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Eat, breathe, ROS: controlling stem cell fate through metabolism

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Abstract

Introduction—Research reveals cardiac regeneration exists at levels previously deemed unattainable. Clinical trials using stem cells demonstrate promising cardiomyogenic and regenerative potential but insufficient contractile recovery. Incomplete understanding of the biology of administered cells likely contributes to inconsistent patient outcomes. Metabolism is a core component of many well-characterized stem cell types, and metabolic changes fundamentally alter stem cell fate from self-renewal to lineage commitment, and vice versa. However, the metabolism of stem cells currently studied for cardiac regeneration remains incompletely understood.

Areas covered—Key metabolic features of stem cells are reviewed and unique stem cell metabolic characteristics are discussed. Metabolic changes altering stem cell fate are considered from quiescence and self-renewal to lineage commitment. Key metabolic concepts are applied toward examining cardiac regeneration through stem cell-based approaches, and clinical implications of current cell therapies are evaluated to identify potential areas of improvement.

Expert commentary—The metabolism and biology of stem cells used for cardiac therapy remain poorly characterized. A growing appreciation for the fundamental relationship between stem cell functionality and metabolic phenotype is developing. Future studies unraveling links between cardiac stem cell metabolism and regenerative potential may considerably improve treatment strategies and therapeutic outcomes.

Keywords

Cardiac progenitor cell; differentiation; glycolysis; metabolism; mitochondria; pluripotency; reactive oxygen species; respiration; self-renewal; stem cell

1. Introduction

Cell metabolism is intimately linked to cell function, a relationship most apparent in cells with high energy demand such as neurons, skeletal myocytes, and cardiac myocytes that

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Declaration of interest

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consume large amounts of fuel to maintain sufficient energy levels for function. Metabolic fuel preference is quite flexible, as muscle cells switch from aerobic metabolism to anaerobic glycolysis when faced with metabolic insufficiency. Metabolic preference also shifts during stem cell differentiation to provide ATP more efficiently in differentiating cells. Correlations between energy demand and production rate suggest that metabolism is merely a consequence of increased energy utilization, but metabolism plays a significant role in determining stem cell phenotype including self-renewal and differentiation potential. Despite growing appreciation for the influence of metabolism upon stem cell phenotype, the field lacks comprehensive understanding of how metabolism of stem cells, and particularly cardiac stem cells, impacts survival, growth, and differentiation during tissue regeneration.

Several stem cell types have been investigated for potential to repair and rejuvenate myocardial tissue including embryonic and induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) derived from bone marrow, resident c-Kit$^+$ cardiac progenitor cells (CPCs), and cardiosphere-derived cells (CDCs) that migrate from cardiac tissue explants. Embryonic stem cells (ESCs) and iPSCs possess ability to differentiate into all three germ layers, form teratomas, and transform into contractile cardiomyocytes *in vitro* [1–3]. MSCs have potential to differentiate into adipocyte, chondrocyte, and osteocyte lineages [4,5], whereas CDCs share many similarities with both MSCs and CPCs including lineage potential [6,7]. CPCs commit to cardiomyocyte, smooth muscle, and endothelial cell lineages *in vitro* [8,9], but studies using c-Kit promoter-driven lineage tracing in mice suggest that CPCs may not give rise to significant numbers of cardiomyocytes *in vivo* [10,11].

Results of research in animal models of heart failure were promising enough to warrant therapeutic application tests but results of clinical trials have been inconsistent. In many cases, basic biological characteristics of stem cells employed in clinical trials are not clearly understood. Rushing cell therapy to clinical trial without a more complete understanding of the biology of stem cell function hampers the likelihood of encouraging clinical outcomes because the tested therapy may not be optimal for treating particular conditions, resulting in marginal efficacy.

This review summarizes how stem cell metabolism influences biology and phenotype, with the goal of identifying gaps in knowledge that may provide key information to improve cardiac regeneration through stem cell therapy.

2. Mitochondrial metabolism provides fuel for life

Mitochondrial respiration provides fuel necessary for cellular function and is especially important in postmitotic cells with large energy demand including neurons and cardiomyocytes. Mitochondrial content and morphology are indicative of a cell's energy requirements. For example, mitochondria comprise approximately 30–40% of the volume of cardiac myocytes [12,13], and their extraordinarily convoluted cristae reflect high membrane potential ($\Delta\psi m$) and ATP output. Similarly, extensive mitochondrial networks observed in neurons are indicative of high energy consumption [14]. The primary function of mitochondria is to produce ATP through oxidative phosphorylation (OXPHOS), which

necessitates formation of a mitochondrial proton gradient. In this process, electron carriers reduced by the tricarboxylic acid (TCA or Krebs) cycle are oxidized and electrons are passed along electron transport chain (ETC) complexes I–IV to move protons into the intermembrane space. In turn, the resulting proton gradient is used by the F_0–F_1 ATP synthase to generate ATP with high efficiency in regard to fuel input. Molecular oxygen (O_2) accepts the final electrons from cytochrome c, a component of Complex IV, forming two molecules of water. Thus, oxygen is required for mitochondrial ATP production, and Complex IV has very high affinity for oxygen. O_2 consumption by mitochondria is unaffected until O_2 availability drops below ~15 μM, which corresponds to roughly 2% in cell culture medium (Reviewed by Solaini et al., 2010 [15]). Most tissues of the body are perfused to pO_2 in the range of 5–10%, and thus pO_2 concentrations below 5% are typically considered hypoxic and may inhibit OXPHOS.

2.1. Embryonic stem cell OXPHOS transitions to glycolysis during development

In addition to being essential for somatic cell function, OXPHOS is also critical in early stages of embryo development. Mouse ESCs derived from the inner cell mass (ICM) of blastocyst stage embryos at day E3.5 have active mitochondria with high membrane potential ($\Delta\psi m$) despite low expression levels of mitochondrial replication factors such as mitochondrial DNA polymerase subunit gamma (Polg) and mitochondrial transcription factor A (Tfam) [16,17]. Early ESCs have immature mitochondria that are small, perinuclear, and globular in morphology [16]. This discrepancy suggests a high energy demand at this stage, and indeed ESCs in culture proliferate faster than later stage cells [18]. Early blastocyst ESCs demonstrate naïve pluripotency (i.e. not primed to differentiate), with expression of markers including *Oct4*, *Sox2*, and *Nanog*, and a high nucleus-to-cytoplasm ratio characteristic of unspecialized highly pluripotent stem cells [19].

