THE ROLE OF METABOLISM IN STEM CELL FATES

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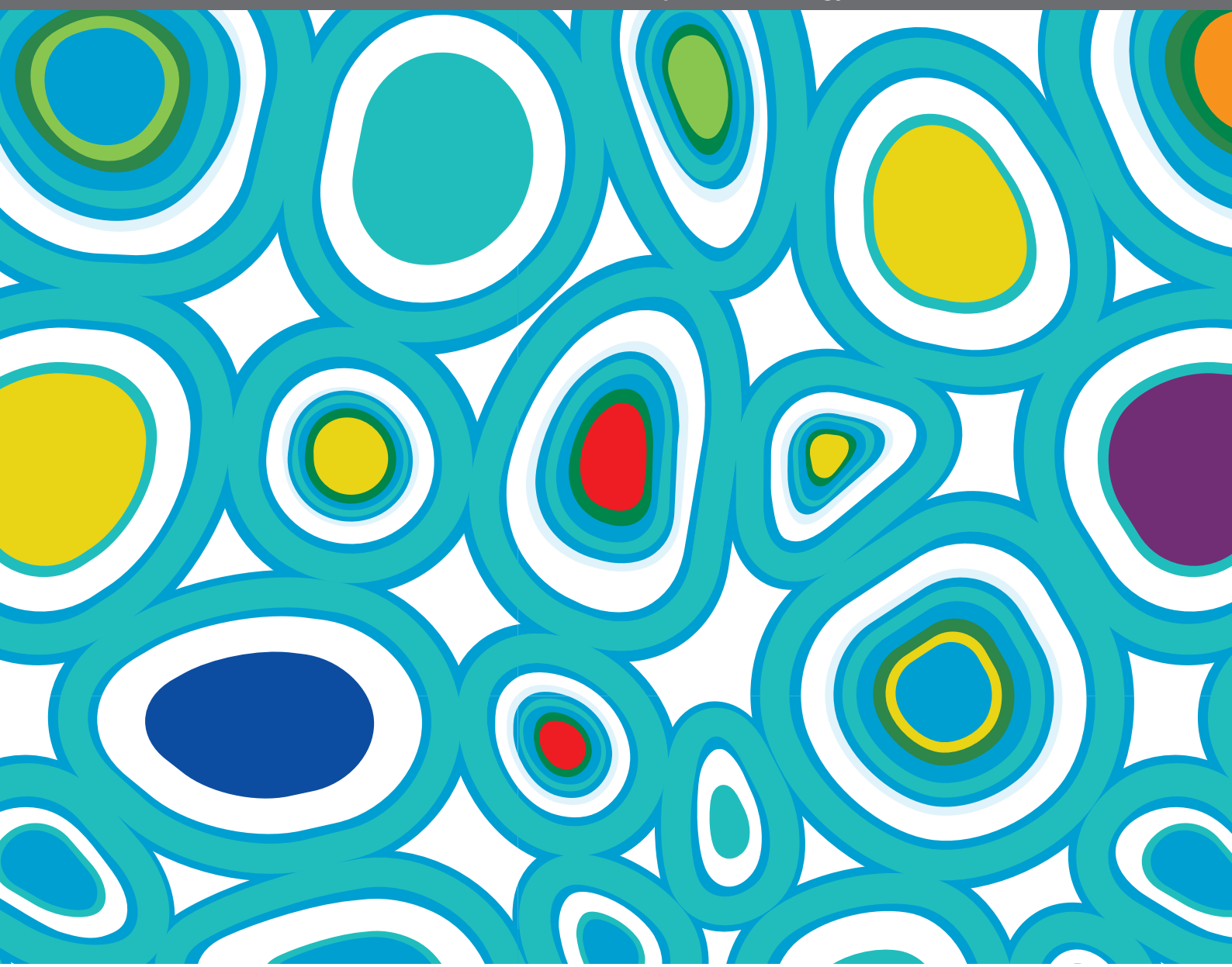

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THE ROLE OF METABOLISM IN STEM CELL FATES

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REVIEW
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The Microenvironment Is a Critical Regulator of Muscle Stem Cell Activation and Proliferation

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Skeletal muscle has a remarkable capacity to regenerate following injury, a property conferred by a resident population of muscle stem cells (MuSCs). In response to injury, MuSCs must double their cellular content to divide, a process requiring significant new biomass in the form of nucleotides, phospholipids, and amino acids. This new biomass is derived from a series of intracellular metabolic cycles and alternative routing of carbon. In this review, we examine the link between metabolism and skeletal muscle regeneration with particular emphasis on the role of the cellular microenvironment in supporting the production of new biomass and MuSC proliferation.

Keywords: injury, repair, skeletal muscle, metabolism, metabolic reprogramming

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INTRODUCTION

Skeletal muscle has a remarkable potential to regenerate following injury, a property conferred by a population of local somatic stem cells termed muscle stem cells (MuSCs). In response to damage or trauma, local MuSCs are quickly activated and undergo extensive rounds of proliferation, differentiation, fusion and maturation in order to repair and/or replace damaged tissue (Charge and Rudnicki, 2004; Relaix and Zammit, 2012; Wosczyna and Rando, 2018). The extent of the MuSC response varies depending on the severity of the initial insult; ranging from a minor strain to major trauma from laceration, ischemia-reperfusion, or myotoxicity. Importantly, the proliferative response of MuSCs to injury is dependent on the capacity of these cells to double their cellular content, requiring synthesis of new biomass in the form of nucleotides, phospholipids, and non-essential amino acids (NEAA) (Koopman et al., 2014; Hosios et al., 2016). Synthesis of these molecules requires a ready supply of carbon-based precursors, satisfied by nutrients in the local extracellular tissue environment.

In this review, we will discuss recent findings linking cellular metabolism and the extracellular environment to cell division, and how efficient carbon routing is critical for MuSC proliferation and successful skeletal muscle regeneration. First, we will provide a brief overview of skeletal muscle regeneration.

AN OVERVIEW OF SKELETAL MUSCLE INJURY AND REPAIR

Muscle injuries can result from physical insults, diseases, toxins, and following ischemia (Souza and Gottfried, 2013). Although mechanical damage to muscle fibers can occur with daily activities and exercise, more severe injury can result in an irreversible loss of functional capacity. These more severe injuries include; contusion, strain, or laceration (Järvinen et al., 2005). Contusions are the most common mechanical insult, arising from a blunt, non-penetrating force that can rupture

blood vessels and cause hematomas (Crisco et al., 1994). Strain injuries arise from high external loads that overstretch activated myofibers, damaging their structure and in more severe cases, the interconnections between muscle-tendon and tendon-bone (Nikolaou et al., 1987). A higher risk of strain injuries comes with advancing age or diseases that render muscles more vulnerable to damage (Lynch, 2004; Baker, 2017). Laceration is caused by a penetrative or crushing force, often leading to tissue loss and formation of scar tissue (Garrett et al., 1984). These severe muscle injuries require longer periods of regeneration and carry an increased risk of incomplete muscle repair.

Muscle regeneration is complex, requiring the coordinated activity of inflammatory cells, fibroblasts, mesenchymal cells, and MuSCs to ensure complete restoration of vasculature, nerves, and myofibers (Christov et al., 2007; Dumont et al., 2015). As mature myofibers are post-mitotic, muscle regeneration is dependent on an adequate population of viable MuSCs.

In the absence of injury, MuSCs typically exist in a quiescent state outside of the cell cycle, residing between the plasma membrane of a myofiber and the basement membrane (Mauro, 1961). During homeostasis, MuSCs do not actively proliferate and typically account for 2–10% of myonuclei, depending on age, sex, and muscle type (Dumont et al., 2015). Upon activation, MuSCs produce a progeny of myogenic cells that can differentiate, culminating in the formation of mature muscle fiber (**Figure 1**). During this process, MuSCs typically become specified to the myogenic lineage after activation and then undergo multiple rounds of proliferation to generate sufficient myonuclei to support protein synthesis and mature muscle formation (Bischoff, 1990). These proliferating myogenic precursors (myoblasts) then exit the cell cycle and terminally differentiate to myocytes which subsequently fuse to form myotubes. Muscle regeneration is completed through further rounds of myoblast fusion and muscle fiber maturation (Knudsen and Horwitz, 1977). Importantly, a small subpopulation of myoblasts return to quiescence so as to restore the MuSC pool.

It is important to note the balance between differentiation and maintenance of the MuSC pool during regeneration. While the majority of MuSCs will undergo activation and proliferation after injury, a sub-population of MuSCs must be maintained for regeneration of subsequent injuries (Collins et al., 2005; Sacco et al., 2008). To maintain this population, MuSCs may undergo symmetric or asymmetric division, respectively, producing either two identical daughter cells or both a single undifferentiated daughter cell and a committed myogenic precursor (Dumont et al., 2015). Control of differentiation versus self-renewal of MuSCs is governed by transcription factors, epigenetics and signaling pathways, and achieving an appropriate balance is key to sustaining muscle plasticity and regenerative capacity (Yin et al., 2013).

When muscle fibers are damaged, MuSCs are activated by both physical and chemical signals. With severe mechanical muscle injuries, ruptured blood vessels cause local hematoma and affected myofibers seal off damaged portions of the cell to prevent the spread of necrosis (Carpenter and Karpati, 1989; Hurme et al., 1991). Resident mast cells release cytokines that increase blood flow and attract circulating inflammatory cells. These cells phagocytose necrotic debris and release cytokines that promote survival of damaged cells (Pillon et al., 2012). The rate of muscle regeneration is also highly dependent on angiogenesis, as blood vessels and endothelial cells supply nutrients and mitogens for MuSC growth (Järvinen, 1976; Christov et al., 2007). Direct damage to the basal lamina or expression of matrix metalloproteinase stimulated by nitric oxide release, may further release trapped growth factors in the extracellular matrix that also encourage activation of MuSCs (Dimario et al., 1989; Tatsumi, 2010). These signals cause MuSCs to leave quiescence, migrate to the site of injury and begin proliferating.

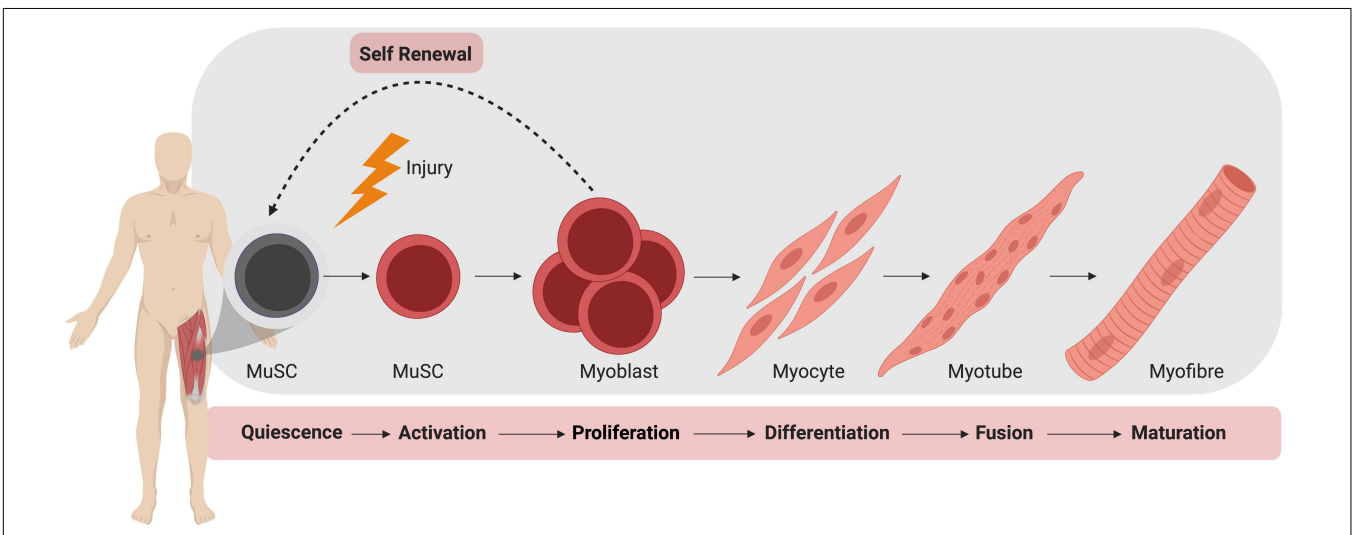

FIGURE 1 | Muscle stem cell (MuSC) mediated skeletal muscle regeneration. Following injury, quiescent MuSCs are activated and undergo rapid proliferation, followed by differentiation into myocytes, which fuse and mature to generate new muscle fibers.

After sufficient proliferation, myocytes fuse to form new, immature myotubes or fuse to existing injured fibers (Knudsen and Horwitz, 1977). Immature myotubes are centrally nucleated and gain functional capacity as they increase in size and express contractile proteins. This fusion is supported by the infiltrated inflammatory cells, as they adopt an anti-inflammatory phenotype that prevents excess damage to remaining healthy tissue (Arnold et al., 2007). The maturation and hypertrophy of myotubes is also supported by the release of insulin-like growth factor 1 (IGF1) from anti-inflammatory M2 macrophages, stimulating protein synthesis through the activation of the Akt-1/mTOR signaling pathway (Rommel et al., 2001; Park and Chen, 2005).

The role of metabolism and the local microenvironment in muscle regeneration has recently received attention (Ryall et al., 2015a; Pala et al., 2018; Yucel et al., 2019), but a comprehensive analysis is lacking. Given the active role of metabolism in the proliferation and differentiation of tumor cells and ESCs (Yanes et al., 2010; Lunt and Vander Heiden, 2011), exploring the link between the microenvironment and MuSC metabolism may help identify novel targets to improve both the rate and extent of muscle repair after injury. Additionally, as many skeletal muscle pathologies are linked to a shift in the local metabolic environment (Chi et al., 1987; Joseph et al., 2018), and many metabolic disorders result in impaired skeletal muscle repair (D'Souza et al., 2016; Monaco et al., 2018), it is critical that we understand the link between MuSCs and their local tissue microenvironment.

A LINK BETWEEN METABOLISM AND SKELETAL MUSCLE REGENERATION

All cells require energy (in the form of ATP) to sustain the critical enzymatic reactions which support life (Bonora et al., 2012; Petersen and Verkhratsky, 2016), with the loss or significant depletion of ATP resulting in necrosis and cell death (Eguchi et al., 1997). Cellular ATP is primarily generated via either glycolysis or oxidative phosphorylation (OxPhos) in the mitochondria, a process linking the acetyl-coA produced from either glycolysis or fatty-acid oxidation to the tricarboxylic acid (TCA) cycle and the electron transport chain. However, in addition to producing ATP dividing cells must double their cellular content, imposing a large demand for the generation of new biomass in the form of nucleotides for DNA/RNA, amino acids for proteins, and phospholipids for cellular membranes (Lunt and Vander Heiden, 2011). Therefore, it is unsurprising that both the local metabolic environment and innate cell metabolism can dictate processes such as the rate of proliferation and/or differentiation (DeBerardinis et al., 2008; Ryall and Lynch, 2018; Zhu and Thompson, 2019).

In one of the first studies to investigate metabolism and MuSC biology, Rocheteau et al. (2012) observed that MuSCs freshly isolated from uninjured skeletal muscle contained variable levels of mitochondria, with an inverse correlation between mitochondria density and the expression of the transcription factor Pax7. The authors observed that $Pax7^{Hi}$ cells contained the lowest level of mitochondria, while $Pax7^{Lo}$ contained the highest. More recently, quiescent MuSCs have been found to transition between quiescence and an intermediate phase termed G_{Alert}, with MuSCs rapidly shifting to this alert phase following injury (Rodgers et al., 2014). Of relevance to the current discussion was the finding that MuSCs in the alert phase were larger and exhibited a greater level of mitochondrial DNA. Whether the $Pax7^{Lo}$ MuSCs identified by Rocheteau et al. (2012) were in the G_{Alert} phase has yet to be confirmed.

In the context of skeletal muscle injury and repair, MuSCs undergo a metabolic switch from fatty-acid oxidation in quiescence to an increased reliance on glycolysis during *in vitro* activation and proliferation (Ryall et al., 2015b). This shift toward glycolysis in activated MuSCs has been confirmed *in vivo* by Pala et al. (2018), who performed an extensive characterization of metabolism in quiescent and active MuSCs and found that the extracellular acidification rate (ECAR, a measurement of glycolytic activity) and oxygen consumption rate (OCR, a measure of OxPhos), was highest in MuSCs isolated from skeletal muscle 3 days post-injury. This peak in metabolic activity occurs during a period of rapid MuSC proliferation (Gayraud-Morel et al., 2009; Quintero et al., 2009; Kimura et al., 2015; Hardy et al., 2016; Xiao et al., 2016). Interestingly, the first 24–48 h of MuSC activation are marked by a significant increase in autophagic flux, with inhibition of autophagy leading to a delay in MuSC activation (Tang and Rando, 2014). The precise role of this acute rise in autophagy, and its importance in terms of MuSC proliferation has yet to be determined.

The peak in ECAR in MuSCs has been observed to decline by day five post-injury, without a concomitant decrease OxPhos, suggesting that a transition toward OxPhos may be required as MuSCs return to a quiescent state (Pala et al., 2018). A similar switch has been observed in many other proliferating cell types including ESCs, hematopoietic stem cells (HSCs), induced pluripotent stem cells (iPSCS) and most notably in cancer cells, and is termed "aerobic glycolysis" or "The Warburg Effect" (Warburg, 1956; Suda et al., 2011; Zhang et al., 2012; Moussaieff et al., 2015).

Professor Otto Warburg first defined the process of aerobic glycolysis in highly proliferative tumor cells, after observing that even in the presence of saturating levels of oxygen, these cells consumed large amounts glucose and extruded lactose (Warburg, 1956). Since this seminal work, researchers have found a link between elevated glucose consumption and cell proliferation in a wide range of cell types including embryonic kidney cells, cancer cells, vascular smooth muscle cells, mesenchymal stem cells, and ESCs (Saki et al., 2013; Han et al., 2015; Shao et al., 2018). While differentiated cells typically convert one molecule of glucose into two molecules of ATP and two molecules of pyruvate which are then used to drive OxPhos in the mitochondria to produce an additional 30–34 molecules of ATP, proliferating cells re-route glycolytic intermediates to drive anabolic reactions and the production of new biomass (Vander Heiden et al., 2009). Under these conditions, each molecule of glucose generates significantly less than two molecules ATP and two molecules of pyruvate. Therefore, proliferating cells must carefully balance their production of biomass with the need for ATP.

Cell division in proliferating cells is achieved via progression through the cell cycle, comprising an initial gap (G_1) phase where cells double their cellular content, an S phase whereby DNA is replicated, a second gap (G_2) phase where replicated DNA is checked, and finally mitosis (M phase) where cells undergo division. Importantly, as cell division is a metabolically demanding process several checkpoints exist, and only allow a cell to proceed when certain conditions are met. One such checkpoint exists in the late G_1 phase where increased glycolytic flux is required prior to the G_1 to S transition (Kalucka et al., 2015). In addition to ensuring sufficient supply of biomass to dividing cells, this increased reliance on glycolysis during cell-division is also likely a mechanism to reduce the production of reactive oxygen species (ROS) to protect against DNA damage.

While cell-cycle progression is regulated by metabolite availability, the cycle itself can directly regulate the activity of several key metabolic enzymes. In one such study, Wang and colleagues found that cyclin D3 activation of cyclin-dependent kinase 6 (CDK6) phosphorylated and inhibited the catalytic activity of phosphofructokinase 1 (PFK1) and pyruvate kinase M2 (PKM2) (Wang et al., 2017). The inhibition of these two enzymes allowed for the accumulation of glycolytic intermediates and increased flux through the pentose phosphate pathway (PPP) to support nucleotide synthesis.

NUCLEOTIDE SYNTHESIS THROUGH THE PENTOSE PHOSPHATE PATHWAY

Nucleotides are essential components of molecules such as ATP, GTP, cAMP, cGMP, and in the synthesis of RNA and DNA (Lane and Fan, 2015), including purines (adenine and guanine) and pyrimidines (cytosine, uracil, and thymine) which differ by the inclusion of either a double carbon and nitrogen ring (purines) or a single carbon ring (pyrimidines). Nucleotide *de novo* generation is achieved via the PPP, one of the first alternate carbon cycles to branch from the main glycolytic pathway and requires simple precursor molecules to be converted to complex nucleic acids (Riganti et al., 2012; Kowalik et al., 2017). In the PPP, glucose-6-phosphate (G6P) undergoes several oxidative carboxylation reactions to form ribose-5-phosphate (R5P) and nicotinamide adenine dinucleotide phosphate (NADPH). R5P serves as a nucleotide precursor, whereas NADPH has a key role in protecting cells from oxidative damage and serves as the major electron donor in many reducing reactions (Meitzler et al., 2014). The flow of glucose into the PPP is first catalyzed by the enzyme glucose-6-phosphate dehydrogenase (G6PD), which irreversibly leads to the oxidative decarboxylation of G6P (**Figure 2**).

The critical importance of G6PD and the PPP in supporting cell proliferation has been confirmed in several studies demonstrating that its inhibition leads to a significant reduction in tumor and plasmodium cell proliferation (Hu et al., 2015; Xu et al., 2016; Zhang et al., 2017). In contrast, increased activity of G6PD such as that observed in tumor cells, is typically linked with rapid cell proliferation (Du et al., 2013). Interestingly, embryonal rhabdomyosarcoma (ERMS), an aggressive form of cancer involving muscle cells that fail to differentiate, express high levels of *G6PD*. In contrast, following the forced differentiation of these tumorigenic cells, *G6PD* was one of the most highly downregulated genes (Coda et al., 2015). In the context of MuSCs, several whole transcriptome studies in mice have revealed a specific enrichment of *G6pd2* and *G6pdx* in proliferating compared to quiescent MuSCs (Liu et al., 2013; Ryall et al., 2015b). These results strongly support a key role for G6PD in regulating myogenic cell proliferation, likely through provision of new nucleotides.

In addition to *de novo* nucleotide synthesis through the PPP, nucleotides can be generated through recycling or salvage pathways (**Figure 2**), which predominate during quiescence and differentiation when only low levels of nucleotide synthesis are required (Fairbanks et al., 1995). The nucleotide salvage pathway recycles intermediates derived from the breakdown of DNA and RNA and converts them to purines and pyrimidines. Importantly, the salvage of nucleotides requires only one molecule of ATP per pyrimidine synthesized, compared with seven molecules required by *de novo* synthesis (Nyhan, 2014; Marsac et al., 2019). The core of the purine salvage pathway relies on the regeneration of nucleobases adenine, inosine, and guanine which can be used to generate ATP, IMP, and GTP, respectively. Of these three nucleotides, IMP exhibits the greatest flexibility with the ability to be converted into GMP or AMP when required (Ljungdahl and Daignan-Fornier, 2012; Peifer et al., 2012). This is important during tissue homeostasis and for cells to utilize a low energy pathway to maintain nucleotide levels. This is observed in terminally differentiated neurons, which rely on salvage pathways to maintain nucleotide homeostasis (Fasullo and Endres, 2015).

Having two distinct pathways to synthesize nucleotides (recycling and *de novo*) is an advantage for mammalian cells (Lane and Fan, 2015), as it allows for cells to adapt based on environmental stimuli such as nutrient and/or substrate availability. During periods of cell stress such as limited nutrient availability, cells utilize the salvage pathway to facilitate nucleotide homeostasis. In contrast, cells undergoing rapid proliferation cannot rely solely on *de novo* synthesis, since this pathway is insufficient to facilitate the demand for new nucleotides.

Nucleotide biosynthesis has received scant attention in skeletal MuSCs, but a recent study by Tran et al. (2019) reported on ribonucleotide reductase (RNR) knockout mouse. In this study the authors developed a mouse model with exon 9 of the M1 subunit of RNR flanked by two loxP sites ($Rrm1^{fl/fl}$) and bred it with a mouse expressing Cre recombinase under the control of muscle creatine kinase (Mck^{cre}), with the resulting mouse expressing a truncated and inactive form of RNR in cardiac and skeletal muscle from embryonic day 13 (Tran et al., 2019). As RNR is a key enzyme for *de novo* nucleotide synthesis, its conditional ablation allowed the investigators to study the importance of this pathway in skeletal and cardiac muscle. Importantly, ablation of RNR was found to be lethal within a few days of birth, with a median survival age of 11.5 days and a maximal age of 27 days. In mice that survived to P15–P17, the hearts were found to contain disrupted nucleotide levels with a threefold decrease in dGTP and a twofold increase in dCTP

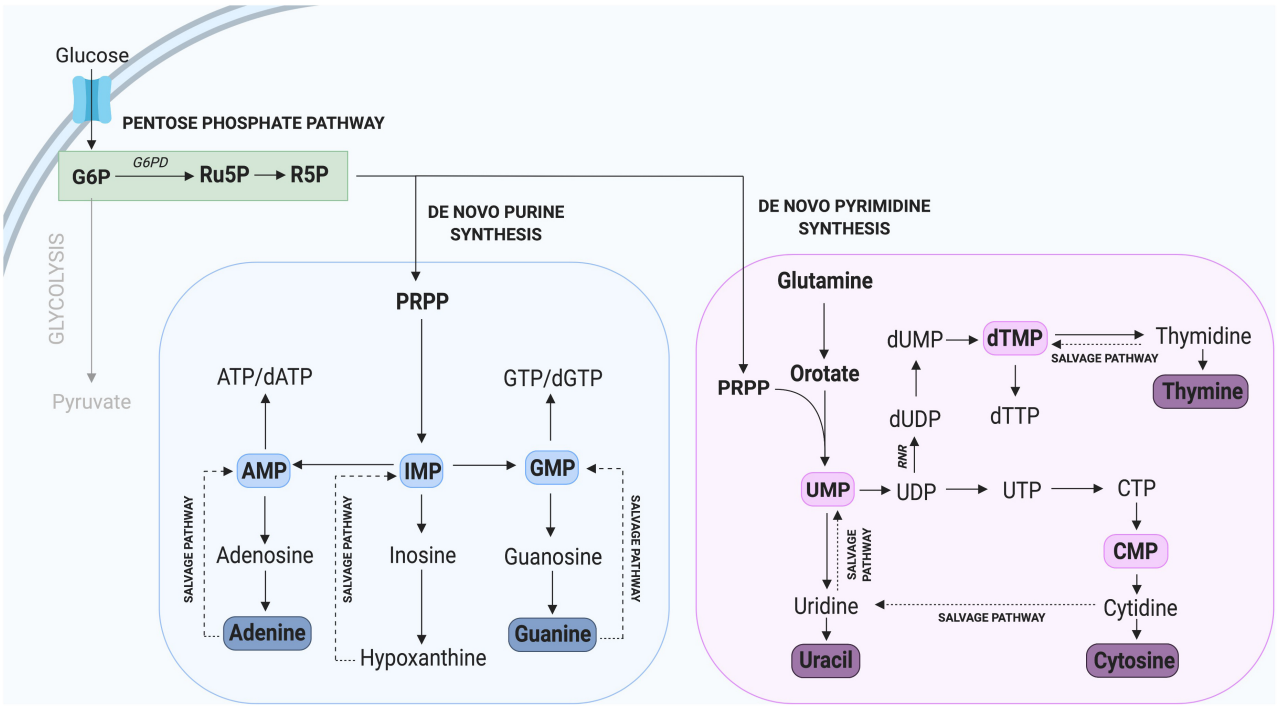

FIGURE 2 | Nucleotide synthesis via the Pentose Phosphate Pathway. Glycolysis contributes to the synthesis of new purine and pyrimidine nucleotides through G6P, which can be converted to the precursor R5P. Not all intermediate steps are shown. AMP, adenosine monophosphate; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate; dTMP, deoxythymidine monophosphate; dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate; GMP, guanosine monophosphate; IMP, inosine monophosphate; UMP, uridine monophosphate.

and dTTP compared to wildtype hearts. These results suggest that (in the heart) *de novo* nucleotide synthesis is only critical for the production of dGTP. While not described in detail, the authors found that muscle fibers in the gastrocnemius muscles of knockout mice were less than half the size of those observed in wildtype mice. Additionally, the number of nuclei per fiber was reduced by more than half (Tran et al., 2019). It will be critical in future studies to determine whether a similar defect in dGTP (as observed in cardiac muscle) is observed in skeletal muscle of RNR knockout mice.

