keratinocytes must be preincubated with the test media to be converted to the fast-migrating type. Without the pre-incubation, the gap would be

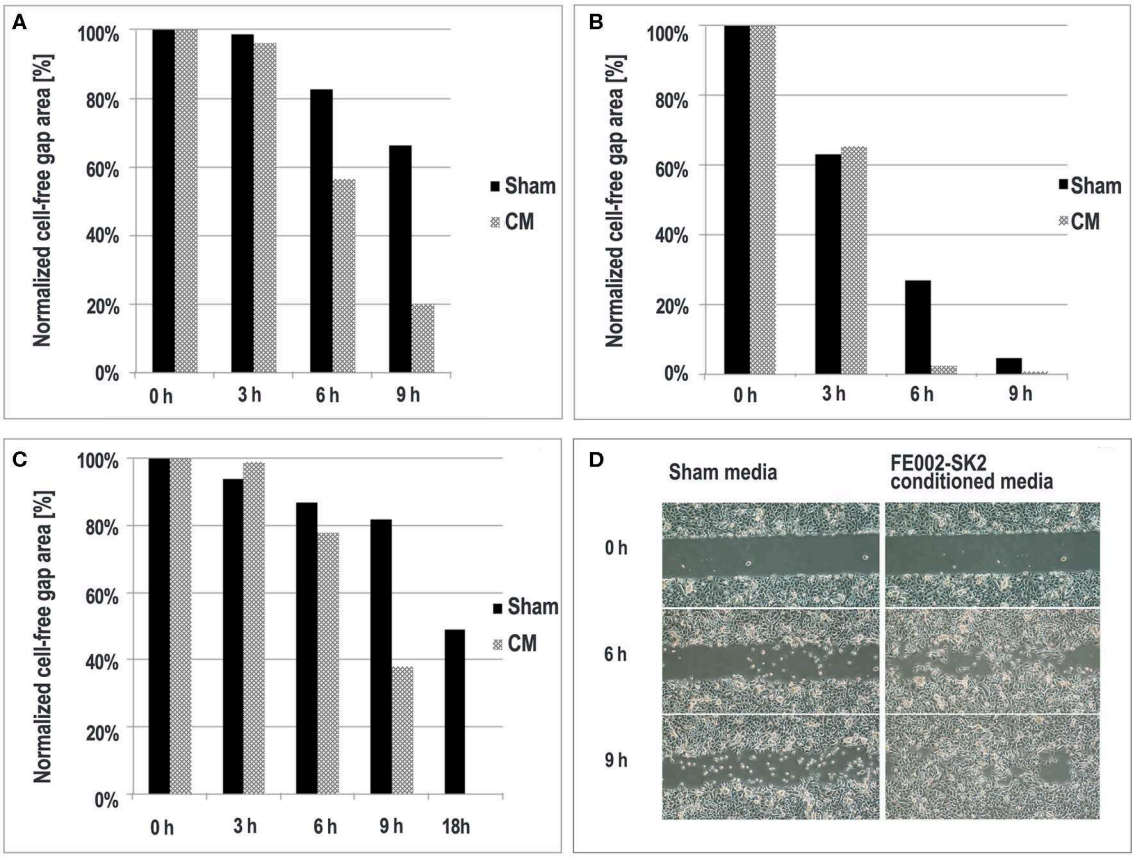

FIGURE 6 | Epithelial migration in the presence of FE002-SK2 conditioned media and sham media. Quantitative results of three experimental repetitions are compared **(A–C)**. At the 9- and 18-h timepoints in the second and third replicates, respectively **(B,C)**, the gaps treated with FE002-SK2 conditioned media were completely filled. Representative imaging of epithelial migration from the first experiment is shown **(D)**. Negligible filling was detectable at the 3-h timepoint. Imaging data was acquired under 40X optical magnification.

filled in both sham and test media before differential migration speeds can be detected (data not shown). Representative imaging of gap-filling at timepoints between 0- and 9-h are shown in **Figure 6D**.

FE002-SK2 Cell Extracts Promote Keratinocyte Proliferation

Keratinocyte proliferation is another critical factor for wound healing (Usui et al., 2008). The potential of FE002-SK2 cell extracts to promote keratinocyte proliferation was assessed, simulating the liberation of active biochemical factors after cell lysis on the patient or in therapeutic constructs. It was found that without the addition of progenitor cell extract, the primary keratinocyte population was reduced after the culture period, indicating that the medium did not support cell maintenance and growth (**Table 2**). It was required to add the cell extracts twice, on both Day 0 and Day 2 to stimulate keratinocyte proliferation (**Figure 7**). Adding 38.4 μL of extract twice clearly and extensively promoted keratinocyte proliferation (**Figures 7N,O**). Multiple adjunctions of cell extract consistently resulted in relatively more potent stimulation of keratinocyte proliferation. In this assay and in the proliferation assays in the following sections, the media used were DK-SFM. This medium had lost its ability to support keratinocyte growth after a short period of appropriate storage, even prior to the indicated expiration date.

Mitomycin C-Treated FE002-SK2 Progenitor Fibroblasts Promote Keratinocyte Proliferation

Although FE002-SK2 cell extracts were capable of promoting keratinocyte proliferation, multiple adjunctions were required for continuous and effective stimulation. The potential for proliferation stimulation of integral and growth-arrested FE002-SK2 cells (MMC-FE002-SK2 cells) constantly interacting with primary human keratinocytes was assessed. The results showed that in the absence of MMC-FE002-SK2 cells, keratinocytes did not proliferate in either collagen-coated or un-coated wells (**Figures 8D–F,J–L**). MMC-FE002-SK2 cells did not proliferate but were able to promote keratinocyte proliferation in co-cultures already since Day 3. The proliferation promotion was evident in both the coated and un-coated wells (**Figures 8G–I,M–O**). The cells that proliferated in the co-culture

TABLE 2 | Primary keratinocyte proliferation represented by mean total population counts in the absence and presence of FE002-SK2 cell extracts.

FE002-SK2 cell extract dosing regimen	Viable keratinocyte mean counts [10^4 cells/well] on Day 7 (Dosed/Sham)		
	Exp 1	Exp 2	Exp 3
Sham Day 0	0.094 (1.0)	0.48 (1.0)	2.22 (1.0)
Single Dose Day 0	0.69 (7.3)	1.24 (2.6)	3.92 (1.8)
Single Doses Day 0 and Day 2	2.41 (25.6)	3.25 (6.8)	9.45 (4.3)
Double Dose Day 0	1.55 (16.5)	1.33 (2.8)	4.53 (2.0)
Double Doses Day 0 and Day 2	19.60 (208.5)	7.68 (16)	12.28 (5.5)

"Sham" conditions corresponded to adding 0 μL of extract, "Single Dose" conditions corresponded to adding 19.2 μL of extract and "Double Dose" conditions corresponded to adding 38.4 μL of extract. The initial viable keratinocyte mean count (Day 0) was of 1.92 × 10^4 cells/well. Mean counts are presented for duplicates and three experimental repetitions (n = 3). Ratios corresponding to total viable cell counts from the dosed wells over those of the sham wells on Day 7 are presented in parentheses (Dosed/Sham).

wells verified to be keratinocytes based on immunostaining (**Figures 8Q,S**) and the MMC-FE002-SK2 cells were verified to have not proliferated.

Proliferation of Keratinocytes in Co-culture With FE002-SK2 Cells and Biomarker Analysis of Conditioned Media

The results presented hereabove indicated that keratinocyte migration and proliferation were activated by the FE002-SK2 cells or derivatives thereof. In order to study the factors involved in such activations, co-culture experiments were carried out. **Table 3** shows that neither pure FE002-SK2 nor keratinocyte populations proliferated over the 6-day culture period. However, in the presence of FE002-SK2 cells, keratinocytes grew to large colonies (**Figures 9M–T**, **Table 3**). Conditioned media biomarker analysis results are presented in **Table 4**. For the DK-SFM control samples, the only factors detected at a significant level (defined as 3-fold of the lower Limit Of Detection, LOD) were insulin and low levels of LIF (**Table 4**). EGF was not detected. For the keratinocyte conditioned media, 14 factors were detected (CXCL16, E-Selectin, Follistatin, Galectin-7, IGFBP2, Insulin, LIF, Lipocalin-2, MIF, PAI-1, PDGF-AA, TIMP1, TIMP2, and Trappin-2). For the FE002-SK2 conditioned media, 12 factors were detected (Angiogenin, CD14, CEACAM1, GDF15, HGF, IGFBP6, Insulin, LIF, Lipocalin-2, PAI-1, TIMP1, and TIMP2). For the co-culture media, several additional factors were detected. **Table 4** lists the factors which were significantly present in at least one of the cell conditioned media. Many of the factors were present at relatively high levels in the co-culture media but at low levels in the media from single population cultures. Galectin-7 was present at high levels in the keratinocyte conditioned media but the concentration was relatively reduced about 5-fold in the co-culture media (**Table 4**).

Porcine *in vivo* Study on Standardized Wounds

During the study period, administered test articles did not affect body weight of pigs, neither did they cause wound infection, edema, maceration, abnormal inflammation or other adverse effects. Serum TNF-α and IL-1 concentrations were not significantly modified throughout the study period. Serum IgG and IgM concentrations were slightly elevated from Day 0 (IgG 199 ± 105 mg/dL; IgM 119 ± 14 mg/dL) to Day 14 (IgG 343 ± 116 mg/dL; IgM 158 ± 17 mg/dL). The monocyte counts were transiently elevated on Day 3 but returned to baseline levels on Day 14. These immunological changes were interpreted as normal for pigs with trauma and treated with human cells. The wound healing rates were similar for all treatment groups (extensive data not shown). However, on Day 6, though statistically non-significant, healing rates of wounds in group D (scaffold and high dose of progenitor fibroblasts) were found to be higher than those of wounds in group A (sham control). On Day 9, considering wound healing rates (presented in [%] with associated standard deviations), the wounds in group B (scaffold alone) (100.00 ± 0.00), C (scaffold and low dose of fibroblasts) (99.78 ± 0.49), and D (scaffold and high dose of fibroblasts) (100.00 ± 0.00) were nearly fully healed while wounds in group A were not fully healed (95.50 ± 9.09). On Day 14, the scars resulting from wounds in group D were less apparent (**Figure 10T**). Microscopic observation of HE-stained tissue biopsies showed normal newly healed wounds (**Figure 11**). Epidermal cells appeared differentiated and stratification was observed in the external layers, while cuboidal cells populated the basal layer. Follicles and dermal papilla were in formation. Interestingly, observation of the tissue sections from all wounds (3 sections *per* wound) revealed that the epithelium appeared to attach better to the dermis in the wounds treated with progenitor cells (**Figures 11C,D**). Statistical significance was not confirmed because of the small number of test subjects.

DISCUSSION

Cellular therapies are valuable assets in the endeavors of repairing, restoring or optimizing tissue and organ functions. Efficient combinations thereof with traditional surgical techniques or tissue engineering can demonstrably lead to additive or synergistic beneficial effects on the patients' health momentum (Montjovent et al., 2004; Bach et al., 2006; Loebel and Burdick, 2018; Costa-Almeida et al., 2019). Various considerations are critical during the cell source selection process, related to availability of said therapeutic cell sources, traceable characterization thereof, inherent expansion potential, regenerative potential and applicability to engineered bio-constructs (Doyle and Griffiths, 1998; Monti et al., 2012). Optimization of this selection process, with major emphasis set on safety and consistency, has led to the identification and recognition of allogenic primary progenitor cell types as highly promising and efficient candidates for cell therapies (De Buys

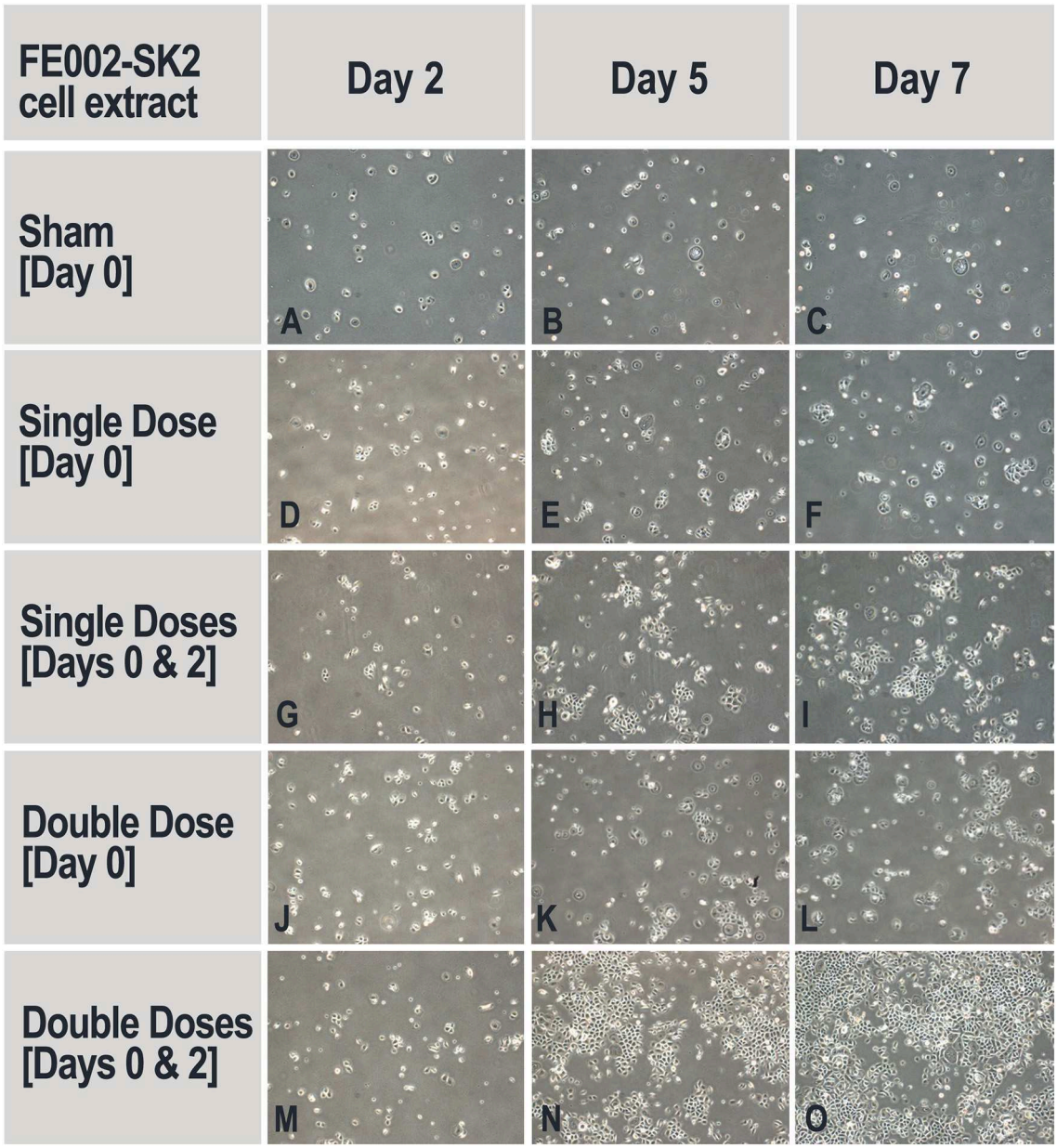

FIGURE 7 | Representative imaging of keratinocyte proliferation in the absence and presence of FE002-SK2 cell extracts. Adjunction of sham medium or cell extracts on Day 0 did not stimulate keratinocyte proliferation and did not ensure maintenance of cell populations **(A–F,J–L)**. Adjunction of cell extracts on Day 0 and Day 2 resulted in augmented keratinocyte proliferation **(H,I,N,O)**. Imaging data was acquired under 40X optical magnification. Mean harvesting total cell counts are listed in **Table 2**.

Roessingh et al., 2006; Mirmalek-Sani et al., 2006; Grognuz et al., 2016; Kim et al., 2018).

Medical needs in the domains of burns and chronic cutaneous wound management are difficult to meet, due to the complex nature of wound bed environments and the delicate process of coordinated responses governing wound closure. Split-thickness skin autografting remains a gold standard of care for numerous complex cutaneous affections, yet negatively contributes to the burden of patients by implying the creation of a donor site wound. High morbidity is the consequence of large surface wounds and delayed healing, as the opportunity for infection is wider (Simman and Phavixay, 2011). Heavy scarring can in turn cause durable and extensive psychological trauma to be borne by patients. Optimized wound management is therefore critical for individual survival and well-being, particularly in burn victim populations, where primary wounds and donor site wounds are constant concerns. An effective therapeutic solution allowing for regeneration of healthy and functional

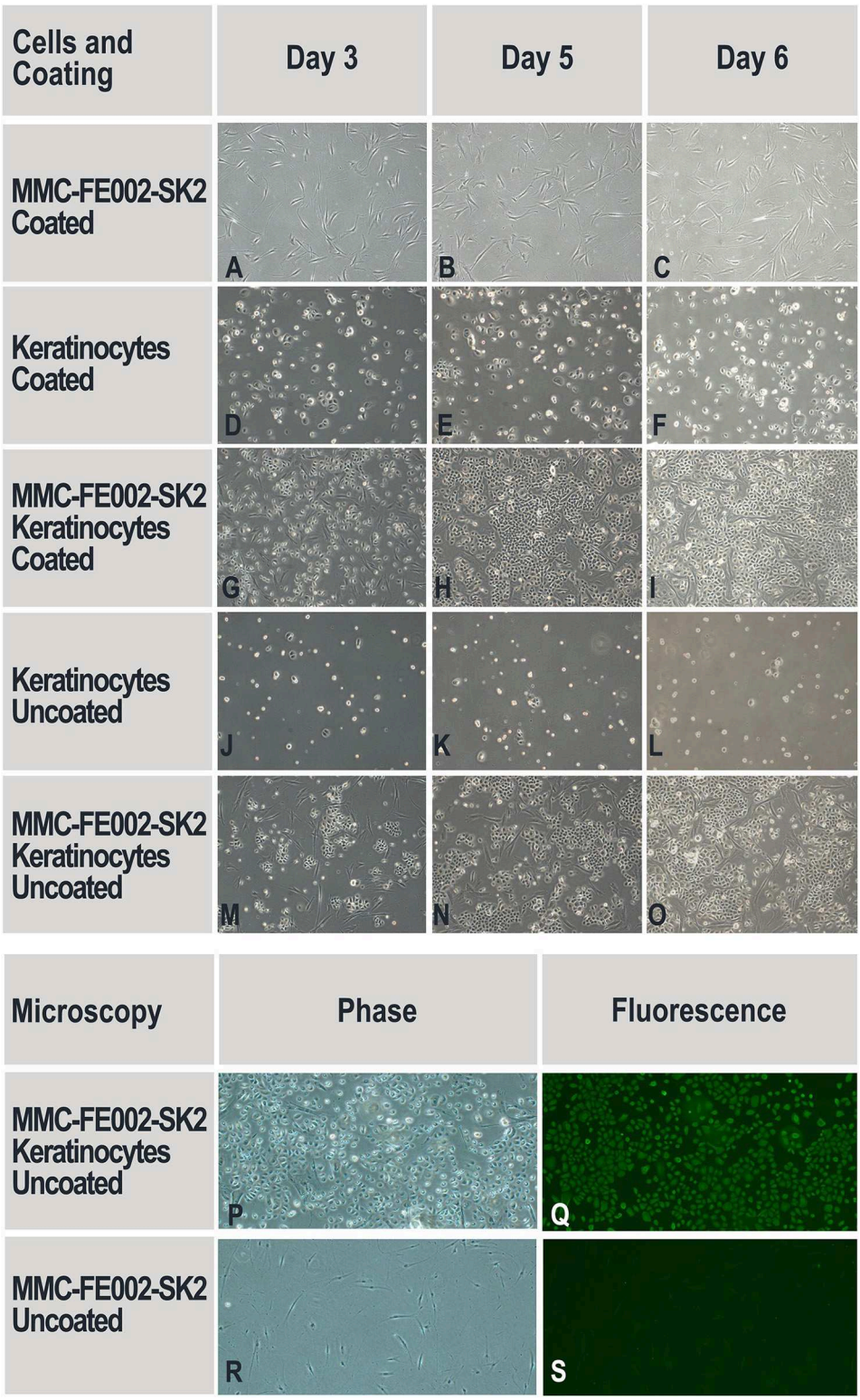

FIGURE 8 | Proliferation of primary keratinocytes in the presence of mitomycin C-treated FE002-SK2 progenitor fibroblasts (MMC-FE002-SK2 cells). DK-SFM is deficient in extracellular matrix (ECM) proteins. Considering that FE002-SK2 fibroblasts can secrete ECM proteins for cell attachment, both collagen-coated **(A–I)** and un-coated surfaces **(J–S)** were comparatively tested. No cell proliferation was observed in the single-population cultures of keratinocytes **(D–F,J–L)** or MMC-FE002-SK2 cells **(A–C)**. Keratinocyte proliferation was observable in co-cultures **(G–I,M–P)**. Selected wells were also stained with anti-CK-14 antibodies **(P–S)**. MMC-FE002-SK2 progenitor fibroblasts appeared negative for immunostaining **(S)**, while proliferating cells in co-culture wells appeared positive **(Q)**. Staining data confirmed that proliferating cells were keratinocytes. Imaging data was acquired under 40X optical magnification and fluorescence microscopy.

TABLE 3 | Primary keratinocyte and FE002-SK2 progenitor fibroblast proliferation in co-cultures.

Cells seeded on Day 0		Exp 1	Exp 2	Exp 3
Keratinocytes [10^4 cells/well]	**FE002-SK2 fibroblasts [10^4 cells/well]**	**Mean viable cumulated cell counts on Day 6 [10^4 cells/well] (Day 6/Day 0)**	**Mean viable cumulated cell counts on Day 7 [10^4 cells/well] (Day 7/Day 0)**	**Mean viable cumulated cell counts on Day 7 [10^4 cells/well] (Day 7/Day 0)**
1.92	0	0.88 (0.46)	0.23 (0.12)	0.27 (0.14)
0	4.80	1.65 (0.34)	2.54 (0.53)	1.99 (0.41)
0	5.76	2.15 (0.37)	4.34 (0.75)	3.36 (0.58)
1.92	4.80	41.60 (6.19)	8.35 (1.24)	31.60 (4.70)
1.92	5.76	47.78 (6.22)	10.78 (1.40)	33.93 (4.42)

Mean cumulated viable counts are presented for duplicates and three experimental repetitions (n = 3). Ratios corresponding to cumulated viable cell counts from the dosed wells on Day 6 or Day 7 over initial cell counts in those same wells on Day 0 are presented in parentheses (Day 6–7/Day 0).

skin within optimal timeframes would widely benefit both patients and medical staff (De Buys Roessingh et al., 2015; Li and Maitz, 2018). Numerous therapeutic solutions already exist on the market with the indication of favoring wound healing processes. Dressings and topical agents for burns and chronic wounds fulfill different patient- and wound-specific needs. Products typically used in burn centers comprise vaseline gauze (e.g., Jelonet™), adhesive film dressings (e.g., Tegaderm™), hydrocolloids (e.g., DuoDERM®), alginate-based products (e.g., Kaltostat®), soft silicon or foam based products (e.g., Mepitel® Ag or PolyMem®), hydrofibers (e.g., Aquacel®), or cell-based therapy products (e.g., OrCel®, Apligraf®, Dermagraft®, TransCyte®) (Dhivya et al., 2015; Varkey et al., 2015; Borda et al., 2016). Clinical evidence indicates that dermal cells used in a therapeutic product promote optimal and durable tissue repair (Spiekstra et al., 2007; Akita et al., 2008). The CHUV Burn Center uses both cultured epithelial autografts (CEAs) and cultured dermal-epidermal autograft (CDEAs) for specific indications and with positive results (Rheinwald and Green, 1975; Abdel-Sayed et al., 2019). Notwithstanding said results obtained with existing cell-based products and aforementioned therapies, cost-effectiveness, availability delays and product homogeneity between production batches remain of major concern. Optimized quality control and economic rationale are therefore main advantages of the described Swiss progenitor cell technology, which in turn positively benefit each step of product development.

Large and heterogenous arrays of biological materials and cultured progeny cells present interesting therapeutic potential for human medical applications in regenerative medicine. Both animal and human donors can be evaluated, at various ages of physiological development, with inherent problematics for each cell type to potentially be used in a therapy. The focus of applied research in academic and industrial settings comprises embryonic stem cells, umbilical cord cells, fetal cells, adult stem cells, platelets, placenta and amniotic fluid cells, amongst many others (Heathman et al., 2015; Mount et al., 2015). Many of these potential sources require specific handling or manipulation to orient or stabilize the differentiation and self-renewal capabilities of cultured progeny cells, which are to be used for defined therapies. Notwithstanding the diversified offer of donor sources and technological advances, major current restrictions on the developmental path of cell-based products remain related to technical limitations. In most cases, sub-optimal intrinsic parameters of selected cell populations increase the complexity and cost of the pathway to product development and market approval (Heathman et al., 2015; Mount et al., 2015). Such hindering parameters comprise feeble cellular proliferation capacity, plasticity in the maintenance of a differentiated phenotype, possible transmission of communicable diseases or low banking consistency and low *in vitro* stability (Rayment and Williams, 2010; Ratcliffe et al., 2011; Abbasalizadeh and Baharvand, 2013; Heathman et al., 2015; Hunsberger et al., 2015). A given example is the use of allogenic fetal keratinocytes for treating burns and chronic wounds (Zuliani et al., 2013; Tan et al., 2014). Although a combination of allogenic cell populations (fibroblasts and keratinocytes) in a therapeutic product is conceptually interesting, many technical barriers arise and hinder the pragmatic establishment of relatively large and consistent keratinocyte cell banks. This aspect is confirmed by the clinical experience around CEA/CDEAs, whereas keratinocytes are relatively more complex and fastidious to obtain in sufficient numbers. The data presented herein further supports the concept that allogenic keratinocytes are not required, as progenitor fibroblasts would exert potent stimulatory effects on resident keratinocytes to facilitate the healing process.

Cultured primary progenitor dermal fibroblasts from one organ donation such as the FE002-SK2 cell source meet the stringent technical requirements pathing the way to development of allogenic biological therapeutic products (Quintin et al., 2007; Applegate et al., 2009; Larijani et al., 2015). Progenitor cells benefit from extensive historical industrial use, attesting to their safety and stability, whereas many vaccines were produced using such biological substrates since the 1950s (Hayflick et al., 1962; Olshansky and Hayflick, 2017). Such cells were and are isolated by bioprocessing specific fetal tissues, which exist following the embryonic stage of development (i.e., after 9 weeks of gestation). Careful selection and screening of the mother-donor coupled with extensive testing of the progeny cultured

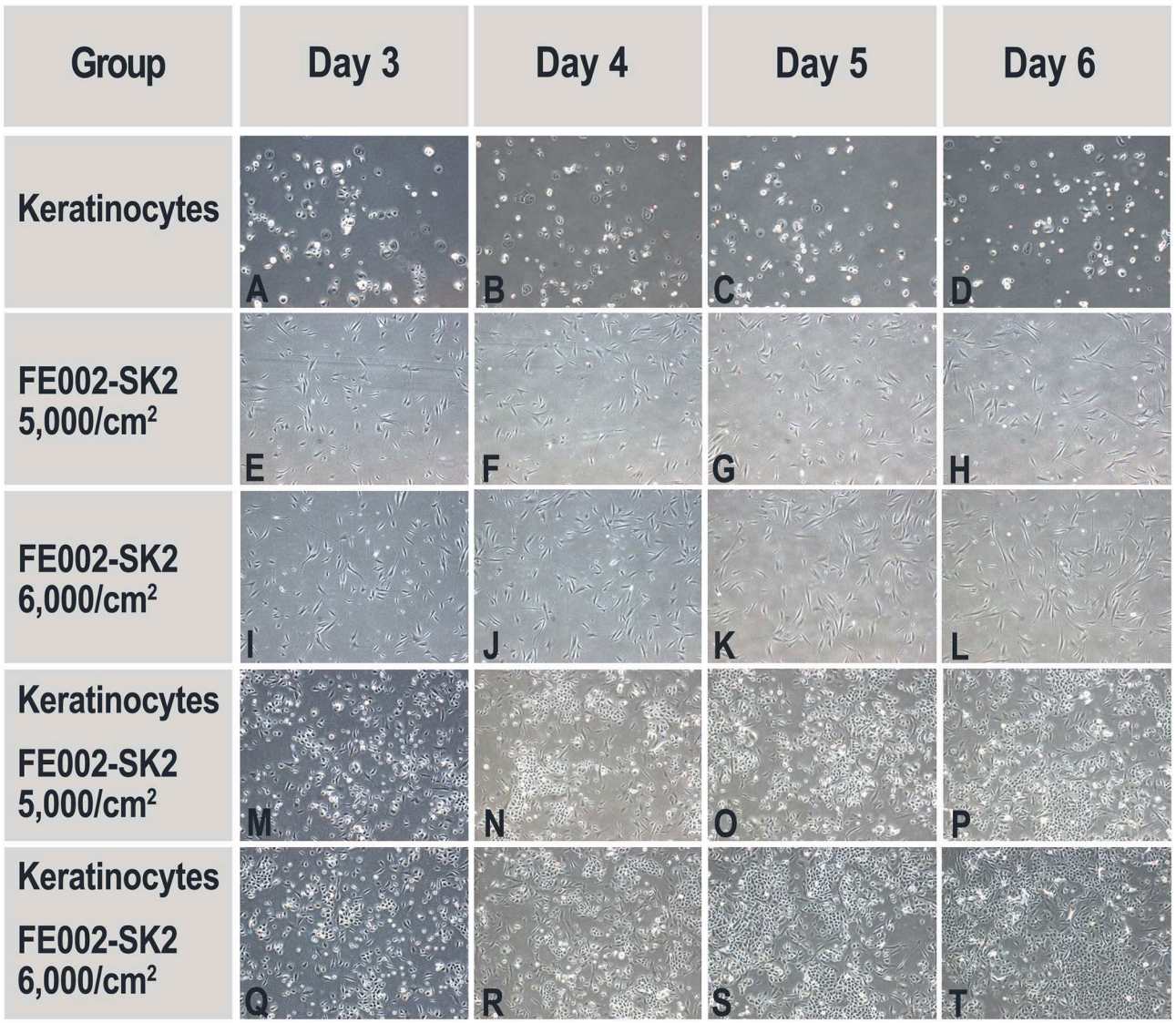

FIGURE 9 | Representative imaging of primary keratinocyte and FE002-SK2 progenitor fibroblast proliferation in single- and co-cultures. Initial viable seeding densities are indicated for each FE002-SK2 group. Initial viable seeding density of keratinocyte was of 2,000 cells/cm^2. No proliferation was observed in the single-population cultures of keratinocytes **(A–D)** or FE002-SK2 fibroblasts **(E–L)**. High proliferation was observable in the co-cultures **(M–T)**. Imaging data was acquired under 40X optical magnification. Mean harvesting cumulated cell counts are listed in **Table 3**.

cells assures minimal risk of viral, fungal or bacterial disease transmission (**Supplementary Figures 1, 2**) (Quintin et al., 2007; Applegate et al., 2013). Progenitor cells are differentiated and characterized by high expansion and regeneration potential, while presenting low immunogenic and tumorigenic properties after transplantation (**Figure 1**) (Doyle and Griffiths, 1998; Quintin et al., 2007). Importantly, these cells do not require growth factor cocktails or feeder-layers for *in vitro* expansion, contrasting with undifferentiated mesenchymal stem cells or primary keratinocytes (Doyle and Griffiths, 1998; Ramelet et al., 2009). The tissue-specific properties of the FE002-SK2 cells and their negligible potential for reverting to a more potent state confer optimal stability and homogeneity to cultured progeny populations under standard conditions (data not shown). Such specificities imply lower technical and financial requirement than embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) for example and widely benefit the industrial product manufacture step. Concretely, end-product benchmarking favors the use of PBBs in the CHUV Burn Center, as previously reported (Abdel-Sayed et al., 2019).

The robustness and advantages of progenitor cell types such as the FE002-SK2 source are therefore mainly based on their isolation process, extensive expansion capacity, minimal growth requirements, excellent biocompatibility with engineered scaffolds and high resistance to oxidative stress. PCBs, MCBs, and WCBs of cultured progenitor cells can be developed rapidly

TABLE 4 | Biomarkers detected in the control media, in single population cultures and in keratinocyte-FE002-SK2 co-culture conditioned media.

	LOD	DK-SFM medium after incubation	DK-SFM from keratinocyte cultures	DK-SFM from FE002-SK2 cultures	DK-SFM from co-cultures
Angiogenin	3	0	1	9	**42**
AR	25	0	51	0	**645**
Axl	13	11	21	27	**76**
CD14	11	8	0	210	12*
CEACAM1	13	26	23	73	5*
CXCL1	5	0	1	1	**105**
CXCL5	12	2	8	4	**3,499**
CXCL8	7	11	12	7	**448**
CXCL16	12	0	41	0	**502**
DKK-1	73	0	0	0	**280**
DR6	4	0	6	0	**72**
EGFR	8	0	6	1	**40**
E-Selectin	80	0	306	0	**608**
Fas	3	0	0	0	**14**
Follistatin	21	3	103	35	**1,453**
Galactin-7	103	0	2,672	0	588*
GCP-2	11	0	0	0	**75**
GDF15	2	0	1	6	**72**
HGF	12	8	5	41	**119**
IGFBP2	23	0	192	0	**1,660**
IGFBP6	241	0	0	3,452	**5,945**
IL-6	17	23	7	25	**2,383**
IL-6R	113	0	0	7	**468**
Insulin	55	11,947	14,882	9,988	9,964
LIF	18	58	56	118	146
Lipocalin-2	1	0	132	3	**429**
MCP-1	25	0	0	0	**382**
MCSF	2	0	0	0	**11**
MIF	15	0	271	27	155*
MIP-3a	1	0	0	0	**17**
MIP-1b	3	0	1	1	**23**
PAI-1	115	0	1,657	13,922	**19,550**
PDGF-AA	3	6	14	0	**40**
PlGF	3	3	1	3	**12**
RANTES	11	2	12	2	**225**
TGFα	6	0	1	0	**35**
TIMP1	23	15	247	6,587	**5,891**
TIMP2	26	9	135	5,098	**4,443**
TNFα	25	21	58	43	76
TNF-R1	24	14	42	27	**274**
Trappin-2	21	0	708	0	**961**
VEGF	8	0	3	0	**105**

Concentration values as well as LODs (lower limits of detection) are presented in [pg/mL]. Values having significantly increased from the single population cultures to the co-cultures are presented in bold in the co-culture column. Values having significantly decreased from the single population cultures to the co-cultures are presented with an asterisk in the co-culture column. Significant increases were defined as levels beyond an additive resulting effect in the co-cultures, normalized to the final mean cumulated cell counts.

and efficiently while safety testing is continuously performed throughout the bioprocessing and manufacturing workflow (**Figure 3**) (Quintin et al., 2007). Therapeutic applications require cultured fetal cells to be used up to two-thirds of their documented and validated *in vitro* life-span, as to assure consistency of important biological properties including total protein concentration, gene expression and biological activity (Quintin et al., 2007). Notwithstanding the latter and due to FE002-SK2 progenitor dermal fibroblast source robustness and expansion possibilities, over 39 billion skin bioengineered

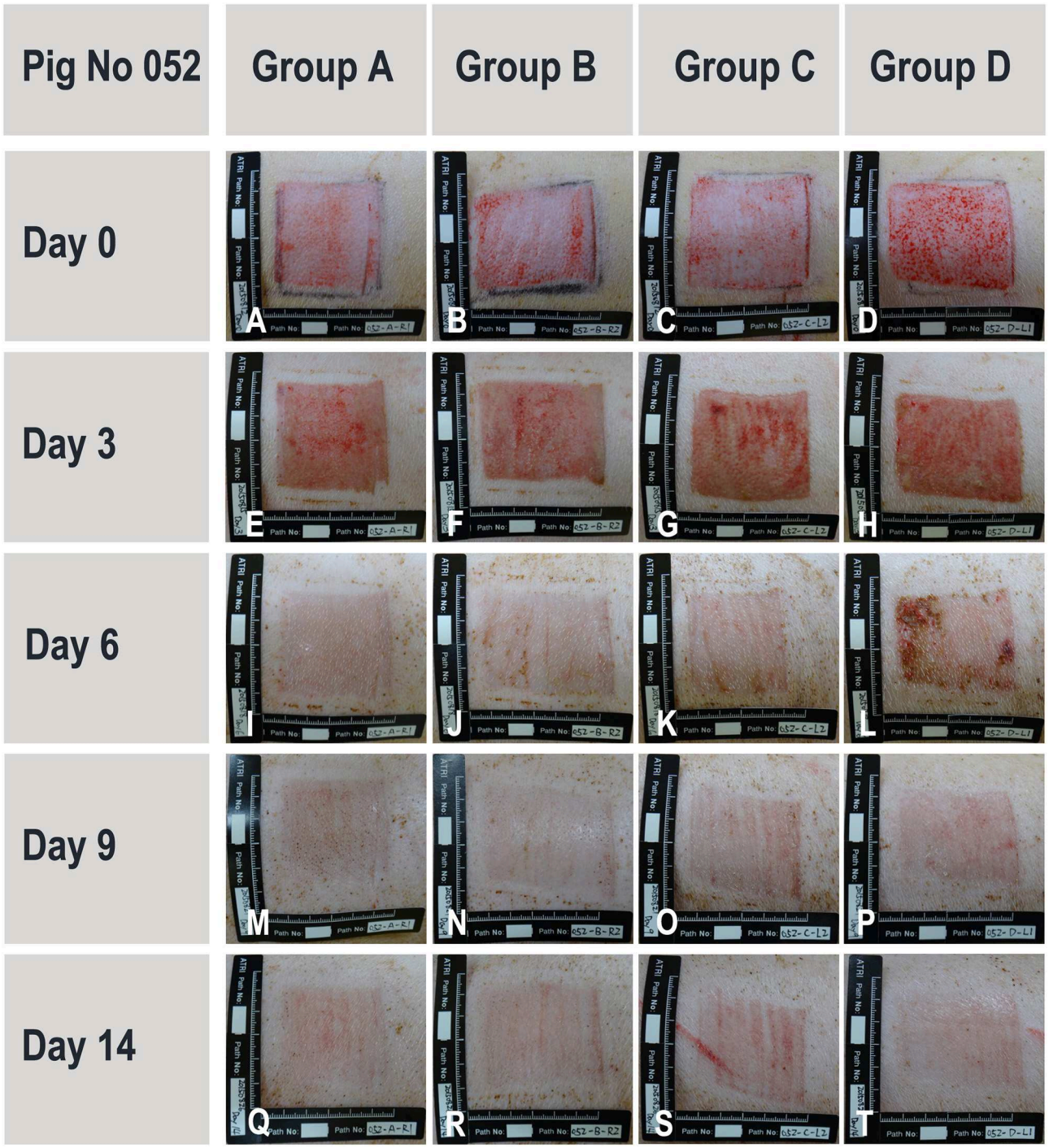

FIGURE 10 | Representative and evolutive imaging of porcine wound models. Data were acquired from pig N°052. All 4 experimental groups are presented at different timepoints ranging from wound creation (Day 0) up until sacrifice (Day 14). Treatment groups [A–D] consisted in the sham control, hydrogel scaffold alone, and hydrogel scaffolds with low and high doses of FE002-SK2 progenitor fibroblasts, respectively.

constructs of 9 × 12 cm can potentially be produced from a dedicated tiered cell bank, which can be cryopreserved for decades. Establishing such cell banks enables the use of consistent and safe starting biological materials, with maximized efficiency and optimized industrial costs, for various applications in tissue engineering and regenerative medicine (Abbasalizadeh et al., 2017; Pigeau et al., 2018). This can assure rapid on-demand availability of consistent end-products once the

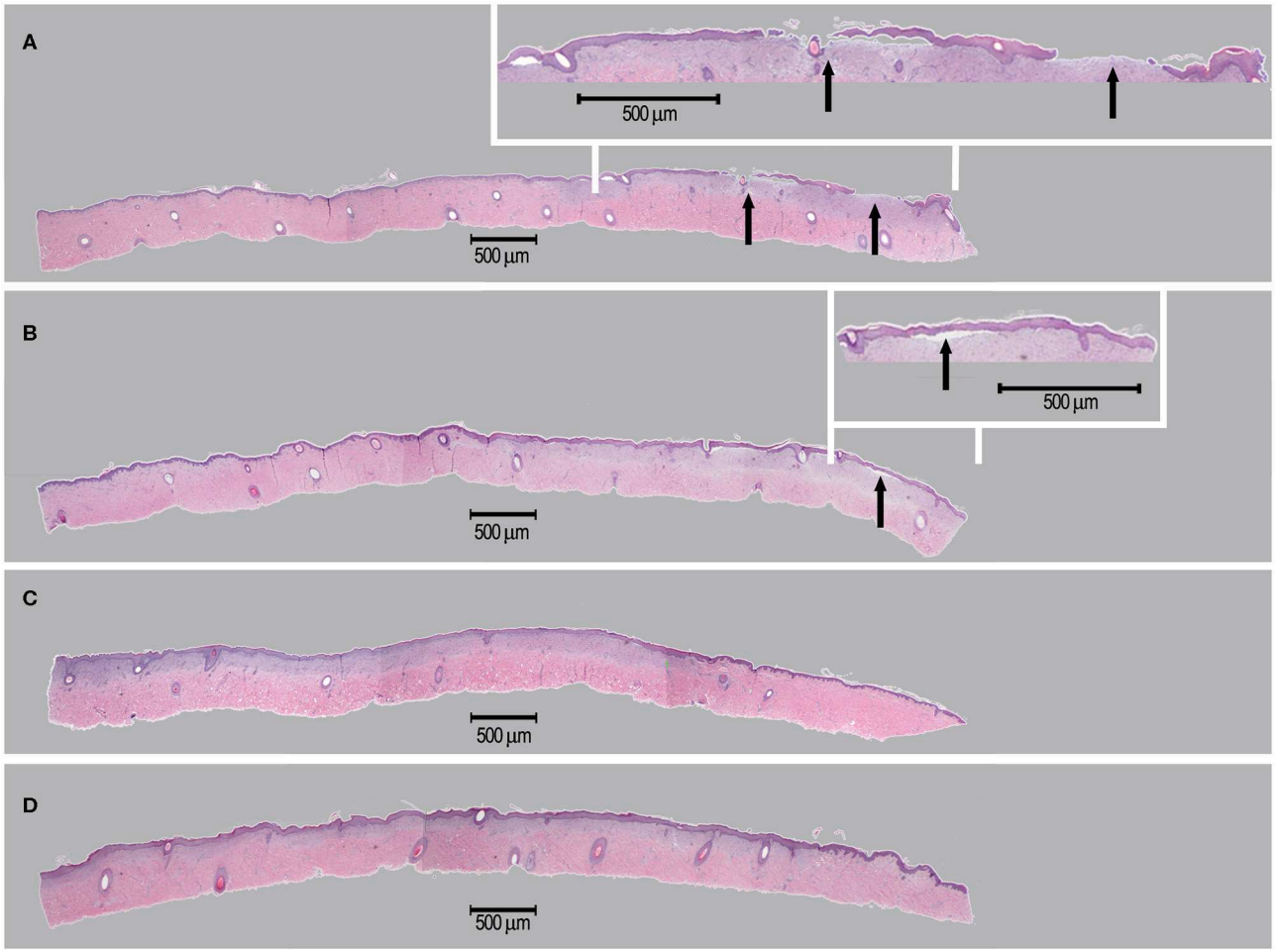

FIGURE 11 | Representative imaging of tissue sections isolated for histological examination. Sections were isolated on Day 14 and stained using HE. Treatment groups [A–D] consisted in the sham control **(A)**, hydrogel scaffold alone **(B)** and hydrogel scaffolds with low **(C)** and high doses **(D)** of FE002-SK2 progenitor fibroblasts, respectively. Arrows in **(A,B)** indicate observable epidermal detachment.

market is reached, as stored vials of cells at defined passages are directly used for therapeutic product preparation in the clinic. Contrasting with autologous cell therapies, extensive culture periods associated with majored costs and risks of contamination would therefore no longer be required (Quintin et al., 2007; Haack-Sørensen and Kastrup, 2011; Hunt, 2019). Inter-individual variability is also taken out of the equation in an allogenic therapeutic approach, allowing for standardization and optimization of bioprocessing technologies and biologic construct manufacture. Indeed, the consistency, stability and robustness in processing the biological starting material is paramount in assuring optimal safety and therapeutic quality of the end-product to be applied to the patient. An optimal cell source therefore allows for realistic clinical translation, industrial transposition and market implementation within modern regulatory frameworks (Quintin et al., 2007; Larijani et al., 2015; Marks and Gottlieb, 2018).

Up-scaling of FE002-SK2 cell banking, continued translational development and preparation of clinical trials has required and requires many steps. Determination and characterization of key elements including identity, purity, sterility, stability, safety, and efficacy are needed for cellular-based products. Regulations impose strict criteria for the production and manufacturing environment used for cell-based therapies destined for clinical trials and treatments (Ratcliffe et al., 2011; Abbasalizadeh and Baharvand, 2013; Hunsberger et al., 2015). Therefore, working with highly consistent biological starting materials allows for efficiency and safety optimization in the manufacturing process of therapeutic products. In order to allow large numbers of patients to be able to benefit from such therapies, the availability of the biological material needs to be optimized. Therefore, the materials and the *ad hoc* technology need to be easily transposable to different countries under consistent quality specifications (Hunsberger et al., 2015).

Based on extensive in-house experience, implementing technology transfers for industrial-scale banking of allogenic progenitor cells remains tedious to date. Each step of the process needs to be optimized and tested, ensuring that the use by

different entities is in accordance with the original banking protocols and legal constraints of the Swiss Fetal Transplantation Program (Applegate et al., 2013). Notwithstanding the technical and administrative barriers, successive and successful technology transfers have been carried out during the last decade with the FE002-SK2 cell source, both in Switzerland and around the globe (**Figure 4**). The first transposition and industrial up-scaling was performed in Scotland (BioReliance) and enabled the establishment of extensive GMP cell banks (**Figures 3**, **4**). In parallel, clinical batches of FE002-SK2 progenitor fibroblasts are continually manufactured in-house in the GMP-certified Cell Production Center for the CHUV Burn Center (**Figure 4**). Close inter-disciplinary collaborations have enabled the local transfer of technology and the continuous treatment of severe burn victims in Lausanne. International research and clinical developments based on the FE002-SK2 cell source are led by TWB, which are currently in clinical trials (approved in Japan, Taiwan, USA; ongoing in Japan, Taiwan). Technological transposition from applied research to commercial industrial settings was successfully carried out during the last decade *via* a CHUV spin-off (Elanix Sàrl). The acquired and specific experience in transposition underlined that a very robust cell source and proper oversight were of utmost importance in ensuring optimal, rational, safe, and efficient use of cell starting materials. Consistency of the cell banks must be met by consistency in the use made of the materials. Achieving multi-center transposition then allows to work with different regulatory frameworks, which stimulates and directs the applied and clinical research in complementary ways.

In the Lausanne University Hospital, progenitor cells were used in clinical settings particularly for severe burns and acute or chronic wounds in human patients, generating over two decades of pioneer clinical experience in pediatric and geriatric populations (Hohlfeld et al., 2005; Ramelet et al., 2009; De Buys Roessingh et al., 2015). The Progenitor Biological Bandages used in the clinic to this day are engineered wound coverage constructs composed of an equine collagen scaffold (9 × 12 cm) carrying clinical-grade FE002-SK2 progenitor fibroblasts (Abdel-Sayed et al., 2016, 2019). On-demand availability to clinicians allows for application of such constructs in the early phases of treatment of trauma victims. The PBBs are used to favor healing of second degree superficial and deep burns, as well as donor site grafts routinely used in the Burn Center. The overall therapeutic goal is to limit the damage to the burned tissues and restrict the need for donor site skin-grafting. This goal was already reached with the treatment of numerous pediatric burn victims to date (Hohlfeld et al., 2005; De Buys Roessingh et al., 2006). The implementation of the Bandages to broader routine clinical use is under examination and will be the object of a new internal clinical trial (Priority Project Bru_PBB). Retrospective studies have already yielded preliminary safety results for the therapy on adult and pediatric patients (data to be published). Standardized comparison with standards of care for burn patients must be performed, in order to assess the positive impacts and therapeutic benefits of the treatment. The Progenitor Biological Bandages developed in the CHUV were also used as a base in the Project Platform *SwissTransMed*, associating antimicrobial factors for the treatment of burn patients (Abdel-Sayed et al., 2016).

Most recent applied research in Switzerland and Taiwan using the FE002-SK2 cell source has aimed toward elucidation of putative mechanisms of action which intervene and possibly mediate therapeutic effects after clinical delivery. Progenitor fibroblast preparations and derivatives thereof (cell extracts, replication-inactivated cells and conditioned media) were found to have demonstrable high bioactivity in defined *in vitro* assays for primary keratinocyte migration and proliferation stimulation. In particular, (a) FE002-SK2 cells facilitated primary keratinocyte migration in Transwell® assays (2.7-fold mean relative increase in migrated population counts, **Figure 5**, **Table 1**), (b) FE002-SK2 conditioned media facilitated *in vitro* epithelial gap-filling (significant decrease in filling time, **Figure 6**), (c) FE002-SK2 cell extracts promoted keratinocyte proliferation (strong increase in total cell counts after 7 days of stimulation, **Figure 7**, **Table 2**) and (d) FE002-SK2 cells and growth-arrested variants promoted keratinocyte proliferation (strong feeder-layer effect, **Figures 8**, **9**, **Table 3**). Hence, quantified *in vitro* evidence of biological effects has been documented for the cell type of interest and is crucial for product characterization and standardization, addressing both manufacturing quality control and regulatory requirements (Hunsberger et al., 2015; Marks and Gottlieb, 2018). By extension, such results support the use of pure fibroblastic populations as allogenic therapeutic agents, without conjugation to technically demanding keratinocytes, due to the potent stimulatory effects described hereabove.

Further analysis of the biomarkers found in co-culture conditioned media may help to elucidate the mechanisms underlying the therapeutic stimulation of wounded tissues. Beneficial effects are indeed suspected to be of trophic nature, relying on patient cell stimulation by the applied engineered constructs and biological materials, rather than engraftment of allogenic cells (Spiekstra et al., 2007; Werner et al., 2007; Barrientos et al., 2008). In addition, liberation of matrix proteins by allogenic therapeutic cells probably supports and promotes structural modifications occurring during tissue remodeling. Biochemical paracrine signaling, through the release of growth factors and cytokines in well-proportioned combinations, probably acts by favoring a return to cutaneous homeostasis after dynamic and multi-step healing of the skin (Wojtowicz et al., 2014). The results presented in **Table 4** suggest clear synergistic effects of cell-cell interactions relative to the expression of biomarkers of interest. These biomarker quantifications allow for hypotheses establishment around the role of the biological therapeutic material in the wound environment, keeping in mind the vast differences between the *in vitro* assay settings and a patient's damaged tissue. Hypotheses are formulated hereunder based on the relative quantities of biomarkers found in the co-culture conditioned media.

PAI-1 (Plasminogen activator inhibitor-1) is a serine protease inhibitor which inhibits degradation of fibrin by plasmin, as well as the activity of matrix metalloproteinases (MMPs). During wound healing, elevated levels of PAI-1 inhibit uPA/tPA/plasmin and plasmin-dependent MMP activities, which may help to facilitate wound healing, by modulation of the intrinsic local

repair mechanisms (Providence et al., 2008). Relatively high levels of PAI-1 in the FE002-SK2 cultures (13,922 pg/mL) and co-cultures (19,550 pg/mL), coupled with an apparent low inter-stimulation of its expression in the co-cultures (resulting additive contributions of both cell populations) indicate that the progenitor fibroblasts are responsible for allogenic contributions of PAI-1 (**Table 4**). TIMP1 and TIMP2 are tissue inhibitors of metalloproteinases, which also inhibit the degradation of tissue matrix. In chronic wounds, the ratio of TIMP/MMP may be too low, which delays wound healing (Krishnaswamy et al., 2017). Co-culture settings had a negligible or slightly antagonistic effect on the production of TIMPs by the cell populations, but it appears again that the progenitor fibroblasts are responsible for the presence of relatively higher levels of these biomarkers in the conditioned media (20-40X higher TIMP levels in fibroblast cultures *vs.* keratinocyte cultures, **Table 4**). Galectin-7 (Gal-7) binds to beta-galactosids and is mainly expressed in stratified epithelial cells, including keratinocytes. Gal-7 is involved in cell-cell and cell-matrix contacts. Gal-7 gene (*LGALS7*) knockdown results in reduced differentiation and increased proliferation of keratinocytes (Chen et al., 2016). This is coherent with the increased keratinocyte proliferation in the co-cultures in this study, whereas Gal-7 levels were decreased. Several additional factors (e.g., AR, CXCL1, CXCL5, CXCL8, CXCL16, Follistatin, IGFBP2, IL-6, IL-6R, MCP-1, and RANTES) were found to be highly expressed in the co-culture conditioned media, while very low levels were detected in the media of single population cell cultures. These factors may be produced by keratinocytes or FE002-SK2 fibroblasts under the conditions of keratinocyte-fibroblast interactions and may play a role in facilitated keratinocyte migration and proliferation *in vitro*. *In vivo* effects can be hypothesized based on the biomarker data but would need more extensive investigation on clinical patient samples. CXCL5 (ENA-78, epithelial-derived neutrophil-activating peptide 78) is produced following stimulation of cells by the inflammatory cytokines IL-1 or TNF-alpha. ENA-78 stimulates the chemotaxis of neutrophils *via* CXCR2, possessing angiogenic properties and stimulating proliferation and migration of keratinocytes (Zaja-Milatovic and Richmond, 2008; Kroeze et al., 2012). The highly synergistic effect of co-cultures on the ENA-78 levels and the naturally highly inflammatory nature of deep skin wounds are coherent with the hypothesis that allogenic fibroblasts potently interact with host keratinocytes in view of repair and regeneration stimulation. CXCL1 [Gro, Gro-alpha, neutrophil-activating protein 3, or chemokine (C-X-C motif) ligand 1], which elicits its effects through the chemokine receptor CXCR2, activates EGFR and stimulates proliferation of epithelial cells. CXCL16 acts as a mediator of innate immunity by attracting CXCR6-expressing cells, such as activated T cells and natural killer T (NKT) cells. CXCL16 is constitutively expressed on the surface of human epidermal keratinocytes, released upon cell activation or photodamage (Scholz et al., 2007).

IL-6 is known to regulate a broad spectrum of immune responses, to stimulate the proliferation of many types of cells and as being vital to wound healing (Gallucci et al., 2000). CXCL8 (IL-8) is a chemokine produced by macrophages and other cell types such as epithelial cells, airway smooth muscle cells and endothelial cells, eliciting its function through CXCR1 and CXCR2. IL-8 is known to recruit neutrophils and to be a potent promoter of angiogenesis, while stimulating proliferation and migration of cultured keratinocytes (Jiang et al., 2012; Sobel et al., 2015). Follistatin (activin-binding protein) has a modest angiogenic effect on endothelial cells and is strongly synergistic with bFGF (basic fibroblast growth factor). Binding of follistatin to activin inhibits the mitogenic function of activin. Follistatin is considered a growth regulator of epithelial cells (Antsiferova et al., 2009). Therefore, synergistic effects of co-cultures on IL-8 and follistatin levels are additional indicators of the trophic dialogues that may take place between allogenic fibroblasts and patient resident keratinocytes in view of therapeutic immune modulation and repair stimulation. The synergistic effects of the co-cultures on the levels of CXCL1, 5, 8, 16, and IL-6 support the hypothesis that under the influence of the progenitor fibroblasts, the patients innate immunity may be stimulated to combat dangerous exogenous pathogens, which is then followed by cell migration and proliferation for regenerating tissues.

For cell-based therapeutic product development submission files, regulatory agencies expect a potency assay pertinent to the clinical indication to be established with the completion of phase II clinical trials, to thoroughly support both proof-of-concept and safety of the product. The quantity of research allocated to biomarker analysis herein was dictated by regulatory requirements, for which stimulation of proliferation and migration coupled to preliminary biomarker analysis was sufficient (for the countries where applications were positively evaluated, i.e., USA, Taiwan, Japan). In developing a cell therapy product, one of the major challenges throughout various product developmental stages (initial research, design of dosage form, development of production process and quality control (QC) method, preclinical toxicology studies, phase I, II, III clinical trials and scale-up manufacturing for marketing) is determining the critical quality attributes (CQAs) or mechanism of action (MOA), which might not be very clear or specific. The keratinocyte migration assay described herein has become part of the actual product release testing. Enhancement of epithelialization rate was targeted as a specific mechanism of action within the indication of treating burns and split thickness wounds, hence the investigation of the ability of fetal progenitor fibroblasts to promote keratinocyte proliferation and migration. Subsequent detailed analysis of culture system protein markers was undertaken in view of further understanding the possible attributing factors of the observed stimulatory effects. However, wounded environments drastically differ from controlled culture conditions of *in vitro* models, whereas results yielded by the biomarker study therefore remain speculative, as markers characterized *in vitro* may or may not affect endogenous keratinocyte behavior on patient wounds. Within the specific context of product development and the relative scarcity of resources and workpower, more advanced biomarker investigations were not yet considered. Indeed, while extremely interesting at a fundamental level, the biomarker investigations were more important for implementing QC assay specifications within the product development process

and approval thereof. The actual product QC is performed by quantification of specific biomarkers to assess cell batch quality and to standardize therapeutic protocols and delivered doses. Similarly, the *in vivo* preclinical preliminary safety and efficacy investigations were not performed within robust or statistically significative settings, as the minimal regulatory requirements were followed, in order to rapidly move along to human clinical trials. The fundamental differences between academic research and industrial-driven development are underlined by this specific issue, as resource constraints and respective objectives differ, yet reconciliation of both aspects is necessary for coherence of the overall message and setting of perspective for considerations about translational therapeutic product development.

Overall, the summarized and probable benefits of progenitor fibroblast therapeutic applications for wound healing induction reside in the secretion of cytokines, growth factors and other ECM proteins onto damaged tissues. In the inflammatory context of cutaneous wounds, cellular proliferation and migration ultimately lead to tissue remodeling (Werner et al., 2007; Barrientos et al., 2008; Providence et al., 2008). Therapeutic stimulation of these physiological processes, which may be complemented by immune-modulation and angiogenesis, can reasonably be conceived after application of progenitor dermal fibroblasts. Data from the *in vivo* porcine study confirmed safety of application of the biological constructs on animal subjects. Low immunogenicity of the products in an inter-species setting strengthened the rationale for specific human biological starting material selection and processing, using immune-privileged sources. Comparative observations between the test groups yielded preliminary indication of superior epidermal attachment at the time of biopsy harvest for the wounds treated with cellular products (**Figures 10**, **11**). While healing rate enhancements were subjectively noted with the use of therapeutic progenitor cells, larger sample groups and specific clinical endpoints shall be further adopted for stringent evaluation of product clinical efficacy. In the specific context of product development, these data will in fact be obtained in the early clinical trial phases (ongoing). Notwithstanding the latter, such *in vivo* evidence complements the extensive clinical experience acquired over the years around the use of PBBs on pediatric and adult human patients suffering from acute and chronic wounds (Hohlfeld et al., 2005; De Buys Roessingh et al., 2015; Abdel-Sayed et al., 2016, 2019). Human safety and comparative efficacy studies are on-going in Asia and pending in the CHUV, in order to assess and quantify the therapeutic gains to be obtained with FE002-SK2-based biologic constructs for wound management.

As cell therapies, the final products yielding FE002-SK2 progenitor fibroblasts must meet numerous standards before being allowed to reach the market. Considerable and continuous efforts are needed to meet the regulatory requirements of respective countries. In parallel, further research needs to be conducted, in order to optimize the logistics and efficiency of the proposed clinical protocols. Indeed, the state-of-the-art protocols detail the use of viable cells preserved in ultra-low temperature freezers or cryogenic storage. A major technical and logistical advantage would be gained by further processing the cellular starting material, in a manner which would respect and maintain biological characteristics, to obtain off-the-shelf stabilized products to be stored at 4°C (Ratcliffe et al., 2011; Abbasalizadeh and Baharvand, 2013; Hunsberger et al., 2015). Further advances in drug delivery options for progenitor fibroblasts will synergistically benefit the regeneration of damaged tissues. Nonetheless, current practices using allogenic progenitor cell-sources enable drastic reduction of clinical availability delays while maximizing consistency and stability of starting biological materials. Indeed, one single defined organ donation in 2009 enabled the establishment of extremely robust and extensive Parental, Master and Working Cell Banks with the FE002-SK2 cell source. Traceability and safety are paramount for Investigational Medicinal Product Dossiers and Investigators' Brochures preparation in view of clinical trials using cellular products (Rayment and Williams, 2010; Heathman et al., 2015). An optimal therapeutic cell source to meet such stringent requirements set forth in this translational work was therefore devised under the Swiss Fetal Transplantation Program. The FE002-SK2 source has supplied research and clinical applications for the last decade and will continue to do so globally. Vast preliminary experience attests to the strong potential of the progenitor technology to be used as a therapeutic product. The ongoing steps to implement FE002-SK2 product use in clinical practice are crucial and well underway, determining the impact and benefits to be gained by patient populations in general.