In contrast, stem cells derived from later-stage mouse epiblasts (EpiSCs) more closely resemble human ESCs in their mitochondrial morphology and metabolism. Mitochondria in human ESCs and mouse EpiSCs are more mature, elongated, and have higher cristae density, yet paradoxically maintain lower mitochondrial respiration and higher glycolysis due to suppression of cytochrome c oxidase (Complex IV), the final respiratory complex [16]. Inhibition of Complex IV can also occur during metabolic gene reprogramming through HIF-1α [16], a concept discussed later in this review. Metabolic distinction between oxidative ESCs and glycolytic EpiSCs and human ESCs is central to the concept of ‘naïve’ versus ‘primed’ pluripotency. Progression from naïve to primed pluripotency, a state of pre-lineage commitment, is characterized by a metabolic switch toward glycolysis [16]. Importantly, resetting primed pluripotent cells to a naïve ground-state necessitates reversing this metabolic switch early during reprogramming [20].

2.2. Mitochondrial function is required for differentiation

Mitochondrial network expansion during differentiation helps meet increased energy demand of lineage commitment and also enables maintenance of sufficient ATP in postmitotic cells including cardiomyocytes. Mitochondrial biogenesis and maturation is prerequisite and occurs early in the process of lineage commitment [17]. Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α and β function as master

regulators of mitochondrial biogenesis by activating nuclear respiratory factor (NRF) 1 and 2 and TFAM to stimulate OXPHOS gene expression and mitochondrial DNA replication [21]. Mouse models of impaired mitochondrial biogenesis demonstrate impaired stem cell differentiation and early mortality [22]. As mitochondrial biogenesis declines with aging [23], diminished regenerative capacity observed during aging is partially due to impaired mitochondrial biogenesis. However, this has not been thoroughly investigated and remains a topic of research interest.

Stem cell mitochondrial function may be a predictor of differentiation capacity, such as in sorting of mouse ESCs based upon fluorescence of mitochondrial membrane potential ($\Delta\psi m$) sensitive dye TMRM into $\Delta\psi m$-low and $\Delta\psi m$-high populations with differential lineage commitment potential. $\Delta\psi m$-high ESCs are generally more pluripotent and naïve, whereas $\Delta\psi m$-low cells show characteristics of primed pluripotency and pre-commitment. $\Delta\psi m$-high cells possessing both higher mitochondrial oxygen consumption and glycolysis than $\Delta\psi m$-low ESCs is in accord with their more naïve status [24]. With the assumption that glycolysis promotes self-renewal of primed adult stem cells, applying the same sorting strategy to T cells also segregates self-renewing cells from pre-committed cells. In this case, high $\Delta\psi m$ T cells exhibit a more differentiated phenotype, whereas low $\Delta\psi m$ T cells possess higher self-renewal potential [25], the inverse of what is observed in naïve ESCs. Mitochondrial membrane potential is therefore predictive of stem cell self-renewal and lineage commitment only with prior knowledge of the cell's primed versus naïve status, and may guide selection of optimal cell metabolic status for regeneration, especially in applications using iPSCs. Further, because $\Delta\psi m$ may have direct bearing on promoting self-renewal versus differentiation, maintenance of optimal mitochondrial function, and therefore $\Delta\psi m$, is essential for maintaining stem cell functionality.

2.3. Mitochondrial quality control is essential for stem cell function

Mitochondrial quality control occurs at both protein and organelle levels. Mitochondrial Lon and AAA proteases degrade individual misfolded or dysfunctional components [26,27]. Alternatively, dysfunctional respiratory proteins may be sequestered via mitochondrial fission, resulting in a daughter mitochondrion with lower functionality and $\Delta\psi m$ that is targeted for degradation by mitochondrial autophagy (mitophagy) via the PINK1/Parkin pathway [28]. The serine/threonine kinase PINK1 becomes stabilized on mitochondria that have lost $\Delta\psi m$ and signals translocation of the E3 ubiquitin ligase Parkin from the cytosol to the dysfunctional mitochondria. Ubiquitination of mitochondrial targets by Parkin marks mitochondria for mitophagy [29,30]. Presence and functionality of these regulatory pathways in stem cells demonstrate critical roles for mitochondrial quality control in stem cell function. Mitophagy precedes mitochondrial biosynthesis and OXPHOS activation and is requisite for differentiation of C2C12 skeletal muscle myoblasts [31]. PINK1 increases during brain development, playing an essential role in neural stem cell differentiation [32]. PINK1 also participates in clearing mitochondria during fibroblast reprogramming, a necessary step for generation of stable iPSCs that do not spontaneously differentiate [33]. Additionally, Parkin expression enhances differentiation of LC3541 noradrenergic neuronal progenitor cells [34]. Thus, mitophagy functions to minimize mitochondrial respiration in primed stem cells to inhibit differentiation and also selects for optimally functional

mitochondria with high $\Delta\psi m$ to enhance differentiation upon lineage commitment. In both cases, mitophagy works to minimize ROS.

