Changing Metabolism in Differentiating Cardiac Progenitor Cells—Can Stem Cells Become Metabolically Flexible Cardiomyocytes?

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The heart is a metabolic omnivore and the adult heart selects the substrate best suited for each circumstance, with fatty acid oxidation preferred in order to fulfill the high energy demand of the contracting myocardium. The fetal heart exists in an hypoxic environment and obtains the bulk of its energy via glycolysis. After birth, the "fetal switch" to oxidative metabolism of glucose and fatty acids has been linked to the loss of the regenerative phenotype. Various stem cell types have been used in differentiation studies, but most are cultured in high glucose media. This does not change in the majority of cardiac differentiation protocols. Despite the fact that metabolic state affects marker expression and cellular function and activity, the substrate composition is currently being overlooked. In this review we discuss changes in cardiac metabolism during development, the various protocols used to differentiate progenitor cells to cardiomyocytes, what is known about stem cell metabolism and how consideration of metabolism can contribute toward maturation of stem cell-derived cardiomyocytes.

Keywords: heart, progenitor cells, substrate metabolism, mitochondria, differentiation, cardiomyocytes

STEM CELL THERAPY FOR THE HEART

Myocardial infarction (MI) is the primary cause of disease-related death in the world with no reliable therapy (1). Acute coronary syndromes, like MI, account for half of all cardiovascular deaths in the industrialized world, with around 20% of patients developing heart failure (HF) and having a 1-year mortality rate (2, 3). Current therapeutic strategies focus on reperfusion, thrombolysis and reducing the workload of the heart using pharmacological agents or surgical procedures (4–6). Recent advances in treatment have improved time to reperfusion, but progress in identifying efficient therapies to offer more than symptom alleviation and support the surviving myocardium is yet to result in substantial clinical benefit (7, 8). The only current long-term solution is heart transplantation but with the limited numbers of donors, and the need for chronic immunosuppressants (9), the search to find an alternative solution to the problem of end stage HF is becoming increasingly urgent. MI can lead to a loss of up to 1 billion cardiomyocytes, which cannot be replaced due to the insufficient degree of regeneration in the adult heart (10). Although the heart is no longer considered a post-mitotic organ, the turnover of cardiomyocytes in the adult heart is around 1% per year (11) which is insufficient to counter the loss caused by MI. Stem cell therapy (SCT) has the potential to regenerate the damaged tissue and restore its

contractility, harnessing the self-renewal and differentiation potential of stem cells (SCs) (12). *In vivo*, the transplanted cells can act *via* a combination of the following mechanisms; (a) replicate themselves and/or differentiate to mature cardiomyocytes; (b) stimulate the endogenous cardiac cells to regenerate; (c) exert a beneficial effect via paracrine mechanisms of action (13) (**Figure 1**).

TYPES OF STEM CELLS FOR THERAPY

A wide range of cells have been tested both in animal models or early-stage human clinical trials in order to find the appropriate source for SCT (14, 15). These include bone-marrow derived cells (16–18), cardiac stem or progenitor cells (19–25), human embryonic stem cell-derived cardiomyocytes (26–29) and human inducible-pluripotent stem cell-derived cardiomyocytes (30, 31).

Bone marrow-derived stem cells were claimed to differentiate into cardiomyocytes that spontaneously beat after 2 weeks in culture (17) or into myotubules that, when injected into infarcted hearts, stimulated angiogenesis and generated cardiac-like cells (16). In addition, it was reported that when bone marrow-derived stem cell growth factor receptor-positive/linage negative (c-kit+/lin-) cells were injected into infarcted tissue, they generated new cardiac cells and blood vessels and re-muscularised the damaged region (18). However, later studies showed that bone marrow-derived cells do not trans-differentiate into cardiomyocytes and that retained transplanted cells adopted a mature haematopoetic fate (32, 33). Bone-marrow derived mesenchymal cells have also been shown to improve cardiac function following MI, although repair is now thought to result from the delivery of a cocktail of beneficial cytokines which induce angiogenesis, limit scar fibrosis and may activate endogenous cardiac progenitors (34–36). Other key types of mesenchymal stem cells (MSCs) such as umbilical cord MSCs (37, 38), adipose-derived MSCs (39–41) and amniotic fluid MSCs (42), chosen for their ease of isolation and differentiation, have also been tested for therapeutic potential after infarction. As with bone marrow cells, any beneficial effect was deemed to be paracrine.
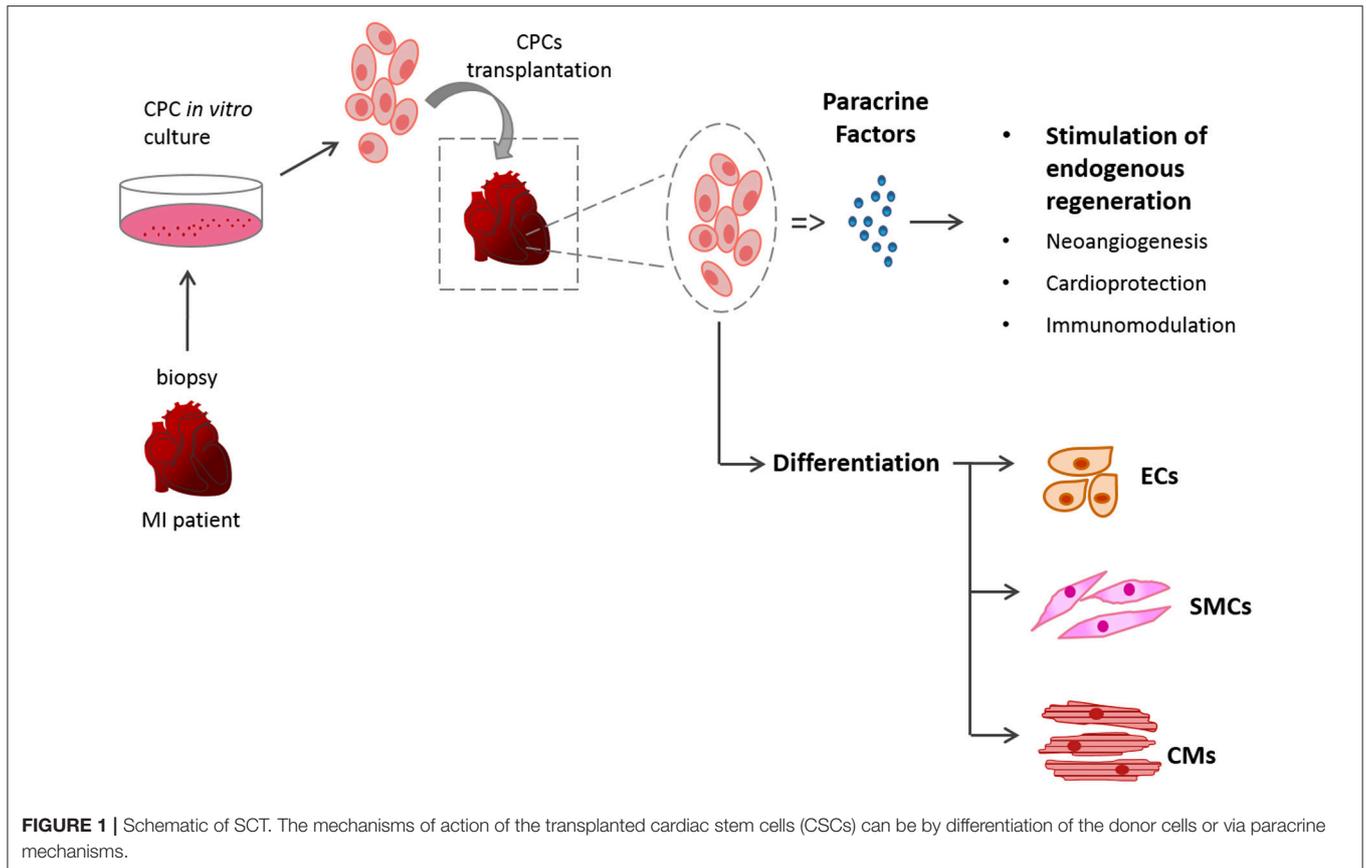
In 2003, a population of cardiac progenitor cells called stem cell growth factor receptor-positive (c-kit+) cells were identified (19). *In vitro*, these proliferative cells can self-renew and differentiate into myocytes. When injected into infarcted hearts, c-kit+ cells were shown to differentiate into cardiomyocytes resulting in myocardium regeneration and improved heart function (19). Subsequent studies supported the beneficial effect after cardiac injury, but suggested that the c-kit+ cells were of bone marrow origin (43) or were mast cells (44), endothelial cells (45) or maybe a mixed population of both (46). Studies looking at the issue from a developmental perspective suggested that c-kit+ cells contribute to new cardiomyocytes after injury in the neonate, but they were unable to do so in the adult heart (47), in line with previous observations regarding the role of c-kit+ cells in the neonatal heart (48). In 2011, the Anversa group used c-kit+ cardiac progenitor cells (CPCs) in a phase-I Stem Cell Infusion in Patients with Ischemic cardiomyopathy

(SCIPIO) clinical trial showing encouraging results for post-MI treatment (49). However, in 2014 the results were questioned for their integrity (50). In 2013, Ellison et al. showed that c-kit+ CPCs were necessary and sufficient for cardiac recovery in rodent models of diffuse myocardial damage causing acute heart failure (51). This was subsequently challenged by studies using c-kit+ lineage tracing mouse models, and reporter lines, where it was shown that cardiac c-kit+ cells contribute to cardiomyocytes only minimally, but mainly and substantially generate cardiac endothelial cells (52–54). The debate continues, with recent publications showing that selected c-kit+ cardiac cells contain a low level (about 1%) of clonal cells that can be expanded and differentiated into spontaneously beating cardiomyocytes (55) and that c-kit expression can be identified on both CPCs and a subpopulation of cardiomyocytes and is upregulated in response to pathological stress (56).