CONCLUSION

Within the well-defined and regulated context of cell therapy product development, the advantages of a safe and consistent biological starting material have been presented herein using the example of progenitor fibroblasts. The large quantity of data which has been generated to date around the FE002-SK2 progenitor cell source is consistently stressing the importance of optimization and standardization in manufacturing processes. The first-hand experience around industrial transposition of pioneer biomedical technology resumed herein attests to the complexity in reaching technical success, a prerequisite for sound product development. Pragmatically harnessing the inherent variability characterizing biologic substrates allows for better implementation of their high therapeutic potentials, both in the regulatory and clinical settings. Technical aspects regarding cell source optimization and whole-cell bioprocessing are not only scientifically relevant but lay the foundations of efficient and widely-available cell-based therapies. A single organ donation was sufficient for the last decade of applied research and clinical investigations. The implementation of optimized bioprocessing methodology and well-devised industrial-scale GMP biobanking of the FE002-SK2 cell source have demonstrated that extensive and consistent clinical-grade cell banks can be established. Although autologous cell therapies remain of high interest and are preferred regulatory-wise, bringing more evidence to the benefits of consistent allogenic products will surely and naturally direct toward a paradigm shift in the near future. The unique clinical experience established in Switzerland combined

with the ongoing clinical trials in Asia will surely confirm the therapeutic benefits of the unique FE002-SK2 cell source. Through further efforts directed at clinical translation and commercial implementation, quality and efficiency of therapeutic care will be optimized. Musculoskeletal tissue affections and overall health of patients worldwide will therefore surely benefit from the unique progenitor cell technology developed in Switzerland.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

FE002-SK2 primary progenitor cells were isolated from the FE002 organ donation according to a validated protocol, approved by the State Ethics Committee (University Hospital of Lausanne—CHUV, Ethics Committee Protocol #62/07: Development of fetal cell banks for tissue engineering, August 2007). The FE002 donation was registered under the Federal Transplantation Program and its Biobank, complying with the laws and regulations within both programs. Obtention and use of progeny cells follow regulations of the Biobank of the Department of Musculoskeletal Medicine in the CHUV. Cell bank safety testing assays performed by BioReliance which involved animal subjects were performed in accordance with the OECD Principles of Good Laboratory Practice (GLP) and individual studies were conducted after proper internal ethical considerations and validation. The porcine study in Taiwan was carried out at the Agricultural Technology Research Institute under GLP standards after proper internal ethical considerations and validation.

AUTHOR CONTRIBUTIONS

AL, PL, NH-B, B-RS, and LA: Study conception and design. AL, PL, CS, MM, AB, NH-B, WR, B-RS, and LA: Acquisition of data. AL, PL, NH-B, B-RS, and LA: Analysis and interpretation of data. AL, PL, and B-RS: Drafting of the manuscript. AB, WR, NH-B, B-RS, and LA: Critical revision. AL, PL, CS, NH-B, MM, AB, WR, B-RS, and LA: Acceptance of final 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/fbioe.2020.00581/full#supplementary-material

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Conflict of Interest: CS, NH-B, AB, WR, and LA were the Founders of Elanix Sàrl, which was constituted for the industrial transposition of the bioprocessing technology out from the CHUV University Hospital. LA is listed as Inventor on the patent describing the bioprocessing technology for progenitor cell banking and is currently the *ad-interim* Administrator of Elanix Sàrl. PL and B-RS are employees of Transwell Biotech Co. Ltd. in Taiwan and hold shares in said Company. AL was employed by company Tec-Pharma SA.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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METHODS
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Phoenix: A Portable, Battery-Powered, and Environmentally Controlled Platform for Long-Distance Transportation of Live-Cell Cultures

*Brittany N. Willbrand, Sylvia Loh, Caitlin E. O'Connell-Rodwell†, Dan O'Connell and Devin M. Ridgley**

SCORPIO-V Division, HNu Photonics LLC, Kahului, HI, United States

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Edited by:
Alain A. Vertes, NxR Biotechnologies GmbH, Switzerland

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Kar Wey Yong, University of Alberta, Canada
Lorenzo Fassina, University of Pavia, Italy

***Correspondence:**
Devin M. Ridgley
dridgley@hnuphotonics.com

†Present address:
Caitlin E. O'Connell-Rodwell, Harvard Medical School, Boston, MA, United States

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Despite the advent of advanced therapy medicinal products (ATMPs) in regenerative medicine, gene therapy, cell therapies, tissue engineering, and immunotherapy, the availability of treatment is limited to patients close to state-of-the-art facilities. The SCORPIO-V Division of HNu Photonics has developed the Phoenix-Live Cell Transport™, a battery-operated mobile incubator designed to facilitate long-distance transportation of living cell cultures from GMP facilities to remote areas for increased patient accessibility to ATMPs. This work demonstrates that Phoenix™ (patent pending) is a superior mechanism for transporting living cells compared to the standard method of shipping frozen cells on dry ice (−80°C) or in liquid nitrogen (−150°C), which are destructive to the biology as well as a time consuming process. Thus, Phoenix will address a significant market need within the burgeoning ATMP industry. SH-SY5Y neuroblastoma cells were cultured in a stationary Phoenix for up to 5 days to assess cell viability and proliferation. The results show there is no significant difference in cell proliferation (∼5X growth on day 5) or viability (>90% viability on all days) when cultured in Phoenix™ and compared to a standard 5% CO_2 incubator. Similarly, SH-SY5Y cells were evaluated following ground (1–3 days) and air (30 min) shipments to understand the impact of transit vibrations on the cell cultures. The results indicate that there is no significant difference in SH-SY5Y cell proliferation (∼2X growth on day 3) or viability (>90% viability for all samples) when the cells are subjected to the vibrations of ground and air transportation when compared to control samples in a standard, stationary 5% CO_2 incubator. Furthermore, the temperature, pressure, humidity, and accelerometer sensors log data during culture shipment to ensure that the sensitive ATMPs are handled with the appropriate care during transportation. The Phoenix™ technology innovation will significantly increase the accessibility, reproducibility, and quality-controlled transport of living ATMPs to benefit the widespread commercialization of ATMPs globally. These results demonstrate that Phoenix™ can transport sensitive cell lines with the same care as traditional culture techniques in a stationary CO_2 incubator with higher yield, less time and labor, and greater quality control than frozen samples.

Keywords: cell therapy, live cell transport, CAR-T, mobile incubator, immunotherapy, SH-SY5Y cell line, stem cell

INTRODUCTION

Significant advances have been made to create advanced therapy medicinal products (ATMPs) in the form of regenerative medicine, cell therapies, gene therapies, tissue engineering, and immunotherapy. ATMPs are a growing treatment category with clinical applications for a variety of diseases and injuries, including cancer immunotherapy (Rosenberg and Restifo, 2015; Hosseinzadeh et al., 2018), brain and spinal cord injuries (Cox et al., 2017; Gabel et al., 2017), heart disease (Fan et al., 2019), pancreatitis (Ahmed et al., 2018), aneurysm (Adibi et al., 2016), paraquat poisoning (He et al., 2018), musculoskeletal disease (Law et al., 2019), erectile dysfunction (Reed-Maldonado and Lue, 2016), sickle cell disease management (Sadat-Ali et al., 2017), perianal fistulas in Crohn's disease (Cheng et al., 2019), and diabetic foot ulcers (Lopes et al., 2018). Though initial animal and case studies appear promising, additional clinical trials are needed to fully characterize the efficacy and limitations of ATMPs in disease management including the standardization of treatment regimens between laboratory research and clinical applications (Borlongan, 2019; Brunet et al., 2019; Fukumitsu and Suzuki, 2019). Unfortunately, these treatment options require state-of-the-art laboratories to produce and maintain the necessary, highly sensitive cell and tissue cultures, dramatically limiting access to patients and researchers. Thus, facilities that offer cell therapy treatment would be inaccessible to patients with restrictive geographic accessibility, limiting market access (Mahalatchimy, 2011).

As cell therapies are becoming more mainstream within the medical community, there is a growing need to reliably transport these sensitive treatments to patients around the world. Currently, access to cell therapy technologies is limited for three primary reasons. (1) Due to the sensitive nature of these cell lines, the time and distance required for transport outside of a controlled environment will result in cell death and render the treatment ineffective. (2) Traditional incubators that are required to keep these cells alive within a R&D laboratory are neither portable, compact, nor energy efficient, rending this equipment ineffective for live-cell transport. (3) Finally, traditional cell transport requires the samples be cryogenically frozen to −150°C in liquid nitrogen (LN_2) for preservation. LN_2 cryopreservation during transport requires laborious, time-consuming biological manipulations prior to therapeutic use, which is neither efficient nor plausible to expect of on-site physicians and remote medical facilities.

Research in cryopreservation methods for bone marrow-derived mesenchymal stem cells (BM-MSCs) varied greatly from 50 to 100% viability depending on the cryopreservation method, cell passage number, and post-thawing testing time/method (Bahsoun et al., 2019). Moreover, these studies suggest that metabolic activity is altered and apoptosis is "evident" in the post-thaw BM-MSCs, though these pathways are not well-characterized (Bahsoun et al., 2019). For t-cells exposed to cryopreservation, recovery rates between 66 and 90% have been observed (Lemieux et al., 2016; Luo et al., 2017). These studies indicate that shipping cell cultures in LN_2 increases the labor prior to and post-shipping as well as reducing the yield by up to 44 and 50% for t-cells and BM-MSCs, respectively. In addition, there is clear evidence to suggest that metabolic pathways are altered, and induction of apoptosis is increased for BM-MSCs. While more research is needed to fully understand the effects of cryopreservation on ATMP-like cells, these studies indicate that cryopreservation may have some negative impacts on cell function and performance. Thus, there needs to be a more efficient (cost and time) and cell culture friendly mechanism to transport ATMPs from production facilities to the patients in need.

Interestingly, there are a few examples of using simple CO_2 incubators to transport reproductive cells from an R&D facility/location. In Asia, there are two examples of these simple incubators being used in veterinary applications to transport mink whale oocytes on a research vessel (Iwayama et al., 2005) and bovine embryos transported from Japan to China for nuclear transport (Dong et al., 2004). In both instances, CO_2 gas was generated in a sealed and negatively pressured box via a chemical reaction with distilled water and effervescent granules. More recently, human pre-implantation embryos were transported in the LEC-960 portable incubator from Ukraine to Isreal (Levron et al., 2014). The LEC-960 was designed specifically for the transport of reproductive cells by using an aluminum heating block for multiple 0.5 mL vials. These studies demonstrate that it is possible to transport living cell cultures over long distances; however, these devices are designed to transport small quantities of reproductive cells to be used for assisted reproductive therapy (ART) applications. Unfortunately, there is no evidence that shows these simple portable incubators can transport sufficient volumes of ATMP-like cell cultures for R&D or therapeutic applications.

The SCORPIO-V team at HNu Photonics has developed a compact transport device that keeps cells healthy during shipments to facilitate live-cell transport of their cell lines for NASA-based research applications. Phoenix^{TM} is a live-cell portable incubator that is composed of the outer container that houses all electronics, power and heating elements, as well as the inner container (cell container) that is a disposable and sealed polymer box to capture the CO_2 atmosphere and maintains the cell culture in a sterile environment (**Figure 1**). Previously, Phoenix^{TM} was developed and utilized to transport living neuroblastoma cells from Maui to the remote West Texas desert for a suborbital flight on Blue Origin's New Shepard vehicle. Indeed, Phoenix technology is derived from the portable, compact, and automated thermal technology developed to perform life science investigations on the International Space Station. These technologies (BioChip SubOrbital Lab and Mobile SpaceLab) have successfully been deployed to Low Earth Orbit (LEO) for biological interrogations. While the Phoenix^{TM} has been used for space biology applications in the past, the technology has been harnessed and adapted to transport ATMP-like cell lines. The ability of Phoenix^{TM} to transport large volumes of ATMP-like cells (up to two t-75 flasks) with automated environmental control make it a viable option for the transport of cell therapies over existing portable incubators.

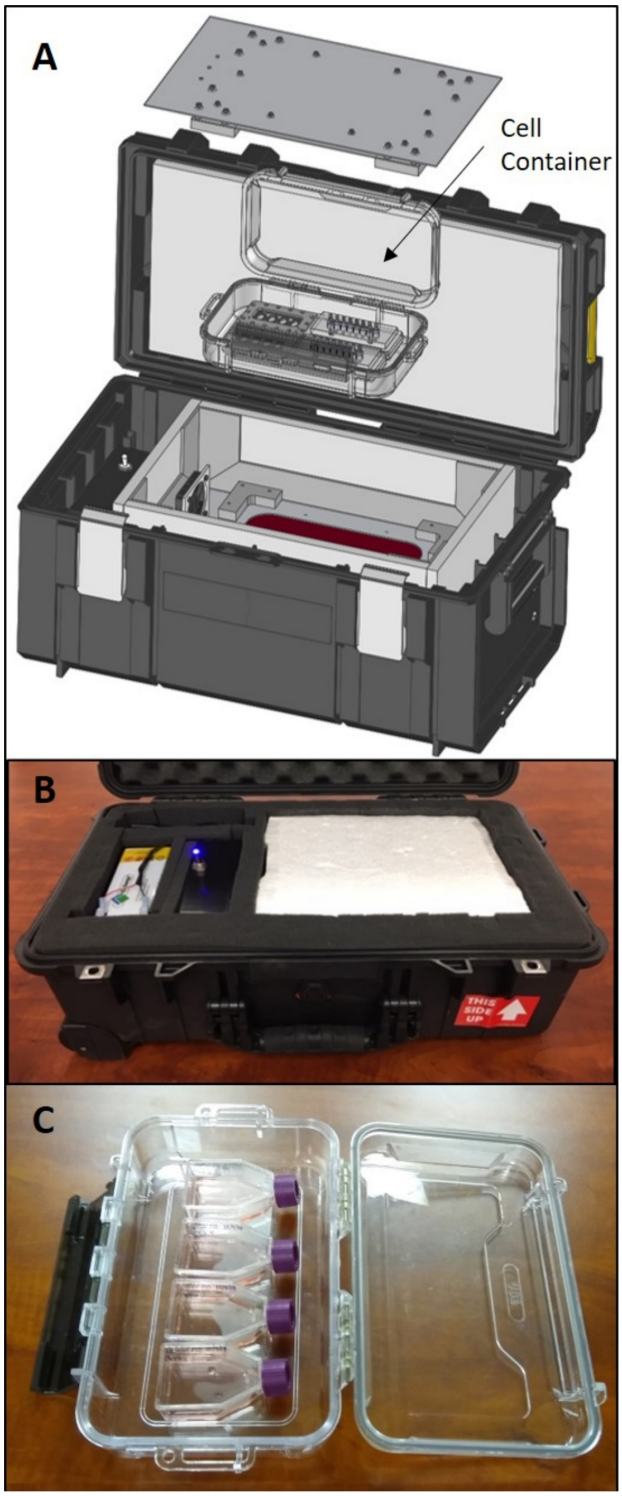

FIGURE 1 | (A) A schematic illustration of the Phoenix system depicting the internal cell container that maintains a sterile environment for t-flasks (SCORPIO-V BioChips shown instead) and captures the 5% CO_2 atmosphere. **(B)** The Phoenix system that was used for testing and to transport living neurons from Maui to West Texas for a test launch on Blue Origin's New Shepard rocket. **(C)** The Phoenix cell container holding four t-12.5 flasks that were used for testing.

Phoenix^{TM} may revolutionize access of ATMPs to clinics and laboratories worldwide to create opportunities for accelerated research, new discoveries, and improved therapeutic accessibility. Although originally developed for space-based applications, Phoenix^{TM} meets a burgeoning market demand by providing a reliable and quality-controlled platform to transport cell therapies over long distances. This study investigates the efficacy of Phoenix^{TM} to maintain a healthy neuron-like cell cultures during several days of incubation and multiple transportation modes. Here, SH-SY5Y neuroblastoma cells were used as a proxy ATMP in order to examine the effects of long-term incubation and transport vibrations on cell proliferation, viability, and morphology. Furthermore, environmental factors such as relative humidity, triaxial acceleration, percent CO_2, and temperature were monitored during each test to quantify the reliability of Phoenix^{TM} to maintain living cell cultures when compared to a standard CO_2 incubator.

MATERIALS AND EQUIPMENT

Cell Culture

SH-SY5Y cells (ATCC) were cultured in DMEM/F12 (Sigma) supplemented with 10% fetal bovine serum (Sigma) and 1% antibiotic-antimycotic (Sigma). Cells were stored in a humidified incubator at 37°C with 5% CO_2 to buffer media solution pH. Cells were cultured in a vented t-12.5 flask (VWR).

Phoenix^{TM} System

An expanded view of Phoenix^{TM} is illustrated in **Figure 1**. Briefly, Phoenix is composed of the outer container that houses all electronics, power and heating elements, as well as the inner cell container that is a disposable and sealed polymer box that captures the CO_2 atmosphere and maintains the cell culture in a sterile environment. It is important to note that the Phoenix system does not contain a CO_2 source to prevent shipping constraints for air travel. The Phoenix system is designed to be transported as a "checked bag" to accompany a traveler if desired.

Cell Viability and Proliferation

A live-dead assay was used to quantify cell viability with calcein AM (Thermo Fisher Scientific) and ethidium homodimer (Thermo Fisher Scientific). The cells were incubated in 4 μM of calcein AM and ethidium homodimer at 37°C for 20 min prior to imaging. Under a microscope, the cells were imaged at 25°C with brief exposures to 450 and 532 nm lasers for excitation of live and dead cells, respectively. Cell proliferation was quantified with a Countess II Automated Cell Counter (Thermo Fisher Scientific).

METHODS

Cell Culture

Cells were passaged when confluency reached 80% with less than 10 passages for the cell cultures used for viability experiments and less than twenty passages for proliferation experiments. For each

experiment, t-12.5 cell culture flasks were seeded at a density of 4.0×10^5 cells per t-flask (5 mL total volume). Please see the Phoenix User's Manual to determine what standard disposable cell culture containers can be used within the Phoenix System. In general, it will be up to the user to determine if a sealed of vented container is appropriate for transport of their cell line. Similarly, medium volume within the container should be considered as low volumes may induce adverse fluid-shear conditions on the given cell line.

Phoenix and Transportation

Phoenix^{TM} was pre-heated to 37°C at least 30 min prior to each experiment. The cell container was sterilized with 70% isopropyl alcohol then stored in an open position inside a standard incubator enriched with 5% CO_2. Prior to loading, media within t-flasks for both incubator treatment groups were supplemented with 25 mM of HEPES buffer solution (VWR) to buffer solution pH without the use of CO_2. Once seeded t-flasks were added, the cell container lid remained in an open position. After 30 min of incubation, the cell container lid was moved to a closed position while inside the standard incubator. The cell container was then transferred to Phoenix^{TM}. Ground transportation occurred between Kahului and Haiku, Hawaii, United States, where Phoenix was loaded inside a motor vehicle then transported 13 miles twice per day. Air transportation occurred in Kahului, Hawaii, United States. Phoenix was loaded into a motor vehicle and driven 4.2 miles to Maui Flight Academy at the Kahului International Airport. After loading Phoenix^{TM} into the cargo hold of a Cirrus SR22, a ∼30-min test flight was performed that included two take-offs (2.1 g), two 45° bank turns (1.6 g), two 60° bank turns (2.0 g), nosedive (0 g), and two landings (0–2.5 g). Additional values can be found in **Figure 8**. All flying aerobatics were performed at an altitude of 1500 ft.

Experimental Design and Statistical Analysis

SH-SY5Y cells cultured in Phoenix^{TM} were compared to SH-SY5Y cells cultured in a traditional CO_2 incubator. Cell viability and proliferation were examined after stationary incubation for 1–5 days, 1–3 days of ground transportation, and viability was examined following ∼30 min of air transportation. Thus, 17 experiments were performed in this study (eight proliferation and nine viability assays). There were four replicates for each experiment, which was defined as a single t-flask. There were eight technical replicates for the live-dead assay, which was defined as a single imaging frame. There were three technical replicates for cell counting, which was defined as a single frame. Technical replicates were averaged within each biological replicate for analysis. The error bars in figures represent the means ± standard error values. Prior to statistical analysis, histograms were examined, and tests were used to determine whether assumptions of normality and homogeneity of variance were violated (Shapiro–Wilk test and Levene's test, respectively). The statistical model was a student's *t*-test with a significance threshold of $p < 0.05$.

RESULTS

Phoenix Data Logging and Stationary Incubation

To assess the ability for Phoenix^{TM} to maintain the required environmental conditions to maintain a healthy cell culture, temperature, relative humidity, and percent CO_2 were measured over 118 h (∼5 days) of live-cell culture. The cell container (as outlined in section "Methods") was sealed in a traditional 5% CO_2 incubator to capture the 5% CO_2 atmosphere. This process is quick, but gentle to enable the capture of ∼5% CO_2 for pH buffering of the cell culture during transport. The Phoenix CO_2 sensor is only able to measure up to 4% CO_2 due to sensor miniaturization requirements. **Figure 2A** shows that during the first 32 h of the experiment, the Phoenix CO_2 sensor was saturated at 4% CO_2. Thereafter, the percent CO_2 decays at a rate of 0.012% CO_2 per hour to reach 3% CO_2 after 118 h (∼5 days). Temperature and humidity were also measured for 118 h (∼5 days). Prior to loading Phoenix^{TM} with a living cell culture, the device is turned on for 30 min to allow all components to reach a 37°C steady state. Thereafter, the cell container is loaded into Phoenix^{TM} and the live cell culture is maintained at 37 ± 0.25°C for the entire duration of the experiment (**Figure 2B**). Furthermore, the relative humidity within the cell container increases from 73 to 80% over the course of the 5-day experiment.

Once it was confirmed that Phoenix^{TM} could maintain the prerequisite conditions to maintain a living cell culture, SH-SY5Y cells were seeded into four T-12.5 flasks ($n = 4$) and incubated within Phoenix^{TM} for 1–5 days stationary and compared to replicate control samples maintained in a standard 5% CO_2 incubator. There was no statistically significant difference in cell proliferation between Phoenix^{TM} and a standard 5% CO_2 incubator for 1–5 days of incubation (**Figure 3A**). Assumptions of normality and homogeneity of variance were not violated, except for the 1-day dataset where the results of statistical analysis suggested that the sample was not derived from a population with a normal distribution (Shapiro–Wilk test, $p = 0.0318$). A *t*-test assuming equal variance was used for each analysis. On day 1, there were $4.6 \times 10^5 \pm 1.4 \times 10^4$ cells per mL in Phoenix^{TM} and $4.9 \times 10^5 \pm 5.0 \times 10^3$ cells in a standard 5% CO_2 incubator; $t(6) = 1.94$, $p = 0.1008$. After 2 days of incubation, there were $2.0 \times 10^6 \pm 3.1 \times 10^5$ cells per mL in Phoenix^{TM} and $2.8 \times 10^6 \pm 4.0 \times 10^5$ cells in a standard 5% CO_2 incubator; $t(6) = 1.49$, $p = 0.1877$. On day 3 of incubation, there were $1.0 \times 10^6 \pm 1.1 \times 10^5$ cells per mL in Phoenix and $1.1 \times 10^6 \pm 8.4 \times 10^5$ cells in a standard 5% CO_2 incubator; $t(6) = 0.98$, $p = 0.3660$. After 4 days of incubation, there were $2.8 \times 10^6 \pm 3.9 \times 10^5$ cells per mL in Phoenix^{TM} and $2.1 \times 10^6 \pm 3.3 \times 10^5$ cells in a standard 5% CO_2 incubator; $t(6) = -1.47$, $p = 0.1922$. On the final, fifth day of incubation, there were $2.4 \times 10^6 \pm 5.3 \times 10^4$ cells per mL in Phoenix^{TM} and $2.5 \times 10^6 \pm 1.6 \times 10^5$ cells in a standard incubator; $t(6) = 0.56$, $p = 0.5966$. Similarly, there were not any morphological anomalies or differences within

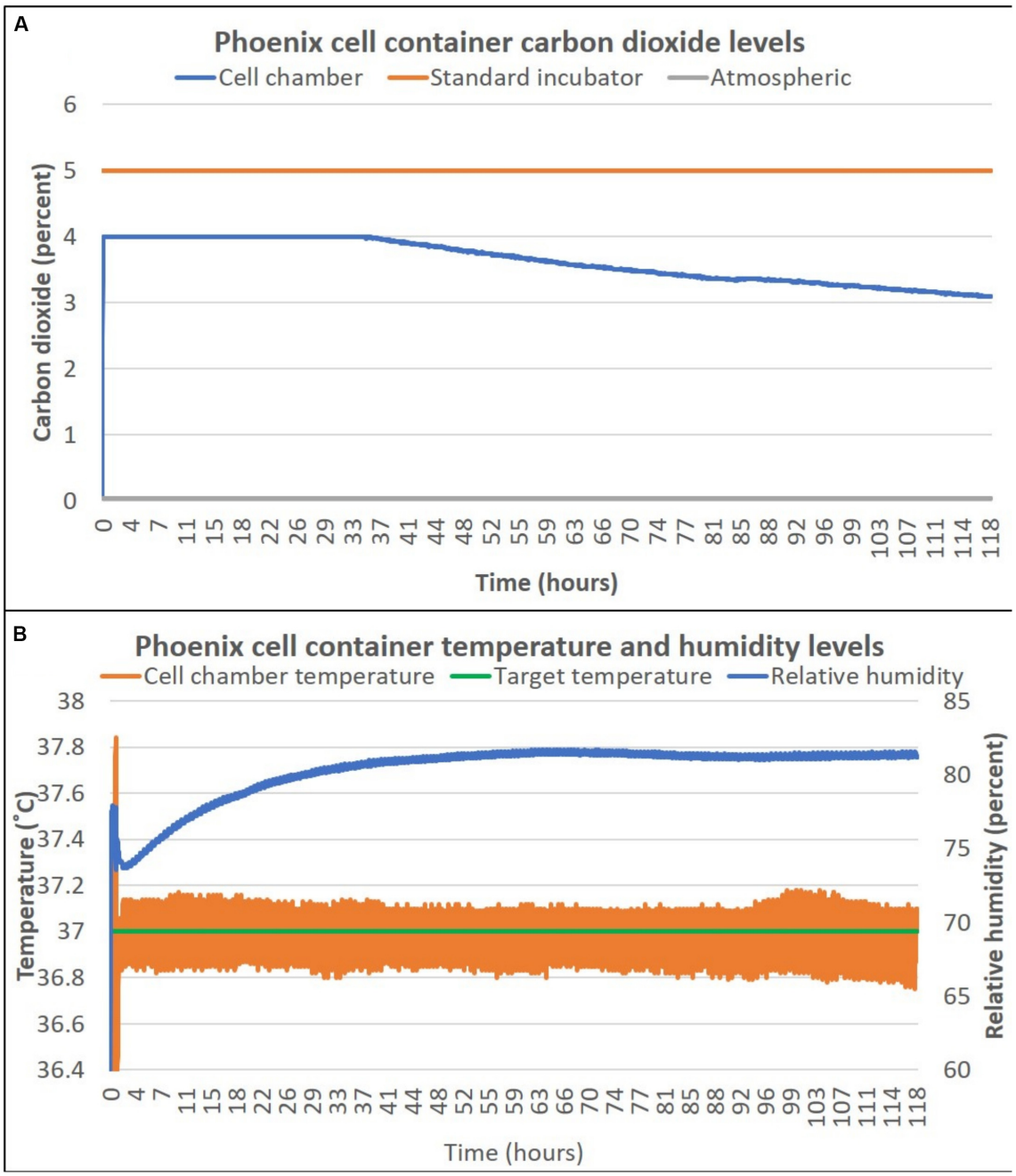

FIGURE 2 | Phoenix^{TM} cell container carbon dioxide **(A)**, temperature and humidity **(B)** levels over 5 days. T-flasks were seeded with SH-SY5Y cells, incubated with media containing HEPES (25 mM), and loaded inside Phoenix^{TM} (N = 4) or a standard CO_2 incubator (N = 4). Phoenix^{TM} was stored on a workbench with an exterior ambient temperature of ∼25°C and remained stationary for the entire 5-day period.

the Phoenix^{TM} treatment or control (5% CO_2 incubator) cell cultures (**Figure 3B**).

While Phoenix^{TM} incubation does not significantly alter cell proliferation, there may be differences in cell viability over extended durations. To assess this, SH-SY5Y cells were seeded into four T-12.5 flasks (n = 4) in the same manner as the cell proliferation experiment and treated with a Live-Dead assay on days 1–5 of stationary incubation within Phoenix^{TM} and compared to control samples (5% CO_2 incubator). There was no statistically significant difference in cell viability between Phoenix^{TM} and a standard 5% CO_2 incubator for 1–5 days of incubation (**Figure 4**). Since assumptions of normality and homogeneity of variance were not violated, a t-test assuming equal variance was used. On day 1 of incubation, there were

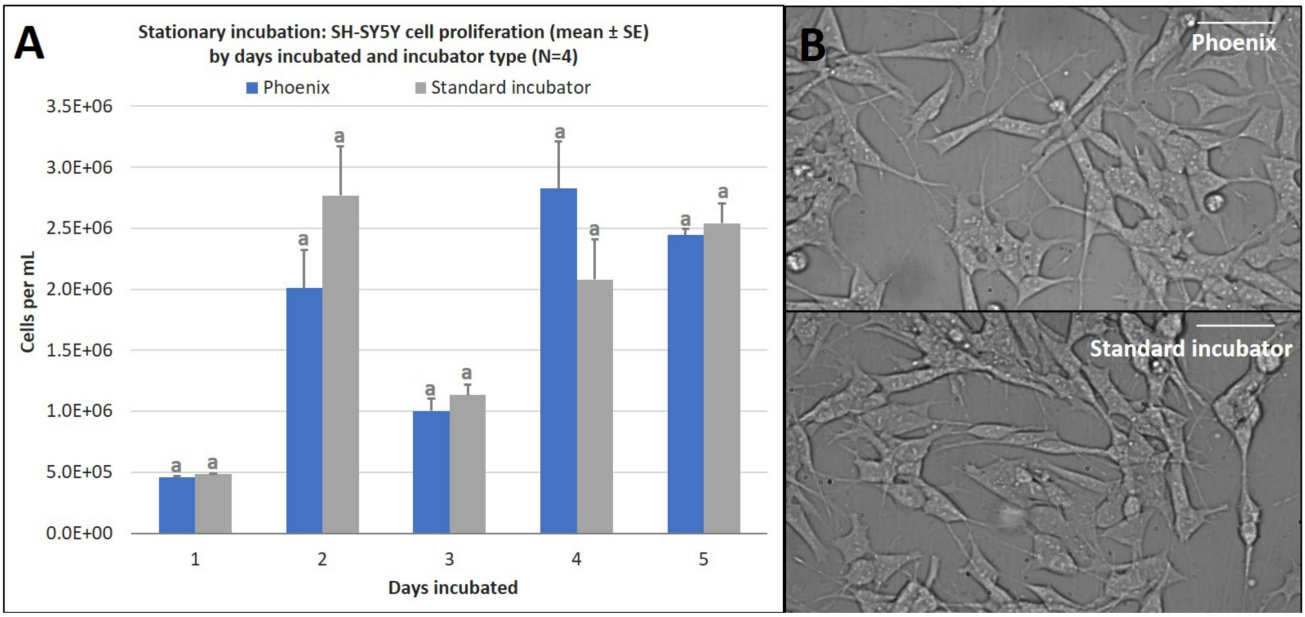

FIGURE 3 | (A) SH-SY5Y cell proliferation (mean ± SE) by days incubated and incubator type following stationary incubation and **(B)** a representative 40x magnification image taken in brightfield after 5 days of incubation. The standard incubator was programmed to 37°C and 5% CO_2 and Phoenix^{TM} was programmed to 37°C without CO_2. Both incubators remained stationary throughout each experiment. Cell media was supplemented with 25 mM of HEPES in both treatment groups to buffer solution pH. Different letters indicate statistically significant differences within each experiment (Student's *t*-test, $p < 0.05$). Scale bars in the upper right side depict 10 μM.

97 ± 1% viable cells in Phoenix^{TM} and 98 ± 1% viability in a standard 5% CO_2 incubator; $t(6) = 0.51$, $p = 0.6290$. After 2 days of incubation, there were 98 ± 1% viable cells in Phoenix^{TM} and 96 ± 1% viability in a standard 5% CO_2 incubator; $t(6) = -1.67$, $p = 0.1450$. On day 3 of incubation, there were 93 ± 1% viable cells in Phoenix^{TM} and 96 ± 1% viability in a standard 5% CO_2 incubator; $t(6) = 2.31$, $p = 0.0606$. After 4 days of incubation, there were 92 ± 1% viable cells in Phoenix^{TM} and 94 ± 1% viability in a standard 5% CO_2 incubator, $t(6) = 1.43$, $p = 0.2039$. On the final, fifth day of incubation, there were 90 ± 2% viable cells in Phoenix^{TM} and 90 ± 1% viability in a standard 5% CO_2 incubator, $t(6) = 0.07$, $p = 0.9489$.

Ground Transportation

In addition to the providing the required temperature, humidity, and CO_2 to a living cell culture, Phoenix^{TM} must also demonstrate that the vibrational loads on the device during transportation do not significantly alter cell proliferation, viability, or morphology. Additional tests were performed to culture cells in a moving vehicle for 1, 2, and 3 days to quantify cell proliferation and cell viability with respect to the acceleration loads the vehicle exhibits on the Phoenix^{TM} device. Triaxial vibration was recorded with an accelerometer over 72 h of the ground transportation experiment with vibration events observed on the *z*-axis between 0 and 2 g with a nominal 1 g static acceleration load. Similarly, in the *x* and *y* direction there was a nominal 0 g load with the majority of all vibrations occurring between −0.5 and 0.5 g for both axes (**Figure 5**).

Just as in the static cell culture tests, SH-SY5Y cells were cultured in four T-12.5 flasks ($n = 4$) and compared to replicate control samples in a standard 5% CO_2 incubator. The results demonstrate that there is no statistically significant difference in cell proliferation for cells incubated in a standard 5% CO_2 incubator when compared to cells exposed to ground transport within Phoenix^{TM} for 1–3 days (**Figure 6**). Since assumptions of normality and homogeneity of variance were not violated, a *t*-test assuming equal variance was used. After 1 day of incubation, there were $5.9 \times 10^5 \pm 4.4 \times 10^4$ cells per mL in Phoenix^{TM} and $5.5 \times 10^5 \pm 2.3 \times 10^4$ cells in a standard 5% CO_2 incubator; $t(6) = -0.84$, $p = 0.4308$. After 2 days of incubation, there were $8.2 \times 10^6 \pm 1.5 \times 10^5$ cells per mL in Phoenix^{TM} and $7.8 \times 10^6 \pm 9.6 \times 10^4$ cells in a standard 5% CO_2 incubator; $t(6) = -0.23$, $p = 0.8285$. After 3 days of incubation, there were $1.6 \times 10^6 \pm 2.1 \times 10^5$ cells per mL in Phoenix^{TM} and $1.4 \times 10^6 \pm 6.7 \times 10^4$ cells in a standard 5% CO_2 incubator; $t(6) = -1.09$, $p = 0.3163$.

Similar to the ground cell proliferation results, there is no statistically significant difference in cell viability for cells incubated in a standard 5% CO_2 incubator when compared to cells exposed to ground transport within Phoenix^{TM} for 1–3 days (**Figure 7**). Since assumptions of normality and homogeneity of variance were not violated, a *t*-test assuming equal variance was used. After 1 day of incubation, there were 95 ± 1% viable cells in Phoenix^{TM} and 96 ± 1% viability in a standard 5% CO_2 incubator; $t(6) = 1.22$, $p = 0.2677$. On day 2 of incubation, there were 92 ± 1% viable cells in Phoenix^{TM} and 91 ± 1% viability in a standard 5% CO_2 incubator; $t(6) = -0.77$, $p = 0.4705$.

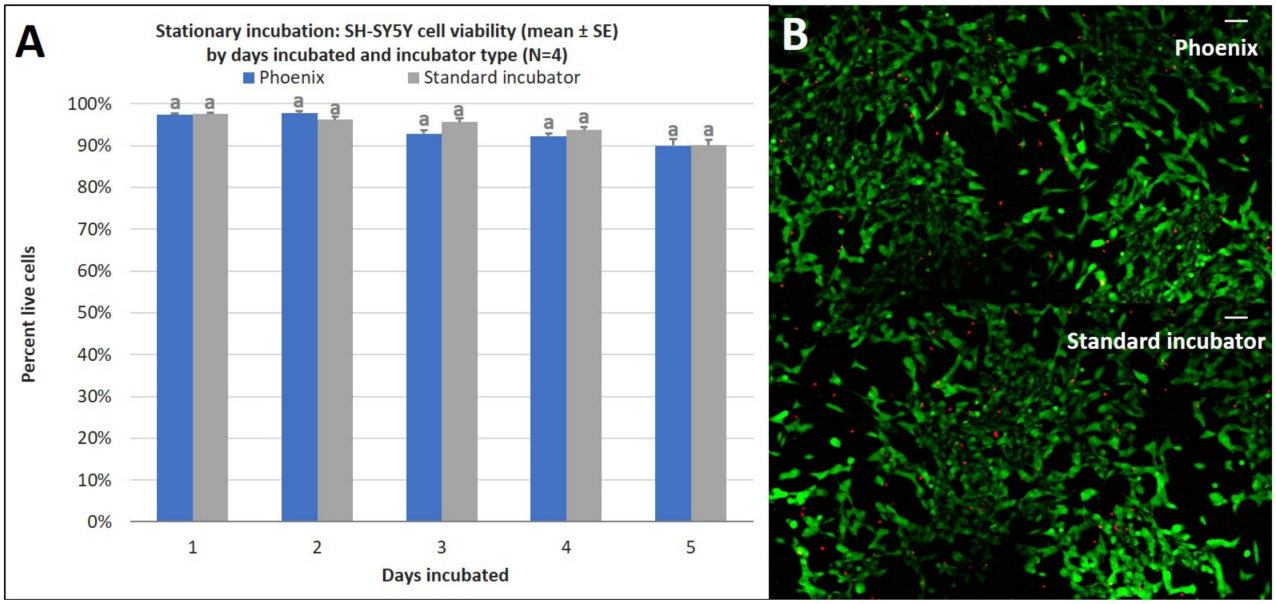

FIGURE 4 | (A) SH-SY5Y cell viability (mean ± SE) by days incubated and incubator type following stationary incubation and **(B)** a representative live-dead overlay image taken at 10x magnification after 5 days of incubation. The standard incubator was programmed to 37°C and 5% CO_2 and Phoenix^{TM} was programmed to 37°C without CO_2. Both incubators remained stationary throughout each experiment. Cell media was supplemented with 25 mM of HEPES in both treatment groups to buffer solution pH. Different letters indicate statistically significant differences within each experiment (Student's *t*-test, $p < 0.05$). A live-dead assay was used to quantify cell viability with calcein AM and ethidium homodimer with image post-processing to produce overlay. Scale bars in the upper right side depict 10 μM.

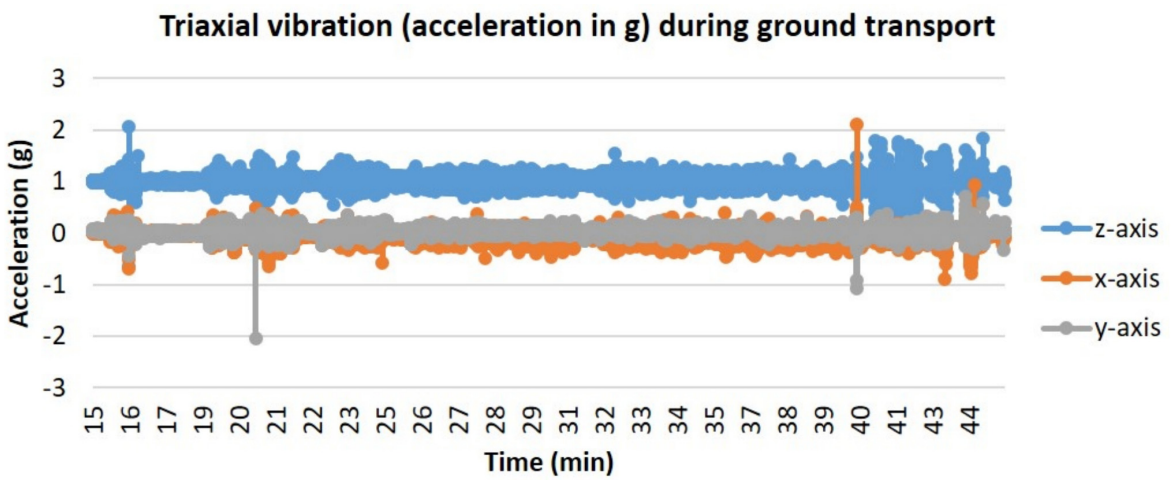

FIGURE 5 | Triaxial vibration (acceleration in g) during ground transport. T-flasks were seeded with SH-SY5Y cells, incubated with media containing HEPES (25 mM), and loaded inside Phoenix^{TM} ($N = 4$) or a standard CO_2 incubator ($N = 4$). Phoenix^{TM} was loaded into a motor vehicle and driven 13 miles between Kahului and Haiku, Hawaii, United States, with the depicted route driven twice per day of transit.

After 3 days of incubation, there were 0 ± 0% viable cells in Phoenix^{TM} and 0 ± 0% viability in a standard 5% CO_2 incubator; $t(6) = 0.00$, $p = 0.0000$.

Air Transportation

In addition to the acceleration loads of ground transport, Phoenix^{TM} will also experience the acceleration loads of air transportation. To assess how the SH-SY5Y cells will react to the acceleration environment of air travel, Phoenix^{TM} was flown on a Cirrus SR22 plane during a pilot training flight to assess the impact of high degree banking turns, nosedives, and multiple landing/takeoffs. Triaxial vibration was recorded with an accelerometer for the full duration of the test flight with peak vibration events observed on the *z*-axis at 0 and 2.5 g (**Figure 8**). Since assumptions of normality and homogeneity of variance were not violated,

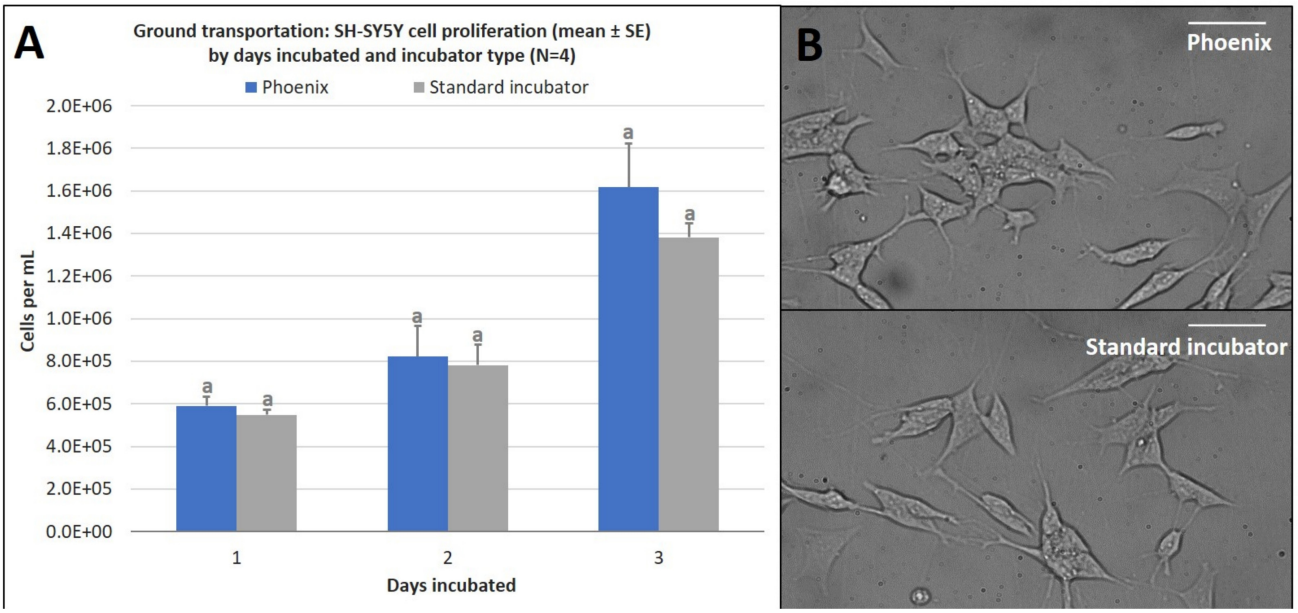

FIGURE 6 | (A) SH-SY5Y cell proliferation (mean ± SE) by days incubated and incubator type following stationary incubation and **(B)** a representative 40x magnification image taken in brightfield after 2 days of incubation. The standard incubator was programmed to 37°C and 5% CO_2 and remained stationary throughout the experiment. Phoenix™ was programmed to 37°C without CO_2 and was exposed to ground transportation. Ground transportation was performed on Maui, Hawaii, United States, with 13 mile segments twice per day. Cell media was supplemented with 25 mM of HEPES in both treatment groups to buffer solution pH. Different letters indicate statistically significant differences within each experiment (Student's *t*-test, $p < 0.05$). Scale bars in the upper right side depict 10 μM.

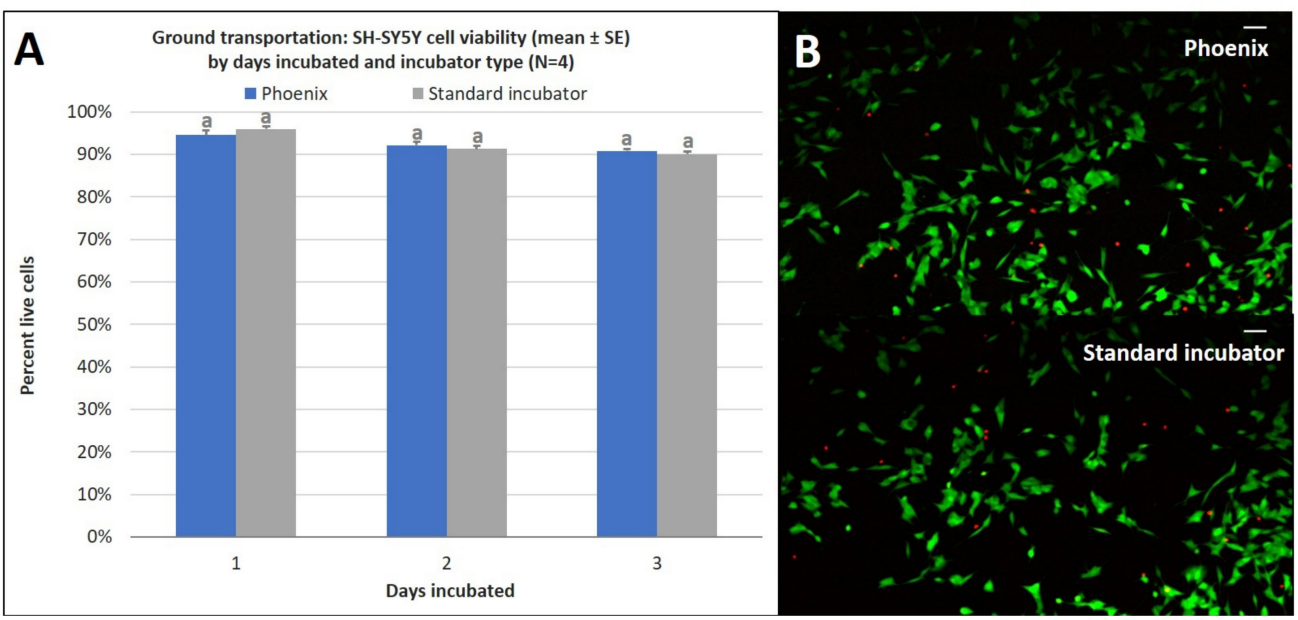

FIGURE 7 | (A) SH-SY5Y cell viability (mean ± SE) by days incubated and incubator type following stationary incubation and **(B)** a representative live-dead overlay image taken at 10x magnification after 2 days of incubation. The standard incubator was programmed to 37°C and 5% CO_2 and remained stationary throughout the experiment. Phoenix™ was programmed to 37°C without CO_2 and was exposed to ground transportation. Ground transportation was performed on Maui, Hawaii, United States with 13 mile segments twice per day. Cell media was supplemented with 25 mM of HEPES in both treatment groups to buffer solution pH. Different letters indicate statistically significant differences within each experiment (Student's *t*-test, $p < 0.05$). A live-dead assay was used to quantify cell viability with calcein AM and ethidium homodimer with image post-processing to produce overlay. Scale bars in the upper right side depict 10 μM.

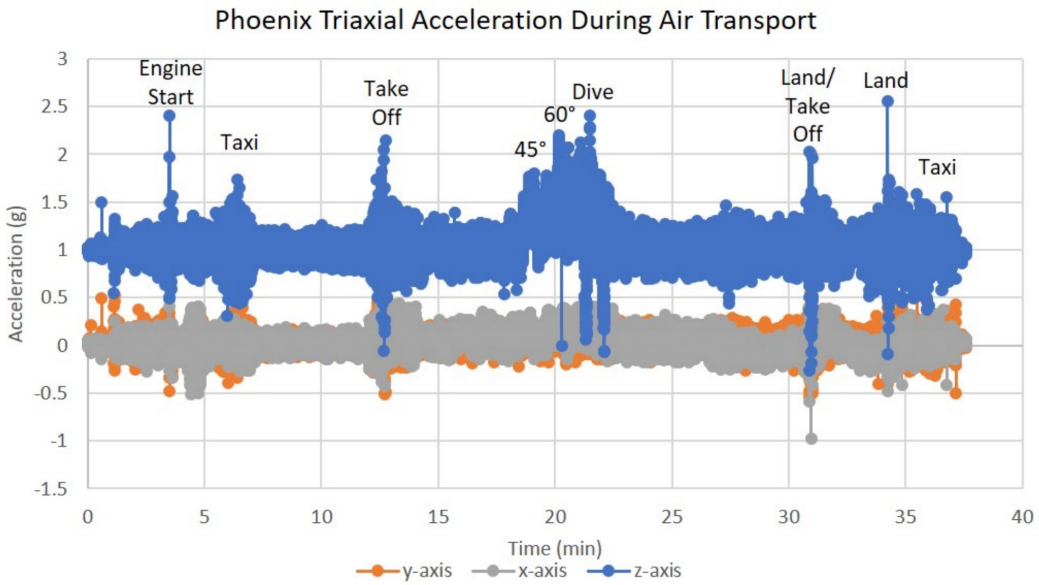

FIGURE 8 | Triaxial vibration (acceleration in g) during air transportation. T-flasks were seeded with SH-SY5Y cells, incubated with media containing HEPES (25 mM), and loaded inside Phoenix (N = 4) or a standard CO_2 incubator (N = 4). Phoenix™ was loaded into a motor vehicle and driven 4.2 miles to Maui Flight Academy in Kahului, Hawaii, United States. After loading Phoenix into the cargo hold of a Cirrus SR22, a ∼30-min test flight was performed that included two take-offs, two 45° bank turns (1.41 g), two 60° bank turns (2.0 g), nosedive (0 –g), and two landings. All flying aerobatics were performed at an altitude of 1500 ft.

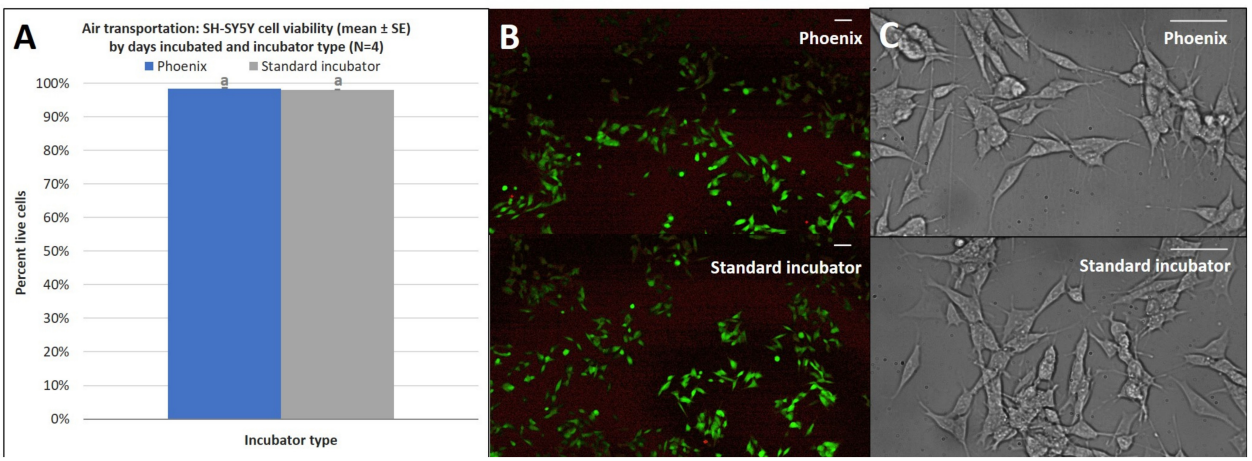

FIGURE 9 | (A) SH-SY5Y cell viability (mean ± SE) by incubator type following air transportation, **(B)** a representative live-dead overlay image taken at 10x magnification, and **(C)** SH-SY5Y cell morphology observed at 40x magnification. The standard incubator was programmed to 37°C and 5% CO_2 and remained stationary throughout the experiment. Phoenix™ was programmed to 37°C without CO_2 and was exposed to air transportation. T-flasks were seeded with SH-SY5Y cells, incubated with media containing HEPES (25 mM), and loaded inside Phoenix™ (N = 4) or a standard CO_2 incubator (N = 4). Phoenix™ was loaded into a motor vehicle and driven 4.2 miles to Maui Flight Academy in Kahului, Hawaii, United States. After loading Phoenix™ into the cargo hold of a Cirrus SR22, a ∼30-min test flight was performed that included two take-offs, two 45° bank turns (1.41 g), two 60° bank turns (2.0 g), nose dive (0 –g), and two landings. All flying aerobatics were performed at an altitude of 1500 ft. Different letters indicate statistically significant differences within each experiment (Student's t-test, $p < 0.05$). A live-dead assay was used to quantify cell viability with calcein AM and ethidium homodimer with image post-processing to produce overlay. Scale bars in the upper right side depict 10 μM.

a t-test assuming equal variance was used. There was no statistically significant difference in SH-SY5Y viability for cells flown and incubated within Phoenix™ when compared to a standard, stationary 5% CO_2 incubator; $t(6) = -0.96$, $p = 3747$ (**Figure 9A**). After a ∼30-min flight, there were 98 ± 1% viable cells in Phoenix™ and 98 ± 1% viability in a standard, stationary 5% CO_2 incubator (**Figure 9B**). There were no observable differences in cell morphology for cells flown and incubated in Phoenix™ when compared to a 5% CO_2 incubator (**Figure 9C**).

DISCUSSION

The neuroblastoma cell line SH-SY5Y was used as a proxy ATMP in order to examine the effects of mobile incubation on cell proliferation, viability, and morphology. This study sought to verify the hardware capabilities of Phoenix^{TM} to maintain the required environmental conditions for healthy cell culture as well as examine SH-SY5Y viability and proliferation after stationary incubation in Phoenix^{TM} for 1–5 days. In addition, experiments were performed to examine SH-SY5Y viability and proliferation during ground and/or air transportation for up to 3 days. This work demonstrated that there were no significant differences in viability or proliferation observed between SH-SY5Y cells incubated in a standard CO_2 incubator to cells transported via ground or air within Phoenix^{TM}, suggesting that Phoenix^{TM} is an effective mobile incubator for live cell transport of ATMPs.

We show that the Phoenix^{TM} system can maintain the required environmental conditions under battery power to promote cell proliferation during both ground and air transport. The data sensors operate through a feedback loop and algorithm to maintain the required temperature (37°C) with minimal fluctuations (±0.5°C) to promote a healthy cell culture at near-physiological conditions. CO_2 is traditionally used to buffer the medium pH and prevent pH shifts that naturally occur as a result of cell metabolism and growth, which can damage the cell culture over long times. In this instance, Phoenix^{TM} was able to capture the CO_2 atmosphere from a standard 5% CO_2 incubator and maintain the CO_2 content over 3% during the 5-day experiment. While the Phoenix CO_2 sensor can only read up to 4% CO_2 due to spatial constraints, linear extrapolation of the percent CO_2 indicates that the initial atmosphere at time 0 h was ∼4.4% CO_2 with a decay rate of 0.012% CO_2/h. When Phoenix^{TM} was operated in a similar mode to a standard 5% CO_2 incubator (i.e., stationary), there were no significant differences in cell proliferation, viability, or morphology when compared to the control samples. Thus, the decrease in percent CO_2 from ∼4.4 to 3% over 5 days did not have any discernable adverse effects on the SH-SY5Y cell culture when compared to the standard 5% CO_2 atmosphere. Thus, from a pure incubator perspective, Phoenix^{TM} is just as effective as the traditional method of culturing mammalian cell lines in a biology laboratory for up to 5 days. It is important to mention, because there is some decrease of CO_2 from day 0 to day 5, there may be some gas exchange (albeit minimal) through the polymer enclosure capturing the CO_2 environment.

However, because Phoenix^{TM} is specifically designed to transport living cell cultures, it is important to investigate the effects of acceleration loads that the cells may experience during ground or air transportation. SH-SY5Y cells were specifically chosen as an ATMP proxy due to their required surface adherence and ability to be differentiated into a neuron-like cell culture. Thus, they would be more susceptible to vibrations or fluid shear stresses that may be present during transport than other non-adherent cell lines or therapies that may require Phoenix^{TM} for transport (t-cells, CAR-T, etc.). Nonetheless, the SH-SY5Y cells incubated in Phoenix^{TM} during transportation did not demonstrate any adverse effects when compared to the control samples. There were no significant differences in viability or proliferation between SH-SY5Y cells incubated in Phoenix^{TM} or a standard incubator regardless of incubation duration (1–5 days) or transportation method (stationary, ground, air). Cell viability remained above 90% for all experiments. Despite the 0 and 2.5 g encountered during air transport, there were no apparent differences in SH-SY5Y morphology observed for cells transported in Phoenix or cells that remained stationary in a standard incubator. Thus, the acceleration and vibration loads of travel do not appear to have detrimental effects on adherent cell lines within Phoenix^{TM} and would have less of an impact on suspension cell lines.

Phoenix^{TM} could alleviate one of the main hurdles of implementing wide-spread access to cell therapies: the freezing and thawing of biological samples, which reduces the quality of the product and requires laborious biological manipulations prior to and post-transport. Phoenix^{TM} will be a significant cost saving measure when ATMPs are deployed to clinics and patients globally by eliminating the time prior to freezing and the cell thawing/recovery period required to traditionally ship cell cultures (dry ice or LN_2) by up to 3–14 days depending on the ATMP. Phoenix^{TM} can facilitate the implementation of parameters to quantify quality-controlled transport of live-cell therapies through data logging of environmental conditions with a multitude of embedded sensors. The need for a portable live-cell incubator is clear, but the exact implementation for all cell therapy applications requires further research. Indeed, multiple methodologies may be required to tailor the transport method of a given cell line/cell therapy product as not all cell lines behave the same. Future research should focus on characterizing additional cell lines such as mesenchymal stem cells and CAR-T cells for Phoenix^{TM} transport. The only way to ensure that this technology will achieve success is to gather input from subject matter experts and to characterize viability and proliferation for multiple ATMPs. Furthermore, additional research is needed to examine cell function at the point of use on a metabolic, rather than a morphological level, to ensure that treatment efficacy remains viable for transported cells.