2.4. Mitochondrial dynamics influence pluripotency

Mitochondria are highly plastic organelles capable of dramatically altering their connectivity, morphology, and subcellular localization to meet changing metabolic demands and respond to cellular stress. Mitochondrial fusion and network elongation protects mitochondria from damage by enabling complementation of compromised mitochondrial DNA and diluting dysfunctional components [28,35]. Fused mitochondria are also protected from degradation by mitophagy [28,36]. In contrast, mitochondrial fission allows for segregation of dysfunctional components and subsequent removal by mitophagy [37], effectively 'culling the herd' of poor-quality mitochondria and enriching for those with highest functionality. Mitochondrial dynamics play a key role in maintaining stem cell pluripotency as high-passage iPSCs with more elongated and networked mitochondria have impaired *in vitro* lineage commitment compared to low-passage iPSCs [38]. In addition, inhibiting mitochondrial fusion in mouse ESCs prevents differentiation and results in a fragmented mitochondrial network and sustained Notch signaling [39]. Last, during stem cell division, older mitochondria are preferentially withheld in the daughter cell that maintains pluripotency, while younger and presumably more ROS-generating mitochondria are apportioned to the daughter cell that is destined to differentiate [40], suggesting yet another mechanism for ROS management. However, effects of mitochondrial fission/fusion dynamics on stem cell metabolism and pluripotency remain unclear and warrant further investigation.

3. ROS signaling promotes differentiation

During mitochondrial respiration, electron leakage primarily from Complexes I and III results in incomplete reduction of O_2, instead generating a superoxide radical ($^{\bullet}O_2^-$) [41]. Superoxide is converted to less reactive but longer-lived H_2O_2 by superoxide dismutase (SOD) and then further metabolized by catalase to yield H_2O and O_2. Superoxide and other reactive oxygen species (ROS) can lead to severe organelle, protein, lipid, and DNA oxidative damage and may eventually culminate in cell death. ROS play a central role in initiating intrinsic apoptotic and necrotic cell death via release of proapoptotic factors or permeability transition pore (mPTP) opening [42,43]. Mitochondrial quality control and efficient ROS scavenging are therefore essential in stem cells to maintain low oxidative stress and preserve cell viability. Antioxidant defenses increase during differentiation, including enzymes such as MnSOD and catalase, as well as nonenzymatic systems including glutathione that function to neutralize ROS and prevent cellular damage caused by elevated mitochondrial respiration [44].

ROS are generally considered detrimental to cellular homeostasis and long-term ROS accumulation is implicated in the aging phenotype attributed to reduced regeneration by stem cells [45]. Indeed, strategies to reduce ROS, including antioxidants and improving mitochondrial quality by upregulating autophagy increase lifespan in C. elegans and mammals [46–49]. Extending lifespan by enhancing mitochondrial quality may partially be

explained as slowing depletion of the stem cell pool and increasing tissue self-renewal. However, ROS are also critical signaling molecules and participate in essential signaling pathways. In this sense, ROS participate as critical mediators between changes in metabolism and downstream effects on cell survival, growth, repair, and differentiation pathways. To act as signaling molecules, ROS modify proteins by oxidation, nitrosylation, or carbonylation, thereby covalently modifying proteins and altering their conformation, binding affinities, and activity [50].

ROS directly participate in initiating lineage commitment of stem cells, and ROS levels correlate with pre-commitment and lineage potential. For example, hematopoietic stem cells (HSCs) with low levels of ROS have higher self-renewal, whereas HSCs with high ROS are more prone to exhaustion after transplantation [51]. Additionally, because mitochondrial ETC Complex III has been identified as a major source of ROS in respiring cells, differentiation of mouse ESCs is prevented by inhibition of Complex III with antimycin A [52]. Although antimycin A increases ROS levels in mouse ESCs in the short term, later time points coincident with differentiation (7–14 days) have not been examined. Thus, the long-term effects of Complex III inhibition on ROS and antioxidant systems remain unknown. In all cases, upregulation of ROS is a driving factor in lineage commitment and differentiation in a variety of stem cells. In adult mouse hippocampal neurogenesis, a peak in mitochondrial abundance and ROS occur in the highly proliferative intermediate progenitor cell state, but ROS are generally low in both undifferentiated neural stems cell due to low OXPHOS and in postmitotic neurons that have switched on oxidative metabolism because of upregulated antioxidant defenses [53]. Alternatively, suppressing mitochondrial ROS generation through hypoxic culture conditions supports self-renewal, inhibits differentiation, and delays cellular senescence of human MSCs, allowing for extended *in vitro* propagation [54]. Thus, ROS link oxidative metabolism with stem cell differentiation, but increases in ROS abundance during lineage commitment are transient. Pathways involved in promoting differentiation by ROS are not well characterized, but one study indicates ROS activate metabolic gene transcription and stimulate mitochondrial biogenesis by stimulating PPARγ [55]. Minimizing ROS is an attractive strategy for maintaining self-renewal of an expanding stem cell population, but is essential during lineage commitment.

Although mitochondria are primary sources of ROS in most cells, NADPH oxidases (NOX) are another major contributor. NOX enzymes generate ROS both as a cellular defense mechanism against pathogens and also as cytosolic signaling molecules. In mouse bone marrow endothelial progenitor cells (EPCs), Nox2 contributes to critical stem cell functions including EPC mobilization from bone marrow and chemotaxis [56]. In addition, H_2O_2 generated by Nox4 is essential for smooth muscle lineage differentiation of mouse ESCs [57]. Thus, non-mitochondrial ROS promote stem cell responses such as chemotaxis and also participate in promoting differentiation, but are not conducive to maintaining a quiescent state.

4. Glycolysis supports stem cell self renewal

Glycolysis involves metabolism of glucose to produce pyruvate and ATP. Pyruvate is further metabolized to lactate or shuttled to the mitochondria and oxidized for use in the TCA cycle.

In addition, glycolytic intermediates can be utilized for biosynthesis of amino acids, or can be shunted to the pentose phosphate pathway (PPP) for generation of reductive NADPH [58]. The PPP subsequently produces precursors for nucleotide synthesis, and also provides antioxidant protection in the form of reduced glutathione. Glycolysis rapidly generates ATP, enriches the intracellular antioxidant pool, and allows for rapid cell proliferation in the absence of oxidative stress as would be beneficial for expansion and self-renewal of a stem cell population [59].

Human ESCs possess many similarities with primed mouse EpiSCs, including morphology, pluripotency gene expression, and cellular metabolism. Both cell types exhibit suppressed mitochondrial respiration despite presence of mature mitochondria, relying instead upon glycolysis for ATP production [16]. Metabolic shift away from OXPHOS may be an adaptive response to low oxygen conditions experienced by EpiSCs within the developing embryo. In contrast, mouse ESCs derived from early-stage blastocysts possess relatively few mitochondria that display immature morphology including low cristae density and rounded appearance, but with elevated membrane potential and oxygen consumption rate relative to human ESCs and mouse EpiSCs [16].