AMINO ACID SYNTHESIS VIA GLYCOLYSIS, THE PPP, AND THE TCA CYCLE

Protein accounts for the majority of dry cell mass and is responsible for the formation of key cellular components including antibodies, enzymes, and cell structures (Hosios et al., 2016). Therefore, in addition to nucleotides, there is strong demand for the synthesis of NEAAs during proliferation. In mammalian cells there are nine "essential" amino acids (EAAs, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) which cannot be synthesized and must be taken up exogenously. The remaining 11 NEAAs (alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine) can be synthesized in the cytoplasm through glycolysis and its sidechains, and in the mitochondria through the TCA cycle.

Besides the generation of ATP, several intermediates of glycolysis can be used to generate amino acids, including 3-phosphoglyceric acid (3PG) and pyruvate (Locasale, 2013). 3PG can contribute carbons to the generation of cysteine, glycine, and serine through the one carbon (1C) cycle while pyruvate can be converted into alanine (Olson et al., 2016; **Figure 3**). Serine derived from the 1C cycle can combine with the folate cycle to form glycine or it can be utilized in the synthesis of phospholipids as phosphatidylserine (PS) (Glinton et al., 2018). The serine biosynthesis pathway is commonly upregulated in highly proliferative tumors to support growth (Mattaini et al., 2016) and is critical to support MuSC proliferation, as its depletion has been found to prevent MuSCs from transitioning from G1 to S-phase of the cell-cycle (Thalacker-Mercer et al., 2019). Furthermore, Ryall et al. (2015b) have found that multiple enzymes in the serine biosynthesis pathway (including *Phgdh*, *Psat1*, *Psph*, *Shmt2*) are all enriched in proliferating MuSCs in mice. Together, these results provide strong evidence for a key role of serine biosynthesis in regulating MuSC proliferation. Whether this pathway may also play a role beyond the simple provision of NEAAs to dividing cells is an exciting topic deserving of further research.

Recently, the EAA methionine was identified as a powerful anabolic agent capable of regulating cell proliferation. In this study, Walvekar et al. (2018) identified that methionine

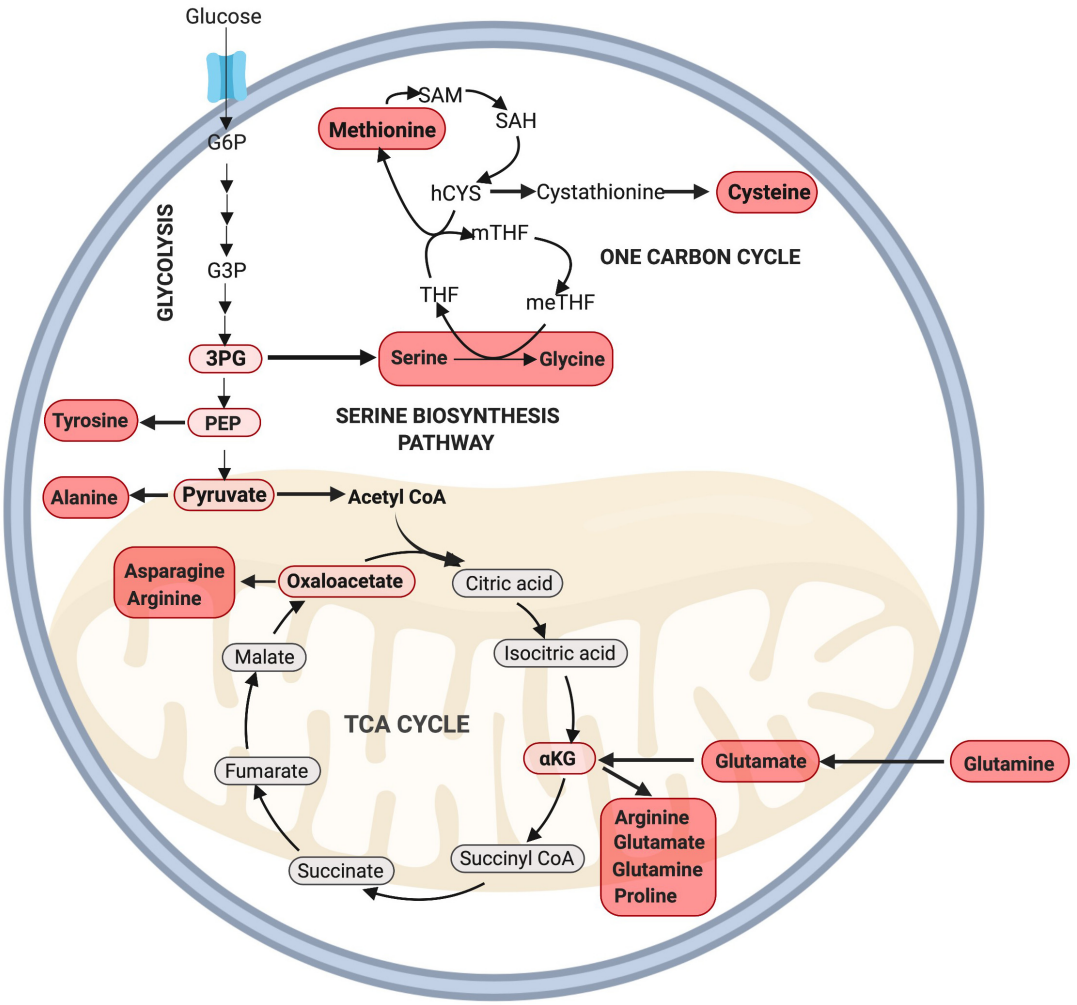

FIGURE 3 | Amino acid synthesis via glycolysis and the TCA cycle. NEAAs can be synthesized through various intermediate metabolites of glycolysis including 3PG, PEP, pyruvate, and the TCA cycle through oxaloacetate and αKG. Note: not all intermediate steps are shown. 3PG, 3-phosphoglyceric acid; hCYS, homocysteine; meTHF, 5,10-methylene THF; mTHF, 5-methyl THF; PEP, phosphoenolpyruvate; SAH, *S*-adenosyl homocysteine; SAM, *S*-adenosylmethionine; THF, tetrahydrofolate.

supplementation alone could increase cell proliferation in yeast cells grown in an amino acid depleted medium. Strikingly, this methionine-dependent increase in proliferation was greater than the anabolic response provided through supplementation of any of the other 18 non-sulfur amino acids. These authors confirmed that methionine increased amino acid synthesis and *de novo* nucleotide synthesis through the PPP and glutamate synthesis pathways (Walvekar et al., 2018). In a related study, Gao et al. (2019) found that dietary methionine restriction alone could significantly reduce tumor growth in mice, and identified a potential mechanism through disruption of *de novo* nucleotide synthesis and the cellular redox balance.

While the importance of methionine in muscle regeneration has yet to be examined, it raises an important question as to whether dietary methionine supplementation alone may improve muscle growth and repair. Interestingly, dietary methionine supplementation in rainbow trout promoted hyperplasia and muscle growth (Alami-Durante et al., 2018), but in chickens only marginal effects on protein synthesis and degradation were reported (Zeitz et al., 2019), indicating that the effect of methionine on skeletal muscle growth and regeneration requires further investigation.

During the proliferating phase of an immortalized murine myogenic cell line (C2C12 myoblasts), glutamine is the second most highly consumed nutrient besides glucose and plays a key role in anaplerosis (Hosios et al., 2016), and amino acid and nucleotide biosynthesis (DeBerardinis et al., 2007). Following transport into the cell, glutamine undergoes a deamination reaction catalyzed by the enzyme glutaminase to produce glutamate. This process, known as glutaminolysis, is critical for cell proliferation (Choi and Park, 2018). Glutamate can then be either converted into glutathione or α-ketoglutarate via oxidative deamination to supply the TCA cycle. Unsurprisingly, glutamine is added to cell culture media to support cell growth as its deprivation leads to cell cycle arrest at the S phase (Gaglio et al., 2009).

PHOSPHO/LIPID SYNTHESIS VIA GLYCOLYSIS AND THE TCA CYCLE

Lipids constitute key components of the cellular plasma membrane, act as an energy source/store, and play key signaling roles through the production of hormones (Watt and Hoy, 2011; Hubler and Kennedy, 2016; Yao et al., 2016). Therefore, lipid metabolism is another critical process for rapidly proliferating cells, including cancer cells and neural stem/progenitor cells (NPSCs) which exhibit elevated exogenous lipid uptake and *de novo* lipid synthesis (Natter and Kohlwein, 2013; Knobloch, 2017; Zhao et al., 2017; Yi et al., 2018).

Lipids can be divided into several separate classes based on their chemical structure and properties, with each having distinct roles within cells. The major classes of lipids incorporated into mammalian cells comprise phosphatidic acid (PA), phosphatidylinositol (PI), PS, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), and cardiolipin (CL; Calzada et al., 2016). Other lipid classes include (but are not limited to) cholesterols, sphingomyelins, cerebroside, gangliosides, phospholipids, and triacylglycerols (TAGs). Importantly, many of these lipid classes have previously been demonstrated to influence rates of cellular proliferation and differentiation of myogenic cells (Mebarek et al., 2007; Gangoiti et al., 2012). For example, Mebarek et al. (2007) found that inhibition of ceramide synthesis in immortalized rat myogenic cells (L6 myoblasts) led to an increase in the rate of differentiation through the upregulation of phospholipase D, an enzyme responsible for the generation of PA. Conversely, addition of exogenous ceramides resulted in a reduction in the expression of the transcription factor *myogenin*, a key regulator of myoblast differentiation (Mebarek et al., 2007). Similarly, ceramides inhibit anabolic growth in mature skeletal muscle through the inhibition of IGF-1/Akt and mTORC signaling (Akhmedov and Berdeaux, 2013; Hsieh et al., 2014). Interestingly, ceramide-1-phosphate (C1P, derived from ceramide) can induce proliferation of C2C12 myoblasts through increased Akt and ERK1/2 signaling (Bernacchioni et al., 2018). In this manner, ceramide can both inhibit myogenic differentiation and, following conversion to C1P, promote proliferation. However, it is important to note that ceramide itself inhibits proliferation, highlighting the complex nature of lipid signaling (Faustino et al., 2008).

In addition to conversion into C1P, ceramide can also be reversibly converted into sphingosine. Both sphingosine and ceramide are negative regulators of cell growth, and have been linked to cell cycle arrest and apoptosis (Woodcock, 2006; Kanno et al., 2014). Similar to ceramide, sphingosine can be phosphorylated to form S1P, which in C2C12 cells is critical for both the inhibition of proliferation and initiation of differentiation (Donati et al., 2004). In contrast to these findings in C2C12 cells, Calise et al. (2012) found that S1P supplementation stimulated proliferation in primary mouse MuSCs. These authors attributed the discrepancy in their results to differences in S1P receptor type availability between C2C12 cells and primary MuSCs (Becciolini et al., 2006; Calise et al., 2012).

In addition to regulating proliferation and differentiation, sphingomyelin levels change following activation, as quiescent MuSCs exhibit high levels of sphingomyelin within the plasma membrane, which subsequently drop after activation (Nagata et al., 2006). These results highlight the importance and complexity of the ceramide/S1P axis in regulating MuSC proliferation and differentiation during regeneration.

Similar to nucleotides and amino acids, proliferating cells can meet the demand for new lipids by utilizing lipids in the local extracellular environment or performing *de novo* lipid biosynthesis from glycolytic intermediates The *de novo* synthesis of all phospholipids (with the exception of PA), requires pyrimidine nucleotide cytidine triphosphate (CTP), which is synthesized in the PPP. Interestingly, CTP synthase, which catalyzes the rate limiting step of *de novo* CTP synthesis, is upregulated in many cancer lines (Williams et al., 1978), and its inhibition reduces cell proliferation through impaired nucleotide and phospholipid synthesis. Therefore, proliferating cells require a coordinated effort of nucleotide synthesis for DNA and phospholipids (Verschuur et al., 2000).

Glyceraldehyde-3 phosphate (G3P), an intermediate metabolite of glycolysis, is intricately involved in the *de novo* synthesis of phospholipids and TAGs (Alves-Bezerra and Gondim, 2012; **Figure 4**). In this pathway, G3P is first converted into dihydroxyacetone phosphate (DHAP), a reaction catalyzed by the enzyme triosephosphate isomerase (TPI), with TPI1 expression correlated with increased rates of proliferation in gastric cancer cells (Chen et al., 2017). Interestingly, overexpression of *TPI1* in hepatocellular carcinoma cells impairs proliferation (Jiang et al., 2017), suggesting the role of TPI may be cell type specific. While the role of *Tpi1* in MuSC proliferation has not been directly assessed, several transcriptomic studies conducted on freshly isolated and proliferating MuSCs have revealed that elevated *Tpi1* expression in proliferating MuSCs (Ryall et al., 2015b). Further research is required to determine the role of Tpi1 in myogenesis and skeletal muscle regeneration.

In another C2C12 based experiment, Lee et al. (2009) demonstrated that supplementing proliferating cells with mono-unsaturated FAs, n-6-polyunstaurated FAs, linoleic acid, gamma-linoleic acid and arachidonic acid all enhanced proliferation. Exogenous arachidonic acid has also been found to promote myoblast differentiation through its conversion to prostaglandin E_2 (PGE2) in a COX-2 dependent-manner (Leng and Jiang, 2019). Of interest, PGE2 has been found to be rapidly synthesized and released into the local muscle microenvironment following damage. Ho et al. (2017) demonstrated that PGE2 is required for successful regeneration, as inhibition of PGE2 synthesis led to impaired MuSC proliferation and weakened muscles.

In contrast to mono-unsaturated fatty-acids, the saturated fatty acid palmitate significantly inhibited C2C12 myoblast proliferation through a reduction in both cyclin A and cyclin D1, while promoting differentiation and increased myotube width (Grabiec et al., 2015). Taken together, these results highlight the importance of a regulated role for fatty acids, as the dysregulation or excessive accumulation of fatty acids in the MuSC microenvironment may negatively affect skeletal muscle regeneration. This is evident in models of diabetes mellitus

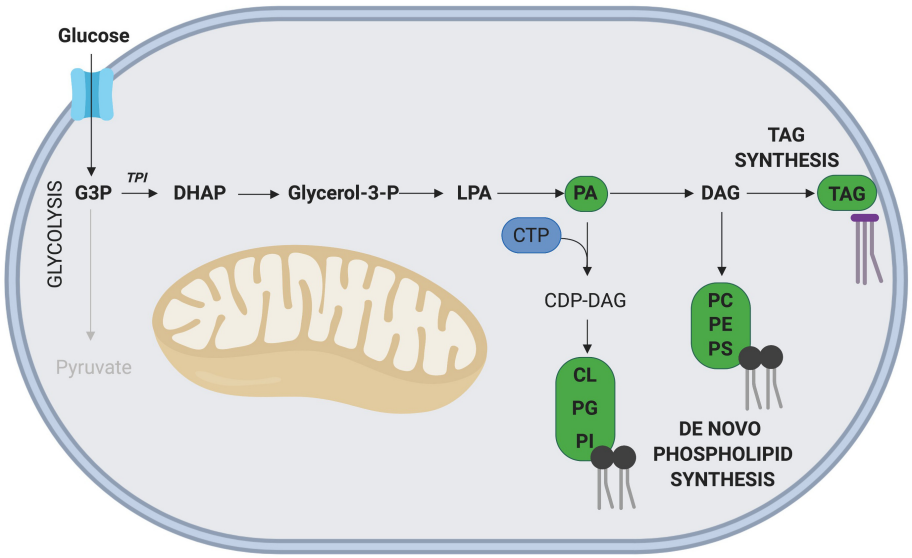

FIGURE 4 | Phospholipid and TAG synthesis via G3P. The glycolytic intermediate G3P, can contribute to phospholipid and TAG synthesis following its initial conversion to DHAP. The nucleoside CTP is required for synthesis of all phospholipids except phosphatidic acid (PA). Key metabolites of this side pathway include PA and diacylglycerol (DAG). Note: not all intermediate steps are shown. CDP-DAG, cytidinediphosphate-diacylglycerol; CL, cardiolipin; LPA, lysophosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

and obesity, which are characterized by excess fatty acids in the microenvironment, insulin resistance and impaired glucose tolerance. Both models display impaired muscle regeneration following injury (Hu et al., 2010; Nguyen et al., 2011; Akhmedov and Berdeaux, 2013; D'Souza et al., 2015; Xu et al., 2018). In one study, MuSC activation and proliferation was impaired in insulin resistant *ob/ob* mice, and in another, myotube maturation was delayed (Hu et al., 2010; Nguyen et al., 2011). Similar to that observed for nucleotides, under nutrient-rich conditions, mammalian cells tend to utilize *de novo* lipid synthesis for cellular proliferation (Palm and Thompson, 2017), but this has yet to be confirmed in MuSCs.

CONCLUSION

While metabolism has previously been thought to play a passive role in myogenesis, it is now established as a key regulator of both cell state and lineage progression. When MuSCs undergo rapid proliferation, efficient carbon routing through glycolysis (including its side branches) and the TCA cycle is required for the generation of precursors such as nucleotides, amino acids and lipids/phospholipids. In addition, an adequate supply of nutrients or precursors within the MuSC microenvironment is critical for these metabolic pathways to proceed. Many studies have demonstrated the regulatory effects of various metabolites on MuSCs and other proliferating cell types *in vitro* (either through the supplementation or deprivation), highlighting the importance of a tightly regulated metabolic microenvironment. However, metabolism and nutrient availability during regeneration remains an understudied topic *in vivo*, with many of these effects yet to be confirmed in regenerating skeletal muscle. Further RNAseq studies examining the expression of genes encoding for enzymes in these metabolic pathways combined with carbon-labeled flux analysis will help identify critical genes and/or metabolites which regulate these processes.

A better understanding of how the local metabolic microenvironment may regulate MuSC biology has important application for a broad range of fields, including synthetic biology studies focused on volumetric muscle loss, regenerative medicine and stem cell based therapies, agricultural research attempting to maximize protein yield and even in the developing field of cellular agriculture where researchers are attempting to generate cultivated meat. Together, the studies discussed in this review highlight an important role for metabolism in MuSC biology, particularly in the regulation of proliferation.

AUTHOR CONTRIBUTIONS

JN and JR conceived the topic for review. JN, JC, GL, and JR wrote the review.

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One-Carbon Metabolism Regulates Embryonic Stem Cell Fate Through Epigenetic DNA and Histone Modifications: Implications for Transgenerational Metabolic Disorders in Adults

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Human (h) and mouse (m) embryonic stem (ES) cells need specific amino acids to proliferate. mES cells require threonine (Thr) metabolism for epigenetic histone modifications. Thr is converted to glycine and acetyl CoA, and the glycine is metabolized specifically to regulate trimethylation of lysine (Lys) residue 4 in histone H3 (H3K4me3). DNA methylation and methylation of other H3 Lys residues remain unimpaired by Thr deprivation in mES cell culture medium. Similarly, hES cells require methionine (Met) to maintain the Met-SAM (S-adenosyl methionine) cycle of 1-carbon metabolism also for H3K4me3 formation. H3K4me3 is needed specifically to regulate and maintain both mES and hES cell proliferation and their pluripotent states. Better understanding of this regulation is essential since treatment of human diseases and disorders will increasingly involve hES cells. Furthermore, since ES cells are derived from their progenitor cells in preimplantation blastocysts, they serve as models of 1-carbon metabolism in these precursors of all mammalian tissues and organs. One-carbon metabolism challenges, such as a maternal low protein diet (LPD) during preimplantation blastocyst development, contribute to development of metabolic syndrome and related abnormalities in adults. These 1-carbon metabolism challenges result in altered epigenetic DNA and histone modifications in ES progenitor cells and the tissues and organs to which they develop. Moreover, the modified histones could have extracellular as well as intracellular effects, since histones are secreted in uterine fluid and influence early embryo development. Hence, the mechanisms and transgenerational implications of these altered epigenetic DNA and histone modifications warrant concerted further study.

Keywords: one-carbon metabolism, folate, threonine metabolism, methionine metabolism, inner cell mass, embryonic stem cells, epigenetic histone modification, metabolic syndrome

INTRODUCTION

Both sperm and egg cells contain DNA and histones that contribute to the genetic and epigenetic complements of mammalian conceptuses. The epigenetic complements of these gametes and early embryos are altered by environmental challenges to their one-carbon amino acid metabolism (Fleming et al., 2018; Clare et al., 2019; Goyal et al., 2019; Velazquez et al., 2019). Such challenges increase the prevalence of metabolic syndrome and related disorders in offspring that develop from the embryonic stem (ES) progenitor cells in these early conceptuses (Van Winkle and Ryznar, 2018).

Within days of conception, embryos give rise to ES progenitor cells and another type of cell in preimplantation blastocysts. A trophectoderm, one cell thick, forms the surface of blastocysts. This trophectoderm attaches to uterine epithelial cells to initiate implantation. Pluripotent cells, termed the inner cell mass (ICM), reside within the trophectoderm layer. After implantation, cells in the ICM differentiate into progenitors of all tissues in the offspring. Thus, environmentally induced epigenetic changes in ICM cells result in such changes in adult tissues and organs (Clare et al., 2019; Velazquez et al., 2019). Since the ICM is used to produce pluripotent ES cell lines, ES cells serve as an experimental model for environmental challenges to their progenitor cells in the ICM of blastocysts.

MOUSE EMBRYONIC STEM (mES) CELLS REQUIRE THREONINE (Thr) METABOLISM TO REMAIN PLURIPOTENT

mES Cells Require Thr One-Carbon Metabolism for Specific, Epigenetic Histone Methylations

Mouse ES cells do not proliferate and begin to differentiate if Thr is not supplied in their culture medium (Wang et al., 2009). These cells catabolize Thr to glycine and acetyl CoA, which are used for and help to regulate epigenetic methylations and acetylations in mES cells (Jog et al., 2017). Di- and tri-methylation of lysine (Lys) residue 4 in histone H3 (H3K4me3) slows dramatically and selectively when Thr is not supplied in the medium, while methylation of DNA and other H3 Lys residues continues normally (Shyh-Chang et al., 2013). Mouse ES cells need H3K4me3 to proliferate and remain pluripotent (Ang et al., 2011; Kilberg et al., 2016; Chen and Wang, 2019). Hence, Thr, taken up from the culture medium by mES cells, seems somehow to be metabolized selectively to provide 1-carbon units for H3K4 methylation.

Might Membrane Transporters Feed Amino Acids to Specific Intracellular Metabolic Compartments?

In the preimplantation blastocyst, leucine taken up by the trophectoderm triggers mammalian target of rapamycin (mTOR) signaling, trophoblast motility, and penetration of the uterine epithelium (Van Winkle et al., 2006). This signaling requires leucine uptake via the $B^{0,+}$ amino acid transporter. Leucine uptake by other transporters does not result in trophoblast motility. $B^{0,+}$ either selectively directs leucine to sites of mTOR signaling, or it generates other signals needed to synergize with mTOR (González et al., 2012).

In mES cells, selective use of Thr for H3K4 methylation may be more complex. Since Thr metabolism begins in perinuclear mitochondria in mES cells (Wanet et al., 2015; Lees et al., 2017; Woods, 2017), we propose that Thr is taken up selectively by a subpopulation of these organelles (**Figure 1**). Thr is then metabolized to formate, which leaves the mitochondria, and is converted to S-adenosyl methionine (SAM) methyl groups needed in the nucleus for H3K4 methylation (Scotti et al., 2013; Harvey, 2019). The formate from this subpopulation of mitochondria must be directed somehow to sites of H3K4 methylation in the nucleus, since only 1-carbon metabolism of Thr can be used to methylate this H3 residue (Shyh-Chang et al., 2013). Other 1-carbon donors cannot be used for this purpose unless supplied in excess to mES cells.

Alternatively, Thr could be directed to the pertinent subpopulation of perinuclear mitochondria by one of at least three plasma membrane Thr transport proteins (Formisano and Van Winkle, 2016). One of these transporters could conceivably direct Thr to the subpopulation of perinuclear mitochondria much like the $B^{0,+}$ transporter might selectively directs leucine to the site of mTOR signaling in the trophectoderm.

Does Thr Also Initiate Signaling by Interacting Directly With One of Its Transporters in Lipid Rafts?