Also in 2003, another population of cardiac progenitor cells was identified, the stem cell antigen 1 (sca-1+) cells, in the mouse heart, having stem-like self-renewal characteristics and the ability to home to the injured myocardium (20). Later it was shown that this cell population led to increased ejection fraction and neoangiogenesis, after injection into the acutely infarcted mouse heart (57). It was also shown that sca-1+ CPCs contribute to the generation of cardiomyocytes during normal aging and after injury sca-1+ cells were induced to differentiate to three cardiac cell types (44). In line with these observations, a genetic deletion of sca-1 caused primary cardiac defects in heart contractility, an impaired damage response and reduced CPC proliferation (58). Although the human sca-1 isoform does not exist, a sca-1-like cell population has been isolated from the human heart using the murine antibody and has been extensively studied by the Goumans group (59). In a similar manner to the isolation of a clonal c-kit+ population, clonal cells have been identified within selected sca-1+, PDGRFα+ mouse cells and have been shown to improve cardiac function after MI, but again by a largely paracrine mechanism (60).

In addition, a population of progenitor cells were derived, via the formation of cardiospheres, from cells migrating from adult human and murine heart explants, called cardiosphere-derived cells (CDCs) (21). It was reported that human CDCs have the ability to self-renew in culture and express the endothelial kinase insert domain receptor (KDR) and other known stem cell markers (CD-31, CD-34, c-kit, and sca-1) (21). This heterogeneous cell population has also been shown to have beneficial effects, both in animal models (22, 61) and in a clinical trial (62), but again via the release of paracrine factors.

CPCs have been also identified by markers of embryonic origin, like Insulin gene enhancer protein 1 (Isl1) and NK2 homeobox 5 (Nkx2.5). Isl1 is a cardiac transcription factor expressed in second heart field progenitors and cardiac neural crest cells, involved in cardiovascular development, and leading to severely deformed hearts in rodents after genetic deletion (24, 63). Nkx2.5, a homeobox-containing transcription factor, has been identified via its involvement in cardiac looping (64, 65). Isl1+ and Nkx2.5+ CPCs have been shown to differentiate into

FIGURE 1 | Schematic of SCT. The mechanisms of action of the transplanted cardiac stem cells (CSCs) can be by differentiation of the donor cells or via paracrine mechanisms.

major cardiac lineages, mainly contributing to proepicardium during development (25).

Human embryonic stem cells (hESCs) are derived from the inner cell mass (ICM) of blastocysts from donated fertilized eggs (66). Human ESCs have the ability to continuously proliferate in an undifferentiated state and, when given the appropriate signals, will differentiate into any cell type. An interesting tool for cell therapy that originated from this field, are the *in vitro*–generated, stem cell–derived cardiomyocytes (SC-CMs). These have been shown to integrate structurally and functionally with healthy host cardiac tissue *in vivo* in various studies (26, 67, 68). These cells show great promise, but there are ethical concerns using hESCs in the clinic and the risk of teratoma formation (69). In 2007, Yamanaka's group were the first to report the reprogramming of human somatic cells into induced pluripotent stem cells (iPSCs), by overexpression of the transcription factors: Oct4, Sox2, KLF4, and c-myc (70). The reprogrammed hiPSCs resembled hESCs and had the ability to self-renew while maintaining pluripotency (70). Human iPSCs can be produced from patient-specific somatic cells, therefore overcoming the problem of immune rejection and the ethical concerns of using hESCs (69). hiPSCs have been shown to improve cardiac function, albeit with limited donor cell retention (30, 31) and used extensively as *in vitro* human-cell-based models to study basic biology and development (71), to model diseases (72) and to screen for drugs (73, 74). This is particularly important for the heart, since

adult cardiomyocytes do not survive *in vitro*, as morphological and functional changes occur in long-term culture and so there has been no easy way to determine whether the effect of genetic mutations or of drug compounds that were observed in animal models would also be seen in a human cardiomyocyte. However, despite the promising *in vivo* results, the initiation of beating in SC-derived cardiomyocytes does not mean that these cells have the maturity or metabolic characteristics of mature cardiomyocytes found in the healthy heart (75). Studies have shown that SC-derived cardiomyocytes have immature calcium handling (76) and a response to drugs more akin to cardiomyocytes from the failing heart (77).

The effect of the transplantation environment on enhancing the maturation of human pluripotent SC-derived cardiomyocytes has been studied in rats. Despite their capacity to survive and form grafts, they failed to improve adverse remodeling or overall cardiac function after chronic MI (28). Approaches to enhance their efficacy, via preconditioning the cells and host environment, are currently being investigated [reviewed here (78)].

CARDIAC METABOLISM

The heart is a fascinating organ that beats 100,000 times a day and pumps 7,200 L of blood through the body, in the same period using 35 L of O_2 for energy production. It requires

about 6 kg of adenosine triphosphate (ATP), which it utilizes at a rate of 30 mg per second to sustain myocardial contraction and maintain ion homeostasis (79, 80). Since the heart has a low capacity for energy storage (81), an array of metabolic networks guides ATP production rates, based on demand. The heart has been characterized as a metabolic omnivore, being able to use a variety of substrates for energy production [see reviews (82, 83)]. Glucose, pyruvate, triglycerides, glycogen, lactate, ketone bodies, fatty acids (FAs) of different chain-lengths and certain amino acids are among the energy-providing substrates of the heart. It is responsible for almost 10% of the whole body fuel consumption; with FAs accounting for 70% of ATP production and carbohydrates for the remaining 30%.

Energy, in the form of ATP, can be produced in the cytosol via glycolysis (**Figure 2**); catabolism of glucose derived from carbohydrates. The end-product of glycolysis is pyruvate, which can be further reduced to produce lactate. In case of carbohydrate shortage, gluconeogenesis of pyruvate, re-oxygenation of lactate or glycerol metabolism, can be used as sources of glucose synthesis (82, 84). Alternatively, pyruvate can enter the mitochondria in the form of acetyl-coenzyme A (Acetyl-CoA) and be oxidized in the TCA cycle (also known as the Krebs cycle), in a process called oxidative phosphorylation (85). The reducing equivalents of this chained reaction act as hydrogen

carriers (Nicotinamide Adenine Dinucleotide Hydrogen; NADH and Flavin Adenine Dinucleotide Hydrogen; $FADH_2$) and enter the electron transport chain (ETC). There the coupled-transfer of electrons and H^+ creates an electrochemical proton gradient that leads to the production of ATP.

ATP can also be generated by the degradation of lipids (including triglycerides) into FAs, which are metabolized in the mitochondria via beta-oxidation (**Figure 2**), which converts Fatty Acyl-CoA to Acetyl-CoA for flux into the TCA cycle and ATP synthesis as above (86). Under aerobic conditions, more than 95% of ATP production comes from oxidative phosphorylation (86) and in the healthy heart 50–80% of the energy is generated via beta-oxidation (87). Oxidative phosphorylation yields 36 ATP/glucose molecule, being more efficient than glycolysis (2 ATP/glucose). Lipids, due to their reduced state are more oxygen-demanding than glucose (producing 2.8 ATP/O_2, vs. 3.7 ATP/O_2), but they are more energy-dense with a much higher yield of ATP/carbon (depending on the chain length of the parent FA) (79, 88).