Human ESCs and iPSCs produce the majority of ATP through glycolysis with lower mitochondrial respiratory reserve capacity [60]. Increased reliance on glycolysis is mediated in part by uncoupling protein 2 (UCP2), which shuttles pyruvate out of mitochondria to inhibit respiration and promote PPP flux. Overexpression of UCP2 increases glycolysis, decreases ROS, and blocks differentiation in primed stem cells [60]. Collective studies indicate that the transition from early ESC to EpiSCs and later to adult stem cells is characterized by a metabolic shift from OXPHOS to glycolysis to restrict mitochondrial respiration and maintain pluripotency [16,60,61].

Maintenance of ESC pluripotency is dependent on metabolic plasticity [62]. Transcriptomic data set analysis revealed that ESCs are heavily reliant upon amino acid and carbohydrate metabolism in comparison with their lineage-selected cardiovascular progeny [62]. Dependence of ESCs upon amino acid metabolism, particularly threonine, lysine, and branched chain amino acid catabolism may result from their need to maintain Krebs cycle intermediates consumed by conversion of pyruvate to lactate. Moreover, the transcriptomic metabolic signature specific to ESC-derived cardiomyocytes includes enrichment in pathways involved in fatty acid metabolism, mitochondrial respiration, and glucose metabolism. This analysis highlights central metabolic changes occurring during cardiac lineage commitment of mouse ESCs, although similar metabolic signatures were not identified for other cardiovascular cell types, such as smooth muscle or endothelial lineages.

ESCs differ from adult stem cells in that they comprise a rapidly proliferating and expanding pool, whereas adult stem cells tend to reside in niches that support quiescence until prompted to begin regeneration or repair in response to tissue injury. Maintaining quiescence is critical for long-term longevity and self-renewal of adult stem cells [63,64]. Glycolysis meets metabolic demands of both highly proliferative EpiSCs and quiescent adult stem cells. In both cases, glycolysis may be the result of the cell's hypoxic microenvironment as evidenced by (1) human ESC proliferation rate is unaffected by low oxygen culture, and (2)

pluripotency marker expression is maintained at higher levels than ESCs cultured in ambient 21% oxygen. Clearly, low oxygen tension does not inhibit energy availability of human stem cells and may be beneficial to preserve regenerative potential [65].

4.1. The hypoxic niche preserves adult stem cell pluripotency through quiescence

Somatic stem cells (SSCs) found in adult human and rodent tissue localize to niches with low oxygen tension. Even in highly vascularized tissues, hypoxic niches harbor stem, and progenitor cells that are metabolically distinct from fully differentiated cells [61,66]. MSCs, HSCs, neural stem cells, and c-Kit$^+$ cardiac progenitor cells reside in niches characterized by oxygen tension of ~1%, a hypoxic condition sufficient to suppress ROS generated by both OXPHOS and NOX enzymes to support stem cell self-renewal and pluripotency. For example, bone marrow HSCs maintain quiescence *in vivo* through preservation of a hypoxic phenotype that includes high levels of hypoxia inducible factor (HIF)-1α protein and positive staining for the hypoxic marker pimonidazole, even when located directly adjacent to arterioles [67,68]. Although bone marrow has a highly complex vascular network the entire bone interior is relatively hypoxic, with pO_2 in the range of 1–4% [69]. In maintaining quiescence within their native hypoxic niche, HSCs must rely on anaerobic glycolysis for energy production [61]. Correspondingly, expansion of MSCs in atmospheric oxygen levels upregulates OXPHOS genes and mitochondrial respiration, and accelerates cellular senescence, while hypoxic culture conditions delay senescence [54]. Differentiation requires upregulation of mitochondrial respiration, so stem cells must leave hypoxic niches for efficient lineage commitment. MSCs are only able to differentiate to osteogenic lineage if oxygen tension is sufficiently high, while chondrogenic differentiation occurs in hypoxia [54]. Stem cell senescence may be delayed through inhibiting mitochondrial respiration with hypoxic culture and possibly through other mechanisms. Human MSCs are particularly susceptible to early senescence and typically halt proliferation at approximately passage 8, which limits their expansion potential and therapeutic value (unpublished observation from author). Ability to enhance MSC culture expansion and delay senescence could substantially improve efficacy for autologous stem cell therapy. Beneficial effects are observed after only transient hypoxic exposure, with 24 h of hypoxic preconditioning increasing HIF-1α and antiapoptotic signaling, improving engraftment and persistence of mouse and primate MSCs after transplantation into animals with myocardial infarction (MI). Moreover, myocardial functional recovery is significantly better with transplantation of hypoxic-preconditioned MSCs compared to non-preconditioned cells [70,71]. Thus, even short-term hypoxia may augment stem cell engraftment and survival through mechanisms independent of metabolic changes.

4.2. Hypoxia and HIF-1α: signaling to maintain pluripotency

Hypoxia activates several signaling pathways involved in cell survival/death and stress response. Canonical mediators of hypoxic responses are HIF family of proteins that are rapidly degraded in the presence of oxygen as a result of hydroxylation of proline residues by prolyl hydroxylase (PHD) and HIF prolyl hydroxylases (HPH) enzymes. Functional heterodimers of HIF-α and HIF-β subunits bind to hypoxia responsive elements (HREs) within promoter regions to initiate transcription of genes involved in cellular metabolism, apoptotic control, DNA damage response, among others. Given sufficient oxygen tension,

HIF-α subunit proline hydroxylation allows for interaction with E3 ubiquitin ligase von Hippel-Lindau protein (pVHL) and potentiates ubiquitin proteasome degradation of HIF-α subunits. When oxygen tension is sufficiently low (<5%), HIF-α subunits become stabilized in the cytosol and translocate to the nucleus. HIF-1α directly regulates metabolic gene transcription and promotes glycolysis in response to hypoxia.