Phoenix^{TM} live cell transport offers a time and cost saving alternative to traditional cell culturing and shipment techniques by providing a mechanism for rapid transportation with minimal biology preparation and exceptional data logging. The results of this study demonstrate that Phoenix^{TM} is an effective mobile incubator for live cell transport which could assist researchers, medical doctors, and patients with improved access to ATMPs. In addition, Phoenix^{TM} may enable long-distance and/or international collaborations to accelerate ATMP research and discovery within the research community. This research validates Phoenix^{TM} for live-cell transport of sensitive cell cultures with negligible effects from acceleration loads, atmospheric conditions, thermal maintenance, and culture pH shifts. Phoenix^{TM} introduces a new paradigm shift to the commercialization and implementation of widespread access to ATMPs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

DR conceived and supervised the study. DR and BW designed the experiments. BW carried out the experiments, performed the statistical analysis, and wrote the first draft of the manuscript. SL developed image analysis macros to quantify response variables. DR, DO'C, and CO'C-R critically revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Predicting and Promoting Human Bone Marrow MSC Chondrogenesis by Way of TGFβ Receptor Profiles: Toward Personalized Medicine

René Rothweiler[1,2†], Valentina Basoli[1†], Fabian Duttenhoefer[2], David Kubosch[3], Rainer Schmelzeisen[2], Brian Johnstone[4], Mauro Alini[1] and Martin James Stoddart[1,2]*

[1] Regenerative Orthopaedics, AO Research Institute Davos, Davos, Switzerland, [2] Department of Oral and Maxillofacial Surgery, Faculty of Medicine, Medical Center - University of Freiburg, University of Freiburg, Freiburg im Breisgau, Germany, [3] Department of Orthopedics and Trauma Surgery, Faculty of Medicine, Medical Center - University of Freiburg, University of Freiburg, Freiburg im Breisgau, Germany, [4] Department of Orthopaedics and Rehabilitation, Oregon Health & Science University, Portland, OR, United States

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***Correspondence:**
Martin James Stoddart
martin.stoddart@aofoundation.org

[†] These authors have contributed equally to this work

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The use of human mesenchymal stromal cells (hMSCs) for cartilage regeneration has been hampered by the inherent donor variation of primary monolayer expanded cells. Although CD markers are typically used to characterize cell populations, there is no correlation between CD marker profile and functional outcomes. Therefore, we aimed to discover novel predictive MSC chondrogenesis markers. The chondrogenic potential of primary human bone marrow MSCs (hBMSCs) over multiple passages was assessed by standard pellet culture. We confirmed that the ratio of TGFβ-RI/TGFβ-RII at the time of cell recovery from the tissue culture plastic reliably predicted chondrogenic potential. Furthermore, it is possible to prospectively characterize any human BMSC cell population as responders or non-responders with respect to chondrogenic differentiation potential. Transient increase of the ratio with siRNA knockdown of TGFβ-RII reproducibly recovered the chondrogenic differentiation ability of non-responsive MSCs. Together this offers an opportunity to produce a more functionally characterized cell population for use in autologous cartilage repair therapies.

Keywords: mesenchymal stem cell, TGF receptor, chondrogenic differentiation, receptor ratio, personalized medicine

INTRODUCTION

Despite the promise of human bone marrow derived stromal cells (hBMSCs) in the field of regenerative medicine, assays predictive of cell function have remained elusive. Several studies show the *in vitro* potential of MSCs to differentiate into chondrocytes under specific stimulations (Wakitani et al., 1994; Cassiede et al., 1996; Yoo et al., 1998); however, the chondrogenic commitment of human MSCs is highly variable. While it has been established that factors such as *in vivo* age and *in vitro* aging, in the case of monolayer expanded/ selected cells, play roles in the variability, there remains much to be gleaned concerning the differences between MSC populations from different individuals. The consequence of our current incomplete picture of the causes of MSC variability is that to date, it is not possible to predict the outcome of chondrogenic differentiation of a specific donor. This is a clinical challenge as cell based therapies are expensive

to administer and without adequate patient stratification they become financially unviable. The expression levels of certain cell surface markers (e.g., Stro-1, CD73, CD105, or CD90) have been associated with MSCs (Stewart et al., 2003; Battula et al., 2009) but none are known to be predictive of stemmness or commitment and do not correlate with the final yield and quality of chondrogenic differentiation [e.g., (Cleary et al., 2015)]. Furthermore, most marker profiles are similar for all cells of mesenchymal origin (Whitney et al., 2009) but hMSCs from different origins have been shown to retain epigenetic memory and display functional differences *in vivo*. These differences are not reflected by the marker profile *in vitro* (Sacchetti et al., 2016). For this reason new methods for predicting the functional potential of hMSCs are urgently needed (McLeod and Mauck, 2017).

During chondrogenic commitment *in vitro*, TGFβ is one of the key factors involved in the determination of cell fate. MSCs express TGFβ receptor type II (TGFβ-RII) on their membrane surfaces, which recognizes and binds TGFβ. This activated complex recruits a type I receptor dimer (TGFβ-RI) creating a phosphorylated hetero-tetrameric complex that progressively activates signaling pathways via SMAD proteins (Grimaud et al., 2002). SMADs translocate into the nucleus and promote gene regulation (Shi and Massague, 2003) through modulation of transcriptional co-activators and co-repressors in a cell type specific manner (Nakao et al., 1997; Massague, 2012). Canonical TGFβ-RI activation is known to mediate the signal via intracellular Smad 2/3; whereas ACVRL-I, also known as ALK1 - an alternative receptor regulated by TGFβ–promotes Smad 1/5/8 phosphorylation (Nakao et al., 1997; Chen and Massague, 1999), a pathway generally associated with hypertrophy.

It has been shown that TGFβ-RI expression and Smad2 phosphorylation decrease dramatically in aged murine cartilage (Blaney Davidson et al., 2005) with a concomitant increase in ACVRL-I expression, resulting in dysregulated chondrogenesis caused by an alteration in TGFβ downstream signaling (Blaney Davidson et al., 2009). Acting on this observation, we investigated the expression of numerous TGFβ receptors in MSCs from a cohort of human donors at different stages of *in vitro* expansion. The aim was to determine a marker profile that reliably predicts the chondrogenic potential of hMSC populations. Based on initial data we then examined in more detail the TGFβR1/TGFβR2 ratio profile in additional donors as a possible predictable indicator of quality and yield. To demonstrate a functional role and influence of different TGFβ receptors involved in the chondrogenic fate, we modulated the ratio in order to improve the chondrogenesis in MSCs that showed a limited chondrogenic potential.

MATERIALS AND METHODS

Human Mesenchymal Stromal Cell Isolation From Fresh Bone Marrow

Bone marrow from 20 different anonymous human donors (range min 18 years, max 85 years, Average 57.71 ± 19.34 years) was harvested after informed consent (Ethical approval: Freiburg, EK-326/08). A known co-morbidity was considered an exclusion criterion. Fresh bone marrow was diluted 1:4 and layered on top of Ficoll, in a proportion of 2.6 ml of Ficoll per ml of undiluted marrow. After centrifugation at 500 g for 20 min, the mononuclear cell-containing interface was recovered, and cells were counted using the Cell Scepter 2.0 Automated Cell Counter (Millipore). Isolated cells were seeded at a density of 50,000 cells/cm² into 300 cm² tissue culture flasks in Minimum Essential Medium Eagle, Alpha Modification (α-MEM; Gibco, UK) containing 10% fetal bovine serum (Sera Plus, PAN-Biotec 3702-P12812 Aidenbach, Germany), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, UK), and 5 ng/ml recombinant human basic fibroblast growth factor (bFGF, Fitzgerald Industries International, Acton, MA, USA). Cells were maintained at 37°C in 5% CO_2, 85% humidity atmosphere. Medium was refreshed every 2nd day. After 4 days, non-adherent hematopoietic cells were removed to select the mesenchymal stromal cell (hMSC) population.

Passaging

hMSCs were cultured from passage 0 up to passage 10, with an initial seeding density of 3,000 cells/cm² in 300 cm² tissue culture flasks, in the conditions described above. Upon reaching 80% confluency, images of cells were taken in order to record their morphology. Cultures were passaged using Trypsin-EDTA (0.5%) (ThermoFisher, UK) for 5 min at 37°C. For the deactivation of trypsin 1:3 growth medium containing 10% fetal bovine serum was used, cells were then centrifuged at 400 G for 5 min. The resultant pellet was used for further expansion or for RNA isolation to evaluate the TGFβ receptor expression.

Chondrogenic Differentiation

Chondrogenic differentiation of hMSCs was performed in 3D pellet culture. A quantity of 2 x 10^5 hMSCs per pellet were seeded in V-bottom 96-well plates (Corning, Corning, NY, USA). To prevent possible cell adhesion on the bottom, the plate was pre-coated with 20 µl of 1% agarose. Cells were centrifuged for 5 min at 500 g in order to form the pellets. Chondrogenic differentiation medium contained DMEM high glucose (Gibco, UK), 1% non-essential amino acids (ThermoFisher, UK), 1% ITS+ (Corning, NY, USA), 100 nM dexamethasone (Sigma-Aldrich, Germany), 10 ng/ml TGF-β1 (Fitzgerald Industries International, Acton, MA, USA) and 50 µg/ml ascorbic acid-2 phosphate (Sigma-Aldrich, Germany). The control growth medium contained DMEM high glucose (Gibco, UK), 1% non-essential amino acids (ThermoFisher, UK), 1% ITS+ (Corning, NY, USA). The medium was replaced every second day and pellets were harvested for further analyses after 28 days.

Transfection and Receptor Silencing

In order to demonstrate the role of TGFβ-Rs during chondrogenic commitment and their relevance during TGFβ signaling pathway activation, we transiently inhibited TGFβ-RI, TGFβ-RII, and ACVRL-I. According to manufactures' protocol using the NEON transfection system: hMSC were resuspended in Buffer R at a final concentration of 0.5 × 10^7 cells/ml. Cells were transfected with either siTGFβ-RI (Ambion, cat#4427038), siTGFβ-RII (Ambion, cat#AM51331), siACVRL-I (Ambion, cat#

4427037) at 25 nM, or siNegative (scramble control) (Ambion, cat# 4390846) by electroporation using a 990 pulse voltage, 40 ms pulse width for one pulse number using a 100 µl pipette tip. Cells were then transferred into chondrogenic medium or control medium in absence of antibiotics.

Real-Time Quantitative PCR Analysis

Total RNA was isolated from adherent hMSC cells after trypsinization during passaging and from 3D chondrogenic induced pellets at day 0 and 28 using TRI Reagent® Solution (Molecular Research Center MRC, cat. # TR-118) according to the manufacturer's protocol. RNA quantity was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher, UK). For reverse transcription, TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, USA) was used. The RT reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C and stopped by heating for 5 min at 85°C. qPCR reactions were set up in 10 µL reaction mixtures containing TaqMan Universal Master Mix (Thermo Fischer, UK), Primer and Probe or AssayOnDemand, DEPC-H20 and cDNA template. The reaction program was set up as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by an annealing/extension step at 60°C for 1 min. qPCR analysis was performed using QuantStudio 6 Flex Real-Time PCR System (Life Technologies, Carlsbad, USA). Duplicates were used for each target gene (technical replicates) and triplicates for each donor (biological replicates).

The relative expression of TGFBR1, TGFBR2, BMPR1A, BMPR1B, BMPR2, ACVR1, ACVR1B, ACVR1C, ACVR2A, ACRV2B, ACVRL1 during expansion in monolayer was determined using the $2^{(-\Delta\Delta CT)}$ method, with ribosomal protein large, P0 (RPLP0) as reference gene and P2 RNA as the baseline.

The relative expression of RUNX2, SOX9, ACAN, MMP13, COL2A1, COL10A1 during chondrogenic differentiation was determined using the $2^{(-\Delta\Delta CT)}$ method, with ribosomal protein large, P0 (RPLP0) as reference gene and day 0 RNA as the baseline. Primer and probe sequences as well as Order Numbers of Assays-on-Demand (Applied Biosystems) are listed in the **Supplemental Table 1**.

To predict the chondrogenic function of an individual cell population the P2 baseline was omitted and the following calculation used.

The ratio of TGFβ-R expression was calculated using ΔCt values:

$$R = 2^{-(\Delta Ct_1 - \Delta Ct_2)}$$

Where ΔCt_1 = Ct hTGFβ-RI – Ct hRPLP0 and ΔCt_2 = Ct hTGFβ-RII—Ct hRPLP0.

Histological Staining

At day 28, samples were fixed in 70% methanol. Cryosections were cut with a thickness of 8–10 µm. For Safranin-O staining, samples were first stained with Weigert's Haematoxylin for 10 min, followed by a 6 min stain with Fast Green and a 15 min stain with Safranin-O. After dehydration with increasing concentrations of ethanol, samples were coverslipped with the use of xylene.

For collagen II staining a monoclonal antibody (CIICI, DSHB, Iowa, USA) was used. After incubating slides in methanol for 30 min, nonspecific binding sites were blocked with horse serum (Vector Laboratories #S-2000; Dilution 1:20) for 1 h. Primary antibody was then added for 30 min (dilution 1:6) followed by an incubation in biotinylated anti-mouse IgG (H+L) secondary antibody (Vector Laboratories #BA-2001; dilution 1:200) and a second incubation in Vectastain Elite ABC Kit (Vector Laboratories #PK-6100). ImmPACT DAB solution (Vector Laboratories #SK-4105) was added as substrate for peroxidase for 4 min. Counterstaining was performed using Mayer's Haematoxylin (Fluka #51275) for 20 s. Samples were dehydration with increasing concentrations of ethanol and coverslipped. For the negative control, the respective samples were stained in parallel without addition of primary antibody.

The histological sections were observed using Zeiss AxioPlan 2 Microscope (Zeiss Microscopy GmbH, Jena, Germany) with objective 10X/0.50. and graded as previously published (Grogan et al., 2006). Pictures were acquired using RGB camera 1X (16 bit) and Axiocam Software (Zeiss Microscopy GmgH, Göttingen, Germany). Grading of the safranin O staining was ranked on a scale of 1–10 as assessed by four independent blinded evaluators according with Bern Score (Grogan et al., 2006) (**Supplementary Table 2**).

Statistics

Statistical analysis was performed using GraphPad Prism 7.03 software. Non-parametric two-way ANOVA in conjunction with Tukey's multiple comparison test was applied. $P < 0.05$ was considered as statistically significant. A two-way ANOVA was used to evaluate distribution and homogeneity variance in the groups; the Tukey's multiple comparison was used to evaluate the means the different groups. For each experimental setup triplicates were done (biological triplicates). Analysis were done in duplicates (technical duplicates). A total number of 20 donors have been cultured from passage 2 to passage 10. Mean and standard deviation were calculated from the results. Spearman's rank correlation coefficient for the non-parametric measurement of the relationship between two rank variables (RATIO, Histological score, Chondrogenic Markers) has been described using a monotonic function using GraphPad Prism 7.03 software. Receiver operating characteristic curve (ROC curve), for the prediction study for each instance has been made based on a continuous random variable created by plotting the donors (based on TGFBR1/TGFBR2 ratio and histological score) responders against non-responders, in order to calculate the cut-off AUR (Arbitrary Unit Ratio). In our system we considered a binary classification, in which the outcomes are labeled either as responder or non-responder. We assumed that values with 100% sensitivity are associated (no false negatives/non-responders) to true good chondrogenesis and 100% specificity (no false positives/responders) are associated to poor chondrogenesis. ROC Curve was performed using GraphPad Prism 7.03 software.

RESULTS

TGFβ-Receptor Expression Screening Over Passaging for Predictable Markers

Initially using 7 donors, we focused on the study of TGFβ-R and BMP-R variation over passage within the donor cohort (considering all donors together) using the $2^{(-\Delta\Delta CT)}$ method and samples at passage 2 as calibrator (**Figure 1**). We compared the means and standard deviations up to passage 7 and investigated whether changes in the expression profiles of TGFβ-Rs and BMP-Rs during cell passaging could be associated with changing chondrogenic potential (**Figure 1**). As expected, there was a high standard deviation (*SD*) among the donors, and several receptors displayed an increase in *SD* with increasing passaging, leading us to investigate each receptor at the individual donor level.

The TGFβR1 (**Figure 1A**) and TGFβR2 (**Figure 1B**) expression levels changed during *in vitro* passaging. TGFβ-R1 expression levels tended to decrease over time, while TGFβ-R2 levels showed a concomitant increase in all donors analyzed, showing a large standard deviation. These changes however were not absolute with the opposite seen for some donors at some passages. These changes did not reach significance. We found that the mean expression BMPR1A, BMPR1B, and BMPR2 was unaltered over time among donors (**Figures 1C–E**). However, the *SD* of BMPR1B and BMPR2 was seen to increase over passages.

The serine/threonine-protein kinase receptors family (ACVR), another family belonging the TGFB superfamily, did not show any significant change in expression over time that would account for possible donor variability (**Figures 1F–K**). In order to assess changes with respect to function, histological analysis was performed at each passage. None of the changes in individual receptor profile correlated with the resulting chondrogenic differentiation (**Figure 1**).

As TGF superfamily receptors heterodimerize, we then investigated whether ratios of expression correlated with chondrogenic differentiation. We observed the TGFBR1/TGFBR2 reliably correlated with the pellet culture histology on day 28 (**Figure 2**). The ratio at the time the cells were taken from the cell culture plastic correlated with the chondrogenic potential as assessed by safranin O staining. Additionally, we calculated the ratio without using P2 as a normalizer to allow a value to be generated from a single population of cells [$R = 2^{-(\Delta Ct_1 - \Delta Ct_2)}$]. The ratio was evaluated directly after cell harvest from tissue culture plastic. As expected, there was a trend toward a general decrease in ratio during *in vitro* aging, although for occasional populations a transient increase was observed. While the rate of change varied depending on the donor (**Figure 2A**), the ratio at the time of cell harvest correlated with the chondrogenic potential as assessed by safranin O staining (**Figure 2B**).

To validate these findings, we further assessed by molecular analysis of the TGFBR1/TGFBR2 ratio and the chondrogenic potential of additional donors from passage 2 to passage 10. Although the cells from different donors were isolated in the same way and maintained in the same conditions, it was evident that high donor variability is independent of passage number. The ratio between TGFBR1/TGFBR2 is highly donor dependent (**Figure 2A**), is not normally distributed over passaging and can be strongly affected from one passage to another (**Figure 2B**). Indeed, as is shown in **Figure 2**, the change in chondrogenic differentiation over time is donor dependent. Some donors considered good (e.g., #170) maintained a high yield of differentiation over time, while other donors considered bad (e.g., #168) already in the early phase showed a poor chondrogenesis (**Figure 2**). In addition, we observed, that some donors that show a high potential at lower passages (#195), drastically decrease the chondrogenic potential in a subsequent passage. This suggests, that although a donor may exhibit functional chondrogenesis at early passages, the change in chondrogenic potential over time cannot be reliably estimated.

To validate the correlation, a further 24 samples from multiple donors and multiple timepoints were analyzed, and the ratio correlated to histological outcomes ranked on a scale of 1–10 as assessed by four independent blinded evaluators according to the Bern Score (Grogan et al., 2006) (**Figure 3**, **Supplementary Table 2**). This score incorporates elements that consider the variability that can be seen within pellets, such as non-uniform staining. (**Supplemental Table 2**). The TGFBR1/TGFBR2 ratio strongly correlated with the histological score (**Figure 3**, $r = 0.8051$, $P < 0.001$). It has been proposed that growth rate in monolayer correlates with MSC function. During monolayer expansion cells were harvested at 80% confluency. We used the number of days to achieve this mark as a proxy of cell doubling time, with more days required to 80% confluency being indicative of a slower growth. A small but statistically significant negative correlation between time to 80% confluence and ratio was detected, with slower growing cells performing worse (**Figure 4**). No correlation between donor age and chondrogenic potential was observed (data not shown).

Identification of Cut-Off Value

To identify the cut-off value below which the population is no longer chondrogenic, the 24 samples were then separated based on the histological evaluation, with histological scores between 1 and 5 considered poorly chondrogenic, while donors with a histological score between 6 and 10 were considered highly chondrogenic (**Figure 5**). We divided the two cohorts based on non-responders and responders for the respective TGFB ratio in order to evaluate a precise cutoff number and by ROC analysis the value of 0.136 was established (**Figure 5C**). This value correctly predicts 23 of the 24 samples (96%), with one sample being a false negative, having a ratio of 0.123991 but was still chondrogenic (**Supplementary Table 3**).

The expression of COL2A1 (**Figure 6A**), a chondrogenic marker associated with chondrogenesis also strongly correlated with the histological score (**Figure 6D**, $P < 0.001$) and the TGFBR1/ TGFBR2 ratio (**Figure 6E**, $P = 0.002$). In contrast, COL10A1 expression was not correlated to the TGFR1/TGFBR2 ratio (**Figures 6B,F**, $P = 0.9488$). That stated, the TGFR1/ TGFBR2 ratio strongly correlated with the COL2/COL10 ratio (**Figures 6C,G**, $P = 0.004$). Combined with the lack of correlation

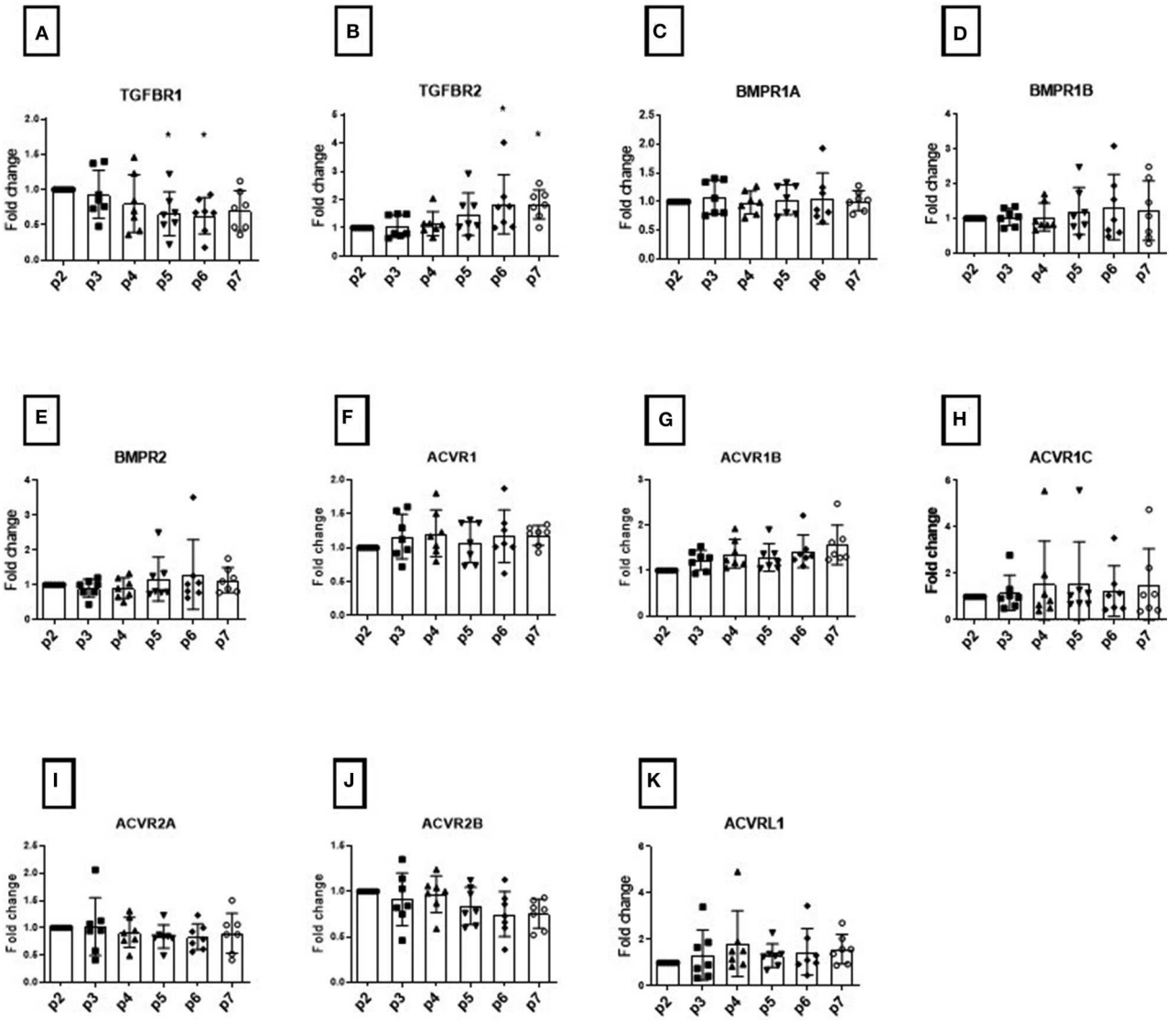


FIGURE 1 | Modulation of TGFB receptors during monolayer expansion of hBMSCs from passage 2 to passage 7. Cells from different donors were cultured in conventional expansion medium. The amounts of TGFBR1 **(A)**, TGFBR2 **(B)**, BMPR1A **(C)**, BMPR1B **(D)**, BMPR2 **(E)**, ACVR1 **(F)**, ACVR2B **(G)**, ACVR1C **(H)**, ACVR2A **(I)**, ACVR2B **(J)**, ACVRL1 **(K)** mRNA was normalized to ribosomal protein large, P0 (RPLP0). The mRNA expression of cells cultured over the passaging were plotted as $2^{(-\Delta\Delta Ct)}$ (mean ± *SD*; $n = 7$) as a fold change to the BMSC in passage 2, the dots shows the different donors, $^{*}p < 0.05$ compared to P2.

to COL10 expression, it suggests that increasing ratio is predictive of a more stable chondrogenesis.

Recovery of Chondrogenic Phenotype

The use of AUR allows the prediction of hMSC chondrogenic outcome prior to the induction of differentiation. Of the 24 samples investigated in detail (**Supplementary Table 3**) 13 did not show any chondrogenic potential, and in each case, this could be predicted by the receptor ratio on the day of cell harvest. However, in order to test whether the receptor ratio was causally associated with the fate of hMSCs, three receptors (TGFBR2, TGFBR1, and ACVRL1) were transiently knocked-down using a single dose of siRNA. The function of the siRNA was confirmed in monolayer culture (**Supplementary Figure 1**).

For those donors that showed a high AUR, no changes during differentiation upon silencing of TGFβ-Rs was observed (data not shown). On the contrary, all donors ($n = 5/5$) with low initial AUR positively responded to the silencing of TGFβ-RII, with a marked enhancement of matrix deposition that was clearly observed by safranin-O staining and COL2A1 protein expression revealed by immunohistochemistry (**Figure 7**). This confirms our previous findings on TGFβ-Rs ratio and demonstrates that it is possible to interfere with the fate associated with the TGFβ-Rs profile by converting hMSCs into a pro-chondrogenic state. It also suggests that high expression of TGFβ-RII may be a reason

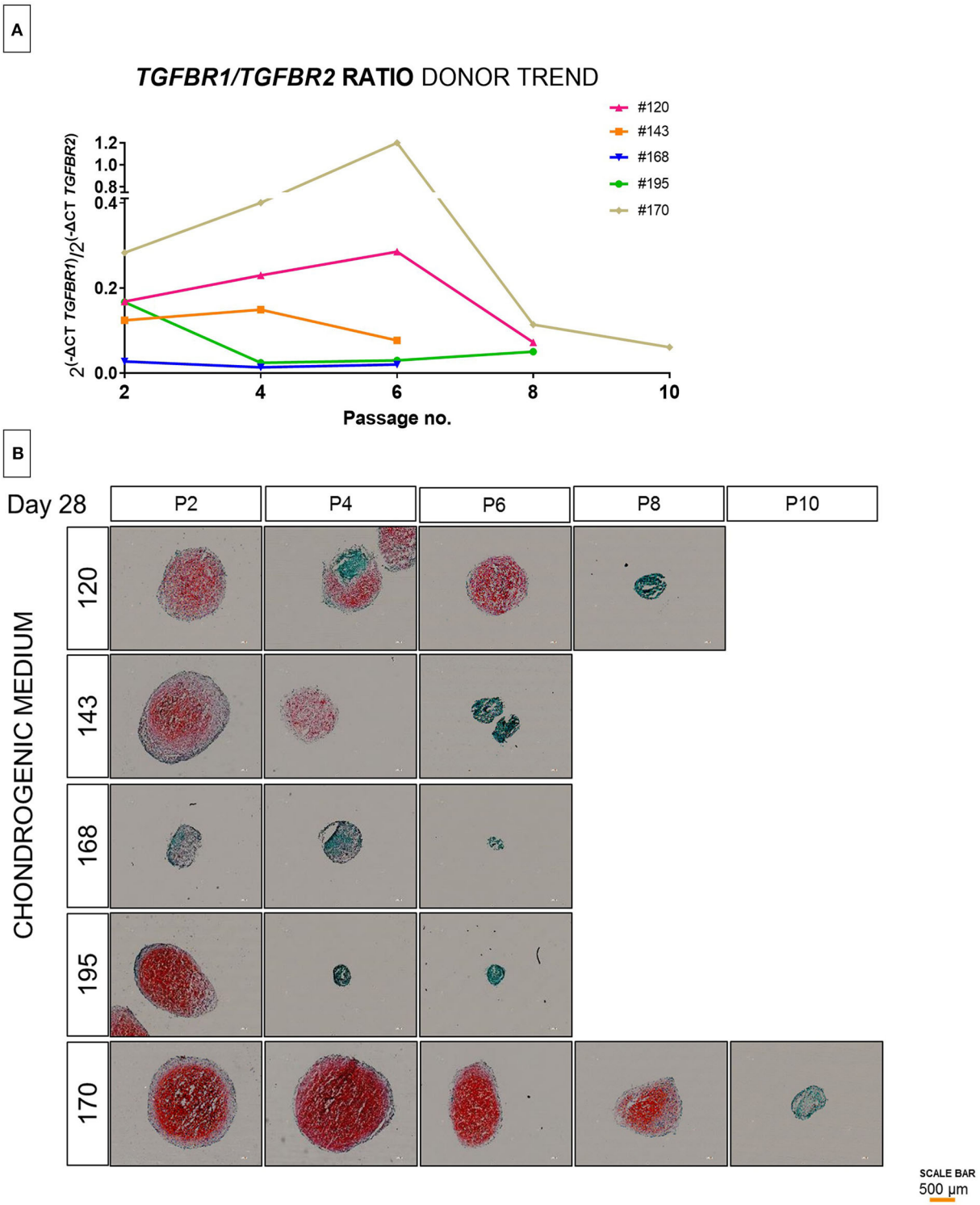
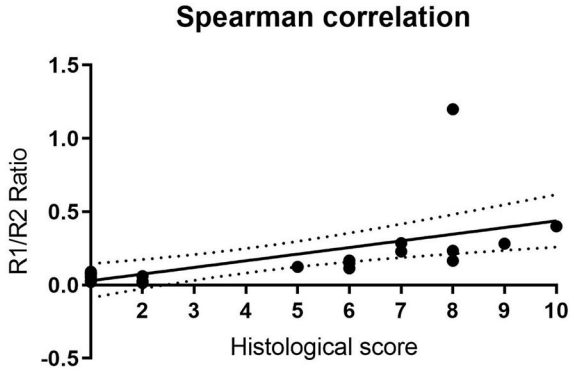
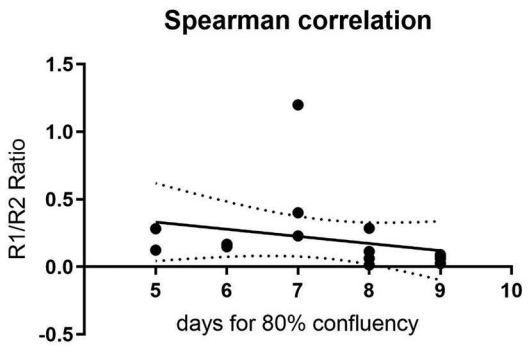


FIGURE 2 | Evaluation of TGFBR ratio in extended population during monolayer expansion of hBMSCs from passage 2 to passage 10. Cells from different donors were cultured in conventional expansion medium. The amounts of TGFBR1/TGFBR2 ratio mRNA was normalized to ribosomal protein large, P0 (RPLP0). The mRNA expression of cells cultured over the passaging were plotted as $2^{[-(\Delta Ct\_1-\Delta Ct\_2)]}$ **(A)**, the lines shows the trend of individual donors over passaging **(B)**. Histological representation by safranin-O and fast green of 5 donors correlating to the initial observation of TGFBR1/TGFBR2 ratio and outcome of differentiation **(C)**. Red scale bar is 200 µm.



Spearman r	
r	0.8051
95% confidence interval	0.5867 to 0.9143
P value	
P (two-tailed)	<0.0001
P value summary	****
Exact or approximate P value?	Approximate
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	24

FIGURE 3 | Histological and molecular Spearman correlation between TGFBR1/ TGFBR2 ratio and histological scores.



Spearman r	
r	-0.6380
95% confidence interval	-0.8602 to -0.2122
P value	
P (two-tailed)	0.0072
P value summary	**
Exact or approximate P value?	Exact
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	17

FIGURE 4 | Time to 80% confluency was correlated to the TGFBR1/ TGFBR2 ratio. A statistically significant negative correlation was observed ($r = -0.638$, $P = 0.0072$).

for poor chondrogenic differentiation, at least in cells aged by *in vitro* culture.

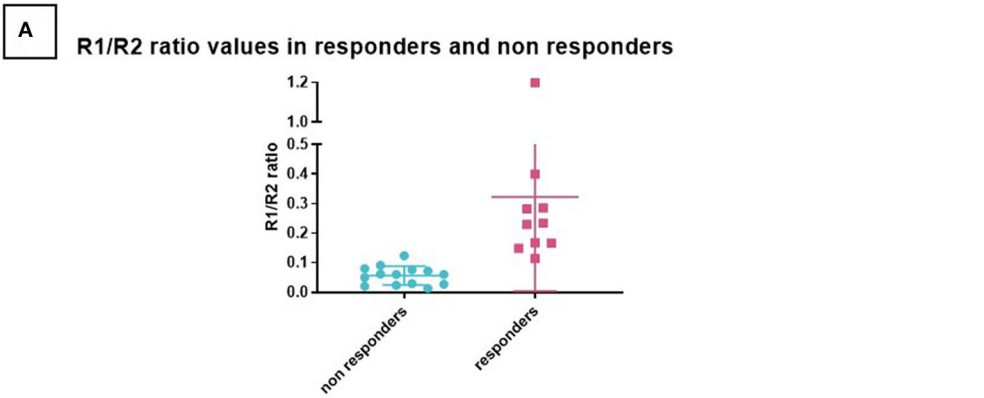
Interestingly, while the silencing of TGFβ-RI did not significantly alter differentiation in comparison with the negative scramble control, ACVRL-I knockdown also led to increased chondrogenic potential (**Figure 7**). Treatment of functional MSC populations with siRNA did not have a noticeable effect (data not shown). We also investigated the osteogenic potential relative to the receptor ratio and no correlation was observed (**Supplementary Figure 2**).

DISCUSSION

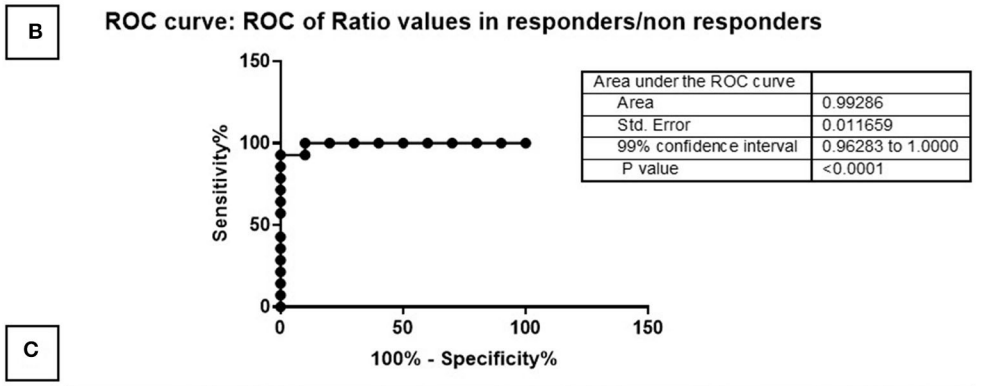
Though widely used, CD markers do not offer insights into MSC function and this has led to the search for more functional outcome parameters. We had previously shown a change in Runx2/Sox9 ratio to be a reliable predictor of MSC osteogenesis (Loebel et al., 2015), however this still requires a osteogenic stimulus to be applied and a period of time to wait for changes to occur. Varas et al. have proposed alpha10 integrin as a marker of chondrogenic potency (Varas et al., 2007). More recently the group of Hollander proposed receptor tyrosine kinase-like orphan receptor 2 (ROR2) as a potency marker for human MSC chondrogenesis (Dickinson et al., 2017). TWIST1 has been proposed as a marker of MSC secretory phenotype that can be manipulated during monolayer expansion by exposure to fibroblast growth factor 2 or interferon gamma (Boregowda et al., 2016). Therefore, the expression of TWIST 1 has been proposed as a clinical indication prediction scale, with high twist leading to a more angiogenic phenotype, while low TWIST1 results in an immuno-modulatory phenotype (Boregowda et al., 2016). The downregulation of TWIST1 has also been demonstrated during chondrogenic differentiation (Cleary et al., 2017).

Real-time PCR is a precise method for determination of gene expression of a target gene. As a consequence, ΔΔCT determinations (Livak and Schmittgen, 2001; Schefe et al., 2006) have become a standard measurement, and are used in cell differentiation experiments to discern the differentiation state of a given cell population. Pooling results of analyses for cells from multiple donors (biological replicates) and within a given experimental setup (technical replicates) to provide overall means and standard deviations for comparison between groups is commonly used to detect a consistent result that should be repeatable. Unfortunately, this approach can hide patterns that are present in subgroups, masking explanations

A



B



Area under the ROC curve	
Area	0.99286
Std. Error	0.011659
99% confidence interval	0.96283 to 1.0000
P value	<0.0001

C

	Sensitivity%	99% CI	Specificity%	99% CI	Likelihood ratio
< 0.016762	7.1429	0.035797% to 42.403%	100.00	58.870% to 100.00%	
< 0.022103	14.286	0.76424% to 51.231%	100.00	58.870% to 100.00%	
< 0.025745	21.429	2.5709% to 58.918%	100.00	58.870% to 100.00%	
< 0.028598	28.571	5.2589% to 65.794%	100.00	58.870% to 100.00%	
< 0.040037	35.714	8.6595% to 72.014%	100.00	58.870% to 100.00%	
< 0.055542	42.857	12.671% to 77.657%	100.00	58.870% to 100.00%	
< 0.061292	57.143	22.343% to 87.329%	100.00	58.870% to 100.00%	
< 0.066918	64.286	27.986% to 91.341%	100.00	58.870% to 100.00%	
< 0.074321	71.429	34.206% to 94.741%	100.00	58.870% to 100.00%	
< 0.078305	78.571	41.082% to 97.429%	100.00	58.870% to 100.00%	
< 0.085587	85.714	48.769% to 99.236%	100.00	58.870% to 100.00%	
< 0.10252	92.857	57.597% to 99.964%	100.00	58.870% to 100.00%	
< 0.11893	92.857	57.597% to 99.964%	90.000	45.571% to 99.950%	9.2857
< 0.13652	100.00	68.492% to 100.00%	90.000	45.571% to 99.950%	10.000
< 0.15781	100.00	68.492% to 100.00%	80.000	35.180% to 98.915%	5.0000
< 0.16745	100.00	68.492% to 100.00%	70.000	26.489% to 96.299%	3.3333
< 0.19909	100.00	68.492% to 100.00%	60.000	19.092% to 92.323%	2.5000
< 0.23206	100.00	68.492% to 100.00%	50.000	12.831% to 87.169%	2.0000
< 0.25850	100.00	68.492% to 100.00%	40.000	7.6768% to 80.908%	1.6667
< 0.28397	100.00	68.492% to 100.00%	30.000	3.7007% to 73.511%	1.4286
< 0.34286	100.00	68.492% to 100.00%	20.000	1.0851% to 64.820%	1.2500
< 0.79978	100.00	68.492% to 100.00%	10.000	0.050113% to 54.429%	1.1111

FIGURE 5 | ROC curve for the determination of a cut-off. Samples were divided in two groups based on the histological score and TGFBR1/TGFBR2 ratio **(A)**. Non-responders have score 1–5 (green), Responders have score 6–10 (red). This division made it possible to generate a ROC curve **(B)** and calculate cut-offs for specificity and sensitivity **(C)**.

for donor to donor variation (Stoddart et al., 2012). Donor variation in primary human isolates of MSCs is widely accepted, yet the classical approach of pooling data and comparing the mean and standard deviation persists. Using such statistical analyses, we initially concluded that there were no changes in TGFβ receptor profiles that correlated with hMSC chondrogenic potential (**Figure 1**). However, when analyzing the data on a donor by donor basis, clear trends started to emerge and an increase of *SD* over time was an indicator that the marker under investigation could be of interest. Chondrogenic media typically contains a standardized concentration of TGFβ, yet the donor response varies. We hypothesized that the variation seen was due to changes in receptor expression, leading to changes in bioavailability. In our experiments we initially started comparing

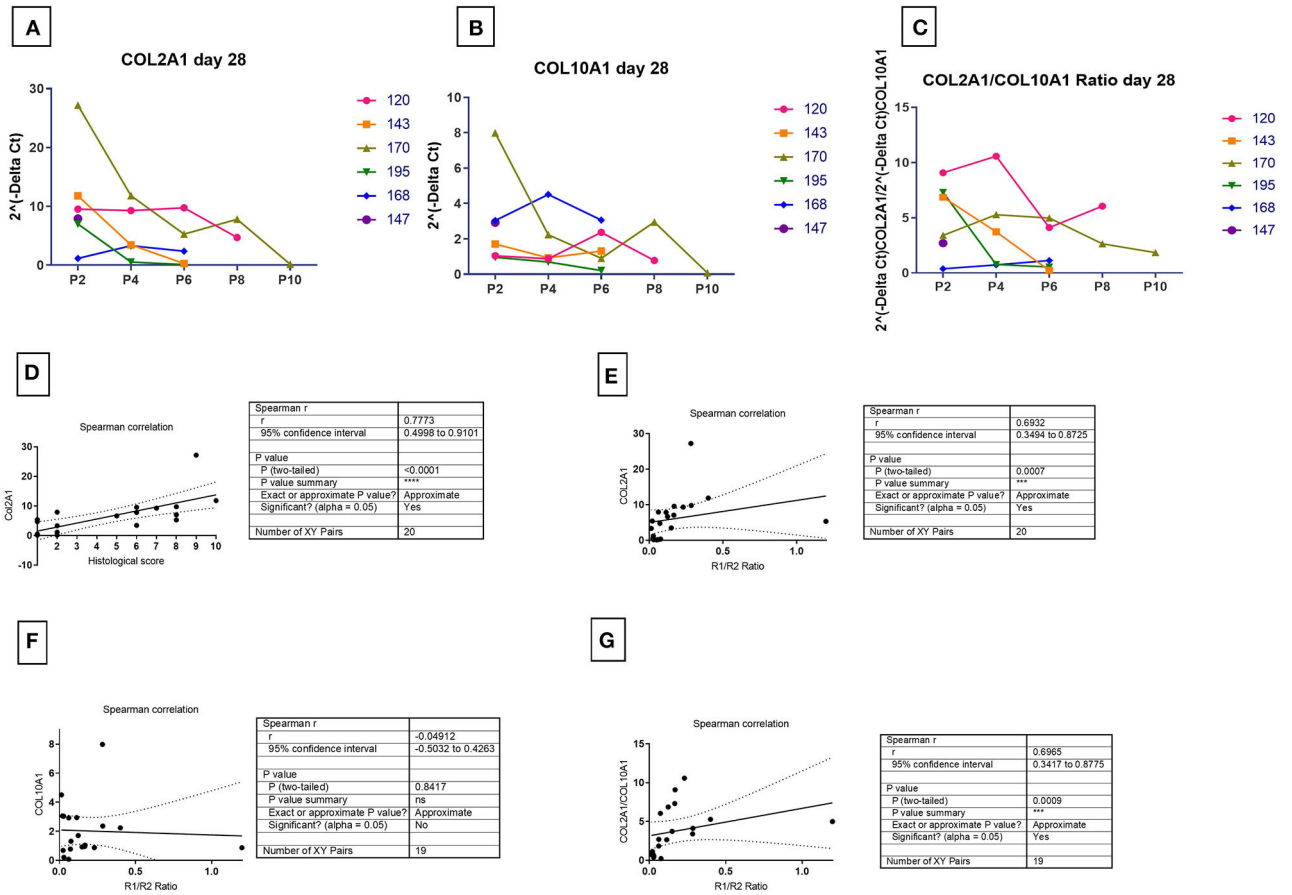


FIGURE 6 | Correlation of marker receptors ratio and chondrogenic outcome markers. The COL2A1 **(A)**, COL10A1 **(B)**, COL2A1/COL10A1 ratio **(C)** for mRNA at 28 days in chondrogenic medium was normalized to ribosomal protein large, P0 (RPLP0). The mRNA expression of receptors over time were plotted as $2^{(-\Delta Ct)}$. Spearman correlation for the detection of significance of hypothesis between R1/R2 and chondrogenic marker expression was done between the amount of COL2A1 and histological score **(D)**, the amount of COL2A1 and R1/R2 ratio **(E)**, the amount COL10A1 and R1/R2 ratio **(F)**, and COL2A1/COL10A1 ratio and R1/R2 ratio **(G)**. One sample had non-detectable levels of COL10A resulting in an $n = 19$ [Donor 195 P6- non-chondrogenic (see **Supplementary Table 3**)].

the changes in BMP and TGFβ receptors in MSCs during passaging using analyses of populations containing multiple donors by comparing means and *SD* of the whole cohort. Using this method, we could not detect significant differences in BMP-receptors except for a higher standard deviation in BMP-R1B and BMP-RII expression with passage (**Figure 1**). While investigating *in vivo* aging, (Moerman et al., 2004) found similar results with MSCs isolated from mice at different ages, with Moerman noting a decrease BMP-R1B. However, no direct link to function could be observed.

For TGFβ-receptors we could identify a slight increase in TGFβ-RII expression with a trend of TGFβ-RI decreasing, but with neither change being consistent, nor individually being correlated with differentiation (**Figure 1**). As the receptors signal as a hetero-tetrameric TGFβ-RI/ TGFβ-RII complex we analyzed the ratio on an individual donor level and noted that cell isolates with lower chondrogenicity expressed a lower TGFβ-RI/TGFβ-RII ratio compared with more chondrogenic cells, as assessed by histology and PCR (**Figure 2**). Moreover, as cells were *in vitro* aged using monolayer expansion the ratio changed over time in a donor dependent manner and a decrease correlated with a decrease in chondrogenic potential. Calculation of the $R = 2^{-(\Delta Ct_{\mathrm{hTGF\beta-RI}} - \Delta Ct_{\mathrm{hTGF\beta-RII}})}$ ratio showed the same effects with the simplification that a value could be calculated at any passage without the need of a calibrator. It was shown that MSCs from aged hearts show a lower TGFβ-RI expression (Cieslik et al., 2014). Dexheimer et al. found that TGFβ-RII decreased in expression in MSCs during chondrogenic differentiation (Dexheimer et al., 2016), which suggests that a low TGFβ-RII might be better for chondrogenic differentiation and might be the reason why older cells tend to be less potent for differentiation than younger.

An AUR > 0.136 AUR (Arbitrary Unit Ratio) for the $2^{-(\Delta Ct_{\mathrm{hTGF\beta-RI}} - \Delta Ct_{\mathrm{hTGF\beta-RII}})}$ ratio is a good baseline for cells with good chondrogenic differentiation potential (**Figure 3**). Only one cell population with a ratio of 0.123991 was incorrectly characterized as non-responsive, when in fact it was chondrogenic (**Supplementary Table 3**). As more donors are investigated the actual cut off value may be further refined, but the principle of a low ratio leading to poor chondrogenesis is

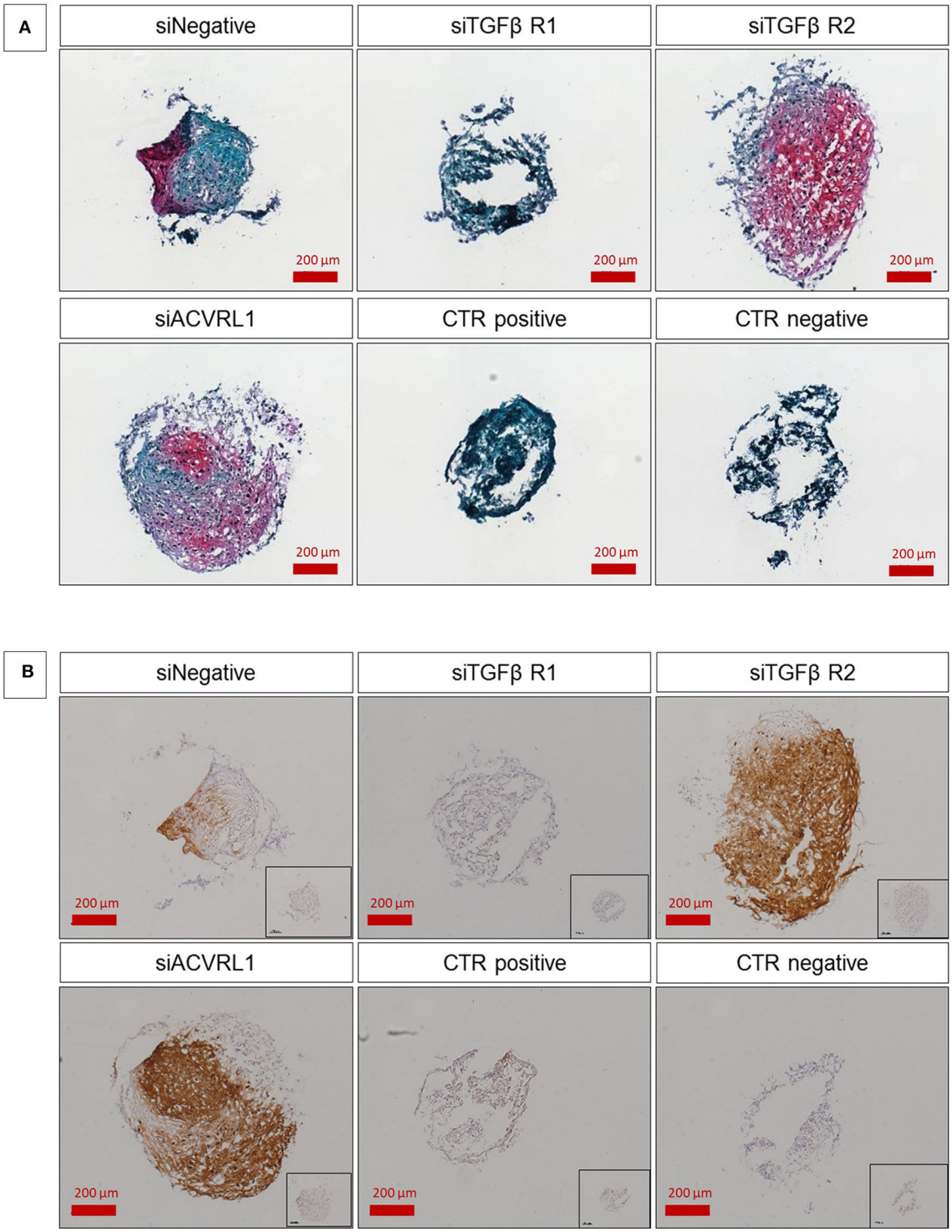


FIGURE 7 | Safranin-O/fast green staining **(A)** and type II collagen/hematoxylin-mayer immunostaining **(B)** of hMSC pellets. SiNegative shows pellet that has been transfected with scramble control and was cultured with TGFβ; siTGFβR1, siTGFβR2, and siACVRL1 were transfected with the respective siRNA and was cultured with TGFβ; CTR positive has been not transfected but was cultured with TGFβ; CTR negative has been not transfected and was not cultured with TGFβ. The intensity of safranin-O (Red) staining is directly proportional to the proteoglycan content inside the pellet, while green structures represent the counterstaining with fast green solution **(A)**. Brown color represents the positive reaction to type II collagen, counter stained with Hematoxylin-Mayer; Inset images show the respective negative controls for the immunostaining **(B)**. The figures are representative of five separate experiments using five different donors. Scale bar for 10× objective = 200 µm.

now established. Whether other laboratories will obtain exactly the same ratio is still to be seen. Furthermore, we are currently investigating other MSC cell sources to determine how broadly applicable to chondrogenesis prediction will be to other cell types. Similarly to the ROR2 chondrogenic marker, this chondrogenic ratio did not correlate with osteogenic potential [(Dickinson et al., 2017) and **Supplemental Figure 2**], suggesting specific markers may need to be discovered for specific phenotypes.

The identification of a threshold above which chondrogenesis reliably occurs offers the potential to enhance chondrogenic potential of previously non-responsive human donors. A transient siRNA interference of TGFBR2 increased the ratio and led to improved chondrogenesis in all donors tested (**Figure 7**, **Supplementary Table 3**). This is an advantage for autologous therapies and would allow cells from all patients to be used with an increased chance of success. It would also offer a method to produce a more reproducible allogeneic cell source. The lack of any detrimental effect on siRNA knockdown on functional cell populations suggests that a receptor manipulation step could become a standardized part of any treatment protocol, thus allowing for a standardized approach. As ACVRL1 knockdown also leads to reversal of poor donor phenotype (**Figure 7**), it suggests the underlying mechanism is limiting the bioavailability of the TGFB/TGFBR2 complex to favor heterodimerization with TGFBR1. siRNA knockdown of TGFBRIII has also been shown to improve TGFβ3 induced chondrogenesis (Zheng et al., 2018). Of note, the siRNA induced knockdown of TGFBRIII was performed using a lentiviral expression system, whereas within this study a single dose applied using electroporation, was sufficient to direct the chondrogenic response over weeks. This would suggest that fate decisions can be taken within the first 24 h, and then become self-propagating, offering the opportunity to manipulate cell differentiation by early and transient modification of cell phenotype.

TGFBRII initially binds TGFβ protein and then forms a complex with an additional receptor. While TGF-β signaling is normally associated with recruitment of the ALK5 (TGFBR1) receptor, activating the SMAD 2/3 pathway, it is also known that TGFβ can signal via ALK1 (ACVRL1), leading to SMAD 1/5/8 activation (Blaney Davidson et al., 2009). Both receptors are present in MSCs [our data and (de Kroon et al., 2015)] and both receptors are needed for chondrogenesis (Hellingman et al., 2011). In other cell types it has been shown that TGF-β signaling is dose dependent with a low dose favoring SMAD 2/3 signals via ALK5, while increasing TGFβ concentration shifts the balance in favor of ALK1 and SMAD 1/5/8 signals (Remst et al., 2014). This has led us to the hypothesis that the relative level of TGFBRII vs. TGFBRI on the cell is defining when classical chondrogenic media cocktails containing 10 ng TGFβ are used. As TGFBRII binds TGFβ, it preferentially recruits TGFBR1 and activates the SMAD 2/3 pathway. If ligand bound TGFBRII is still available once the TGFBRI has been recruited, it is then able to recruit increasing numbers of ALK1 receptors, with increasing SMAD 1/5/8 signaling. By downregulating TGFBRII the balance is shifted in favor of the TGFBRI recruitment, and despite the excess of TGFβ there is no available TGFBRII to bind the ligand and complex with ALK1.

CONCLUSION

In the era of personalized medicine, patient specific quantitative measures will be required that do not rely on statistical evaluation. Determination of the TGFβ-RI/TGFβ-RII ratio in hMSCs during expansion is a relatively easy method to screen MSCs for their chondrogenic differentiation properties. While MSCs at higher passages with lower differentiation potential show a lower quotient, the quotient is higher in cells with greater chondrogenicity. We defined a $2^{-\Delta CtTGF\beta-RI}/2^{-\Delta CtTGF\beta-RII}$ ratio value of ∼0.136 as the threshold for indicating an hMSC cell preparation has poor chondrogenicity. Once a potential mechanism was established, there was the opportunity to modify the receptor profile and assess the effect of chondrogenic differentiation. Transient inhibition of TGFBRII expression by siRNA treatment reliably enhanced the chondrogenic differentiation of all non-chondrogenic donors. Increasing the TGFβ-RI/TGFβ-RII ratio by temporary knockdown of TGFβ-RII via siRNA prior to differentiation can increase the differentiation potential of hMSCs. Donors with good differentiation quality are not affected by this knockdown. The ability to increase chondrogenicity of a given preparation will enhance the possibility to use a patient's own cells for autologous cell therapies involving cartilage tissue engineering.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by ethics board of the University of Freiburg, permission number 135/14. All samples were harvested after signed informed consent.

AUTHOR CONTRIBUTIONS

RR, VB, and MS: conception and design, collection and assembly of data, data analysis, and interpretation. DK, FD, RS, and BJ: sample collection and interpretation, final approval of manuscript. RR, VB, BJ, and MS: writing and final original draft of manuscript. DK, FD, RS, BJ, and MA: review & editing of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Conflict of Interest: The AO Foundation has a patent pending on the TGF-receptor ratio and reversal of poor donor chondrogenesis. None of the authors will personally benefit should the patent application be successful.

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ORIGINAL RESEARCH
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Preparation and Characterization of an Optimized Meniscal Extracellular Matrix Scaffold for Meniscus Transplantation

Yong He[1,2†], Yunbin Chen[1,2,3†], Xinyu Wan[1,2,4†], Chenchen Zhao[1,2], Pengcheng Qiu[1,2], Xianfeng Lin[1,2], Jianfeng Zhang[1,2*] and Yue Huang[1,2*]*

[1] Department of Orthopaedic Surgery, Sir Run Run Shaw Hospital, Medical College of Zhejiang University, Hangzhou, China, [2] Key Laboratory of Musculoskeletal System Degeneration and Regeneration Translational Research of Zhejiang Province, Hangzhou, China, [3] Department of Neurology, The First Affiliated Hospital of Wenzhou Medical College, Wenzhou, China, [4] First Clinical College, Wenzhou Medical University, Wenzhou, China

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Edited by:
Tracy Tong Li Yu,
NxR Biotechnologies GmbH,
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Adetola B. Adesida,
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Caroline Curtin,
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Ireland

***Correspondence:**
Xianfeng Lin
xianfeng_lin@zju.edu.cn
Jianfeng Zhang
zhangjianfengxs@163.com
Yue Huang
2000huangyue@zju.edu.cn

† These authors have contributed equally to this work

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Many studies have sought to construct a substitute to partially replace irreparably damaged meniscus. Only the meniscus allograft has been used in clinical practice as a useful substitute, and there are concerns about its longevity and inherent limitations, including availability of donor tissue and possibility of disease transmission. To overcome these limitations, we developed an acellular xenograft from whole porcine meniscus. Samples were treated with 2% Triton X-100 for 10 days and 2% sodium dodecyl sulfate for 6 days. The DNA content of extracellular matrix (ECM) scaffolds was significantly decreased compared with that of normal porcine menisci ($p < 0.001$). Histological analysis confirmed the maintenance of ECM integrity and anisotropic architecture in the absence of nuclei. Biochemical and biomechanical assays of the scaffolds indicated the preservation of collagen ($p = 0.806$), glycosaminoglycan ($p = 0.188$), and biomechanical properties (elastic modulus and transition stress). The scaffolds possessed good biocompatibility and supported bone marrow mesenchymal stem cells (BMSCs) proliferation for 2 weeks *in vitro*, with excellent region-specific recellularization *in vivo*. The novel scaffold has potential value for application in recellularization and transplantation strategies.

Keywords: meniscus, extracellular matrix, biomechanics, region-specific recellularization, decellularization

INTRODUCTION

The meniscus of the knee is fibrocartilaginous in nature, with an organized arrangement of collagenous fibers. The main functions of human menisci are load transmission, stress distribution, stability, and lubrication of the joint, which collectively prevent cartilage damage (Stapleton et al., 2011). Damage or degeneration of the meniscus is usually followed by loss of these functions and development of knee arthritis (Ding et al., 2007). Meniscus lesion repair has always been a great challenge in orthopaedic surgery because of its limited vascularization and capacity for self-regeneration. When damage involves the non-vascularized areas, meniscus preservation or restoration is difficult to achieve. Development of novel therapeutic methods for meniscus repair is both timely and necessary (Maier et al., 2007).

Many materials have been used to repair meniscus defects after (partial) meniscectomy. These include natural polymers (Stone et al., 1992), synthetic polymers (Klompmaker et al., 1996),

allogenic meniscus (van Arkel and de Boer, 2002; Verdonk, 2002), and autologous tissue (Kohn et al., 1997; Bruns et al., 1998). A goal of polymer research in this regard is to mimic the organization and interactions among the major tissue constituents of natural meniscus. However, long-term studies have indicated the limited success of this approach. Meniscal allograft transplantation has been applied in patients worldwide, since the first successful operation in 1984 (Milachowski et al., 1989). Meniscal allografts produced good to excellent results regarding pain and function (Cameron and Saha, 1997; Rath et al., 2001; Noyes et al., 2005; Cole et al., 2006; Ha et al., 2011). Nevertheless, concerns over longevity and inherent limitations of the allografts, including limited graft supply and possible disease transmission (Noyes and Barber-Westin, 2010), mean they have limited wide application. Furthermore, while they are safe and available, properties of fat pad, quadriceps, and Achilles tendon autografts have been inferior to the allograft (Messner et al., 1999).