The heart has a remarkable ability to adapt to changes in its physiological state by selecting the most efficient substrate, depending on the conditions of its environment (82). For example, as FAs require more oxygen than glucose, to generate the same amount of ATP (82, 89), upregulation of the hypoxia-inducible factor (HIF) under hypoxia has been shown to increase

FIGURE 2 | Schematic overview of basic cell metabolic pathways for energy production.
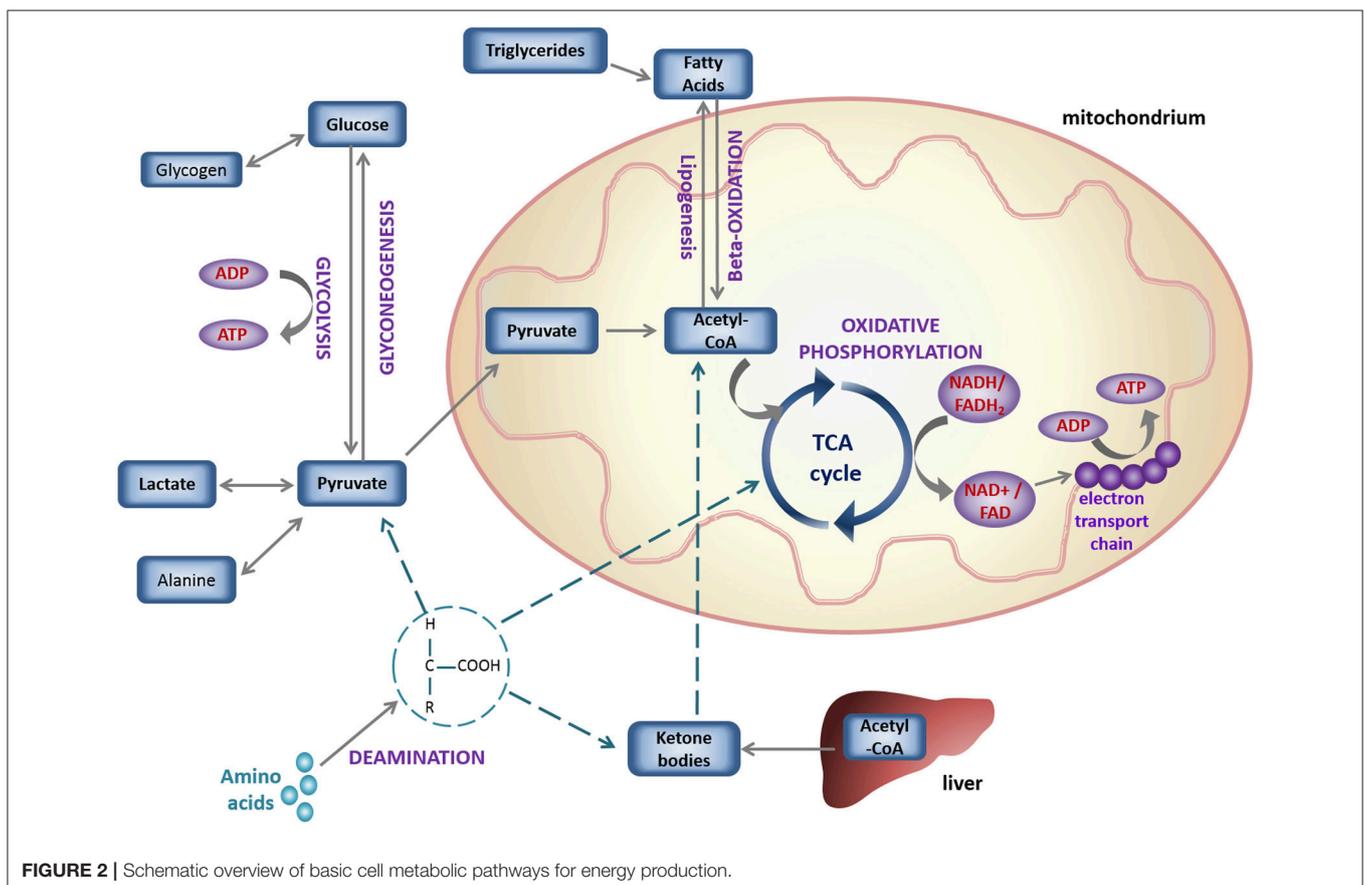
glycolysis and suppress mitochondrial oxidative metabolism (83, 90), shifting toward the more oxygen-efficient fuel: glucose. A network of interrelated signaling pathways control the flux of glucose and fatty acid metabolism to enable the heart to switch substrates rapidly. This was first described as the glucose-fatty acid cycle by Randle in 1963 (91), but the complexity of this network is yet to be fully explored (92). Under conditions of starvation, acetyl-CoA in the liver can form ketone bodies, that are water-soluble forms of this metabolite, and can be efficiently used by the brain and other oxidative organs (82) (**Figure 2**).

Cardiac Metabolism During Development—Metabolic Switch (1)

The fetal heart is adapted to an environment of low oxygen and low fatty acid content, so fetal cardiomyocytes are highly dependent on glycolysis for ATP production (93). In addition, lactate availability allows for energy production via lactate oxidation. During development, the heart undergoes a major metabolic alteration; the main physiological changes during the transition to the post-natal stage are the increased workload, and the demand for growth, that cannot be met by glucose and lactate consumption alone (94, 95). Interestingly, immediately after birth some studies suggest that the main energy biogenesis mechanism is still glycolysis (96). The post-natal increase in both circulating levels of free FAs (due to dietary alteration and lipid content in maternal milk) and in O_2 levels mediates a switch from glycolysis-dependence, to predominantly relying on oxidative metabolism as mature cardiomyocytes (93, 97) (**Figure 3**). After birth, the "fetal switch" to oxidative metabolism

of glucose and fatty acids has been linked to the loss of the regenerative phenotype (98). Neonatal mouse hearts can regenerate in the first postnatal week but this is lost after day 7 (99). Puente et al. investigated exposure of neonatal mice to hyperoxia (100% oxygen) or mild hypoxia (15% oxygen) and found that hyperoxia induced cardiomyocyte cell cycle arrest after birth whereas hypoxia prolonged the regenerative window (98). They hypothesized that this effect resulted from increased oxidative stress that accompanied the induction of oxidative metabolism and showed that treatment with an antioxidant resulted in a significant increase in cardiomyocyte mitosis in the first weeks after birth. During the early postnatal period, as cardiac energy demands increase, the number of mitochondria in cardiomyocytes increases dramatically (100, 101). A recent study has shown that the HIF1 signaling localisation pattern controls the embryonic switch toward oxidative metabolism, disruption of which affects cardiac maturation. The cardiac compartment where HIF was absent, the trabeculae, has increased oxidative metabolism, as well as higher mitochondrial content (102). Several studies demonstrated an increase in PPAR coactivator 1α (PGC-1), as well as Peroxisome Proliferator-Activated Receptor α (PPARα), mRNA levels in mice or rats during development (103, 104). The regulatory mechanism involves genes encoding several key mitochondrial ETC proteins, specifically the transcription factors nuclear respiratory factors-1 and−2 (NRF-1 and−2) (103, 105). Disruption of the ETC function, during cardiac development, leads to disrupted mitochondrial organization in the cardiomyocytes, resulting in perturbed sarcomere formation and contraction (106). Various