HIF-1α maintains expression of pyruvate dehydrogenase kinase 1 (PDK1), which inhibits the TCA cycle enzyme pyruvate dehydrogenase and prevents mitochondrial acetyl-Co-A oxidation. The net result is a metabolic shift that favors glycolysis and suppresses ROS generation in hypoxia, preventing apoptotic cell death [72]. Activation of HIF-1α also drives mouse ESCs to adopt a more EpiSC-like phenotype and metabolism, including upregulation of glycolysis and glycolytic genes, and downregulation of cytochrome *c* oxidase (Complex IV) [16]. In addition, HIF-1α perpetuates the undifferentiated state of mouse neural precursor cells, C2C12 myoblasts, and human ESCs via activation of Notch signaling and enhanced expression of pluripotency genes [73,74]. Notch signaling is highly evolutionarily conserved, but its function in either promoting or inhibiting differentiation is dependent on cell type and microenvironment. HIF-1α directly interacts with Notch as part of a complex and promotes transcription of Notch-responsive stemness genes [73]. Last, stabilization of HIF-1α is mediated by ROS generated by mitochondrial Complex III during hypoxia [75], a putative mechanism for modulating glycolytic metabolism based on mitochondrial function and further tying HIF-1α signaling with cellular metabolism. HIF-1α therefore supports stem cell self-renewal by both directly activating pluripotency genes, and also indirectly by shifting metabolism from OXPHOS toward glycolysis.

5. Induced pluripotency through metabolic reprogramming

Ability to dedifferentiate cells into an induced pluripotent state has revolutionized approaches for tissue repair and rejuvenation. Somatic cell reprogramming into pluripotent cells through expression of pluripotency transcription factors induces vast remodeling of cellular structure and requires complete metabolic reprogramming from oxidative to glycolytic phenotype. During reprogramming, the mitochondrial network is substantially pared as mitochondria lose cristae and adopt rounded morphology, essentially the reverse of mitochondrial biogenesis observed during differentiation, representing unexpected metabolic plasticity. Trimming the mitochondrial network is a critical step in reprogramming to pluripotency and inhibition of this process impairs de-differentiation [20,33]. Simultaneously, OXPHOS protein expression decreases and glycolytic gene expression increases [33]. Metabolic reprogramming is a fundamental factor in obtaining induced pluripotency. Interestingly, several genes associated with induced pluripotency directly modulate metabolism, such as Nanog that directly represses OXPHOS genes and limits ROS generation, increasing self-renewal of tumor-initiating stem-like cells [76]. Reduced expression 1 (REX1) is another widely used pluripotency marker highly expressed in mouse and human ESCs that substitutes for KLF4-mediated pluripotency induction. REX1 enhances mitochondrial fission, facilitating removal of mitochondria during reprogramming and promoting the glycolytic switch [77]. Additionally, enhancing glycolysis during reprogramming enhances iPSC induction efficiency. Several human somatic cell lines were successfully reprogrammed using a small molecule inhibitor of PDK1 in combination with

histone deacetylase inhibition and Oct4 overexpression [78]. PDK1 inhibition upregulates glycolytic gene expression and inhibits mitochondrial respiration, thereby enhancing reprogramming efficiency by about 15-fold [78].

Lin28A and Lin28B are highly expressed RNA-binding proteins in ESCs used as part of a gene cocktail (Oct4, Nanog, Sox2, and Lin28A) to reprogram somatic cells. Lin28 controls expression of *let-7* tumor suppressor miRNAs; Lin28 declines with postnatal development in adult somatic tissues, while expression of *let-7* increases [79]. Leaky expression of Lin28A in transgenic mice suppresses global *let-7* levels and enhances regeneration of tissues including ear wound closure, hair regrowth, and distal digital amputation [80]. Interestingly, enhanced regeneration was mediated by upregulation of OXPHOS in stem cells via a mechanism not dependent on *let-7* inhibition, supporting the hypothesis that augmenting oxidative metabolism in stem cells enhances differentiation and tissue repair. However, whether Lin28A overexpression leads to premature aging or senescence due to expedited exhaustion of the stem cell pool remains unknown. During reprogramming of hiF-T human fibroblasts, endogenous *LIN28B* gene expression precedes *LIN28A* expression by several days, implying an earlier role for LIN28B in pluripotency induction [81]. Loss of Lin28A and B results in mouse iPSCs with ‘naïve’ pluripotency and higher glycolysis, indicating that Lin28 expression is important for pluripotent cell priming and the shift from glycolytic to oxidative metabolism during priming. Importantly, this study did not compare effects of *LIN28* deficiency in human ESCs versus mouse ESCs and EpiSCs, a comparison that may be critical in discerning differences between human and mouse naïve versus pluripotent cell states. ESCs and EpiSCs have a great deal in common, including ability to differentiate into all three germ layers, teratoma formation, a high nucleus-to-cytosol ratio, and expression of many core stem cell marker genes including Oct4, Sox2, and Nanog [82,83]. However, mouse ESCs and EpiSCs also have many distinctions, including exclusive expression of Rex1 and Klf4 only in ESCs, while EpiSCs express Fgf5 and Nodal that are considered differentiation markers in ESCs. Culture conditions necessary to maintain pluripotency are another distinguishing feature, as ESCs must be maintained in the presence of leukemia inhibitory factor (LIF), whereas EpiSCs require Activin A and fibroblast growth factor 2 (FGF2) to maintain pluripotency [82,83]. These distinctions reflect different embryonic development stages as well as distinct cellular metabolisms.