Acellular xenogenic meniscal tissue has similar anatomy and matrices to the human meniscus and is easy to access, and thus, it might be a promising alternative for transplantation. Many different protocols have been described for preparing meniscus extracellular matrix (ECM) scaffolds (Maier et al., 2007; Stapleton et al., 2008; Sandmann et al., 2009; Stabile et al., 2010; Azhim et al., 2013; Chen et al., 2015; Wu et al., 2015). The dense fibrous structure of the meniscus makes it difficult to undergo decellularization while retaining the maximum amount of bioactive molecules and biomechanical potential (Stapleton et al., 2008; Ionescu et al., 2011). Maier et al. (2007) reported the complete cell removal in whole ovine meniscus using an enzymatic solution. However, there was appreciable disruption and digestion of the ECM. In 2008, Sandmann et al. (2009) incubated human meniscus tissue in 2% sodium dodecyl sulfate (SDS) for 2 weeks and achieved cell removal. However, such a long SDS treatment resulted in greater cytotoxicity and ECM injury than treatment with Triton X-100 or tri-(n-butyl) phosphate (Cartmell and Dunn, 2004; Gilbert et al., 2006). Acellular ECM meniscal hydrogels as well as ECM meniscal fragments based on dissection and sonication have also been explored (Azhim et al., 2013; Wu et al., 2015), but the lower biomechanical potential of these materials is an important limitation.

In this study, we optimized the previous methods and developed a novel protocol for the decellularization of whole porcine meniscus, which is very similar in size to human menisci. The scaffolds preserved the bioactivity, biomechanics, and cytocompatibility features of the tissue, and excellent region-specific recellularization was observed *in vivo*.

MATERIALS AND METHODS

Preparation of Acellular Meniscus Scaffolds

Six male pigs (6-month-old, Large White) were obtained from the Animal Experimental Center of Zhejiang University. All animal experiments were approved by the Animal Experimental Ethics Committee of Zhejiang University, and the animals were treated according to approved experimental protocols. Both medial and lateral menisci were harvested from pigs and divided into two groups, the normal group ($n = 6$) and decellularized group ($n = 6$). The menisci were then washed in phosphate buffered saline (PBS) to remove excess blood. Samples in the control group were immediately stored at 4°C for biochemical analysis, in 4% formaldehyde for histological analysis, and in 4% glutaraldehyde for scanning electron microscopy (SEM). Before determining the optimal decellularization protocol, Menisci were suspended in 2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, United States) for 10 days and 2% SDS (Sigma-Aldrich) for 6 days, followed by deionized water (48 h) and PBS treatment (24 h) to wash out all remnants of detergent. To minimize the DNA content in the ECM, 100 U/ml DNAase (Sigma-Aldrich) in PBS was applied (**Figure 1**). All steps were performed at room temperature with agitation (100 rpm). Samples were stored in PBS at 4°C within 2 weeks of processing.

Histological Analysis

Normal menisci (NM) and decellularized menisci (DM) were fixed for 24 h in a 10% neutral buffered formalin solution at room temperature for 24 h, then dehydrated automatically by gradient concentrations of ethanol (50, 70, 80, 90, and 100%, for 5 min each), and treated by xylol for 20 min before embedding in paraffin wax. Sections 5 mm in thickness were used for histological staining. Standard hematoxylin and eosin (H&E) staining was used to evaluate tissue microstructure and remaining cells. Alcian blue staining (1% w/v, pH = 2.5) was used for the qualitative analysis of glycosaminoglycan (GAG) content. Cell nuclei were counterstained with 1% neutral red stain. Masson trichrome staining was used to visualize the collagen distribution and orientation. Cell nuclei were stained using hematoxylin. Images were captured digitally using an ECLIPSE 80i microscope (Nikon, Tokyo, Japan) and qualitatively analyzed.

Immunohistochemical (IHC) Analysis

IHC staining was performed by placing the slides (transverse and longitudinal) in an antigen retrieval solution consisting of citrate buffer at 95°C in a steamer for 10 min. After cooling, the sections were incubated in 3% perhydrol solution for 15 min to block the endogenous peroxidase reaction and non-specific binding was blocked using 1% bovine serum albumin. Sections were incubated with primary antibodies against type collagen I (Abcam, Cambridge, MA, United States, 1:200) and II (Abcam, 1:200) at 4°C overnight. Following the incubation, the slides were washed three times in PBS. Goat anti-mouse IgG1 biotinylated secondary antibody (Abcam, 1:1000) was then applied for 30 min and the slides were then subjected to three more washes in PBS followed by treatment with a streptavidin-horseradish peroxidase complex, diaminobenzidine solution, and counterstaining with haematoxylin. NM and DM samples were incubated for 5 min in 4′, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for fluorescent staining of nuclei to evaluate the presence of residual nuclei.

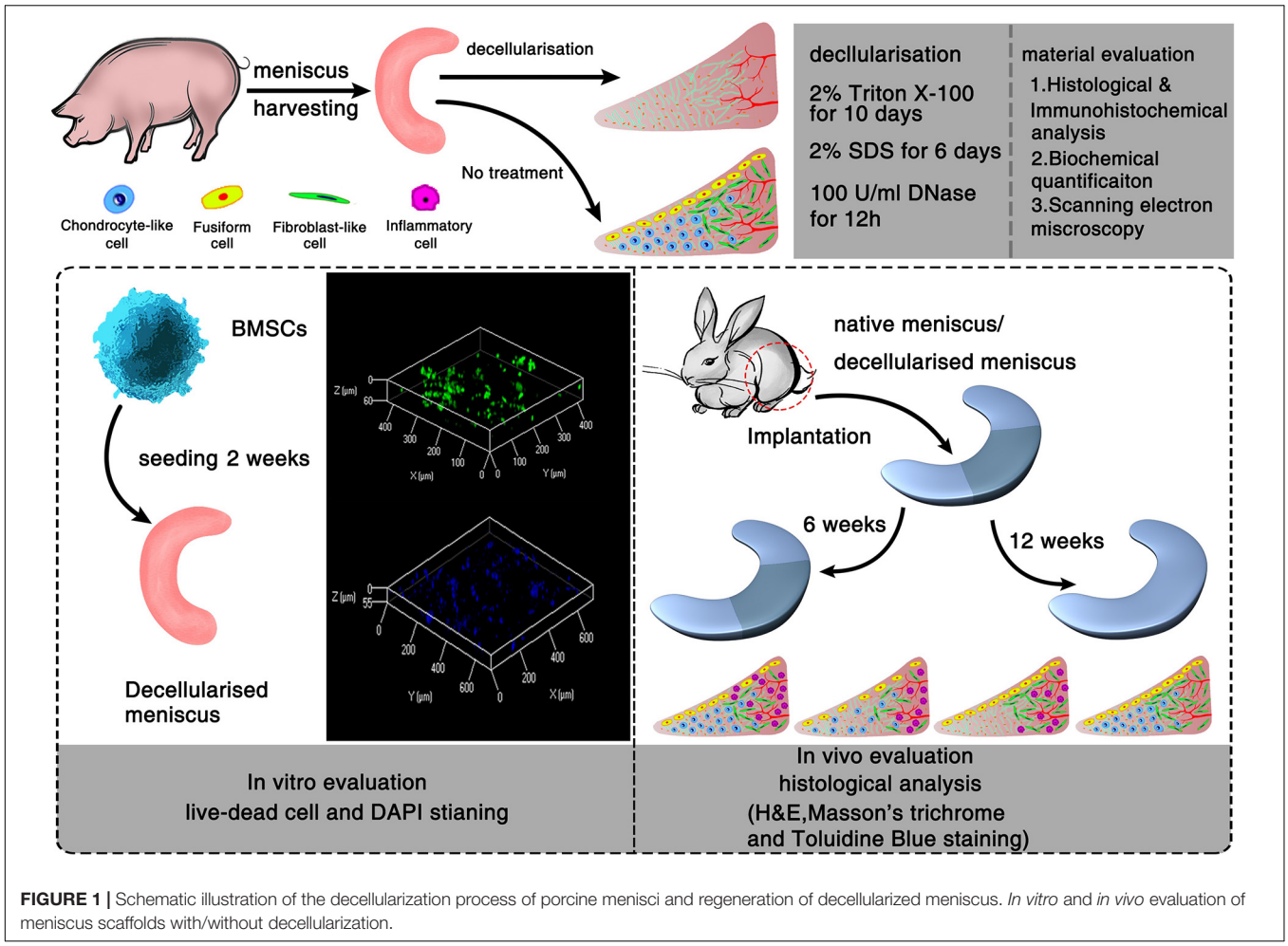

FIGURE 1 | Schematic illustration of the decellularization process of porcine menisci and regeneration of decellularized meniscus. *In vitro* and *in vivo* evaluation of meniscus scaffolds with/without decellularization.

Analysis of DNA Content

Total genomic DNA in the samples was extracted using a Genomic DNA Extraction Kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. Briefly, NM and DM samples were lyophilized at −80°C to thoroughly remove residual moisture until achieving a constant weight, and were then weighed, cut into thin strips and digested with proteinase K and RNase for 4 h. The digested samples were centrifuged and purified with two phenol/chloroform/isoamyl alcohol (25:24:1, v/v) 33extractions. The remaining DNA was collected using an elution buffer. The extracted genomic DNA was quantified by measuring absorbance using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). DNA quantity was normalized to the initial dry weight of the tissue and expressed as ng/mg.

Determination of GAG, Collagen, and Water Content

NM and DM samples were weighed, freeze-dried and weighed again. The weights before and after freeze-drying were recorded to evaluate water content. GAG content was determined using a modified dimethylmethylene blue (DMMB) method as previously described (Farndale et al., 1986). NM and DM samples were lyophilised to a constant weight and then digested at 60°C in papain buffer (125 mg/mL papain, 5 mM cysteine/HCl, 5 mM disodium EDTA in PBS) for 12 h. The absorbance values of the samples were determined at 525 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, United States) immediately after the addition of a 1,9-dimethylmethylene blue solution (Sigma-Aldrich). GAG content was calculated using a standard curve, which was made using different concentrations of chondroitin sulfate sodium salt (Sigma-Aldrich). Final values were expressed as μg of GAG per dry weight of sample. Collagen content was determined based on hydroxyproline (HYP) content, which was measured using a spectrophotometric method (Chan et al., 1998). The amount of HYP in the samples was then determined using a calibration curve prepared using HYP assay (Sigma-Aldrich), and total collagen content per mg dry weight of sample as calculated using a HYP-to-collagen ratio of 1:7.2.

SEM

SEM was performed to examine the microarchitecture of the meniscus before and after decellularization. Meniscal samples were fixed with 2.5% (v/v) glutaraldehyde in PBS, post-fixed with

1% (w/v) OsO_4, dehydrated using a graded alcohol series and dried using hexamethyldisilazane (Sigma-Aldrich). The dried samples were sputter-coated with gold-palladium and viewed using a model S-3000N microscope (HITACHI, Tokyo, Japan). The porosity of each group was tested by ImageJ (National Institutes of Health, United States).

Cytotoxicity of DM Scaffold

To assess the chemical toxicity of decellularized scaffolds, cytotoxicity testing was conducted as previously described (Xu et al., 2014). Briefly, DM was incubated in standard Dulbecco's modified Eagle medium (DMEM)/F12 containing 10% fetal bovine serum for 24 h, and the extracts was collected for subsequent use. Bone marrow mesenchymal stem cells (BMSCs) extracted from lumbar vertebra of New Zealand white rabbits were trypsinized, centrifuged and resuspended in the same medium and were maintained in culture at 37°C. And the medium was exchanged every 3 days. After successive cycles, the third-passage cells were harvested. Cells were then seeded in 96-well cell culture plates at a concentration of 2.5×10^4 cells/well that would allow confluency after 24 h in standard culture medium. The medium was removed and replaced with leach liquor at varying concentrations (25, 50, and 100%). Cells cultured in the standard medium served as the control group. Cell proliferation and metabolic activity were then assessed using a Cell Counting Kit-8 (CCK-8, DOJINDO, Tokyo, Japan). CCK-8 was added to each well of the plate and incubated for 3 h on days 1, 3, 4, and 5. Absorbance was measured at 450 nm using an Epoch Microplate Spectrophotometer (BioTek, Instruments). Five replicates were evaluated per sample.

Biomechanical Testing

All mechanical tests were performed using a model Z2.5 computer-controlled test machine (Zwick/Roell, Ulm, Germany) as we described previously (Zhao et al., 2009; Junhui et al., 2015). Mechanical uniaxial tensile tests were conducted to calculate the tensile initial elastic modulus, elastic modulus, transition stress, transition strain, and ultimate tensile strength of NM and DM. On the day of testing, frozen slices were cut from the central portion of the menisci with a cross-sectional area of 3×3 mm (thickness $\times$ width). In addition, to rule out the influence of damage caused by the mechanical grips, the tensile test specimens were then cut with a dumbbell-shape with a gauge length of 10 mm between the grips. Only specimens with dimensions within $\pm$ 10% of the pre-specified dimensions were considered. Specimens were kept hydrated at 37°C in PBS until testing and were then clamped to the grips in the mechanical apparatus. Tests were conducted at a strain rate of 10 mm/min, similar to our previous study (Lin et al., 2016). The initial elastic modulus and the elastic modulus were calculated from the slope of the linear curve fit up to 1% strain and the slope of the linear curve, based on the best R-square value using linear curve fitting. The intersection point of the two slopes defined the transition stress and strain. The ultimate tensile strength was calculated from the highest point on the stress strain curve.

Recellularization *in vitro*

The cytocompatibility of the ECM scaffolds was assessed using recellularization *in vitro*. After γ-rays radiation sterilization (24KGy), the whole scaffolds were immersed in DMEM/F12 containing 10% fetal bovine serum and 1% antibiotics for 24 h and were dried using sterile gauze. New Zealand white rabbit-derived BMSCs were passaged continuously after the third generation as mentioned above. The cells were resuspended to the density of 10×10^6 cells/mL. The scaffolds were then completely immersed in the cell suspension and incubated for 5 to 10 min and then transferred to a 12-well cell culture plates. Then, third-passage BMSCs were harvested and seeded onto the surface of per specimen at the same density of 2×10^6 cells/cm^2 and incubated for 2 h before the addition of supplemented culture medium. The culture medium was changed every 12 h. To generally observe the BMSCs on the scaffold on week 2 after cell seeding, cells were washed with PBS, fixed for 24 h in 10% neutral buffered formalin solution, and then stained with DAPI for 15 min at 37°C. Meanwhile, cell viability was assessed by Live-Dead cell staining, which indicated survival condition of BMSCs on scaffold after 2 weeks of culturing. The cells were washed with PBS and stained by Live-Dead cell staining kit (Biovision, Milpitas, CA, United States). After staining, cells were observed immediately using a Nikon ECLIPSE 80i microscopy (Nikon, Japan) equipped with a band-pass filter.

In vivo Animal Study

The intermediate part of the meniscus was shaped into a wedge-shaped semilunar disk. Ten skeletally mature male New Zealand white rabbits weighing between 3.0 and 3.5 kg were included in this study. Partial medial meniscectomy (one-third of the meniscus) was performed on the knees of all rabbits. The right knees were transplanted with DM (DM group) and the left knees underwent operation with the NM as a control (NM group). In this procedure, the rabbits were anesthetized with intravenous urethane (1 g/kg). After skin disinfection, the right knee was approached using a medial parapatellar incision. The meniscal tissue was thawed by immersion in sterile saline solution and then sutured to the adjacent meniscus with non-resorbable No. 5-0 sutures (Ethicon, Somerville, NJ, United States) after the meniscectomy. The capsule, periarticular tissues and skin were closed with No. 3-0 sutures (Ethicon). After the operation, the animals were immediately allowed free movement without any restriction. Five of the animals were killed with pentobarbital sodium at the end of weeks 6 and 12, and samples were evaluated by histological analysis as described *above*.

Semi-Quantification for Histological Staining

To further evaluate the repair condition of explants, semi-quantification of four typicalcells found in meniscal repair tissue was analyzed using Image J software 12 weeks H&E stained samples. Briefly, three sections of different points in both NM and DM group were chosen, respectively. The multipoint counting function in Image J was used to mark four kinds of cells, including inflammatory cells, remnant chondrocyte-like cells, fusiform cells

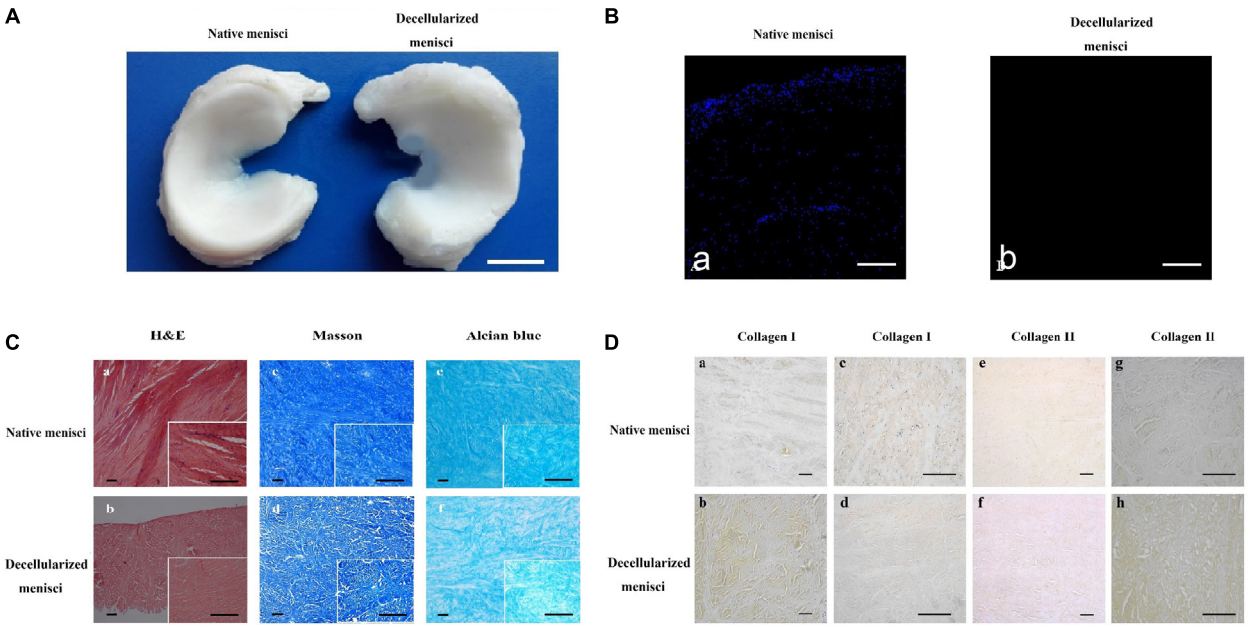

FIGURE 2 | Histological and immunohistochemical analysis of native meniscus and decellularized meniscus. **(A)** Gross morphological inspections of native meniscus and meniscus scaffold. Overall shape of the scaffold was maintained after processing. Scale bar: 10 mm. **(B)** DAPI staining of longitudinal section of native and acellular meniscus scaffold **(a,b)**. Scale bar: 100 μm. **(C)** H&E staining of **(a)** native and **(b)** decellularized meniscus. Masson's trichrome staining: **(c)** native and **(d)** decellularized meniscus; collagen is blue, cytoplasm is pink and nuclei are brown. Alcian blue staining of GAG: **(e)** native and **(f)** decellularized meniscus. Scale bar: 100 μm. **(D)** Collagen I labeling of tissue cross-sections: **(a)** native and **(b)** decellularized. Collagen I labeling of longitudinal section: **(c)** native and **(d)** decellularized. Collagen II labeling of tissue cross-sections: **(e)** native and **(f)** decellularized. Collagen II labeling of longitudinal section: **(g)** native and **(h)** decellularized. Scale bar: 100 μm.

and elongated fibroblast-like cells. Then the numbers of these cells per counting area (0.28 square millimeters) were calculated, respectively, and converted to percentage for statistical analyses.

Statistical Analyses

All numerical data were analyzed using SPSS 19.0 software (SPSS Inc, Chicago, IL, United States). The mean and standard deviation were calculated using the Descriptive Statistics function with a 95% confidence interval. Data from NM and DM, as well as that from different test configurations were compared using two-tailed Student's *t*-test. A p-value < 0.05 was considered significant.

RESULTS

Histological Analysis of DM

Gross inspection (**Figure 2A**) revealed that the decellularization process did not change the general shape of the menisci. Histological analyses after decellularization showed complete cell removal. Compared with the NM (**Figure 2Ca**), the DM (**Figure 2Cb**) was free of cell nuclei, and the collagenous fibers were well preserved with much loose arrangement, since loosening agent was used to make decellularization easier. Masson's trichrome staining revealed maintenance of the collagenous fibers (**Figures 2Cc,d**), as well as a slight increase in porosity, which is beneficial for recellularization and cell growth. Alcian blue staining (**Figures 2Ce,f**) revealed that GAG content was slightly reduced after decellularization, with the reduction of GAG distributed homogeneously.

Evaluation of Collagen Organization and Expression in DM

IHC staining indicated the presence of a well-preserved collagen bundle with much loose arrangement in the DM group (**Figure 2D**). There was no evident change in expression of either collagen I (**Figures 2Da–d**) or II (**Figures 2De–h**) after decellularization. Strong positive staining for collagen I could be observed across the whole meniscus, while collagen II was only labeled in the fibrocartilaginous section. Besides, much stronger staining for collagen I and II could be observed in DM group compared to NM group (**Figures 2Da,b,g,h**). It is most likely because decellularization detergents could partially break up peptide bonds and expose more amino and carboxylic groups to form hydrogen bonding with water, which results in higher HYP and water content. A similar paradoxical phenomenon was also observed in other studies (Grauss et al., 2005; Yuan et al., 2013).

Analysis of Residual DNA Within the Scaffold

DAPI staining of nuclear content revealed an abundance of nuclei in the intact meniscus and an absence of nuclei in the

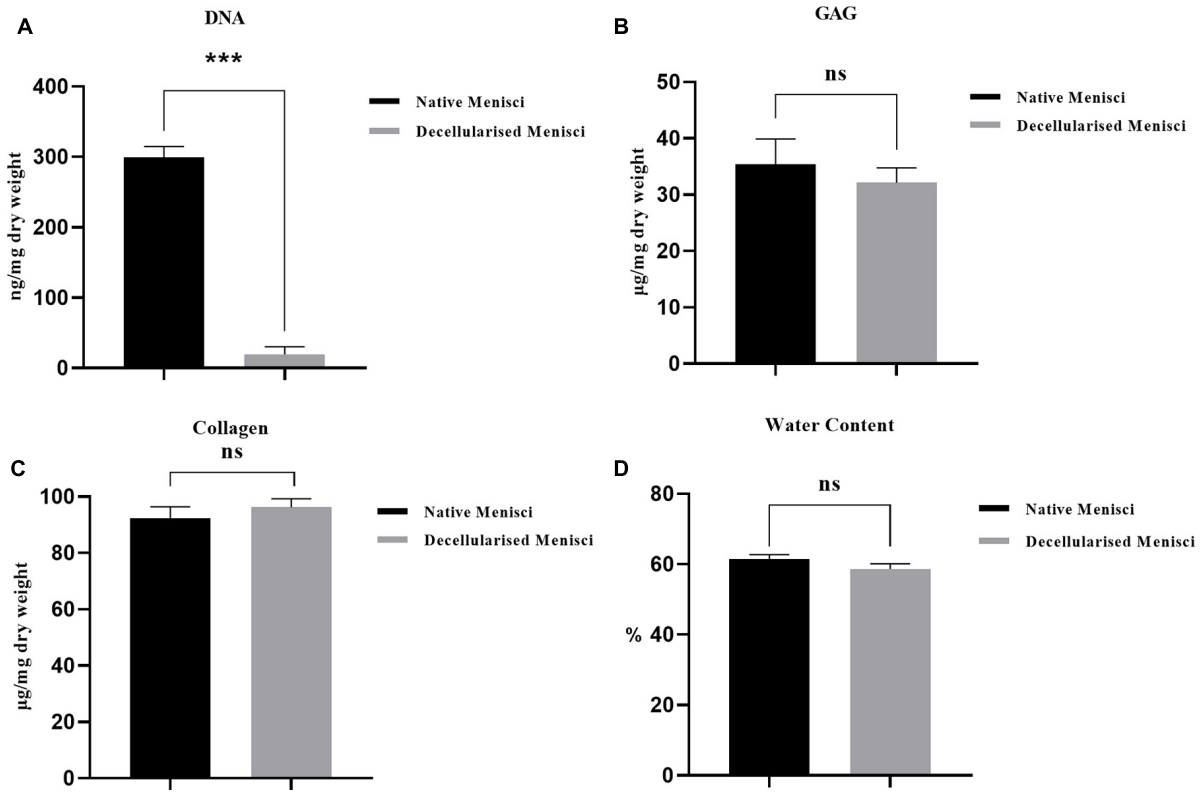

FIGURE 3 | Biochemical quantification of **(A)** water ($p < 0.05$), **(B)** glycosaminoglycan (GAG) ($p > 0.05$), **(C)** hydroxyproline (HYP) ($p > 0.05$) and **(D)** DNA content ($p < 0.001$) of native and DM ($n = 3$). ***$p < 0.001$.

scaffolds (**Figure 2B**). The amount of residual DNA within the DM was quantitatively evaluated using spectrophotometry, in which the peak of absorbance at 260 nm was measured. Results were normalized to the dry weight and compared with those of the NM treated with PBS (**Figure 3A**). The amount of DNA in NM was 299.61 ± 15.2 ng/mg dry weight, which was decreased to 19.54 ± 10.94 ng/mg dry weight ($p < 0.001$) after decellularization.

Biochemical Analyses in DM

The DMMB assay revealed no significant difference in GAG content between NM and DM ($p > 0.05$, **Supplementary Table 1** and **Figure 3B**). The amount of total collagen, a major component of porcine menisci, was calculated using the HYP assay. The collagen content of NM was 92.40 ± 3.98 μg/mg dry weight. There was no significant decrease in collagen content in DM (96.18 ± 3.02 μg/mg) compared with normal tissue ($p > 0.05$, **Figure 3C**). The percentages of water content within the menisci were 61.39 ± 1.3 vs. 58.63 ± 1.4% before and after decellularization ($p > 0.05$, **Figure 3D**).

Microstructure of Scaffolds

ECM structure was evaluated using SEM. The microstructure of NM and DM is shown in **Figure 4**. NM was very dense and the collagen fibers were aligned in parallel. The order of collagen fibers in the DM group was preserved, and displayed open pores after processing. The porosity in NM group and DM group was 6.5 and 19.8%, respectively.

Cytotoxicity Testing

The cytotoxicity of ECM scaffolds was evaluated using a cytotoxicity assay. The metabolic activities of cells incubated in different leaching solution concentrations were assessed. CCK-8 analysis indicated that the proliferation of cells cultured in a standard medium were similar to those of cells cultured in leaching solutions ($p > 0.05$, **Figure 5A**).

Biomechanical Evaluation

Representative curves for the results of the tensile tests are shown in **Figure 6A**, and the biomechanical properties of NM and DM are shown in **Figure 6B**. The measured initial elastic modulus, elastic modulus, ultimate tensile strength and transition stress for the NM ranged from 18.1 to 26.7, 131.0 to 167.1, 50.2 to 68.1, and 1.62 to 1.69 [MPa], respectively (**Supplementary Table 2**). The corresponding transition strain ranged from 3.6 to 5.8%. Compared with the NM group, the DM group showed a higher transition stress (1.66 ± 0.07 vs. 1.87 ± 0.06, $p < 0.01$) and elastic modulus (149.05 ± 36.25 vs. 182.70 ± 38.74, $p < 0.05$). The ultimate strength (59.20 ± 17.94 vs. 59.98 ± 16.93) and the transition strain (4.55 ± 0.69 vs. 4.24 ± 0.56) of DM were not significantly different from those of NM.

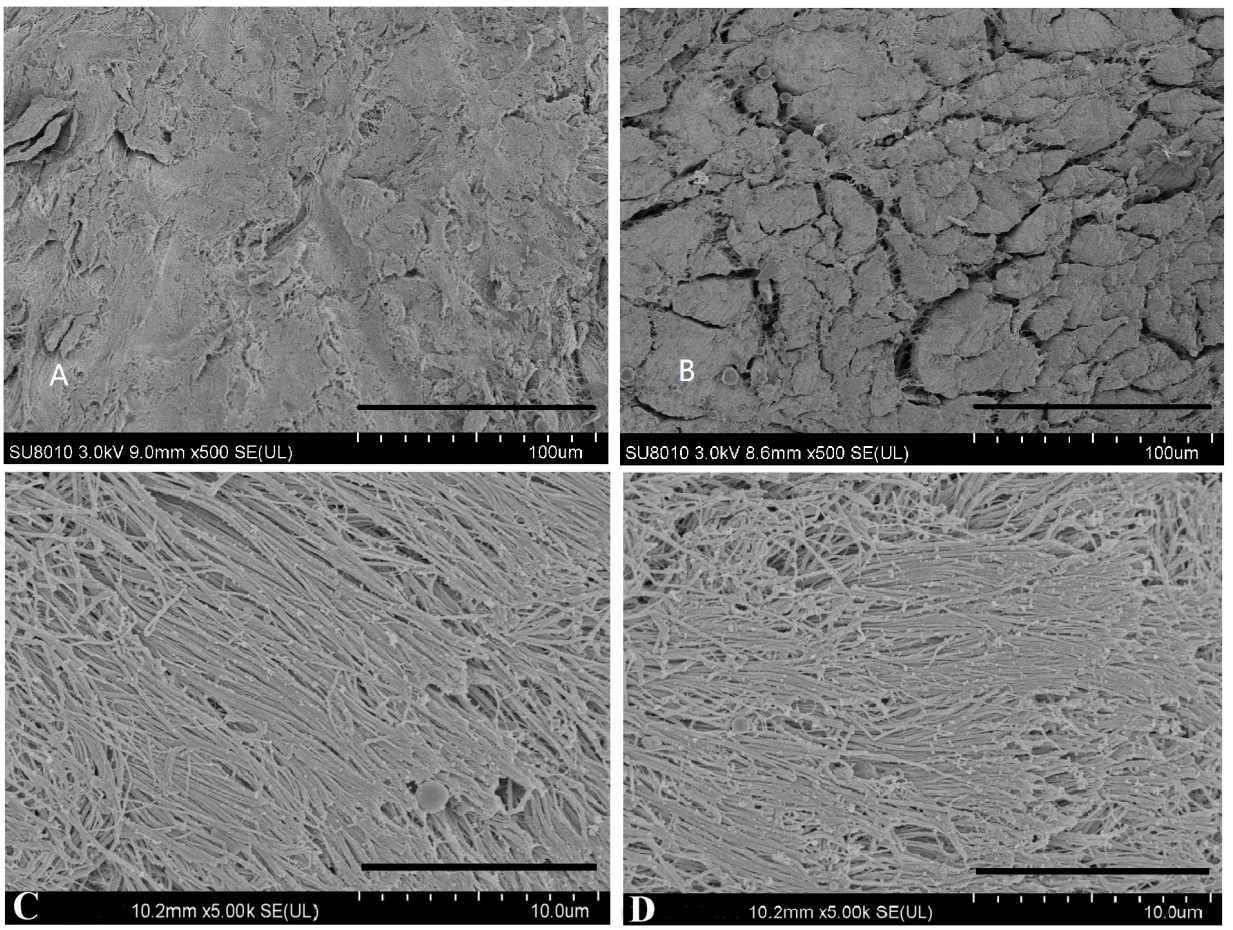

FIGURE 4 | Scanning electron microscopy photographs of native menisci **(A,C)** and decellularized porcine menisci **(B,D)**. Panels **(A,B)** show the cross sections through the menisci. Scale bar: 100 μm. And the horizontal sections are demonstrated in **(C,D)**. Scale bar: 10 μm.

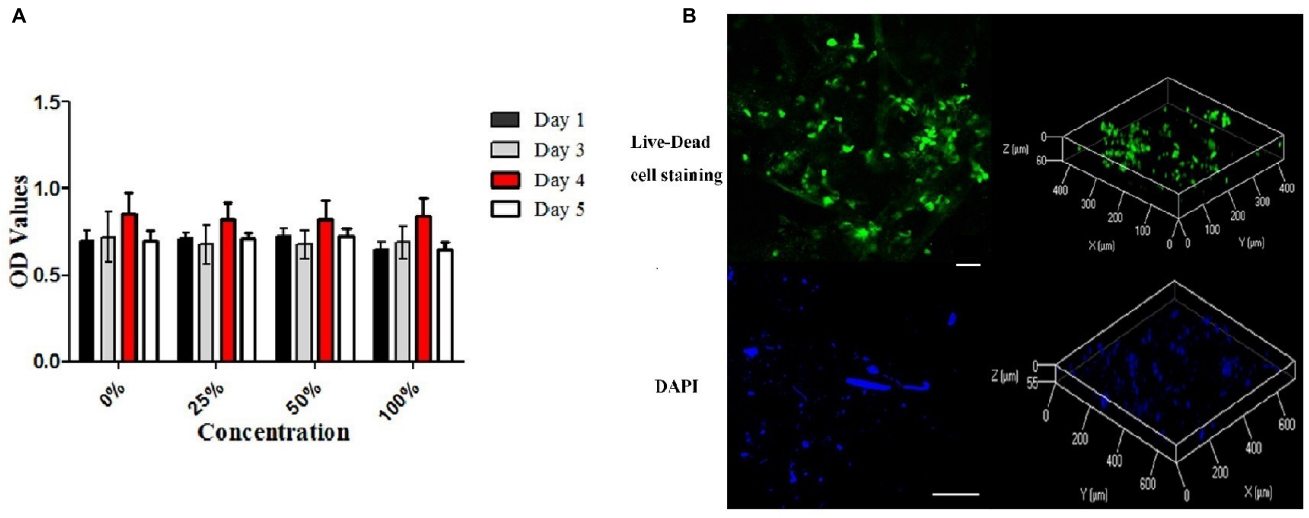

FIGURE 5 | Cytotoxicity of the decellularized meniscus ECM scaffold and recellularization *in vitro*. **(A)** Cytotoxicity assay of DM: the proliferative activities of the cells cultured in standard medium and those cultured in the extracts at different concentrations. **(B)** Fluorescence Live-Dead cell (Scale bar: 30 μm) and DAPI (Scale bar: 100 μm) staining images (two and three dimensions) demonstrating seeding of BMSCs on DM ECM scaffolds at 2 weeks.

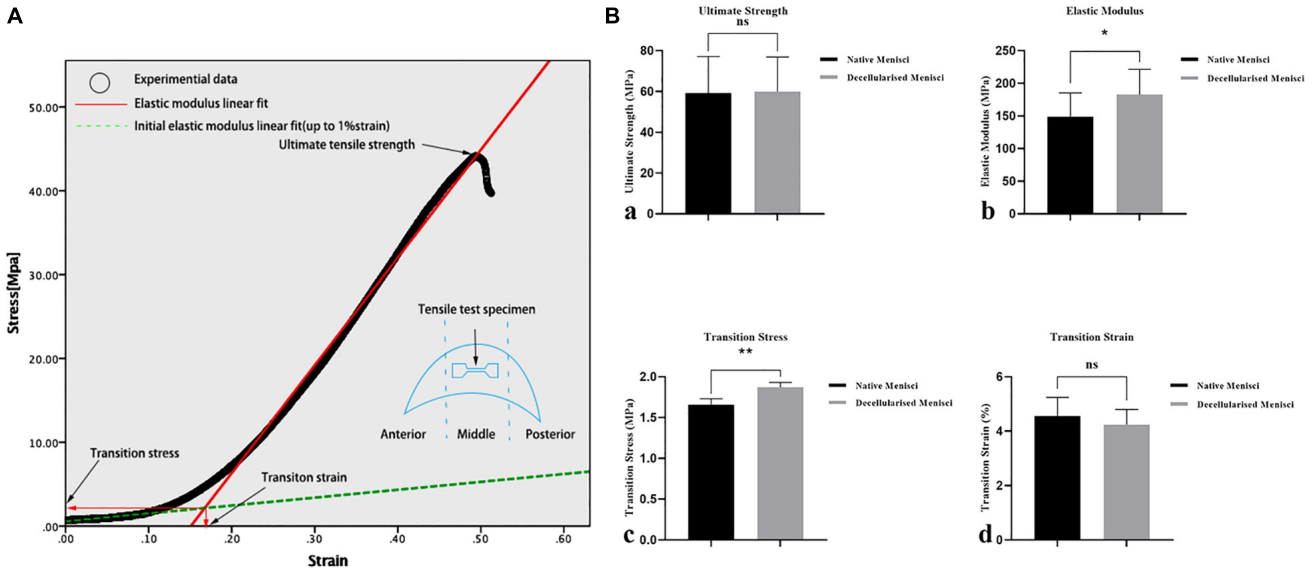

FIGURE 6 | Biomechanical properties of the specimens of the native and DM (n = 3). **(A)** Representative curves for the tensile tests and biomechanical properties of the specimens. **(B)** Tensile mechanical properties: **(a)** ultimate strength, **(b)** transition stress, **(c)** elastic modulus and **(d)** transition strain. *$p < 0.05$ and **$p < 0.01$.

Evaluation of Seeded BMSCs Integration With the ECM Scaffolds *in vitro*

After cell seeding, Live-Dead cell staining confirmed that the meniscal ECM scaffolds could support the proliferation of BMSCs *in vitro* (**Figure 5B**). BMSCs attached well to the surface of scaffolds and proliferated over 2 weeks, which was evident at the image of three dimensions. Also, DAPI staining demonstrated the dense distribution of BMSCs on the surface of specimens over 2 weeks. The findings further verified the cytocompatibility of the ECM scaffolds.

In vivo Animal Study

The scaffolds were harvested after 6 and 12 weeks, respectively. Histological analysis (H&E, Masson's trichrome and Toluidine Blue staining) of the NM group after 6 weeks demonstrated the appearance of infiltrates of inflammatory cells, remnant chondrocyte-like cells and elongated fibroblast-like cells in clumps and collagenous fibers arranged in order. In contrast, the DM group 6 weeks after surgery displayed few chondrocyte-like cells and fibroblast-like cells with infiltrates of inflammatory cells (**Figure 7**). After 12 weeks, fibrocartilage differentiation was detected with cells of three phenotypes surrounded by ECM in the DM group, while poor differentiation was observed for the NM group (**Figure 7**).

Semi-Quantification for Histological Staining

After 12 weeks, three sections of different points in both NM and DM group were used for semi-quantification of H&E analysis. Cell numbers were calculated and converted to percentage of total cells (**Figure 7**). There was no statistical difference for fusiform cells and fibroblast-like cells. However, there were more chondrocyte-like cells in the DM group than NM group ($p < 0.05$). Additionally, there were more inflammatory cells in the NM group than DM group ($p < 0.05$). The results showed the better repair ability and milder inflammatory reaction of the scaffolds *in vivo*.

DISCUSSION

In this study, a new protocol was developed for the decellularization of whole porcine meniscus. It was confirmed that BMSCs attached well to the surface of scaffolds and proliferated over 2 weeks of culture on the decellularized meniscus scaffold *in vitro*. Additionally, after 12 weeks, region-specific recellularization was detected in the DM group *in vivo*, while poor differentiation for the NM group. Finally, better repair ability and milder inflammatory reaction of the scaffolds were confirmed using histological semi-quantification.

To date, menisci from a variety of species have been decellularized (Maier et al., 2007; Stapleton et al., 2008; Sandmann et al., 2009; Stabile et al., 2010; Schwarz et al., 2012; Azhim et al., 2013; Chen et al., 2015; Wu et al., 2015). However, entire meniscal ECM scaffolds were developed in only a few studies (Maier et al., 2007; Sandmann et al., 2009; Stabile et al., 2010). Also, considering the properties of these scaffolds for whole meniscal replacement, no optimal scaffold that could maintain tissue ECM and preserve biomechanical properties together with minimum DNA residues in parallel has been developed. In 2008, Sandmann et al. (2009) decellularized human menisci with 2% SDS for 2 weeks, and complete removal of cells was achieved without compromising biomechanical properties. However, there was no evaluation about the *in vivo* performance of the scaffolds. It was confirmed that SDS was

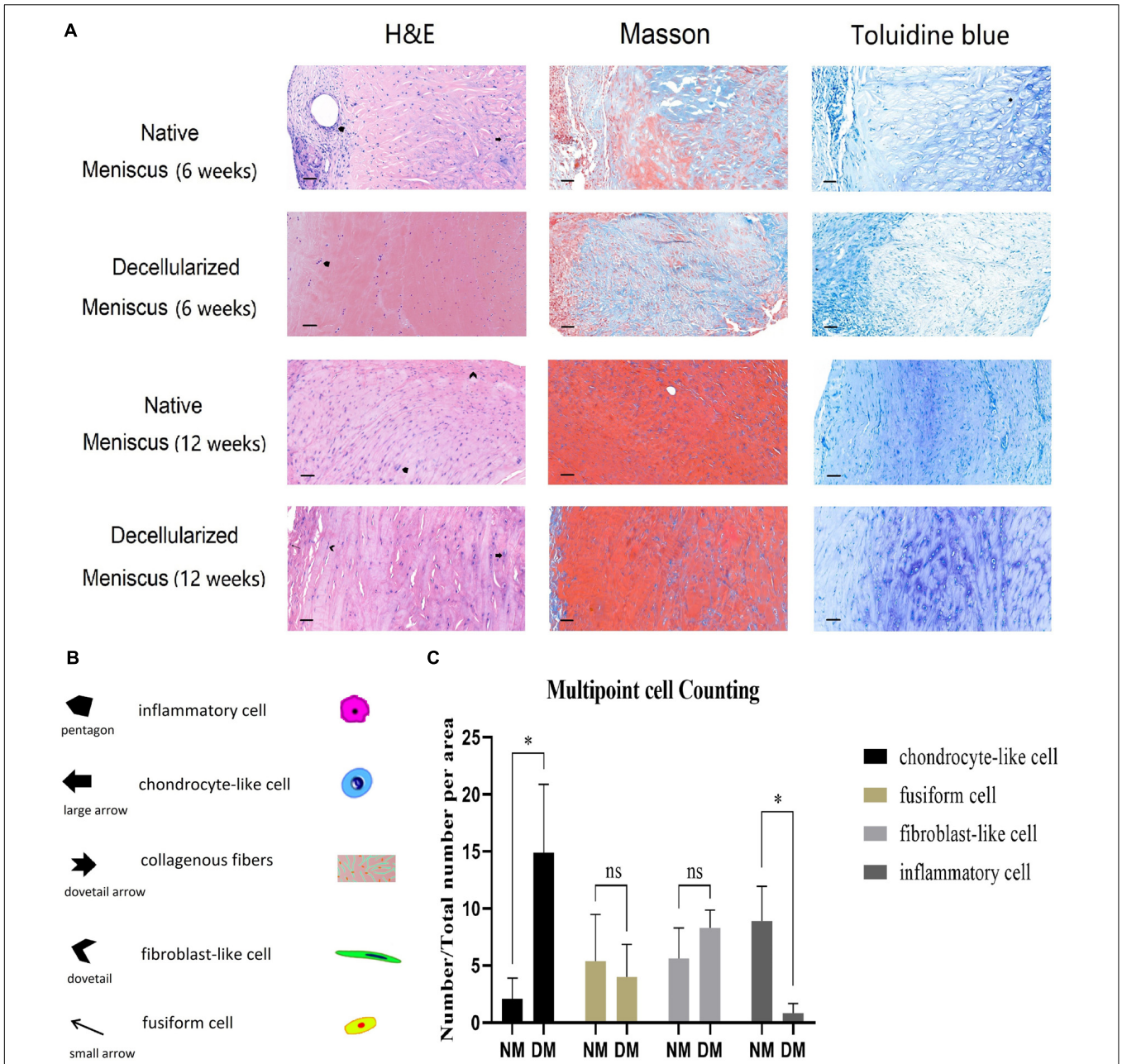

FIGURE 7 | (A) Histological analysis (H&E, Masson's trichrome and Toluidine Blue staining) of the native and decellularized meniscus ECM scaffolds *in vivo* after 6 weeks are showed in the 1st and 2nd row, respectively ($n = 5$). Native and decellularized meniscus ECM scaffolds after 12 weeks are in the 3nd and 4th row ($n = 5$). **(B)** The pentagon and dovetail indicate the inflammatory cell and the elongated fibroblast-like cell, respectively. The large arrow denotes the chondrocyte-like cell, and the small arrow indicates the fusiform cell. The dovetail arrow indicates collagenous fibers. Scale bar: 40 μm. **(C)** Semi-quantification of four typical cells over 12 weeks is demonstrated in the histogram ($n = 3$). The percentage of chondrocyte-like cell, fusiform cell, fibroblast-like cell and inflammatory cell are showed in column 1–4, respectively. $^*p < 0.05$.

more efficient in cell lysis than Triton X-100, but the former was observed to cause extreme fragmentation and swelling of collagen fibers (Andrew, 1986; Courtman et al., 1994). Also, SDS separates GAGs from proteins in the ECM and is less supportive of cell reseeding of the scaffolds (Maier et al., 2007). Therefore, the time of SDS treatment should be reduced to ensure GAG retention.

In this study, two kinds of chemical detergents (SDS and Triton X-100) were used for decellularization. We used different detergent concentrations and processing time and determined an optimal protocol, which effectively removed all the macroscopic nuclei and preserved the ECM. In this protocol, non-ionic detergents such as Triton X-100 are more effective than ionic detergents such as SDS. Therefore, utilizing Triton X-100

as decellularization reagent first is more conducive to the subsequent decellularization process. SDS appears more effective than Triton X-100 for removing nuclei. Such an optimal protocol can make the difference between complete and incomplete cell residues and nuclei removal. To minimize the DNA content in the ECM, DNAase I was applied to decompose the residual nuclear fragments in the ECM after the detergent processing.

The DNA content within ECM scaffolds decreased by more than 90% and was reduced to an acceptable level according to minimal criteria which suffice to satisfy the intent of decellularization (<50 ng/mg dry weight) (Crapo et al., 2011). H&E and DAPI staining confirmed the relatively successful removal of cell nuclei. Immunohistochemical staining indicated the preservation of collagen I and II. In addition, biochemical assays showed a favorable maintenance of water, GAG and collagen content. SEM was conducted to evaluate the microstructure. The main microstructure characteristics were well preserved while porosity of the menisci was increased, which may be beneficial for cell attachment and proliferation. Cytotoxicity assays demonstrated similar growth trend of cells in a standard medium and leaching solution of DM, which confirmed the absence of chemical residue within DM after decellularization.

As for the biomechanical properties, the tensile biomechanical properties of DM were compared with NM. The elastic modulus of DM was higher than that of NM, which was similar to that of a previous study (Abdelgaied et al., 2015), mainly because of the loss of tissue components within the accepted range. The ultimate strength and the transition strain of DM were not significantly different from those of NM. These results demonstrated that the biomechanical properties of porcine menisci were successfully maintained during decellularization. In previous studies, GAG extraction changed the structure of the ECM, increasing compressive stiffness and compressibility and slightly decreasing the residual force (Maier et al., 2007; Azhim et al., 2013). The extraction of GAGs resulted in the loss of water and thus contributing to the increase of stiffness (Chen et al., 2017). Thus, the comprehensive analyses performed to evaluate this scaffold were more extensive than those in previous studies.

The biomechanical property of the meniscus is deeply influenced by its anisotropic architecture. The meniscus is characterized by regional differences in composition, structure and cell phenotype. Generally, the outer region of the meniscus is characterized by highly aligned collagen type I fibers and is full of elongated fibroblast-like cells. Whereas the inner region becomes less aligned and has more GAGs than the outer zone, which has much higher GAG content and rounded chondrocyte-like cells and is dominated by collagen type II network (Makris et al., 2011). The microarchitecture plays an important role in regulating the behaviors of endogenous or exogenous stem/progenitor cells and subsequent tissue formation (Kumar et al., 2011; Neffe et al., 2015). Efforts have been made in recent studies to mimic the organization and interactions among the major tissue constituents of natural meniscus. In this study, region-specific extracellular matrix was preserved well after decellularization, providing biomechanical properties that were more similar to the native porcine meniscus than that of the other acellular scaffolds previously reported (Stapleton et al., 2008; Chen et al., 2015). In addition, both *in vitro* and *in vivo* studies have confirmed that the mean pore size of scaffold would also influence fibrochondrogenic differentiation and subsequent tissue formation (Makris et al., 2011). Larger pores of scaffolds benefit cell diffusion and migration, while smaller ones provide a higher surface area for cell adhesion (O'Brien et al., 2005). An anisotropic architecture of these pores serves as templates to guide extracellular matrix deposition to mimic its native counterpart, which may contribute to the biochemical and mechanical properties of regenerated constructs. In this study, the porosity of meniscus was promoted significantly after decellularization. Excellent region-specific recellularization was achieved *in vivo*.

CONCLUSION

We prepared a novel porcine meniscal ECM scaffold that preserved bioactivity, biomechanics and cytocompatibility features, and promoted excellent region-specific recellularization. In conclusion, the acellular xenogeneic meniscal scaffold has excellent potential for development of a tissue-engineered solution for meniscal repair.

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 Experimental Ethics Committee of Zhejiang University.

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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00779/full#supplementary-material

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Automation, Monitoring, and Standardization of Cell Product Manufacturing

*Meletios-Nikolaos Doulgkeroglou[1,2], Alessia Di Nubila[1,2], Bastian Niessing[3], Niels König[3], Robert H. Schmitt[4], Jackie Damen[5], Stephen J. Szilvassy[5], Wing Chang[6], Lynn Csontos[6], Sharon Louis[5], Patrick Kugelmeier[7], Vincent Ronfard[8,9,10], Yves Bayon[11] and Dimitrios I. Zeugolis[1,2]**

[1] Regenerative, Modular & Developmental Engineering Laboratory, National University of Ireland Galway, Galway, Ireland, [2] Science Foundation Ireland, Centre for Research in Medical Devices, National University of Ireland Galway, Galway, Ireland, [3] Fraunhofer Institute for Production Technology, Aachen, Germany, [4] Production Engineering Cluster, RWTH Aachen University, Aachen, Germany, [5] STEMCELL Technologies Inc., Vancouver, BC, Canada, [6] STEMCELL Technologies Ltd., Cambridge, United Kingdom, [7] Kugelmeiers Ltd., Zollikerberg, Switzerland, [8] College System of Pharmacy, University of North Texas Health Science Center, Fort Worth, TX, United States, [9] Cutiss AG, Zurich, Switzerland, [10] HairClone, Manchester, United Kingdom, [11] Medtronic – Sofradim Production, Trévoux, France

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***Correspondence:**
Dimitrios I. Zeugolis
dimitrios.zeugolis@nuigalway.ie

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Although regenerative medicine products are at the forefront of scientific research, technological innovation, and clinical translation, their reproducibility and large-scale production are compromised by automation, monitoring, and standardization issues. To overcome these limitations, new technologies at software (e.g., algorithms and artificial intelligence models, combined with imaging software and machine learning techniques) and hardware (e.g., automated liquid handling, automated cell expansion bioreactor systems, automated colony-forming unit counting and characterization units, and scalable cell culture plates) level are under intense investigation. Automation, monitoring and standardization should be considered at the early stages of the developmental cycle of cell products to deliver more robust and effective therapies and treatment plans to the bedside, reducing healthcare expenditure and improving services and patient care.

Keywords: cell therapy, scalability, manufacturing, monitoring, spheroid culture, biorectors

INTRODUCTION

Cell and cell-based tissue engineering products have an extraordinary clinical potential by offering unique therapeutic solutions to disease conditions without any effective treatments yet, such as non-curable cancers or non-healing or hard to heal tissues (Perez et al., 2018; Abreu et al., 2019). So far, their promises have been successfully translated only in few commercial products, primarily due to difficulties in reproducible and economical scalability, regulatory hurdles, and reimbursement issues (Morrow et al., 2017). For example, it is still challenging to translate labor-intense academic-based discoveries (automated systems often come at a prohibitive cost for academic setting and, by nature, academia is more research, as opposed to development, orientated) to automatedly manufactured industrial products. Further, the prolonged culture times required to develop a cell-based tissue engineering implantable device are associated with cell phenotypic drift and high manufacturing costs (Cigognini et al., 2013; Schrock et al., 2017; Vormittag et al., 2018). Yet again, cell therapies market size continuously raising, considering that they have the potential to transform

patient care. As a fact, the global market size of cell therapies was estimated at US$ 5 billion in 2017 and it is expected to increase at a 5.34% compound annual growth rate (CAGR) until 2025 (Grand-View-Research, 2018). Although the market is shaped mainly by allogeneic therapies, autologous cell therapies are expected to rise to more than 33.3% of the total cell therapy size. Stem cell therapy market share was valued at US$ 0.8 billion in 2018 and is expected to impressively grow to US$ 11 billion by 2029 (Kanafi et al., 2013).

While there are major differences between autologous (e.g., immuno-compatible) and allogeneic (e.g., relatively readily available in large numbers) cell therapies, they share limitations in manufacturing (e.g., cell harvesting, expansion and purification; cell phenotype preservation; and development of a reproducible formulation) that may compromise the administration of a successful therapy to patients and increase costs (Aijaz et al., 2018). For example, scalable, reproducible, and biomimetic culture conditions are required to maintain cellular function during *ex vivo* culture (Liu et al., 2017; Stephenson and Grayson, 2018; Ruiz et al., 2019; Serra et al., 2019). Further, large-capacity and automated bioreactor systems have the potential to reduce batch-to-batch variability and the use of expensive highly skilled labor (Peroglio et al., 2018; Costariol et al., 2019; de Sousa Pinto et al., 2019; Hamad et al., 2019). In the case of allogeneic therapies, the aim is to scale up processes for numerous patients. In the case of autologous therapies, however, where a single patient is treated from his/her own cells, there is no need for large scale production of multiple batches with high expansion rates. Instead, manufacturers aim to culture simultaneously cells from different patients in an attempt to level up production and make it viable. An option would also be to continue culturing the cells for other patients, should appropriate consent forms be granted. Nonetheless, autologous cell therapies are still produced at small-scale, in dedicated suites, in centralized or localized manufacturing facilities at the point-of-care, which results in very expensive production costs.

In any case, both autologous and allogeneic therapies require skilled and expensive personnel, often susceptible to error, resulting in increased batch-to-batch variability, manufacturing costs and risk of contamination, which represents the biggest part of the cost of goods (COGs) for manufacturing, including tissue procurement, material acquisition, facility operation, production, storage, and shipment (Lipsitz et al., 2017). Although decentralization (Harrison et al., 2018b) and micro-factories (Harrison et al., 2018a) approaches have been proposed, automation is key for rendering these therapies more attractive, reducing the COGs, de-risking the supply chain and establishing a reliable batch-to-batch reproducibility (Hunsberger et al., 2018; Moutsatsou et al., 2019). Yet again, many questions have to be answered. For example, if the manufacturing process is scalable and suitable for automation, how can be fitted in the user requirement specifications (URS)? Regulatory considerations and ease of implementation in industrial/scalable environment are also essential. The business model should be well defined and adapted to the final product and market. The automation program should be considered as part of the full life cycle of the product, integrated into overall product development plan and its commercial manufacturing, while every potential impact of automation into the final product should be investigated. European agencies, such as the European Medicines Agency's Innovation Task Force may assist with the development of automated processes starting with the designation of the automation, whether it should be a device or laboratory equipment. Automation challenges, coupled with lack of reliable and effective standardization, process monitoring, product reproducibility, and inadequate donor availability increase the production and reimbursement costs. It is imperative to address automation challenges in an effective way and implement process modifications with minimal disruption of the bioprocess to ensure delivery of a safe product in a commercially viable manner.

Automation offers control over a bioprocess, leading to a more accurate and faster process optimization, de-risking the supply chain, via optimized quality control, quality assurance, ultimately making the process more regulatory compliant. Although biological variations are difficult to tackle due to the complexity of the products, in-process human variation must be addressed to ensure consistent product quality. Indeed, automated pipetting, for example, can timely, accurately, repeatedly, and consistently perform liquid handling, including mixing and transferring of liquids, reducing variability within and between batches. Automation of monitoring processes (e.g., advanced algorithmic approaches, such as machine learning, coupled with image acquisition and processing) eliminates the need of subjective human judgments (e.g., cell morphology assessment, confluency assessment) further enhancing control over reproducible product development. As cell-based therapies are maturing, it is imperative to standardize and control manufacturing engineering strategies and implement robust automation and process monitoring and control for safety (above all), consistency and reproducibility purposes (Ball et al., 2018; Hunsberger et al., 2018; Pigeau et al., 2018; Moutsatsou et al., 2019). This manuscript will describe some real-life indicative examples of automation and monitoring designed to address manufacturing issues in cell-based therapies domain.

AUTOMATING PRECISE PIPETTING

Pipettes are laboratory tools used in the areas of chemistry, biology, and medicine, where precise, accurate and reproducible transfer of small volume of liquids is required. The accuracy of the pipetted volume can vary significantly due the quality of pipettes and tips, calibration and performance checking, environmental conditions (e.g., the temperature and density of the liquid), pipetting methods (e.g., forward or reverse), as well as the individual ability of the operator (Lippi et al., 2017). Examples of inadequate operator techniques include usage outside of pipette range, volume selection inaccuracy, fast or careless aspiration and dispensing, over-aspiration and barrel contamination. Considering the potentially high levels of user-dependent inaccuracy, in recent years, the use of automated pipetting systems has increased significantly to meet the need for high accuracy and high throughput in biomedical laboratories.

Robots work without fatigue, perform consistently, increase the production and ensure accuracy and precision. A typical liquid-handling robotic workstation (**Figure 1**) consists of a control center, dispensing apparatus, robots, washing modules, and sensors (Kong et al., 2012). The robot, coordinated by the control center, moves between the dispensing part and the washing station. Dispensing tools include dispensing heads, actuators, and substrates. The dispensing head expels liquid samples on the substrates for further processing. The washing station cleans the dispensing head to lengthen its life and to ensure the integrity of the sample. Sensors monitor the status of the dispensing component to ensure that feedback control can be performed by the control center. One of the main challenges regarding automated pipetting systems is the viscous material handling. The key factor is the distance between the dispensing needle tip and the base of the well (Peddi et al., 2007; Yaxin et al., 2011). If the distance is too great, the sample from the needle forms a continuous cylinder and is not delivered to the well, whereas, if the distance is too short, the sample remains attached to the needle. In order to choose an adequate distance, it is necessary to consider relevant parameters, such as needle size, syringe volume, pumping temperature, flow speed, and viscosity grade. Moreover, dusty and viscous materials, air bubbles or the accumulation of liquid debris may cause clogging in tubes, valves and dispensing heads; thus, clogging detection is required (Kong et al., 2012).

Automated precise pipetting plays a central role in cell culture automation. Automated cell culture systems enable large-scale production of cells and enhance technical precision, reproducibility and efficiency (Konagaya et al., 2015). Monitoring the flow rate, for example, during media change, is an important operation to ensure that shear forces on cells are contained (Ly et al., 2013). A fine-tuning of the pipetting settings could decrease the shear stress, but very slow aspirating steps are associated with a long duration of the process. However, a benefit of automation should be a reduced process time compared to manual operations (Lehmann et al., 2016). A prerequisite for the successful implementation of automated procedures in cell culture experiments is a complete and adequate validation, during which automated pipetting systems are directly compared to manual pipetting, conducted by an experienced laboratory technician. In a previously published case study (Rothmiller et al., 2020), toxicity studies in HaCaT cells were conducted using two epMotion® automated pipetting systems (Eppendorf, Germany), which were validated / contrasted against an experienced. Validation analysis revealed that automated seeding was faster and more precise than manual seeding, with a significantly lower variability and equivalent intraday variability. Collectively, automated pipetting, if it is not already, should become an industry standard for accurate, reproducible and cost-effective development of cell-based products.

AUTOMATION AND SCREENING

In an increasing and demanding tissue engineering market, advanced automation and screening for quality control are essential for sustainability. In this direction, recent commercial efforts have made available automated systems for cell manufacturing (e.g., CliniMACS Prodigy®, Miltenyi Biotec; Sefia S-2000, GE Healthcare, Life Sciences). For industrialization and manufacturing, quality control requires well-characterized, fully reproducible and safe products to ensure delivery of the expected medical benefits. Considering that cell morphology is indicative of phenotype, effective monitoring of cells' and cell clusters' morphology are prerequisites for standardization and homogenous product delivery (Maddah et al., 2014; Boutros et al., 2015; Nagasaka et al., 2017). Microscopic observations are the routine method used for the assessment of cell culture. The need for automated and fast evaluation has led to the development of machine learning algorithms and artificial intelligence, able to assess morphological and functional properties of cell culture. The principle of machine learning includes the development of algorithms that are being trained by data input, thus improving their intrinsic processes and providing more accurate outputs. Since many single or populational characteristics would indicate the suitability of cells for further experimentation, it is imperative that techniques can fast and accurately process large volume of data (e.g., images). Indeed, image processing machine learning techniques have been successfully implemented and validated in oncological studies to predict specific function based on gene phenotype similarities (Sailem et al., 2020), in predicting cell growth per passage from batches obtained from donors varying in age (Mehrian et al., 2020) or phenotypically and structurally evaluating different cell types (Logan et al., 2016; Van Valen et al., 2016; Wakui et al., 2017; Buskermolen et al., 2018; Radio and Frank, 2018; Lam et al., 2019; Orita et al., 2019). Following successful implementation of machine learning for cell morphology analysis, automation on the level of cell production and screening is the next vital step, which systems, such as the StemCellFactory, aspire to achieve. This system automates reprogramming and expansion of induced pluripotent stem cells (iPSCs) for disease modeling and drug screening (Jung et al., 2018). The system is comprised of various devices (**Figure 2**), which are functionally joined and integrated into a central control system orchestrating the process execution and data handling.