In addition to providing metabolites for epigenetic DNA and histone methylation and acetylation (Jog et al., 2017), Thr

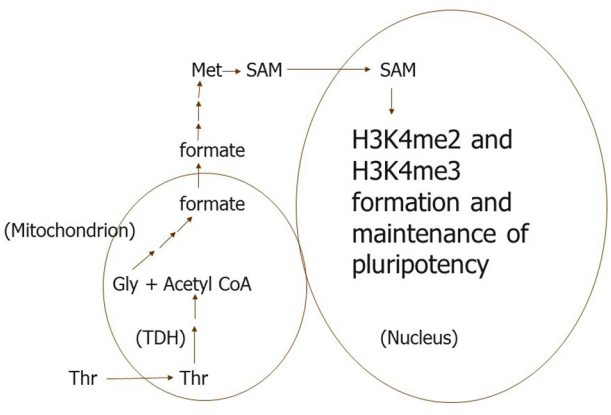

FIGURE 1 | We propose that a subpopulation of perinuclear mitochondria is specialized to take up only Thr and metabolize it to formate in *mouse* ES and their progenitor cells. The formate is then converted to 1-carbon units in a pool of SAM used specifically for H3K4me di- and tri-methylation. TDH, Thr dehydrogenase.

itself is likely needed for proliferation and maintenance of an undifferentiated state in mES cells (Ryu and Han, 2011). Thr promotes expression of cMyc through processes mediated by mTOR in these cells. Intact lipid rafts containing a Thr transporter seems essential for this signaling, since the signaling cannot occur in mES cells when these rafts are disrupted. Either an intact raft supports Thr transport and resultant intracellular signaling by the amino acid itself, or raft caveolae initiate signaling during Thr interaction with its transporter in the plasma membrane. As an alternative to a present thesis (e.g., **Figure 1**), perhaps the latter signaling and Thr catabolism together foster H3K4 methylation by somehow selectively activating pertinent histone modifying enzyme(s).

By interfering with Thr transport (Van Winkle et al., 2014; Formisano and Van Winkle, 2016) as well as catabolism (Wang et al., 2009), the Thr analog, 3-hydroxynorvaline (3-HNV), inhibits proliferation of mES cells. Similarly, this Thr transport and resultant signaling as well as catabolism in the ICM of blastocysts would help maintain pluripotency in these mES progenitor cells.

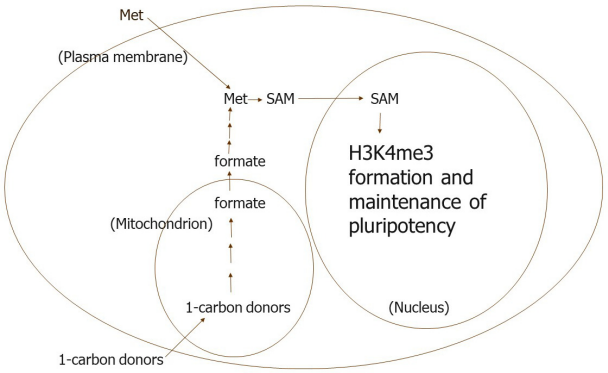

FIGURE 2 | We propose that Met is needed in the culture medium to maintain the intracellular concentrations of metabolites in the Met-SAM methylation cycle that are needed for H3K4me3 formation in *human* ES and their progenitor cells. In our view, other methyl transferases are able to use SAM preferentially when its intracellular concentration is lower possibly because the K_m values of these other transferases are lower than the transferases that methylate H3K4.

HUMAN EMBRYONIC STEM (hES) CELLS NEED METHIONINE (MET) METABOLISM FOR PROLIFERATION

Met Metabolism for Specific, Epigenetic Histone Methylations May Also Be Partially Compartmentalized in hES Cells

Naïve and primed mES cells are epigenetically different, and hES cells seem to better correspond to primed mES cells (Takahashi et al., 2018). Nevertheless, 1-carbon metabolism regulates hES cell proliferation. Met is required by hES cells, and their progenitors in the inner cell masses of blastocysts, for specific, epigenetic histone methylations. If Met is not in the culture medium, hES cells do not proliferate but undergo apoptosis (Shiraki et al., 2014). Exogenously supplied Met is metabolized to SAM in hES cells; these cells utilize SAM to carry out epigenetic methylations. A lack of Met (or supplemental SAM) in the medium nearly eliminates di- and tri-methylation of Lys residue 4 in histone H3 (H3K4me3). Global DNA methylation is also decreased by a modest amount through Met deprivation of hESC cells (Shiraki et al., 2014), but methylation of other H3 Lys residues remains unimpaired (Kilberg et al., 2016). As for mES cells, H3K4me3 maintains hES cell proliferation and pluripotency.

Met is needed in the culture medium apparently to maintain adequate concentrations of metabolites in the Met-SAM methylation cycle in hES cells (**Figure 2**). This cycle seems to be used preferentially by methyltransferases other than those catalyzing methylation of H3K4 when the concentration of metabolites in the cycle are low. Moreover, Met might be easily depleted from hES cells when not present in the culture medium owing to exchange of Met for other bulky zwitterionic amino acids in the medium. The only good transporter of Met in hES cell membranes appears to be one or more of those corresponding to the Na^+-independent system L, which is used for such exchange (Van Winkle and Ryznar, 2019).

An Enigma: Thr Recuses hES Cells From Growth Inhibition by the Thr Analog, 3-HNV, but Not Because Thr Is Needed for One-Carbon Metabolism or Protein Synthesis

According to Wang and associates Wang et al. (2009), the Thr analog, 3-HNV, inhibits mES cell proliferation because it inhibits Thr dehydrogenase (TDH) and metabolism of Thr to glycine and acetyl CoA. We found, however, that 3-HNV also inhibits hES cell proliferation (Van Winkle et al., 2014). Unlike mouse cells, hES cells lack functional TDH and so do not catabolize Thr to the 1-carbon units needed for epigenetic H3K4 methylation and maintenance of pluripotency. Somewhat surprisingly, 3-HNV inhibits hES cell proliferation in a manner morphologically similar to the way it arrests mES cell growth (Van Winkle et al., 2014).

It has been proposed that 3-HNV inhibits proliferation of cells lacking TDH activity through 3-HNV incorporation into protein in place of Thr (Najafzadeh, 2018). But 3-HNV does not inhibit proliferation of other mouse cell lines or HeLa cells (a human cell line; Wang et al., 2009), so it is unlikely to inhibit hES cell growth owing to its incorporation into protein. An excess of Thr in the culture medium does, however, rescue hES proliferation from 3-HNV inhibition. These findings support the theory that Thr fosters hES and mES cell proliferation through signaling, such as that requiring intact lipid rafts, that is separate from (and in addition to) regulation of epigenetic DNA and histone modifications by 1-carbon metabolites of Thr in mES cells (Van Winkle et al., 2014).

METHYL DONOR PERTURBATIONS, INCLUDING A MATERNAL LOW PROTEIN DIET, LIKELY ALTER TRANSGENERATIONAL EPIGENETIC DNA AND HISTONE MODIFICATIONS IN mES PROGENITOR CELLS

Does a More Invasive and Pinocytic Trophectoderm Supply More Thr to mES Progenitor Cells and Modify Placentation?

When female mice consume a LPD during preimplantation development of their conceptuses, trophoblast cells adapt in several ways in blastocysts. The trophectoderm exhibits more proliferation, motility during penetration of the uterine epithelium, and endocytic sequestration of uterine fluid proteins (reviewed by Fleming et al., 2018). These proteins are likely hydrolyzed to supply Thr to mES progenitor cells (Van Winkle and Ryznar, 2019), so increased accumulation of the proteins should result in delivery of more Thr to these pluripotent cells. More protein hydrolysis could also supply more leucine and arginine and, thus, augment trophoblast motility (González et al., 2012).

Other changes may also cause mES progenitor cells to accumulate more Thr. Mouse ES cells take up Thr via amino acid exchange (Formisano and Van Winkle, 2016). Thus, Thr can be accumulated against its chemical potential gradient only if other amino acids are first taken up against their gradients. For instance, mES cells should be able to accumulate alanine against its gradient via the Na^+-dependent amino acid transport system A (Van Winkle and Ryznar, 2019). This alanine could then exit the cells via system ASC and drive uptake of Thr into mES cells. In this regard, alanine may accumulate in the environment of mES progenitor cells when their mothers are consuming a LPD. When blastocysts develop from the 2-cell stage in culture medium without added amino acids, their alanine concentration is six times higher than in morphologically similar blastocysts developing *in vivo* (Van Winkle and Dickinson, 1995). While this *in vitro* culture system is an extreme model, it might mimic the effects of LPD well enough to indicate how mES progenitor cells could accumulate more Thr in exchange for alanine. Greater Thr accumulation could alter the supply of 1-carbon units to SAM and its regulation of H3K4me di- and tri-methylation.

Maternal consumption of a LPD during the pre- and peri-implantation stages of development also results in development of more efficient placentas (Fleming et al., 2018). More vigorous trophoblast invasion of the uterine epithelium owing to maternal LPD consumption likely leads to this development. Likewise, greater delivery of nutrients by the yolk sac placenta occurs, owing to altered epigenetic histone modifications in the primitive endoderm that gives rise to this placenta (Fleming et al., 2018). In addition, switching from LPD to control diet consumption by pregnant mice at the time of blastocyst implantation causes the somatic cell lineages of mES progenitor cells to decrease then raise, respectively, the level of some of their epigenetic DNA modifications relative to the control rate. Such changes in the nutrient supply to conceptuses also likely change epigenetic histone modifications during early embryo development.

Altered Epigenetic Histone Modifications in mES Progenitor Cells Likely Persist in Future Generations Without Further Methyl Donor Perturbations

Dysfunction of endothelial cells and elevated blood pressure occur in grandprogeny of rat dams consuming a LPD during pregnancy, in the absence of protein restriction of the F_1 generation, probably owing to transgenerational epigenetic DNA and histone modifications (Torrens et al., 2008). For the same reasons, impaired placental function or older age of F_0 mothers result in transmission of cardiorenal and metabolic alterations to their grandprogeny (Gallo et al., 2012; Master et al., 2015). Moreover, extracellular as well as intracellular actions of histones could have occurred, owing to uterine secretion of the modified histones by F_1 females (Van Winkle and Ryznar, 2018).

Epigenetic methyl donor challenges can also be genetic. For example, impairment of folate metabolism through genetic mutation in F_0 mice results in suppression of epigenetic methylation, with transgenerational effects on development of wild-type progeny mice, for at least five generations (Padmanabhan et al., 2013). Epigenetic modifications in the germline of F_1 females cause congenital malformations, whereas alterations in the uterine environment of F_1 mothers produce growth defects. The altered uterine environment likely includes epigenetically modified histones, since uterine cells of F_1 wild-type mice exhibited hypomethylation of DNA and probably histones, and uterine fluid contains histones that likely influence early embryo development (Van Winkle, 2017; Van Winkle and Ryznar, 2018). Histones act both intracellularly and extracellularly, however, so the effects of their hypomethylation probably include both congenital malformations and growth defects.

The proposed actions of extracellular histones are also illuminated by methyl donor supplementation that prevents transgenerational obesity amplification in F_3 agouti viable yellow mice (Waterland et al., 2008). In this study, germ cells of F_0 mice were formed in the unsupplemented control environment since their parents received no methyl donor supplementation. As a result, mES progenitor cells that produced F_1 mice had the epigenetic modifications associated with the control diet, so the organs that developed in F_1 mice did not benefit from methyl donor supplementation. We propose that the resultant uterine secretion of altered histones by F_1 mice (Van Winkle and Ryznar, 2018) contributed to development of obesity in the F_2 generation. In contrast, methyl donor supplementation of F_0 mice improved development of F_1 germ cells, so organs, such as the uterus, also developed

more normally in the F_2 offspring of F_1 mice. As a consequence, transgenerational obesity amplification was not observed in F_3 mice, owing to methyl donor supplementation beginning in the F_0 generation (Waterland et al., 2008).

Other Sources of Extracellular Histones in the Reproductive Tract

In addition to histones secreted in uterine fluid (Van Winkle, 2017; Van Winkle and Ryznar, 2018), other sources of extracellular histones include seminal fluid and dead sperm cells (Fung et al., 2004; Miller et al., 2010; Miller, 2018). Paternal as well as maternal environmental challenges adversely affect peri-implantation development of embryos through intergenerational epigenetic modifications (Clare et al., 2019; Su and Patti, 2019). These changes are likely present in extracellular and intracellular histones, thus providing one mechanism by which paternal diet could program offspring health through both seminal plasma- and sperm-specific pathways (Watkins et al., 2018).

Perturbations in Methyl Donor Supply Also Likely Alter Epigenetic DNA and Histone Modifications in Pre- and Peri-Implantation *Human* ES Progenitor Cells

Epigenetic DNA methylation marks change during formation of early embryos, and these changes foster normal tissue development. Human oocytes have high levels of the folate receptor FOLR1, suggesting that folate uptake for 1-carbom metabolism is important during the maternal to zygotic transition (Strandgaard et al., 2017). Babies born after IVF procedures have higher rates of disorders related to imprinting and epigenetic changes, maybe because commercial IVF media does not contain intermediates of 1-carbon metabolism, such as folates and methionine (Menezo et al., 2019). Moreover, epigenetic changes occurring during the activation of induced pluripotent stem cells require an optimal SAM pool for appropriate reprograming back to the pluripotent state (Fernández-Arroyo et al., 2015). This reprograming can be influenced by nutritional deficiencies of needed cofactors derived from vitamin B complexes. The altered methyl donor metabolism affects DNA methylation, histone modifications, and miRNAs. In addition, methyl donor challenges in humans, such as a LPD, alters epigenetically programed ES cell progenitor differentiation (Wu et al., 2019).

Furthermore, the differentially methylated CpG islands that regulate genomic imprinting may be highly susceptible to changes in diet during pre- and peri-implantation development. With over 150 imprinted genes identified in the human genome, and many human diseases associate with dysregulation of these areas, changes in diet affecting these regions could have profound effects on the offspring. For example, folic acid supplementation before versus during pregnancy showed changes in DNA methylation patterns specifically at the imprinted H19/IGF2 locus in infants. The authors proposed that since methylation marks regulating imprinted genes are acquired at differentially methylated DNA regions before gastrulation, their methylation patterns may reveal environmental exposures such as a change in diet or toxin exposure (Hoyo et al., 2011).

Other locations within the human genome may be affected by changes in epigenetic marks as the result of diet fluctuations. For example, active H3K4me3 and repressive H3K27me3 maintain a pluripotent epigenetic state in ES and their progenitor cells (Wu et al., 2019). In hES cells, there are also abundant non-CpG methylated cytosines that may be important to early development (Lister et al., 2009).

A maternal LPD also predisposition human as well as rodent offspring to metabolic syndromes by modifying genes including H19, IGF2, UBE3A, POMC, GR, and PPAR-α (Gong et al., 2010; Lillycrop and Burdge, 2011). These modified genes are related to imprinting, metabolism, or the stress response. Such changes in methylation patterns of the genome in the early embryo, after birth, or early in life provide evidence of how environment contributes to transgenerational human disease states.

CONCLUSION

Epigenetic DNA and histone modifications in ES and their progenitor cells in early embryos require membrane transport and compartmentalized metabolism of amino acids. One-carbon metabolites produced specifically to regulate H3 methylation in mES and hES cells require Thr and Met from the culture medium. ES cells require these 1-carbon units to produce H3K4me3 and remain undifferentiated. Further understanding of this regulation is essential. All adult tissues and organs arise from ES progenitor cells, and treatment of human diseases and disorders will increasingly involve hES cells.

Furthermore, metabolic syndrome and related disorders often result from altered 1-carbon metabolism in ES progenitor cells. This altered 1-carbon metabolism produces modified epigenetic DNA and histone methylations. Such epigenetically produced diseases and disorders can become transgenerational phenotypes in humans. Extracellular as well as intracellular histones may alter development beginning in early embryos when these histones are produced, through perturbation of 1-carbon methyl donor metabolism, and secreted in uterine fluid.

AUTHOR CONTRIBUTIONS

Both authors created sections of the manuscript, reviewed and provided feedback to each other that facilitated its critical revision, and approved the final version of the manuscript for publication.

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REVIEW
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Role of Metabolic Stress and Exercise in Regulating Fibro/Adipogenic Progenitors

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Obesity is a major public health concern and is associated with decreased muscle quality (i.e., strength, metabolism). Muscle from obese adults is characterized by increases in fatty, fibrotic tissue that decreases the force producing capacity of muscle and impairs glucose disposal. Fibro/adipogenic progenitors (FAPs) are muscle resident, multipotent stromal cells that are responsible for muscle fibro/fatty tissue accumulation. Additionally, they are indirectly involved in muscle adaptation through their promotion of myogenic (muscle-forming) satellite cell proliferation and differentiation. In conditions similar to obesity that are characterized by chronic muscle degeneration, FAP dysfunction has been shown to be responsible for increased fibro/fatty tissue accumulation in skeletal muscle, and impaired satellite cell function. The role of metabolic stress in regulating FAP differentiation and paracrine function in skeletal muscle is just beginning to be unraveled. Thus, the present review aims to summarize the recent literature on the role of metabolic stress in regulating FAP differentiation and paracrine function in skeletal muscle, and the mechanisms responsible for these effects. Furthermore, we will review the role of physical activity in reversing or ameliorating the detrimental effects of obesity on FAP function.

Keywords: obesity, metabolic syndrome, FAPs, differentiation, skeletal muscle, physical activity, exercise, mesenchymal stem cell

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INTRODUCTION

Over the last decades, lifestyle changes in western societies such as diet and physical inactivity are a major global public health problem leading to metabolic syndrome (MetS) (Saklayen, 2018). MetS is a cluster of different conditions including central adiposity, hypertension, insulin resistance, inflammation, and dyslipidemias, among others, which are themselves risk factors for type 2 diabetes (T2D), cardiovascular disease and even increasing the risk of cancer (Manuel et al., 2014). According to the World Health Organization (WHO), obesity (body mass index (BMI) $\geq$ 30 kg/m^2) has almost tripled since 1975, such that as of 2016 over 603 million adults and 107 million children are obese (WHO, 2019). This increased incidence of MetS is associated with an increase in the prevalence of musculoskeletal diseases and disorders (Wearing et al., 2006; Collins et al., 2018). Emerging evidence has shown that metabolic complications are positively correlated to reduction of muscle mass, impaired muscle repair, and increase in fibro/fatty tissue

accumulation (Akhmedov and Berdeaux, 2013). These pathological changes ultimately result in increased morbidity and disability (Hoy et al., 2014; March et al., 2014).

Underlying these pathological changes are alterations in the heterogeneous stem/progenitor cell populations that reside within skeletal muscle. The regenerative potential of skeletal muscle relies primarily on myogenic stem cells, called satellite cells (MuSCs), residing under the myofiber basal lamina (Wang and Rudnicki, 2011; Bentzinger et al., 2013). Upon muscle injury MuSCs enter the cell cycle, proliferate, and differentiate to repair damaged myofibers, while self-renewing to repopulate the reserve pool (Feige et al., 2018). Recently, a novel mesenchymal cell population of non-myogenic cells, named fibro/adipogenic progenitors (FAPs), has been identified in the skeletal muscle interstitium (Joe et al., 2010; Uezumi et al., 2010). FAPs are critical during muscle regeneration in order to sustain MuSC differentiation via paracrine mechanisms, and to maintain the MuSCs pool (Wosczyna et al., 2019). However, in pathological conditions FAP expansion continues unchecked, resulting in the production of fibro/fatty infiltrations, and impaired myogenesis (Rodeheffer, 2010; Uezumi et al., 2010, 2011; Mozzetta et al., 2013; Dong et al., 2017; Madaro et al., 2018; Stumm et al., 2018). Metabolism plays a crucial role in controlling the fate of progenitor cells, including MuSCs in tissue development, homeostasis, regeneration, and disease (Ryall et al., 2015a; Knobloch et al., 2017; Pala et al., 2018); however, the effects of metabolic stress and the metabolic regulation of FAPs has only recently begun to be explored. As such, the purpose of the present review is to provide an overview of the current state of the literature regarding to the effects of metabolic stress, induced by disease or exercise, on FAP differentiation and paracrine function.

CONNECTION BETWEEN METABOLIC STRESS AND ECTOPIC ADIPOSE TISSUE ACCUMULATION IN MUSCLE

Skeletal muscle is an important tissue for the regulation of whole-body metabolic homeostasis. In most individuals, skeletal muscle comprises 40–60% of the total body mass, accounts for ∼30% of the resting metabolic rate in adult humans (Zurlo et al., 1990), is a key contributor to whole body lipid utilization (Egan and Zierath, 2013), and ∼80% insulin-stimulated glucose disposal (Brüning et al., 1998). Skeletal muscle has a high capacity for substrate oxidation and a relatively high potential for substrate storage (DeFronzo et al., 1981). Under conditions of metabolic stress, detrimental changes to skeletal muscle occur, including muscle loss, intra- and inter-myofibrillar lipid accumulation, and connective tissue deposition. Eventually, these changes lead to a detrimental effect on contractile function and/or metabolic properties of skeletal muscle having an important impact on human health and contribute to insulin resistance (Sakuma and Yamaguchi, 2013). In the obese state, muscle lipid accumulation may occur as a result of insufficient adipose tissue expansion, in which the excess lipid are stored in non-adipose tissue compartments such as liver and skeletal muscle (Szendroedi et al., 2014; Cuthbertson et al., 2017; Czech, 2017; Conte et al., 2019). One result of the ectopic adipose tissue accumulation is the expansion of intramyocellular lipids (IMCLs) located within muscle cells (Sinha et al., 2002; Boesch et al., 2006). Paradoxically, endurance trained individuals also demonstrate an accumulation of IMCLs that are distinguished from the obese state by their location (Samjoo et al., 2013). Whereas in athletes IMCLs provide a local store of substrate for aerobic ATP generation, in persons with obesity, IMCLs are linked to insulin resistance and increased risk of T2D (Kautzky-Willer et al., 2003; Brumbaugh et al., 2012).

Intermuscular adipose tissue is distinguished from IMCLs as the former represents adipocytes that form between muscle fibers and muscle groups (Hamrick et al., 2016). Intermuscular adipose tissue increase with age in humans (Kirkland et al., 2002; Addison et al., 2014) and is highly correlated with a decrease in muscle mass, muscle strength, and insulin-sensitivity (Visser et al., 2005; Miljkovic-Gacic et al., 2008; Delmonico et al., 2009). Similarly, in participants with obesity, intermuscular adipose tissue accumulates and is associated with systemic insulin resistance (Goodpaster et al., 2000; Goss and Gower, 2012). Although not yet directly tested, the local accumulation of intermuscular adipose tissue may impair muscle metabolism by producing high intramuscular concentrations of adipokines, adipose-derived hormones, and free fatty acids. In support of this notion, some studies have reported positive correlations between intermuscular adipose tissue accumulation and the decrease in insulin sensitivity observed during aging and obesity (Goodpaster et al., 1997, 2000; Ryan and Nicklas, 1999; Sachs et al., 2019). Interestingly, the majority of studies on intermuscular adipose tissue accumulation under conditions of metabolic stress have been conducted in humans. Three recent studies indicated that intermuscular adipose tissue accumulates in rodents in both obesity (Khan et al., 2015; Zhu et al., 2019) and aging (Cui et al., 2019). This is relevant because most of the work investigating the mechanisms regulating intermuscular tissue adipose tissue accumulation have been conducted in rodents. Thus, similar responses in human and rodent skeletal muscle have been observed in the limited studies that have evaluated intermuscular adipose tissue accumulation in metabolic stress.

FIBRO/ADIPOGENIC PROGENITORS AS THE CELLULAR SOURCE OF INTERMUSCULAR ADIPOSE TISSUE

Fibro/adipogenic progenitors are muscle-resident non-myogenic progenitors of mesenchymal origin, which express stem cell antigen 1 (Sca-1), platelet-derived growth factor receptor α (PDGFRα), and also high levels of CD34 (Joe et al., 2010; Uezumi et al., 2010, 2014). FAPs are distinct from MuSCs as they lack Pax7 expression (Joe et al., 2010; Uezumi et al., 2010, 2014). FAPs have been defined as multi-potent progenitors, residing on the abluminal side of the capillaries in the interstitial spaces between the myofibers in both humans and mouse skeletal muscle (Joe et al., 2010; Uezumi et al., 2010; Arrighi et al., 2015). These cells are defined by their ability to differentiate

into fibroblasts, adipocytes, and osteoblasts, and originate from a non-myogenic (Myf5-) cell population which is supported by their lack of myotube formation *in vitro* (Joe et al., 2010; Uezumi et al., 2010). Following muscle injury, FAPs transiently become activated, proliferated, and expand (Lemos et al., 2015; Wosczyna et al., 2019). Via primarily paracrine mechanisms, FAPs promote MuSC proliferation (Fiore et al., 2016) and differentiation (Joe et al., 2010; De Lisio et al., 2014; Zou et al., 2015; Contreras et al., 2016; Dammone et al., 2018; Madaro et al., 2018), thus participating in muscle repair. Conversely, in pathological conditions characterized by myofiber damage or atrophy, FAPs undergo unchecked expansion and differentiation causing fibrosis, fat deposition an impaired myogenesis (Lemos et al., 2015; Dammone et al., 2018; Madaro et al., 2018).

Metabolic stress has been linked to FAP accumulation and fibro/adipogenic differentiation (Dammone et al., 2018; Gorski et al., 2018; Kang et al., 2018; Buras et al., 2019). Using several different genetic and diet-induced mouse models of diabetes, Mogi et al. (2016) demonstrated that ectopic adipocyte accumulation in skeletal muscle was derived from PDGFRα^+ progenitors. Similarly, Arrighi et al. (2015) isolated a population of FAPs, identified as CD56$^-$CD15$^+$/PDGFRα^+, that formed functional adipocytes *in vitro*. These FAP-derived adipocytes may have reduced insulin sensitivity compared to conventional adipocytes, as indicated by lack of phosphorylation of insulin receptor, suggesting that accumulation of FAP-derived adipocytes may contribute to a compromised peripheral insulin sensitivity (Arrighi et al., 2015). However, given the relatively small contribution of intermuscular adipose tissue relative to whole body adipose depots, the negative effects of intermuscular adipose tissue on glucose disposal is likely via secondary mechanisms that reduce the ability of myofibers to uptake glucose. Similar to findings in limb skeletal muscle, 6 months of high-fat feeding induced FAP proliferation, increased adipocytes, and type I collagen-depositing fibroblasts in the diaphragm leading to respiratory dysfunction (Buras et al., 2019). Together, these data indicate that in obesity and related metabolic disorders, FAPs directly contribute to intermuscular adipose tissue accumulation in skeletal muscle.