6. Implications for cardiac stem cells

During cardiomyogenesis, cardiac progenitors undergo mitochondrial network expansion with increases in mitochondrial cristae density and genes associated with mitochondrial respiration and decreased glycolysis [84]. Cardiomyocyte differentiation shares many features in common with differentiation of other high energy somatic cells. First, cardiac lineage commitment requires upregulation of oxidative metabolism, as mitochondrial inhibition or downregulation of mitochondrial mass significantly reduces cardiomyocyte differentiation [84,85] while promoting mitochondrial biogenesis augments differentiation [85]. Next, inhibiting mitochondrial fusion in mouse ESCs prevents cardiomyocyte differentiation and results in a fragmented mitochondrial network [39]. Indeed, selection of highly pure cardiomyocytes derived from mouse ESCs is possible based on their high $\Delta\psi m$, indicating a distinct requirement for high mitochondrial function for cardiomyocyte lineage

commitment [86]. Additionally, an enriched population of fetal-like cardiomyocytes can be selected from pluripotent stem cell precursors based upon their unique capability to metabolize lactate and survive in the absence of glucose, indicative of considerably altered fuel preference following differentiation [87]. Last, similar to differentiation toward other lineages, ROS are essential for cardiomyocyte lineage commitment of ESCs. Inhibiting mitochondrial respiration or scavenging ROS with antioxidants impairs cardiomyocyte differentiation, while promoting ROS enhances cardiac lineage commitment [84,88]. Nox4 also promotes *Mef2c* and *Gata4* cardiac gene expression to stimulate cardiomyocyte differentiation of mouse ESC and embryonic carcinoma cells via a mechanism involving p38 MAPK and c-Jun [89,90], indicating a role of non-mitochondrial ROS in cardiac lineage commitment. Hence, basic metabolic requirements for cardiomyocyte differentiation are common to lineage commitment of other well-studied cell types.

Amino acid metabolism control is essential for maintenance of undifferentiated state in mouse ESCs. Threonine, lysine, and branched chain amino acid catabolism are particularly important for maintaining Krebs cycle intermediate levels [62]. Glutamine metabolism also enhances CPC proliferation and resistance to oxidative stress, thus emphasizing the importance for anaplerosis in maintaining and possibly enhancing CPC regenerative function [91]. Further transcriptomic analyses specifically directed toward dissecting cardiac stem cell metabolism will be key to compiling a complete understanding of how self-renewal and lineage commitment are influenced by heretofore poorly understood metabolic pathways.

Resident cardiac stem cells are of particular interest for autologous cell therapy for heart failure. Several lines of evidence indicate commonalities with other stem cells that can be used to infer CPC metabolic phenotype, although very few studies have directly investigated the metabolism of resident cardiac stem cells. Much like other adult stem cells, c-Kit$^+$ CPCs reside within hypoxic niches [66]. Consequently, CPCs rely on glycolysis in their undifferentiated state, but differentiation drives mitochondrial biogenesis and expression of OXPHOS genes. Mutations in mitochondrial DNA of CPCs inhibit OXPHOS protein upregulation during differentiation, particularly Complex IV, resulting in cell death [92]. Hypoxic culture of CPCs increases glycolysis, decreases mitochondrial content, and delays senescence. CPC migration is also improved by hypoxia, suggesting enhanced mobilization [93]. However, excessive glucose may also be detrimental to CPCs, as culture of human c-Kit$^+$ CPCs in supra-physiological glucose conditions (up to 25 mM) results in mitochondrial fragmentation, impaired endothelial-lineage differentiation, and cell death [94]. Importance of ROS in CPC differentiation is demonstrated by inhibition of Nox2 or Nox4 in freshly isolated CPCs suppressing cardiac and smooth muscle lineage commitment while maintaining stem cell marker expression [95]. However, in contrast with ESCs, Notch signaling in CPCs enhances metabolic activity and promotes their differentiation [96], reflective of their primed state versus naïve ESCs.

Much like CPCs, CDCs expanded under 2% O_2 have higher self-renewal and express higher levels of pluripotency and stem cell migratory genes, while suppressing expression of OXPHOS genes [97]. Hypoxia preconditioned CDCs are also more resistant to apoptosis, show improved engraftment, and improved cardiac contractile recovery prior to

transplantation into hearts subjected to acute MI [98,99]. Cardiac lineage differentiation of CDCs is also inhibited in hypoxic culture, again highlighting the importance of oxidative metabolism for cardiomyogenesis [97]. On the other hand, CDCs also require a minimal amount of ROS for proper maintenance of genomic stability, as suppressing ROS to sub-physiological levels results in augmented DNA damage and abnormal karyotypes [100]. Thus, an optimal level of ROS is essential to balance cellular housekeeping while maintaining pluripotency and quiescence in resident cardiac stem cells.

Considering all available information on resident cardiac stem cell metabolism, it is likely that HIF-1α signaling plays a crucial role in long-term maintenance of cardiac stem cells, possibly through modulation of Notch signaling. Suppressing oxidative metabolism appears critical for maintaining self-renewal and quiescence, especially within a hypoxic niche. Thus, maintaining cardiac stem cells under their native oxygen tension *in vitro*, not only during expansion but also particularly during isolation, may provide the most 'stem-like' cells possible for transplantation into patients. Alternatively, transplantation of an un-stimulated and self-renewing stem cell may not be the best approach for optimal cardiac regeneration. Relocation to a relatively higher oxygen environment may initiate mitochondrial biogenesis and ROS generation essential for lineage commitment, and thus may prove critical for optimal regeneration of cardiac tissue. In addition, characterization of critical pathways activated by ROS will unveil key players in determining lineage specificity.

Whether cardiac stem cells maintain a glycolytic phenotype or shift toward oxidative metabolism to promote differentiation following transplantation remains a critical unanswered issue and an important topic to address if stem cell-based therapies are to succeed clinically. Research has recently begun to address how the injured microenvironment affects ability of stem cells to proliferate and differentiate. Injured spinal cord delays proliferation and migration of engrafted stem cell neurospheres and alters their differentiation fate [101], while human ESCs are only able to become cardiac precursors in uninjured myocardium [102]. Although research has so far failed to characterize changes to individual cell metabolism following engraftment, one would predict lineage commitment to be inhibited by low oxygen availability. However, low serum culture conditions significantly inhibit formation of beating embryoid bodies from ESCs, while 5% O_2 has no effect [102]. Because of the complexity of the microenvironmental alterations in the injured myocardium, including nutrient and oxygen deprivation, pH acidification, and cytokine release, more stringent studies are necessary to determine which factors influence progenitor cell fate.