Each device has its local software agent, which serves as middleware interface and abstracts the hardware heterogeneity by offering data and functionality in a service-oriented way to the control unit. Local information and functionality from the individual device are processed in the middleware up to the higher-level of the control system, such that the user only operates one software with control over the complete system. In order to expand and monitor the iPSCs, the system is equipped with an automated microscope to assess their morphological structure and confluency level. The control system utilizes data handling and flexible process control to perform the tasks. For example, the user can input a confluence level that will lead to cell splitting or media change. Due to the high amount of data generated (20 GB per media transfer protocol) and the needed high computational power for evaluation, deep learning algorithms are used. These algorithms classify an image into six different classes that are color-indicated (**Figure 3**). Automation can also be achieved in genome editing or reprogramming during

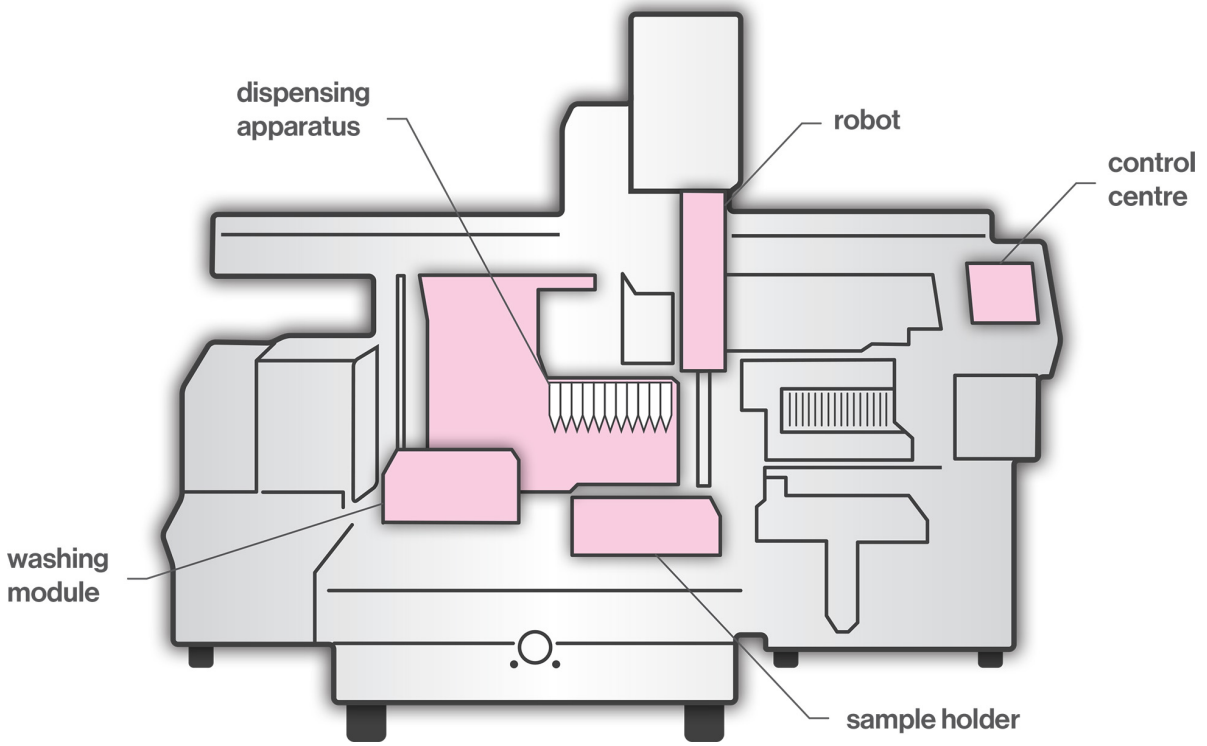

FIGURE 1 | A typical liquid-handling robotic workstation.

cell culture steps. To this end, for the automated detection of iPSC colonies, the CellCelector system is implemented, which allows automated picking of clones for subsequent clonal expansion on the StemCellFactory. Even more, the Nucleofector device allows automated genome editing. So far, the StemCellFactory has been used for the automated reprogramming of human dermal fibroblasts, clonal selection and expansion of primary iPSC clones and scaled enzyme-free sub-cultivation of iPSC lines. To summarize, the StemCellFactory can provide reproducible results, growth behavior monitoring, high throughput through parallelization. These automated platform and novel software tools address the technological challenges for automation of complex stem cell culture processes and are expected to meet the challenges of the increasing demand for patient-derived iPSCs and their derivatives.

AUTOMATED CLASSIFICATION AND QUANTITATION OF COLONIES OF BLOOD CELLS

The transplantation of hematopoietic stem and progenitor cells (HSPC) from human bone marrow (BM), adult mobilized peripheral blood (MPB) or umbilical cord blood (CB) has for more than 50 years been employed as an effective treatment for a variety of blood disorders and malignancies (Juric et al., 2016; Takami, 2018; DeFilipp et al., 2019). An important approach to assess their potency and predict the likelihood of robust engraftment is to determine the number and quality of lineage-specific progenitor cells and multipotent stem cells among the cells to be transplanted. Many studies have shown that the number of HSPCs is directly correlated with engraftment outcomes (Prasad et al., 2008; Page et al., 2011). Several criteria are commonly used to establish graft potency and quality, including the total number of viable nucleated cells, the number of cells expressing the CD34 antigen and the number of cells able to produce discrete colonies of mature blood cells upon culture in semi-solid growth media. Hematopoietic cells with the latter capability form colony-forming units (CFU) and the CFU assay is the current gold standard for determining the number of functional HSPCs. Hematopoietic progenitor cells can differentiate into several blood cell lineages in the CFU assay. Depending on the growth factors present in the culture medium, the assay can identify (a) erythroid progenitor cells that produce either very small or medium to large colonies comprised of pure red blood cells [i.e., colony-forming units-erythroid (CFU-E) and burst-forming units-erythroid (BFU-E), respectively]; (b) uni- or bi- potent myeloid progenitor cells [i.e., colony-forming units granulocyte (CFU-G), colony-forming units macrophage (CFU-M), and colony-forming units granulocyte-macrophage (CFU-GM)]; or (c) multipotent progenitor cells that generate large colonies comprised of all four major non-lymphoid cell types [colony-forming units granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM)]. Shown in **Figure 4** are examples of colonies derived from CFU-GEMM and CFU-GM with each exhibiting distinct morphological features, most notably

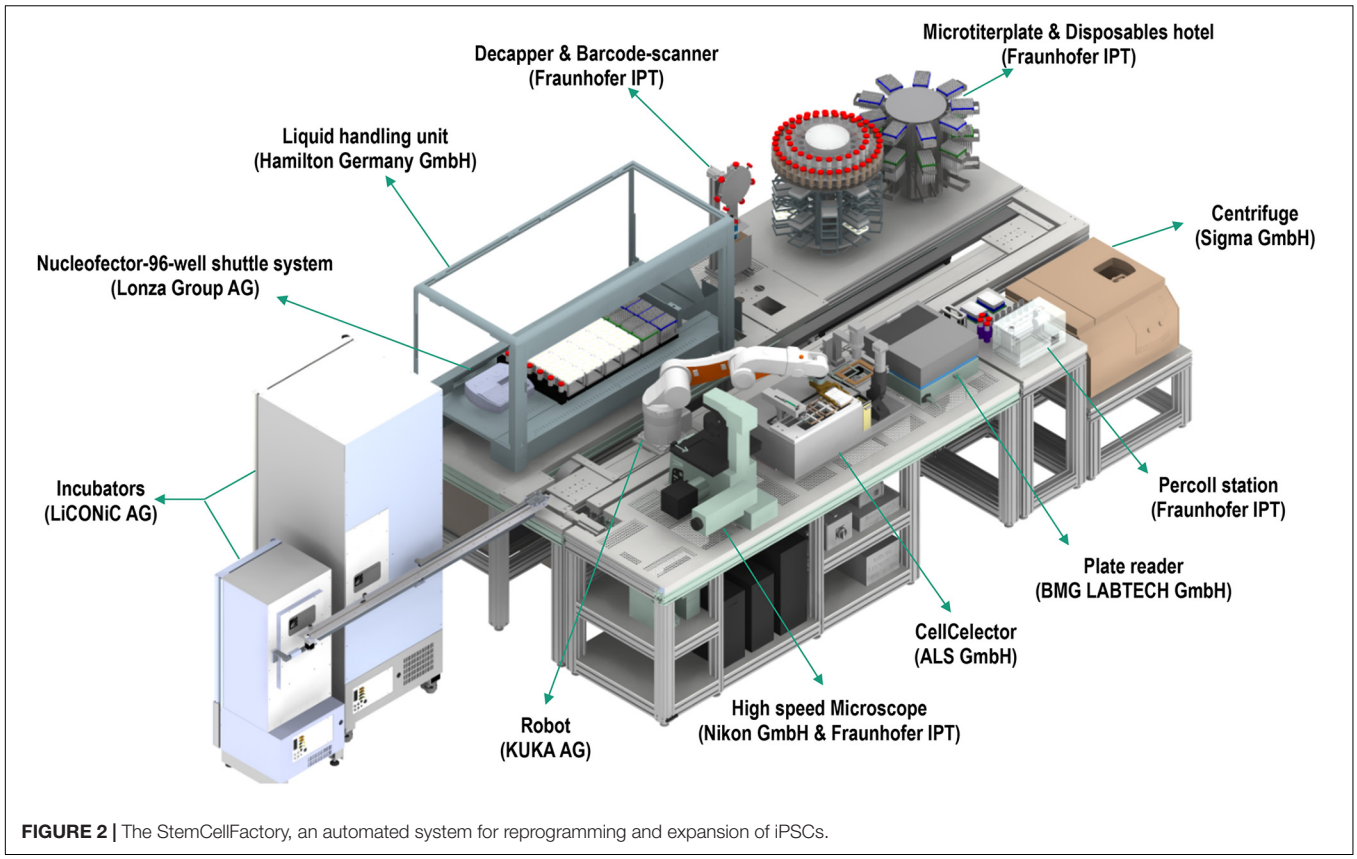

FIGURE 2 | The StemCellFactory, an automated system for reprogramming and expansion of iPSCs.

different size and cellular composition. These colonies produced by CFU *in vitro* are usually counted manually using an inverted microscope by trained personnel with (ideally) extensive experience, but who nevertheless must often make difficult judgements on the boundaries and composition of the discrete colonies that they observe. For example, colonies produced by BFU-E or CFU-GEMM share overlapping characteristics that pose challenges to colony classification. This can contribute to a degree of inter-individual variability in CFU assay scoring accuracy, typically between 10 and >100% depending on the colony sub-type (Pamphilon et al., 2013). In addition, manual counting and characterization of CFU colonies is labor intensive. Thus, an automated solution would increase both the speed and accuracy and facilitate standardization in performing the CFU assay.

Toward these objectives, STEMCELL Technologies Inc. developed STEMvision™ (**Figure 4**), a bench-top instrument designed specifically for imaging, classifying and counting hematopoietic colonies produced by human or mouse progenitor cells in the CFU assay. The instrument separately counts and identifies colonies generated by CFU-E, BFU-E, CFU-GM, or CFU-GEMM that develop in the conventional 14-day CFU assay performed using MethoCult™, a line of semi-solid methylcellulose-based culture media supplemented with combinations of hematopoietic growth factors that stimulate the survival, proliferation and differentiation of all sub-types of CFUs. STEMvision™ eliminates the inter- and intra-individual and laboratory variations associated with manual colony counting by using sophisticated image acquisition and analysis software to identify and classify hematopoietic colonies. The morphological criteria that facilitate classification of the different sub-types of CFUs are applied consistently, facilitating standardization of the CFU assay to ensure accuracy and reproducible results. All of the common and particularly challenging phenomena encountered when counting CFU assays are addressed. For example, colonies can occasionally present with multiple foci or clusters, which some individuals may consider to be separate colonies thus erroneously skewing the total count to higher CFU numbers that in turn may lead to an overestimation of HSPC graft potency. Conversely, colonies at the edge of the culture dishes may be missed in the shadow produced by the meniscus of the MethoCult™ medium, leading to under-counting of CFUs and under-estimation of graft potential. By performing the assay in SmartDish™ culture plates that prevent meniscus formation and employing standardized imaging and analysis software that are specifically developed and validated for counting all of the different types of colonies produced by CFU from BM, MPB or CB, use of this platform results in significantly greater accuracy and less variability in colony counts. Improved colony characterization is also accomplished by analyzing colony features from both dark-field (i.e., black and white) and bright-field (i.e., color) images (**Figure 4**) to improve automated decision-making. Following analysis with STEMvision™, data can be visualized in a pdf

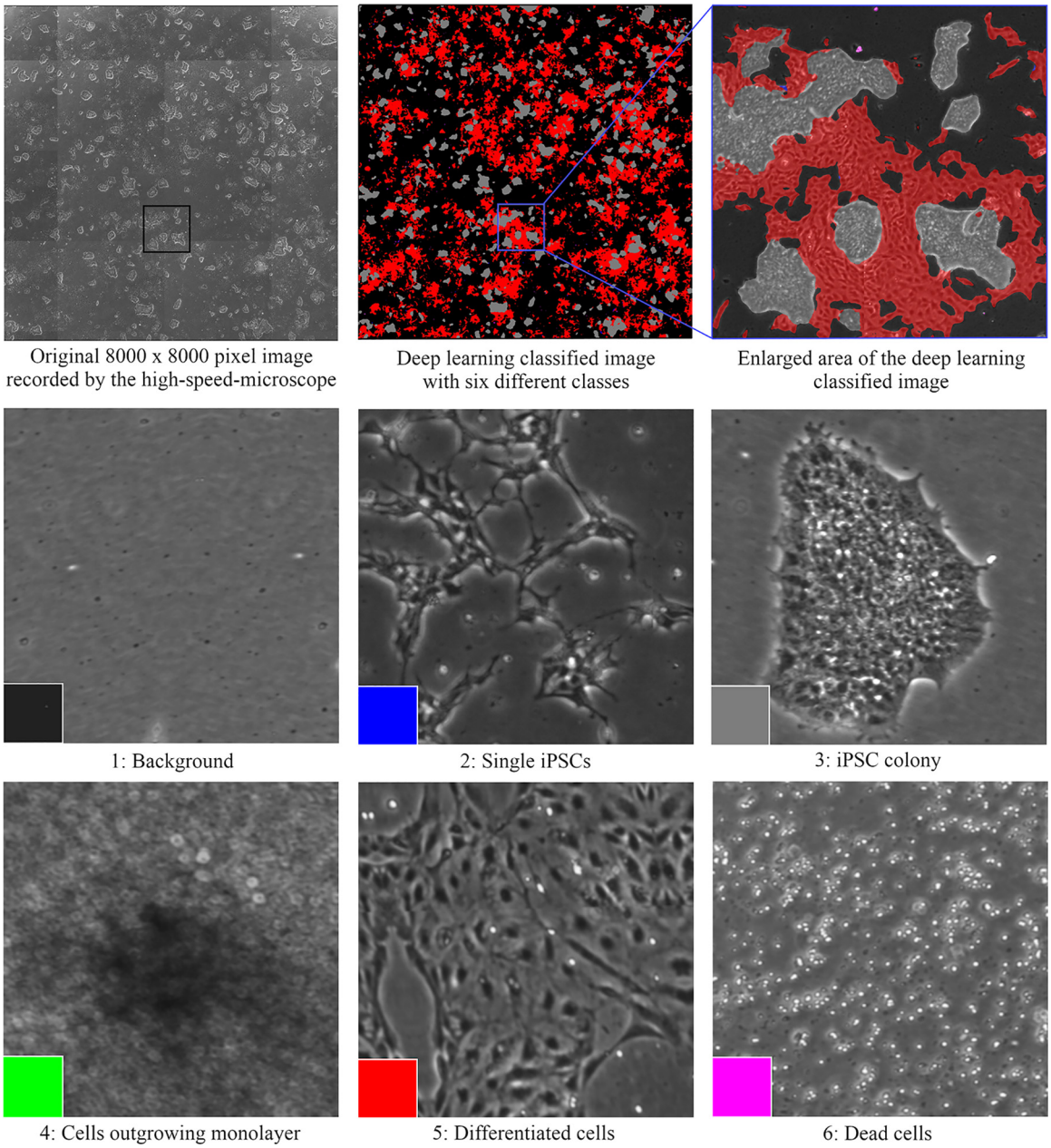

FIGURE 3 | Cell classification with the deep learning algorithms that are color-indicated (background is depicted in black, single iPSCs in blue, iPSC colonies in gray, cells in 3D structure in green, differentiated cells in red, and dead cells in purple).

report format that can be pre-filled with information, such as donor ID, sample ID, number of cells plated and additional qualifiers defined by the user. The report is automatically generated and results are expressed as CFU frequencies with digital images of the analyzed cultures available for manual review and long-term archiving. The plate and sample ID are linked to each image for traceability and time stamped.

The development of new gene-editing tools such as CRISPR/Cas9 technologies has opened up new avenues for gene therapy approaches to blood disorders and researchers are vigorously testing and optimizing new protocols for correcting genetic defects in HSCs (Dever et al., 2016; Naldini, 2019). Given the current guidelines for quality and process control for all types of manipulated HSCs, advancement of HSPC-based cellular therapies will certainly depend increasingly on the use of standardized potency assays, such as the CFU assay, especially when these cells are modified through CRISPR/Cas9 targeting prior to transplantation. Current guidelines set out by the FDA specify that frozen CB units must be tested not only for cell viability, but also for potency since cryopreservation and thawing

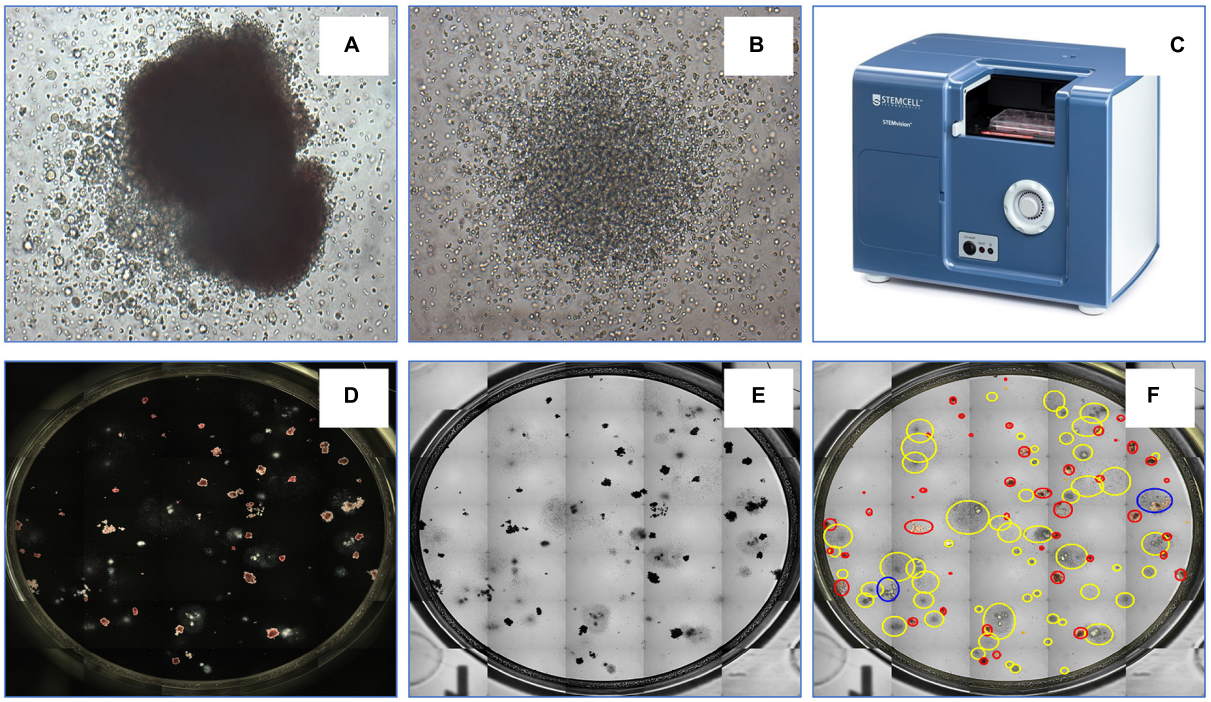

FIGURE 4 | An example of a CFU-GEMM **(A)** and CFU-GM **(B)** in a typical 14 day CFU assay from bone marrow-derived hematopoietic stem cells. The automated colony-forming unit counting and characterization instrument, STEMvision™ **(C)**. STEMvision records both dark-field **(D)** and bright-field **(E)** images of CFUs and utilizes state-of-art software to identify colonies. **(F)** Yellow circles represent CFU-GM; blue circles represent CFU-GEMM; and red circles represent BFU-E.

are often associated with reduced growth and differentiation capacity of HSPCs (Watts and Linch, 2016). Several investigators have validated STEMvision™ for standardizing the CFU assay within and across labs (Velier et al., 2019) and it is clear that STEMvision™ provides an effective automated approach to classify and count CFUs during the evaluation of hematopoietic stem cell viability and potency.

3D CELL CULTURE SYSTEMS: THE CASE OF PANCREATIC ISLET CELL CLUSTERS FOR TYPE I DIABETES

Diabetes affects globally >382 million individuals with expected increase almost to 600 million in the next 15 years (Guariguata et al., 2014; Cho et al., 2018). There are different types of diabetes, of which type 1 diabetes has gained more attention due to its autoimmune nature. Type 1 diabetes is associated with malfunction of the pancreatic islets and more specifically with the destruction of the insulin-producing cells (beta cells), which reside inside the islets. Destruction of beta cells leads to insufficiency of insulin production from the body, leading to inability of glucose entering the cells, which leads to elevated level of sugars into the bloodstream (Fu et al., 2013; Kettunen and Tuomi, 2020). Moreover, diabetes is the main cause for kidney disease with a correlation of 25% of diabetic people resulting in kidney failure (Guariguata et al., 2014; Marshall, 2014). To be safe and effective, islet cell transplantation needs size standardization, which would lead to much higher cell survival due to better oxygenation of the islet (Papas et al., 2019). In addition, limited availability, immuno-rejection and procedure issues should be addressed to alleviate islet cell loss and to improve engraftment outcomes (Shapiro et al., 2017; Gamble et al., 2018).

In vitro cell culture platforms have the potential to standardize islets (Hilderink et al., 2015; Ichihara et al., 2016; Vlahos et al., 2019) and also provide an environment to prepare autologous or allogeneic stem cell therapies for diabetes (Lilly et al., 2016; Cierpka-Kmiec et al., 2019; Kumar et al., 2019). In recent years three dimensional spheroid culture systems have emerged that simulate more effectively the physiological tissue microenvironment due to the cell-cell and cell-ECM contact and interaction (Mitchell, 2017; Langhans, 2018). Cells spheroids have shown improved osteogenic, adipogenic and chondrogenic potential over conventional culture systems (Yoon et al., 2012; Yamaguchi et al., 2014; Cesarz and Tamama, 2016; Miyamoto et al., 2017; Moritani et al., 2018; Tsai et al., 2019), improved vascularization in ischemic tissue (Bhang et al., 2012) and constitute the first choice in cancer models and evaluation of anti-cancer drugs (Chatzinikolaidou, 2016; Zanoni et al., 2016; Rodrigues et al., 2018). Various scaffold-free [e.g., seeding cells in a porous microwell agarose microchip (Colle et al., 2020), seeding cells in 3D printed well inserts (Boyer et al., 2018) or the hanging drop method (Kapur et al., 2012)] and scaffold-based [e.g., natural or synthetic hydrogels are used as substrates for spheroids growth (Murphy et al., 2014; Chang et al., 2018; Lee et al., 2018; Kim et al., 2019)] have been described in the literature.

Regarding scalability, of significant importance are recent studies that describe scaffold-free cell spheroids production using the hanging drop method performed by a robotic device (Gutzweiler et al., 2017) and a robotic automated droplet microfluidic platform (Langer and Joensson, 2020). We should also mention that automated production of cell spheroids in outer space has also been documented (Pietsch et al., 2017).

In diabetes field, cell spheroids can provide an inducive environment for islet differentiation from stem cells, upregulate stemness factors and allow production of angiogenic and non-thrombogenic therapies (Moritz et al., 2002; Oh et al., 2018; Lo et al., 2019). Early data of islets/mesenchymal stem cell co-cultures in spheroids demonstrated improved islet long term viability, but not function (Rawal et al., 2017). Recent data however study demonstrated that incorporation of human amniotic epithelial cells into islet organoids to markedly enhance engraftment, viability and graft function in a mouse type 1 diabetes model (Lebreton et al., 2019). Although the potential of spheroids in regenerative medicine has already been demonstrated in preclinical models for most organ systems (Hagemann et al., 2017; Petrenko et al., 2017; Polonchuk et al., 2017; Ong et al., 2018), their slow clinical translation may be attributed to variable cluster size, which affects cell response (Moritz et al., 2002; Van Hoof et al., 2011; Anitha et al., 2020). Thus, spheroid production must be standardized to bridge the gap between preclinical testing and clinical translation.

The link between islet transplantation and regenerative medicine is that islet transplantation is the only spheroid/cluster/organoid transplantation in the world that is being performed in a routine clinical fashion for 20 years (Bottino et al., 2018). Therefore, the clinical experience of islet transplantation can be taken as a 'blueprint' for future cell therapies with spheroids. The format and thus handling, challenges and principles are literally the same. Having said that, even in classical islet transplantation, the formed cell clusters are not flawless, mainly due to anoxia occurring in the center of large clusters due to the high diffusion distance (Brandhorst et al., 2016). After transplantation, the only oxygen supply path is through diffusion whereas they remain in hypoxic condition in the portal system (Moritz et al., 2002). This is the reason why currently 80–90% of the transplanted islet cells are not surviving the first days of transplantation (Suszynski et al., 2016). Oxygen consumption is also directly correlated to the insulin production (Porterfield et al., 2000) and cluster size (Labuschagne et al., 2019). Indeed, *in vitro* and clinical data in patients suffering from type 1 diabetes have shown in large clusters less insulin-expressing cells both in normoxic and hypoxic conditions and the larger islets were significantly reduced in size under

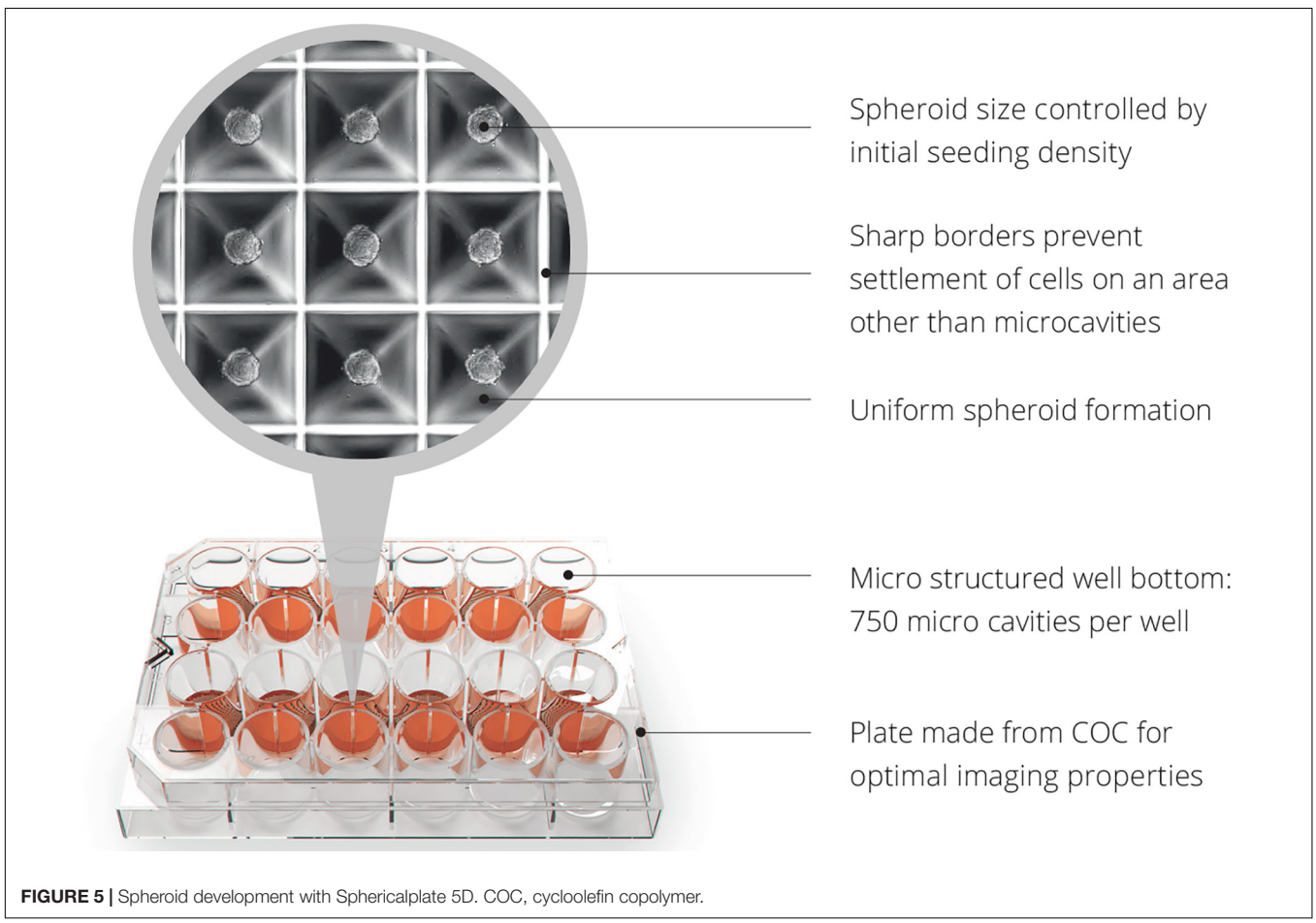

FIGURE 5 | Spheroid development with Sphericalplate 5D. COC, cycloolefin copolymer.

hypoxia (Lehmann et al., 2007). The Sphericalplate 5D is an example of a cell culture platform that can produce regular cell clusters in the desired numbers and quality for improved clinical islet transplantation and future applications with spheroids (Zuppinger, 2019; Schulze-Tanzil et al., 2020). The shape of the Sphericalplate 5D platform allows size standardization and also correct stem cell communication within the formed spheroids by recreating the physiological niche environment in thousands of microwells (**Figure 5**). Regarding scalability and automation, the Sphericalplate 5D platform fulfils necessary first principles of clinical cell transplantation, such as reproducibility, medium change capacity and optimized mechanobiology for every single spheroid (Kugelmeier et al., 2010). So far, diverse populations of cells (e.g., islets, embryonic stem cells, iPSCs, BM stem cells, prostate cancer cells, hepatocytes) have been successfully expanded in this platform (Schmidhauser et al., 2019) and the first clinical trial is planned for 2021 to improve current islet cell transplantation by standardizing spheroid/cluster size and consequently cell survival in the Sphericalplate 5D. More such scalable and, hopefully, effective technologies will enable the development of functional and affordable cell therapies.

CONCLUSION

Cell-based therapies have the potential to offer an effective treatment to still uncurable disease conditions. Their broad commercialization has been jeopardized by limitations (e.g., scaling up and automating labor-intense academic discoveries, high manufacturing costs and variation between batches) in large scale automated manufacturing. Herein, we discussed examples in the field of cell manufacturing automation, monitoring and standardization. Such successful examples of automated and controlled cell product manufacturing and monitoring should inspire the development of cost-effective cell products for the benefit of patients still suffering from uncurable diseases.

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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The Few Who Made It: Commercially and Clinically Successful Innovative Bone Grafts

*Ignacio Sallent[1,2], Héctor Capella-Monsonís[1,2], Philip Procter[3,4], Ilia Y. Bozo[5,6], Roman V. Deev[5,7], Dimitri Zubov[8,9], Roman Vasyliev[8,9], Giuseppe Perale[10], Gianni Pertici[10], Justin Baker[11], Peter Gingras[11], Yves Bayon[12] and Dimitrios I. Zeugolis[1,2]**

[1] Regenerative, Modular & Developmental Engineering Laboratory, National University of Ireland Galway, Galway, Ireland, [2] Science Foundation Ireland Centre for Research in Medical Devices (CÚRAM), National University of Ireland Galway, Galway, Ireland, [3] Division of Applied Materials Science, Department of Engineering Sciences, Uppsala University, Uppsala, Sweden, [4] GPBio Ltd., Shannon, Ireland, [5] Histograft LLC, Moscow, Russia, [6] Federal Medical Biophysical Center of FMBA of Russia, Moscow, Russia, [7] I.I. Mechnikov North-Western State Medical University, Saint Petersburg, Russia, [8] State Institute of Genetic & Regenerative Medicine NAMSU, Kyiv, Ukraine, [9] Medical Company ilaya®, Kyiv, Ukraine, [10] Industrie Biomediche Insubri, Lugano, Switzerland, [11] Viscus Biologics LLC, Cleveland, OH, United States, [12] Sofradim Production, A Medtronic Company, Trévoux, France

Bone reconstruction techniques are mainly based on the use of tissue grafts and artificial scaffolds. The former presents well-known limitations, such as restricted graft availability and donor site morbidity, while the latter commonly results in poor graft integration and fixation in the bone, which leads to the unbalanced distribution of loads, impaired bone formation, increased pain perception, and risk of fracture, ultimately leading to recurrent surgeries. In the past decade, research efforts have been focused on the development of innovative bone substitutes that not only provide immediate mechanical support, but also ensure appropriate graft anchoring by, for example, promoting *de novo* bone tissue formation. From the countless studies that aimed in this direction, only few have made the big jump from the benchtop to the bedside, whilst most have perished along the challenging path of clinical translation. Herein, we describe some clinically successful cases of bone device development, including biological glues, stem cell-seeded scaffolds, and gene-functionalized bone substitutes. We also discuss the ventures that these technologies went through, the hindrances they faced and the common grounds among them, which might have been key for their success. The ultimate objective of this perspective article is to highlight the important aspects of the clinical translation of an innovative idea in the field of bone grafting, with the aim of commercially and clinically informing new research approaches in the sector.

Keywords: bone grafting, bioadhesives, bioactive scaffolds, cell therapies, clinical trials, commercialization

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****Correspondence:***
Dimitrios I. Zeugolis
dimitrios.zeugolis@nuigalway.ie

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INTRODUCTION

Bone's extraordinary healing capacity can be challenged by complex fractures (i.e., injuries above critical size) or health conditions (i.e., diabetes, genetic factors, poor lifestyle), resulting in non-union fractures that can lead to long-term disability and pain (Keating et al., 2005). Bone grafting is one of the most frequently used procedures in traumatology, orthopedics, oral and maxillofacial surgery, intending to form new bone tissue at the target area (e.g., skeletal defect, atrophy region, a space between bones to be fused). Annually, half a million patients require bone repair intervention in the US and Europe (Amini et al., 2012). The global annual expenditure in bone fractures and

orthobiologics is estimated at US$ 5.5 billion and US$ 4.7 billion, respectively, whilst the total cost of bone repair-related expenditure is estimated at US$ 17 billion per year (Ho-Shui-Ling et al., 2018).

Bone repair interventions are based on autografts, allografts, xenografts, and artificial scaffolds. Autografts are considered the gold standard due to their osteoinductive and osteoconductive properties, but entail important drawbacks, such as limited availability and donor-site morbidity (Fillingham and Jacobs, 2016; Morris et al., 2018; Haugen et al., 2019). Allografts and xenografts, although efficiently overcoming the aforementioned limitations, are prone to trigger immune rejection, disease transmission and their osteoinductive potential is frequently impaired due to disruptive processing (Fillingham and Jacobs, 2016; Klifto et al., 2018; Haugen et al., 2019). Alternative approaches are based in the use of artificial scaffolds specifically designed to maintain physical integrity and promote bone ingrowth at the defect site. Artificial scaffolds, also denominated bone graft substitutes, can be divided in three groups: natural or synthetic scaffolds alone, scaffolds combined with bioactive molecules and cell-based combination products (Ho-Shui-Ling et al., 2018).

Although a huge range of bone grafts and substitutes is available for clinical use, the problem of effective reconstructive treatment remains extremely challenging. Further, despite the extensive pre-clinical investigations, the translational pathway for novel technologies is slow and commonly results in minor improvements of established clinical treatments. The major impediments reside in scalability, high economic requirements and safety concerns that some of these new therapies entail (Hollister and Murphy, 2011; Bara et al., 2016).

Herein, we discuss some of the therapies that have successfully reached the clinic, serving as models in clinical translation of bone tissue engineering. This manuscript covers the use of glues, cements, tissue grafts, biohybrid scaffolds, bioactive matrices, and stem cell-loaded constructs in reported clinical trials or in well-settled clinical practices.

FROM SCREWS TO BONE CEMENT AND GLUE

The implantation of plates and screws to fix bone fractures is a common practice in orthopedic surgery since the beginning of the 20th century. These implants have evolved substantially with the development of new materials, designs, and clinical implantation strategies (Augat and von Rüden, 2018). The primary function of plates and screw implants is to provide mechanical stability to bone fracture fragments. In non-locked plates as the screws are tightened and go into tension, the resulting friction between the plate and the bone stabilizes the fracture and results in a load-sharing device (Egol et al., 2004). The Gamma Locking Nail® (Stryker) is an example of an intramedullary fixation system whose design has been shown effectiveness in providing mechanical stability as an intramedullary fixation device in hundreds of thousands implantations to date (Queally et al., 2014). However, implant complications still occur at considerable rates (up to 20%) and include screw cut-out through poor bone quality, re-fractures and infections that necessitate revision surgeries (Ahrengart et al., 2002; Schliemann et al., 2015; Ma et al., 2017). In such complications, bone quality plays a vital role and osteoporotic or low-density bone leads inevitably to higher risks of implant failure. Other factors, such as implant position and anatomical fracture reduction, influence this complication rate independently of the screw design (Mueller et al., 2013). Therefore, for several decades, it has been accepted that there is a clinical need for augmentation systems, which will improve the performance of the current fixation devices, in terms of osteointegration and biomechanical support, particularly in osteoporotic bone.

To satisfy this unmet clinical need, bone cements have been further developed to increase the area of contact between the screws and the bone, providing better anchoring and mechanical support (DeKeyser et al., 2019). Such bone cements have proved their clinical value in fractured osteoporotic bones (Moroni et al., 2006; DeKeyser et al., 2019; McCoy et al., 2019). This is the case in HydroSet™ (Stryker), a cement employed in clinics to augment screws in cancellous bone (approved in the EU only) in both *ex vivo* (Kainz et al., 2014; Ruddy et al., 2018) and *in vivo* (Larsson et al., 2012) studies. This and other calcium cement-based products, such as BoneSave™ (Stryker), BoneSource® (Stryker) and Norian SRS® (Synthes), have built their path into the clinic and have improved the mechanical stability of screws and outcomes in the treatment of poor quality bone (Van der Stok et al., 2011). However, limitations exist and there is still room for improvement (Van der Stok et al., 2011; DeKeyser et al., 2019; McCoy et al., 2019). Specifically, there is a major clinical need in bone surgery to attach the implant to bone and/or bone to bone, whilst improving biomechanical properties and promoting *de novo* osteoinduction (Sánchez-Fernández et al., 2019). However, there is a gap in the clinical translation of an adhesive biomaterial to meet this specific need. Surgical adhesives, such as cyanoacrylates, have been investigated and shown good mechanical properties *in vitro* (Kandalam et al., 2013), but they lack the osteoinductive potential and their degradation products induce local and systemic toxicity (Hochuli-Vieira et al., 2017; Sánchez-Fernández et al., 2019). Other adhesives, such as fibrin-based glues, present poor mechanical properties (Noori et al., 2017; Sánchez-Fernández et al., 2019). To fill this unmet need, functionalized bone cements have been developed, such as the OsStic™ (GPBio), which is a bioceramic glue composed of tricalcium phosphate combined with phosphoserine, an amino acid. The amino acid triggers rapid (minutes) bonding, providing a strong fixation between tissues and biomaterials (Pujari-Palmer et al., 2018). This occurs through a hierarchical organization of an organic/inorganic interphase, where phosphoserine acts as a nucleation initiator, forming a fibrillar network and allowing the aggregation of calcium phosphate. This bone adhesive technology has already proved its safety and effectiveness in initial pre-clinical *in vivo* tests (Procter et al., 2019) and seems to have a clear pathway to clinical translation, considering that it combines a clinically used material (calcium in bone cements) and an amino acid, whose mechanism

of action has been interpreted (Pujari-Palmer et al., 2018). Should further *in vivo* studies confirm the initial positive results, this is an assured pathway (combining materials with successful clinical history) to effective clinical translation to address a significant unmet clinical need.

TISSUE GRAFTS AS BONE GRAFT AUGMENTATION DEVICES IN DENTISTRY – COMMERCIAL DEVELOPMENT CHALLENGES

Bone grafting is an extensive practice in dentistry with an increasing trend, where implant failure due to the poor fixation or loosening of the implanted grafts is a common complication (Liaw et al., 2015; Kang et al., 2019). To reduce these complications, containment materials are employed to increase the contact interface with the graft and to facilitate cellular in-growth and targeted high quality bone formation, which results in a better fixation and stabilization of the bone graft material (Larsson et al., 2016). Initially, non-resorbable synthetic polymers, such as polytetrafluoroethylene, were employed with this goal, however, they require a second surgical intervention for removal, which unavoidably increases patients' distress and expenditure. This encouraged the employment of decellularised tissue scaffolds (Elgali et al., 2017). The significant advantages of decellularised scaffolds include high cytocompatibility and remodeling potential, which promote osteointegration and regeneration of surrounding soft tissue (Vignoletti et al., 2014; Elgali et al., 2017). Tissue grafts employed in this clinical scenario include allografts (e.g., decellularised pericardia and skin) (Adibrad et al., 2009) or xenografts (e.g., processed porcine and bovine dermis) (Wessing et al., 2017; Arunjaroensuk et al., 2018), which are extensively and successfully employed in other fields, including wound healing or hernia repair (Slater et al., 2013; Brett, 2015; Chen and Liu, 2016). Examples of these products include Creos^{TM} Xenoprotect (Nobel Biocare^{TM}) or BioGuide® (Geistlich), which have proved to promote bone gain and implant support in 46 patients undergoing dental surgery (Wessing et al., 2017).

For the clinical translation of these products in dentistry, special attention must be paid to their source and processing. The raw material (i.e., the tissue graft) requires extensive screening to reduce the risk of disease transmission in both allografts and xenografts, as regulated by FDA in the recognized standard ASTM F2212-11 or CE with EU Regulation 722/2012 and ISO 22442-2015, still valid also under the new European Medical Device Regulation EU 2017/745. In addition, processing of these materials (debriding, decellularization, crosslinking, etc.) must be carried out under strict cGMP or ISO standards to ensure safety, reproducibility, and scalability of the process. Examples of these standards of processing and source control include the 2014 FDA Guidance: Medical Devices Containing Materials Derived from Animal Sources (except for *in vitro* diagnostic devices), FDA's Quality System Regulations 21 CFR 820 and the Quality Management System standard for medical devices ISO 13485-2016. Another point of stress is the sterilization of these products, which must ensure concurrently maximum safety and minimum risk of infection upon implantation (Delgado et al., 2014), in an already susceptible to infection location, the human mouth. All this processing steps must be accompanied with the preservation of the structure and composition of the graft (Delgado et al., 2015; Liaw et al., 2015); after all, these properties rationalize their use and offer them a competitive advantage over synthetic materials. Should these commercial development requirements be met, tissue grafts would have their niche ensured in the clinical translation of dentistry as augmentation systems.

BIOHYBRID GRAFTS – NATURE-INSPIRED BONE SUBSTITUTES

Advances in bone tissue engineering have resulted in a constant decline in the use of autografts, the gold standard in clinical practice (Miller and Chiodo, 2016; Zorica et al., 2016; Klifto et al., 2018; Haugen et al., 2019), and a parallel increase in artificial scaffolds (Morris et al., 2018; Haugen et al., 2019; Stark et al., 2019). However, the new products are far from optimal as low fusion rates and adverse effects have been reported (Zorica et al., 2016; Haugen et al., 2019). To overcome these limitations, nature-inspired bio-hybrid bone grafts (e.g., calcium-phosphate/poly-ε-caprolactone particles (Neufurth et al., 2017), silicon carbide/collagen scaffolds (BioSiC) (Filardo et al., 2014), poly(*N*-acryloyl 2-glycine)/methacrylated gelatin hydrogels (Gao et al., 2019) have been developed. These materials combine the mechanical properties of tailored synthetic polymers and the bioactive element of natural polymers or minerals. A successful example in the clinical translation is a bovine-derived mineral matrix reinforced with resorbable poly(lactic-co-caprolactone) block copolymer embedding RGD-exposing collagen fragments onto its surface (SmartBone®, IBI) (Pertici et al., 2014). Its design follows the "safety by design" paradigm, that is now considered one of the pillars of the new European Medical Devices Regulation. This means that each single component used is sourced in its medical-grade supply form, and its role in the overall mechanism of action is well established and supported by clinical evidences. However, such design must be accompanied by an extensive characterization (e.g., composition, microstructure, mechanical performance, cytocompatibility, preclinical model assessment) to show the safety and efficacy of the device according to international standards (e.g., ISO and ASTM). Also, production under an ISO13485:2016 standard is required to reach the clinic and market scalability. More relevantly, under the new Medical Devices Regulation, and also having in mind that the very ultimate goal is to improve patients' health, clinical performance of innovative products, without existing equivalent products in the market, has to be evaluated during the pre-market approval process (Haugen et al., 2019).

In the case of SmartBone®, such a path resulted in a fully positively characterized material *in vitro* (Pertici et al., 2014, 2015), *in vivo* (Pertici et al., 2015) and in clinical trials (Abuelnaga et al., 2018; Ferracini et al., 2019), ultimately granting device certification (i.e., CE marking), which was complemented

with a post-marketing surveillance in its clinical applications related to bone regeneration in various skeletal disorders. Many other bio-hybrid composites are following the same path with positive results *in vitro* and *in vivo* (Ceccarelli et al., 2017) and in preliminary clinical trials, like in the case of hydroxyapatite/collagen scaffolds (Kon et al., 2011). However, the number of successful bone substitutes in clinical translation remains very low, considering the number of research studies (e.g., 9,313 papers appear in PubMed; terms searched: "bone" and "scaffold" in Title/Abstract). The key to survive the "med-tech valley of death" is in an evidence-based approach from start to finish. This spans from identifying and understanding the unmet clinical need through to measurable clinical outcomes that prove the market differentiation value of the biohybrid medical device to both patient and payer.

ENHANCING BONE REGENERATION WITH BIOACTIVE MATERIALS

Among bone autografts and substitutes, "bioactivated materials" with growth factors are considered as combination products or drugs in FDA and EMA terms, respectively. Alternative to growth factors have been developed with formulations containing cells or gene constructs that are capable of stimulating reparative osteogenesis with different regulatory status, for example, falling into the category of Advanced Therapy Medicinal Products (ATMPs) in Europe (Ho-Shui-Ling et al., 2018).

Bone regeneration is a multi-step process spatiotemporally coordinated by an array of growth factor signaling pathways (De Witte et al., 2018). Bone morphogenetic proteins (BMPs) were the first growth factors to be identified as osteoconductive and osteoinductive, in other words, being able to differentiate stem cells toward osteoprogenitor cells and promote scaffold bone tissue ingrowth (Evans, 2013). Since their FDA approval in the early 2000s, BMP-2 and BMP-7 remain the most commonly used growth factors for bone graft functionalization and constitute the active molecules of two major devices: Infuse® (Medtronic) and Osigraft® (Olympus), respectively (Evans, 2013). These two collagen-based bone grafts have repeatedly shown to promote bone ingrowth in FDA approved clinical spine and tibia trauma indications (Friedlaender et al., 2001; Govender et al., 2002). However, Osigraft® production was discontinued and the off-label use of Infuse® has resulted in reported complications (Simmonds et al., 2013; Vukicevic et al., 2014). The BMP solution component must exclusively be used with a legally approved carrier/scaffold component and for the legally approved indication. This highlights the importance of a new or improved technology that will allow for a controlled release of the bioactive cargo, to support the regulatory approval of extended clinical uses (Geiger et al., 2003; Chatzinikolaidou et al., 2010; Carragee et al., 2011).

Despite promising results in research for "smart" formulations to support improved control over growth factor release to bone regeneration sites, the reality is that most of these ambitious materials never go beyond the animal study stage (Bessa et al., 2010; De la Riva et al., 2010; Webber et al., 2015). Indeed, such products have to compete with autografts and demineralized bone matrix (DBM), in terms of bone repair or fusion efficacy, particularly when they are processed to maintain the osteoinductive and osteoconductive properties of the native bone matrix (Miron and Zhang, 2012). Most of the activated devices that made it to the clinics in the past decade are based in allografts or collagen/tricalcium phosphate scaffolds. This is the case of OsteoAMP® (Bioventus Surgical) and Augment® bone graft (Wright Medical Group), the former made of an allogeneic-derived matrix, especially treated to preserve a cocktail of native growth factors and the latter composed of a collagen/tricalcium phosphate composite combined with platelet-derived growth factor (PDGF-BB). Both devices promoted bone ingrowth without the need of autograft harvesting in two clinical trials with patients undergoing transforaminal lumbar or lateral interbody fusion and ankle or hindfoot arthrodesis, respectively (DiGiovanni et al., 2013; Roh et al., 2013; Krell and DiGiovanni, 2016).

The short-lived activity of growth factors in medical devices may be a concern for optimal clinical efficacy (Jabbarzadeh et al., 2008). A sophisticated new approach able to circumvent this limitation is based on the exogenous delivery of plasmid DNAs from gene-activated matrices to the host cells in the site of bone defects, to induce the endogenous production of reparative growth factors. In a recent clinical trial, a combination product based on a collagen-hydroxyapatite medical device and a plasmid DNA encoding for vascular endothelial growth factor-A (VEGF-A), showed to promote bone ingrowth in maxillofacial bone defects without causing adverse effects (Bozo et al., 2016). Similarly, Histograft (Russia) has developed an octacalcium phosphate scaffold carrying VEGF-A coding plasmid that has completed a clinical trial (NCT03076138).

Taken together, devices with bioactive molecules have proven to match or increment the regenerative capabilities of traditional bone grafts. However, the flow of technology from benchtop to clinic appears to be slow, since any new bioactive candidate should strictly comply with regulatory requirements, notably in terms of safety and efficacy (Vukicevic et al., 2014). The 510(k) process of FDA allows devices characterized as "substantially equivalent" to an existing approved device to enter fast into the market. However, bioactive scaffolds with poorly defined degradation products require significant effort to establish safety, substantially increasing time and cost associated with preclinical and clinical assessment (Webber et al., 2015; Ho-Shui-Ling et al., 2018). The design of a bioactive molecule(s) delivery system that can serve as a scaffold for cell attachment and matrix deposition, whilst promoting the active migration of cells, angiogenesis, and osteogenic cell differentiation, all in the right time and amount, appears as a mission impossible, unless more elegant, yet regulatory compliant systems, are developed (Abbah et al., 2015; Thomas et al., 2016). It is also time to realize that a single molecule approach is unlikely to result in functional repair and regeneration (Pugliese et al., 2018). However, regulatory approval of multi-cargo delivery systems is rather onerous, which encourages the use of cell therapies, considering that cells can act as a factory of trophic/bioactive molecules at the site of implantation.

BIOMATERIAL AND STEM CELLS COMBINING TECHNOLOGIES

Cell-based strategies for bone tissue engineering have a long trajectory in the research stage but have minimally contributed to current clinical practices (Mishra et al., 2016). Indeed, the introduction of cells as a component in tissue engineering entails important economic and safety concerns; the former is related to the logistics, technology and human resources necessary and the latter is related to possible immunogenicity, teratoma formation and disease transmission risks (Webber et al., 2015). As a result of the second, only those therapies that involve minimal *ex vivo* manipulation of autologous cells are FDA approved, whilst those that follow the traditional tissue engineering paradigm (*in vitro* expansion of autologous/allogeneic cells and *ex vivo* development of a cell-based construct), present a more tortuous regulatory pathway that commonly results in the abandonment of the technology, in the best scenario, after clinical trials (Jager et al., 2010).

Cell source in bone tissue engineering is a matter of debate, where the type of stem cell chosen for *in vitro* and *in vivo* experimentation notably differs in the literature (Gao et al., 2017). Bone marrow stromal cells (BMSCs), however, have been the preferred choice in clinical studies due to their intimate involvement in bone physiology and pathology, osteogenic potency, and anti-inflammatory properties (Zheng et al., 2019). Cell therapy for bone regeneration using freshly extracted BMSCs is a technique with 30 years of history. The first reported clinical study using bone marrow aspirates dates from 1991 (Connolly et al., 1991). In 2003, composite grafts of DBM serving as scaffolds and autologous bone marrow showed similar results as compared to autografts in the spinal fusion of 77 patients (Price et al., 2003). The same type of scaffold-cell graft showed positive results when implanted in the unicameral bone cysts of 23 patients (Rougraff and Kling, 2002). Further work showed that a collagen/hydroxyapatite composite (Healos®, DePuy) incubated for 20 min with autologous bone marrow aspirate promoted similar posterolateral spine fusion rates as bone autografts, avoiding any autograft-related donor-site morbidity (Neen et al., 2006). Similarly, the use of tricalcium phosphate scaffolds pre-incubated for 2 h with bone marrow aspirate, showed positive results in the spine fusion of 41 patients, 34.5 months after the procedure (Gan et al., 2008).

Despite the issues related to the *ex vivo* expansion of autologous stem cells prior to implantation, the use of this technique might also entail considerable benefits. For instance, cell expansion substantially increments cell numbers and allows for the *ex vivo* treatment of cells with growth factors or other biochemical/biophysical stimuli to increment their therapeutic potential (Cigognini et al., 2013). In a recent study, *ex vivo* expanded autologous adipose-derived stem cells (ADSCs) seeded on bioactive glass or β-tricalcium phosphate scaffolds and, in some cases, pre-incubated with BMP-2, showed integration of the constructs and tissue formation in 10 out of 13 patients suffering from large cranio-maxillofacial hard-tissue defects (Sandor et al., 2014). Furthermore, a clinical study performed in 2017 utilized a cocktail of expanded autologous BMSCs, periosteal progenitor cells and endothelial progenitor cells on a fibrin hydrogel-DBM composite, to restore critical-sized bone defects of 47 casualties with complicated gunshot bone wound. X-ray examination determined that within 4–6 months post-operatory, 90.4% of the treated defects regained native integrity (Vasyliev et al., 2017).

Taken together, tissue engineering approaches and, more precisely, the use of stem cells in combination with biomaterials has proven, in most of the clinical studies, to match or surpass the clinical outcomes of autografts. The extra step of *in vitro* cell expansion entails numerous risks and cost-related burdens but, if well designed, can increment the therapeutic outcomes. The major impediment of cell-based technologies for their clinical translation is and will be the costs and risks associated, making the address of these issues, at an early stage of research, fundamental.

CONCLUSION

Despite the huge scientific efforts to develop safe and functional bone substitutes, bone tissue grafts remain the gold standard in clinical practice. The prevalence and market size of bone repair and regeneration encourage the development of therapeutic technologies to overcome limitations of bone tissue grafts and fill clinical gaps in a wide spectrum of applications (e.g., from traumatology to dentistry). Yet again, only few products have demonstrated safety and efficacy in clinical setting. Their success has been largely attributed to the precise understanding of the mechanism of action of the various device components and their compliance with regulatory frameworks. In fact, this is key in translating new concepts from lab-bench to bedside, overcoming regulatory hurdles, and normative framework changes, whilst providing a safe and functional therapy.

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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REVIEW
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Mesenchymal Stem Cell-Derived Extracellular Vesicles: Opportunities and Challenges for Clinical Translation

*Marie Maumus[1,2], Pauline Rozier[1], Jérémy Boulestreau[1], Christian Jorgensen[1,3] and Danièle Noël[1,3]**

[1] IRMB, University of Montpellier, INSERM, CHU Montpellier, Montpellier, France, [2] Bauerfeind France, IRMB, Montpellier, France, [3] Clinical Immunology and Osteoarticular Diseases Therapeutic Unit, Department of Rheumatology, Lapeyronie University Hospital, Montpellier, France

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***Correspondence:**
Danièle Noël
daniele.noel@inserm.fr

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Extracellular vesicles (EVs), including exosomes and microvesicles, derived from mesenchymal stem/stromal cells (MSCs) exert similar effects as their parental cells, and are of interest for various therapeutic applications. EVs can act through uptake by the target cells followed by release of their cargo inside the cytoplasm, or through interaction of membrane-bound ligands with receptors expressed on target cells to stimulate downstream intracellular pathways. EV-based therapeutics may be directly used as substitutes of intact cells or after modification for targeted drug delivery. However, for the development of EV-based therapeutics, several production, isolation, and characterization requirements have to be met and the quality of the final product has to be tested before its clinical implementation. In this review, we discuss the challenges associated with the development of EV-based therapeutics and the regulatory specifications for their successful clinical translation.

Keywords: mesenchymal stem cells, extracellular vesicles, regenerative medicine, therapy, clinical translation

INTRODUCTION

Extracellular vesicles (EVs), including exosomes and microvesicles, are nanoscale vesicles that are released by all cell types and act as signaling/communication agents between adjacent or distant cells. The transmission of information to a multitude of cells and locations confers them important roles in both physiological and pathological processes. EVs derived from mesenchymal stromal/stem cells (MSCs) display similar functions as their parental cells and show therapeutic efficacy in many non-clinical models (Keshtkar et al., 2018). MSC-derived EVs (MSC-EVs) exert their functions through the transfer of their cargo (i.e., proteins, lipids, and nucleic acids such as mRNA, micro-RNAs, long non-coding RNAs, DNA, and metabolites) (Busatto et al., 2019; Qiu et al., 2019). They can be used as therapeutic tools either in naïve form, as substitutes of intact cells, or after modification for targeted drug delivery. However, for clinical applications, EV safe and effective production systems and rigorous quality control are needed before the release of clinical batches. The *International Society for Extracellular Vesicles* (ISEV) and the *European Network on Microvesicles and Exosomes in Health and Diseases* (ME-HaD) have highlighted a number of safety and regulatory requirements that must be considered for the clinical applications of EV-based

therapeutics (Lener et al., 2015). In the present review, we summarize the recent developments in EV production for pharmaceutical manufacturing, and discuss the regulatory issues associated with their clinical application.

DEFINITION AND CHARACTERIZATION OF EXTRACELLULAR VESICLES

Extracellular vesicles are defined as particles that are delimited by a lipid bilayer, cannot replicate, and are released from the cell (Thery et al., 2018). At least three EV types can be characterized on the basis of their biogenesis pathway: (i) exosomes (small particles of endocytic origin with a diameter of 30–150 nm), (ii) microvesicles or microparticles (generated from the plasma membrane by direct budding; diameter of 150–500 nm), and (iii) apoptotic bodies (vesicles of 800–5000 nm in diameter, formed via membrane blebbing of apoptotic cells) (He et al., 2018). However, due to the overlapping sizes among EV subtypes and the frequent use of isolation techniques that rely on size-based separation, the ISEV recently recommended to define them as small vesicles (EVs $<$ 200 nm), and medium or large EVs (EVs $>$ 200 nm) (Thery et al., 2018). This definition is not comprehensive, particularly concerning EV biogenesis, but probably it represents the best option for classifying EVs that are mainly isolated according to their size. A more accurate definition will require the development of devices that allow EV isolation with high yields based on the presence of specific biomarkers and that are compatible with large-scale production.

Extracellular vesicles are secreted by all human and non-human cell types and can be divided into plant-derived EVs, bacterial/fungal/parasitic EVs, and animal product-derived EVs (Schuh et al., 2019). They are key components of the local environment through intercellular communication pathways, and also of the systemic environment through their release into the fluids of complex organisms. In animals and humans, they can be isolated from all body fluid types: blood, urine, breast milk, synovial liquid, amniotic fluid, cerebrospinal fluid, saliva,... (Schuh et al., 2019). EVs convey large numbers of molecules (e.g., proteins, mRNA, non-coding RNAs, and lipids) that mediate different functions, depending on the cells from which they originate. They transfer signals to recipient cells through different mechanisms: receptor-ligand interactions, direct membrane fusion, and endocytosis/phagocytosis (van Niel et al., 2018). They act in a paracrine and endocrine manner, and can be also taken up by their cells of origin. Therefore, EVs have important roles in both physiological and pathological processes.