FIGURE 3 | Schematic of metabolic switches during cardiac development (left) and cell differentiation (right).
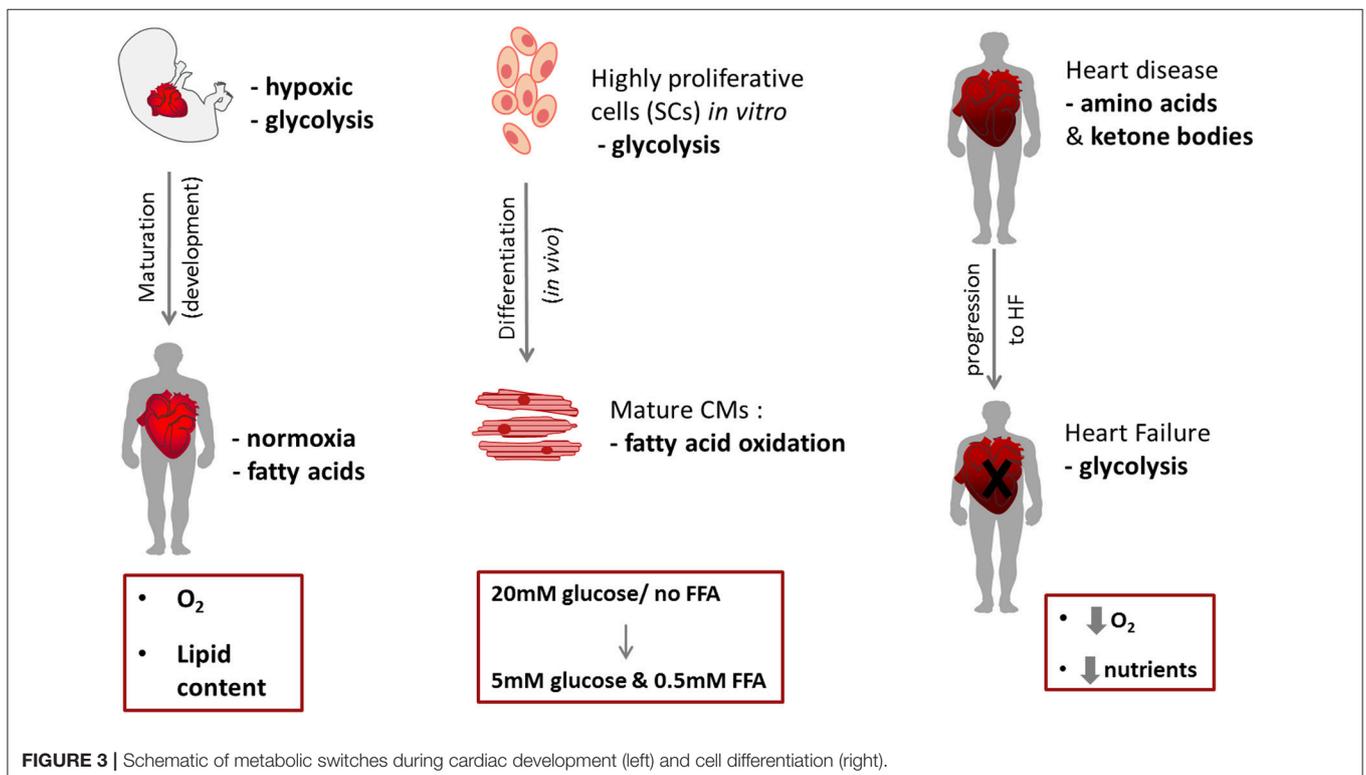
mouse models have revealed that disruption in mtDNA processes leads to embryonic lethality (107). In addition, signaling pathways involving the Nuclear factor of activated T-cell (NFAT) family of transcription factors play a role in mitochondrial biogenesis and cardiac function. Mice with nfatc3 and nfatc4 deletion had abnormal mitochondrial structure and reduced oxidative capacity, eventually dying after E10.5 (108). The ERRα/ERRγ axis plays a crucial role in mitochondrial energy production, as well, and their deletion leads to premature lethality in mice (109). Disruption of the ERRγ gene leads to impaired mitochondrial function, eventually blocking the switch from glycolysis to oxidative phosphorylation and leading to early prenatal death in mice (110).

Cardiac Metabolism in MI and HF—Metabolic Switch (2)

Following MI, tissue ischaemia leads to hypoxia, which in turn activates long-term HIF signaling in the myocardium (111). During MI, the ischemic region relies solely on glycogen, as an anaerobic fuel for energy production (112). The absence of oxygen for OXPHOS means that NADH and FADH2 are accumulated, affecting fatty acid enzyme reactions and leading to a build-up of fatty acid intermediates and resulting in disruption of mitochondrial cristae and function (87). Reduction in fatty acid oxidation upregulates anaerobic glycolysis and lactate production leading to intracellular acidosis (87). HIF1α activation upregulates the transcription of Bcl-2 adenovirus E1B 19 kDa-interacting protein 3 (BNIP3), which triggers selective mitochondrial autophagy (113, 114). Under normal conditions, BNIP3 expression is suppressed by the nuclear factor kappa beta (NF-κB) pathway (114). In addition, HIF1α upregulates the transcription of pyruvate dehydrogenase kinase 1 (PDK1), which inactivates pyruvate dehydrogenase, thereby blocking the entry of pyruvate into the mitochondria for oxidative phosphorylation (113). These HIF-1 mediated changes play key roles in reducing mitochondrial oxidative metabolism and thereby in reducing the generation of reactive oxygen species (ROS) (115). Following reperfusion, oxidative metabolism is restored but the resulting induction of ROS can lead to mitochondrial damage (116). As the heart progresses to failure, flux from pyruvate into the TCA cycle decreases (117). Mitochondria can be found with membrane and ETC defects (118, 119), as well as reduced respiratory capacity (119, 120) and reduced oxidative phosphorylation (121–123). These findings are consistent with the concept that, during HF, the metabolic state of the heart resembles that of the fetal stage, switching to glycolysis rather than mitochondrial oxidative metabolism (124); with increased glucose uptake (125) and glycolysis (86) and either no change or a decrease in glucose oxidation (125, 126).

The aforementioned perturbations in cardiac metabolism lead to a dangerous environment, due to the excess of lactate and oxidative metabolic intermediates. Progressive heart failure induces, at a cellular level, increased conversion to lactate, increasing cell acidosis (127). However, amino acids such as aspartate play a role in reducing damage that occurs as a result of lactate accumulation via upregulating the conversion of pyruvate

to alanine. Glutamate and aspartate improve the production of ATP as they produce intermediates that feed the TCA cycle for further oxidation (128). Studies have reported the reduced availability of amino acids in heart failure patients, leading to the depletion of some valuable amino acid derivatives that are important for normal cardiac function (l-carnitine and creatine) (129). However, there is also evidence of their accumulation during HF, as seen in a study on failing mouse hearts where genes associated with amino acid catabolism were downregulated during compensated hypertrophy and overt failure (130). Transcriptomic analyses reflected downregulation of genes involved in amino acid degradation pathways [proline, alanine, tryptophan, and mainly branched-chain amino acids (BCAAs)] (131–133). Using a genetic mouse model, Sun et al. demonstrated that a deficiency in BCAA catabolism induced heart failure under mechanical overload, resulting from increased oxidative stress (132). Similarly, Li et al. found that whilst chronic accumulation of BCAAs did not affect cardiac energetics and function in the healthy mouse heart, glucose oxidation was decreased which increased ischaemic injury after myocardial infarction (134).

The importance of mitochondrial integrity for maintenance of cardiac function has been also highlighted through several conditions which are characterized by mitochondrial mutations or abnormalities, causing among others: cardiomyopathy, neuromuscular dysfunction, diabetes mellitus and even sudden death (122, 135). In addition, the efficiency of the ETC is found to decline with age, which in turn decreases ATP generation (135, 136).

Cardiac Metabolism From Stem Cells to Adult Cells and Vice Versa—Metabolic Switch (3)

Metabolic changes during embryonic development have been reviewed in detail by Johnson et al. (137). Mammalian oocytes contain low levels of glycogen and fat but have substantial stores of amino acids and protein which have been estimated to be sufficient for the energetic needs of the first few days of development (137). The preimplantation embryo relies on the mitochondria inherited from the oocyte and on pyruvate oxidation for ATP production (138, 139). There is a gradual switch in metabolism during the preimplantation stage (morula, blastocyte) from aerobic oxidation to anaerobic glycolysis in preparation of the low-oxygen environment in the uterine wall (137). As the preimplantation embryo undergoes cell division there is a reduction in mitochondrial DNA copy number (mtDNA) and mitochondrial density (138) and an upregulation of glucose transporter 3 and hexokinase gene expression until anaerobic glycolysis increases to maximal rates as the blastocyst implants into the uterus (137). ESCs originate from the inner cell mass of preimplantation blastocyst, therefore, these cells show high rates of glycolysis and low oxidative phosphorylation to support the rapid cell proliferation (138, 140). Similarly, ESCs derived from in vitro cultured ICMs (141) or ESCs cultured in vitro (142) show high rates of glycolysis and low oxidative phosphorylation. A comparison of the metabolomics and energetics of iPSCs with those of ESCs showed many

similarities and confirmed that, during reprogramming, somatic cells convert from an oxidative state to a glycolytic state in pluripotency (143).

The reprogramming factors, c-Myc and Lin28, have been shown to enhance glycolysis and nuclear reprogramming (140) and reprogrammed pluripotent stem cells show upregulation of glycolytic enzymes such as the glucose transporter GLUT1, hexokinase (HK), phosphofructokinase (PFK) and LDH (144). In addition, hiPSCs have increased levels of intracellular glucose-6-phosphate, which feeds into the pentose phosphate pathway (PPP) to generate the reducing factor NADPH, which is used for biosynthesis of FAs and nucleotides necessary for rapid proliferation (144). Stimulation of glycolysis with D-fructose-6-phosphate or by PDK1 activation has been shown to enhance the efficiency of iPSC reprogramming (145). Mitochondrial morphology changes from being elongated and tubular-shaped with well-developed electron-dense cristae in somatic cells, to being spherical with small-undeveloped cristae in hiPSCs, with a transition from an extensive cytoplasmic networks in somatic cells, to a predominantly peri-nuclear location in hiPSCs (146). Prieto et al. found that this fragmentation was not associated with mitophagy but was induced by activation of dynamin-related protein 1 (Drp1), which fragments mitochondria in a GTPase-dependent manner (147). They showed that Erk activation in early reprogramming induced Drp1 phosphorylation which was critical to the reprogramming pathway. Studies in human and mouse ESCs have also found a small number of rounded and immature mitochondria with under-developed cristae (106, 148). Moreover, multipotent stem/progenitor cells show the same characteristics; for example, mitochondria in HSCs are relatively inactive and ATP content increases in lineage-committed progenitors compared with HSCs (149).