Alternatively, maintenance of progenitor cell state following transplantation may also promote myocardial healing by prolonging salutary effects, particularly through release of trophic factors. Undifferentiated MSCs have a robust cardioprotective secretory profile [103]. Conditioned medium from human MSCs grown under hypoxic conditions contains higher levels of angiogenic factors compared to medium from MSCs grown under atmospheric oxygen tension [104], and attenuates adult rat cardiomyocyte death induced by hypoxia (0.5% O_2) [105]. However, the relationship between cytokine/growth factor secretion and cellular metabolism remains largely unexplored. In the absence of glycolytic flux, GAPDH is made available to bind mRNA 3′ UTRs to limit translation and secretion of IFN-γ and IL-2 cytokines [106]. Conversely, rat bone marrow-derived MSCs subjected to

1% O_2 for 24 h upregulate expression of cytokines and growth factors including VEGF-D, PGF, PBEF1, and HB-EGF [107]. Therefore, multiple unexplored regulatory pathways may govern paracrine factor secretion in response to changing fuel preference. The secretory profile of CPCs in relation to metabolism remains to be investigated to advance understanding of transplanted CPC paracrine effects.

7. Clinical implications

Nearly 50 clinical trials to determine the efficacy of MSCs on improving contractile function and patient outcomes in heart failure patients were underway by 2014 [108]. Many of these studies found promising improvements in ejection fraction in patients with heart failure, whereas others reported small or negligible outcomes. The large variance in functional outcomes may be due to poorly understood biological variability of both the patient cohorts and the cells used. Particularly in studies using autologous stem cells, patient age, medical background, and genetics may strongly influence the potency of the transplanted cells. Metabolism of stem cells clearly is a major determining factor in their functionality and potential as a therapy, yet no studies thus far have correlated stem cell metabolism with clinical outcomes. Additional basic science will eventually fill the gaps in our understanding of why stem cells are only sometimes efficacious, and studies on how cellular metabolism affects regenerative potential are fundamental to optimizing cell-based therapy for cardiac regeneration.

Methods for noninvasive metabolic tracing are continually improving, allowing for *in vivo* assessment of fuel preference and utilization in injured myocardial tissue through nuclear magnetic resonance (NMR) spectroscopy, PET, or MRI [109]. Although current resolution limitations remain inadequate for metabolic analyses of single cells, combining advances in noninvasive tracing of adoptively transferred cells may soon allow for tracking changes to metabolism of engrafted stem cells [110]. Translation of *in vivo* single-cell metabolic tracking to clinical applications remains a significant challenge, but is essential for understanding and improving cardiac regenerative therapy.

8. Conclusion

Understanding of how metabolic changes alter regenerative potential of cardiac stem cells remains limited, but findings of stem cell metabolism in other organs will serve as an important guide to apply those concepts for improvement cardiac regenerative therapy. Most stem cells rely upon glycolysis to maintain quiescence, and biogenesis of high-quality mitochondria is essential for differentiation. ROS play a central role in signaling lineage commitment and are suppressed in hypoxia to maintain quiescence (See Figure 1 for summary). Researchers are now identifying distinguishing metabolic features of particular stem cell types, such as maintenance of pluripotency in naïve cells requiring high $\Delta\psi m$ whereas primed cells require low $\Delta\psi m$. Inducing pluripotency with reprogramming factors reverses mitochondrial expansion and reverts cell metabolism. Metabolic features such as ROS or mitochondrial abundance may also distinguish stem cells committed to a lineage from those self-renewing. Therefore, metabolism is not merely a consequence of stem cell phenotype, but may fundamentally define a stem cell. Identifying unique metabolic

signature(s) of cardiac stem cells will provide important clues for novel cardiac regenerative strategies.

9. Expert commentary

Isolation and expansion of cells, particularly those that reside in hypoxia *in vivo* invariably alters their metabolism compared to their native state. Considering that most adult stem cells maintain quiescence *in vivo* via metabolic suppression, rapid proliferation observed *in vitro* is artificial and likely detrimental to stem cell self renewal. Measurements of mitochondrial respiration and glycolysis performed *in vitro* in atmospheric oxygen tension do not accurately reflect the metabolism of the cells residing within their niche *in vivo*. Discrepancies between *in vivo* and *in vitro* microenvironment manifests as limited expansion potential of adult stem cells, low engraftment rates during transplantation, and inefficient differentiation, particularly toward cardiac lineage. Strategies to isolate and expand stem cells under constant hypoxic conditions potentiate pluripotency and delay senescence, while inhibiting spontaneous differentiation. The development of novel tools to assess cellular metabolism and fuel preference *in vivo* will be essential for a more thorough understanding of stem cell maintenance and function.

Goals for stem cell-based cardiac treatment include approaches to: (1) repair myocardial tissue after acute injury, and (2) rejuvenate hearts with chronic, diffuse injury, such as that caused by aging. Both objectives are achievable either through more traditional stem cell-based approaches that involve *in vitro* expansion and subsequent transplantation, or through approaches aimed at recruiting and enhancing the endogenous stem cell population. Identifying metabolic pathways and methods to enhance stem cell self-renewal *in vitro* will certainly improve efficacy of cell transplantation, but greater understanding of the metabolic changes that occur *in vivo* as stem cells transition from quiescence to a highly active differentiating state will enable augmentation of endogenous repair mechanisms. The ambitious goal of empowering innate regenerative machinery may be achievable by understanding the inseparable link between stem cell metabolism and function. To this end, *in vivo* transdifferentiation approaches for the replacement of scar fibroblasts with fully functional cardiomyocytes have already shown great promise. Focusing on methods to improve transdifferentiation, possibly through metabolic modulation with small-molecule chemicals rather than gene transfer will accelerate clinical implementation.

Hopefully a primitive resident cardiac stem cell capable of high-efficiency cardiomyocyte differentiation remains to be identified. The rarity of fully functional cardiomyocytes derived from currently available cardiac stem cells suggests a high degree of stem cell heterogeneity and perhaps only a fraction of those cells are bonafide cardiac precursors. Given the unique metabolic demands of cardiomyocytes and the degree of alteration that must occur for an undifferentiated stem cell to become so highly specialized, it may be possible to identify particular metabolic traits to enrich for cardiac precursors.