Several potential mediators of the effects of obesity on FAP differentiation have been investigated. Adipokines released from expanded adipose tissue such as thrombospondin 1 (THBS1), was increased in obese mice and promoted FAP proliferation (Buras et al., 2019). Similarly, TGFβ which is produced in many organs including adipose tissue (Lee, 2018) controls FAP proliferation and differentiation to a fibrogenic lineage *in vitro* (Ito et al., 2013; Lemos et al., 2015). Conversely, inhibition of PDGFRα and TGFβ signaling resulted in reduced FAP number and a reduction in collagen deposition (Ieronimakis et al., 2013; Ito et al., 2013; Lemos et al., 2015; Fiore et al., 2016). Thus, several adipocyte-derive factors increase FAP adipogenesis, indicating a direct mechanism whereby adipose tissue expansion in obesity may stimulate intermuscular adipose tissue accumulation.

In contrast to adipokines, factors synthesized by myofibers play an important role in limiting adipogenesis during muscle regeneration. Nitric oxide (NO), which is increased in response to muscle injury and exercise, inhibits FAP adipogenic differentiation by down-regulation of the peroxisome proliferator-activated receptors gamma (PPARg) (Cordani et al., 2014). Marinkovic et al. (2019) showed that suppression of myofiber-derived NOTCH signaling via inhibition of γ-secretase or by interfering with the expression of NOTCH stimulates FAP differentiation in a dose-dependent manner, whereas activation by the NOTCH ligand DLL1 leads to significant inhibition of adipogenesis in *mdx* mice. Kopinke et al. (2017) demonstrated a critical role of cilia in modulating the adipogenic fate of FAPs by controlling the activity of the Hedgehog signaling pathway. Pharmacological inhibition of matrix metalloprotease (MMP)-14 represses C/EBPδ and PPARγ in FAPs by way of cilia Hedgehog signaling and this reduces the adipogenic fate of FAPs. As a result, this enhanced muscle regeneration during acute muscular injury and in a model of muscular dystrophy (Kopinke et al., 2017). Collectively, these data indicate that regenerating muscle releases several factors that inhibit FAP adipogenesis, providing a potential mechanism whereby exercise-induced muscle damage may prevent ectopic intermuscular adipose tissue accumulation under metabolic stress.

Cell metabolism is also a driver of mesenchymal progenitor cell fate during differentiation. For instance, during induction of adipogenesis mesenchymal progenitors need to enhance reliance on oxidative phosphorylation in order to continue differentiation into pre- and mature adipocytes (Shyh-Chang et al., 2013). This may explain why incubating fibroblasts from human skeletal muscle with fatty acids is a potent inducer of adipogenesis (Agley et al., 2013). Similarly, generation of osteoblasts is also associated with high reliance on oxidative phosphorylation. In contrast, fibrogenesis and chondrogenesis seems to require utilization of glycolysis during differentiation (Shyh-Chang et al., 2013; Zhao et al., 2019). FAPs from regenerating *mdx* muscle have an increase in glycolytic proteins and a reduction of mitochondrial proteins compared to control mice (Marinkovic et al., 2019) resulting in *mdx* FAPs favoring glycolysis over oxidative metabolism (Reggio et al., 2019). Interestingly, these metabolic changes were associated with greater proliferative capacity and adipogenic potential *in vitro* which was reversed by inhibiting glycolysis and forcing oxidative metabolism (Reggio et al., 2019). This impaired metabolic phenotype was reversed *in vivo* by providing a short-term high fat diet which stimulated oxidative metabolism in FAPs (Reggio et al., 2019). Conversely, long-term high fat diet, and obesity are associated with increased muscle adiposity and fibrosis (Goodpaster et al., 2000). Hogarth et al. (2019) identify FAPs and their adipogenic differentiation as a major contributor to dysferlin-deficient muscle loss in limb-girdle muscular dystrophy 2B (LGMD2B); a disease associated with mitochondrial dysfunction (Vincent et al., 2016). Together, these interesting findings indicate that mitochondrial function and metabolism are important regulators of FAP fate, and that the FAP response to metabolic stress may be distinct from other interstitial cells in skeletal muscle or from mesenchymal cells in different tissues. Further, they suggest that FAP fate may be regulated by substrate availability, which provides novel areas for therapy.

The effects of exercise on FAP differentiation have yet to be fully elucidated. Endurance exercise training is associated with

an increase in IMCLs, but not intermuscular adipose tissue (van Loon et al., 2004; Dubé et al., 2008). Furthermore, acute resistance and endurance exercise is known to increase muscle extracellular matrix synthesis with a similar increase in breakdown resulting in matrix remodeling without excessive accumulation, unlike pathological conditions (Martinez-Huenchullan et al., 2017). Using a preclinical model of radiation exposure, recent work from our group showed a differential FAP response of obese and exercise trained mice (D'Souza et al., 2019). Interestingly muscle fibrosis and adipose tissue accumulation were higher in high fat-fed mice with no effect of treadmill exercise. However, we were not able to distinguish between intermuscular and intramyocellular adipose tissue in our analyses. Conversely, FAP content trended to increase in high fat-fed, sedentary mice, and trended to be reduced in high fat-fed, exercise trained mice (D'Souza et al., 2019). While these results are preliminary, they suggest that exercise training may inhibit FAP expansion in obesity. Similarly, Zeve et al. (2016) reported that endurance exercise suppressed adipogenic progenitor proliferation and differentiation into mature adipocytes *in vitro* and suppressed adipogenesis *in vivo* in mice fed a high fat diet. These effects were mediated in part through secretion of R-spondin 3 from slow myofibers, which may activate Wnt signaling to suppress adipogenesis (Zeve et al., 2016). In the bone marrow, exercise training and mechanical forces inhibit adipogenic differentiation of mesenchymal stromal cells (Emmons et al., 2017; Rubin et al., 2018), a population similar to skeletal muscle FAPs. Future work should investigate if similar effects of exercise are present in skeletal muscle FAPs. Under conditions of metabolic stress, exercise may directly regulate oxidative capacity of FAPs by stimulating mitochondrial biogenesis as it does in skeletal muscle, or counteract the pro-adipogenic effects of adipokines by increasing secretion of anti-adipogenic factors from myofibers.

THE RELATIONSHIP BETWEEN METABOLIC STRESS AND THE FAP SECRETOME

Fibro/adipogenic progenitors exert much of their functional effects on muscle regeneration and repair via responding to and secreting paracrine factors in their local microenvironment (Joe et al., 2010; Lemos et al., 2015). The most complete list of factors that are secreted by FAPs, and that FAPs respond to have been reviewed elsewhere (Biferali et al., 2019). Interestingly, despite the importance of paracrine factor signaling on FAP function, relatively little attention has been paid to the role of metabolic stress on the FAP secretome. One myogenic paracrine factor that has received some attention in the literature is follistatin (Mozzetta et al., 2013; Reggio et al., 2019). Follistatin is a myostatin inhibitor, and thus promotes muscle growth (Lee and McPherron, 2001) and glucose uptake (Han et al., 2019). Recent studies determined that FAPs are a major source of follistatin in skeletal muscle, and FAP-derived follistatin is a key mechanism responsible for FAP-induced myoblast differentiation (Mozzetta et al., 2013). Further, HDAC inhibitors blocked adipogenic differentiation of FAPs and improved their ability to promote differentiation of MuSCs, through upregulation of the soluble factor follistatin in early stage, but not late stage, *mdx* mice; which are also characterized by metabolic defects (Mozzetta et al., 2013). Using the same *mdx* mouse model of muscular dystrophy, Reggio et al. (2019) demonstrated that follistatin is diminished in FAPs due to reduced β-catenin signaling. However, short-term high fat diet feeding reversed this defect by increasing β-catenin levels which promoted IGF-1 and follistatin expression and release leading to improved ability of FAPs to support myogenesis and muscle regeneration (Reggio et al., 2019). Thus, short-term metabolic reprograming of FAPs, via high fat diet feeding may ameliorate some of the regenerative defects observed in dystrophic mice (Reggio et al., 2019). Similarly, recent work from our laboratory using a model of *in vivo* radiation exposure demonstrated that high fat feeding increased levels of phosphorylated NF-κB, particularly in interstitial nuclei (D'Souza et al., 2019). However, it was not evident if this marker of increased interstitial NF-κB activation was localized specifically to FAPs (D'Souza et al., 2019). In the context of aging, Lukjanenko et al. (2019) determined that diminished FAP secretion of the matricellular protein WNT1 inducible signaling pathway protein 1 (WISP-1) underlies the impairment in MuSC regenerative capacity. However, in this same study, the authours determined that adipogenic differentiation of FAPs is diminished (Lukjanenko et al., 2019). Thus, the signals regulating FAP function may differ between aging and obesity. These data indicate that in models associated with metabolic dysfunction, short-term high fat feeding, can increase paracrine factor secretions from FAPs, which contrast some of the detrimental effects of long-term high fat feeding on FAP differentiation. Mechanistically, it will be important to determine if the specific composition of the diet is relevant to regulating the FAP secretome, or if alterations in FAP paracrine factor secretion are due to increased adiposity associated with the diet, and what regulates the apparent differential responses of FAP differentiation/secretome to long- versus short-term high fat feeding.

Exercise, and exercise-induced signals, have been suggested to alter FAP paracrine function (Boppart et al., 2013). Exercise used to induce a physiologically relevant adaptive response has been shown to increase FAP content in humans (Farup et al., 2015), and a heterogeneous population of muscle mesenchymal stromal cells which contains FAPs in mice (Valero et al., 2012). In these studies, increased FAP content was associated with increases in MuSCs and an enhanced muscle adaptive response to exercise (Valero et al., 2012; Farup et al., 2015). Using a model of *in vitro* "exercise" where isolated muscle mesenchymal cells, including FAPs, were exposed to mechanical forces, it was determined that the strained cells produced a mix of paracrine factors that enhanced myoblast proliferation *in vitro* (De Lisio et al., 2014). Further, mechanical strain applied to these stromal cells *in vitro* prior to injection into muscle *in vivo* improved the muscle response to exercise (Huntsman et al., 2013, 2018; Zou et al., 2015). The relative contribution of FAPs to the overall secretome

of the heterogeneous population of muscle-derived mesenchymal cells at rest and after exercise remains to be determined.

THE EFFECTS OF METABOLIC STRESS ON FAP ACTIVATION

In relation to obesity and T2D one of the most obvious changes in the systemic environment is the chronically elevated levels of metabolic substrates such as glucose and triglycerides. Activation (exit from quiescence) and first cell division of stem/progenitor cells (e.g., MuSCs or FAPs) has in recent years been described as a highly unique process compared to the following (second, third, etc.) cell divisions (Rodgers et al., 2014; Liu et al., 2018). For instance, in mice time-to-first division in MuSCs is approximately 60 h, whereas the following divisions are approximately four times faster. Although not well-described in FAPs yet, we speculate that the same may be true for FAPs since most of these also reside in a quiescent/non-cycling state in normal mouse muscle (Joe et al., 2010; Rodgers et al., 2014). The time before first cell division is characterized by a major increase in cell size, anabolic signaling, mitochondrial content and ATP levels (Rodgers et al., 2014). In MuSCs these changes have been associated with an increased reliance on glycolysis as well as increased autophagic flux likely to support sufficient generation of ATP and biomass to prepare for the cell cycle (Tang and Rando, 2014; Ryall et al., 2015b). In contrast, genetic deletion of major enzymes involved in this anabolic process (e.g., mTORC1) or blocking autophagy delays the cell cycle entry (Rodgers et al., 2014; Tang and Rando, 2014; Ryall et al., 2015b). Glucose and related insulin signaling are major upstream regulators of these pathways making them likely candidates for perturbing the balance between maintaining and exiting quiescence. Although there is yet no direct evidence linking changes in substrate availability in the FAP niche to activation of FAPs it is interesting to note how the major FAP regulator, platelet-derived growth factor (PDGF), is driving changes in cell metabolism. For instance, PDGF treatment of muscle FAPs leads to increased cell proliferation as well as transcriptional changes related to glycolysis (Mueller et al., 2016). Moreover, PDGF signaling in mesenchymal progenitors or fibroblasts from other tissues is known to increase reliance on glycolysis and thereby also increasing lactate production (Ran et al., 2013; Xiao et al., 2017). In fact, fibrosis in multiple tissues is associated with increased glycolytic flux compared to non-fibrotic areas and blocking glycolysis seems to ameliorate some of these pathological events and reduce extracellular matrix accumulation (Zhao et al., 2019). This suggest that increased glycolysis is not merely a result of cell activation, but likely have a causal relationship to cell activation. Collectively, evidence is accumulating that cell metabolism is intimately involved in fibrosis development, in skeletal muscle potentially through priming FAPs for exiting quiescence. Since the normal FAP clearance (as observed during skeletal muscle regeneration from TNFα induced FAP apoptosis) (Lemos et al., 2015) is likely not present under these circumstance, one can speculate that this could lead FAPs to accumulate over time, contributing to increased fatty-degeneration of the skeletal muscles in obesity and T2D.

Exercise provides a unique, physiological stimulus to examine the role of mechanical and metabolic stress on skeletal muscle that results in efficient and complete repair and adaptation. Early work demonstrated that a single bout of damaging exercise increased the content of a heterogeneous population of muscle-derived mesenchymal progenitors, which likely included FAPs (Valero et al., 2012). These findings mirrored the effects of acute exercise on mesenchymal progenitors in other tissues, such as the bone marrow (Emmons et al., 2016). More recent work has suggested that the effects of exercise might be dependent on the population of mesenchymal progenitors investigated. Muscle pericytes did not increase in human muscle following eccentric exercise (De Lisio et al., 2015) or in mice following electrical stimulation (Dvoretskiy et al., 2019). This response is different from mesenchymal progenitors from other tissues, as bone marrow-derived mesenchymal progenitors are activated by an acute exercise (Emmons et al., 2016). Conversely, resistance training was associated with an increase in the content of FAPs expressing markers of active cell cycle (Farup et al., 2015). As such, the available data suggest that the effects of exercise FAP/mesenchymal progenitor activation may be dependent on the tissue of origin and specifics of the exercise stimulus, among other, yet-to-be investigated factors. Moreover, in mouse and in particular in human skeletal muscle, more in-depth phenotyping is needed in order to distinguish the specific overlapping interstitial cell populations in muscle.

THE ROLE OF CHRONIC LOW-GRADE INFLAMMATION IN METABOLIC STRESS ON FAP FUNCTION

The inflammatory response following muscle injury is a well-orchestrated, time-dependent process necessary to obtain complete muscle regeneration (Tidball, 2017). This response begins with the infiltration of the earliest immune cells such as neutrophils and eosinophils (Tidball, 2011). During muscle injury, IL-4 and IL-13-secreting eosinophils are recruited to the injured site (Heredia et al., 2013). These inflammatory signals act through IL-4Rα to stimulate signal transducer of transcription 6 (STAT6), which promotes FAP proliferation and inhibits FAP differentiation into adipocytes (Heredia et al., 2013). The early immune response is followed by an infiltration of macrophages with a M1 phenotype (pro-inflammatory) followed by the expansion of M2 macrophages (anti-inflammatory), which are associated with tissue repair and MuSC differentiation (Chazaud et al., 2003). Therefore, the polarization of M1 and M2 macrophages play a crucial role in successful muscle regeneration. Recently, it has been suggested that in response to acute muscle damage, macrophage-derived TNF-α plays a crucial role in regulating FAP apoptosis (Lemos et al., 2015; Fiore et al., 2016). Indeed, Lemos et al. (2015) demonstrated that in the absence of TNF-α-producing macrophages, FAPs accumulate and aberrantly differentiate into a fibrogenic lineage. Pagano et al. (2019) showed that TNF-α mediated FAP apoptosis might

be perturbed in a glycerol model of muscle injury, leading to intermuscular adipose tissue development. However, treatment with the TGF-β inhibitor decorin decreases intermuscular adipose tissue development and might restore FAP apoptosis (Pagano et al., 2019). Thus, inflammatory cell-derived factors are required for proper FAP regulation, and any dysregulation of the timing or concentrations of these factors could contribute to pathological extracellular matrix accumulation by FAPs.

In contrast to the acute inflammatory response to muscle damage, myopathies, dystrophies, aging, diabetes, and obesity are associated with a chronic low-grade inflammation. This chronic, low-grade inflammation is associated impaired function of MuSCs, immune cells, and FAPs, leading to fibrosis, and poor skeletal muscle regeneration (Rostasy et al., 2008; Mann et al., 2011; D'Souza et al., 2015; Wang et al., 2015). Moreover, chronic inflammation in these conditions results in an increase in cytokine release that is responsible for the extracellular matrix production (Van Linthout et al., 2014). Consequently, muscle fibrosis develops which disrupts the cell niche for proper skeletal muscle regeneration (Murphy et al., 2011). Specifically, during chronic muscle damage, macrophage-derived TGF-β1, inhibits TNF-mediated FAP, and instead induce their fibrogenic differentiation and consequent extracellular matrix deposition (Lemos et al., 2015; Davies et al., 2016; Fiore et al., 2016; Juban et al., 2018). Similarly, Moratal et al. (2018) report that IL-1β-activated macrophages and IL-4-polarized macrophages have opposite effects on FAP differentiation into adipocytes *in vitro*, which was dependent on Smad2 phosphorylation in FAPs. Thus, under chronic inflammatory conditions associated with several metabolic disorders, signals that regulate FAP apoptosis and inhibit proliferation are perturbed, leading to a chronic state of remodeling which ultimately results in fibro/fatty tissue accumulation.

Exercise is a well-known modulator of the inflammatory response (Febbraio, 2007; Lancaster and Febbraio, 2014). In response to acute exercise, skeletal muscle produces a myriad

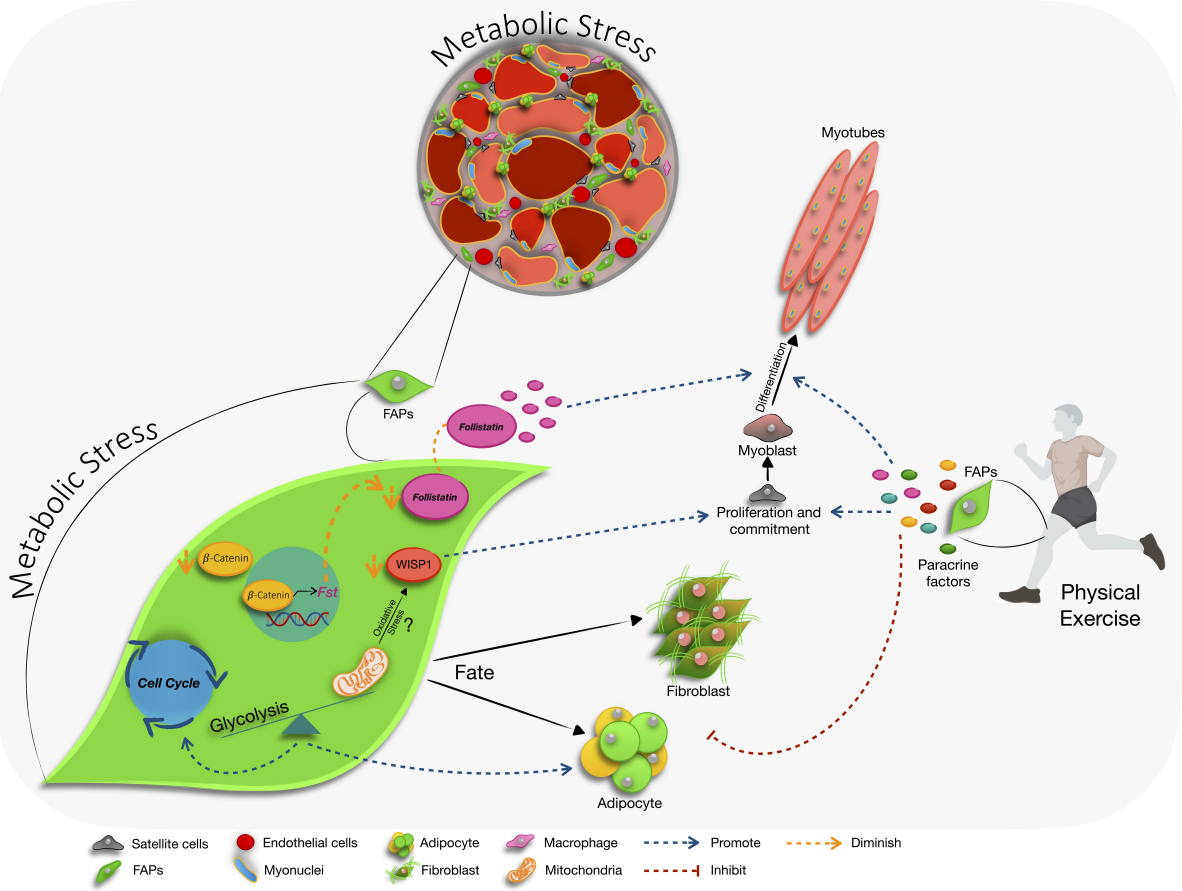

FIGURE 1 | The influence of metabolic stress on fibro/adipogenic progenitor (FAPs) cell function. In skeletal muscle, metabolic stress leads to an accumulation of intermuscular adipose tissue, extracellular matrix production, and inflammation. FAPs are the primary cellular source of intermuscular adipose tissue, extracellular matrix proteins, and interact with immune cells to participate in the inflammatory response. Available literature indicates that during metabolic stress FAPs favor glycolysis during proliferation and adipogenic differentiation, and downregulate the production of the pro-myogenic factor follistatin (*fst*) via modulation of the β-catenin signaling pathway. In aging, altered secretion of WNT1 inducible signaling pathway protein 1 (WISP1) by FAPs, which may be induced by mitochondrial dysfunction, reduces their capacity to support MuSC activation and commitment. Exercise reduces the characteristic changes in skeletal muscle that occur during metabolic stress. Further, exercise stimulates FAP production of pro-myogenic factors and may inhibit adipogenic and fibrogenic potential of FAPs.

of pro-inflammatory cytokines, including but not limited to IL-6 (Pedersen and Steensberg, 2002), IL-1ra (Ostrowski et al., 1998), IL-8, and IL-15 (Nielsen and Pedersen, 2007). The role of these cytokines in relation to skeletal muscle is to participate in the muscle repair/adaptive response to exercise (Serrano et al., 2008; McKay et al., 2009), and modulate whole-body and muscle glucose metabolism (Febbraio et al., 2004; Carey et al., 2006). Additionally, cellular mediators of the inflammatory response, increase in skeletal muscle after acute exercise (Paulsen et al., 2010), and macrophages with a pro-regenerative phenotype also increase in skeletal muscle following 12 weeks of endurance training (Walton et al., 2019). The increase in phenotypically pro-regenerative macrophages was associated with greater increases in fiber cross-sectional area and increases in MuSC content (Walton et al., 2019). Mesenchymal stromal cells in other tissues are known to regulate inflammatory responses (Munir et al., 2018); however, whether they regulate muscle inflammation, and how exercise can influence this relationship remains unknown.

PERSPECTIVES AND CONCLUSION

Obesity, diabetes, and other metabolic disorders are reaching epidemic proportions in western countries. Impairment of skeletal muscle is a central player in the detrimental effects of metabolic stress leading and metabolic disorders. Under conditions of metabolic stress, muscle dysfunction is characterized by excessive intermuscular adipocytes, extracellular matrix accumulation, and inflammation (Lawler et al., 2016). In these conditions, the accumulation of fibro/fatty tissue in skeletal muscle is associated with a loss of muscle mass, a reduction in muscle strength (Delmonico et al., 2009), insulin resistance, and inflammation (Szendroedi et al., 2014). As the cellular source of intermuscular adipose tissue, primary producers of the extracellular matrix, and key regulators of muscle mass, FAPs are central to these detrimental changes in skeletal muscle under metabolic stress (**Figure 1**). Exercise is an effective first line therapy for metabolic disorders; however, the role of exercise on FAP fate and function are just beginning to be identified. As the effects of metabolic stress and role of metabolism in regulating FAP function begin to be unraveled in the coming years, as well as the mechanisms responsible for exercise-induced FAP regulation, novel avenues for therapy will emerge to maintain muscle function, metabolic health, and reduce morbidity.

AUTHOR CONTRIBUTIONS

NC and MD conceived the topic for review. NC, JF, and MD wrote the review.

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Metabolic Coordination of Pericyte Phenotypes: Therapeutic Implications

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Pericytes are mural vascular cells found predominantly on the abluminal wall of capillaries, where they contribute to the maintenance of capillary structural integrity and vascular permeability. Generally quiescent cells in the adult, pericyte activation and proliferation occur during both physiological and pathological vascular and tissue remodeling. A considerable body of research indicates that pericytes possess attributes of a multipotent adult stem cell, as they are capable of self-renewal as well as commitment and differentiation into multiple lineages. However, pericytes also display phenotypic heterogeneity and recent studies indicate that lineage potential differs between pericyte subpopulations. While numerous microenvironmental cues and cell signaling pathways are known to regulate pericyte functions, the roles that metabolic pathways play in pericyte quiescence, self-renewal or differentiation have been given limited consideration to date. This review will summarize existing data regarding pericyte metabolism and will discuss the coupling of signal pathways to shifts in metabolic pathway preferences that ultimately regulate pericyte quiescence, self-renewal and *trans*-differentiation. The association between dysregulated metabolic processes and development of pericyte pathologies will be highlighted. Despite ongoing debate regarding pericyte classification and their functional capacity for *trans*-differentiation *in vivo*, pericytes are increasingly exploited as a cell therapy tool to promote tissue healing and regeneration. Ultimately, the efficacy of therapeutic approaches hinges on the capacity to effectively control/optimize the fate of the implanted pericytes. Thus, we will identify knowledge gaps that need to be addressed to more effectively harness the opportunity for therapeutic manipulation of pericytes to control pathological outcomes in tissue remodeling.