POTENTIAL THERAPEUTIC USE OF MESENCHYMAL STEM CELL-DERIVED EXTRACELLULAR VESICLES

Extracellular vesicles from various cell sources have different physiological functions and therefore, may have different therapeutic applications. They were first investigated as vaccines to enhance the antitumor response using antigen-presenting cells, primarily dendritic cells loaded with tumor antigens, and then as vaccines for infectious and allergic diseases (for review, see Markov et al., 2019; Zurita et al., 2019). Subsequently, the detection of EVs with increased concentrations and differential cargoes in body fluids from patients with different pathological conditions led to much research on the potential use of EV proteins and RNA molecules as biomarkers of different diseases (Lasser, 2015). Finally, due to their capacity to carry large numbers of active molecules, EVs can be exploited as drug delivery systems, and can be chemically or biologically engineered to deliver enhanced or broaden therapeutic agents. Indeed, EVs act as "logistics shuttles" that show high stability in the bloodstream, specific targeting capacities (like their parent cells), and capacity to pass through physiological barriers (the blood brain barrier, for example). Indeed, MSC-derived EVs were shown to strongly inhibit lymphocyte proliferation and antibody production by targeting B-cells in heart failure (van den Hoogen et al., 2019). Chemical modification of EVs by addition of RGD peptides conjugated onto EV surfaces led the EVs to pass the blood brain barrier and target brain cells after ischemic stroke (Tian et al., 2018). EVs can also be loaded with drugs by cell transfection and genetic expression of a candidate gene, or by drug encapsulation after their isolation [(Mao et al., 2019; He et al., 2020; Ou et al., 2020); for review, see Crivelli et al. (2017)]. MSC-EVs were extensively characterized as drug delivery platform and shown to have greater internalization capabilities than commercial liposomes (Le Saux et al., 2020). The interest of using MSC-EVs loaded with doxorubicin by electroporation to target murine breast cancer cells or osteosarcoma cells has been demonstrated (Gomari et al., 2019; Wei et al., 2019). Interestingly, MSCs can entrap drug-loaded nanoparticles and release EVs that contain the nanoparticles enabling to combine MSC-based regenerative therapy to pharmaceutical nanomedicine (Perteghella et al., 2017).

The interest of using MSC-EVs for clinical applications is related to the variety of molecules with therapeutic functions they can carry and the fact that their cargo is naturally protected from degradation in the circulation (Tsui et al., 2002). EVs isolated from autologous MSCs are non-immunogenic. Although they should be poorly immunogenic in the case of allogenic injection thanks to the immunomodulatory molecules they convey, it is still unclear whether EVs contain major histocompatibility complex (MHC) molecules that might elicit alloimmune responses (Crivelli et al., 2017; Lohan et al., 2017). Numerous reports have highlighted the functional properties of MSCs and MSC-EVs using *in vitro* assays, and identified many factors involved in their functions (for review, see Doorn et al., 2012; Maumus et al., 2013; Clark et al., 2014; Glenn and Whartenby, 2014; Burrello et al., 2016; Abbasi-Malati et al., 2018). The possibility of using MSC-EVs to eliminate or reduce the clinical symptoms of several diseases has been widely assessed in animal models. A recent review of the literature discussed the applications of EVs from umbilical cord-derived MSCs (UC-MSCs) in various diseases (Yaghoubi et al., 2019). EVs isolated from different MSC sources have shown efficacy in non-clinical models of neurological diseases, particularly epilepsy (Xin et al., 2013; Long et al., 2017), post-traumatic brain

injury (Zhang et al., 2015), brain damage in pre-term neonates (Ophelders et al., 2016; Drommelschmidt et al., 2017; Sisa et al., 2019), and stroke (Doeppner et al., 2015). In animal models, MSC-EVs have been used to treat myocardial infarction (Lai et al., 2010; Bian et al., 2014) and for ischemic injury prevention in chronic renal failure (Gregorini et al., 2017). MSC-EVs are also efficient for the management of acute conditions, such as acute renal failure (Bruno et al., 2009) and respiratory failure (Zhu et al., 2014; Monsel et al., 2015; Monsel et al., 2016). MSC-EVs can reduce clinical symptoms in murine models of osteoarthritis and rheumatoid arthritis (Cosenza et al., 2017, 2018). Finally, MSC-EVs are effective in liver regeneration, as well as in experimental infectious conditions and ophthalmic diseases (Li et al., 2013; Tan et al., 2014; Tan et al., 2016; Bai et al., 2017; Chen et al., 2017; Chang et al., 2018). In conclusion, many studies have demonstrated the therapeutic efficacy of MSC-EVs in animal models and their potential is now evaluated in human clinical trials.

As it is generally accepted that MSCs from different tissue sources and from different donors display qualitatively different functional capacities, EVs isolated from different MSCs also should present differences in their cargo and related properties (Baglio et al., 2015). However, only few studies compared EVs from MSCs isolated from different sources. It has been recently reported that EVs from adipose tissue-derived MSCs (AD-MSCs) and from cardiac MSCs exhibit more potent angiogenic capacities than bone marrow-derived MSCs (BM-MSCs) (Chance et al., 2020; Kang et al., 2020). In addition, the capacity of EV production and secretory profile are higher in BM-MSCs than AD-MSCs, supporting a differential activity of EVs from different MSCs (Villatoro et al., 2019). Similarly, the expression of surface markers and function vary in MSCs from different species. Indeed, although MSC immunosuppressive capacity may cross the species barriers, different mechanisms of action are reported: through soluble factors for human MSCs and through cell–cell contacts for rodent MSCs (Uder et al., 2018). To our knowledge, no study has compared EVs from different species yet. Nevertheless, it is obvious that EVs from different species are different. This implies that the conclusions of pre-clinical studies that use human EVs in animal models have to be taken with cautions.

Another important notion for EV therapeutic applications is the definition of the minimal dose for effective clinical outcome in patients. Like parental MSCs, the effective dose of EVs is dependent on the biological activity, which can be defined on the basis of the number of particles or the quantity of bioactive proteins or RNAs. However, the protein or RNA content may quantitatively and qualitatively vary in function of MSC culture conditions or activation status and EV production method. In general, a dose-dependent effect of MSC-EVs has been observed using different functional assays (Cosenza et al., 2018; Bari et al., 2019a; Dal Collo et al., 2020). For instance, 50 μg of MSC-EVs are needed to induce the proliferation and differentiation of neural stem cells to oligodendrocytes (Otero-Ortega et al., 2020), while 10 μg of placenta-derived MSC-EVs are sufficient to increase the migration and tube formation of placental microvascular endothelial cells (Salomon et al., 2013). These differences in the dose needed for bioactivity can be related to the MSC source, the method used for EV isolation, or the mechanism of action (MoA) of EVs. The definition of the MoA for a specific therapeutic indication should allow designing a reproducible and reliable functional *in vitro* assay to determine the EV protein or RNA effective concentration, as proposed in Dal Collo et al. (2020). However, *in vitro* potency assays do not necessarily predict the therapeutic effect *in vivo*, and even less the patients' outcome in clinical trials.

The minimal effective dose of EVs could be determined using a relevant pre-clinical model (ideally a large animal model) for a specific therapeutic application. The therapeutic dose of EVs is usually in the range of 10–100 μg of proteins in mouse models (Riau et al., 2019). For example, a dose of 50 μg of EVs was sufficient to enhance protection and brain repair in a rat model of subcortical ischemic stroke, compared with 100 and 200 μg of EVs (Otero-Ortega et al., 2020). This dose was also the smallest effective dose identified in a functional *in vitro* assay. However, the most efficient dose is not always the highest dose, as shown for MSCs in a model of systemic sclerosis (Maria et al., 2016). Interestingly, it was reported that EVs isolated from non-pigmented ciliary epithelium display enhanced pro-MMP9 activities at high doses, but significantly reduce β-catenin expression and GSK-3 phosphorylation only at low doses (Tabak et al., 2018). This concentration-dependent effect of EVs might be related to different interaction modes with the target cells (e.g., direct binding to cell membrane receptors or internalization).

Moreover, investigating different administration routes may help to reduce the effective dose, if the accessibility of the target tissue is increased. Indeed, as the route of administration determines EV biodistribution, increasing the uptake of exogenous EVs by a targeted organ can enhance their efficacy (Di Rocco et al., 2016). Unlike intravenous injection, EV administration by the intraperitoneal or subcutaneous route results in higher accumulation in pancreas and gastrointestinal tract and in lower concentrations in liver and spleen (Wiklander et al., 2015). In addition, EV uptake is potentiated by the concomitant presence of extracellular proteins, for instance albumin (Schneider et al., 2017). Moreover, EV dose also can affect their biodistribution, as indicated by the inverse correlation between intravenous injection of increasing EV concentrations and their accumulation in liver (Wiklander et al., 2015).

In conclusion, the minimal effective doses of EVs can be determined by *in vivo* studies and these findings can be extrapolated for human use. EV dose and also the route, timing, and frequency of administration need to be carefully investigated for optimal and safe EV delivery in patients, as discussed elsewhere (Bari et al., 2019b).

CLINICAL APPLICATIONS OF MESENCHYMAL STEM CELL-DERIVED EXTRACELLULAR VESICLES

A total of nine clinical trials can be identified in the ClinicalTrials.gov database when using the keywords "exosomes"

and/or "extracellular vesicles" and focusing on MSC-EVs (**Table 1**). Six of them are still recruiting or are completed, two trials are not recruiting yet, and one has an unknown status.

The first phase I clinical trial was initiated in 2014 with the aim of evaluating the safety of EVs isolated from UC-MSCs in 20 patients with type 1 diabetes. Patients received a systemic injection of exosomes at day 0 and of microvesicles at day 7, and the effect on the total daily requirement of insulin was evaluated at 3 months. The status of the trial is unknown. In 2017, another phase I study enrolled patients with large and refractory macular holes (MH). This randomized and controlled study has included 44 patients who received 20 or 50 μg of UC-MSC-derived exosomes in the vitreous cavity close to the MH, after pars plana vitrectomy and internal limiting membrane peeling. This study is still recruiting. The treatment efficacy is evaluated by assessing MH closure by optical coherence tomography, at 24 weeks post-treatment. More recently, a safety and tolerability study was performed in pre-term neonates (born before gestational week 27) at high risk of bronchopulmonary dysplasia (BPD). This multi-center controlled double-bind trial included 3 to 14-day-old neonates (n = 18) who received 20, 60, or 200 pmol phospholipid of BM-MSC-EVs (UNEX-42)/kg body weight by intravenous injection. Safety was the primary endpoint, but BPD incidence and severity were also determined (secondary endpoints). A phase 1/2 multi-center randomized study to evaluate the effectiveness and safety of daily local injections of MSC-EVs (AGLE-103) in 30 patients with dystrophic epidermolysis bullosa was registered in November 2019. The primary endpoint is the safety and efficacy of wound closure at 8 months after treatment. The last phase 1/2 clinical study recorded in December 2019 will assess the alleviation of dry eye symptoms in 27 patients with chronic graft versus host disease (GVHD) after local treatment with UC-MSC-derived exosomes four times per day for 14 days. The treatment safety and efficacy will be evaluated at different time points by measuring the changes in the ocular surface disease index.

Two phase I clinical trials evaluate genetically engineered MSC-EVs. The first trial assessed EVs isolated from miR-124-overexpressing MSCs in five patients with acute ischemic stroke. Patients received 200 μg of total EV protein by stereotaxis, 1 month after the stroke. The incidence of adverse events was the primary outcome measure, but efficacy was also assessed using a Modified Rankin Scale after 12 weeks of treatment. The trial has recently been completed, but results are not available yet. The

TABLE 1 | Clinical trials evaluating MSC-EV therapies.

Disease	EV type	Administration route	Injected dose and time of injection	Cell source	Trial phase	Control group	Status	Number of patients	Ref/NTC
Type 1 diabetes	Exo and MV	IV	Eq of SN from 1.2-1.5 × 10^6 cells/kg body weight (day 0)	UC-MSCs	1	No	Unknown	20	NCT02138331
Macular holes	Exo	Local	20 μg or 50 μg Eq proteins (day 0)	UC-MSCs	1	Yes	Recruiting	44	NCT03437759
Bronchopulmonary dysplasia	Not indicated	IV	20, 60, or 200 pmol/kg (day 0)	BM-MSCs	1	Yes	Recruiting	18	NCT03857841
Dystrophic epidermolysis bullosa	Exo	Local	Not indicated (once a day for 60 days)	Not indicated	1/2	Yes	Not yet recruiting	30	NCT04173650
Dry eye (graft versus host disease)	Exo	Local	10 μg Eq proteins/drop, 4 times a day, 14 days	UC-MSCs	1	No	Recruiting	27	NCT04213248
Ischemic stroke	Exo	Local	200 μg Eq proteins (day 0)	miR-124 over expressing MSCs	1/2	No	Completed	5	NCT03384433
Pancreatic cancer	Exo	IV	Unspecified	MSCs loaded with KrasG12D siRNA	1	No	Not yet recruiting	28	NCT03608631
Graft versus host disease	Small size EVs	IV	4 units (1 unit = Eq of SN from 4 × 10^7 cells) (day 0)	BM-MSCs	NA	NA	Completed	1	Kordelas et al., 2014
Chronic kidney disease	Total EVs	IV (1st)/IA (2nd)	100 μg Eq proteins /kg (x2) (day 0)	UC-MSCs	2/3	Yes	Completed	40	Nassar et al., 2016

EVs, extracellular vesicles; Eq, equivalent dose; Exo, exosomes; IV, intravenous; IA, intra-arterial; MV, microvesicles; SN, supernatant; BM-MSC, bone marrow-derived mesenchymal stem cells; UC-MSC, umbilical cord-derived mesenchymal stem cells; NA, not applicable.

second clinical trial will evaluate MSC-derived exosomes loaded with small interfering RNAs against KRAS G12D (iExosomes) in patients with metastatic pancreatic cancer. Patients (n = 28) will receive the treatment by intravenous route at days 1, 4, and 10, and then every 14 days for up to three courses in the absence of adverse events or unfavorable disease outcome. The study aim is to identify the maximum tolerated dose and the dose-limiting toxicities of iExosomes, but has not recruited patients yet.

There are only two publications on the use of MSC-EVs in the clinic. The first article reported the case of one patient with therapy-refractory GvHD who received four units of BM-MSC-derived small EVs by intravenous injections (Kordelas et al., 2014). One unit of EVs was defined as the EV fraction recovered from the supernatant of 4×10^7 BM-MSCs conditioned for 48 h and isolated by filtration using 0.22 μm filter membranes, precipitation with polyethylene glycol, and a final ultracentrifugation at 100.000 g for 2 h. To reduce the potential side effects, the patient initially received one tenth of a unit, and then progressively increasing unit amounts every 2–3 days to a total of 4 units. No adverse event was observed and clinical symptoms were remarkably improved within 14 days after EV administration, suggesting the safety and potential efficacy of this EV-based treatment. The second article concerned a randomized placebo-controlled clinical trial that evaluated the safety and efficacy of UC-MSC-EVs in 40 patients with stage III and IV chronic kidney disease (Nassar et al., 2016). EVs were collected from UC-MSC conditioned supernatant using two ultracentrifugation steps at 100,000 g for 1 h. Patients received two injections of 100 μg EVs/kg body weight 1 week apart, the first one by the intravenous route and the second one through the intra-renal arteries. No adverse event was recorded. The overall renal function significantly improved during the 12-month follow-up period. Interestingly, TGFβ1 and IL10 levels significantly increased concomitantly with the clinical improvement, suggesting immune modulatory regulation. Although few clinical results are available, pre-clinical data and early clinical results on EV-based therapeutics are very encouraging. However, it is important to stress that these clinical trials are mostly phase 1 studies on the feasibility and safety of EV administration for different clinical applications. Only one phase 2/3 trial has been completed and showed the safety (primary endpoint) and efficacy (secondary endpoint) of UC-MSC-EVs in patients with chronic kidney disease, as indicated by the reduction of serum creatinine level by 50% and the twofold increase in eGFR (Nassar et al., 2016). Randomized, double-blinded phase 2 and 3 clinical trials are required to definitively demonstrate MSC-EV efficacy and therapeutic interest.

CHALLENGES FOR THE INDUSTRIAL PRODUCTION OF GMP-GRADE EXTRACELLULAR VESICLES

The main challenge linked to the industrialization of EV-based therapeutics for regenerative medicine is to define new manufacturing strategies under Good Manufacturing Practice (GMP) for EV scalable production and isolation. Standardized operating procedures (SOPs) using reproducible and standardized assays are mandatory to manufacture a defined and qualified EV product because each manufacturing procedure will generate a different product. To reach this goal, major questions have to be addressed early in the product development: (i) how to manufacture EVs; (ii) how to characterize and qualify the final product; and (iii) how to organize the product storage in order to maintain its stability.

Manufacturing

MSC Sources

Several tissue sources of MSCs can be used, such as BM, adipose tissue, synovial membrane, UC. These MSCs have been tested in various *in vitro* functional assays and in a large number of non-clinical disease models where MSC-EVs have shown therapeutic efficacy [for review see D'Arrigo et al. (2019)]. Nevertheless, no comparative study identified the most efficient MSC source for EV production, in terms of quantities or functional activities. Primarily two sources of MSCs (UC- and BM-MSCs) have been tested in clinical trials, but for different applications (**Table 1**). Therefore, the first question for EV manufacturing is the identification of the best MSC source(s) for a specific clinical application, and more data are necessary to answer this question. The best MSC source can be determined by identifying the most relevant MoA for the targeted therapeutic activity. For example, if an anti-inflammatory or pro-angiogenic function is envisioned, AD-MSCs or UC-MSCs might be preferred to BM-MSCs. It is nevertheless recommended to determine the best MSC source experimentally by comparing MSCs from different sources in a pre-clinical model relevant for the therapeutic application, by testing different batches of different MSC sources or by comparing pools of MSCs to avoid inter-donor variability. EV production is also influenced by the features of the producing cells. For instance, it has been shown that cell aging (replicative senescence and donor age-associated senescence) and cell–cell contacts (confluence and seeding density) affect EV production. Specifically, senescent MSCs secrete greater numbers of EVs than non-senescent MSCs (Huang et al., 2019; Fafian-Labora et al., 2020). Conversely, confluent MSCs produce lower amounts of EVs than proliferating MSCs (Patel et al., 2017). The impact of senescence on the production and functionality of MSC-EVs in different therapeutic applications has recently been reviewed (Boulestreau et al., 2020). EVs from aging MSCs did not exhibit the protective effect of EVs from young MSCs in an acute lung injury model (Huang et al., 2019). In consistency, intercellular transfer of EVs from young MSCs are more potent than EVs from aged MSCs to rejuvenate aged hematopoietic cells and restore their function through the uptake of autophagy-related mRNAs (Kulkarni et al., 2017). Production process will therefore have to include quality controls to evaluate the percentage of senescent cells and its impact on the functionality of EV batches.

Immortalized MSC lines could be used to ensure batch reproducibility, to avoid inter-individual donor variability, and

to maintain bioactivity during culture expansion. For instance, EVs released by embryonic stem cell-derived MSCs immortalized by transfection of a lentivirus carrying the c-Myc oncogene reduced the infarct size in a mouse model of myocardial injury (Chen et al., 2011). Of course, the immortalized MSC stability and absence of the transgene protein in the derived EVs must be demonstrated. Nevertheless, this strategy ensures an infinite supply of EVs with high inter-batch reproducibility.

EV Production

A second question is the choice of culture system (e.g., medium composition and cell-adhering support) for EV production. Indeed, several cell culture parameters influence EV production and cargo composition. For clinical purposes, the use of xeno- and EV-free culture media is recommended to remove any source of variability and animal-associated contaminations. It has been shown that xeno- and serum-free culture media support sustained MSC proliferation without loss of viability and promote the cell secretory functions (Lee et al., 2017; Mochizuki and Nakahara, 2018; Palama et al., 2020). Platelet lysates can be used at the place of fetal calf serum in GMP manufacturing conditions, although defined media are more appropriate (Pachler et al., 2017; Bari et al., 2018). To scale up EV production for industrialization, 3D-culture in bioreactors has been tested, such as multilayered cell culture flasks, hollow fiber bioreactors, stirred-tank bioreactors, and spheroidal aggregates of MSCs. Hollow fiber and stirred-tank bioreactors are the more promising approaches because they are closed and GMP-compatible scalable systems that provide a high surface-to-volume ratio for MSC growth (Mendt et al., 2018; Mennan et al., 2019; Vymetalova et al., 2020). EV production in bioreactors is increased at least by 40-fold compared with 2D culture systems (Watson et al., 2016). Furthermore, the duration of EV production and the frequency of medium collection have to be tested to determine the optimal parameters for cell proliferation, confluence and EV re-uptake by producing cells.

EV production can be stimulated using different biochemical or biophysical strategies. Among the biophysical strategies, hypoxia can be controlled and modulated. It has been reported that MSC culture and EV production in hypoxic conditions (1–5% O_2) increase the number of EVs released and their cargo composition (growth factors and miRNAs), thus enhancing their pro-angiogenic, immunomodulatory, cardioprotective and neuroprotective effects (Cui et al., 2018; Xue et al., 2018; Zhu et al., 2018). Another option is to take advantage of bioreactors to mechanically stimulate EV production by applying fluid shear stress or compression [for review, see Piffoux et al. (2019)]. Although, the underlying mechanisms are not known, one hypothesis is that in this condition, MSCs inhibit their own re-uptake of EVs. Recently, it has been reported that ultrasonication of ultracentrifuged MSC-EVs followed by regular centrifugation and filtration allows increasing the EV yield by 20-fold (Wang et al., 2019). Moreover, the authors demonstrated that these EVs are functional and promote wound healing in animal models. However, this technique might release a fraction of vesicles that normally remain tethered at the plasma membrane, or vesicles that have been recaptured by the producing cells, or even other components from secretory pathways (van Niel et al., 2018). Therefore, EVs isolated after ultrasonication need to be better characterized.

Different strategies have been considered to modulate EV content and biological activities, including biochemical stimuli and genetic modification of MSCs to overexpress specific proteins or miRNAs [for review, see Park et al. (2019)]. MSC activation with lipopolysaccharides before EV production does not change the number of released EVs, but influences their content. These EVs have been used to modify macrophage polarization, procoagulant properties, or the ability to support wound healing (Ti et al., 2015; Zeuner et al., 2016; Fiedler et al., 2018). The importance of miRNAs in MSC-EV therapeutic effects suggested that genetic engineering of MSCs to overexpress the miRNAs of interest might improve their efficacy. For example, miR-92a-3p overexpression in MSCs allowed producing EVs with higher protective effect against cartilage destruction in an osteoarthritis model (Mao et al., 2018).

In conclusion, all parameters that can influence EV number and content must be clearly identified to define the best balance between production conditions and EV functions. Improvement of EV functions can be obtained through genetic modification or pre-activation of MSCs. Because different manufacturing procedures and culture conditions can affect the characteristics and functionalities of EVs, the production process will have to be clearly defined for optimal use of EVs for specific clinical indications.

EV Isolation

There is no unique or standardized method to isolate EVs. This might explain the variability in EV characteristics and bioactivities among laboratories. For clinical applications, the challenge is to isolate EVs with high yield and purity, while preserving their structure and activity. In addition, the isolation method should be scalable, cost-effective, compatible with a high-throughput production process, and ideally, in a closed system. Differential ultracentrifugation-based techniques are the most common EV isolation methods in basic research, but they are not scalable, do not give pure EV preparations, may lead to EV aggregation, and are time-consuming. However, sequential centrifugation steps have been used for large-scale production of clinical-grade MSC-EVs (Mendt et al., 2018). Size-based fractionation methods that include tangential flow filtration and size exclusion chromatography are GMP-compliant and scalable systems for EV isolation [for reviews, see Agrahari et al. (2019); Paganini et al. (2019)]. Ultrafiltration also reduces the isolation times and costs compared with other techniques (Saxena et al., 2009; Bari et al., 2018, 2019b,c). In our opinion, currently, this is the method of choice for high-scale and GMP-compliant isolation of EVs. A comparative analysis of the secretome from BM- and AD-MSCs enriched by ultrafiltration or sequential ultracentrifugation indicated that ultrafiltration results in higher particle yield with higher protein content, in GMP-compliant conditions (Bari et al., 2019c). Finally, the choice of the isolation technique will have to be a compromise

between EV yield and cost. In addition, it is important to keep in mind that each variation in the production process generates a product modification that will require a new functional qualification.

Quality Controls

Quality controls concern MSC characterization and expansion, EV production and isolation, and the release criteria of EV batches [recently updated in Thery et al. (2018); Rohde et al. (2019)]. Attempts to standardize the methods of EV isolation and characterization are regularly discussed within ISEV. To measure the production yield, the number of isolated EV particles needs to be determined by Nanoparticle Tracking Analysis (NTA) or Tunable Resistive Pulse Sensing (TRPS). These methods allow measuring the number of particles in a solution. The production yield should be expressed as the particle number in cell equivalents because it takes into account the number of viable cells at harvest time and allows a better evaluation of the inter-batch reproducibility. Although there is no standard size for EV preparations, a size of $\leq$200 nm characterizes small EVs and could be defined as the standard to ensure better inter-batch reproducibility. EVs must also be profiled by flow cytometry or western blotting: expression of EV markers (CD9, CD63, TSG101, and CD81) and MSC markers (CD44, CD73, CD90, and CD105), and absence of signal for immune cell markers (CD14, CD34, and CD45). The presence of at least three different markers enriched in EVs should be a major criterion for batch release: CD9, CD63, CD81, Tsg101, Alix, and the ganglioside GM1, which has been described as an exosome marker (Tan et al., 2013). Finally, standard safety tests to exclude microbial impurities should be performed to determine the endotoxin levels, sterility, absence of mycoplasma, and absence of viral enrichment in the final product.

Additional information on protein and RNA concentration, which is not part of the released criteria, could be added to the quality control list. This information allows expressing the number of EVs as particles per μg of protein or RNA, and could be used to assess inter-batch reproducibility. In addition, specific microRNAs or proteins, known to be relevant for EV therapeutic effect, could be identified and quantified by quantitative PCR and ELISA assays to provide supportive data on EV functional properties. This will be relevant to define potency assays for EVs in relation to the dedicated clinical applications.

Storage and Stability

The preservation of EV biological activity during storage is both critical and challenging. Few studies have reported consistent data on EV storage and formulation. Siliconized vessels are recommended for EV storage to prevent their adherence to surfaces and their loss (Jeyaram and Jay, 2017). Phosphate buffered saline is habitually used for EV resuspension. Storage at −80°C is encouraged, although it can affect EV size, number and function (Lorincz et al., 2014; Cosenza et al., 2018). It has been reported that EV concentration (quantified by NTA) remains stable after 1 week of storage at +4, −20, and −80°C (Jeyaram and Jay, 2017). Nevertheless, storage at +4°C causes EV aggregation, and the amount of the associated proteins and miRNAs dramatically decreases at +4°C and −20°C. For clinical applications, EV products need to be suspended in sterile 0.9% NaCl and stored at −80°C. Moreover, they should be frozen and thawed rapidly to preserve their morphology and function. EV products should be formulated for single-use because it has been observed that their number decreases, and their morphology and content are altered after two cycles of freezing and thawing (Kusuma et al., 2018).

The possibility to freeze-dry the EV products for long-term storage at room temperature has been investigated. Freeze-drying preserves EV characteristics and function, and thus might represent a cost-effective storage strategy. It also reduce transport costs (Charoenviriyakul et al., 2018). The characteristics and functionality of peripheral blood mononuclear cell-derived secretomes remain stable for up to 6 months after lyophilization and high dose γ-irradiation when stored between −20 and +25°C (Laggner et al., 2020). However, such lyophilized secretomes contained albumin, cholesterol and triglycerides that might have preserved the sample bioactivity. Another study showed that the exosome number and size distribution and biological activity are not affected after storage at −80°C for 45 days or 6 months (Mendt et al., 2018). Disaccharide stabilizers could be added in the storage buffer to improve EV preservation. Trehalose is a natural, non-reducing disaccharide sugar used as a cryo-preservative for labile protein drugs, vaccines, and liposomes. Its safety and tolerance have been demonstrated in mice and humans after oral, gastric and parenteral administration (Sato et al., 1999; Richards et al., 2002). It has been reported that addition of trehalose to EV samples improves their stability when stored at −80°C and when lyophilized, by preventing EV aggregation and lysis (Bosch et al., 2016; Charoenviriyakul et al., 2018). Mannitol is another cryoprotectant that maintains the functionality of freeze-dried secretomes stored at −20°C for at least 2 months (Bari et al., 2019c). The addition of 5–10% dimethylsulfoxide (DMSO) also maintains EV integrity and function (Romanov et al., 2019). The possibility to develop an off-the-shelf lyophilized product is a huge strength compared with the parental cell product that must be frozen for preservation and must be transported fresh after revitalization and/or expansion, or frozen under stringent requirements. Finally, whatever the storage formulation and conditions, batch stability will have to be carefully examined and monitored during storage. Stability can easily be assessed by quantifying the particle number, the quantity of total RNA and proteins, and the MoA-associated bioactive factor at different times during storage.

REGULATORY ASPECTS FOR THE INDUSTRIAL AND CLINICAL USE OF EXTRACELLULAR VESICLES

The regulatory aspects for manufacturing and clinical applications of EVs as new therapeutics have to be implemented. In 2015, an ISEV position paper discussed the classification of EV-based products as biological medicine or biological drugs, and categorized EVs based on the anticipated active substance(s)

(Lener et al., 2015). It also provided a detailed discussion on the regulatory issues associated with EV-based therapeutics. According to the regulatory frameworks for manufacturing and clinical trials in Europe, United States and Australia, quality and safety control data must be provided, as underlined in the previous section. In addition, the existing guidelines require the identification, quantification and characterization of the main substance(s) of a biological drug to indicate the MoA. The active substance determines the pharmaceutical classification and the MoA, and will define the potency assay to be used (Rohde et al., 2019). However, we can expect that the MoA will not be limited to a single molecule, as it has been shown for MSCs, and it will be difficult to precisely define the active substance in EVs. We can also anticipate that the MoA, and associated bioactive factors, of a same MSC-EV batch may also depend on the targeted clinical application and the related therapeutic function. Interestingly, a review paper discussed the respective role of miRNAs and proteins as major factors in the MoA and in mediating EV therapeutic effect (Toh et al., 2018). A prerequisite for their potency is the presence of biologically relevant amounts of molecules. By analyzing the average quantity of miRNAs in MSC-EVs and the possible number of EVs taken up by a cell, the authors concluded that miRNAs are not likely to be in the right concentration or configuration to have a relevant biological activity. A similar analysis for proteins indicated that they were more likely to elicit a biologically relevant response, suggesting that proteins could be the main drivers of MSC-EV MoA. Nevertheless, it has been reported that several miRNAs are important actors, at least as mediators, of MSC-EV immunoregulatory effects (Martin-Rufino et al., 2019). More studies are needed to bring firm conclusions on the role of proteins and miRNAs in the MoA of MSC-EVs. Although not required in the early stages of clinical development, the definition of the active substance(s) that supports the MoA and the efficacy of EV-based treatments is a requirement to develop appropriate pharmaceutical control strategies. Indeed, in the early phases 1 and 2 of pharmaceutical development, the batch-to-batch consistency must be checked using biochemical, biophysical and functional assays (Rohde et al., 2019). Due to their complex nature, the specific MoA of EVs may be difficult to identify; however, in contrast to cells, it could be easier to set-up quality control tests for EV characterization and inter-batch homogeneity assays (Riazifar et al., 2017). The regulatory requirements will also be different for EV-based drugs derived from cells (genetically engineered or not) and for EVs used as drug-delivery systems [for review see Lener et al. (2015)]. Compliance with the regulatory frameworks is pivotal for the approval of EV-based therapies and their large-scale implementation.

CONCLUSION: CHALLENGES AND PERSPECTIVES

MSC-EVs exert comparable therapeutic functions as their parental cells, but have some advantages over MSCs because they lack nuclei and cannot abnormally proliferate or differentiate. Moreover, small-size EV preparations that are isolated using protocols including a filtration step through 0.22 μm membranes can be considered as sterile and do not require an additional sterilization step. However, there are still many challenges to be addressed concerning the scalable production, standardization, and characterization of EV products for the successful translation of EV-based therapeutics in the clinic (Agrahari et al., 2019). The heterogeneity of MSCs used for EV production (BM, adipose tissue, other tissues; non-manipulated or immortalized) and of the obtained EVs (production process; EV size; contents of EV fractions) makes difficult to select the EV drug with the highest therapeutic efficacy. Several companies have already developed EV- or secretome-based products for different clinical applications using diverse cell sources, and MSC-derived EVs represent around 40% of such products (Gimona et al., 2017). The best sources for reproducible, safe and cost-effective production must be identified using the relevant non-clinical models for each specific clinical applications. Large-scale processes to manufacture EV therapeutics in GMP conditions (mainly bioreactor technologies for EV production, and ultrafiltration technologies for EV purification) are being implemented using preferentially closed systems for higher safety to ensure robust production procedures. Several GMP-compliant processes for the production of MSC-EV or secretome products have been developed (Pachler et al., 2017; Bari et al., 2018; Mendt et al., 2018; Laggner et al., 2020). Importantly, although quality controls for cell production include cell viability and apoptosis rate measurements, they do not assess cell senescence. Yet, EV yield is higher when using senescent cells, and their cargo composition is altered (abnormal levels of some miRNAs) [for review, see Boulestreau et al. (2020)]. The proportion of senescent cells in the production batches and their effect on EV content should be taken into account in quality control procedures. Moreover, EV standardization (protein or RNA quantification, particle determination) should be improved. In addition, some undesirable miRNAs, such as miR-410 that promotes carcinoma cell growth and aging-associated miRNAs, should be quantified (Fafian-Labora et al., 2017; Dong et al., 2018). Analytical methods to accurately characterize EVs at the single-vesicle level are under development and are needed for reliable standardization. Lyophilization could be used for the long-term storage of EVs and to develop off-the-shelf products with high stability. This would represent a real advantage compared with MSCs because it would facilitate and reduce the costs of storage and transport (at room temperature). The formulation of EVs into a standardized biological drug has to be defined for each clinical application in terms of dosage, excipients (use and type of cryoprotectant, for example) and pharmaceutical forms (powder or liquid) (Bari et al., 2019b). The formulation may depend on the administration route. While liquid formulations can be used for systemic or parenteral injection of EVs, powder formulations might be preferred for oral or aerosolized administrations. The dosages for a specific application will determine the batch sizes for production (Rohde et al., 2019). The procedure for the characterization of the active substance(s), which can be localized in the inner and/or outer

part of EVs, and of the "excipient" non-biologically active moiety of the EVs will have to be established before the industrialization step. It is now crucial to address the challenges related to the production and the regulatory and clinical aspects of EV-based biological products in order to pave the way to their commercialization.

AUTHOR CONTRIBUTIONS

All authors contributed to the design and writing of the manuscript. MM, PR, JB, and CJ proofread and given comments as well as suggestions. DN supervised and finalized the manuscript.

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Holistic Approach of Swiss Fetal Progenitor Cell Banking: Optimizing Safe and Sustainable Substrates for Regenerative Medicine and Biotechnology

*Alexis Laurent[1,2,3], Nathalie Hirt-Burri[1], Corinne Scaletta[1], Murielle Michetti[1], Anthony S. de Buys Roessingh[4], Wassim Raffoul[5] and Lee Ann Applegate[1,6,7]**

[1] Regenerative Therapy Unit, Lausanne University Hospital, University of Lausanne, Épalinges, Switzerland, [2] Tec-Pharma SA, Bercher, Switzerland, [3] LAM Biotechnologies SA, Épalinges, Switzerland, [4] Children and Adolescent Surgery Service, Lausanne University Hospital, University of Lausanne, Lausanne, Switzerland, [5] Plastic, Reconstructive and Hand Surgery Service, Lausanne University Hospital, University of Lausanne, Lausanne, Switzerland, [6] Oxford Suzhou Center for Advanced Research, Science and Technology Co., Ltd., Oxford University, Suzhou, China, [7] Competence Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zurich, Switzerland

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Ivan Martin,
University of Basel, Switzerland

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Paul Emile Bourgine,
Lund University, Sweden
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Karolinska Institutet (KI), Sweden

***Correspondence:**
Lee Ann Applegate
Lee.Laurent-Applegate@chuv.ch

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Safety, quality, and regulatory-driven iterative optimization of therapeutic cell source selection has constituted the core developmental bedrock for primary fetal progenitor cell (FPC) therapy in Switzerland throughout three decades. Customized Fetal Transplantation Programs were pragmatically devised as straightforward workflows for tissue procurement, traceability maximization, safety, consistency, and robustness of cultured progeny cellular materials. Whole-cell bioprocessing standardization has provided plethoric insights into the adequate conjugation of modern biotechnological advances with current restraining legislative, ethical, and regulatory frameworks. Pioneer translational advances in cutaneous and musculoskeletal regenerative medicine continuously demonstrate the therapeutic potential of FPCs. Extensive technical and clinical hindsight was gathered by managing pediatric burns and geriatric ulcers in Switzerland. Concomitant industrial transposition of dermal FPC banking, following good manufacturing practices, demonstrated the extensive potential of their therapeutic value. Furthermore, *in extenso,* exponential revalorization of Swiss FPC technology may be achieved *via* the renewal of integrative model frameworks. Consideration of both longitudinal and transversal aspects of simultaneous fetal tissue differential processing allows for a better understanding of the *quasi*-infinite expansion potential within multi-tiered primary FPC banking. Multiple fetal tissues (e.g., skin, cartilage, tendon, muscle, bone, lung) may be simultaneously harvested and processed for adherent cell cultures, establishing a unique model for sustainable therapeutic cellular material supply chains. Here, we integrated fundamental, preclinical, clinical, and industrial developments embodying the scientific advances supported by Swiss FPC banking and we focused on advances made to date for FPCs that may be derived from a single organ donation. A renewed model of single organ donation bioprocessing

is proposed, achieving sustained standards and potential production of billions of affordable and efficient therapeutic doses. Thereby, the aim is to validate the core therapeutic value proposition, to increase awareness and use of standardized protocols for translational regenerative medicine, potentially impacting millions of patients suffering from cutaneous and musculoskeletal diseases. Alternative applications of FPC banking include biopharmaceutical therapeutic product manufacturing, thereby indirectly and synergistically enhancing the power of modern therapeutic armamentariums. It is hypothesized that a single qualifying fetal organ donation is sufficient to sustain decades of scientific, medical, and industrial developments, as technological optimization and standardization enable high efficiency.

Keywords: biotechnology, cell therapy, clinical cell banking, fetal cell transplantation, primary fetal progenitor cells, regenerative medicine

INTRODUCTION

Evolution of Regenerative Medicine and Cell Therapies

Changes in demographics and lifestyle worldwide lead to drastic modifications in the incidence and prevalence of degenerative diseases afflicting the musculoskeletal system and cutaneous structures in general. Accidental trauma continuously yields high numbers of acute clinical cases necessitating considerable therapeutic resources. High demand for efficacious preventive and curative treatments has prompted increasing effort and resource allocation in translational medical research and development. A specific focus area has been the development and implementation of innovative products or protocols designed to optimize biological functions or complement traditional surgical management (Déglise et al., 1987; Vacanti and Langer, 1999; Marks and Gottlieb, 2018). In this context, regenerative medicine currently diversifies into vast arrays of novel therapeutic leads, among which cell therapies and cell-based products represent prime prospects. Indeed, such therapies or products, initially proposed over a century ago by Dr. Brown-Séquard and later by Dr. Niehans in Switzerland, constitute multiple potential clinical implementations toward tissue repair optimization and normal organ and system function restoration (Abdel-Sayed et al., 2019b). The reconstitution of maximal patient health can be efficiently implemented through synergistic combinations of tissue engineering, specialized surgical techniques, or classical pharmaco-therapeutic management (Montjovent et al., 2004; Bach et al., 2006; Loebel and Burdick, 2018; Costa-Almeida et al., 2019).

Importance of Standardized Therapeutic Cell Sources

For classic and novel biological active pharmaceutical ingredients (API), preliminary considerations and prerequisites for biological product development or cell therapy studies reside in the starting materials and cell sourcing. Numerous heterogeneous biological sources have been considered by researchers in human regenerative medicine. Autologous, allogenic, or xenogenic biopsies of various developmental stages may be processed, whereas specific cultured progeny cells retain inherent multifactorial problems to be projected in therapeutic product development processes. Imperative technical, biological, clinical, and sustainability considerations, therefore, help condition and orient cell source selection procedures. Requirements for potential transformation and widespread therapeutic use comprise safety and consistency, availability in adequate quantities, traceable characterization, sufficient inherent expansion capacity, and compatibility with acceptable delivery methods such as engineered bioscaffolds (Doyle and Griffiths, 1998; Monti et al., 2012). Diverse classes of cell sources fit these restrictive criteria, including, but without being limited to, fetal progenitor cells (FPC), embryonic stem cells (ESC), adult stem cells [adipose stem cells (ASC), bone marrow-derived mesenchymal stem cells (BM-MSC), itMSC (ischemia-tolerant mesenchymal stem cells)], neural stem cells (NSC), limbal stem cells (LSC), hematopoietic stem cells (HSC), endothelial progenitor cells (EPC), umbilical cord cells, neonatal foreskin cells, platelets, placenta, and amniotic fluid

Abbreviations: API, active pharmaceutical ingredient; ASC, adipose stem cell; ATMP, advanced therapy medicinal product; BMSC, bone marrow stromal cell; BPyV, bovine polyomavirus; cATMP, combined advanced therapy medicinal product; CD, cluster of differentiation; cGMP, current good manufacturing practices; CHUV, centre hospitalier universitaire vaudois; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; EBV, Epstein-Barr virus; ECM, extracellular matrix; EOPCB, end of production cell bank; ePBB, equine progenitor biological bandage; EPC, endothelial progenitor cell; FBS, fetal bovine serum; FPC, fetal progenitor cell; GF, growth factor; GMP, good manufacturing practices; HA, hyaluronic acid; HAV, hepatitis A virus; HBoV, human bocavirus; HBV, hepatitis B virus; hCMV, human cytomegalovirus; HCV, hepatitis C virus; HSC, hematopoietic stem cell; HE, hematoxylin and eosin; HHV-6/7/8, human herpes viruses types 6, 7 and 8; HIV-1/2, human immunodeficiency viruses types 1 and 2; HLA, human leukocyte antigen; HPL, human platelet lysate; HPV, human papillomavirus; HTLV-1/2, human T-cell leukemia-lymphoma viruses types 1 and 2; HuPyV, human polyomavirus; IB, investigator's brochure; IMPD, investigational medicinal product dossier; iPSC, induced pluripotent stem cell; itMSC, ischemia-tolerant mesenchymal stem cell; KIPyV, KI polyomavirus; LSC, limbal stem cell; MCB, Master Cell Bank; MoA, mechanism of action; mRNA, messenger ribonucleic acid; MSC, mesenchymal stem cell; NSC, neural stem cell; PBB, progenitor biological bandage; PCB, Parental Cell Bank; PCR, polymerase chain reaction; PDT, population doubling time; PDV, population doubling value; PS, penicillin-streptomycin; Px, passage number x; RNA, ribonucleic acid; SV40, simian virus 40; TEM, transmission electron microscopy; TEP, tissue engineering product; WCB, Working Cell Bank; WUPyV, WU polyomavirus.

cells (Vertelov et al., 2013; Heathman et al., 2015; Mount et al., 2015; Muraca et al., 2017; Li and Maitz, 2018; Sacchetti et al., 2018; Jayaraj et al., 2019; Torres-Torrillas et al., 2019). Most available cell sources are technically demanding, as progeny cells require dedicated processing or biochemical manipulation to orient or stabilize their potency and self-renewal capacity. Technical limitations related to sub-optimal intrinsic biological parameters significantly hinder the development of therapeutic cellular products. Increased complexity and costs have belated the development or lengthened the pathways for product market approvals (Heathman et al., 2015; Mount et al., 2015). Potential obstacles comprise low cell proliferation potential, the relative scarcity of the source within donors, high phenotypic plasticity or highly variable differentiation potential, tendency to serve as a communicable disease vector, or mediocre *in vitro* stability and lifespan (Rayment and Williams, 2010; Ratcliffe et al., 2011; Abbasalizadeh and Baharvand, 2013; Heathman et al., 2015; Hunsberger et al., 2015).

Allogenic FPC Technology for Translational Research

Pragmatic optimization of cell source selection and processing is crucial within translational development and clinical implementation of cell therapies and related products. Iterative amelioration and successful application of standardized workflows have led to identify allogenic primary FPC sources as highly promising and efficient candidates for regenerative medicine (Hebda and Dohar, 1999; De Buys Roessingh et al., 2006; Mirmalek-Sani et al., 2006; Metcalfe and Ferguson, 2007, 2008; Larijani et al., 2015; Grognuz et al., 2016b; Kim et al., 2018). Upon adequate isolation from fetal tissues (i.e., enzymatic or mechanical methods), culture-expansion and cryopreservation, progeny cells and derivatives present numerous advantages. Fetal progenitor cells differentiate until acquiring stable phenotypic (i.e., tissue-specific) characteristics, while retaining intrinsic feeble immunogenic potential, high longitudinal expansion capabilities, and potent stimulatory effects (Quintin et al., 2007; Laurent et al., 2020d). Additionally, such cell types possess few growth requirements to establish an adherent monolayer culture, have high cytocompatibility with various bio-constructs, are resistant to oxidative stress, and have trophic or paracrine mediator effects toward scarless wound healing (Shah et al., 1994; Cass et al., 1997; Doyle and Griffiths, 1998). Furthermore, validation of consistent and robust FPC banking at an efficient industrial scale following good manufacturing practices (GMP) is enabled by continued evaluation of sterility, safety, identity, purity, potency, stability, and efficacy (Quintin et al., 2007). Such prerequisite characteristics defined under restrictive regulations and quality standards for biologicals and starting materials for cell therapies or cell-based products must be investigated rapidly within product development pathways (Doyle and Griffiths, 1998). Allogenic FPC therapies may therefore demonstrably minimize delays in medicinal product availability, as extensive cell banks may serve for direct clinical application or further product developments. Although certain FPCs have yet to demonstrate potential performance advantages when compared to adult cell types in large *in vivo* settings, clinical insights from the past two decades in our Lausanne Burn Center have outlined the superiority of dermal FPCs versus standard cell therapy products and therapies in use (i.e., autologous platelet-rich plasma, cultured epithelial autografts, cultured dermal-epidermal autografts). Multiple clinical trials in Switzerland and in Asia (i.e., Japan, Taiwan) have confirmed the potential for diversified therapeutic uses of dermal FPCs (e.g., FE002-SK2 cell type) as cell therapies. Additionally, our group has three decades of clinical experience with cell-based cell-free topical formulations (i.e., ovine FPC-based cell-free products) classified as cosmetics or medical devices, which were and are used by clients and patients around the world, with positive feedback related to numerous diversified cutaneous affections.

Translation, Industrial Development, and Commercialization of Swiss FPC Technology

Cell therapies have been the focus of many public and private sponsors, whereas successful development is highly dependent on interprofessional collaboration integrating all complementary dimensions of novel products and protocols (Marks and Gottlieb, 2018). Allogenic cell-based therapies comprising cell culture steps may be classified as advanced therapy medicinal products (ATMP), and derivatives, as medical devices, whereas using correctly harnessed, consistent, and robust cell sources yields enormous advantages (Applegate et al., 2009; Marks and Gottlieb, 2018). Indeed, fundamental safety and traceability elements are required to prepare investigational medicinal product dossiers (IMPD) and investigator's brochures (IB), whereas optimal biological starting materials may be procured and processed through well-defined Fetal Transplantation Program workflows (Rayment and Williams, 2010; Heathman et al., 2015; Laurent et al., 2020f). Additionally, the robustness of multi-tiered primary FPC biobanks ensures optimal and cost-effective manufacturing for processes which require biological material sourcing. Pragmatic devising and implementation of Fetal Transplantation Programs can realistically be achieved in less than six months, with investment costs around a million Swiss Francs (CHF), to establish a GMP parental cell bank (PCB). Assuming total valorization of progeny cellular materials, industrial development efforts may be sustainably equipped for decades and potentially generate trillions of CHF in revenues following a single organ donation. In addition, direct costs of active principles (i.e., viable cells or cell-free extracts) are negligible within market-approval and commercialization steps of standardized bioengineered therapeutic agents. Unique conjunctures of high innovation and local incentives toward industrial development and commercialization of life science products in Western Switzerland (i.e., Health Valley) have led to the development and marketing of Swiss FPC banking and therapeutic/regenerative derivatives in the past decades. Swiss FPC technology is well adapted to tackle regulatory and industrial manufacturing challenges, while safely and effectively supplying arrays of core and adjuvant therapeutic components for highly innovative Swiss-made products globally. Notably, several

patents and two University Hospital spin-offs (i.e., ELANIX Sàrl and Neocutis SA) have contributed to translational developments or commercialization of tissue engineering products (TEPs) or cosmeceutical products around the world.

Hypothesis Formulation: One-Shot Fetal Transplantation Program

Optimal management of safety and consistency of therapeutic cell sources is attained by avoiding the pooling of numerous heterogeneous biological samples. Therefore, pragmatic devising and exploitation of Fetal Transplantation Programs present unique characteristics and considerable advantages, outlined throughout two decades of translational research on FPCs in Switzerland. Indeed, ethical and controlled revalorization of a single qualifying therapeutically aborted fetus and donated tissues enables, in a unique way, the differential and simultaneous establishment of multiple primary FPC types (e.g., derived from skin, cartilage, tendon, muscle, lung, bone, connective tissue, intervertebral disc). Furthermore, such transversal conceptual approaches to biobanking have been successfully experimentally validated and iteratively optimized for human, equine, and ovine FPC types in Switzerland (**Table 1**; Applegate et al., 2013; Laurent et al., 2020b,e). Thereby, each individual and tissue-specific cell source may be selectively applied to complementary cutaneous or musculoskeletal regenerative medicine applications and biotechnological developments. Here, we integrated fundamental, preclinical, clinical, and industrial implementational developments representing the scientific advances supported by multi-tiered FPC banking in Switzerland. Overall, cultured FPCs appear as optimal fits for modern regulatory framework development and stringent GMP industrial transposition in a rapid, safe, effective, and traceable manner (Laurent et al., 2020e,g). The benefit of the Swiss FPC technology described herein is the safe, standardized, ethical, and continual high-value supply chain design for unique diversified biological assets. It is hypothesized that a single qualifying fetal organ donation is sufficient to sustain decades of scientific, medical, and industrial developments, as related technological optimization and standardization enable high efficiency. The range of possible valorization applications levels with the *quasi*-indefinite potential material yield of multi-tiered FPC biobanks. The core therapeutic value of optimized and comprehensive Fetal Transplantation Programs enables sustainable and widespread treatment of millions of patients suffering from cutaneous and musculoskeletal diseases with affordable and effective therapeutic products. The main goal of this work was to substantiate, convey, and broaden awareness and interest around the use of standardized protocols for translational regenerative medicine utilizing FPCs. The renewed transversal and longitudinal model of single organ donation bioprocessing described herein shall continue to provide persistent contributions to modern translational regenerative medicine and biopharmaceutical therapeutic product manufacturing, increasing the power of modern therapeutic armamentariums. An overview of implemented therapies used for managing burns and wounds over the past two decades will be highlighted. In addition, progress on characterization and preclinical work on other tissue-specific FPC types will be reviewed, in order to show parallels in pathways to implement new clinical treatments.

CLASSIC CURRENTS OF THOUGHT: SCARCITY AND POOLING OF THERAPEUTIC CELL SOURCES

In human organ transplantation, the relative scarcity of high therapeutic value biological materials often requires compromise, while maintaining adequate safety and quality standards (Glantz et al., 2008). In the case of blood banks for medical transfusion or industrial-scale manufacturing of human platelet lysate (HPL) and fetal bovine serum (FBS), pooling of multiple donor samples is necessary to achieve the required lot size to produce coherent deliverable quantities after adequate safety and quality testing is performed. Similarly, production of homogenized cell pools for industrially commercialized therapeutic products (e.g., pooled neonatal foreskin keratinocytes) assumes the integration of many variables and potentially heterogeneous components, albeit meeting the specifications for lot qualification and liberation, achieved due to large numbers of donors. Such practices and related technical considerations are well accepted and detailed in pharmacopeia sections on blood-related products, for example. Considerable advantages of focusing efforts on a single donor yielding homogenously derived cell sources enable the abolition of the variability mentioned above, while enabling extensive and rational testing of biological materials. Indeed, screen-testing of donors for pools is then replaced by extensive safety testing of the mother-donor in the Fetal Transplantation Program, followed by routine testing of cell production lots, inherently implemented in GMP workflows, resulting in relatively low overall normalized costs. The consistency, robustness, and extensive cellular expansion capacities within FPC biobanks allow maximal characterization and standardization of biological substrate variables. These crucial aspects were most helpful in the early route to such optimized sources for vaccine or recombinant protein production by the pharmaceutical industry (Applegate et al., 2010). Additionally, optimal conservation and persistence of cellular characteristics throughout whole-cell bioprocessing and maintenance of extensive *in vitro* lifespans negate the necessity of primary cell immortalization into cell lines, thereby minimizing artificial manipulation of the biological materials (Applegate et al., 2009). Low heterogeneity exists between different fetal organ donations and between different samples consistently processed from the same biopsy (Quintin et al., 2007). Optimal consistency in cellular expansion parameters and endpoint cell yields may be achieved, as FPCs do not rely on growth factor supplementation for phenotypic modulation. A paradigm shift toward the replacement of pooled biological materials by cultured FPCs would surely result in optimized availability and affordability of therapeutic products or biotechnological substrates, while maximizing both consistency and safety, due to the numerous relative advantages of FPC biobanking, as described hereafter.

TABLE 1 | Overview of primary FPC types established and studied within the Swiss FPC Transplantation Programs, with respective applications and gathered experiences.

FPC types	Scope of work and gathered experience	Cell type lifespan characteristics	Selected references
Human dermal FPCs (e.g., FE002-SK2 cell type)	The most clinical experience around cutaneous tissue regeneration has been gathered using such cell types, effectively applied for managing severe burns, refractory ulcers, or donor-site wounds. Safety and efficacy of such therapeutic materials have been demonstrated in various clinical trials. Thorough experience has been gathered around industrial GMP manufacturing transposition for commercialization of cell-based or cell-derivative products. The extensive industrial biobanking potential was validated using the FE002-SK2 cell type	In preclinical works, FE002-SK2 cells were studied up to P18–P20 In clinical settings, FE002-SK2 EOPCBs were established and validated at P12 Current clinical protocols describe the use of cells at P8 When using the same isolation and culture methods as described for FPCs, adult dermal fibroblasts are generally characterized by a lifespan of 6–7 passages	Hohlfeld et al., 2005 Quintin et al., 2007 Hirt-Burri et al., 2011 De Buys Roessingh et al., 2015 Laurent et al., 2020e,h
Human tendon FPCs (e.g., FE002-Ten cell type)	Such cell types have been extensively characterized *in vitro* and were shown to optimally adapt to drug delivery solutions for whole tissue replacement or localized regeneration stimulation of wounded tendons. *In vivo* applications in rabbit models have preliminarily confirmed safety of such cell types	In preclinical works, FE002-Ten cells were characterized by a lifespan of 12–15 passages Recommended passages for therapeutic applications are P6 (cell therapies) to P8 (cell-based cell-free formulations) When using the same isolation and culture methods as described for FPCs, adult tenocytes are generally characterized by a lifespan of 7–8 passages	Grognuz et al., 2016a,b Aeberhard et al., 2019 Grognuz et al., 2019
Human cartilage FPCs (e.g., FE002-Cart.Art cell type)	Optimal homogeneity, phenotypic plasticity, and chondrogenic potential have been demonstrated for such cell types, whereas application in caprine models for articular reconstruction has yielded preliminary evidence of safety. Detailed investigation of biochemical and biomechanical parameters of extracellular matrix deposition were performed using such cell types	In preclinical works, FE002-Cart.Art cells were characterized by a lifespan of 10-12 passages, whereas optimal functionality (i.e., ECM generation) was confirmed up to P5 Recommended passages for therapeutic applications are P5 (cell therapies) to P8 (cell-based cell-free formulations) When using the same isolation and culture methods as described for FPCs, adult chondrocytes are generally characterized by a lifespan of 6–8 passages	Quintin et al., 2010 Darwiche et al., 2012 Broguiere et al., 2016 Studer et al., 2017 Cavalli et al., 2018 Li et al., 2020
Human bone FPCs (e.g., FE002-Bone cell type)	Detailed investigation of phenotype modulation and matrix production activities were performed on such cell types, providing extensive insights on the multiple parameters within optimization of skeletal tissue engineering. Murine and rat models have demonstrated safety of application of such cell types	In preclinical works, bone FPCs were studied up to P8–P9 Recommended passages for therapeutic applications are P5 (cell therapies) to P7 (cell-based cell-free formulations)	Montjovent et al., 2004, 2007, 2008, 2009 Hausherr et al., 2017, 2018
Human muscle FPCs (e.g., FE002-Mu cell type)	High interest for applications in tissue reconstruction was evidenced for such cell types, whereas application in murine models has demonstrated safety and absence of immunogenicity for such cell types	In preclinical works, muscle FPCs were studied up to P4–P5	Hirt-Burri et al., 2008a Laurent et al., 2020c
Human intervertebral disc FPCs (e.g., FE002-Disc cell type)	*In vitro* characterization has allowed to establish the tangible potential of such sources for application in skeletal tissue engineering and amelioration of patient quality of life	In preclinical works, intervertebral disc FPCs were studied up to P4–P6	Quintin et al., 2009, 2010
Human lung FPCs (e.g., FE002-Lu cell type)	Such cell sources were studied and benchmarked with currently used biotechnological cellular substrates (e.g., MRC-5), demonstrating high potential for implementation in industrial workflows with augmented safety, consistency, stability, and output. Therapeutic exploitation of anti-inflammatory properties is considered	In preclinical works, FE002-Lu cells were studied up to P20	NA
Ovine FPCs (e.g., AG001-AG005 cell types)	Combination of ovine FPC banking and biotechnological processing has demonstrated the potential for stabilization of tremendous healing stimulation properties and application thereof for topical regenerative effects. Extensive *in vitro* lifespans and high consistency were demonstrated for various primary ovine FPC types, constituting tangible advantages for biological product supply chain sustainability	In preclinical works, ovine FPCs were studied up to P40	Lapp et al., 2013

(Continued)

TABLE 1 | Continued

FPC types	Scope of work and gathered experience	Cell type lifespan characteristics	Selected references
Equine FPCs (e.g., ED001-ED002 cell types)	The simultaneous multi-organ harvest workflow adopted for human fetal donations was conceptually confirmed and experimentally validated using equine fetal tissues. Subsequent characterization and therapeutic applications of equine FPC therapies have demonstrated high similarities with human regenerative medicine and further broaden the potential therapeutic applications of primary FPC banking	In preclinical works, equine FPCs were studied up to P10	Laurent et al., 2020b

*Specific cell type lifespan characteristics were included, expressed as passages (see **Supplementary Material**). Data succinctly summarize primary and secondary published works, for which selected references are provided. Due to the renewal of regulatory frameworks and successive adaptations of the Transplantation Programs throughout the years, data accumulated over two decades was generated with primary FPC types isolated from different donations. Unification and standardization of FPC clinical use was operated after processing of the FE002 fetal donation in 2009. The overarching conclusions are the high consistency, extensive banking potential, and proven safety of various primary FPC types of mammalian origin. NA, Non-Applicable.*

SWISS FETAL TRANSPLANTATION PROGRAMS

Usefulness and adequacy of Fetal Transplantation Programs are most easily demonstrable, and the utilization of robust FPC banks may contribute to the alleviation of the constant organ transplant demand or shortages. The practical design of optimal workflows for cell source selection and processing is paramount when developing cell therapy, tissue bioengineering, or cell-based products. Along with biological material homogeneity, consistency, and robustness, documented traceability and quality also ensure safety and efficacy for clinical applications (Kent and Pfeffer, 2006; Pfeffer and Kent, 2006). Optimization must, therefore, be undertaken for the identification of cell sources, material procurement, and subsequent processing. Transplantation Programs are highly regulated and adaptable frameworks optimally suited for such exhaustive and descriptive activities. Swiss FPC Transplantation Programs were devised in the early 1990s in Lausanne to establish cell banking of primary FPC types after regulated voluntary pregnancy terminations and subsequent organ donations (Applegate et al., 2013). Initially registered in 1991 and reorganized in 2007, the successive Transplantation Programs remain regulated by Swiss federal laws, pertaining to organ transplant procedures, and are registered with the Swiss National therapeutic products agency (i.e., Swissmedic, Bern, Switzerland). Key stakeholders in the Program collaboratively pool complementary professional expertise and capabilities to fulfill respective duties and ensure adequate compartmentalization (**Figure 1**). Adequate documentation enables appropriate Program validation and follow-up, comprising technical specifications, fetal biobank regulations, and mandatory license documents. Highly regulated and sequentially defined voluntary and therapeutic pregnancy interruptions serve as the operating base for mother-donor recruitment. Regulatory vetting and GMP constraints relative to traceable tissue procurement, testing, and bioprocessing favor an up-stream medical and serological testing approach (i.e., repeated bloodwork for HIV-1/2, HTLV-1/2, hCMV, EBV, HHV-6/7/8, HSV, HBV, HCV, HPV, West Nile virus, syphilis) of mother-donors for inclusion in the Program, positively impacting long-term testing costs (**Supplementary Figure 1**; Quintin et al., 2007; Applegate et al., 2013). Practically, optimized workflows and specifications eventually enabled traceable simultaneous isolation of various FPC types (i.e., FPCs isolated from fetal tissues such as skin, cartilage, tendon, bone, muscle, intervertebral disc, lung) from a single fetal organ donation (i.e., codename FE002, 2009) for rapid and efficient PCB establishment and subsequent industrial GMP processing (Laurent et al., 2020e). Specific bioprocessing methodologies enable safe and sustained use of original cell sources for extended periods, as adequate testing implementation ensures maximal safety of the end-products or substrates (De Buys Roessingh et al., 2015). One single qualifying fetal organ donation, yielding specific tissue biopsies, is sufficient for the derivation of multi-tiered cryopreserved cell stocks, which may be preserved for decades, minimizing the need for multiple organ donations, ultimately lowering constraint levels related to timeframes and costs.