Due to the ongoing controversy as to whether the heart contains resident progenitor cells (150), little is known about the metabolism of cardiac stem cells in the adult heart. However most adult endogenous progenitor/stem cells reside in hypoxic niches (151), in a quiescent state, and therefore the metabolic rate is presumed to be low. CPCs isolated from the heart, by marker selection, tissue digestion or from explants, and expanded *in vitro*, are proliferative and grown in culture medium containing high levels of glucose and little or no FAs. Basal cellular homeostasis involves processes like protein turnover, DNA repair, and vesicle trafficking and therefore proliferating cells, in addition to homeostasis maintenance, need energy for anabolic processes such as cell division and growth. Proliferative cells, such as cancer cells, have been suggested to predominantly rely on glycolysis for ATP production, irrespective of oxygen presence; this metabolic paradox, first observed by Warburg in 1956 (152) has been termed "aerobic glycolysis." Apart from Warburg's observations on cancer cells, mouse fibroblasts (153) and human (154) and mouse (155) lymphocytes have been shown to utilize "aerobic glycolysis," when stimulated to proliferate. Various studies have supported the conclusion that the major function of aerobic glycolysis is to supply glycolytic intermediates for anabolic reactions in cells, thus being the metabolic pathway of choice during cell proliferation [for Review see (156)]. Although cells in high glucose medium predominantly metabolise glucose, they can use other substrates if provided. Human bone marrow MSCs have an active oxidative metabolism with a range of substrates and can produce more ATP from substrate oxidation than glycolysis with certain substrates (157). For example, the ketone body, acetoacetate, can be oxidized at up to 35 times the rate of glucose. Many proliferating mammalian cells, such as human MSCs, also consume glutamine to provide material for biosynthesis (158). Glutamine, as a carbon source, can supply the TCA cycle with intermediates that can be used for the production of new macromolecules in cells. A recent study by Hosios et al., in 2016, argued that glutamine contributes most to protein synthesis, suggesting that anaplerosis of glutamine in the TCA cycle is serving mainly amino acid biosynthesis (159).

When stem/progenitor cells differentiate to cardiomyocytes they need to increase the number of their mitochondria and upregulate FA metabolism (**Figure 3**). A study comparing hiPSC-derived cardiomyocytes (hiPSC-CM) to hiPSCs, showed an increase in mitochondrial relative abundance and activity (mitochondria membrane potential) as a result of cardiac differentiation (160) and that additional mitochondrial maturation in hiPSC-CMs could be achieved by long-term culture (3 months). Comparison of substrate metabolism in ESCs with that in ESC-derived cardiomyocytes has shown increased oxidative metabolism after differentiation (161) and that the respiratory capacity of cardiomyocytes was higher than in ESCs, resulting in an increased ADP:ATP ratio in the cardiomyocytes (106). The mitochondria become elongated with abundant and organized cristae in cardiomyocytes and formed into networks which filled the cytoplasm (106).

WHY STUDY STEM CELL METABOLISM

The metabolism of any cell type *in vitro* depends on energy requirements and substrate availability. Cardiac progenitor cells, when expanded *in vitro*, reside in a high glucose environment and rely on aerobic glycolysis for energy generation. CPCs will experience a shift in substrate availability following transplantation *in vivo* (**Figure 3**), being transferred from the culture medium, which contains about 5–25 mM glucose (depending on the culture protocol) and no FFA, to substrates in plasma that vary substantially. Glucose levels in mice have been measured at between ∼3.4 and 9.6 mM (2.8–7.5 mM in rats) (162, 163) and ∼0.18–0.6 mM FFA (164). In humans, healthy plasma glucose levels are around 5 mM and FFA 0.5 mM (165). This alteration is bound to cause changes in the metabolic machinery of the cells which might be one of the stimuli that induce differentiation following transplantation, but may also result in increased release of ROS and cellular damage.

The various protocols used for CPC differentiation focus on pharmacological reagents and cytokines, and do not refer to or take into account the substrate composition. This is a striking fact especially knowing how metabolic changes affect the function of the cells (166) and how transition from glycolysis to FA oxidation affects cell maturation (93), and *vice-versa* (144). iPSC-CM have been shown to integrate structurally and functionally with healthy host cardiac tissue *in vivo* in

various studies (26, 67, 68). Despite the promising *in vivo* results, the initiation of beating in iPSC-CM does not mean that these cells have the metabolic characteristics of mature cardiomyocytes found in the heart. Studies have shown that SC-CMs have immature calcium handling (76, 167) and a response to drugs more akin to cardiomyocytes from the failing heart (77). Despite the observed mitochondrial remodeling and upregulation of oxidative metabolism previously discussed, newly differentiated iPSC-CM in culture have been shown to retain a predominantly glycolytic metabolism (168). One of the major roles of iPSC-CM is that of a drug-testing platform and this requires the differentiated cells to acquire a fully mature phenotype (74). For example, a recent iPSC-CM study showed that the electrophysiological responsiveness of iPSC-CM was dependent on their maturation state (169).

ASSESSING CELL METABOLISM

The main approaches for the investigation of substrate metabolism include the measurement of metabolic fluxes using radio-labeled substrates and of oxygen consumption.

Radio-Labeled Substrates Assays

Glucose oxidation rates are commonly measured using the method of the Collins et al. (163) with cell culture media containing D-U-^{14}C-glucose. Glucose oxidation results in the production of $^{14}CO_2$ which is trapped for analysis using a scintillation counter. For ^{3}H-FA oxidation the cells are incubated in media supplemented with the radioactive FA tracer of interest. FA oxidation rates are determined by the production of ^3H_2O from the mitochondria (170, 171). Media aliquots contain both ^3H_2O and ^{3}H-FA, so the ^3H_2O is separated via a Folch extraction (170). Glycolytic rates are determined through the conversion of ^{3}H-glucose to ^3H_2O via enolase which converts 2-phosphoglycerate to phosphoenolpyruvate and releases ^3H_2O as a by-product that is collected using a Dowex mesh (Sigma, UK) anion exchange column, allowing for the ^{3}H-glucose to bind to the column and ^3H_2O to be eluted (171).

Oxygen Consumption

The rate of mitochondrial oxygen consumption (OCR) can be measured using the XF Extracellular Flux Analyzer (Seahorse Bioscience), the Oroboros O2K or the Clark-type oxygen electrode (172, 173). Seahorse XF Analyzers measure the concentration of dissolved oxygen and pH to quantify the oxygen consumption and extracellular acidification rate in the media in multi-well plates. Four injection ports in each well allow for addition of reagents. However, the Seahorse requires cells to be adhered to the wells, multiple additions of substrates are not possible and the individual wells are not self-contained and thus cells can be affected by gases from adjacent wells (172). The Clark-type oxygen electrode is embedded in individual reaction chambers and cells are added in suspension in the respiration media (173). Unlimited reagents and substrates can be added by manual addition. This system is more automated in the Oroboros O2K which also includes optical sensors to allow for detection of fluorescent dyes so that parameters such as ATP production or mitochondrial membrane potential may be measured (172).

The OCR can be measured under baseline un-stimulated conditions in media containing the substrate of interest (such as pyruvate; palmitate; malate; or oleic acid). Addition of the ATP synthase inhibitor oligomycin provides a measure of contaminating ATP synthase activity from damaged mitochondria and of proton leak, whilst the metabolic uncoupler, carbonylcyanide-p-trifluoromethoxy-phenylhydrazone (FCCP) permits measurement of fully uncoupled or maximal respiration (173). Finally, mitochondrial inhibitors such as rotenone (complex I) and antimycin (complex III) will completely inhibit mitochondrial respiration. More exquisite interrogation of the function of mitochondrial metabolism can be performed using substrates such as glutamate which assesses the second span of the Krebs cycle, succinate which enters the electron transport chain at complex II, or β-hydroxybutyrate, a fatty acid-derived substrate which bypasses β-oxidation and enters the Krebs cycle as acetyl CoA (116, 174).

Extracellular Measurement of Glucose Consumption and Lactate Production

Lactate production can be measured using the Seahorse Flux Analyzer from the extracellular acidification rate (ECAR) of the media. Glucose and lactate levels can also be determined using enzymatic assays or the ABX Pentra 400 Chemistry Analyser (Horiba Ltd. USA). Glycolysis yields two molecules of pyruvate, which can be converted to either lactate or acetyl CoA. Therefore, the ratio of glucose consumption to lactate production can be used as an indicator of the level of utilization of pyruvate in oxidative metabolism (175).