Keywords: adult stem cell, metabolism, *trans*-differentiation, proliferation, quiescence, fibrosis, regenerative medicine

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INTRODUCTION: WHAT IS A PERICYTE?

Pericytes are a heterogeneous population of mural vascular cells that typically reside within the basement membrane on the abluminal surface of most capillaries and some larger blood vessels. The pericyte basement membrane is contiguous with that of the endothelial cells and is composed of extracellular matrix proteins (predominantly collagen IV and the glycoprotein laminin) secreted by both cell types. Morphologically, pericytes are characterized by numerous

cytoplasmic processes that emanate from a prominent cell body, tracking along several endothelial cells and occasionally spanning adjacent capillaries (**Figure 1**). Pericyte protrusions (pegs) insert into endothelial cell invaginations (sockets) at occasional interruptions in the basement membrane, providing structural support as well as direct heterotypic cell–cell communications (Armulik et al., 2005). The current review focuses on the traditionally defined pericytes, but it should be noted that specialized pericytes that deviate from these conventional characteristics reside within various tissues (i.e., stellate cells in liver, mesangial cells in kidneys, reticular cells in bone marrow) (Díaz-Flores et al., 2009).

During development, pericytes are first attracted to newly formed capillaries via the endothelial-secreted chemoattractant platelet-derived growth factor (PDGF)-BB, which binds to PDGF receptor β (PDGFRβ) on pericytes (Lindahl et al., 1997; Benjamin et al., 1998). Integrin-mediated adhesion of pericytes to laminin helps to maintain expression of PDGFRβ (Durbeej, 2010; Reynolds et al., 2017). Interference with PDGF-BB/PDGFRβ signaling is sufficient to disrupt endothelial-pericyte interactions (Lindahl et al., 1997), indicating that perpetual signaling through PDGFRβ is critical to maintain pericyte localization to capillary endothelial cells. Several other proteins, such as Notch receptors, support the continued close interaction of pericytes with the underlying endothelial cells and play important roles in maintaining pericyte identity (Armulik et al., 2005; Geevarghese and Herman, 2014; Kofler et al., 2015; Ando et al., 2019) (**Figure 2**). This tight interaction between endothelial cells and pericytes is critical to the stability and maintenance of the integrity of mature capillary networks. Pericytes restrict capillary expansion and promote endothelial cell viability and quiescence through their physical connections and via secretion of paracrine factors (i.e., Ang-1, TIMP-3) (Papapetropoulos et al., 2000; Saunders et al., 2006). Conversely, loss of pericyte contact (following pericyte detachment or apoptosis) reduces endothelial cell survival and promotes capillary regression. Pericytes play additional functions in the vascular compartment, including preservation of capillary barrier function, blood flow regulation, and immunomodulation (Bergers and Song, 2005; Armulik et al., 2011; Geevarghese and Herman, 2014; Navarro et al., 2016). Of note, pericytes also contribute to different cellular processes involved in tissue homeostasis through the potential of differentiating in other cell types (discussed in detail below).

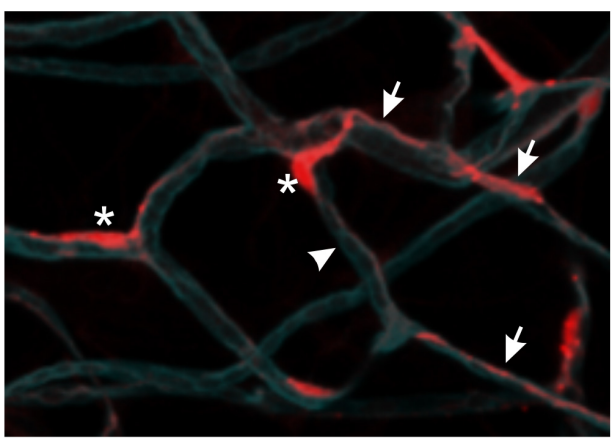

FIGURE 1 | Pericyte morphology. NG2^{+} pericytes are localized to the abluminal surface of adipose tissue capillaries (arrowhead) in mice that express red fluorescent protein (DsRed) under the control of the promoter of chondroitin sulfate proteoglycan 4 (encoding NG2). Pericyte cell bodies (asterisks) are prominently visible due to nuclear localization of DsRed. Thin cytoplasmic processes extend along one or more capillaries (arrows). Capillaries were visualized using Isolectin B4 (cyan).

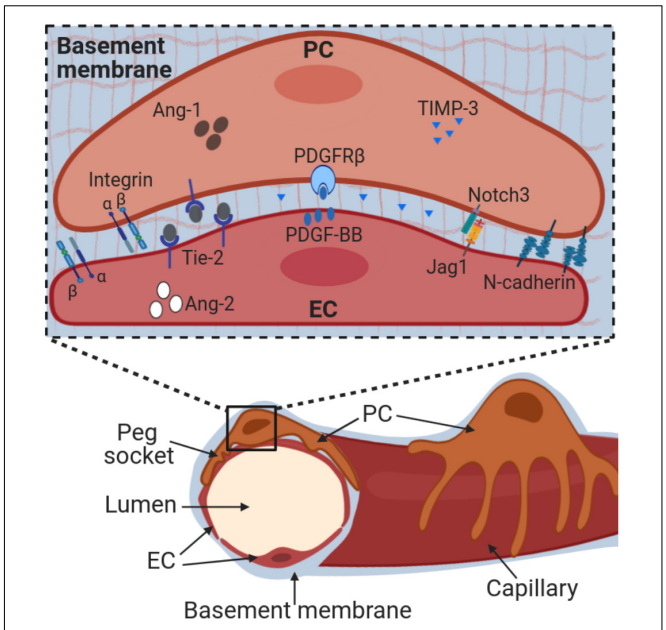

FIGURE 2 | Pericyte-endothelial interactions. Pericytes are embedded into a basement membrane that is shared with endothelial cells and composed primarily by collagen IV and laminins. Adherence to the basement membrane occurs via integrins. Pericyte adherence to endothelial cells at 'peg and socket' connections is mediated by N-cadherins and other junctional proteins. Pericyte-endothelial cell cross-talk occurs through multiple molecular pathways, including PDGF-BB/PDGFRβ, Notch receptors and ligands, Angiopoietin-Tie2 and TIMPs. Alteration of these interactions directly impacts pericyte-endothelial interactions and ultimately vessel stability. Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; EC, endothelial cells; Jag1, Jagged1; PC, pericytes; PDGF-BB, platelet derived growth factor; PDGFRβ, PDGF receptor β; TIMP-3, tissue inhibitor of matrix metalloproteinases-3.

To date, there is no molecular marker known to be unique to pericytes. Thus, a combination of general criteria are commonly used to define pericyte populations, such as perivascular localization, morphology and the expression of one or more recognized molecular markers such as Neural/glial antigen 2 (NG2), PDGFRβ or cluster of differentiation 146 (CD146) (Armulik et al., 2011; Holm et al., 2018). However, these pericyte markers lack specificity. They are expressed to some extent in other cell types (i.e., smooth muscle cells and interstitial cells such as fibro-adipocyte progenitors) and they display variable expression patterns on pericytes across tissues, location within the vascular tree, developmental state and pathological setting (van Dijk et al., 2015; Sacchetti et al., 2016; Kumar et al., 2017).

ACTIVATION, MULTIPOTENCY AND FATE SPECIFICITY OF PERICYTES

Pericytes that reside within an established microvessel network are dominantly quiescent. However, physiological expansion of the capillary network and a variety of pathological conditions trigger their activation and proliferation, which may result in self-renewal or in differentiation. During sprouting angiogenesis, pericytes initially detach from the vessel wall and assist in remodeling the basement membrane to enable endothelial cell sprout formation (Carmeliet and Jain, 2011). Proliferation and migration of pericytes during capillary sprouting ensures pericyte coverage of nascent capillaries. Following sprout formation, pericytes either re-establish pericyte-endothelial cell contacts and return to a quiescent state or they undergo differentiation into smooth muscle cells, resulting in arteriolarization of capillaries (Skalak et al., 1998; Peirce and Skalak, 2003; Volz et al., 2015). Despite the substantial amount of angiogenesis research in past decades, moderately little is known about the molecular pathways that dictate the transition of pericytes between quiescence, proliferation or differentiation.

The differentiation of pericytes into multiple lineages (osteoblasts, chondrocytes and adipocytes) is observed when these cells are cultured (Crisan et al., 2008; Geevarghese and Herman, 2014). This multipotency is analogous to cells belonging to the heterogeneous multipotent stromal population previously referred to as "mesenchymal stem cells" but more commonly described now as "mesenchymal progenitors." In fact, cultured pericytes display broader multipotency compared to mesenchymal progenitors, including differentiation into vascular smooth muscle cells, myofibroblasts as well as parenchymal cells such as skeletal and cardiac myocytes and neuronal cells (Barron et al., 2016; Ji et al., 2016; Birbrair et al., 2017; Siedlecki et al., 2018; Alarcon-Martinez et al., 2019). The broad multipotency reported for pericytes is the basis on which some researchers postulate that pericytes are the predominant source of tissue resident mesenchymal progenitors (Crisan et al., 2008; da Silva Meirelles et al., 2008). These multipotent characteristics also has led to their growing use for cell therapies to promote tissue healing and regeneration.

Multipotency is not a universal property of all pericytes. For instance, pericytes expressing T-Box transcription factor 18 (Tbx18) failed to display *trans*-differentiation capacity *in vivo* in multiple tissues assessed, including skeletal, cardiac and adipose tissues (Guimarães-Camboa et al., 2017). Notably, not all pericytes express Tbx18 and thus it has been proposed that multipotent pericytes are marked by the absence of Tbx18 (Birbrair et al., 2017; Wörsdörfer and Ergün, 2018). As well, there is evidence that pericyte subsets within and across tissues exhibit distinct transcriptomes and differentiation potentials that may correspond with pre-programed commitment to specific lineages (Birbrair et al., 2013; Sacchetti et al., 2016; Yianni and Sharpe, 2018). This idea is supported by recent single cell profiling of brain and lung derived pericytes[1] (He et al., 2018; Vanlandewijck et al., 2018) that revealed a non-overlapping expression profile of lineage specific regulators including Runx2 (osteogenesis), Ppar γ (adipogenesis) and Sox-9 (chondrogenesis). Further, single cell sequencing identified sub-populations of adult brain-derived pericytes that exhibited distinct competencies for induced reprograming to a neuronal lineage (Karow et al., 2018).

Effective strategies to modulate pericyte function and *trans*-differentiation are currently lacking. One significant challenge is that pericyte differentiation potential varies uniquely dependent on their tissue/organ of origin (Chen et al., 2015; Pierantozzi et al., 2016; Yianni and Sharpe, 2018). For example, PDGFRβ$^+$ZFP423$^+$ pericytes within murine adipose tissue readily undergo adipogenesis, thus contributing to adipocyte hyperplasia (Vishvanath et al., 2016). Type-1 and Type-2 pericytes within skeletal muscle, which are classified based on their expression of PDGFRα or Nestin, exhibit exclusive adipogenic or myogenic potential, respectively (Birbrair et al., 2013, 2014). In the brain, pericytes are a potential source of precursors that regenerate neuronal cells (Nakata et al., 2017; Farahani et al., 2019). A recent study revealed a role for epigenetic regulation of pericyte stemness and differentiation potential by demonstrating tissue-specific histone modification patterns within genes that regulate pericyte phenotype, metabolism and fate specificity (Yianni and Sharpe, 2018). Thus, available data support the concept that subsets of pericytes exhibit a degree of pre-programed commitment to specific lineages. Although pericyte differentiation into distinct lineages is achievable *in vitro*, it is still under debate whether pericytes *in vivo* receive the necessary microenvironmental cues, under physiological or pathological conditions, to promote these differentiation events (Wörsdörfer and Ergün, 2018). Overall, mechanisms that regulate the multipotency and tissue-specific pre-programing that contribute to pericyte diversity require further elucidation.

METABOLIC SUPPORT OF PERICYTE STATUS

Recent studies have revealed the relevance of metabolic pathways in controlling the acquisition of different phenotypes of vascular and stem cells, indicating promise of novel metabolism-based therapeutic strategies to manipulate the activation status, functions and the fate decisions of native pericytes and those used for regenerative therapies. The contribution of specific metabolic programs to the regulation of cell-state decisions has been investigated extensively in endothelial cells (De Bock et al., 2013; Schoors et al., 2015; Kim et al., 2017; Diebold et al., 2019), whereas the metabolism of pericytes has undergone very limited analysis to date. Below, we will discuss the current knowledge of pericyte metabolism when these cells are instructed to switch from a quiescent to a proliferative state (also illustrated in **Figure 3**) and in differentiation. Where known, we identify the species and tissue source of pericytes used in each study.

Cells increase the uptake and catabolism of nutrients, particularly glucose, to undergo proliferation. While it was initially believed that the uptake of glucose in pericytes was mediated entirely by the GLUT1 glucose transporter (Mandarino et al., 1994), more recent data demonstrate that transcripts

[1] http://betsholtzlab.org/VascularSingleCells/database.html

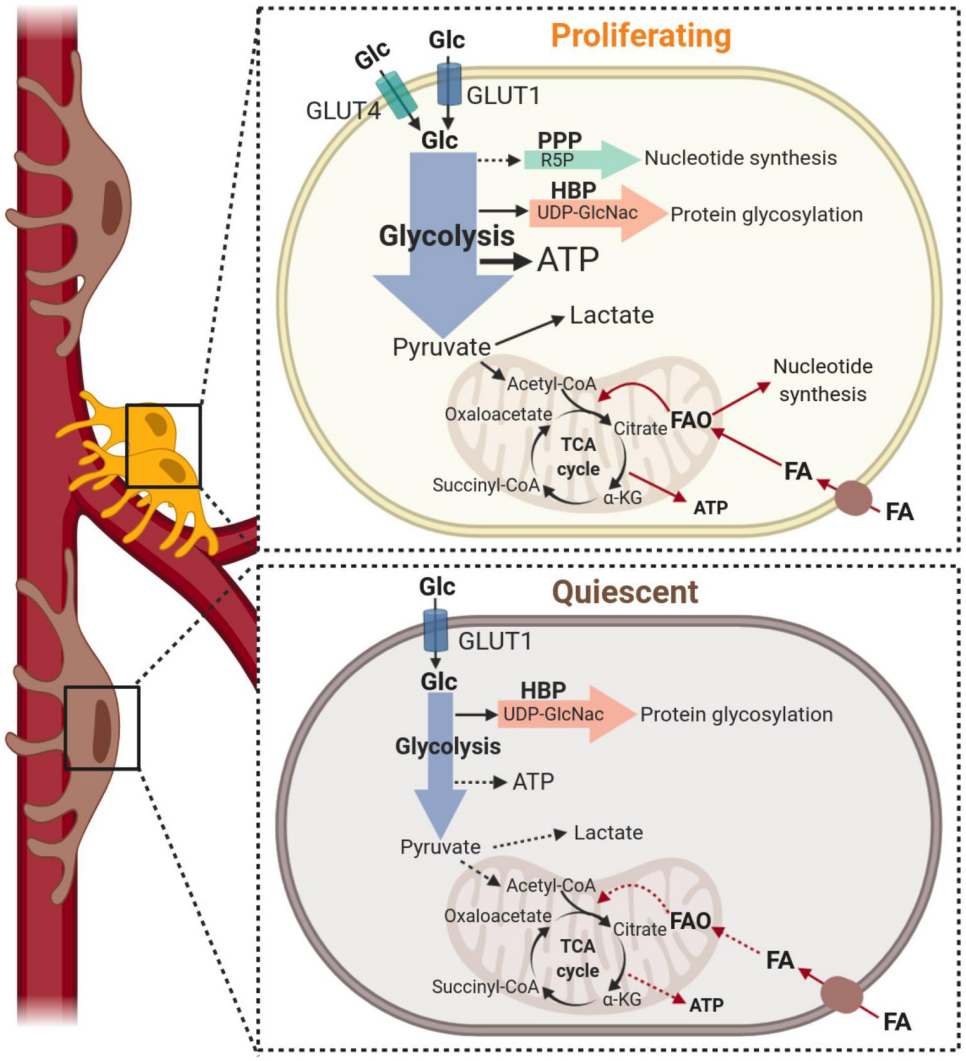

FIGURE 3 | Metabolism of pericytes during proliferation and quiescence. **Top:** Under growth factor stimulation, pericytes increase glucose metabolism (black arrows) to support proliferation. Pericytes increase glucose uptake via GLUT1 and GLUT4 transporters and glucose is metabolized to produce ATP predominantly through glycolysis. This metabolic pathway also provides precursors to protein glycosylation that are essential to the production of capillary basement membrane proteoglycans. Although nucleotide synthesis may be supported by glycolysis via the pentose phosphate pathway (dashed arrow), current data indicate that fatty acid-derived carbons are incorporated into dNTP synthesis in pericytes. Fatty acid oxidation (red arrows) also contributes to the bioenergetic demand during proliferation, generating up to 15% of the total ATP content. **Bottom:** Quiescence is associated with a low metabolic state and downregulation of all metabolic programs. However, the relative contributions of glycolysis, glucose oxidation and fatty acid oxidation (illustrated with dashed arrows) within quiescent pericytes have not been established. α-KG, alpha-Ketoglutarate; ATP, Adenosine triphosphate; FA, fatty acid; FAO, fatty acid oxidation; Glc, glucose; HBP, hexosamine biosynthesis pathway; PPP, pentose phosphate pathway; R5P, Ribose 5-phosphate; TCA, Tricarboxylic acid; UDP-GlcNac, Uridine 5′-diphospho-*N*-acetylglucosamine.

for both GLUT1 and GLUT4 are detectable in human and murine brain pericytes (Castro et al., 2018; He et al., 2018; Vanlandewijck et al., 2018), indicating that glucose uptake can occur via both insulin-dependent and independent pathways. This may explain the greater maximum glucose transport capacity observed in bovine retinal pericytes as compared to endothelial cells (which uptake glucose exclusively through GLUT1) (Mandarino et al., 1994).

Pericyte metabolism has been assessed to the greatest extent in primary cultures of human placental and bovine retinal pericytes (Schoors et al., 2015; Cantelmo et al., 2016). In the proliferative state that exists in cell culture, placental pericytes rely heavily on glycolysis to meet energy demands, with ∼85% of their ATP generation coming from this metabolic pathway (Cantelmo et al., 2016). In fact, only a small decrease in oxygen consumption was observed when proliferating retinal pericytes were treated with oligomycin, an inhibitor of H^+-ATP-synthase, further illustrating their reliance on glycolytic rather than mitochondrial ATP production (Trudeau et al., 2011). Notably, the inhibition of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), which impairs glycolysis, was shown to restrain both proliferation and migration of pericytes

and to enforce pericyte quiescence *in vivo* and *in vitro* (Cantelmo et al., 2016). This indicates that glycolysis is vital to orchestrate the exit from quiescence in these cells.

Besides providing ATP and reducing cofactors to support anabolic reactions, the catabolism of glucose generates precursors to sustain lipid production, the biogenesis of nucleotides and non-essential amino acids and the synthesis of glycolipids, proteoglycans and substrates for protein glycosylation. Since pericytes have greater maximal rate of glucose transport than endothelial cells, it has been suggested that pericytes channel a greater proportion of glycolytic intermediates into the hexosamine biosynthesis pathway (HBP), which generates *N*-acetylglucosamine for *O*- and *N*-glycosylation of proteins and supports the production of capillary basement membrane proteoglycans (Mandarino et al., 1994). The HBP also directly increases cellular biomass (Wellen et al., 2010) and, therefore, diverting glucose into this side branch of glycolysis may play key roles in pericyte self-renewal by sustaining proliferation and also re-establishing the quiescent state of these cells.

Fatty acid oxidation is estimated to contribute to ∼15% of human placental pericyte ATP production (Cantelmo et al., 2016). Pericytes express CD36 and several fatty acid transporter proteins (FATPs) (Winkler et al., 2014), but the relative contribution of these transporters or others in fatty acid uptake remain to be defined. The cultured placental pericytes use fatty acid-derived carbons for the synthesis of deoxyribonucleotides, which implies that fatty acid oxidation supports the proliferation of pericytes (Schoors et al., 2015). While fatty acid oxidation is the predominant metabolic pathway in quiescent endothelial cells (Kalucka et al., 2018), its contribution to the metabolic activity of quiescent pericytes remains to be established. Moreover, the relevance of glutamine metabolism in supporting the proliferative or quiescent states of pericytes is also unknown, although this generally constitutes a major nutrient that is consumed by proliferating cells.

It is well-recognized that the differentiation of multipotent cells is accompanied by shifts in cellular metabolism. Glycolysis is associated with stem cell pluripotency (Folmes et al., 2013; Gu et al., 2016) as it fuels cytosolic acetyl-CoA synthesis, which is essential to maintain histone acetylation required for a multipotent epigenetic state (Moussaieff et al., 2015). Furthermore, glycolysis limits cellular reliance on oxygen and the generation of reactive oxygen species (ROS) (Chen et al., 2008; Suda et al., 2011), which in turn play a critical role in the differentiation of stem cell into various cell populations (adipocytes, osteocytes, chondrocytes, myocytes) (Boopathy et al., 2013; Higuchi et al., 2013; Mateos et al., 2013). Accordingly, stem cell differentiation is usually associated with upregulation of mitochondrial capacity and a substantially higher use of OXPHOS (Funes et al., 2007; Zhang J. et al., 2012; Coller, 2019), which leads to increased levels of ROS. Thus, modulating the glycolytic metabolism of pericytes may not only influence the switch from a quiescent to proliferative state but may also be centrally involved in maintaining the stemness of pericytes. However, no study to date has elucidated the metabolic alterations that accompany the cell-type-specific fate decisions in pericytes.

MOLECULAR PATHWAYS COORDINATING PERICYTE STATE AND METABOLISM

The metabolic programing that supports pericyte status is under the control of signal transduction pathways that in turn are governed by local levels of stabilizing and mitogenic factors. Below, we will discuss molecular mechanisms that enforce quiescence, favor proliferation or induce commitment and differentiation of pericytes by driving changes in cellular metabolism. These pathways are summarized in **Figure 4**.

Quiescence

The quiescent state of pericytes is preserved by cell-intrinsic programs but also by cell-cell interactions. This is achieved in large part by the cell adhesion protein N-cadherin, which mediates heterotypic cell contacts between endothelial cells and pericytes (Gerhardt et al., 2000). Human placental pericyte glycolytic activity is inversely associated with the expression level of N-cadherin (Cantelmo et al., 2016). It has been shown that N-cadherin sequesters β-catenin to the plasma membrane, which may lower β-catenin-dependent promotion of proliferation as was shown to occur in tumor cells (Nadanaka et al., 2018). This effect may be mediated through the regulation of metabolic pathways since c-Myc, a major target gene of the β-catenin/TCF transcription factor complex, is a well-established effector of glycolysis (Li and Wang, 2008). Metabolic state of the cell also influences N-cadherin expression in tumor vessel pericytes, as glycolytic inhibition using the PFKFB3 inhibitor increased N-cadherin protein level and enhanced pericyte adhesion to endothelial cells (Cantelmo et al., 2016).

Besides the regulation imposed by cell adhesion molecules, pericyte quiescence is promoted by Notch signaling. Notch3 on pericytes interacts directly with Jagged-1 on endothelial cells to promote pericyte-endothelial cell adhesion while also inhibiting pericyte proliferation and migration (Liu et al., 2009, 2010; Schulz et al., 2015; Ji et al., 2016). Notch signaling is implicated in the regulation of quiescence and metabolism in many cell types (Iso et al., 2003; Liu et al., 2010; Koch et al., 2013), through diverse actions that include downregulated expression of glycolytic enzymes such as PFKFB3 (De Bock et al., 2013; Bayin et al., 2017). Thus, pericyte quiescence enforced by interactions with endothelial cells via Notch may involve metabolic reprograming driven by this signal pathway.

A similar enforcement of quiescence may be promoted by the Forkhead box O (FOXO) transcription factors. This family of transcriptional regulators maintains cellular quiescence in adult stem cells through repression of metabolic pathway genes and cell cycle activators (Liang and Ghaffari, 2018). FOXO3A was shown to be the predominant FOXO family member produced by pericytes (Teichert et al., 2017). In muscle satellite cells, FOXO3A promotes quiescence, in part through enhancing the expression of Notch1 and 3 (Gopinath et al., 2014; Yue et al., 2017), suggesting a similar function may occur in pericytes. However, elevated levels of nuclear FOXO3A in pericytes (due to Ang2-dependent decrease in Tie-2 activation) was associated

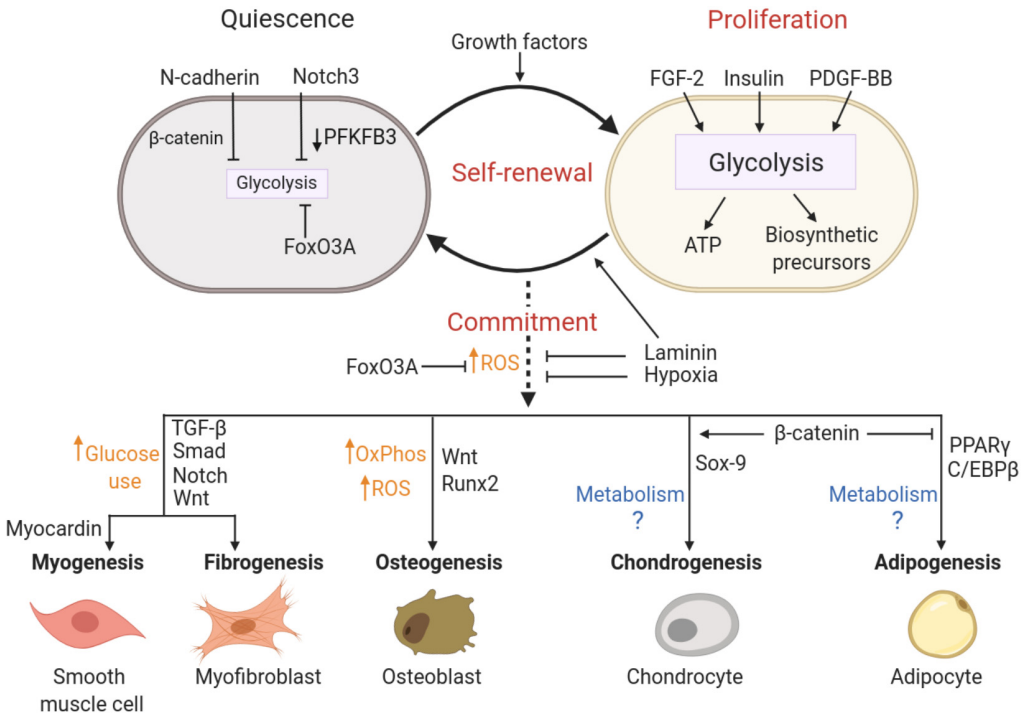

FIGURE 4 | Putative molecular pathways coordinating pericyte state and metabolism. Activation, multipotency and fate specificity of pericytes are governed by different molecular pathways that may involve coordinated metabolic shifts. N-cadherin, Notch3 and FoxO3A signaling are associated with the maintenance of a quiescent state in pericytes by limiting cellular metabolism (mainly glycolysis), whereas pericyte proliferation is stimulated by growth factors and supported by a concomitant increase in glycolytic activity. Self-renewal is achieved by cycles of proliferation followed by the return to a quiescent state, which may be promoted by cell adhesion to laminin. Commitment and *trans*-differentiation may be triggered by an increase in oxidative stress, while unique lineage specifications are determined by a combination of distinct lineage drivers and metabolic reprograming. Conversely, FoxO3A signaling, antioxidant agents, laminin and hypoxia can repress commitment and *trans*-differentiation events. See text for details on the relevant studies. ATP, Adenosine triphosphate; FGF-2, Fibroblast growth factor; FoxO3A, Forkhead BoxO3A; OxPhos, oxidative phosphorylation; PDGF-BB, platelet derived growth factor; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; ROS, reactive oxygen species.

with a phenotype shift in pericytes that promoted migration and overall, increased capillary growth (Teichert et al., 2017). Thus, although the function of FOXO proteins in governing quiescence via suppression of metabolic activity has been well-established in many other cell types including endothelial cells, these functions require further investigation in pericytes.