PRIMARY FPCs: STRONG SCIENTIFIC AND MEDICAL INNOVATION BACKGROUND

Historical Use of FPCs or Embryonic Cell Types and Cell Lines

Fetal and embryonic cells have been extensively used throughout history in the biomedical industry, starting back in the 1930s with the continuous development of numerous vaccines (e.g., chickenpox, Ebola, hepatitis A, HIV, influenza, Japanese encephalitis, polio, rabies, rubella, and smallpox), which are still currently in use (Jacobs et al., 1970; Reisinger et al., 2009; Applegate et al., 2013, 2017). A Nobel Prize in medicine was given in 1954 for the polio vaccine, developed using human fetal cell cultures. Such industrial uses demonstrate the *quasi*-universal applicability of fetal cells as substrates in therapeutic product manufacturing, providing excellent in-use safety and stability (Hayflick et al., 1962; Jacobs et al., 1970; Zimmerman, 2004; Olshansky and Hayflick, 2017). Specific human embryonic/fetal tissues and/or animal biopsies led to the establishment of well-known cell types or cell lines (e.g., HEK-293, MDCK, MRC-5, PER.C6, and WI-38/CCL-75) (Palache et al., 1997;

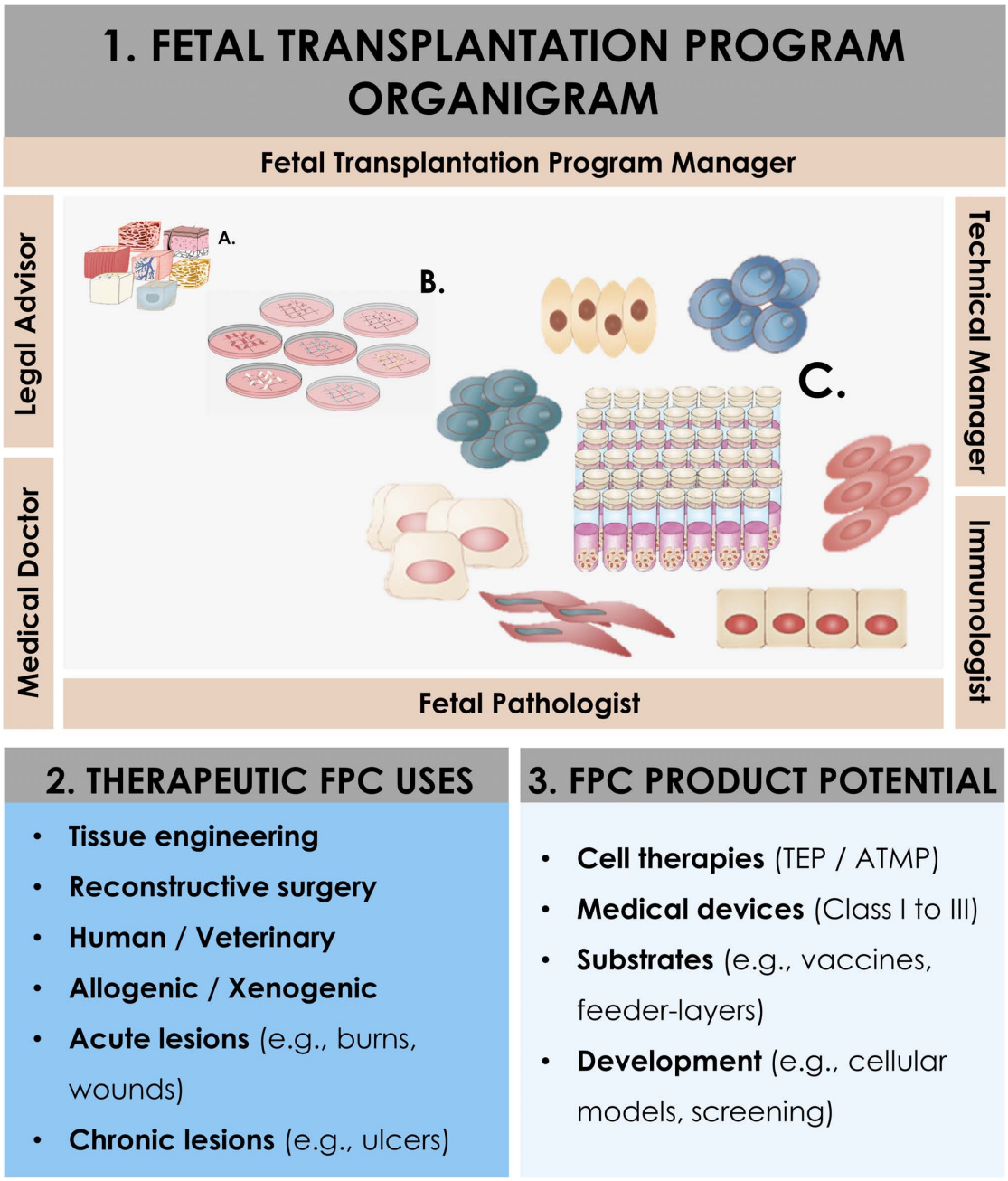

FIGURE 1 | Schematic overview of the components and ramifications of a Fetal Transplantation Program and of primary FPC banking in general, assorted to potential applications and benefits in view of product development. The high core-value is created throughout adequate biopsy procurement, bioprocessing thereof, and establishment of homogenous PCBs of primary FPC types. Essential multidisciplinary building blocks of a human Fetal Transplantation Program comprise complementary expertise and experience, mutualized between the Program Manager (i.e., establishment and coordination of the Program, usually a pharmacist or biologist with extensive experience in tissue processing and cell banking for optimized cell source selection and technical specifications establishment), the Legal Advisor (i.e., interpretation of regulatory frameworks for transplantation practice and therapeutic product use, design and validation of the Program within local and national laws, and regulations on research and medicine), the Technical Manager (i.e., oversight of the bioprocessing and cell banking steps, usually a biologist or senior laboratory technician with extensive experience in tissue processing and cell banking), the Medical Doctor (i.e., experienced gynecologist, performs donor identification, screening, consent obtention, and donation procurement, preferably from a secondary independent hospital), the Fetal Pathologist (i.e., oversight of coded autopsy, preferably experienced in fetal histopathology), and the Immunologist (i.e., pathogen screening of mother-donor biological samples and of established cell banks). A defined organigram enables optimal anonymous traceability within the information flow. Iterative validation steps ensure optimal quality and safety of all processed materials. Pathology and serology reports are evaluated to confirm requirement fulfillment and admissibility of the donor in the Program. Established PCBs are quarantined until the three-month bloodwork results exclude seroconversion of the donor for the target pathogens. Potential applications of banked FPC types are diverse, comprising cell therapy or medical device development for arrays of soft-tissue and musculoskeletal acute and chronic affections (i.e., human and veterinary), *in vitro* fundamental research, and industrial biotechnological manufacturing processes (e.g., viral vaccine production, feeder-layer roles).

Zimmerman, 2004). Early therapeutic use of fetal tissue or derived FPCs focused on neurology (e.g., Huntington's or Parkinson's disease, strokes, spinal cord injuries) (Freeman, 1997; Clarkson, 2001; Rosser and Dunnett, 2003; Reier, 2004; Savitz et al., 2004). Fetal hepatic cells were studied and transplanted to manage severe hematological disorders, immunodeficiencies, liver failure, diabetes, and congenital metabolic disorders (Touraine et al., 1993; Gridelli et al., 2012; Montanucci et al., 2013; Cardinale et al., 2014). In clinical settings, fetal hepatocyte infusions have been performed in more than 30 patients so far in view of alleviating transplant shortages, with promising results yielded mostly by one research group in India (Habibullah et al., 1994; Khan et al., 2010).

Specific Characteristics and Therapeutic Potential of FPCs

Fetal wound healing before mid-gestational stages is specifically and characteristically orchestrated, leading to regeneration without scar tissue formation in several organs and structures (e.g., skin, bone, cartilage, tendon) (Adzick and Longaker, 1992; Longaker et al., 1992; Namba et al., 1998; Beredjiklian et al., 2003; Bullard et al., 2003; Dang et al., 2003; Favata et al., 2006; Rodrigues et al., 2019). Cultured FPCs isolated after nine weeks of gestation are pre-terminally differentiated, possessing finite high expansion capacities, and scarless regeneration stimulation potentials, while presenting low risks of immunogenicity or tumorigenicity after transplantation (**Figure 2**; Doyle and Griffiths, 1998; Quintin et al., 2007; Markeson et al., 2015; Laurent et al., 2020d). Differential gene expression (e.g., genes coding for TGF-β2, BMP-6, GDF-10, midkine, or pleiotrophin) and related proteomic fingerprints may explain specific healing patterns mediated by adult cells and FPCs (Hirt-Burri et al., 2008b). As early descendants of stem cells, FPCs are found in diverse developed tissues (e.g., skin, intestine, blood system, brain), mediating tissue homeostasis and repair (Nakatomi et al., 2002). Along with the absence of self-renewal capacity, relatively restricted potency distinguishes FPCs and stem cells, as FPCs are reportedly unipotent or oligopotent, providing relatively superior phenotypic stability. Technically, FPCs are independent of growth factor supplementation or presence of cellular feeder-layers for *in vitro* cultures (Asahara et al., 1997; Seaberg and van der Kooy, 2003). This specific inherent advantage over undifferentiated MSCs or induced pluripotent stem cells (iPSC) primarily benefits consistency in manufacturing and industrial scale-up processes (Doyle and Griffiths, 1998; Ramelet et al., 2009; Zuliani et al., 2013; Tan et al., 2014; Lee et al., 2020). Constraints on production timelines and economic factors additionally favor the use of low-maintenance and robust cell types such as primary FPCs.

Immune Privileges of FPCs

Fetal progenitor cells are pre-immunocompetent and fail in eliciting immunological responses due to the lack of post-thymic T-lymphocytes in the first 13 gestational weeks (Gabbianelli et al., 1990; Crombleholme et al., 1991). Major histo-compatibility complex (MHC) antigen expression during fetal development is organ- and gestational age-specific (Foglia et al., 1986). Primary FPCs generally lack MHC class II proteins (e.g., HLA-DP, DQ, DR) and exhibit relatively low levels of MHC class I counterparts (e.g., HLA-A, B, C), approaching cell surface marker panels characterizing MSCs or neonatal foreskin keratinocytes, for example (Tsujisaki et al., 1987; Streit and Braathen, 2000; Le Blanc et al., 2003; Grognuz et al., 2016b). Specific fetal tissues were shown to express HLA-G, a known mediator of tolerogenic effects (Piccinni, 2010; Deschaseaux et al., 2011). Fetal progenitor cells therefore evade immune responses, possibly through immune-modulation and inhibition of $TCD8^{+}$ lymphocyte proliferation (Bartholomew et al., 2002; Le Blanc et al., 2003). Absence during normal human gestation of an immune reaction, despite *in utero* recognition of paternal HLA-C markers and modulatory effects of HLA-G on lymphocytic activity, additionally characterize the particular immune status of fetal tissues and FPCs (Rouas-Freiss et al., 1997; Ober, 1998; Carosella et al., 2008; Piccinni, 2010).

Technical Simplicity, Stability, and Robustness of FPCs

The ability of therapeutic cells to maintain inherent biological characteristics, when isolated *in vitro,* presents considerable potential for tissue engineering. Differential requirements for processing and clinical delivery specifically characterize ESCs, adult MSCs, and FPCs, whereas numerous technical advantages favor the use of the latter (Bhattacharya, 2004; Ostrer et al., 2006; Capes-Davis et al., 2010). Embryonic stem cells can be derived from the blastocyte (i.e., constituted by approximately 100 cells) between zero and two weeks after ovum fertilization. These "immortal" cells require growth factor support in culture or appropriate feeder-layers to sustain growth, potentially introducing inconsistencies in progeny cell populations. Additionally, ethical concerns, propensity toward tumorigenicity, and high potency render the obtention and use of such populations difficult. Embryonic fetal cells can be derived at timepoints between five and eight weeks of gestation (i.e., total size of $>10^3$ cells/embryo). Relatively restricted potency compared to ESCs characterizes these populations, but all other disadvantages remain, assorted to onerous culture and maintenance requirements. Fetal tissues (i.e., total size of $>10^6$ cell/fetus) exist in the developing organism between weeks number nine and sixteen of the gestational period. Fetal progenitor cells yielded by various fetal tissues are therefore pre-terminally differentiated and present defined tissue-specific properties and behaviors, which are conserved in monolayer *in vitro* cultures. In contrast, MSCs are scarce or difficult to isolate and to purify for obtention of adequate cell populations, are patient-specific because of immunological and safety factors, and therefore necessitate multiple organ donations, whereas culture scale-up is difficult to implement. Legal distinctions categorize work around cellular material existing before and up to eight weeks of gestation, as a federal license is required in Switzerland. Starting at nine weeks of gestation, studies with specific fetal tissue biopsies are regulated under Federal Transplantation Laws, and such tissues are defined as organ donations. Standardized isolation methods for FPCs in defined gestational timeframes

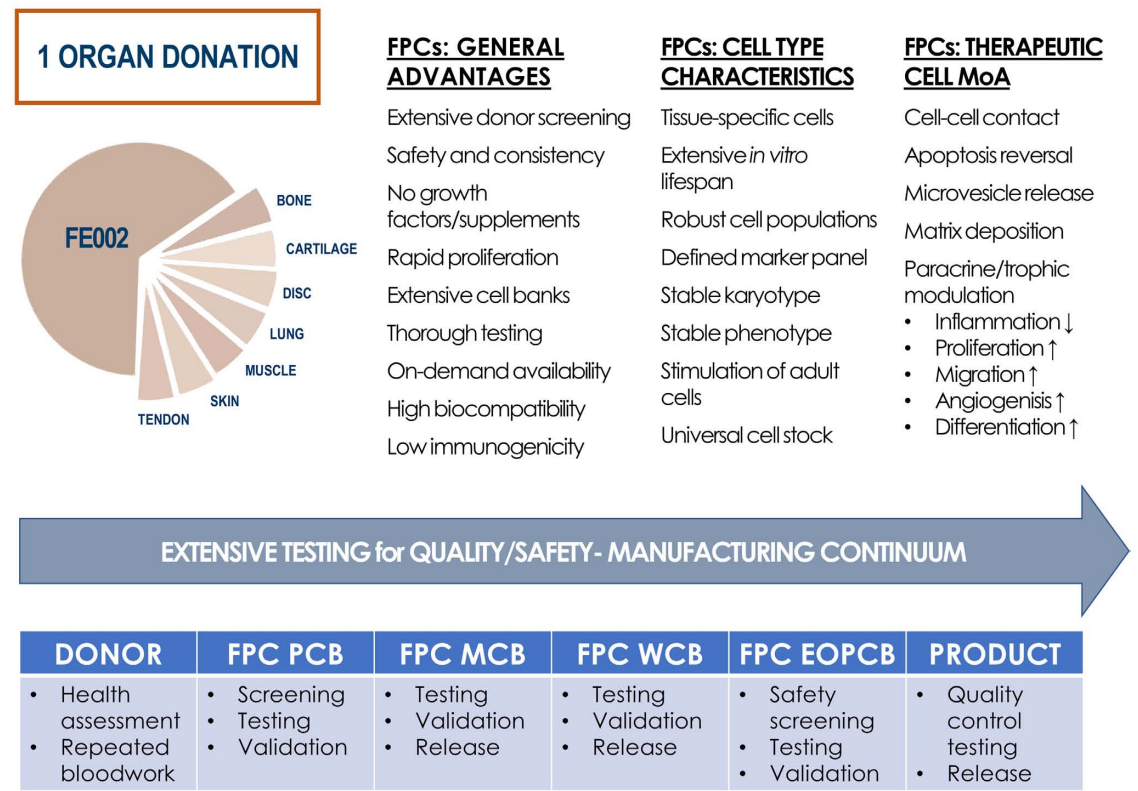

FIGURE 2 | Summary of biological and technological advantages of processing and using FPCs as therapeutic agents, production intermediates, or substrates. From one single fetal organ donation (i.e., FE002, 2009), various tissue samples (e.g., bone, cartilage, intervertebral disc, lung, muscle, skin, tendon) were bioprocessed for FPC isolation using both enzymatic and non-enzymatic methods. Inherent technical and clinical advantages are attributed to FPCs. Various proposed mechanisms of action (MoA) of allogenic FPCs are summarized. Optimized and consistent tissue procurement, cell isolation, and biobanking workflows allow for thorough testing throughout the manufacturing continuum, ensuring quality and safety of end-products.

yield uniform preliminary cultured populations characterized by homogenous and stable tissue-specific properties, without the need for specific cell-sorting (**Figures 2–5** and **Supplementary Figures 2–7**; Quintin et al., 2007, 2009, 2010). Progeny FPCs are characterized by their relatively high and consistent division potential *in vitro* before reaching senescence due to their relatively longer telomeres (Decary et al., 1997). Therapeutic applications in clinical protocols or product manufacturing workflows in regenerative medicine restrict the use of progeny cell sub-cultures to two thirds of the documented and safety-validated *in vitro* lifespans of specific cell types. Such regulations ensure end-product consistency and maintenance of paramount cellular biological properties, such as cumulative or specific protein content (e.g., MDK, MMP, TGF, TIMP, and VEGF levels), gene expression levels, and bio-stimulatory activities to be assessed *via* quantitative quality controls or functional assays (Vuadens et al., 2003; Quintin et al., 2007). A benefit of using allogenic banked cellular substrates instead of autologous sources is the drastic reduction in availability delays, as off-the-freezer cell therapies or stabilized cell-derivatives may be available upon request. Maximized safety and quality of end-products are demonstrable with banked FPCs, allowing realistic clinical translation, transposition to industrial settings, and commercial implementation in leading markets, well within current regulatory frameworks and sustainable developmental economic burdens (Quintin et al., 2007; Larijani et al., 2015; Marks and Gottlieb, 2018).

Swiss Multi-Tiered Biobanking Model for Primary FPCs

Optimal stability and consistency of FPCs derived from one single organ donation present a vast potential toward scalable and extensive biobanking, while following stringent safety- and quality-driven regulations for therapeutic product manufacturing (Abbasalizadeh and Baharvand, 2013; Hunsberger et al., 2015; Laurent et al., 2020e,g). Albeit finite, *in vitro* lifespans and expansion potentials of primary

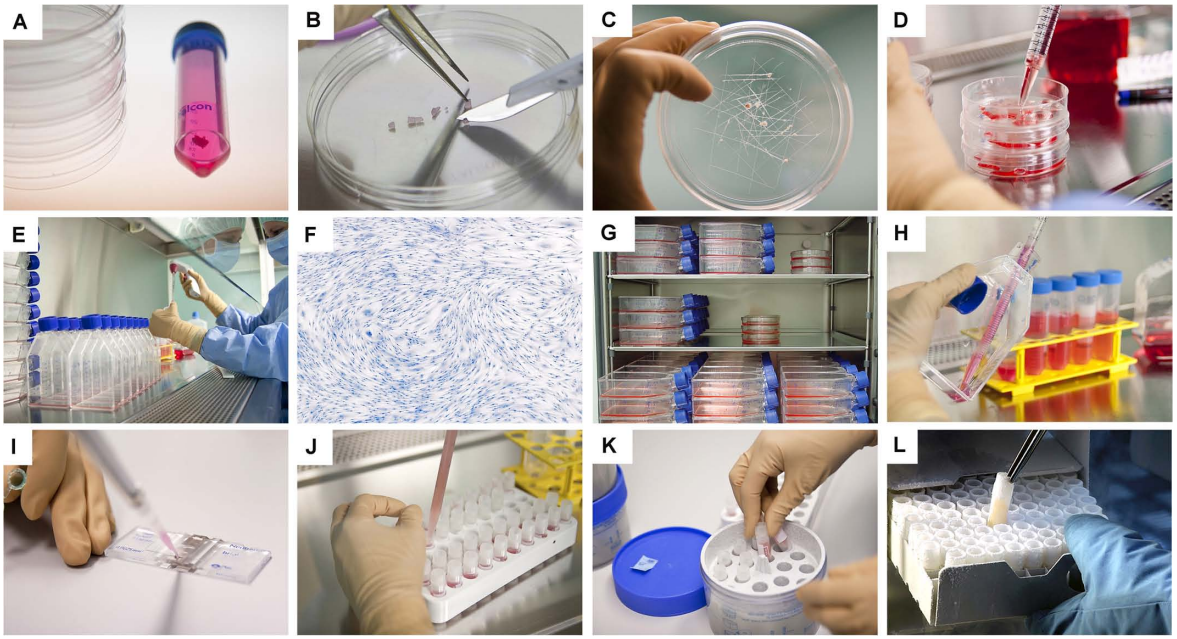

FIGURE 3 | Overview of the simple and standardized mechanical procedure for primary FPC type isolation from organ donation tissue biopsies and Parental Cell Bank establishment. Highly similar simultaneous processing of multiple tissues yielded by one organ donation enables maximal consistency throughout progeny cell populations. **(A)** Individual anonymized tissue biopsies are provided by the pathology department in transport medium. Each specific tissue of interest is separately conditioned. Fetal skin is used as an example herein. **(B)** Tissue biopsies are further processed into small fragments. **(C)** Tissue fragments are minced and placed within a checkboard pattern created on the culture surface by scoring with a sterile scalpel. **(D)** Cultures are initially fed with small amounts of growth medium in order to avoid early flotation of fragments. **(E)** Adherent cells are further expanded in culture flasks. **(F)** Cells are regularly microscopically assessed to verify adequate morphology or growth and to exclude contamination. **(G)** Multiple FPC types are simultaneously culture-expanded in humidified incubators set at 37°C under 80% relative humidity and 5% CO_2. **(H)** Confluent cells are harvested by trypsin detachment and pooled. **(I)** Total and viable relative cell counts are determined by microscopic enumeration using Trypan blue exclusion dye. **(J)** Cells are resuspended in a cryopreservation solution (i.e., DMEM, FBS, DMSO) and homogenously dispensed in individual cryovials (i.e., 10^6–10^7 viable cells/vial). **(K)** Vials are transferred to controlled-rate freezing devices (e.g., Mr. Frosty™ or CoolCells®) and placed in ultra-low temperature freezers (i.e., −80°C) overnight. **(L)** Cryovials are then transferred to Dewar storage tanks in the gaseous phase of liquid nitrogen for long-term storage. Some technical limitations in large-scale cell bank manufacturing are outlined and must be the object of continuous optimization. Such limits comprise, without being limited to, operator-related cell quantification, relatively important occupied volumes in conventional incubators with limited airflow and oxygenation, or relative contamination risks (e.g., open vessels for cryopreservation).

FPCs are sufficient for industrial-scale GMP manufacturing with minimal processing requirements. Standardized multi-tiered cell banking model establishment (i.e., sub-tiering cryopreserved cell stocks in Parental, Master, Working, and End of Production Cell Banks, PCB-MCB-WCB-EOPCB, with tier nomenclature based on *in vitro* passages) allows for efficient constitution, transposition, and utilization of consistent biological sources of high therapeutic value (**Figures 6**, **7**; De Buys Roessingh et al., 2013; Laurent et al., 2020e). Rapid establishment of such cryopreserved materials allows for *quasi*-infinite research and development, as each FPC type from the original organ donation may be valorized to provide $>10^7$–10^9 product doses. Local applications (e.g., skin, tendon, or cartilage tissue repair) of relatively small doses of cells or derivative equivalents (i.e., 5×10^5–10^6 units, cell type-specific) are optimal and preferable to systemic delivery, as they allow sparing use of biological materials, compared to alternative therapeutic cell sources (e.g., 10^8 cells/dose for MSCs or 10^9 cells/dose for pluripotent stem cells) (Hohlfeld et al., 2005; Pigeau et al., 2018; Pittenger et al., 2019). At the same time, safety testing and quality controls are easily implemented throughout bioprocessing workflows (**Figure 8**; Quintin et al., 2007). Derivation of multiple FPC types from a single organ donation and the development of robust analytical technologies drastically simplify screening and testing processes during manufacturing (e.g., tests for sterility, isoenzyme typing, mycoplasma, viruses, prions, endotoxins, virus-like particles, retroviral activity, fungi, yeasts, bacteria, and tumorigenesis assays) (Applegate et al., 2009). Maximized safety, efficiency, and optimized industrial manufacturing schemes cost-enable innovative therapeutic developmental research and ensure on-demand availability of end-products (Haack-Sørensen and Kastrup, 2011; Abbasalizadeh et al., 2017; Pigeau et al., 2018; Hunt, 2019).

Human Dermal FPCs (e.g., FE002-SK1, FE002-SK2 Cell Types)

Cell Therapies for Cutaneous Regenerative Medicine

Sub-optimal pharmacotherapeutic management of severe and complex cutaneous affections and complications (e.g., chronic ulcers, burns, donor-site wounds) has prompted the development of numerous skin graft solutions (e.g., amniotic membrane,

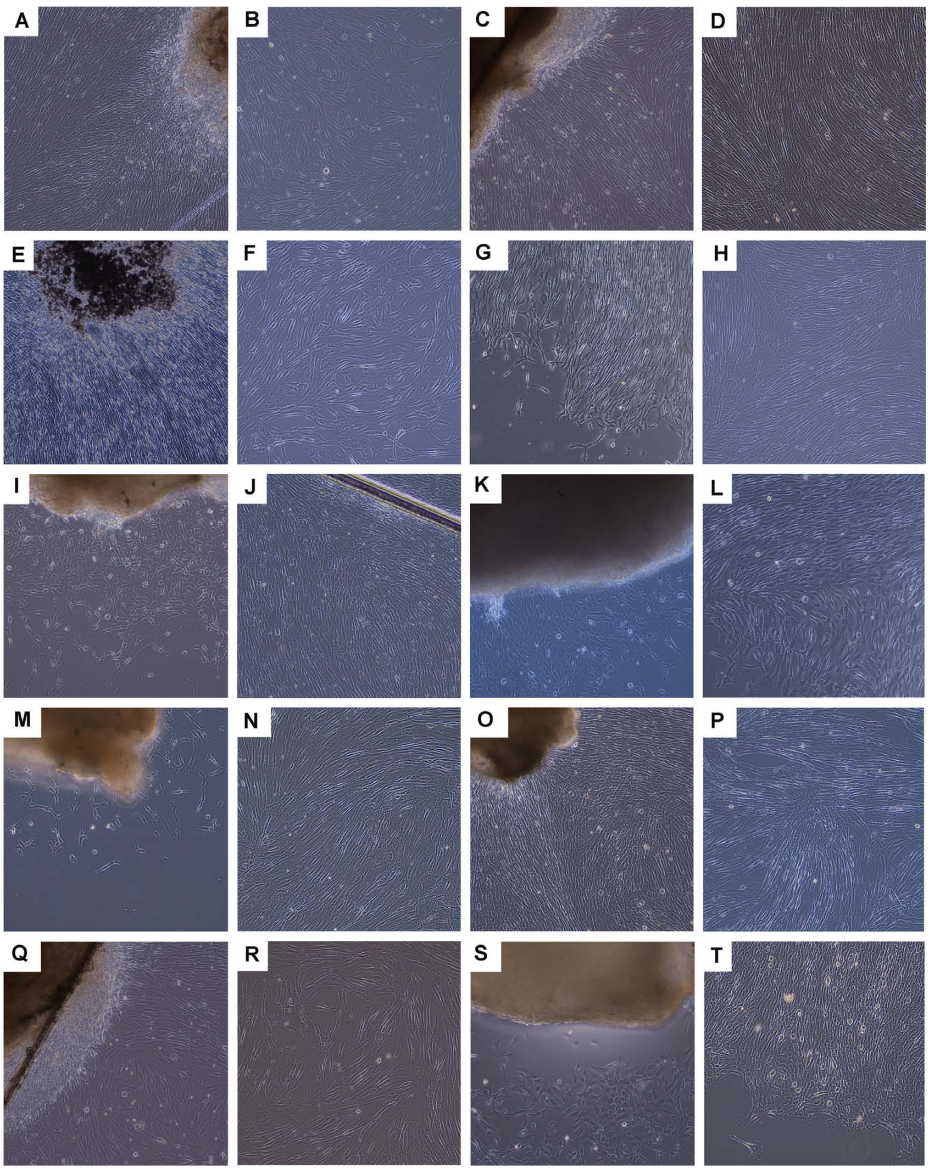

FIGURE 4 | Photographic imaging of culture initiation and culture-expansion steps performed following a fetal organ donation within updated legislative frameworks (i.e., post-2007). Various tissue biopsies were procured from the same organ donation (i.e., FE002, 2009) and simultaneously differentially processed following enzymatic and non-enzymatic methodologies. Pictures were obtained under 100X optical magnification on a phase contrast microscope and represent the non-enzymatically isolated primary FPC types. **(A,B)** Ventral skin with emitting dermal FPCs (i.e., FE002-SK1 cell type, P0). **(C,D)** Dorsal skin with emitting dermal FPCs (i.e., FE002-SK2 cell type, P0) and confluent cells at P2. **(E,F)** Tendon tissue with emitting tendon FPCs (i.e., FE002-Ten cell type, P0). **(G,H)** Articular cartilage with emitting cartilage FPCs (i.e., FE002-Cart.Art cell type, P0) and confluent cells at P2. **(I,J)** Cartilage tissue with emitting cartilage FPCs (i.e., FE002-Cart cell type, P0). **(K,L)** Bone tissue with emitting bone FPCs (i.e., FE002-Bone cell type, P0). **(M,N)** Intervertebral disc tissue with emitting disc FPCs (i.e., FE002-Disc cell type, P0) and confluent cells at P1. **(O,P)** Lung tissue with emitting lung FPCs (i.e., FE002-Lu cell type, P0) and confluent cells at P1. **(Q,R)** Muscle tissue with emitting muscle FPCs (i.e., FE002-Mu cell type, P0) and expanding cells at P2. **(S,T)** Connective tissue with emitting connective tissue FPCs (i.e., FE002-CT cell type, P0). For higher magnification, see **Supplementary Figure S5**.

cadaver grafts, fish skin), innovative bioengineered cellular therapy solutions (e.g., cultured autografts), or autologous and allogenic cell-based products (e.g., Allox®, Apligraf®, Epicel®, Lyphoderm®, OrCel®, ReCell®, TransCyte™) that complement surgical care and support tissue structural integrity and functional recovery (Lukish et al., 2001; Limat and Hunziker, 2002; Kumar et al., 2004; Amani et al., 2006; Hartmann et al., 2007; Zaulyanov and Kirsner, 2007; Akita et al., 2008; Hirt-Burri et al., 2008b; Guerid et al., 2013; Zuliani et al., 2013; Malhotra and Jain, 2014; Tan et al., 2014; Debels et al., 2015; Akershoek et al., 2016; Abdel-Sayed et al., 2019b; Lima-Junior et al., 2019; Momeni et al., 2019; Climov et al., 2020). Further optimization of biological starting materials for such advanced solutions may primarily benefit

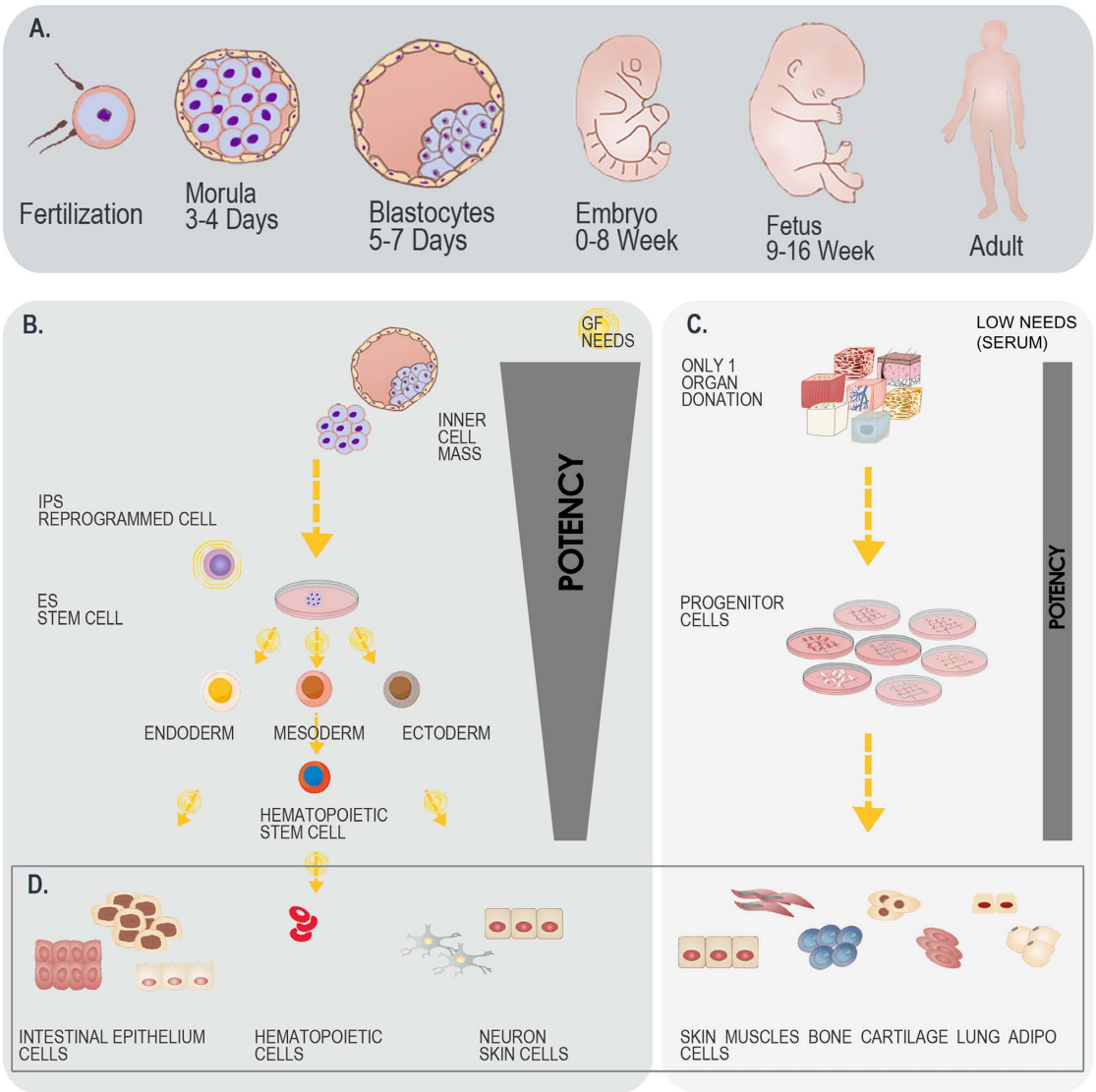

FIGURE 5 | Differential overview highlighting the similarities and differences between stem cells and primary FPC types. **(A)** Schematic representation of developmental stages within the human biological continuum, assorted to classes of cells to potentially be isolated. **(B)** Schematic representation of the isolation and culture-expansion of stem cells from blastocytes. Such cell types may serve for subsequent processing and generation of various stem cell types (e.g., iPSCs). **(C)** Schematic representation of the simultaneous isolation and culture-expansion of primary FPCs. Such procedures are relatively simpler and more robust than when working with stem cells, as a single organ donation enables differential isolation of several tissue-specific cell types, without the resort to growth factor (GF) cocktails in culture-expansion and maintenance steps, which largely and positively impact the consistency of progeny cellular materials. **(D)** Schematic representation of cellular materials obtained after biopsy processing and cell bank establishment. Differentiated cell types are eventually obtained when using both starting materials (i.e., blastocytes versus fetal tissue organ donations), with specificities to each strategy. A single isolation procedure is necessary when working with blastocytes, whereas differential biochemical manipulation enables generation of various cellular phenotypes maintaining designed relatively restricted potency. A single isolation procedure is equally necessary when working with fetal tissues, whereas standardized parallel processing enables generation of homogenous FPC types, inherently relatively restricted in terms of potency. Overall, while both strategies for therapeutic cell type obtention may be compared, the use of primary FPCs is relatively more robust, may be standardized, is cost-effective and sustainable.

from banked dermal FPCs (e.g., FE002-SK2 cell type), which have displayed clinical benefits in topically managing complex dermatological conditions, such as actinic dermatitis, eczema, or psoriasis. Cell-laden bioengineered constructs and cell-derivative formulations using dermal FPCs present potent therapeutic results (Hirt-Burri et al., 2011; Moore et al., 2018; Lorant et al., 2019; Poinas et al., 2019). Adapted pharmaceutical forms and delivery scaffolds are moldable and biocompatible with wounded tissues and therapeutic cells, providing optimal physical characteristics (e.g., porosity and mechanical stability). These scaffolds also allow the development of cell contraction forces and homogenous distribution of therapeutic biological substrates. Possible matrices comprise nylon mesh, silicone, collagen (i.e., bovine, equine, or porcine), polyglycolic acid, or hyaluronic

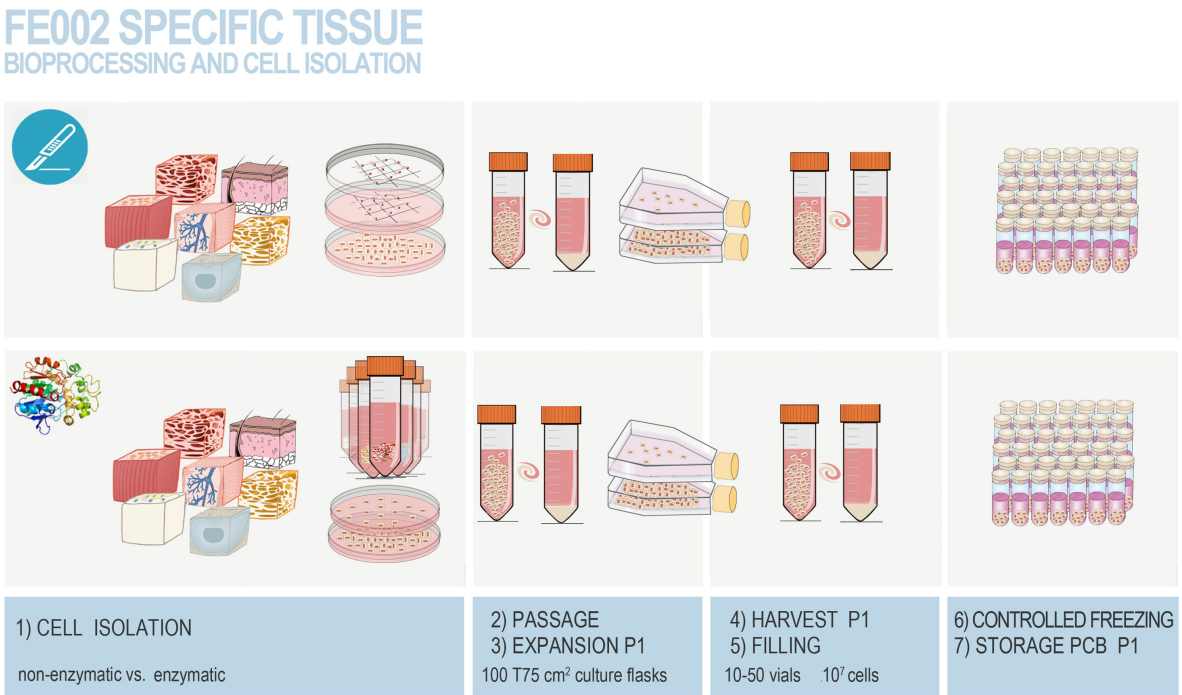

FIGURE 6 | Overview of the simultaneous differential biopsy processing methods devised for the FE002 fetal organ donation in view of adherent FPC culture initiation. The different fetal tissues were simultaneously either submitted to enzymatic or non-enzymatic processing. Individual tissue biopsies from the FE002 donation were procured by the pathology department and further dissected into fragments, providing starting material for both cell isolation methods. All fragments were washed thrice in phosphate buffered saline supplemented with 1% penicillin–streptomycin. **(1)** Fragments were then either appropriately dissected and placed in scored sterile culture dishes (i.e., non-enzymatic workflow) or subjected to appropriate trypsin digestion (i.e., enzymatic workflow) before plating in culture dishes. Sufficient amounts of seeded culture vessels were prepared for each individual tissue type and both cell isolation methods. Cells and tissue fragments were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% clinical-grade fetal bovine serum (FBS). Cultures were incubated in a 37°C humidified incubator under 5% CO_2 and the growth medium was renewed every other day. **(2)** After rapid cell emission or free proliferation, preliminary cultures (i.e., P0) were harvested by trypsinization after attaining 90 % confluency. **(3)** Cells were then enumerated and used to seed sufficient amounts of vented cell culture flasks for further expansion (i.e., P1). Culture medium was thereafter composed of DMEM, FBS, and additional L-glutamine. **(4,5)** Once optimal banking confluency was reached, cells at P1 were harvested, enumerated, and conditioned in individual 1 mL aliquots in a DMSO-based cryopreservation solution for long-term storage. **(6,7)** Cryovials were frozen following a controlled rate and were transferred to the vapor phase of separate level-alarm-fitted locked Dewar storage tanks to constitute the Parental Cell Banks. Figure adapted with permission from Laurent et al. (2020e).

acid (HA). Additionally, synergistic *in vitro* effects are yielded by combining polycationic dendrimers and collagen matrices, providing potent anti-microbial effects coupled with keratinocyte migration stimulation and direct angiogenic effects (Abdel-Sayed et al., 2016). Further optimization of biological material processing will enable the transition from off-the-freezer to off-the-shelf therapies, with shortened production and availability delays, simplified logistics, and maintained therapeutic potential (Hunsberger et al., 2015; Li and Maitz, 2018). Probable therapeutic mechanisms of action of FPCs comprise paracrine signaling, with the release of well-proportioned arrays of growth factors or cytokines, and deposition of extracellular matrix (ECM) proteins in wounded environments (Spiekstra et al., 2007). Modulation of inflammation, cell migration and proliferation, immune system, and angiogenesis induction then leads to facilitated tissue repair or regeneration (Werner et al., 2007; Barrientos et al., 2008; Providence et al., 2008; Wojtowicz et al., 2014; Varkey et al., 2015). Due to the robustness of dermal FPCs, many alternative applications are envisioned for *in vitro* standardized models of screening assays or biotechnological manufacturing processes (e.g., feeder-layers, growth supplements for keratinocytes or MSCs, therapeutic cell-free extracts) (Hirt-Burri et al., 2011; Krähenbühl et al., 2015; Patrulea et al., 2015, 2019; Laurent et al., 2020e,i).

Swiss Tools for Cutaneous Regeneration: Progenitor Biological Bandages

Progenitor biological bandages (PBB) consist of moldable, single-use, non-invasive bioresorbable wound coverages composed of dermal FPCs yielded by equine collagen scaffolds (9 cm × 12 cm), which are currently GMP-manufactured and clinically delivered on-demand in less than 48 h to the Lausanne Burn Center (**Figure 9**). Advantages of PBBs comprise a simple and relatively painless one-step application, without staples, providing cost-effective healing promotion within different types of cutaneous lesions (Abdel-Sayed et al., 2019a,b). Such constructs were successfully applied for various cutaneous conditions such as pediatric and adult severe burns, sharp-force trauma wounds, geriatric refractory chronic ulcers, and donor-site wounds, yielding unique reconstructive results (**Figures 10**, **11**;

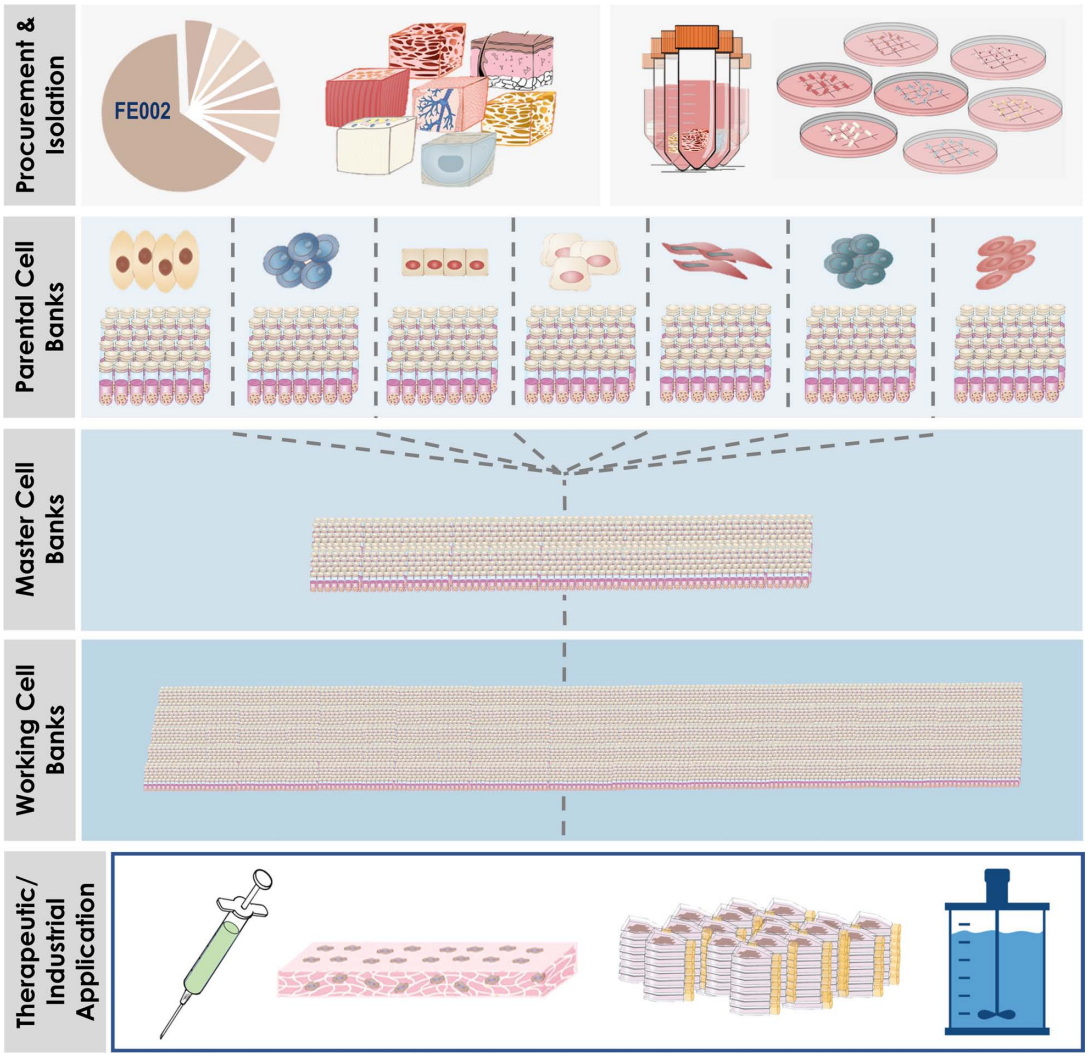

FIGURE 7 | Overview of simultaneous differential establishment of various primary FPC types following specific processing of biopsies from the same single organ donation (i.e., FE002, 2009). Procurement of the donation and micro-dissection enabled the specific tissue processing workflows to be implemented (i.e., enzymatic or non-enzymatic adherent cell culture initiation). Following the establishment of the tissue-specific FPC types, multi-tiered cell banking was performed in parallel for each specific cell type. Materials from Working Cell Banks were then used for diversified applications, which comprised or may comprise therapeutic live-cell product manufacture, use of FPCs or cellular materials as feeder-layers or culture supplements, and use of FPCs as substrates for biotechnological applications (e.g., viral vaccine production).

Hohlfeld et al., 2005; Ramelet et al., 2009; De Buys Roessingh et al., 2015). Skin regeneration was achieved extremely rapidly, with the restoration of high elastic properties and improved pigmentation balance, which was without pain, hypertrophy, retraction, inflammation, or the necessity for additional skin grafts. Bioengineered PBB constructs were observed to promote proliferation, adhesion, and migration of endogenous cells, without atrophic skin formation (Ramelet et al., 2009). Over two decades of clinical experience and multicentric studies have shown the safety or beneficial therapeutic effects of dermal FPCs in PBBs, notably within phase I and II clinical trials in Switzerland and Asia (i.e., ClinicalTrials.gov identifiers: NCT02737748 & NCT03624023) (Hohlfeld et al., 2005; Ramelet et al., 2009; Laurent et al., 2020e). In view of further optimization of burn wound or ulcer care in particular, high therapeutic benefits may be gained by stabilizing and formulating active cell-derivative components in pharmaceutical creams, ointments, or gels, as these are used for the maintenance therapy to accelerate wound healing (i.e., potentially scarlessly) after primary wound closure.

Human Cartilage FPCs (e.g., FE002-Cart, FE002-Cart.Art Cell Types)

Cartilage FPCs in Regenerative Medicine

Due to frequent cartilage defects caused by degenerative diseases or excessive wear, cell therapies, cell-based approaches, or combined bioengineered constructs are of high interest for translational medicine applications and predominate

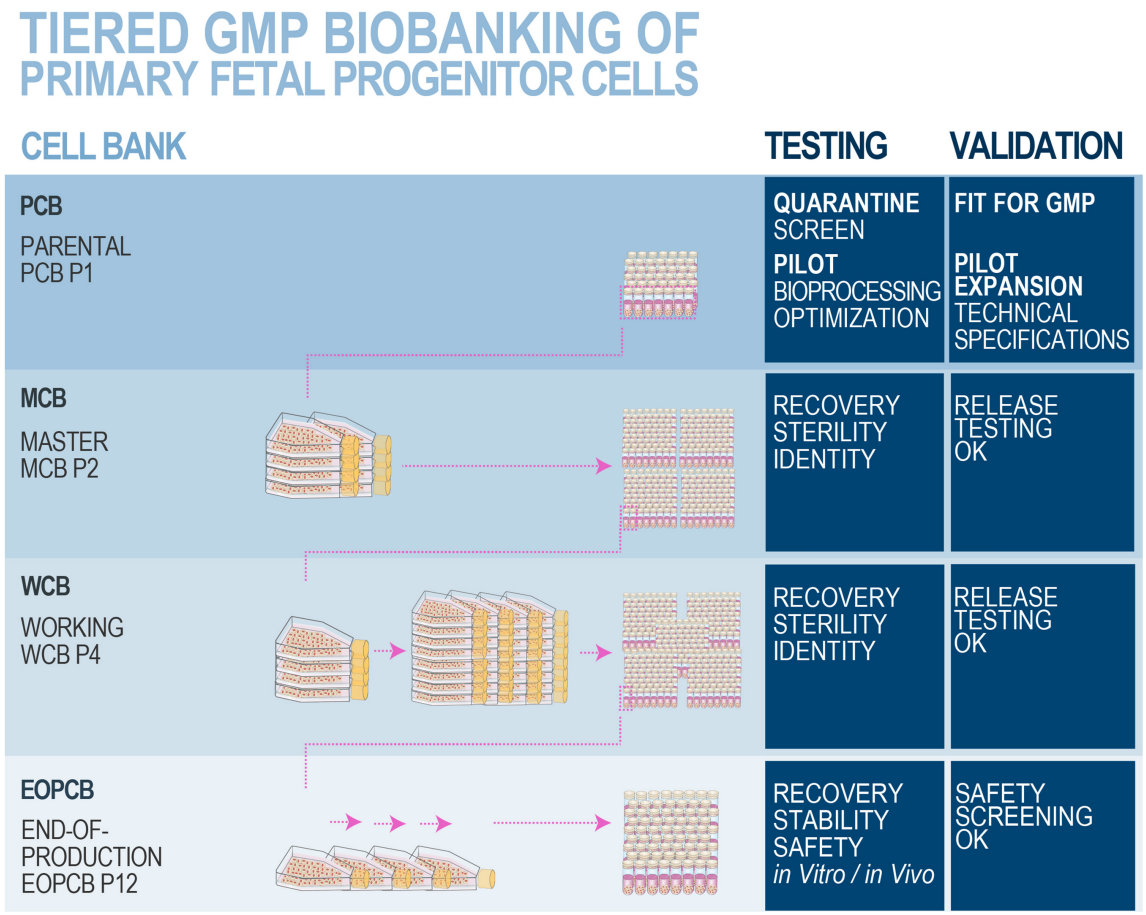

FIGURE 8 | Schematic overview of optimized and standardized multi-tiered cell banking workflows for primary FPCs. *In vitro* optimization steps performed during the pilot study may comprise serum lot choice, culture surface, and brand comparison, in order to maximize cell viabilities and population doubling values within minimal timeframes, obtaining the highest endpoint cell yields and best efficiency of manufacturing. Specific product release and characterization testing for the manufactured cell bank lots may comprise cell growth, isoenzyme testing to confirm cell type origin, DNA fingerprinting of the cell type, qualification/testing for sterility, specific testing for the absence of endotoxins, mycoplasma, viral contaminants (e.g., picornavirus, orthomyxovirus, paramyxovirus, adenovirus, reovirus, West Nile virus, BPyV, HuPyV, HPV, HBoV, WUPyV, KIPyV, EBV, HAV, HBV, HCV, hCMV, HIV-1, HIV-2, HTLV-1, HTLV-2, HHV-6, HHV-7, HHV-8, SV40, and B19 parovirus), evaluation of reverse transcriptase activity, and quantitative transmission electron microscopy (TEM) of cell sections for the detection of viruses, virus-like particles, mycoplasma, yeasts, fungi, bacteria (i.e., ≥200 cell profiles). Safety assessments may be performed on EOPCB materials and comprise *in vivo* tumorigenicity assays and karyology studies. Nomenclature for correlated bank tier and passage numbers is provided here as an example, as it has been validated for dermal FPCs (i.e., FE002-SK2 cell type). The devised technical specifications, testing, and validation strategies are optimally adapted for banking FPCs, due to the inherent high robustness, consistency, and stability of the considered cellular materials. Industrial transposition towards GMP production is therefore tangibly attained with such materials, as extensive multi-tiered cryopreserved cell banks may be rapidly and efficiently established. Figure adapted with permission from Laurent et al. (2020e).

developmental efforts (Vrahas et al., 2004; Flanigan et al., 2010; Makris et al., 2015; Carluccio et al., 2020). The avascular and alymphatic nature of cartilage tissues confers relative immune privileges (i.e., isolation from antigen-presenting cells, migratory macrophages, and dendritic cells) and renders allogenic cell therapy approaches possible for tissue regeneration chaperoning (Quintin et al., 2010; Studer et al., 2017). Autologous cartilage cell therapy implementation remains hindered or delayed due to the induction of hypertrophic tissue phenotypes, fibrocartilage formation, high-cost cell expansions, *in vitro* de-differentiation, two-step surgery, donor-site morbidity, and high variability in functional outcomes (Brittberg et al., 1994; Horas et al., 2003; Lu et al., 2006; Katopodi et al., 2009; Vinardell et al., 2012). Differential autologous and allogenic approaches comprise high cellular variability, and related inhomogeneous potency restricts potential therapeutic benefits (Wakitani et al., 2007; Stolzing et al., 2008; Prockop, 2009; Pelttari et al., 2014; Pleumeekers et al., 2014; Steinwachs et al., 2014). Neonatal chondrocytes or cartilage FPCs are optimal candidates for cell therapies, possessing relatively superior chondrogenic potential (i.e., constitutive immature chondrodifferentiation for the latter cell types) than adult chondrocytes (Almqvist et al., 2009; Adkisson et al., 2010a,b; Quintin et al., 2010; Acosta et al., 2011; Darwiche et al., 2012; Dhollander et al., 2012; Cavalli et al., 2018). Fetal progenitor cells also present relatively low hypertrophy marker expression (e.g., type X

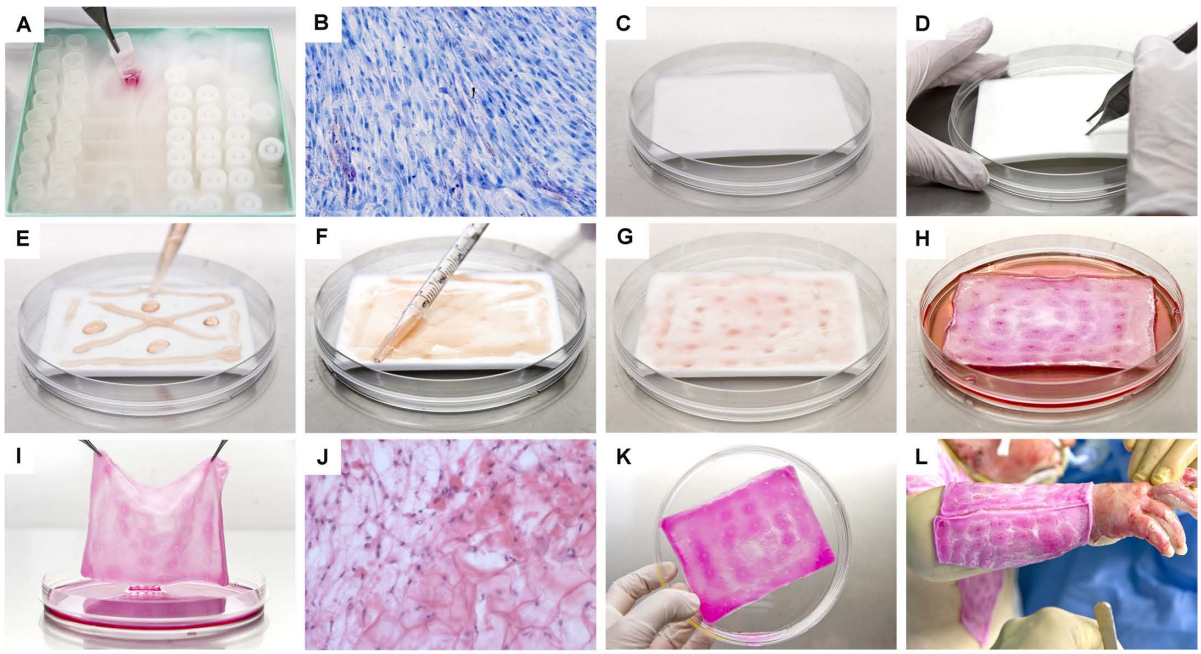

FIGURE 9 | Photographic illustrations providing an overview of the supply chain and manufacturing steps necessary for the preparation of Progenitor Biological Bandages (PBBs), following GMP standards, for clinical application in the Lausanne Burn Center. **(A)** Upon manufacturing order receipt from the clinic, vials from the dermal FPC Working Cell Bank (i.e., FE002-SK2 WCB, P7-P8) are selected and initiated for therapeutic construct preparation. **(B)** Cell suspensions are thawed and cellular viability is assessed. **(C)** Sufficient amounts of equine collagen scaffolds are procured. **(D)** Scaffolds are pre-conditioned by symmetrical puncture of the whole surface. **(E)** Cells are rinsed and seeded on the scaffolds. **(F)** Cell suspensions are further homogenously distributed over the integral surface of the scaffold, to allow optimal cell colonization and integration. **(G)** Seeded scaffolds are further processed to allow uptake of cell suspensions. **(H)** Constructs are incubated for 24–48 h at 37°C under 5% CO_2. **(I)** After incubation, the scaffolds are checked following quality assurance specifications. **(J)** Histological investigation of a cell-seeded construct (i.e., PBB) after snap-freezing and staining with hematoxylin and eosin. **(K)** PBBs are rinsed and delivered to the operating theater in isotherm containers. **(L)** After standard surgical wound care and disinfection, the constructs are applied and subsequently overlaid with bandages to favor wound healing rate acceleration.

collagen), possibly due to epigenetic modulations *in vivo* (Zimmermann et al., 2008; Tompkins et al., 2013). Clinical translation of therapeutic cartilage FPCs is appealing due to the potential to consistently treat large numbers of patients (i.e., $>10^8$ individual therapies consisting of cell-seeded biocompatible implants following a single fetal organ donation) (Darwiche et al., 2012).