CARDIAC DIFFERENTIATION *IN VITRO*

Various methods and strategies have been applied to develop the optimal protocol for directing *in vitro* cardiac differentiation of stem cells. *In vitro* differentiation of adult endogenous CPCs is very challenging, due to their limited plasticity. Despite a variety of differentiation studies, the ability of adult progenitors for differentiation, is still under debate (176, 177). Different approaches, utilizing various differentiation factors have been used on both pluripotent SCs and CPCs, with the main ones being; DMSO, 5-Azacytidine, Ascorbic Acid, members of the TGF-β superfamily, oxytocin, dexamethasone and retinoic acid (see **Table 1**).

More specifically, 5-azacytidine (5-Aza) is a demethylating agent that allows for the exposure of genes that are normally silenced, due to hypermethylation, by inhibiting DNA methyltransferase (191, 192). Several *in vitro* studies suggested that 5-Aza can induce cardiac differentiation, on different MSC types, such as human umbilical cord-derived MSCs (186) and adult human bone marrow-derived MSCs (17, 187). Other studies have demonstrated the inefficiency of 5-Aza as a cardiac differentiation agent, showing transdifferentiation to skeletal muscle cells, rather than cardiac cells (193) as well as unsuccessful differentiation of adipose-derived stem cells (ASCs) (194) and adult mouse Sca-1+ CPCs (182). Ascorbic Acid (A.A.), is an antioxidant compound which has been shown to increase the expression of cardiac genes and their proteins

TABLE 1 | Factors and media used to differentiate adult stem cells to cardiomyocytes.

Differentiation approaches	References	Differentiated cell type	Cell culture medium and differentiation factors	Glucose concentration
TGF-β1 family	(178)	Human atria Sca-1$^+$; clonally isolated & magnetic-sorting	5 μM 5-Aza, TGF-β1 1 ng/ml, 10^{-4} M AA, IMDM/Ham's F12 GlutaMAX, 2% serum, 1% MEM amino acids, 1% insulin–transferrin–selenium	
	(179)	Human fetal and adult atrial biopsies, Sca-1$^+$ magnetic-sorting		12.5 mM
	(180)	Whole mouse heart Sca-1$^+$/ CD45$^-$ magnetic-sorting CDCs (Isl1$^+$)		
Oxytocin	(181)	Mouse or rat whole hearts, c-kit$^+$/CD45$^-$/Tryp$^-$ magnetic-sorting	DMEM 100 nM oxytocin acetate, 50 μg/ml AA, 2% serum, 1 μM dexamethasone, beta-glycerol phosphate 10 mM, TGF-β1, 5 ng/ml, BMP2 10 ng/ml, BMP4 10 ng/ml, Dkk1 150 ng/ml	5.5. mM
	(182)	Mouse whole heart Sca-1+ magnetic-sorting	IMDM, 10% serum, 100 nm oxytocin	22.5 mM
	(183)	Rat, mouse ventricles side population cells		
5-Azacytidine	(184)	Mouse bone marrow stroma MSCs	IMDM, 20% serum, 3 μmol/L 5-Aza	20 mM
	(185)	Human adipose MSCs	RPMI + 15% FCS, 1–9 μmol/L 5-Aza	9.4 mM
	(20)	Mouse whole heart Sca-1$^+$ magnetic-sorting	Medium 199, 2% FBS, 3 μM 5-Aza	5.5. mM
	(186)	Human umbilical cord MSCs	LG-DMEM, 10% FBS, 10 μM 5-Aza	5.0. mM
	(187)	Human bone marrow MSCs	LG-DMEM, 20% serum, 3 μM 5-Aza	4.4 mM
	(188)	Rat bone marrowMSCs	DMEM, 10% FBS, 10 μM 5-Aza	5.0. mM
Dexamethasone	(189)	Human atrial or ventricular c-kit$^+$ -sorted	F12 medium 10% serum, 10 nM dexamethasone	9 mM
	(19)	Rats c-kit$^+$/ Lin$^-$ magnetic-sorting	F12 medium 10% serum, 10 nM dexamethasone	9 mM
	(190)	Dog left ventricle c-kit$^+$/ Lin$^-$ and Sca-1$^+$/ Lin$^-$ magnetic-sorting	F12 medium 10% serum, 10 nM dexamethasone	9 mM
	(53)	mouse whole heart c-kit$^+$	DMEM, 10% serum, 10 nM dexamethasone	9 mM

5-Aza, 5-azacytidine; AA, Ascorbic acid; BMP, Bone morphogenic protein; Dkk1, Dickkopf-related protein 1; DMEM, Dubecco Modified Eagle Medium; MDMD, Iscove's Modified Dulbecco's Medium; LG-DMEM: low glucose DMEM; MEM, Minimum Essential Medium; RPMI, Roswell Park Memorial Institute medium; TGFβ, transforming growth factor β.

and to lead to beating cardiomyocytes in mouse ES cells (195). Cao et al. in 2012 demonstrated that A.A. was able to induce cardiac differentiation and maturation in several human and mouse iPSC lines (196). In contrast, treatment of BM-derived MSCs with A.A. triggered their proliferation and differentiation into osteoblasts and adipocytes (197). Another key player in cardiac differentiation is transforming growth factor-beta 1 (TGF-β1) which is thought to drive cardiac differentiation by inducing the cardiac transcription factor Nkx2.5 (198). Goumans et al. used TGF-β1 to induce differentiation of adult atrial Sca-1$^+$ CPCs, in combination with 5-Aza and A.A (178, 179). The mechanism of action involves phosphorylation of Smad2, that leads to the expression of cardiac-specific proteins (179). Dexamethasone is a glucocorticoid compound with immunomodulatory properties. Initial studies showed that it stimulates differentiation and maturation of osteogenic progenitor cells (199). The osteogenic effect of dexamethasone has since been demonstrated on MSCs (200–202). Interestingly,

various groups have used it as the main agent of differentiation of adult selected c-kit+ CPCs to cardiomyocytes (19, 181, 189, 190). One of the most successful *in vitro* differentiation protocols for adult cardiac progenitors uses staged treatment with oxytocin, BMP2/4, TGF-β, and DKK1 which has been shown to induce cloned c-kit+ progenitor cells to form beating cardiomyocytes (181)

Cardiac directed differentiation protocols used for pluripotent SCs can be divided into two main groups, the Embryoid Body (EB)-based method (203, 204) and the monolayer-based method (30, 205–208). These protocols are discussed briefly here but a more comprehensive review has been given by Mummery et al. (209). Although cardiac differentiation protocols vary, most monolayer-based methods involve stage-specific activation and inhibition of signaling pathways that control heart development, replicating the early cardiac developmental stages in the early embryo (mesoderm induction, mesoderm cardiac specification, and generation of cardiomyocytes). The signaling pathways that

are involved in most directed cardiac differentiation protocols are Activin/Nodal and bone morphogenetic protein (BMP) signaling (203), which are members of the (TGFβ) signaling pathway and the fibroblast growth factor (FGF) (30, 203), and the Wnt signaling pathways (205–208). Activin A, BMP4, and Wnt3 induce the mesoderm, and upregulate the expression of Brachyury T, whereas inhibition of Wnt signaling at later stages of differentiation has been shown to induce mesoderm cardiac specification (209). Following these steps, cells are generally cultured with media in the absence of growth factors, to allow cardiomyocyte maturation to give spontaneous beating. The distinct effects of Wnt signaling during various stages of cardiac development has been thoroughly investigated, both *in vitro* and *in vivo* (210). More specifically, it was shown with *in vivo* gene function studies in the mouse that Wnt initially enhances mesoderm commitment, while it later hinders the induction of cardiogenesis, and that it stimulates the proliferation of Isl1+ cardiac progenitors (211, 212). In addition, a study in zebrafish embryos demonstrated the switch from the inductive to the inhibitory role, of Wnt on cardiac formation, during a short 1-hour window prior to gastrulation (213). In chick embryos, treatment with Wnt antagonists *in vivo* enhanced expression of cardiac muscle differentiation markers and increased expression of Isl1 and Nkx2.5 in splanchnic mesoderm (214). *In vitro*, Murry's lab in 2010 showed that supplementation with exogenous Wnt at the point of initiation of cardiogenic differentiation of hESC enhanced the cardiac marker expression, while the same effect was induced by antagonism of endogenous Wnts at a later stage (208).