Proliferation

Growth factors that stimulate pericyte proliferation increase the uptake and utilization of available nutrients. For example, insulin promotes glucose uptake and induces glucose-mediated proliferation of bovine retinal pericytes (King et al., 1983). PDGF-BB is another major growth factor for pericytes. Excessive PDGFR activation is responsible for driving the proliferation of pericytes in kidney disease (Chen et al., 2011). Since this growth factor is a strong driver of glycolysis in cancer cells and smooth muscle cells (including increased expression of glucose transporters) (Wang et al., 1999; Ran et al., 2013; Xiao et al., 2017) it is very likely that PDGFRβ signaling enhances glycolysis to meet the energy and biomass demands of proliferating pericytes. Fibroblast growth factor (FGF)-2 also exerts mitogenic influences on pulmonary artery-associated pericytes and can provoke excessive pericyte proliferation in pathological contexts (Ricard et al., 2014). These effects may be mediated by metabolic reprograming, as it has been shown that FGF-2 can coordinate endothelial cell proliferation and migration by increasing the glycolytic capacity of these cells (Yu et al., 2017).

Commitment and Differentiation

Generally, "stemness' of pericytes is retained through cycles of proliferation. Maintenance of the stemness and multipotent state of pericytes is highly dependent on interactions with the basement membrane protein laminin. In the absence of this protein, brain pericytes develop the properties of a contractile cell (Yao et al., 2014). Stemness of pericytes is also tuned by other microenvironmental cues and transcription factors that are established regulators of genes encoding metabolic pathway components. Thus, changes in metabolism are expected to accompany and to facilitate differentiation of pericytes.

For example, it has been reported that hypoxia represses the differentiation of $CD146^{+}$ human umbilical cord perivascular cells (presumptive pericytes), allowing them to proliferate and maintain their multipotency (Tsang et al., 2013). Since hypoxia upregulates glycolytic activity, these observations imply that a

metabolic switch to oxidative metabolism could provoke lineage commitment of pericytes. Accordingly, signaling pathways involved in the regulation of antioxidant defenses, as well as antioxidant compounds, may directly impact the differentiation of pericytes. For example, besides regulating metabolic pathways, FOXO proteins protect cells against oxidative stress via transcriptional upregulation of antioxidant defense proteins (superoxide dismutase, catalase) (Carter and Brunet, 2007). Depletion of FOXO3A results in limited self-renewal and greater commitment of neural stem cells, hematopoietic stem cells, satellite cells (Miyamoto et al., 2007; Tothova et al., 2007; Paik et al., 2009; Gopinath et al., 2014). Antioxidant treatment was shown to improve self-renewal in FoxO3$^{-/-}$ hematopoietic stem cells and in neural precursors, highlighting a critical role for the regulation of oxidant stress in determining commitment (Tothova et al., 2007; Paik et al., 2009). However, the implementation of metabolic switching is lineage-specific (Cliff et al., 2017) and cannot be generalized across all stem cell populations. Importantly, fate decisions also rely on the concomitant upregulation of specific lineage drivers that guide cell-type specific transcriptional programs. Some knowledge of these signal pathways has been established for pericyte differentiation, which we summarize below for three of the most-studied pericyte fates.

Myofibroblast/Smooth Muscle Cell Differentiation

The transcriptional regulator myocardin is a major lineage driver for smooth muscle cell differentiation (Li et al., 2003; Long et al., 2008; Alexander and Owens, 2012). In turn, TGF-β -Smad/Notch signaling augment pericyte differentiation into smooth muscle cells (Meyrick et al., 1981; Chambers et al., 2003; Alexander and Owens, 2012; Volz et al., 2015). Similarly, the TGFβ, Notch and Wnt signaling pathways contribute to pericyte conversion to myofibroblasts (Kirton et al., 2006; Cheng et al., 2008; Andersson-Sjöland et al., 2016; Aimaiti et al., 2019). Wnt signaling is robustly activated in lung and kidney pericytes following tissue injury and correlates temporally with the onset of fibrosis (Kirton et al., 2007; Andersson-Sjöland et al., 2016). Given that TGF-β-dependent differentiation of fibroblasts to myofibroblasts was associated with increased glucose uptake and glutaminolysis (Bernard et al., 2015, 2018; Andrianifahanana et al., 2016), it is tempting to speculate that this also applies to the conversion of pericytes to myofibroblasts, although this remains to be demonstrated experimentally. In addition, epigenetic modulation of histone marks may 'lock in' myofibroblast differentiation, as seen in pericytes that exhibited both repressive marks on PPARγ and activating marks on fibrotic genes (Mann et al., 2010; Perugorria et al., 2012; Zeybel et al., 2017).

Osteo/Chondrogenic Differentiation

Expression of the transcription factor Runx2 (Osf2/Cbf1a) is a hallmark of pericytes that are poised to undergo osteogenic or chondrogenic differentiation (Doherty and Canfield, 1999; Farrington-Rock et al., 2004). Osteo-inductive factors such as bone morphogenetic proteins (BMP) increase Runx2, which then interacts with osteoblast-specific *cis*-acting elements to promote the expression of osteogenic genes (Ducy et al., 1997; Zebboudj et al., 2003; Gao et al., 2013). During chondrogenesis, Runx2 is downregulated while Sox-9, the master regulator of chondrogenesis, is upregulated (Bi et al., 1999; Akiyama et al., 2002). Interplay between Sox-9 and β-catenin drives chondrogenesis while suppressing other lineages, such as adipogenesis (Akiyama et al., 2004; Kirton et al., 2007).

Metabolic state may contribute to determining pericyte fate decision toward osteogenesis. Runx2 expression itself is glucose-dependent (Wei et al., 2015) and its gene regulatory influences favor glycolysis over oxidative respiration (Choe et al., 2015). However, osteogenesis in mesenchymal cells involves a progressive switch toward oxidative metabolism, which is necessary for complete differentiation (Chen et al., 2019). In line with this, pericyte osteogenic differentiation was markedly reduced under hypoxic conditions, suggesting a reliance on oxidative phosphorylation and/or oxidant stress to elicit complete osteogenic differentiation (Byon et al., 2008; Tsang et al., 2013). FOXO1, which represses glycolysis, plays a significant role in restraining osteoblast differentiation. It does this through lowering oxidant stress as well as by interfering with Wnt-dependent transcriptional programs (Dowell et al., 2003; Rached et al., 2010; Chen et al., 2019). The influence of FOXO transcriptional regulators in pericyte lineage differentiation remains to be established.

Adipogenesis

The peroxisome proliferator activated receptor gamma (PPARγ) transcription factor is recognized as a master regulator of pre-adipocyte differentiation and metabolism and its expression is strongly stimulated by exposing cultured pericytes to adipogenic factors (Farrington-Rock et al., 2004; Lefterova et al., 2014). C/EBPβ is proposed to play a key transcriptional priming role through binding to closed chromatin 'hotspots' with which PPARγ subsequently interacts (Lefterova et al., 2014). The majority of pericytes in lung and brain express C/EBPβ, while only a small percentage of these cells express PPARγ (He et al., 2018; Vanlandewijck et al., 2018), which appears consistent with the concept that a portion of quiescent pericytes are poised to respond to adipogenic cues. In mouse skeletal muscle, Type-1 (PDGFRα^{+}) pericytes exhibit adipogenic potential and were shown to contribute to the generation of adipocytes when transplanted into glycerol-injured muscles (Birbrair et al., 2013). Further, pericytes in human and murine skeletal muscle were reported to express leptin mRNA, the levels of which increased under obesogenic conditions (Nwadozi et al., 2019). PPARγ also may be expressed in activated but non-adipogenic pericytes (i.e., proliferating pericytes *in vitro*) and several studies point to additional roles of PPARγ beyond driving adipogenesis. In hematopoietic stem cells, PPARγ suppresses self-renewal while promoting cell differentiation, in large part through strong suppression of glycolysis (Guo et al., 2018). However, hepatic stellate cells (specialized pericytes within the liver) rely on PPARγ signaling to promote fatty acid oxidation, which serves to both maintain their quiescence

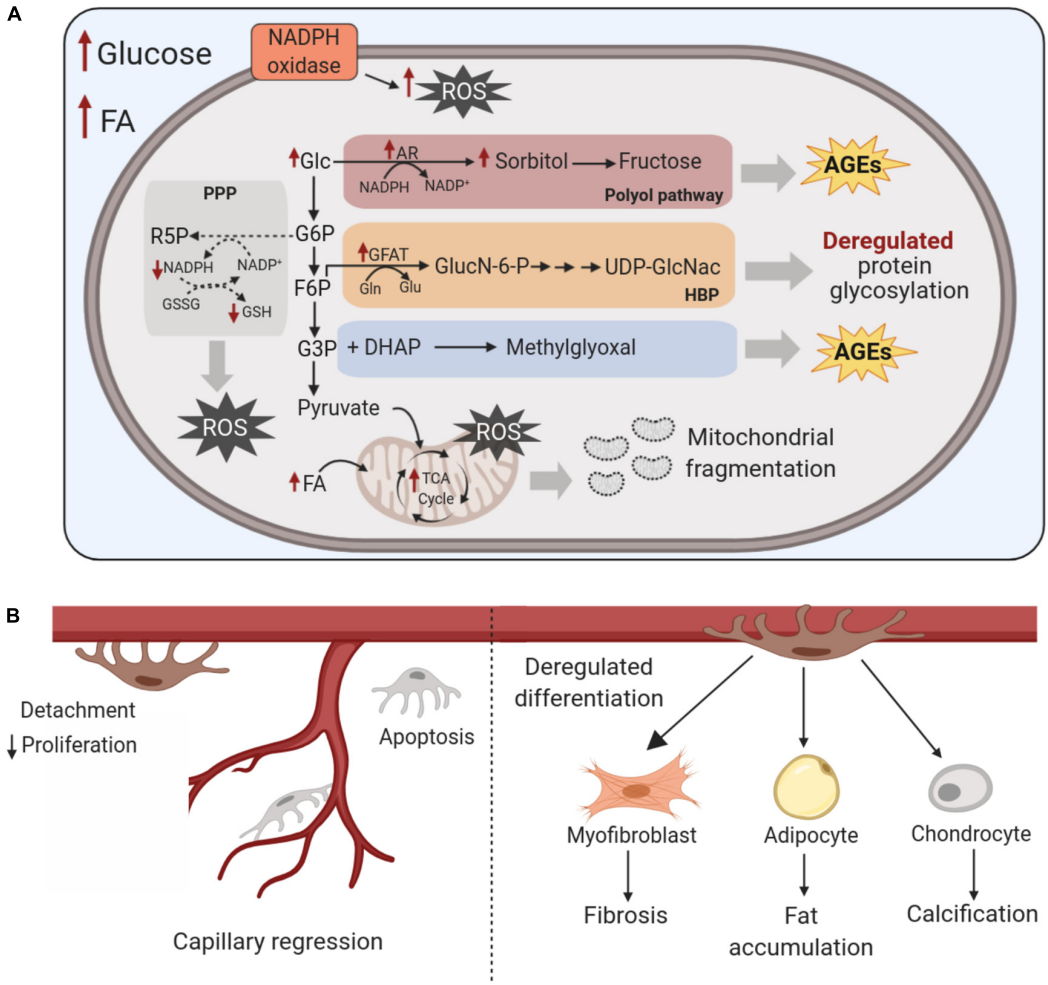

FIGURE 5 | Effects of chronic nutrient excess on pericyte metabolism and *trans*-differentiation. **(A)** Chronic exposure to high glucose levels of pericytes disrupts glucose metabolism and increases diversion of glycolytic intermediates into the polyol and hexosamine biosynthesis pathways, elevates NAD(P)H oxidase activity and increases cellular oxidative stress; This gives rise to augmented ROS levels with the exhaustion of antioxidant defenses, the formation of harmful AGEs, enhanced glycosylation of proteins and fragmentation of mitochondria. Increased fatty acid levels also exacerbate cellular oxidative stress, with concomitant disruptions to metabolic pathways including mitochondrial function. **(B)** These disruptions in the metabolism of pericytes increase their detachment from the vessel wall, lower their proliferation and increase apoptosis, ultimately leading to capillary regression. These pathological metabolic shifts also impact pericyte multipotency by promoting their *trans*-differentiation to myofibroblasts, adipocytes and chondrocytes, which contribute to interstitial fibrosis, fat accumulation and vessel calcification, respectively. AR, aldose reductase; AGEs, advanced glycation end products; DHAP, dihydroxyacetone phosphate; F6P, fructose-6-phosphate; FA, fatty acids; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; GFAT, glutamine fructose-6-phosphate amidotransferase; Glc, glucose; GSSG, glutathione disulfide; GSH, glutathione; HBP, hexosamine biosynthesis pathway; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species, TCA, Tricarboxylic acid; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine.

and prevent myofibroblast differentiation (She et al., 2005; Zhu et al., 2010).

EFFECTS OF NUTRIENT EXCESS ON PERICYTE METABOLISM AND DIFFERENTIATION

Survival and Activation

Nutrient availability can impact pericyte state in several ways. Hyperglycemia acutely increases glucose metabolism (Giacco and Brownlee, 2010). In the short term, increased levels of glucose enhance glycolysis and glucose oxidation, thus supporting the activation of cellular processes associated with proliferation and migration in cultured rat retinal pericytes (Shah et al., 2013). However, glucose oxidation increases ROS production. In turn, ROS-mediated inhibition of glycolytic enzymes (i.e., GAPDH, PFK) causes the glycolytic intermediates to stall and divert through side-branches of glycolysis (PPP, hexosamine, polyol), thus increasing synthesis of advanced glycation end-products (AGE) (Giacco and Brownlee, 2010; Tang et al., 2012; Mullarky and Cantley, 2015). Increased flux through the polyol pathway consumes NADPH in the process of aldose

reductase generation of sorbitol. The continual depletion of NADPH may impair the regeneration of reduced glutathione (GSH), which lowers overall cellular oxidant buffering capacity. High glucose-induced oxidative stress also is sustained by elevated cytosolic NAD(P)H oxidase activity (Giacco and Brownlee, 2010; Tang et al., 2012). These disruptions are illustrated in **Figure 5**. In pericytes, the associated increase in cellular ROS that accompanies these metabolic disturbances leads to decreased proliferation, fragmentation of mitochondria (with an associated decline in oxygen consumption) and increased apoptosis (Manea et al., 2004, 2005; Mustapha et al., 2010; Trudeau et al., 2011). In rodent retinal and cerebral cortex pericytes, increased aldose reductase-dependent production of sorbitol (Kennedy et al., 1983; Berrone et al., 2006) enhances cellular osmotic stress and endoplasmic reticulum- stress. This may contribute to increased pericyte apoptosis, as was documented in porcine retina and rat lens explants (Takamura et al., 2008; Zhang P. et al., 2012). In fact, NADPH oxidase production of ROS, rather than mitochondria-derived ROS, was found to be instrumental in driving glucose-induced apoptosis of retinal pericytes (Mustapha et al., 2010).

Hyperglycemia also elevates flux through the hexosamine pathway in pericytes by increasing production of the rate-limiting enzyme glutamine:fructose-6-phosphate aminotransferase (GFAT) (Semba et al., 2014). The resultant increase in *O*-GlcNac-modification of proteins, including insulin receptor and PDGFRβ, interferes with mouse retinal pericyte migration (Gurel et al., 2013). FOXO1 may exert a pro-apoptotic function in pericytes in diabetic retinopathy based on the observation that cultured bovine retinal pericytes stimulated with TNFα or AGE products exhibited FoxO1-dependent apoptosis and that FOXO1 RNA interference lowered retinal pericyte apoptosis in diabetic rats (Behl et al., 2009; Alikhani et al., 2010). This suggests that increased conversion of glycolytic intermediary glyceraldehyde 3 phosphate to AGEs can influence a transcription factor that itself is a cornerstone in the regulation of cell survival, metabolism and proliferation/differentiation. Within the retina, prolonged hyperglycemia ultimately is associated with pericyte migration away from the capillary or pericyte 'dropout,' which decreases pericyte coverage of the capillary structure, compromising capillary integrity and provoking capillary regression (Pfister et al., 2008; Corliss et al., 2019; Hayes, 2019).

Prolonged exposure to increased levels of fatty acids causes insulin resistance and lipid toxicity in many cell types (Yazıcı and Sezer, 2017). To date, there has been limited investigation of these effects in pericytes. One report indicated that exposure of cultured bovine retinal pericytes to high doses of the fatty acid palmitate increased NAD(P)H oxidase–mediated oxidative stress, with concomitant disruptions to metabolic pathways including mitochondrial function (Cacicedo et al., 2005). Palmitate treatment of cultured retinal pericytes also appears to exacerbate the anti-proliferative, pro-apoptotic effects of AGEs on pericytes, indicating additive effects of these metabolic perturbations (Yamagishi et al., 2002; Cacicedo et al., 2005; Ding et al., 2014). These studies imply that high fatty acids will contribute to oxidative stress-induced damage to pericytes.

Differentiation Potential

Exposure to excess glucose influence pericyte multipotency in several ways. Neural pericytes cultured in high glucose have increased production of AGEs and up-regulated expression of pro-fibrotic TGF-β, favoring myofibroblast differentiation (Shimizu et al., 2011). Notably, hyperglycemia also increases core fucosylation of the TGF receptors-type I and II (TGFβRI and TGFβRII), which enhances its activation of downstream signals, resulting in hypersensitivity to TGFβ ligands (Sun et al., 2017; Wang et al., 2017). This enhanced core fucosylation of TGFβRI is a key determinant of mesangial pericyte myofibroblast *trans*-differentiation and interstitial fibrosis in mouse models of kidney disease (Lin et al., 2011; Shen et al., 2013; Wang et al., 2017). Diabetes also increases the propensity of muscle- and bone-marrow-derived pericytes to differentiate into adipocytes *in vitro*, while limiting their capacity to promote myogenesis or angiogenesis (Vono et al., 2016; Ferland-McCollough et al., 2018; Mangialardi et al., 2019). These effects were proposed to be linked to elevated ROS. Increased cellular ROS (via NADPH oxidase) also contributes to myofibroblast and osteogenic differentiation in smooth muscle cells and pericytes in diabetic vessels (Byon et al., 2008; Barnes and Gorin, 2011; Raaz et al., 2015). Fatty acid exposure may provoke a similar transition of mesangial pericytes to myofibroblasts by increasing TGF-β secretion (Mishra and Simonson, 2008). In sum, these studies indicate the potential for nutrient imbalances that disturb intracellular metabolism and elevate oxidative stress to trigger inappropriate lineage commitment and *trans*-differentiation of pericytes.

CLINICAL RELEVANCE

Correcting the Functions of Native Pericytes

Establishing the molecular and metabolic switches that control pericyte behavior has the potential to direct the development of new therapeutic opportunities for diseases involving pericyte dysfunction. Targeting pathways that regulate pericyte survival/apoptosis are under exploration as potential therapies in diabetic retinopathy. As previously described, the accumulation of ROS and AGEs induces pericyte apoptosis, ultimately contributing to the capillary loss and fluid leakage that occur in diabetic vasculopathies (Armulik et al., 2011; Ferland-McCollough et al., 2017; Hayes, 2019). Thus, identification of enzyme modifiers that effectively limit glucose uptake or divert the flux of glucose away from "damage-inducing" metabolic pathways (i.e., polyol; AGE-producing) may protect pericytes from high glucose-induced damage and apoptosis by minimizing ROS and AGE accumulation.

On the opposite spectrum, excessive pericyte proliferation promotes disease progression in pulmonary artery hypertension (PAH), hypoxia-induced retinopathies (characterized by excessive neovascularization), and kidney and liver fibrosis (Rabinovitch, 2012; Ferland-McCollough et al., 2017; Dubrac et al., 2018). Studies using pericytes derived from PAH patients indicate that aberrant pericyte proliferation and migration

is fueled by glycolysis, as these cells had elevated glycolytic capacity and lower mitochondrial activity (Rabinovitch, 2012; Yuan et al., 2016). Thus, repression of pericyte glycolytic activity could provide a useful therapeutic approach in this disease. Recently, siRNA-mediated knock-down of PDK4 (which suppresses mitochondrial activity in favor of glycolysis by inhibition of pyruvate dehydrogenase) was shown to increase PAH-derived pericyte mitochondrial function, lower their proliferation and improve their interactions with endothelial cells (Yuan et al., 2016), demonstrating promise of this conceptual approach. Similar strategies may be useful in regulating pericyte proliferation in chronic kidney disease or retinopathies, although these approaches remain to be tested.

Excessive differentiation of pericytes into smooth muscle cells also occurs in PAH and contributes to the pathogenesis and severity of the disease (Rabinovitch, 2012; Ricard et al., 2014). In general, the pathological differentiation of pericytes into myofibroblasts is a hallmark of fibrotic kidney and liver diseases (Humphreys, 2018; Parola and Pinzani, 2019). Notably, lineage tracing also demonstrated that *trans*-differentiation of PDGFRβ+ pericytes into fibroblasts occurs within a tumor environment, which causes alterations to capillary structure and interstitial matrix components that ultimately enhance tumor invasion and metastatic potential (Hosaka et al., 2016). *In vivo* pathological activation of osteogenesis/chondrogenesis within pericytes can lead to ectopic ossification (Farrington-Rock et al., 2004; Kirton et al., 2007). This also contributes to large vessel calcification in atherosclerosis (Canfield et al., 2000; Tintut et al., 2003). While efforts to date have focused almost exclusively on disrupting the initiation of ligand-activated receptor signaling (i.e., disproportionate TGF-β or PDGF-BB signaling that provokes smooth muscle or myofibroblast differentiation in these diseases) (Humphreys et al., 2010; Duffield et al., 2013; Hung et al., 2013; Hosaka et al., 2016), it is plausible that coordinated manipulation of metabolic pathways has the potential to achieve more effective repression of these pathological forms of pericyte differentiation. However, more discrete definition of the metabolic switches associated with specific lineage differentiation will be required to take advantage of these opportunities.

Improving the Use of Pericytes as a Tool for Regenerative Medicine

The capacity to fine-tune pericyte metabolism may provide mechanisms to optimize the function of pericytes used in regenerative medicine, through enhancing the survival and participation of these cells despite the unfavorable conditions found within the host ischemic or injured tissues. Pericytes, and related cell populations that have been referred to as 'pericyte-like adventitial cells,' 'fibro-adipocyte progenitors' and 'mesenchymal stromal cells' have been tested in various types of cell-based therapies and as tools for tissue bioengineering (Zamora et al., 2013; König et al., 2016; Avolio et al., 2017; Murray et al., 2017). To date, pericyte injections have been utilized in pre-clinical studies to promote muscle repair, recovery from muscle or cardiac ischemia, bone/skin injury, diabetic retinopathy (including the clinical trial RETICELL) (Dar et al., 2012; Mendel et al., 2013; Siqueira et al., 2015; Thomas et al., 2017; Alvino et al., 2018; Munroe et al., 2019). The expectation is that pericyte delivery will improve outcomes by one or more actions: secretion of factors that support functions of native cells; replacement of existing 'damaged' or 'dead' pericytes; differentiation into functional vascular or muscle cells (Caplan, 2009; Cathery et al., 2018).

The value of pericytes as a tool for cellular therapy is highly dependent on their capacity to survive and function within the environment to which they are delivered. The relative reliance of proliferating pericytes on glycolytic vs. oxidative metabolism may help to support pericyte survival when they are injected into ischemic cardiac or skeletal muscle (Cathery et al., 2018). However, conditions within the host tissue microenvironment (i.e., oxidative stress, hyperglycemia, excess TGF-β or PDGF-BB) may impede appropriate activation, proliferation, differentiation and function of healthy donor pericytes, as has been demonstrated within the ischemic limbs of db/db mice (Hayes et al., 2018). Further, the pericyte secretome will vary between pro and anti-inflammatory dependent on the local environment/stimulus (Chen et al., 2013; Gaceb et al., 2018). For example, inflammatory stimuli and AGE will enhance secretion of pro-fibrotic TGF-β, and this could influence the extent to which transplanted pericytes would evoke a fibrotic response in the host tissue.