Phenotypic Stability, Chondrogenic Potential, and Biomechanics

High phenotypic stability and chondrogenic potential (i.e., elevated sulfated GAG content, Sox9:Scleraxis ratios, *IHH* and *PTH1R* gene expression, TGF-β3-induced production of aggrecan, types I+II collagen) of cartilage FPCs are differential advantages supporting their application in tissue engineering (Broguiere et al., 2016; Studer et al., 2017). Despite expressing stem cell surface markers, cartilage FPCs present relatively lower adipogenic and osteogenic differentiation capacities on a site-specific basis (Stokes et al., 2002; Quintin et al., 2010). Conjugation with alginate optimally stimulates and maintains ECM production, while resisting mineralization and circulatory vessel infiltration *in vivo*, thereby drastically improving stability and therapeutic potential of cartilage FPCs, along with optimal structural parameters (Häuselmann et al., 1994; Mellor et al., 2014; Mhanna et al., 2014; Studer et al., 2017). Polyethylene glycol, chitosan, albumin, or hyaluronan scaffolds have been investigated as functional cell vectors for injectable applications, yielding adhesive, chondrogenic, and mitogenic properties (Madeira et al., 2015; Mardones et al., 2015). For combination product assembly, impermeable, tortuous, and hydrophobic scaffolds often present resistance to liquid phase infiltration, despite high porosity and relative void volume, which negatively affect cell integration, colonization, and persistence (Wendt et al., 2003; Solchaga et al., 2006; Melchels et al., 2010). Various dynamic cell seeding protocols for the induction of active infiltration (e.g., perfusion, centrifugation, orbital shaking, spinner flasks) allow cell distribution uniformity and optimal preservation of cellular integrity and function (Burg et al., 2000; Alvarez-Barreto et al., 2007; Roh et al., 2007; Thevenot et al., 2008). An equilibrium must be reached between cell proliferation and adequate chondrogenesis (i.e., responsiveness versus stability) following homogeneous scaffold seeding, directly defining adequate seeding density, methods for construct obtention, and preculture conditions (Roche et al., 2001; Moretti et al., 2005; Hasegawa et al., 2010; Erickson et al., 2012; Nasrollahzadeh et al., 2017; Studer et al., 2017). This ultimately results in the integration of structural and mass transport properties with the functional chondrogenesis components

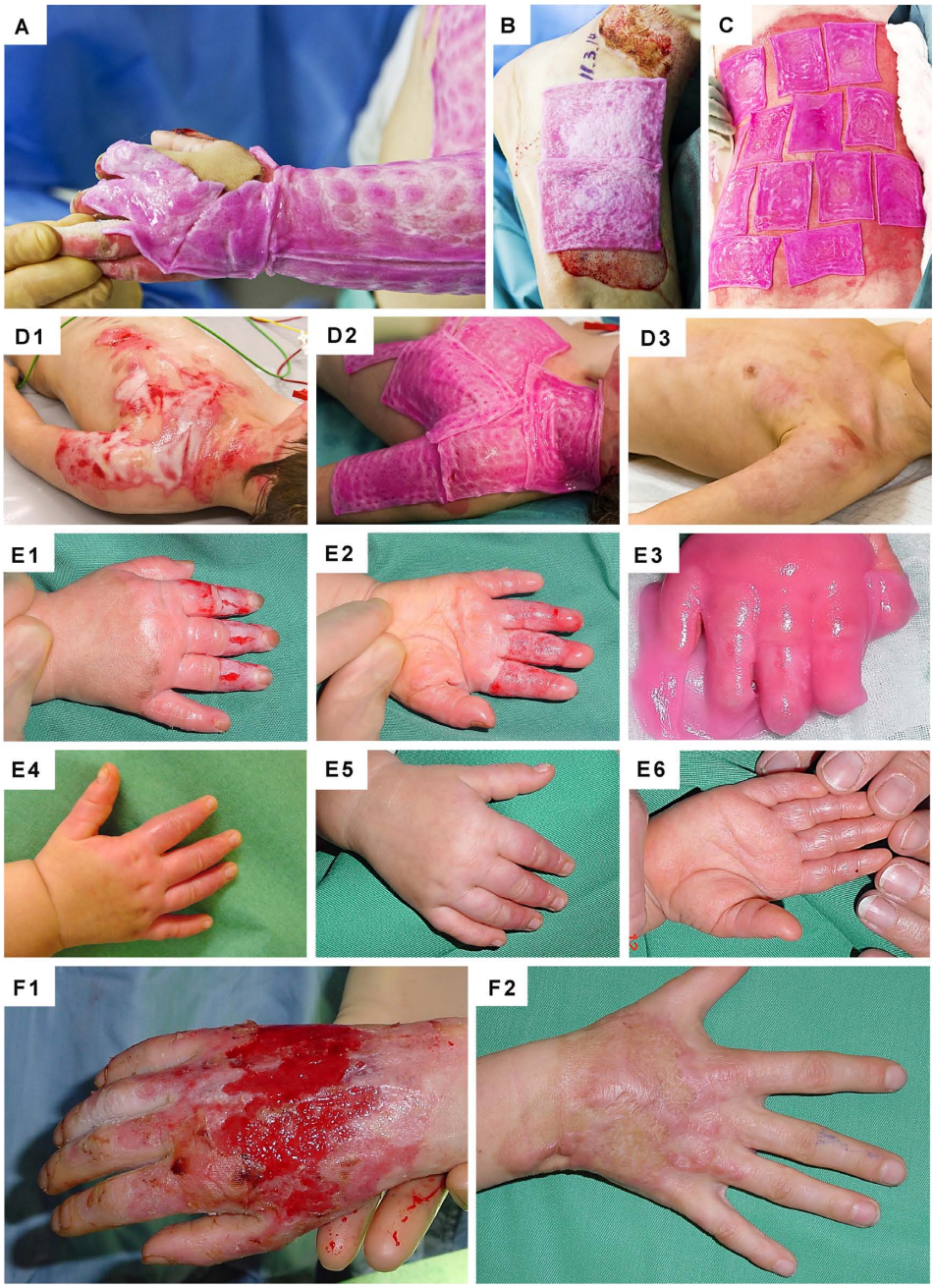

FIGURE 10 | Clinical case-reports illustrating the use and efficacy of Progenitor Biological Bandages for the management of human pediatric burns and donor-site wounds. **(A–C)** Photographic representation of Progenitor Biological Bandages used for primary lesions of a pediatric burn victim and donor-site graft secondary wounds. Unlike skin autografts or synthetic wound coverage solutions, PBBs do not need to be stapled to the patient, as they are simply applied and overlaid with Vaseline gauze before standard bandages are adjusted. **(D1–D3)** Second-degree deep pediatric burn wound (i.e., scalding liquid). Photographic representations of the lesions after early debridement, after PBB application, and after six weeks of treatment. **(E1–E6)** Second and third-degree pediatric burn wound (i.e., scalding liquid). Photographic representations of the lesions after early debridement, after PBB application, and after six weeks of treatment. **(F1–F2)** Second-degree pediatric burn wound (i.e., scalding liquid). Photographic representations of the lesions after early debridement and after ten years during patient long-term follow-up. Figures modified with permission from Hohlfeld et al. (2005) and Laurent et al. (2020a).

of the cells, which enable load bearing after successful implantation and integration (Hollister, 2005; Kemppainen and Hollister, 2010). External or internal biochemical modulation, specific processing (e.g., microgel encapsulation), and scaffold mechanical stimulation differentially constitute potent cues for chondrogenesis and structural or functional improvement in bioengineered constructs (Huang et al., 2005, 2010; Campbell et al., 2006; Terraciano et al., 2007; Levinson et al., 2019;

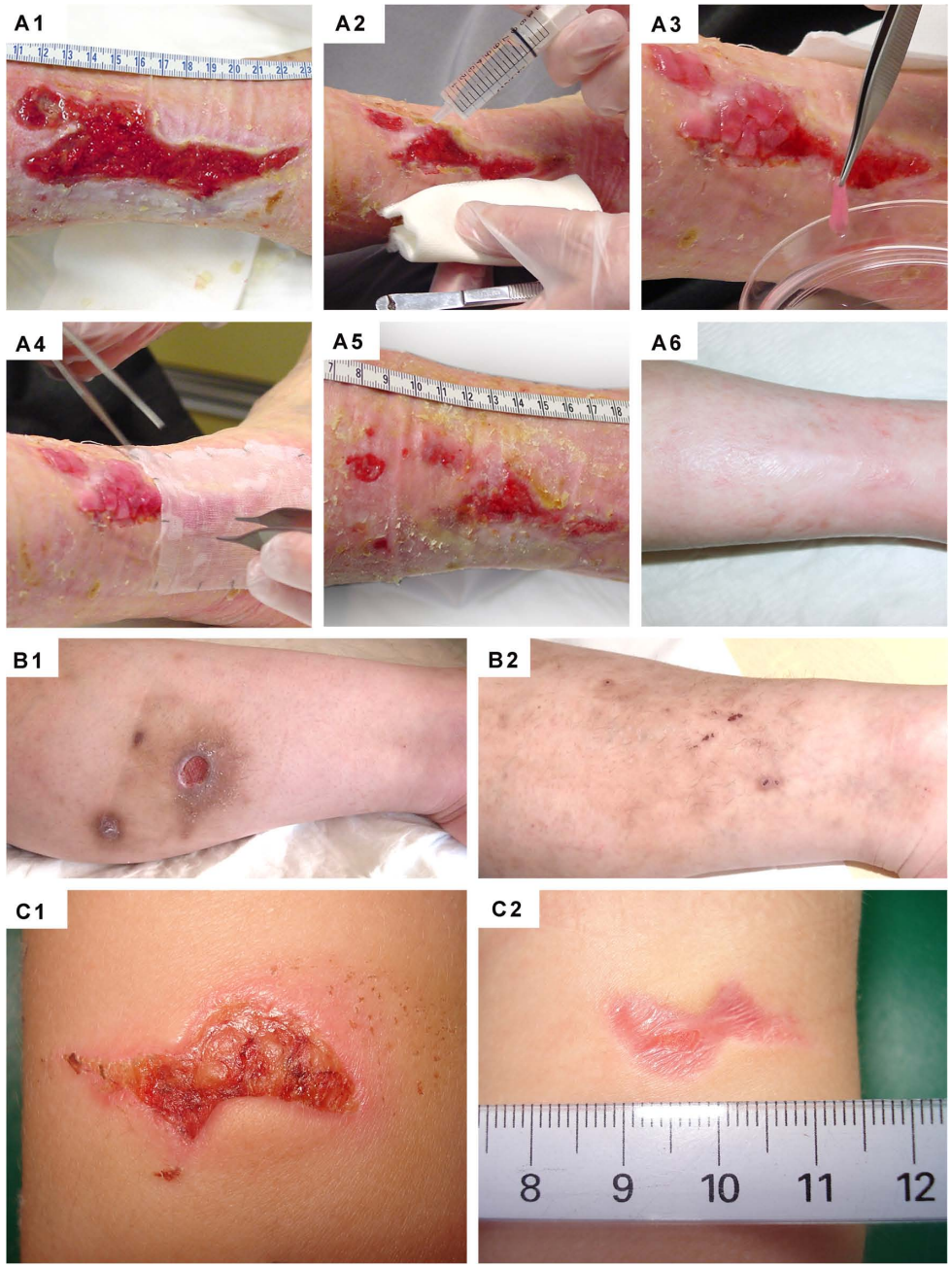

FIGURE 11 | Clinical case-reports highlighting the efficacy of primary FPCs and derivatives thereof for the management of human acute and chronic cutaneous affections. **(A1–A6)** Refractory painful post-thrombotic ulcer lesions were treated weekly with Progenitor Biological Bandages and evolutive photographic representations were acquired at the time of treatment initiation, 11 weeks later, and 15 months later for follow-up. **(B1–B2)** Refractory atypical lower-leg ulcer lesions were treated as for the previous patient, and evolutive photographic representations were acquired at the time of treatment initiation and five weeks later for follow-up. **(C1–C2)** Sharp-force trauma wounds were treated daily with ovine FPC derivatives formulated in a cell-free pharmaceutical cream, and evolutive photographic representations were acquired at the time of treatment initiation and two weeks later. Figures modified with permission from Hirt-Burri et al. (2011) and Lapp et al. (2013).

Li et al., 2020). Scaffold stiffness improves with ECM deposition and may approach physiological ranges in clinically relevant timeframes (Broguiere et al., 2016). Controlled and function-oriented energy dissipation modulation within native viscoelastic cartilage-like materials favors optimal chondrogenic expression under dynamic loading and subsequent load-bearing (Hunter et al., 2004; Shaw and MacKnight, 2005; Li et al., 2010; Abdel-Sayed et al., 2014). Relatively high energy dissipation levels lead to the upregulation of specific chondrogenic markers (e.g., mRNA of Acan, Col2a1, Sox9, and TGF-β3), while lower

dissipation is linked to downregulation (Mauck et al., 2007; Thorpe et al., 2008; Abdel-Sayed et al., 2014).

Human Tendon FPCs (e.g., FE002-Ten Cell Type) for Regenerative Medicine

Tendinous tissue disorders (e.g., tendinosis, lipoid degeneration, and calcification), along with imperfect inherent tissue healing capacities and iatrogenesis, result in disability, chronic pain, functional, and productivity deficits, particularly in sporting and manual labor areas. These diseases or injuries implicate highly specialized professional care and high burdens for public healthcare systems (Verdan, 1972; Kannus and Józsa, 1991; Maffulli et al., 2003; Sharma and Maffulli, 2005; Tuncali et al., 2005; Reinking, 2012). Adhesions and high rates of secondary ruptures are current clinical concerns, as functionally defective fibrotic scar tissue accumulates (James et al., 2008). Slow inherent tissue metabolism, delayed inflammation, effector recruitment, ECM deposition, tissue architectural reorganization, and alignment render the modulation of tendon regeneration complex (Sharma and Maffulli, 2005; Voleti et al., 2012). The efficacy of tendon transfer is hindered by accelerated graft degeneration and would largely benefit from therapeutic cell stimulation, ideally leading to optimal elasticity, mobility, and tensile strength restoration (O'Brien, 1997; Kannus, 2000). Bioengineering scaffolds of interest, such as human cadaveric and equine decellularized tendons or artificial equivalents, enable optimal maintenance of biocompatibility, mechanical properties, and susceptibility for cell seeding, whereas autologous vestigial tendons remain as the standard of care (Wehbé, 1992; Chong et al., 2009; Jakubietz et al., 2011; Pridgen et al., 2011; Burk et al., 2016; Lovati et al., 2016; Valentin et al., 2016; Aeberhard et al., 2019). Vast arrays of potential therapeutic cell types have been investigated in tendon bioengineering for regeneration enhancement, including tendon sheath fibroblasts, adult tenocytes, stem cells, placenta cells, amniotic cells, and platelet-derivatives (Kadner et al., 2002; Kaviani et al., 2002, 2003; Awad et al., 2003; Chen et al., 2009; Akhundov et al., 2012; Xu et al., 2013; Petrou et al., 2014). Tendon FPCs present tremendous therapeutic potential due to high stability of their tenogenic and karyotypic properties in culture, low propensity for de-differentiation, expansion characteristics, therapeutic stimulatory potential, and the ability to maintain cell viability along with rheological properties of bioengineered hydrogel constructs (Grognuz et al., 2019). Their similarities with stem cells but lack of specific tendon markers require *in vitro* characterization of tendon FPCs using marker panels (e.g., type I collagen, scleraxis, and tenomodulin) (Hulmes, 2002; Le Blanc et al., 2003; Docheva et al., 2005; Murchison et al., 2007; Banos et al., 2008; Taylor et al., 2009). Extensive tendon FPC cell banks may be consistently established and yield approximately 2×10^{14} cells within the clinically relevant *in vitro* lifespan, potentially serving for the manufacture of more than 10^8 treatment units (e.g., reseeded biocompatible scaffolds for localized tendon replacement) (Grognuz et al., 2016b). Relatively increased ECM production is achieved by tendon FPCs under appropriate conditions, as compared to primary adult tenocytes. Development of injectable products designed for tissue regeneration stimulation (e.g., degenerative diseases, small hand injuries, fissures or partial ruptures) using registered medical devices without cell preculture periods enables tangible translational development (Petrou et al., 2014; Grognuz et al., 2016a).

Human Muscle FPCs (e.g., FE002-Mu Cell Type) for Regenerative Medicine

Intrinsic potential for functional rearrangement and healing is low in human muscle tissue, further diminishing with the advancement of biological age (Grasman et al., 2015; Passipieri and Christ, 2016). Without effective therapeutic management, severe and extensive tissue structural bias (e.g., volumetric muscle loss) is often predictive of poor clinical outcome, as spontaneous optimal healing is hindered or negated, which results in diminished contractility associated with fibrotic tissue formation (Montarras et al., 2005; Ciciliot and Schiaffino, 2010; Grogan et al., 2011; Sicari et al., 2014; Duffy et al., 2016). Muscular tissue engineering is designed to effectively manage and restore structure and function in the aftermath of intense soft tissue trauma, burns, malformations, or tumor ablation, while minimizing volumetric loss and donor-site morbidity consequences (Laurent et al., 2020c). Traditional reconstructive surgical care may tangibly and synergistically benefit from supplementation with cell therapies. Immune rejection, poor distribution, and extremely restricted cell persistence after implantation have been significant challenges limiting the potential of myoblast transfer therapy in muscular loss, Duchenne muscular dystrophy, or cardiac surgery (Partridge et al., 1978; Mendell et al., 1995; Miller et al., 1997; Skuk and Tremblay, 2000; Smythe et al., 2000; Huard et al., 2002; Menasché, 2005). Such obstacles dramatically hamper therapeutic efficacy, as eventual functional benefits are dependent on cell survival *in situ* (Fan et al., 1996; Beauchamp et al., 1997, 1999; Qu et al., 1998; Hodgetts et al., 2000, 2003; Tambara et al., 2003; Sammels et al., 2004). Multimodal development efforts have been allocated to optimize persistence and therapeutic effects of implanted cells, comprising differential cell source choice, cell population purification and pre-treatment, or modulation of existing pharmacotherapeutic care protocols (Huard et al., 1994; Pavlath et al., 1994; Guérette et al., 1997; Qu et al., 1998; Jankowski et al., 2001; Maurel et al., 2005; Schäfer et al., 2006). Defined cell population identity and high purity of human muscle FPCs (i.e., stable desmin expression) or *in vivo* persistence were demonstrated in immunocompetent murine models, excluding immunogenicity and tumorigenicity, while positively affecting contractile recovery potential (Hirt-Burri et al., 2008a; Laurent et al., 2020c). Specific estimations indicate that a single fetal organ donation can potentially yield more than 10^{12} progeny cells at a low passage (i.e., P4), enabling subsequent safe industrial-scale manufacturing of off-the-freezer therapeutic cellular products. High FPC robustness and adaptability to bioengineered scaffolds, such as equine collagen sheets, were shown, with rapid colonization and proliferation of therapeutic cells *in vitro*, and persistence thereof *in vivo* (Hirt-Burri et al., 2008a). Optimal restoration of muscle tissue function was

demonstrated, concerning functional endpoints of tissue repair, following engraftment of human muscle FPCs in a murine model for volumetric muscle loss (Laurent et al., 2020c).

Human Bone FPCs (e.g., FE002-Bone Cell Type)

Bone FPCs for Skeletal Tissue Engineering

Conventional specific surgical management strategies for bone injuries or diseases include autografting, allografting, or xenografting, which retain relatively elevated risks of contamination and immune response eliciting, leading to subsequent invasive procedures (Younger and Chapman, 1989; Strong et al., 1996; Vacanti et al., 2001; Schantz et al., 2002; Tenorio et al., 2011). Bone replacement and skeletal regenerative cell therapies focus mainly on orthopedic medicine, osteogenesis imperfecta, and mandibular care (Horwitz et al., 1999; Ohgushi and Caplan, 1999; Yildirim et al., 2000; Bianco et al., 2001; Patino et al., 2002; Rose and Oreffo, 2002; Mauney et al., 2005; Oreffo et al., 2005; Yoshioka et al., 2007; Mendes et al., 2008). Use of FPCs for skeletal tissue engineering eliminates the need for extensive population selection and complex biochemical phenotype manipulation, while cells maintain sustained differentiation states, with relevant mineralization activities *in vitro* and *in vivo* (Petite et al., 2000; Parikh, 2002; Gronthos et al., 2003; Mendes et al., 2004; Montjovent et al., 2004, 2008, 2009). Allogenic FPC supplementation in artificial bone constructs facilitates cell migration, proliferation, and differentiation at the injury site after implantation, in order to favor tissue regeneration (Caplan and Goldberg, 1999; Shea et al., 2000).

Bone FPC Modulation and Drug Delivery

Osteogenic activity (e.g., dexamethasone-induced cbfa-1, ALP, type I collagen, and osteocalcin gene expression) and mineralization processes are comparatively superior in magnitude or more rapid in FPCs than in stem cells and adult osteoblasts, whereas orientation toward mature osteoblast differentiation is relatively simple (Zernik et al., 1990; Franceschi, 1999; Karsenty, 2000; Pioletti et al., 2006). Fetal progenitor cell expansion and migration are culture medium-dependent and sensitive to PDGF-BB, FGF-2, or BMP-2 stimulation (Krattinger et al., 2011). Constitutive expression of TGF, VEGF-A, EDN1, IL-6, and MCP-1 in FPCs was shown, along with characteristic markers (e.g., Stro-1, ALP, CD10, CD44, CD54, β2-microglobulin, HLA-I, CD80) (Montjovent et al., 2009). Fetal progenitor cells present a tendency toward osteogenic differentiation, whereas specific modulation is achieved using ascorbic acid, glycerophosphate, 1α,25-dihydroxyvitamin D3, or dexamethasone, and may be evaluated by monitoring the expression levels of *RUNX2*, *OSX*, or *SOX9* (Aubin, 1998; Gallagher, 2003; Krattinger et al., 2011). Bone FPCs display the characteristics of osteoprecursor cells, relatively more advanced in terms of differentiation than stem cells, and produce relatively superior quantities of ECM, whereas fully-induced differentiation processes result in the appearance of specifically mineralized bone-like nodules (Montjovent et al., 2004; Krattinger et al., 2011). Phenotypic maturation *in vivo* was shown to not carry the immune privileges of therapeutic FPCs in rodent models (Hausherr et al., 2017). Chemical functionalization (e.g., click chemistry, bioorthogonal chemical reactions, covalent binding) of therapeutic cell surfaces allows optimal conjugation with bioengineered scaffolds, while maintaining and optimizing cellular viability, adhesion, persistence, and function (Borcard et al., 2011, 2012; Comas et al., 2012; Krauss Juillerat et al., 2012). Optimal mechanical properties and efficient vascularization capacity of implanted constructs are essential, while biodegradable hydrogels may enable local cell maintenance (Tenorio et al., 2011; Amini et al., 2012). For critical-size bone tissue replacement, cyto- and histo-compatible permanent bone-mimicking substitute materials (e.g., bioceramics) must comprise trans-scaffold micro-structure channels enabling nutrient diffusion and migration (i.e., pore size-dependent osteoconduction) of therapeutic cells, to ensure permanent cellularization and sustained functionality (Triplett and Schow, 1996; Ducheyne and Qiu, 1999; Griffith and Naughton, 2002; Montjovent et al., 2007, 2008; Klenke et al., 2008; Krauss Juillerat et al., 2012). The temporal onset of construct preculture mechanical loading influences and regulates bone architectural properties, whereas early or delayed loading may be beneficial for bone tissue formation within short timeframes (Carter et al., 1989; Huiskes et al., 2000; Roshan-Ghias et al., 2010; Boerckel et al., 2012). Based on *in vivo* experiments, it was established that low predictability characterizes the specific behavior of a given cell type and scaffold conjugate, concerning the intensity and temporal onset of mechanical loading (Hausherr et al., 2018). High cellular resistance to shear stress enables extrusion of cell-laden hydrogels through small-bored needles without compromising cellular viability, whereas HA constitutes a versatile and functional scaffold, allowing relatively enhanced cell migration at the delivery site and ameliorated therapeutic stimulation (Drury and Mooney, 2003; Weinand et al., 2006). Similar valuable characteristics (i.e., absorption, biocompatibility, chemotactic activities, void filling, and migration enhancement) are shared by collagen scaffolds (Patino et al., 2002).

Human Intervertebral Disc FPCs (e.g., FE002-Disc Cell Type) for Regenerative Medicine

The widespread prevalence of intervertebral disc degeneration mainly contributes to back pain-related surgical management and spine surgeries (Urban and Roberts, 2003; Anderson and Tannoury, 2005; Haefeli et al., 2006). Intervertebral disc tissue is characterized by mediocre intrinsic regenerative potential, further complicating therapeutic management and advancing the onset of degenerative disease. Cell therapy approaches for disc degeneration prevention present considerable potential for replacing autologous nucleus pulposus transplantation (Ganey et al., 2003; Sato et al., 2003; Crevensten et al., 2004; Meisel et al., 2006, 2007; Sakai et al., 2006). After intervertebral FPC isolation and during subsequent characterization, both structure and composition of ECM (e.g., aggrecan, type I and II collagen, sulfated GAGs), spontaneously produced by intervertebral disc FPCs, approach those of adult origin, as observed in alginate

bead culture, outlining the full chondrogenic differentiation potential (Häuselmann et al., 1994; Mok et al., 1994; Chiba et al., 1997; Melrose et al., 2000; Quintin et al., 2009). Absence of specific markers enabling population purity assessment prompts, for each new fetal organ donation and derived primary cell type, close evaluation of phenotypic consistency and stability for intervertebral disc FPCs, as they represent mixed populations isolated from whole spine units (Quintin et al., 2009). Therefore, based mainly on the initial dissection and culture initiation methods, some cell types may be unfit for further processing and should be excluded at an early stage, based on characterization results. Interestingly, intervertebral disc FPCs presented relatively lower adipogenic differentiation potential than comparable cartilage FPCs (Quintin et al., 2010). Overall, accumulated data strategically positions intervertebral disc FPCs for further research and development in skeletal tissue regeneration applications.

Human Lung FPCs (e.g., FE002-Lu Cell Type) for Biotechnological Manufacturing or Regenerative Medicine

Lung FPCs present tremendous potential and vast hindsight for applications in biotechnology, as the vaccine industry has been using such cell types for half a century. The finite human diploid MRC-5 cell type was initially isolated in the 1960s from a male fetal lung (i.e., 14-week gestational age), donated following a pregnancy interruption, and has been used as a substrate for manufacture of chickenpox, hepatitis A, polio, smallpox, and rabies vaccines (Jacobs et al., 1970; Lewis and Tarrant, 1972; Petes et al., 1974). Safety, stability, and quality of substrate cell types are of paramount importance, as defects may be passed down to therapeutic products and eventually endanger patients. Some concerns have emerged following reports that MRC-5 fibroblasts could de-differentiate under specific conditions and exhibit different markers typically found in ESCs or MSCs and neural tissue, or further become osteoblasts (Rieske et al., 2005; Zhang et al., 2014; Wan et al., 2018). Such capabilities tend to indicate a relative instability of the considered cell type, potentially creating problems in modern-day industrial validation. Additionally, aging of the MRC-5 cells, recurrent doubts about the identity of currently marketed MRC-5 cells, and unavailability of these in different geographical regions have led to the establishment of replacement cell types. Modern alternatives were reportedly developed (e.g., Walvax-2 cell type, PRC), with particular attention being paid to the ethnicity of the donor, in order to optimize industrial outputs by exploiting shorter doubling times, improved robustness, or cell viability (Ma et al., 2015). Recently established primary lung FPCs, such as the FE002-Lu cell type, benefit from all the aforementioned technical advantages of FPCs, and may be expanded at full industrial scale within specific multi-tiered cell banking workflows, therefore potentially constituting a tangible candidate for the replacement of the MRC-5 cell type. Optimization of novel and safe cellular substrates shall allow for the optimal replacement of biotechnological intermediates for vaccine production, therefore indirectly contributing to augmenting the quality of therapeutic products, benefiting populations globally. Additionally, primary lung FPCs may present substantial therapeutic utility in treating lung tissue inflammatory diseases. Recent clinical studies (i.e., ClinicalTrials.gov identifiers: NCT04315987, Brazil; NCT04313322, Saudi Arabia; NCT04333368, France; ChiCTR2000029990, PRC) are advancing with the use of multiple therapeutic stem cell sources for managing COVID-19 patients (Zhao, 2020). Similarly, it is hypothesized that lung FPCs may provide enhanced anti-inflammatory and tissue regeneration stimulation, as observed within cutaneous regenerative applications of related dermal FPCs. Meanwhile, the tissue-specific origin and high consistency or stability of such cell types may prove to be the optimal parameters for standardized therapeutic success.

Single Tissue Donation for Multiple Mammalian FPC Types

Ovine FPCs and Cell-Based Cell-Free Topical Preparations

In addition to therapeutic cell roles for tissue-engineered products, banked primary FPCs are well adaptable as intermediates/substrates in the supply chain of therapeutic and medical (e.g., medical devices) or cosmetic/cosmeceutical products, targeting mild to moderate cutaneous diseases or states, such as acne scars, post-laser maintenance, physiological aging marks, burns, and wounds (Limat et al., 1996; Fitzpatrick and Rostan, 2003; Wu et al., 2003; Gold and Biron, 2006; Gold et al., 2007). Various cutaneous/ectodermal and musculoskeletal ovine FPC types (i.e., isolated from skin, muscle, connective tissue) have been established in collaboration with the food industry for further processing, culture-expansion, multi-tiered banking, and the eventual inclusion of cell-free derivatives in stabilized biopharmaceutical topical preparations, achieving further optimization of primary FPC banking for regenerative cutaneous applications (Lapp et al., 2013). Ovine primary FPC types were found to adapt to standardized whole-cell bioprocessing and out-scaling frameworks optimally (i.e., efficiently outperforming human FPC types), characterized by optimal expansion kinetics and remarkable *in vitro* stability (i.e., extensive lifespan, protein concentration regularity), and normalized efficacy in co-culture models. Carefully balanced derivative combinations in near homeopathic relative quantities yielded optimal stimulatory results, indicating complementary or synergistic effects of various specific active principles. Pharmaceutical-grade cell-free preparations were applied for veterinary and human case studies (i.e., wounds and burns), yielding efficient results for aiding tissue repair (**Figure 12**). Additionally, a significant technological advantage exists in the stabilization of the therapeutic potential of ovine FPCs, consistently retaining and preserving initial physiological properties and therapeutic attributes, *via* derivation of cell-free extracts. In addition, the formulation of the latter in ready-to-use topical pharmaceutical delivery forms with extensive shelf lives compared to fresh living cells is another advantage (Lapp et al., 2013). Such preparations appear well suited for maintenance therapies within consolidated wound repair strategies, or as specific topical regenerative solutions, depending on dosage and formulation type.

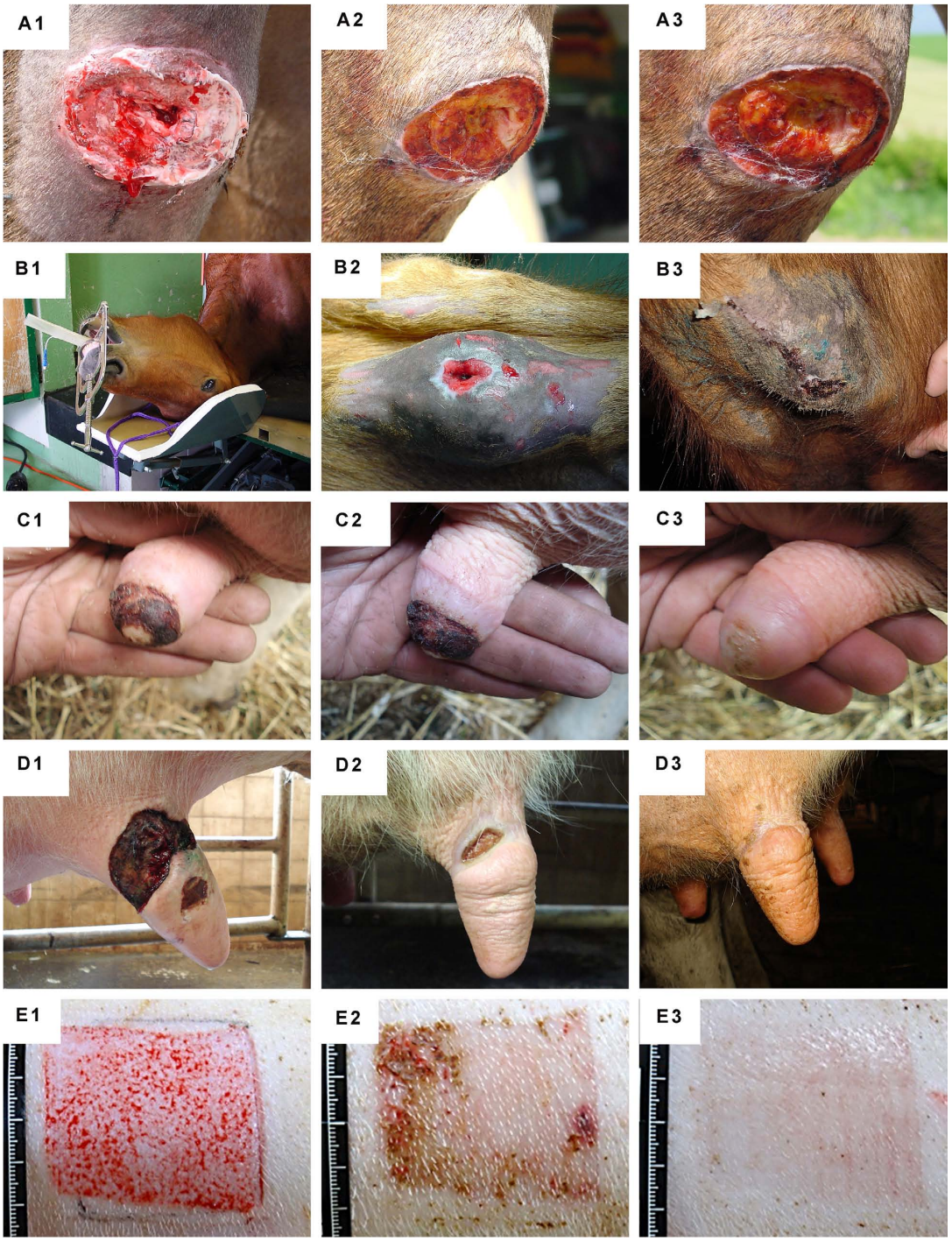

FIGURE 12 | Clinical veterinary case-reports illustrating the use and efficacy of equine Progenitor Biological Bandages, ovine FPC derivatives formulated as creams, and human dermal FPCs formulated in hydrogels for the management of animal traumatic injuries and donor-site wounds. **(A1–A3)** Profound distal limb articular lesion on the right hind knee of a French Saddlebred Pony (i.e., caused by a severe fall against barbed wire). The lesion was treated with ePBBs and bandages were removed after three days. **(B1–B3)** Mandibular fistula created by an abscessed tooth on a Franche-Montagne horse. The lesion was treated with ePBBs and bandages were removed after nine days. **(C1–C3)** Cow udder having suffered compression force trauma. Cell-free cream containing ovine FPC derivatives was applied daily. Photographic representations of the lesions at the beginning of treatment, after two weeks, and after seven weeks. **(D1–D3)** Cow udder having suffered sharp force trauma. The same cream was applied daily. Photographic representations of the lesions at the beginning of treatment, after seven weeks, and after nine weeks. **(E1–E3)** Porcine skin model for donor-site wound healing stimulation evaluation using human dermal FPCs (i.e., FE002-SK2 cell type) formulated in hydrogels. Photographic representations of the lesions at the beginning of treatment, after six days, and after 14 days. Figures modified with permission from Lapp et al. (2013) and Laurent et al. (2020e).

Equine FPCs in Hippiatric Regenerative Medicine

Based on the extensive experience in the regenerative potential of primary human and ovine FPC types isolated from various cutaneous and musculoskeletal tissues (i.e., skin, muscle, cartilage, tendon, connective tissue, and bone) of single fetal organ donations, the optimized fetal transplantation framework was applied to equine tissue biopsies (i.e., individual organ donations yielding multiple tissue sources) (Laurent et al., 2020b). There is high demand for large animal (e.g., horses or camels) innovative bioengineered therapeutic solutions in tissue reconstruction, which might be extrapolated from human regenerative medicine, due to strong similarities in respective wound healing processes (also see the "One Health Initiative") (Bigbie et al., 1991; Litzke et al., 2004; Carstanjen et al., 2006; Koch et al., 2009). Primary equine musculoskeletal FPCs were found to optimally and rapidly adapt to standardized bioprocessing and robust multi-tiered biobanking frameworks in view of optimized hippiatric medicine applications (i.e., tissue reconstruction and wound healing). Consistency, safety, and cytocompatibility were demonstrated with collagen and HA constructs, as well as the absence of immunogenicity or tumorigenicity in several case studies of reconstructive surgeries (Laurent et al., 2020b). Indeed, bioengineered equine PBBs (ePBB, formulated as magistral preparations) yielded efficient preliminary results in stimulating healing resurgence and stimulation of animal tissue repair. In particular, equine FPC therapies seemed to effectively stimulate epidermal and soft tissue regeneration, while limiting granulation tissue formation. Allogenic equine FPC therapy products derived from a single equine fetal organ donation may, therefore, be applied for the multifactorial translational musculoskeletal regenerative treatment of millions of veterinary patients in a safe, effective, and cost-effective manner (Laurent et al., 2020b).

ITERATIVE OPTIMIZATION OF FPC BIOBANKING AND DRUG DELIVERY OPTIONS

High clinical and regulatory pressure prompts iterative optimization of bioprocessing methods involving cell culture steps, mainly to replace animal-derived materials or substrates with defined products or consumables compatible with GMP manufacture, for safety and quality maximization (e.g., avoiding risk of contamination by transmissible spongiform encephalopathies in animal serum) (De Corte et al., 2012). Products such as Accutase® or TrypLE™ and Biofreeze®, human serum albumin or sugar-based solutions have been proposed to replace porcine trypsin and DMSO-based cryopreservation media, respectively, and numerous producers tentatively develop serum-free culture media or HPL-based supplements, with variable results. The imperative nature of such changes must be relativized, as extensive industrial use of FBS has not yet yielded critical adverse effects. For industrial-scale manufacturing of cell bank lots, stringent optimization must be conducted regarding raw materials, reagents, and contact-process consumable selection. In particular, the make, model, and lot identity of culture vessels and nutrient supplements must be optimized by thorough benchmarking before use in a GMP environment, as these elements may bear significant impacts on endpoint cell yields or population doubling times, thereby tangibly affecting the overall cost of production (Laurent et al., 2020e). Novel culture vessel systems may be investigated for two-dimensional (e.g., Corning®HYPERFlasks®, Nunc™ Cell Factories™, or Greiner CELLdiscs™) or three-dimensional (e.g., Terumo Quantum®, roller bottles, spinner flasks) cell culture efficiency, but should be thoroughly validated before adoption at industrial scales. Extensive optimization of polymeric biomaterials and novel biophysical processing methods continuously provide delivery scaffold options (i.e., inert, functionalized, or bioactive) for drug delivery of conjugated therapeutic cells. Acceptable cell survival and relative engraftment *in vivo* may be desired in specific applications, as wound environments adversely affect these parameters, due to anoikis, hypoxia, and local inflammatory effectors (Hyun et al., 2013). High resistance to oxidative stress, cryogenic shock, and physical constraints, such as shear stress, enable the effective coupling of primary FPCs with vast arrays of biomaterials, whereas, concerning cytocompatibility, the choice of therapeutic cell type often proves to be a major limiting factor (Ng et al., 2004; De Buys Roessingh et al., 2006; Grognuz et al., 2016a). Importantly, future efforts in the development of therapeutic biological constructs will need to include ancillary, yet necessary, modalities of tissue reconstruction, such as anti-microbial factors to combat infectious complications (Abdel-Sayed et al., 2016; Valerio et al., 2016). In order to pursue continued product optimization and abolition of logistical dependency to cold chains, further biochemical or physical processing of therapeutic cellular materials may be implemented for integral cells or cell-free derivatives, in order to obtain preparations such as desiccated powders (e.g., lyophilizates) or semi-solid topical or injectable formulation (e.g., viscous hydrogels or creams) (Hirt-Burri et al., 2011; Lapp et al., 2013; Aldag et al., 2016; Grognuz et al., 2016a; Bari et al., 2019). For facilitated regulatory submissions, the combination of therapeutic cells should be considered with existing and marketed products, such as medical devices, benefiting from clinical validation. Such approaches, whenever possible, contribute to diminishing the validation efforts of novel TEPs and combination products such as combined advanced therapy medicinal products (cATMPs) in particular (Tenorio et al., 2011). Cell–scaffold interactions, creation of functional tissues, or absence of cytotoxicity and toxic by-products, must then be demonstrated, as well as biodegradability in specific cases (Hirt-Burri and Applegate, 2013; Jafari et al., 2017). Safety risks (i.e., immunogenicity or tumorigenicity) associated with the use of viable therapeutic cells (i.e., stem cells or FPCs) may be completely averted or mitigated by using devitalized cells or cell-free products (e.g., cell-based cell-free formulations). Alternative processing options for cell populations during cellular therapy or product manufacture comprise various physical (e.g., direct cryopreservation, lyophilization) or chemical processes (e.g., controlled lysis), resulting in devitalization and/or loss of cellular structural integrity, which may be followed by extraction or purification steps. Alternatively, conditioned media may be

used for cell-free approaches. Devitalization or use of cell-free derivatives may be of considerable interest from a regulatory standpoint, as resulting therapies or products may be classified as cosmetics or medical devices, based on the nature of the intended effects and the relative importance of said effects (i.e., main or ancillary effects). Overall, tangible benefits favor the specific workflows of simultaneous and differential isolation of multiple FPC types from single fetal tissue donations, as culture initiation conditions are highly similar for all considered biopsies, may be controlled in parallel, and may be adjusted iteratively. Further optimization of FPC biobanking shall focus on epigenetics and influences of ethnic diversity on comparative efficiency for therapeutic product design or biotechnological manufacturing optimization.

ETHICS, MORALS, RELIGION, AND POLITICS AROUND FPCS

The unique approach of multiple-organ harvest following fetal organ donations, as practiced for adult solid organ transplantation, additionally restricts the need for multiple cell type isolation procedures. Technical or logistical availability of fetal tissue is theoretically not an issue, given the high relative rates of voluntary pregnancy termination in modern societies (e.g., six to nine terminations *per* 1,000 women in Switzerland over the past two decades) (Addor et al., 2003; Swiss Federal Statistical Office, 2019). In many countries including Switzerland, procurement of fetal tissue is classified as an organ donation and is highly regulated, as it requires Federal Authorities and Ethics Committee approvals (Applegate et al., 2009, 2010). Furthermore, regulated methodological aspects of Fetal Transplantation Programs and donor consent obtention ensure that related biomedical research does not increase either the number of pregnancy terminations nor the moral value thereof, and does not influence the termination date within the gestational period. Ethical examinations of programs seeking access to such fetal tissues embody a large place in the actual proceedings, whereas large variability exists in this respect throughout different countries and even between different states in Switzerland. Much like work with embryonic cells, the use of FPCs is deeply linked to moral questions, which are most prone to elicit debate. Depending on the technical availability of donated tissues, it is clear that some countries may not establish sufficient amounts of therapeutic treatments, but therapies or cell-based treatments may be imported, where they are allowed for use. In our own personal views, the whole-cell bioprocessing of fetal tissues for the establishment of primary FPC types, as described herein, might appear "unnatural" from conservative or strong religious standpoints. Nevertheless, this workflow requires minimal manipulation and ensures optimal conservation of initial tissue-specific biological characteristics for progeny cell populations. Such cell types therefore require relatively less human and biochemical intervention than phenotypically oriented MSCs or manipulated iPSCs, in order to obtain therapeutic cell populations or cell substrates fit for eventual clinical use.

It is also our personal belief that scientific and ethical advantages may be established around the use of fetal tissue or FPCs for therapeutic purposes, such as the potential medical benefits for millions of patients following one single organ donation, the restricted need for resorting to autograft harvest, and the respectful revalorization of high therapeutic value tissues otherwise destined for destruction. Considered clinical applications and therapeutic benefits resulting from the use of fetal organ donations may be quantified, and their weight may be clearly examined by appropriate regulatory and ethics bodies. Without the original fetal or embryonic cell lines established in the 20th century, many vaccines would not have been developed, potentially costing millions of lives. However, similarly to research on embryonic cells and despite the clear technical and clinical benefits from a scientific point of view, profound ethical and emotional aspects indirectly govern the practice of FPC therapy and the use of such substrates in the biotechnological industry (Lawler, 1981; Sanders et al., 1993; Rahman et al., 1998; Jost, 2002; Zimmerman, 2004; Greely, 2006; Panikkar et al., 2012). In particular, remarkable dissertations by the Catholic Church and Vatican-related groups extensively discuss the use of fetal cells from pregnancy terminations and applied for vaccine production, listing the "incriminated" products and companies and deeming the use of such materials as nuanced between and within the scope of "licit and illicit cooperation in Evil," under the influence of pharmaceutical companies and pertained to "social/medical moral coercion" (Furton, 1999; Maher et al., 2002; Pontifical Academy for Life, 2006). Interestingly, such positions are not maintained around the use of perinatal stem cells, as their exploitation for therapeutic purposes benefits from more leniency (Abbaspanah et al., 2018; Gaggi et al., 2019). On a political side, direct modulation of fetal cell research (i.e., including *in vitro* fertilization) has been achieved in the United States by cyclic restrictions on federal funding, with conservative positions aiming at banning such practices, while liberals have historically promoted women's health and freedom of choice, directly and indirectly benefiting medical progress (Manier, 2002). In a broader perspective, it is to note that perceived obstructionism to specific therapeutic cell source exploitation is not limited to religious or radical positions, as the US government has, through various and evolving polices, banned many aspects of research around ESCs for example, as this specific topic remains in heated debate (Murugan, 2009).

LEGAL AND REGULATORY FRAMEWORKS FOR FPCs AND PRODUCT DEVELOPMENT

Development and commercialization of therapies or cell-derived products are highly regulated in order to ensure safety and quality for the recipient. Respective regulatory landscapes and frameworks have been disruptively updated in Europe recently, creating labyrinthian procedures with mitigated outcomes on advances in the field of regenerative medicine, while potentially creating many regulatory pathway complications and deadlocks (Bertram et al., 2012; De Wilde et al., 2016; Dimitropoulos et al., 2016; Hartmann-Fritsch et al., 2016; Abdel-Sayed et al.,

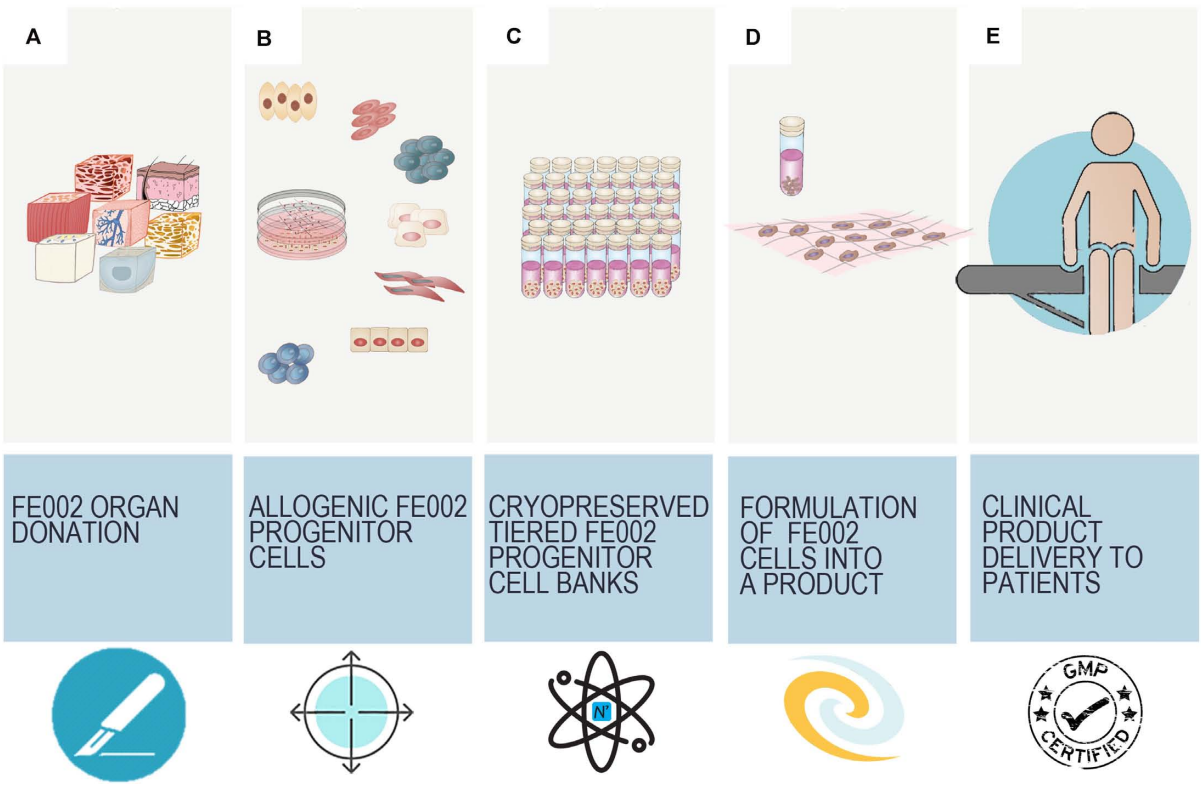

FIGURE 13 | Workflow schematic from initial fetal organ donation biopsy processing to FPC drug delivery to the patient within regenerative medicine settings. Following procurement of the FE002 donation **(A)** within the redefined regulatory framework (i.e., post-2007), tissue-specific allogenic primary FPC types were differentially and simultaneously derived **(B)**, and used to constitute multi-tiered cryopreserved cell banks **(C)**. In view of clinical delivery of therapeutic cells, appropriate vials may be initiated from storage and conjugated with adequate bioengineered scaffolds **(D)**. The resulting constructs are standardized and safety is ensured by GMP processing from raw materials to final products. Following liberation, the products are transferred to the clinic for application on patients **(E)**.

2019b). Bioengineered products (e.g., cell-laden scaffolds), as considered herein for primary FPC delivery, are classified as combined ATMPs or TEPs, implying inherent substantial manipulations for standardized transplant elaboration, for which GMP requirements are derived from classical pharmaceutical industry guidelines (Johnson et al., 2011; Fisher and Mauck, 2013; Esteban-Vives et al., 2018, 2019). Such dangerous or hampering constraints have limited and eventually reduced the number of products and therapies reaching the market in Europe and are particularly problematic for University Hospitals in particular, as local regulators enforce supranational regulatory frameworks often in detrimental or jeopardizing ways concerning historically used and clinically proven therapies (e.g., cultured autografts for burn patients) (Gallico et al., 1984; Gallico and O'Connor, 1985; Hickerson et al., 1994; Wood et al., 2006; Cirodde et al., 2011; Auxenfans et al., 2015; Eder and Wild, 2019; Laurent et al., 2020i). Faced with pharaonic costs of GMP manufacture and regulatory submissions burdening all public and private stakeholders, hospitals have developed differential approaches to implement in-house cell therapies (Gaspar and Swift, 2013; Cuende et al., 2014). Such undertakings were essential in order to comply with overarching legal frameworks, while continually providing the best therapies available to patients and conducting tangible innovative translational research for highly specialized medical applications. Such approaches of legal exposure mitigation comprise hospital exemptions, compassionate use, exceptional authorizations, orphan drug pathways, magistral or officinal preparations, possibly paving the way for the inclusion of cell-based therapies or cell/cell-derived APIs in official recognized repositories such as pharmacopeias (Pirnay et al., 2013, 2018; World Medical Association, 2013; Pearce et al., 2014; Dimitropoulos et al., 2016; Laurent et al., 2020i). Conjugation of high innovation and virtuosic interpretation of restrictively rigid or unharmonized legal and regulatory frameworks are current necessities, in order to ensure the progress of translational therapeutic developments for the benefit of patients worldwide.

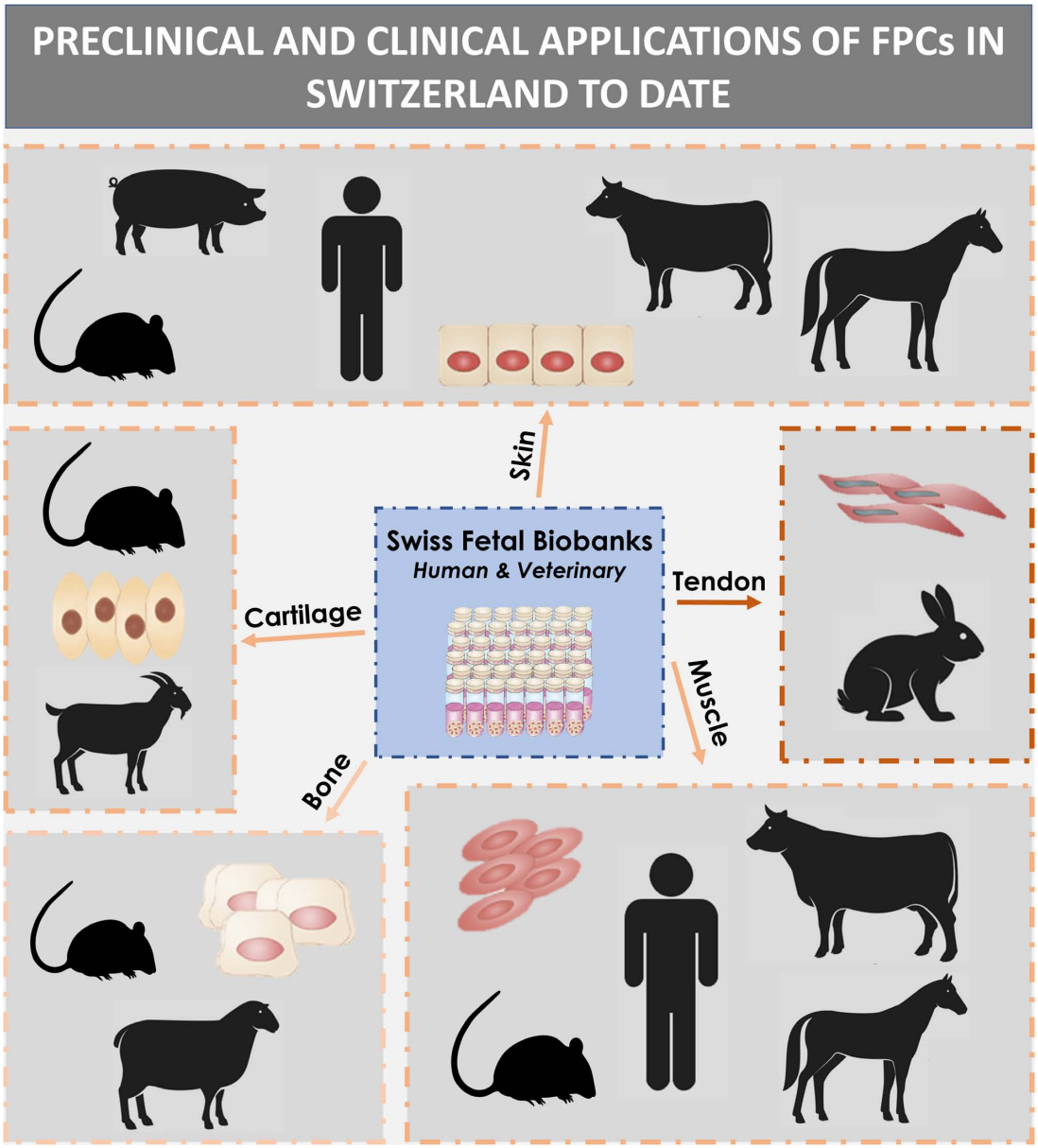

FIGURE 14 | General overview of *in vivo* experimentation and clinical applications of banked primary FPC types in Switzerland during the past two decades. Therapeutic cells constituted successive human Fetal Biobanks, as well as equine and ovine Fetal Biobanks. Therapeutic products comprised combined cell therapy products (i.e., PBBs, ePBBs, viable cells seeded in alternative polymeric scaffolds) or cosmeceutical/medical device-type semi-solid topical formulations of cell-free extracts (i.e., creams and hydrogels). Each FPC type is associated with the different models (i.e., human, porcine, murine, ovine, caprine, bovine, equine, lagomorph) which were part of preclinical investigations or clinically treated with FPCs or stabilized derivatives thereof. Accumulated clinical experience and hindsight attest to the absence of immunogenicity or tumorigenicity of mammalian FPCs, in their bio-integral, viable, or cell-free extract form, in both allogenic and defined xenogenic settings.

GENERAL DISCUSSION

The present work describes fundamental, preclinical, clinical, and industrial developments embodying the scientific advances supported by Swiss FPC banking. Such comprehensive reformulation and update of the past three decades of multidisciplinary work aimed to substantiate and convey interest, broadening awareness and use of standardized protocols for translational regenerative medicine, potentially impacting millions of patients suffering from cutaneous and musculoskeletal wounds and diseases. The high utility potential of recently derived primary FPC types (e.g., FE002-Lu cell type) for biopharmaceutical therapeutic product manufacturing was also addressed, allowing for potential direct, indirect, and synergistic improvement of modern therapeutic armamentariums. The necessity for safe and consistent biological

material sources is of paramount importance, in view of applicable regulatory, technical, and economic requirements existing within cell therapy product or biotechnological substrate development. In such regulated and defined contexts, optimization and standardization are of prime concern and should be the key steps in any translational workflows and manufacturing processes. Optimal management of safety and consistency of therapeutic cell sources is accomplished by avoiding pooling of numerous heterogeneous biological samples, and alternatively, exploiting sustainable multi-tiered FPC biobanks, simultaneously and differentially established after single fetal organ donations. Iterative therapeutic optimization and customized Fetal Transplantation Programs, enabling ethical and controlled biological material revalorization, have constituted the core innovative and developmental base for FPC therapy in Switzerland throughout three decades (**Figure 1**). Straightforward workflows were devised for tissue procurement, maximizing traceability, safety, consistency, and robustness of progeny cellular materials (**Figures 6–8**). The overall perception generated by translational work on FPC banking and transposition of related innovative biomedical technologies has comprehensively detailed the complexity of technical and therapeutic success obtention, which remains as a founding prerequisite in commercial product development. Banked FPCs have been historically used and thoroughly investigated throughout three decades in Switzerland, and have been deemed to adapt exceptionally well to specific therapeutic product developmental pathways. Extensive clinical experience has demonstrated the safety and usefulness of multiple primary FPC types to date. In the Lausanne University Hospital, pioneer contributions to innovative cutaneous regeneration solutions using dermal FPCs (e.g., FE002-SK2 cell type) have constituted the unified clinical flagship and eventual translational embodiment of the Swiss FPC Transplantation Program (**Figures 9–12**). These undertakings have yielded plethoric insights into the adequate conjugation of modern biotechnological innovation with current constraining legislative, ethical, and regulatory frameworks.

Transversal works on soft tissue and musculoskeletal FPC types of human and animal origin have provided diversified and differential insights into the potentials of FPC banking and supported further translational work in clinical testing and implementation. Most importantly, a single human fetal organ donation (i.e., FE002) qualifying for the Swiss Fetal Transplantation Program in 2009 yielded multiple unique FPC types (e.g., skin, cartilage, tendon, muscle, bone, and lung FPCs), validating the sustainable model of single donation for simultaneous differential organ harvest, subsequently presenting the *quasi*-infinite potential of applied research, clinical studies, and product development (**Figures 7**, **13**). Widespread optimized and standardized sustainability constitute the core therapeutic value of FPC material sourcing and biobanking workflows supported herein, allowing the potential derivation of billions of affordable and efficient therapeutic product doses. As demonstrated herein by the comprehensive and detailed holistic approach of Swiss FPC biobanking technology, a single voluntary fetal organ donation is sufficient to support translational research encompassing the cutaneous and musculoskeletal systems for several decades (**Figure 14** and **Table 1**). Further formulation and delivery system optimization, preclinical work, and clinical translation of therapies using FPCs will further enhance quality and efficiency of therapeutic care, benefiting overall health of patients worldwide.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the CHUV University Hospital: State Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AL and LA: study conception and design. AL, CS, MM, and NH-B: acquisition of data. AL and NH-B: analysis and interpretation of data. AL, NH-B, MM, and LA: drafting of the manuscript. CS, NH-B, AB, WR, and LA: critical revision. AL, CS, MM, AD, WR, NH-B, and LA: acceptance of final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.557758/full#supplementary-material

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