Differentiation protocols were first developed using hESCs and then were translated to iPSCs. **Table 2** summarizes and compares some of the published differentiation protocols and shows a shift from the EB-based model using hESCs to the monolayer-based model using hiPSCs, which is the commonly model used currently. **Table 2** also takes note of the fact that all these protocols are differentiated in variable concentrations of glucose (4.5–25mM) and insignificantly low amounts to no fatty acids (at most 2 μM). In general, spontaneously beating EBs are generated from cells in suspension using a serum-based media to induce spontaneous cardiac differentiation (27, 204, 216). The beating areas are then hand-picked from the rest of the EB or flow-sorted. Laflamme et al., played a key role in the enhancement of cardiac differentiation protocols by shifting from the serum-induced EB-based differentiation protocol that generated 10–15% spontaneously beating EBs (218) to the serum-free monolayer-based differentiation generating >30% cardiomyocytes using Activin A and BMP4 (27). In 2011, Burridge et al. systematically compared 45 variables added to EBs formed by forced aggregation which they tested on four hESC and seven hiPSC lines (222). Their optimized method included addition of BMP4 and FGF2, with polyvinyl alcohol to aid EB formation, serum and insulin to induce oxidative metabolism and with staged exposure to physiological (5%) oxygen. The growth factor-directed differentiation enabled cells to be differentiated as monolayers, thereby introducing more straightforward, and hopefully more reproducible, methods of differentiation (209). Lian et al. also

optimized the Wnt-based protocol by removing insulin during early stages of differentiation (205). They had found that when iPS cells were differentiated using a Gsk3β inhibitor, Activin A, and BMP4, the presence of insulin in the early stages had a strong inhibitory effect but that this was not seen when cells were differentiated by manipulation of Wnt signaling. They identified an interplay between insulin signaling and Wnt signaling which coordinates to influence differentiation to cardiomyocytes and therefore introduced staged removal and re-addition of insulin to their differentiation protocol (205).

MEDIA COMPOSITION DURING/AFTER DIFFERENTIATION

As shown in **Tables 1**, **2**, the majority of differentiation protocols for CPCs to cardiomyocytes had a no lipids in the media composition whilst the glucose concentration in culture media ranged from 4.5 mM to 25 mM (Here it should be mentioned that the addition of serum at percentages ranging from 2 to 20% does not allow for an absolutely clear image of the media composition). The variability of glucose concentration is striking, especially bearing in mind that a glucose level of 25 mM *in vivo* is considered hyperglycemic and leads to loss of mitochondrial networks (224, 225).

The Role of Hypoxia and of Reactive Oxygen Species in Differentiation

Stem cells *in vivo* occupy an hypoxic niche and their energy yielding metabolism is likely to be hypoxic with a high reliance on glycolysis for ATP generation (226, 227). There are conflicting reports on the relationship between hypoxia and differentiation of stem cells. Hypoxia alone can revert hESC- or iPSC-derived differentiated cells back to a stem cell-like state, by re-activation of an Oct4-promoter reporter (228). In contrast, exogenous expression of HIF has been shown to promote cardiac differentiation of ESC (229). Transient hypoxia during *in vitro* cardiac differentiation upregulated the Wnt signaling pathway with increased expression of the endogenous Wnt proteins (wnt3, wnt3a, wnt9a, and wnt11), which was lost when the cells were transferred back to normoxia. This resulted in increased expression of cardiac markers such as Isl-1 and Troponin C but decreased expression of βMHC and a failure to develop the contractile phenotype (230). To further complicate the picture, Gaber et al. found a dose-dependent increase in expression of the DNA damage marker γH2AX and of senescence in ESCs differentiated under increasing exposure to hypoxia (231). These differential reports may result from the different cell types (ESC or iPSC) and species studied. Fynes et al. found that hypoxic culture of mESCs primed the cells for differentiation and resulted in increased differentiation along the mesoderm and endodermal lineages whereas hiPSCs were pushed toward a more naïve pluripotent state by hypoxia and were then primed for ectoderm differentiation (232).

As previously discussed, the loss of regenerative potential of the heart in the first weeks of life has been attributed

TABLE 2 | Factors and media used to differentiate pluripotent stem cells to cardiomyocytes.

Different-iation method cell type	Growth factor or small molecules	Culture media	Glucose concent-ration	% of Cardiac markers	References
EB-Method hESCs iPSc	Cells in suspension -optionally differentiated with serum-based media, DMSO, all-trans retinoic acid or 5-Aza	80% KO-DMEM, 1 mMol L-glutamine, 1.1-1.2mMol B-ME,1% NEAA, 20% FBS	4.5mM	8.1% spontaneously beating EBs. In beating EBs, 29.4% cTnI+ cells. 25% spontaneously beating EBs by day 8 and 70% by day 16 of differentiation 5-Aza enhanced levels of cardiac α-MHC 10 to 15% positive for sarcomeric MHC	(204) (215) (216) (217) (218)
Monolayer hESCs hiPSC	Guided differentiation: h-Activin A, h-BMP4 +/- staged addition of h-Wnt3a & h-DKK1	RPMI+B27	11.1mM Note: 2uM of oleic acid	> 30% CMs Differentiated cells underwent Percoll gradient centrifuge for CM enrichment (69 ± 10% CMs) Increase in sarcomeric MHC+ cells from 4 to 27%	(27) (70) (208)
EB-Method hESCs hiPSC	END-2 method (Insulin depletion, PGI2, p38 inhibition)	80% KO-DMEM, 2 mMol L-glutamine,10ng/ml bFGF, 1.2 mMol B-ME, 7.5% FCS	4.5mM	50% beating CM	(219) (220)
EB-Method hESCs	Guided differentiation: h-Activin A, h-BMP4, h-bFGF, h-VEGF, h-DKK1	StemPro-34 (Base) + [L-glutamine, AA, optional MTG, P/S]	>25mM	KDR+ selected cells 35 ± 6% cTNT+, enriched to 57±4% by monolayer culture. DKK1 on day 4 gave 2-fold enrichment of CTNT+ cells KDR+/PDGFRA+ cells, 50–70% cTNT+ cells in beating EBs. 80% cTNT+ cells by monolayer culture	(221) (203)
EB-method iPSCs	Forced aggregation EBs with guided differentiation:BMP4, FGF2, Staged O$_2$ levels	RPMI (L-glutamine) 20% FBS on day 3 Insulin on days 0-2 and day 4	9–11mM Note: 2uM FA	Contracting EBs contained 64–89% of cardiac troponin I$^+$cells	(222)
Monolayer hiPSCs	Guided differentiation: h-Activin A, h-BMP4, h-bFGF	StemPro-34 + [L-glutamine,MTG, AA, P/S]	>25mM	Spontaneously beating sheets of CMs 40 ± 15% CMs	(30)
Monolayer hESCs hiPSCs	Guided differentiation method: h-Activin A, h-BMP4, h-bFGF, + Matrigel or CHIR99021, IWP4/IWP2	RPMI+B27(-insulin) [d0-6 of differentiation]; RPMI+B27 [from d7 of differentiation]	11.1mM Note: 2uM oleic acid	Matrigel addition on day−2 and day 0 generated 80% cTnT+ CMs Spontaneously beating sheets of CMs, 87% cTNT+ CMs	(223) (207) (206) (205)

B-ME, beta-mercaptoethanol; CM, cardiomyocytes; MTG, monothioglycerol; P/S, penicillin/streptomycin; AA, ascorbic acid; NEAA, non-essential amino acids.

to DNA damage resulting from the increase in ROS that accompanies upregulation of mitochondrial metabolism (98). However, ROS have also been shown to signal differentiation to several cell types, including cardiomyocytes. Transient expression of an NADPH oxidase-like enzyme with induction of ROS during embryoid body development of ESCs enhanced cardiomyogenesis, which was shown to occur *via* PI-3-kinase regulation and to be inhibited by the addition of radical scavengers (233). Similarly, NADPH oxidase (NOX)-derived ROS induced cardiac differentiation via a p38 mitogen-activated protein kinase (MAPK)-dependent pathway (234). Inhibition of mitochondrial biogenesis using shRNA targeting of PGC-1α, in hESC differentiation to cardiomyoytes, repressed mitochondrial respiration, and beating frequency (161). Levels of ROS increased during differentiation but were repressed by knockdown of PGC-1α. However, decreasing ROS levels by differentiating cells under hypoxia decreased the rate of mitochondrial biogenesis, which was stimulated by induction of ROS (161). Interestingly, in addition to reducing the beating frequency, decreasing ROS levels increased the action potential and calcium transient amplitude, but made the cells vulnerable to metabolic stress. ES cells cultured in physiological levels of glucose (5 mmol) maintained their stemness and showed reduced levels of ROS, but failed to differentiate to fully-formed cardiomyocytes (235). This was associated with reduced levels of NOX4 and MAPK which were rescued by addition of the pro-oxidant ascorbic acid.