Moreover, the success of pericyte-based therapies may be limited by the patient's pre-existing disease. Although autologous transplantation is preferable to use, these cells may be deficient in number and/or function (Fadini et al., 2017, 2019; Teng and Huang, 2019). For example, diabetic individuals have fewer skeletal muscle and bone-marrow derived pericytes than age-matched non-diabetics (Tilton et al., 1981; Mangialardi et al., 2019). Additionally, the 'stemness' of these cells may be compromised. Muscle-derived pericytes from diabetic patients exhibited greater adipogenic potential and reduced capacity to promote angiogenesis, which was related to enhanced oxidative stress-dependent damage (Vono et al., 2016). Similarly, bone marrow-derived pericytes from type 2 diabetic patients displayed less Akt activation, which associated with lower survival and proliferation (Mangialardi et al., 2019). Ideally, manipulation of these cells *in vitro* could be used to correct these deficiencies and to condition the cells for more effective functions once transplanted.

CURRENT CHALLENGES AND FUTURE DIRECTIONS

An ongoing challenge in the field of pericyte biology is the inherent heterogeneity of this cell population. This has led to substantial study to study inconsistencies in the classification of pericytes, which in turn confounds the interpretation of research on pericyte functions. Thus, a comprehensive characterization of pericyte subtypes and validation of appropriate identifying markers for these populations are crucial next steps for advancing our knowledge of pericyte functions and differentiation potential.

Single cell sequencing offers tremendous promise in establishing global gene expression signatures and novel molecular identifiers of pericytes, as well as enabling the discovery of subtype, tissue and disease-specific patterns of pericyte gene expression. With this knowledge, it will be possible to evaluate the extent to which pericyte subpopulations are pre-programed with discrete and tissue-specific lineage potentials and to identify which pericyte subgroups have the most favorable characteristics for use in regenerative cell therapies.

Emerging evidence indicates that the regulation of metabolic pathways may offer the potential to manipulate the cell-state and fate decisions of pericytes, which could enable the development of novel strategies to control pericytes contributions to pathological conditions and their use in regenerative medicine. However, knowledge of pericyte metabolism is rudimentary to date and the overall understanding of how pericytes integrate signal transduction, metabolic programing and cell state/fate decisions currently lags far behind the state of knowledge of these relationships in other vascular and stem cells. Therefore, many fundamental questions remain to be answered. Considering the extent of pericyte heterogeneity *in vivo*, it will be important to determine if there is variability in metabolic programing that coincide with specific gene expression signatures. With the evolving advancements in metabolomics technologies, there is now unprecedented opportunity to map the metabolome of pericytes even to the single cell level and to identify the metabolic pathways that are integral to transitions in pericyte state and lineage commitment. Lastly, it is crucial to consider the impact of various pericyte isolation and culturing conditions on the signaling and metabolic pathways that regulate activation and differentiation potential, since this could greatly influence the reproducibility and efficacy of cell-based therapeutic treatments. Addressing these avenues of research not only will provide valuable insight into the contribution of specific metabolic pathways in shaping pericyte phenotype but it may also reveal new tools for the optimization of pericyte characteristics in pathological conditions and when used in cell therapies.

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

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REVIEW
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Lipid Mediated Regulation of Adult Stem Cell Behavior

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Adult stem cells constitute an important reservoir of self-renewing progenitor cells and are crucial for maintaining tissue and organ homeostasis. The capacity of stem cells to self-renew or differentiate can be attributed to distinct metabolic states, and it is now becoming apparent that metabolism plays instructive roles in stem cell fate decisions. Lipids are an extremely vast class of biomolecules, with essential roles in energy homeostasis, membrane structure and signaling. Imbalances in lipid homeostasis can result in lipotoxicity, cell death and diseases, such as cardiovascular disease, insulin resistance and diabetes, autoimmune disorders and cancer. Therefore, understanding how lipid metabolism affects stem cell behavior offers promising perspectives for the development of novel approaches to control stem cell behavior either *in vitro* or in patients, by modulating lipid metabolic pathways pharmacologically or through diet. In this review, we will first address how recent progress in lipidomics has created new opportunities to uncover stem-cell specific lipidomes. In addition, genetic and/or pharmacological modulation of lipid metabolism have shown the involvement of specific pathways, such as fatty acid oxidation (FAO), in regulating adult stem cell behavior. We will describe and compare findings obtained in multiple stem cell models in order to provide an assessment on whether unique lipid metabolic pathways may commonly regulate stem cell behavior. We will then review characterized and potential molecular mechanisms through which lipids can affect stem cell-specific properties, including self-renewal, differentiation potential or interaction with the niche. Finally, we aim to summarize the current knowledge of how alterations in lipid homeostasis that occur as a consequence of changes in diet, aging or disease can impact stem cells and, consequently, tissue homeostasis and repair.

Keywords: lipid, metabolism, fatty acids, stem cells, niche

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Abbreviations: ACC, Acetyl-CoA carboxylase; AGPAT, 1 -acylglycerol-3-phosphate O-acyltransferase; AMPK, AMP activated kinase; Cer, Ceramide; CPT1, Carnitine palmitoyltransferase I; CSC, Cancer stem cell; DHCer, Dihydroceramides; EB, Embryoid body; ETC, Electron transport chain; FA, Fatty acid; FAO, Fatty acid oxidation; FASN, Fatty acid synthase; FSC, Follicle stem cell; GSC, Germline stem cell; hESC, Human embryonic stem cell; HFD, High fat diet; HSC, Hematopoietic stem cell; iPLA1, Intracellular phospholipase A1; iPSC, Induced pluripotent stem cell; ISC, Intestinal stem cell; LD, Lipid droplet; Lpcat3, Lysophosphatidylcholine acyltransferase 3; mESC, Mouse embryonic stem cell; MS, Mass spectrometry; MSC, Mesenchymal stem cell; NSC, Neural stem cell; NSPC, Neural stem/progenitor cell; PC, Phosphatidylcholine; PDK1, PI-dependent kinase 1; PE, Phosphatidylethanolamine; PI, Phosphoinositides; PI3K, PI3 kinase; PI4KIIIa, PI4 kinase IIIa; PIP2, Phosphatidylinositol-4,5-bisphosphate; PIP3, Phosphatidylinositol-3,4,5-trisphosphate; PPAR, Peroxisome proliferator-activated receptor; PPRE, Peroxisome proliferator hormone response element; PS, Phosphatidylserine; PSC, Pluripotent stem cell; PUFA, Polyunsaturated fatty acid; RXR, Retinoid X receptor; S1P, Sphingosine-1-phosphate; SREBP, Sterol regulatory elements binding protein; SVZ, Subventricular zone; TAG, Triacylglycerol; TCA, Tricarboxylic acid; TOR, Target of Rapamycin; UPLC, Ultra performance liquid chromatography.

INTRODUCTION

Adult stem cells, also referred to as "tissue" stem cells, are relatively undifferentiated cells that have the ability to self-renew and produce differentiating progeny. Consequently, they are crucial for maintaining tissues that must be constantly replaced and also serve as a reservoir of cells that can be used to repair damaged tissues after wounding or exposure to environmental insults. Stem cell behavior is influenced by the integration of cell-intrinsic factors, extrinsic cues provided by the local microenvironment (or "niche") and systemic factors (Voog and Jones, 2010). Furthermore, the capacity of stem cells to self-renew or differentiate can be attributed to distinct metabolic states and it is now becoming apparent that metabolism plays instructive roles in stem cell fate decisions (Ito and Suda, 2014; Chandel et al., 2016; Folmes and Terzic, 2016; Mana et al., 2017; Shyh-Chang and Ng, 2017).

Lipids can be broadly defined as any organic compound insoluble in water but soluble in organic solvent. Therefore, lipids constitute an extremely vast class of biomolecules, which is reflected in the high diversity of roles they assume in a cell (Fahy et al., 2011) (**Table 1**). Glycerophospholipids, in particular phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), as well as sphingolipids and cholesterol, primarily serve as building blocks for membranes and organelles (van Meer and de Kroon, 2011). Fatty acids (FAs) provide a source of energy, as they can be stored in the form of triglycerides (TAG) in lipid droplets (LDs). When FAs are needed, TAG undergoes lipolysis, and the resulting FAs can be broken down through β-oxidation in mitochondria or in peroxisomes in the case of very long chain FAs, to produce energy under the form of ATP and reducing power (Houten and Wanders, 2010). In addition, lipids can act as signaling molecules through specific lipid-protein interactions. Signaling lipids, acting as extracellular ligands or intracellular second messengers, participate in the regulation of various biological processes, including cell proliferation, cell death, cell migration, gene expression or immune reactions such as inflammation. An increased appreciation of the involvement of lipids in metabolic diseases such as obesity, atherosclerosis, stroke, hypertension, diabetes and cancers (Wymann and Schneiter, 2008), together with technological advances in mass spectrometry and computational methods and global efforts like the LIPID MAPS (Metabolites And Pathways Strategy) consortium (Fahy et al., 2005), have pushed lipid biology to the forefront of metabolism research.

In this review, we intend to summarize growing evidence implicating lipids in the regulation of stem cell behavior. A better understanding of the mechanisms by which lipids act will contribute to improve the appreciation of how imbalances in lipid homeostasis can cause or contribute to alterations in tissue homeostasis and regeneration. In addition, a better appreciation for the roles that lipids play in stem cells will facilitate the development of novel approaches to enhance stem cell expansion and differentiation *in vitro* for use in regenerative medicine. Taken together, this knowledge may ultimately allow for the control of stem cell behavior in patients, by modulating lipid metabolic pathways pharmacologically or through diet.

LIPIDOMICS AND LIPIDS ENRICHED IN STEM CELLS

The lipidome is the complete set of lipids present within a cell, a tissue or an organism. It is a subset of the metabolome, which also includes the three other major classes of biological molecules: amino acids, sugars and nucleic acids (Fahy et al., 2011). It has become clear that the lipidome, similar to the transcriptome and the proteome, is dynamic and can be actively remodeled upon different physiological conditions, diets and stimuli (García-Cañaveras et al., 2017; Lydic and Goo, 2018). Thus, improved approaches for lipidomics have contributed significantly to the development of diagnostic tools and therapeutic strategies for metabolic diseases (Lydic and Goo, 2018).

Lipidomics

Approaches to provide global profiles of lipid species, referred to as lipidomics, recently experienced significant advances, due to the advent of next-generation mass spectrometry (MS) instruments in combination with bioinformatics (Wenk, 2005, 2010; German et al., 2007; Shevchenko and Simons, 2010). Lipidomics involves multiple steps (Lydic and Goo, 2018) (**Figure 1**). First, lipids are extracted from the biological sample using organic solvents. Lipids can then be ionized and directly infused into a mass spectrometer (as in the case of "shotgun" lipidomics) or separated by chromatography, prior to detection by MS. Both methods are complementary, as the shotgun method allows lipid profiling from a smaller amount of biological sample and the simultaneous analysis of various classes of lipids, while chromatography/MS enables a more targeted analysis with the detection of structurally close lipids within a single class. Finally, identified lipids are quantified, using a ratio against internal standard(s). In the case of targeted lipidomics, labeled lipids can be included for absolute quantification. For shotgun lipidomics, exogenous lipids representative of the main lipid classes of interest are generally used, with lipid cocktails being commercially available for this purpose.

Lipidomics in Stem Cells

Pluripotent Stem Cells

In 2010, Yanes and colleagues were one of the first to provide a characterization of stem cells with an untargeted metabolomics approach. When comparing the metabolomes of mouse embryonic stem cells (mESCs) and differentiated neurons and cardiomyocytes, lipid messengers and inflammatory mediators, such as arachidonic acid, linolenic acid, diacylglycerols, glycerophosphocholines, glycerophosphoglycerols, and eicosanoids, were among the most upregulated metabolites in mESCs, relative to differentiated cells. In addition, the degree of unsaturation was significantly higher in mESCs compared to differentiated cells. Differentiated cells showed increased levels of saturated free FAs and acyl-carnitines, which consist of fatty acyl–CoA conjugated to carnitine and are intermediates

TABLE 1 | Classes of lipids.

Categories	Composition	Function	Classes or examples
Fatty acids	Carboxylic acid + hydrocarbon chain; synthesized by chain elongation of an acetyl-CoA with malonyl-CoA	Building blocks to complex lipids	SFA, MUFA, PUFA
Glycerolipids	FA + glycerol; may have sugar residues	Energy storage, cell signaling	TAG, DAG, MAG
Glycerophospholipids	Polar head group + glycerol group, may contain LCFA	Membrane composition, cell signaling	PC, PE, PS, PI, PA
Sphingolipids	Sphingoid base + LCFA-CoA	Membrane and lipoprotein composition, cell signaling	Ceramides, Phosphosphingolipids, glycosphingolipids
Sterols	Hydroxyl group + steroid	Membrane, precursor to hormones and vitamins	Cholesterol, bile acids
Prenols	Isopentenyl diphosphate + dimethylallyl diphosphate	Antioxidants, vitamin precursor	Quinone, ubiquinone, Vitamin E, Vitamin K
Saccharolipids	FAs + sugar backbone	Membrane components	Glucosamine
Polyketides	Acetyl + propionyl polymerization	Secondary metabolites	Tetracycline, erythromycin

List of the different categories of lipids, as defined by the LIPID MAPS consortium, with summaries of their average composition, functions and classes. FA, fatty acid; SFA, short chain fatty acid; MUFA, mono-unsaturated fatty acid; PUFA, poly-unsaturated fatty acid; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; PC, phospatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatiylinositol; PA, phosphatidic acid; LCFA-CoA, long-chain fatty acid-coenzyme A.

for the transport of FAs into the mitochondria for β-oxidation. Because carbon-carbon double bonds are highly reactive under oxidative conditions, the authors propose that the high degree of unsaturation observed in mESCs could allow for the maintenance of "chemical plasticity." As oxidative pathways, such as the eicosanoid signaling pathway for which substrates were found to be enriched in mESCs, promote differentiation, control of the reduction-oxidation (redox) status of mESCs could be a mechanism to regulate stem cell fate (Yanes et al., 2010). Accordingly, inhibition of the eicosanoid pathway promoted pluripotency and the maintenance of high levels of unsaturated FAs, while metabolites that promote fatty acid oxidation (FAO), such as acyl-carnitine, enhanced differentiation (Yanes et al., 2010). Importantly, human ESCs also appeared to be enriched in metabolites with a high degree of unsaturation and preliminary data suggested that human ESC differentiation involves oxidative reactions as for mESCs (Yanes et al., 2010). Of note, an untargeted metabolomics approach was also applied to induced pluripotent stem cells (iPSCs) and the metabolomics profile of iPSCs shared much more similarities with ESCs than with the cell of origin, which may be referred to as a pluripotent metabolomics signature (Panopoulos et al., 2012). Interestingly, however, the levels of unsaturated FAs were lower in iPSCs than in ESCs.

In 2010 as well, Park and colleagues performed a more targeted lipidomics analysis of mESCs focused on changes in sphingolipids and ceramides (Cer) species during the differentiation to embryoid bodies (EBs), a model that recapitulates the early stages of embryonic development (Park H. et al., 2010). They found that C16-Cer, a lipid species involved in apoptosis, generally decreased during transition from ESCs to EBs. A higher level of apoptosis in ESCs was suggested to participate in the elimination of residual pluripotent cells. In addition, there was an increase in very long chain dihydroceramides (DHCer), which was proposed to play a role in developmental autophagy taking place during the formation of the inner cell mass. In parallel, changes in the expression of genes encoding enzymes involved in the biosynthesis of sphingolipids and ceramides were assessed and appear to confirm the lipidomics data.

Another study reported the changes of the lipid profile of stem cells during differentiation of iPSCs into hepatocyte-like cells (Kiamehr et al., 2017). During hepatic differentiation and maturation, an overall increase in the FA chain length of sphingolipids was observed. Among phospholipids, polyunsaturated fatty acid (PUFA)-containing lipid species increased in the more mature stages. Importantly, the amount of FAs and lipids available in the different culture media successively used to induce differentiation and maturation of the iPSCs into hepatocyte-like cells was greatly reflected in the lipid composition of the cells at different stages. This was also observed in mesenchymal stem cells (MSCs; see below) (Chatgilialoglu et al., 2017) and constitutes a possible caveat when analyzing the lipidome of isolated stem cells maintained *ex vivo*.

Mesenchymal Stem Cells

Several studies analyzed the lipidome of MSCs (Kilpinen et al., 2013; Campos et al., 2016; Chatgilialoglu et al., 2017; Lu X. et al., 2019). Specifically, the glycerophospholipid profiles of human bone MSCs from young and old donors and across passages during *in vitro* culture were assessed (Kilpinen et al., 2013; Chatgilialoglu et al., 2017; Lu X. et al., 2019). Small changes in membrane glycerophospholipids can have important consequences in terms of signaling mediated by lipid derivatives. Hence, it is particularly relevant to determine changes induced by *in vitro* culture conditions, especially if the stem cells were to be used for therapy.

During long-term culture, total PI and total lysoPC consistently increased, with a more pronounced effect when MSCs were isolated from young donors (Kilpinen et al., 2013). Freshly isolated MSCs had high omega-6 FA content, which decreased in culture (Chatgilialoglu et al., 2017). In addition, the proportion of individual saturated FAs increased in late passages, whereas individual mono-unsaturated FAs (Kilpinen et al., 2013)

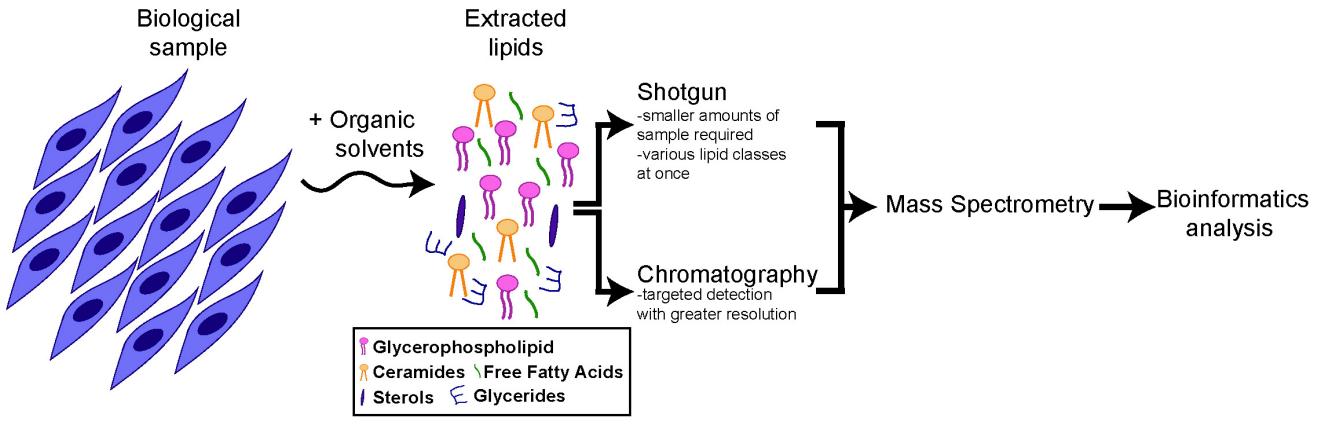

FIGURE 1 | Schematics of lipidomics analysis. All main lipids categories can be extracted from cells or tissue samples through organic solvents. After extraction the lipid composition of the samples can be analyzed directly ("shotgun" approach) or after chromatography, by mass spectrometry and bioinformatics analysis (for more details, see section "Lipidomics and Lipids Enriched in Stem Cells").

and poly-unsaturated FAs (Chatgilialoglu et al., 2017) decreased. Based on these findings, a tailored culture medium that minimizes changes in the membrane FA composition of MSCs across passages was proposed (Chatgilialoglu et al., 2017).

Recently, Lu et al. also characterized alterations of the lipidome during passaging of MSCs, as a way to uncover changes that may play an important role in the aberrant differentiation of MSCs during aging (Lu X. et al., 2019). Aging is one condition that biases MSC differentiation toward adipocyte fate at the expense of osteoblasts, which may contribute to age-related loss of bone mass and osteoporosis. This study used ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS), as this lipidomics approach is more sensitive than the shotgun-based analysis and able to effectively cover lipid species with low abundance. The results obtained were largely consistent with previous studies described above (Kilpinen et al., 2013), as lysoPC and saturated FAs were similarly found to increase with passaging (Lu X. et al., 2019). In contrast, whereas PI species exhibited a significant increase with passaging in the previous study (Kilpinen et al., 2013), in this study a decrease was observed. The reduction of PIs observed by Lu et al. is somewhat inconsistent with the transcriptomics analysis conducted in parallel that showed an increase in expression of the enzymes involved in the conversion of phosphatidic acid to PIs, suggesting that PI biosynthesis is likely more active.

Finally, to better understand the mechanisms underlying MSCs anti-inflammatory properties, a primary reason for the use of MSCs in therapy, Campos et al. (2016) examined the variations of the MSC lipidome under pro-inflammatory conditions. Overall, pro-inflammatory stimuli caused no significant differences in phospholipid class levels, but variations were found in specific molecular species within all classes, except PI. These variations can be correlated with MSCs immunomodulatory properties. The lipidome of the untreated MSCs was consistent with previous results (Kilpinen et al., 2013), with the exception of the presence of sphingomyelins (Campos et al., 2016), which had not been previously identified.

Adult Stem Cells

Despite improvements in lipidomics, the number of studies that have analyzed specifically the lipidome of adult stem cells remains rather limited, especially in comparison to the numerous studies investigating the transcriptome and the proteome. In 2007, what appears to be the first lipidomics analysis in stem cells focused on glycerophospholipids in adult mouse retinal stem cells (Li et al., 2007). Interestingly, among PE, PS and PI, proliferating retinal stem cells showed a significant enrichment for saturated FAs and a decrease in very long chain PUFAs, when compared to differentiated retinal cells. The changes correlated with lower membrane fluidity in retinal stem cells. The same year, another study performed lipidomics on hematopoietic progenitor cells undergoing apoptosis in response to growth factor deprivation (Fuchs et al., 2007). Specifically, this study characterized the extent of changes occurring in membrane glycerophospholipids during apoptosis and found a decrease in diacyl-PC and diacyl-PE species, with a concomitant increase in ether-linked glycerophosphocholines and -ethanolamines in the membranes of the hematopoietic progenitors.

Imaging MS can be applied to tissue sections to characterize the lipidome *in situ* and obtain information on the local distribution of lipid species. This approach was used to characterize the lipidome of the human subventricular zone (SVZ), an important center for neurogenesis in the brain, and showed variations in lipid composition among the four layers of the SVZ (Hunter et al., 2018). However, they were not able to identify lipids specific to the layer that contains neural stem cells (NSCs), possibly due to the heterogeneity of cell types within this region and a greater diversity of lipids. Yet, this may provide a basis for further, more refined, lipidomics analysis in neural stem and progenitor cells.

Lipids That Are Enriched in Stem Cells

Lipidomics in stem cells potentially offers the possibility to identify specific lipid signatures that could be used as biomarkers to identify and sort stem cells. In addition, changes in the

lipidome throughout differentiation of stem cells could reflect changes in substrate availability and could shed light on the mechanisms underlying the differentiation process (Pébay and Wong, 2013). Lipidomics analyses should be performed quickly upon isolation of the stem cells, whenever possible, to provide a snapshot of the lipid profile *in vivo*, prior to culture and expansion. Indeed, current lipidomics data suggest that the composition of the media should be taken into account when analyzing the lipid profile of stem cells maintained in culture. For example, a recent study found that maintaining hPSCs in a lipid-deprived culture medium was responsible for the activation of a lipid biogenesis program in hPSCs that maintained these cells in a more naïve pluripotent state (Cornacchia et al., 2019). Consequently, the authors propose that manipulating lipid availability could be used to modulate the pluripotent state.

More lipidomics analyses are required, particularly in adult stem cells, to establish the extent of the similarities between different types of stem cells and whether this could allow to identify lipid species that would regulate "universal" stem-cell properties, such as self-renewal or maintenance in a multipotent state. Remarkably, the existing data described here suggest that different types of stem cells, including ESCs, iPSCs and MSCs, show an enrichment for lipids containing unsaturated FAs, although this may not be true for retinal stem cells that have less poly-unsaturated FAs than their differentiated counterparts. It would be interesting to determine in this model whether the overall unsaturation level is higher in stem cells. Nevertheless, if this trend is confirmed in other types of adult stem cells, this could corroborate the model proposed by Yanes et al. (2010), according to which the degree of unsaturation would be correlated with a "chemical plasticity" required to maintain stem cells in a pluri- or multipotent state.

LIPID METABOLISM IN STEM CELLS

Fatty Acid Oxidation

When FAs are taken up for catabolism, they can be transported into peroxisomes, in case of very long chain FAs, or the mitochondria, where a step-wise series of redox reactions, collectively called FAO or β-oxidation, breaks down FAs into acetyl-CoA (**Figure 2**). This process is tightly regulated by a set of transporters and enzymes that facilitate the entry of FAs into the mitochondria. The rate-limiting step of this process is controlled by carnitine palmitoyltransferase I (CPT1), a mitochondrial enzyme that catalyzes the transfer of acyl groups from fatty acyl-CoA molecules to L-Carnitine for further entry into the mitochondrial matrix, where oxidation of the fatty acyl groups happens (**Figure 2**). When inside the mitochondrial matrix, acetyl-CoA enters the tricarboxylic acid (TCA) cycle and generate NADH and $FADH_2$, which in turn are oxidized in the electron transport chain (ETC) to fuel oxidative phosphorylation (**Figure 2**) (Houten and Wanders, 2010).

In vertebrates, the peroxisome proliferator-activated receptor (PPAR) family of transcription factors acts as major regulators of FA metabolism. PPARs bind to co-factors, usually lipids, and translocate into the nucleus, where they dimerize with the retinoid X receptor (RXR) to bind to peroxisome proliferator hormone response elements (PPREs). These sequences are most commonly found in the promoter regions of genes involved in lipid metabolism. There are three major classes of PPARs, with PPARα and PPARβ/δ being associated with genes involved in lipid catabolism, while PPARγ is associated with genes involved in lipid anabolism. PPAR families and splice variants are differentially expressed in a tissue specific manner (Berger and Moller, 2002; Pawlak et al., 2015).