10. Five-year view

The development and refinement of single-cell analysis techniques *in vivo* and improved proteomic, transcriptomic, and metabolomic analyses will give us a much clearer understanding of how stem cell metabolism affects phenotype and function, particularly in regard to disease states such as myocardial injury, cardiomyopathy, and aging. The ability to isolate and profile individual cells from their resident niches using single-cell approaches such as RNA-Seq and laser capture microdis-section techniques will remove artifacts of extended culture and expansion, clarify the endogenous state, and reveal unrecognized heterogeneity of the cell population. Noninvasive single-cell metabolic tracers will also elucidate deficiencies in regeneration caused by suboptimal metabolic status. Within 5 years, many discrepancies between human and mouse pluripotent cell models may be resolved and the field may gain a greater appreciation for how differences in naïve versus primed pluripotency are reflected in differing metabolic needs. Ultimately, regenerative potential of known and yet-to-be-discovered cardiac stem cells will be revealed through single-cell analyses and delineation of metabolic nuances.

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Key issues

- Naïve pluripotent stem cells such as embryonic stem cells derived from early embryonic stages have a high metabolic demand and consequentially have both high oxidative metabolism and glycolysis to provide adequate ATP.
- At later stages, embryonic stem cells and adult somatic stem cells shift their metabolism from oxidative to more exclusively glycolytic to reduce reactive oxygen species generation and preserve pluripotency by protecting the cell from oxidative damage.
- Adult stem cells maintain quiescence by residing in hypoxic niches, where oxidative metabolism is suppressed. Typical isolation conditions relocate stem cells from hypoxia to relative hyperoxia in atmospheric oxygen and drive a shift from quiescence to active proliferation. Relative hyperoxia accelerates senescence and limits *in vitro* cell expansion potential. Isolation and expansion of stem cells under hypoxic conditions preserves their self-renewal and delays senescence, but also impairs differentiation that requires reactivation of oxidative metabolism.
- Hypoxia inducible factors (HIFs) control the expression of many metabolic and pluripotency genes. HIF-1α stabilization promotes glycolysis and 'stemness,' while simultaneously inhibiting oxidative metabolism. Likewise, reactive oxygen species generated by mitochondria under hypoxia contribute to HIF-1α stabilization and promote the metabolic shift from oxidative metabolism towards glycolysis to maintain stemness.
- Differentiation requires mitochondrial biogenesis and a metabolic shift from glycolysis to oxidative phosphorylation (OXPHOS), and can be enhanced by improving OXPHOS. However, differentiation and activation of OXPHOS accelerates ROS production and speeds stem cell senescence, depleting the stem cell pool. Conversely, inhibiting OXPHOS and enhancing glycolysis promotes an undifferentiated phenotype and improves self-renewal of stem cells.
- Structural and metabolic changes that occur during differentiation, including mitochondrial biogenesis and maturation, mitochondrial fusion, and expression of OXPHOS genes, are reversible through expression of pluripotency transcription factors. During reprogramming, induced pluripotent stem cells (iPSCs) must degrade and revert their mitochondrial phenotype to an immature state. Incomplete removal of mitochondria inhibits iPSC generation.

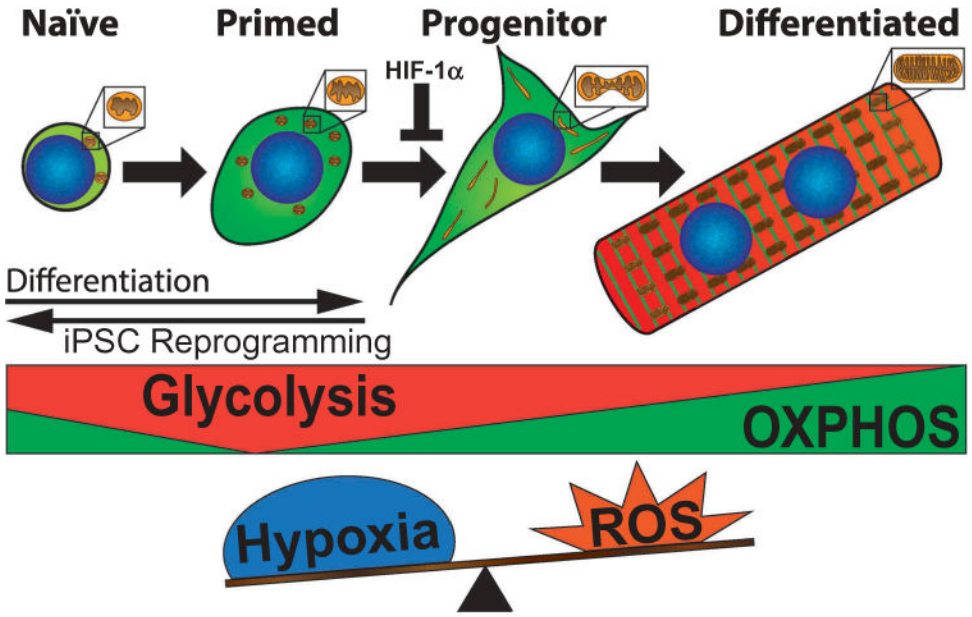

Figure 1.

Metabolism is central to stem cell fate. Naïve stem cells have immature mitochondria and rely on both glycolysis and oxidative phosphorylation (OXPHOS) for energy. The transition to primed pluripotency is accompanied by decreased dependence on OXPHOS. Glycolysis helps maintain pluripotency within the stem cell's hypoxic niche through HIF-1α signaling, but differentiation is dependent upon OXPHOS upregulation and ROS generation. Mitochondrial morphology (shown in expanded view boxes) is indicative of mitochondrial membrane potential ($\Delta\psi m$) and reliance on OXPHOS. Mitochondrial maturation is characterized by increased cristae density, mitochondrial fusion, and mitochondrial movement away from the nucleus.