STRATEGIES TO MATURE CELLS

A range of strategies have been used to mature iPS- and ESC-CMs, including time in culture (236), mechanical and electrical stimulation (237, 238), addition of small molecules (239), substrate stiffness (240), genetic approaches (241–243) and growth in 3D tissues (244–246). These are reviewed in detail elsewhere (74, 247) but very few consider the medium in which the cells are cultured.

CHANGING MEDIA COMPOSITION TO BE MORE PHYSIOLOGICALLY RELEVANT

There is a change in energy substrate availability and metabolism during heart development from the embryo into the adult stage. However, this change in metabolism is not mimicked in most published differentiation protocols. Maturation of ESC-CMs was induced by treatment with the thyroid hormone, tri-iodo-L-thyronine, which induced an enhancement in contractile kinetics, in rates of calcium release and reuptake and in sarcoendoplasmic reticulum ATPase expression, and a significant increase in maximal mitochondrial respiratory capacity and respiratory reserve capability (239). However, in that study the newly formed cardiomyocytes were cultured in serum-free medium with no fatty acids. Similarly, where maturation of ESC-CMs was induced using members of the Let-7 family of microRNAs, increasing cell size, sarcomere length, force of contraction, and respiratory capacity, no fatty acids were added to the serum-free culture medium (243).

A few recent studies have begun to change media composition to induce a particular disease phenotype. Kim et al. used a lipogenic medium comprising insulin, dexamethasone and 3-isobutyl-1-methylxanthine to induce fatty acid metabolism in iPSC-CM derived from patients with arrhythmogenic right ventricular dysplasia. They saw a mild increase in lipogenesis with minimal apoptosis after 4–5 weeks of treatment and increased expression of PPARα. Cell maturation revealed that metabolic derangement was implicated in the onset of arrhythmia (168). Drawnell et al. induced a diabetic phenotype by treating differentiated iPSC-CM with a maturation medium containing insulin and fatty acids, but no glucose for three days, followed by treatment with a diabetic milieu of glucose (10 mM), endothelin-1 (10 nM), and cortisol (1 μM) (248). This induced an increase in sarcomere length, and in velocity and duration of action potential, in addition to increases in the myosin light chain genes, MYL2, MYL3 and MYL4; of genes involved in regulation of sarcoplasmic reticulum calcium content and an associated repression of fetally enriched genes. Correia et al. reported that shifting hPSC-CMs from glucose-containing to galactose- and fatty acid-containing medium induced fast maturation into adult-like cardiomyocytes with higher oxidative metabolism, enhanced contractility and more physiological action potential kinetics (249). Perhaps the most comprehensive investigation into the effects of changing media is that of Rana et al., who modified a basal medium with combinations of high or low glucose with galactose or fatty acids (250). They found that

exclusion of glucose from the medium was needed to induce iPSC-CM to switch from glycolysis to OXPHOS for their ATP production.

TISSUE ENGINEERING APPROACHES

It is hypothesized that culturing cells in 3D would better mimic conditions *in vivo*, such that differentiated cells would adopt a more mature phenotype. There are four main types of tissue engineering strategies that have been developed to construct contractile heart muscle equivalents: stacking monolayer cell sheets to form multi-layered heart muscle (251), cells seeded onto decellularized native tissue, cells seeded onto synthetic or biologic scaffolds (252–257) and entrapment of cells in naturally occurring biogels or hydrogels (258–260). Bian et al. cultured neonatal rat cardiomyocytes in a 3D environment and after 3 weeks saw aligned, electromechanically coupled cardiomyocytes with capillary-like structures, improved calcium handling properties. The cells had action potentials which showed enhanced conduction velocities and directional dependence on the local cardiomyocytes orientation (261). Cardiac muscle strips which were fabricated from hESC-CMs and stromal cells in collagen-based biomaterials, showed higher passive and active twitch force, aligned sarcomeres, regularly dispersed connexin-43 and N-cadherin and increased expression of maturation markers (262).

Scaffolds can be formed from synthetic polymers or from natural materials such as the cardiac extracellular matrix (ECM). Besides its structural role of giving support to surrounding cells, the ECM also has important signaling roles in cardiac development and remodeling. In recent years, it has been shown to help regulate cell survival, proliferation, migration and differentiation, for example by modulating the activity, bioavailabilty or presentation of growth factors to cell surface receptors (263, 264). ECM macromolecular proteins such as collagen (259, 265, 266), elastin (267), fibrin (268, 269) and glycoaminoglycans (270, 271) can be extracted from tissue and integrated in 3D scaffolds (267, 271) or hydrogels (268) to explore further the retention and maturation of SCs seeded. Material properties are more controllable in synthetic polymers which can be modified to incorporate adhesion peptides or release biological molecules (272).

In the hydrogel approach, cells are encapsulated in the scaffold during synthesis of the gel which allows homogenous seeding. Hydrogels have also been shown to induce cell maturation and differentiation which makes them an attractive system for basic studies of cardiac development and potential for the delivery of therapeutics to the heart (273, 274). Suspension of CDCs in a hydrogel formed from serum and the glycosoaminoglycan hyaluronic acid, increased the oxygen consumption rate from that of cells in suspension and increased cell retention after transplantation *in vivo* (275). Porous scaffolds can be generated by freeze-drying suspensions poured in molds (267, 276). This type of manufacturing gives flexibility in shape and composition, but limits cell seeding efficacy as most seeded cells remain attached to the scaffold surface (267). Fibrous

scaffolds can be manufactured from a large variety of materials by electrospinning which gives control over the nano-scale structure and mechanical properties of the scaffold, but again limits the seeding efficiency (277). Zhang et al. found that, compared to 2D hESC monolayers, hESC-CM in a 3D fibrin-based cardiac patch exhibited significantly higher conduction velocities, longer sarcomeres and enhanced expression of genes involved in cardiac contractile function (244). Potentially the "ultimate scaffold material" is that of decellularized heart tissue since it has the potential to give rise to appropriately structured scaffolds for organ replacement (278).

One noteworthy 3D construct is the engineered heart tissue (EHT) which is a three-dimensional, hydrogel-based muscle construct that is restrained between posts, thereby allowing the cells to contract against a force. EHTs can be generated from isolated heart cells from adult hearts, such as chicken (259) and rat (258) as well as from hESC (279) and hiPSC (280). iPSC-CM EHTs have showed to increase mitochondrial mass, DNA content, and protein abundance (proteome) compared to their 2D counterparts. Moreover they were found to generate more energy via oxidation of glucose, lactate, and fatty acid with a decreased reliance on anaerobic glycolysis, generating 2.3-fold more ATP by oxidation than 2D hiPSC-CMs (245). Mills et al. have developed a technique for growing 3D cardiac organoids in 96 well plates (281). The cells form dense muscle bundles in serum-free conditions developed to promote metabolic and proliferative maturation. They found that addition of palmitate increased the force of contraction and expression of ventricular myosin light chain 2. Interestingly, addition of insulin promoted cell cycling and so was not included in the maturation medium comprising 1 mM glucose and 0.1 mM palmitate. By comparing contractile properties of organoids grown in maturation medium with those in control medium, they found that maturation medium reduced activation time and relaxation time, thereby recapitulating changes seen during human cardiomyocytes development.

Thus, three-dimensional culture conditions have induced binucleation, rod-like cell shape, increased sarcomere alignment, more mature electrophysiology, and calcium handling properties (247). Complex, multi-cellular cell sheets are now being developed from mixtures of iPSC-CMs with fibroblasts and endothelial cells (282) or with mesenchymal cells (283) which exhibit more mature physiology or drug responses but these are still grown in non-physiologic culture media (282, 283). What is needed now, is routine modulation of the culture medium, as discussed above, to cells in 3D constructs to fully induce metabolic maturation.

CONCLUSIONS

The field of CPC biology has come a long way over the last 20 years. Beating sheets of cardiomyocytes can now be generated routinely from ESCs and iPSCs in large numbers. However, the maturity of the cardiomyocytes remains a cause for concern if they are to be used to validate new drug compounds, detect cardiotoxicity or recapitulate the cardiac physiology of patients with genetic disorders. Long term culture can aid maturation, as can culture in 3D constructs, but a fully mature cardiomyocytes should express the appropriate level of substrate transporters and mitochondrial proteins. Further work is required to determine appropriate cell culture conditions to enable pluripotent stem cell-derived cardiomyocytes to achieve the metabolic flexibility of the adult heart.

AUTHOR CONTRIBUTIONS

All authors contributed conception and design for the manuscript. SM-M and CL wrote the first draft of the manuscript. All authors wrote sections of the manuscript and contributed to manuscript revision, read and approved the submitted version. SM-M and CL are joint first authors.

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