Recently, a model has emerged to suggest that FAO plays a crucial role in the maintenance of several adult stem cell populations. Although the precise mechanisms through which FAO acts to maintain stem cells may differ depending on the nature and behavior of the stem cell population (e.g. quiescent versus highly proliferative stem cells), data have shown that adult stem cells are negatively affected by pharmacological or genetic ablation of components of the FAO machinery.

Hematopoietic stem cells (HSCs) have been shown to rely on glycolysis for energy homeostasis (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010; Simsek et al., 2010; Takubo et al., 2010). However, in 2012, Ito and colleagues made the surprising discovery that HSCs rely on FAO for asymmetric division and, thus, maintenance. Indeed, HSCs displayed higher rates of FAO than differentiated progeny, and FAO inhibition by the CPT1 inhibitor etomoxir in HSCs led to a significant reduction in long-term HSC occupancy in bone marrow of irradiated mice upon transplantation. Inhibition of FAO *in vitro* triggered loss of quiescence in HSCs combined with excessive commitment, resulting in HSC exhaustion and inability to sustain the hematopoietic compartment (Ito et al., 2012). Moreover, loss of PPARδ in HSCs led to decreased ATP levels and exit from quiescence, while activation of PPARδ resulted in an increase in ATP levels and enhanced HSC function, as measured by colony formation and differentiation potential (Ito et al., 2012). Using a combination of PPARδ agonists and etomoxir, FAO was shown to act downstream of PPARδ in the regulation of HSC maintenance. The same group further detailed the cellular mechanisms controlling HSC maintenance by showing that Pink1/PARKIN-mediated mitophagy was also important for controlling FAO rates and the maintenance of HSCs (Ito et al., 2016).

Adult neural stem/progenitor cells (NSPCs) are also quiescent and rely on FAO for their maintenance (Knobloch et al., 2017). Similar to what was observed for HSCs, quiescent NSPCs express higher levels of CPT1A and have higher rates of FAO than proliferating NSPCs (Stoll et al., 2015; Knobloch et al., 2017). Strikingly, the presence of malonyl-CoA, which inhibits the rate-limiting step in FAO, was sufficient to induce exit from quiescence to proliferation (Knobloch et al., 2017), indicating that the tight regulation of FAO plays an important role in the regulation of NSPC behavior. FAO is also required in embryonic NSCs, where CPT1A and the breakdown of FAs from LDs have been shown to regulate the asymmetric divisions and maintenance of these stem cells (Xie et al., 2016).

Male germline stem cells (GSCs) in *Drosophila melanogaster* have been recently characterized to also rely on the mitochondrial uptake of lipids to prevent the switch to lipid anabolism and consequent loss of stem cell identity (Sênos Demarco et al., 2019).

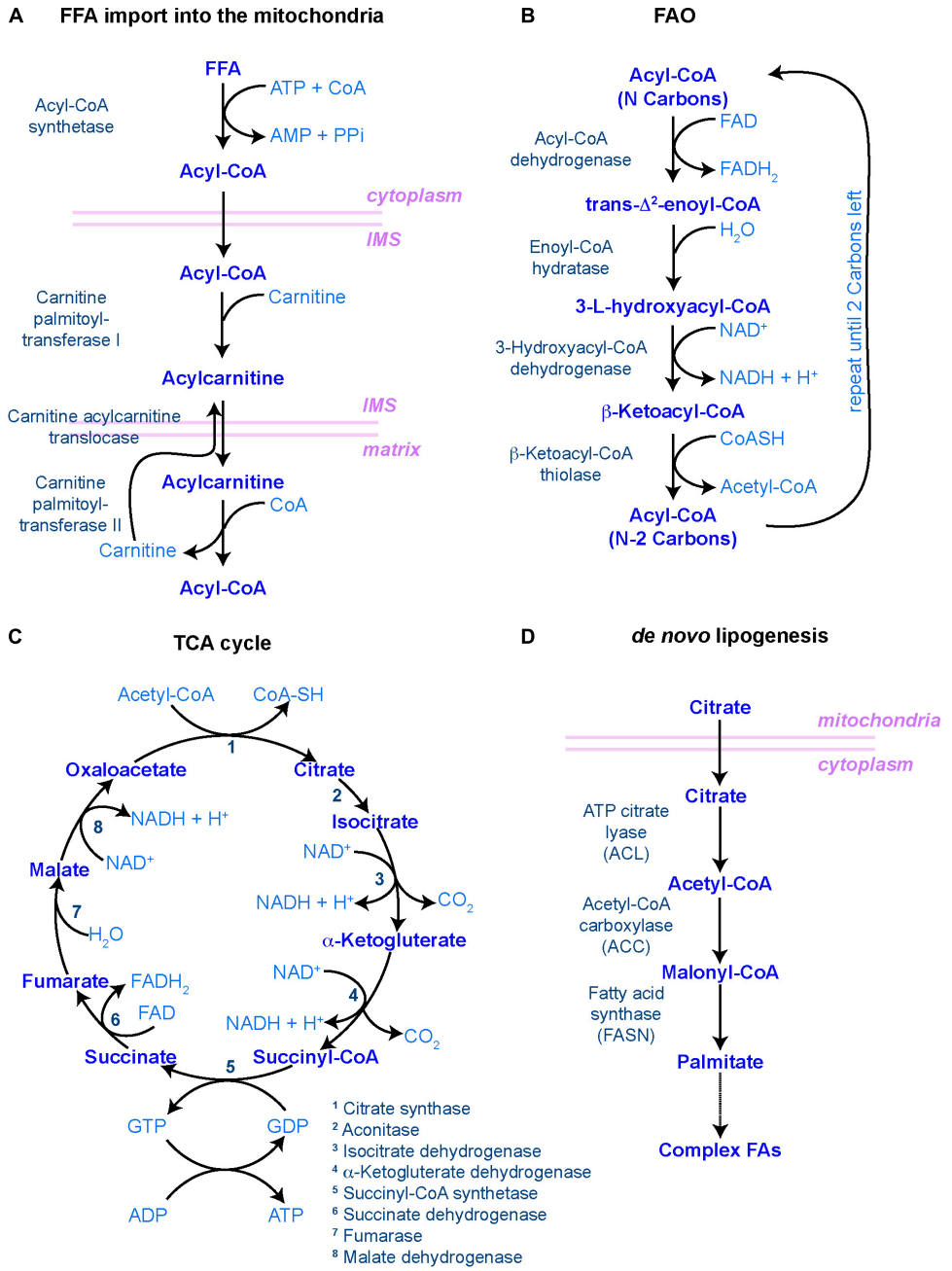

FIGURE 2 | Main biochemical pathways involved in lipid metabolism. **(A–C)** Pathways involved in lipid catabolism. **(A)** In order to be catabolized, free fatty acids (FFAs) must first travel to the mitochondrial matrix through the carnitine shuttle. IMS – inner mitochondrial space. **(B)** Once incorporated into the matrix, fatty acyl-CoA molecules undergo rounds of FAO, resulting in one molecule of acetyl-CoA (with two carbon atoms) and another molecule of fatty acyl-CoA with the original number of carbon atoms minus 2. This process is repeated until only two carbon atoms are left. **(C)** The resulting acetyl-CoA molecules are then incorporated into the tricarboxylic acid (TCA) cycle, where the carbon atoms will be fully reduced into CO_2, generating NADH, FADH2, GTP (and hence, ATP) and H^+ protons to fuel the oxidative phosphorylation process in the mitochondrial cristae, regulated by the electron transport chain. **(D)** In order to make new FAs (i.e. lipid anabolism), citrate leaves the mitochondria to start the *de novo* lipogenesis pathway. In all diagrams, enzymes are in dark blue to the left [in panel **(C)**, they are numbered]; main metabolites are in the middle in blue; and co-factors/resulting products are in light blue to the right. Mitochondrial membranes are represented in pink.

Similar to the proposed mechanism of mitochondrial control of FAO utilization seen in HSCs (Ito et al., 2016), active mitochondria are required to promote lipid catabolism and GSC maintenance. Disruption of mitochondrial activity in these cells, via blocking mitochondrial fusion, led to the accumulation of LDs, activation of Target of Rapamycin (TOR), and precocious germ cell differentiation (Sênos Demarco et al., 2019).

Other stem cell populations, such as intestinal stem cells (ISCs) and skeletal muscle stem cells (also known as satellite cells), also rely on FAO for maintenance. In *Drosophila*, inhibition of lipolysis or FAO led to ISC necrosis (Singh et al., 2016). In mammals, both a high fat diet (HFD) and fasting regimens have been shown to promote FAO through PPARδ, and CPT1A in the case of fasting. Both led to an increase in ISC number and function (Beyaz et al., 2016; Mihaylova et al., 2018). However, while fasting improved ISC function during aging and regeneration (Mihaylova et al., 2018), HFD enhanced the capacity of ISCs to promote tumors upon loss of *Apc* (Beyaz et al., 2016). A recent study confirmed that FAO is required for ISC maintenance under normal conditions in mice (Chen et al., 2019). This study showed that cells in the intestinal crypts, including ISCs, displayed a higher capacity to import FAs than differentiated cells in villi. In addition, ISCs express high levels of hepatocyte nuclear factor 4 (HNF4) transcription factors. These transcription factors activate the transcription of FAO genes, which are required for the maintenance of ISCs (Chen et al., 2019). ISC loss was rescued by supplementation of acetate, suggesting that the essential role of HNF4 factors for ISC maintenance involves their role in promoting FAO. Therefore, in ISCs, high levels of FAO are regulated, at least in part, at the transcriptional level.

In muscle, quiescent satellite cells undergo a transition from FAO to glycolysis during activation and proliferation, which mediates a decrease in NAD^+ levels and consequent decreased SIRT1 activity. Reduced SIRT1 activity resulted in changes in the epigenome, particularly elevated H4K16 acetylation, and the expression of differentiation genes (Ryall et al., 2015).

The promotion and maintenance of cells that seed and maintain tumors, known as cancer stem cells (CSCs), also rely on FAO, a process that could be exploited as anti-tumor therapy. As mentioned above, high FAO rates are associated with the development of intestinal tumors (Beyaz et al., 2016; Singh et al., 2016). Additionally, FAO also supports CSC maintenance in the blood, liver and breast (Samudio et al., 2010; Chen et al., 2016; Wang T. et al., 2018).

In sum, in contrast to initial assumptions that stem cells would rely disproportionately on glycolysis, FAO seems to be an essential factor in the maintenance of many adult stem cells. Inhibition of FAO often results in a reduction in stem cell number, although the precise mechanism(s) by which stem cells are lost may differ; changes in asymmetric division, proliferation rates, gene expression, and cell death have all been implicated in a decrease in stem cell number. It remains to be seen whether a requirement for specific FAO-derived metabolites will be similar or different among adult stem cell populations.

De novo Lipid Synthesis in Stem Cells

De novo lipogenesis is the formation of FAs from acetyl-CoA, malonyl-CoA and NADPH. This process takes place in the cytoplasm and involves two key enzymes: acetyl-CoA carboxylase (ACC) and fatty-acid synthase (FASN) (**Figure 2D**). The activity of ACC is rate-limiting and, as such, highly regulated both at the level of ACC expression, through transcription factors such as Sterol Regulatory Elements Binding Protein 1 (SREBP-1) and PPAR-γ, and at the level of its activation through multiple signaling pathways involving for instance AMP-activated kinase (AMPK) or thyroid hormones (Brownsey et al., 2006). ACC catalyzes the production of malonyl-CoA, an essential substrate for FASN and FA synthesis and a potent inhibitor of CPT1, the rate-limiting enzyme of mitochondrial FAO. Because of this dual function, the availability of malonyl-CoA, downstream of ACC, has been proposed to act as a rheostat by regulating the balance between anabolic lipogenesis and catabolic FAO (Folmes et al., 2013). *De novo* synthesized FAs can then serve as substrates for production of membrane lipids, be stored in the form of TAG to later generate energy through β-oxidation, or provide metabolites implicated in protein modification and signaling networks. Therefore, lipid anabolism is likely to contribute to stem cell function via multiple mechanisms. Yet, only a few studies investigated its impact on adult stem cells directly.

In a key study, *de novo* lipogenesis, and in particular FASN activity, was shown to be specifically elevated in mouse NSPCs, relative to differentiated neuronal cells (Knobloch et al., 2013). Lipogenesis was required for stem cell proliferation and, therefore, for normal neurogenesis to proceed. On the other hand, Spot14 (also called thyroid hormone responsive protein, THRSP) was identified as a repressor of NSPC proliferation. Mechanistically, Spot14 decreases FASN activity by dimerizing with Mig12, an activator of ACC, thereby inhibiting its function and causing a decrease of ACC-mediated malonyl-CoA production, leading in a reduction in FA synthesis (Knobloch et al., 2013). Of note, it was suggested that cholesterol biosynthesis, which occurs through an independent pathway, is also required for NSPC self-renewal and maintenance in the developing mouse forebrain, as NSPCs with mutations in enzymes involved in this pathway exhibit premature differentiation into neurons, causing exhaustion of the stem cell pool (Driver et al., 2016). Importantly, as in the case of NSPCs, increased lipid anabolism in *Drosophila* GSCs through the activation of SREBP also resulted in stem cell loss (Sênos Demarco et al., 2019), suggesting that a conserved mechanism may be at play across stem cell populations.

Fatty acids produced by FASN can then be elongated and desaturated, through the action of various enzymes, and participate in the formation of more complex lipids, such as membrane glycerophospholipids. Diverse forms of membrane lipids can be produced by adding different degrees of unsaturation. Lysophosphatidylcholine acyltransferase 3 (Lpcat3) is one enzyme participating in such reactions, catalyzing the incorporation of poly-unsaturated FAs on lysophospholipids. Remarkably, specific loss of function of Lpcat3 in mouse intestinal crypts led to hyperproliferation of ISCs and progenitors without affecting differentiated enterocytes, both *in vivo* and in organoids (Wang T. et al., 2018). Lipidomics on isolated Lpcat3 deficient crypts showed a selective decrease of polyunsaturated PC and increase in saturated and monounsaturated PC, which could affect membrane fluidity. Loss of Lpcat3 induced a strong transcriptional upregulation of sterol biosynthesis enzymes, driven by an increase of SREBP-2 activity and, accordingly, an increase of cholesterol in the crypts. Inhibition of cholesterol

synthesis in Lpcat3-deficient ISCs rescued the hyperproliferation phenotype, while increasing cholesterol was sufficient to induce ISC proliferation (Wang B. et al., 2018).

Interestingly, *de novo* FA synthesis is also essential for pluripotency in mESCs and during reprogramming (Wang et al., 2017): ACC and FASN are upregulated during reprogramming to pluripotent cells and lipogenesis is enhanced, while lipogenesis decreased during induction of differentiation. Likewise, single human pluripotent stem cells (hPSCs) require lipid synthesis for their survival, although treatment with lipid synthesis inhibitors does not affect already established hPSC colonies (Romani et al., 2019).

Taken together, current data suggest that while FAO seems to be required for the maintenance of adult stem cells, *de novo* lipogenesis appears required and sufficient to promote stem cell activity and differentiation. One possibility is that *de novo* lipogenesis and lipid incorporation enhance the ability of stem cells to rapidly expand the plasma and organellar membranes, which is crucial for cell division and often associated with membrane remodeling during differentiation. Another possibility is that lipid biosynthesis may generate signaling lipids playing a role in the differentiation program. Moving forward, lipidomics analyses in purified stem cells will help revealing whether energy and redox (in the forms of ATP and NADH/NAD^+) or specific lipid metabolites play a role in the maintenance of adult stem cell populations. Metabolomics analyses should also provide insights into the utilization of *de novo* generated lipids in response to proliferation and differentiation cues.

MECHANISTIC INSIGHTS INTO HOW LIPIDS REGULATE STEM CELL BEHAVIOR

Adult stem cells are capable of dividing in such a way that the daughter cells produced can adopt different fates. This "asymmetry," with respect to cell fate, can be regulated by both intrinsic factors and extrinsic cues, often provided by the surrounding microenvironment, known as the "niche" (Watt and Hogan, 2000; Morrison and Kimble, 2006; Knoblich, 2008). However, stem cells must also be able to divide symmetrically to produce two stem cell daughters or daughter cells destined to differentiate (i.e. for tissue repair). The ability of stem cells to maintain tissue homeostasis by switching from asymmetric to symmetric outcomes, as the tissue demands, is achieved by "populational asymmetry." In this section, we will describe some of the potential mechanisms by which lipid metabolism or specific lipid species can influence either self-renewal or maintenance of a multipotent state, through regulating asymmetric stem cell divisions and/or interaction with the niche or through regulating signaling and gene expression (**Figure 3**).

Lipids and Asymmetric Cell Division

The establishment of cellular polarity is one mechanism by which an asymmetric outcome can be achieved, and cell polarity often depends on specific membrane phospholipids and more specifically phoshoinositides (PIs). Although not a stem cell *per se*, the early *Caenorhabditis elegans* embryo has served as an excellent model to uncover mechanisms of asymmetric division (reviewed in Schneider and Bowerman, 2003), and several studies have shown the involvement of lipids in generating polarity in this model. First, depletion of FAs or loss of ACC activity in the *C. elegans* one-cell embryo caused mislocalization of the PAR-3/PAR-2 cell fate determinants and a loss of asymmetry during the first division (Rappleye et al., 2003). In addition, PPK-1, a PI(4)P5 kinase, was found to accumulate at the posterior side of the one-cell embryo, where it would be responsible for an asymmetric generation of phosphatidylinositol-4,5-bisphosphate (PIP2), which in turn would lead to the recruitment at the posterior of LIN-5 and GPR-1/2, two factors required for asymmetric spindle positioning, in an unknown manner (Panbianco et al., 2008). The asymmetric distribution of plasma membrane PIP2 along the antero-posterior axis was confirmed recently and found to be dependent upon PAR determinants and F-actin (Scholze et al., 2018). Interestingly, PIP2 reciprocally regulates F-actin enrichment at one pole, which also appears to be required for proper polarity establishment and spindle positioning (Scholze et al., 2018).

The signaling activities of phospholipids are often mediated by phospholipases (Dennis, 2015). The seam cells in *C. elegans* have features of epithelial adult stem cells. In these cells, the catalytic activity of intracellular phospholipase A1 (iPLA1) is required for spindle orientation and asymmetry of cell fate specification during asymmetric divisions (Kanamori et al., 2008). This would be mediated, at least in part, by controlling the subcellular localization of β-catenin, which is involved in the establishment of polarity, through regulation of endosome-to-Golgi retrograde trafficking (Kanamori et al., 2008). *C. elegans* mutants for iPLA1 exhibit altered FA composition of PI, similar to mutants for 1-acylglycerol-3-phosphate O-acyltransferase (AGPAT) enzymes (Imae et al., 2010). As these AGPAT mutants present the same defects in seam cell asymmetric division and trafficking of β-catenin, this suggests a model in which altered FA composition of PI would cause abnormal localization of PIs in the membrane bilayer, leading to the mis-sorting of cortical proteins, such as β-catenin, by retrograde membrane trafficking (Imae et al., 2010).

In *Drosophila*, larval neuroblasts act as NSCs to generate all of the neurons and glia in adult flies. Recent data have shown that the PI regulating enzymes Vibrator and PI4 kinase IIIa (PI4KIIIa) are required for the asymmetric distribution of cell fate determinants in neuroblasts (Koe et al., 2018). Mutations that affect the PI binding and PI lipid transfer activities of Vibrator induce defects in neuroblast asymmetric division, leading to either the formation of ectopic neuroblasts or their loss through differentiation into neurons. The proposed model is that Vibrator stimulates PI4KIIIa to promote synthesis of phosphatidylinositol-4-phosphate (PI(4)P) at the plasma membrane, which, in turn, binds and anchors myosin to the neuroblast cortex, where it could recruit fate determinants.

The involvement of PIs in establishing polarity axis for asymmetric division of stem cells appears to be conserved in mammals. For instance, the asymmetric division of mouse

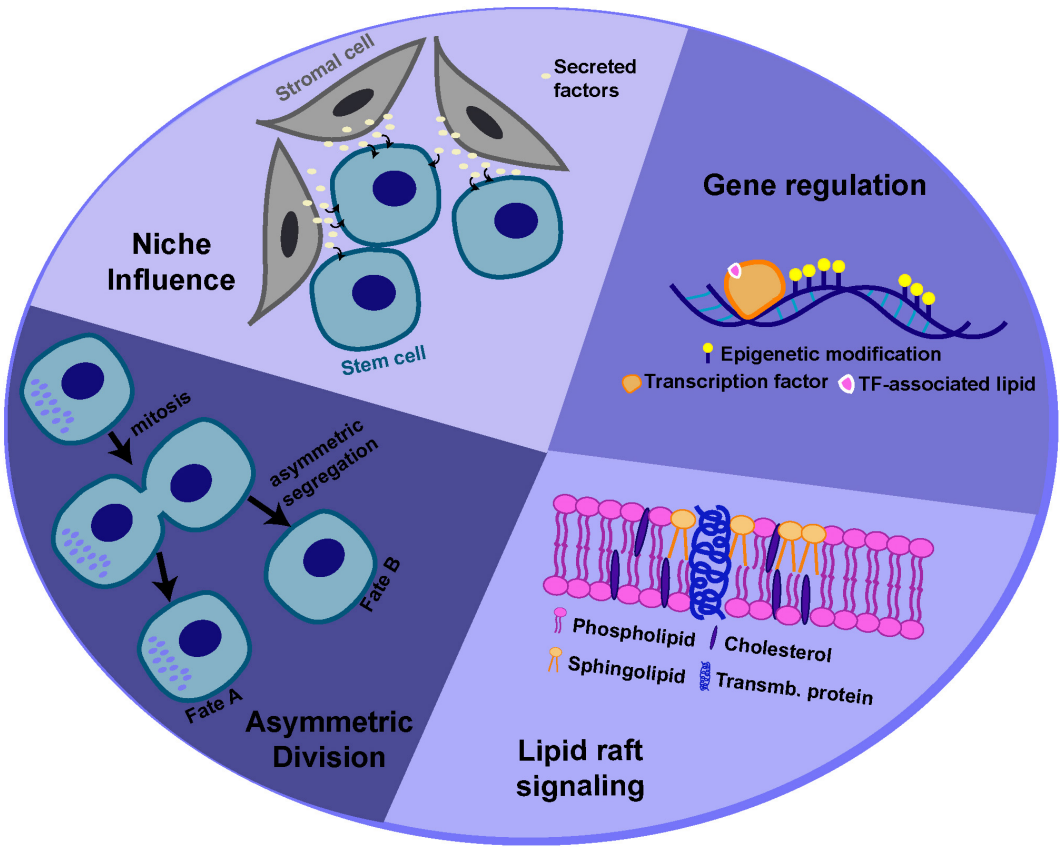

FIGURE 3 | Potential mechanisms for lipid-mediated regulation of stem cell behavior. Lipids and lipid metabolism may regulate stem cell behavior through different mechanisms, including modulation of asymmetric cell division, interactions with the niche, cell signaling and gene expression.

epidermal stem cells depends on PI-dependent kinase 1 (PDK1). PDK1 is recruited to the apical side of the stem cell where phosphatidylinositol-3,4,5-trisphosphate (PIP3) is enriched, through the local activation of the PI3 kinase (PI3K) by interaction with E-cadherin at cell-cell contacts (Dainichi et al., 2016).

In addition to phospholipids, post-transcriptional lipid modifications, which constitute a prevalent mechanism for targeting proteins to membranes, may also participate in the establishment of cell polarity and asymmetric division. N-terminal N-myristoylation, the addition of a 14-C saturated FA, facilitates protein-plasma membrane interactions and is found on many proteins involved in asymmetric cell division (Benetka et al., 2008). Thus, myristoylation could possibly mediate the targeting of polarity and fate determinants to a given region of stem cell plasma membrane, although this remains to be investigated in more detail.

The above examples involve specific lipid species that directly maintain polarity of stem cells through interaction with cytoskeletal and other proteins. However, there are also some intriguing cases in which lipid metabolic pathways were shown to influence symmetric versus asymmetric outcomes of stem cell divisions, but the precise mechanisms involved remain to be elucidated. As noted above, in HSCs, a decrease in FAO leads to an increase of symmetric divisions giving rise to differentiating cells, which ultimately leads to exhaustion of the stem cell pool (Ito et al., 2012). Remarkably, deficiencies in FAO also increase the rate of mouse NSC symmetric differentiating divisions, at the expense of asymmetric divisions that normally maintain the pool of stem cells (Xie et al., 2016).

Lipids and Interactions With the Niche

Adult stem cells reside in a specialized microenvironment, known as the niche, that provides signals essential to maintain "stemness" and survival (Voog and Jones, 2010; Scadden, 2014). Lipids could affect the interactions between stem cells and supporting cells within the niche in a variety of ways. Perhaps one of the most intuitive mechanisms would involve secretion of signaling lipids by niche cells; however, to our knowledge, there is only little evidence for such a mechanism. Osteoblasts are present in bone marrow and can participate in the regulation of HSCs by producing prostaglandin E2 (reviewed in Domingues et al., 2017). This lipid of the eicosanoid family binds to specific receptors at the surface of HSCs (Hoggatt et al., 2009) that mediate activation of the Wnt signaling pathway, which is important for HSC specification during development (Goessling et al., 2009) and for expansion of adult HSCs (Reya et al., 2003). In addition, HSCs also

express receptors for the signaling lipid sphingosine-1-phosphate (S1P) that regulate localization within the bone marrow niche (Ogle et al., 2017).

Lipids can also influence niche-stem cell communication through regulation of signaling in niche cells. In *Drosophila*, subsets of niche cells in the ovary sense dietary cholesterol, which triggers the release and secretion of the signaling ligand Hedgehog to activate follicle stem cell (FSC) proliferation (Hartman et al., 2013). Interestingly, two studies in NSCs showed that LDs accumulating in adjacent cells affect NSC behavior in different ways. In a mouse model of Alzheimer's disease, an accumulation of LDs was observed specifically in ependymal cells, the main support cells of the forebrain NCS niche, which correlated with a concomitant loss of NSCs (Hamilton et al., 2015). Through imaging MS-lipidomics in intact tissue sections, the authors determined that these LDs were selectively enriched in TAG containing oleic acid (18:1). Both *in vitro* and *in vivo*, an increase in oleic acid concentration negatively affects proliferation of NSCs via a mechanism that remains to be elucidated in detail but appears to involve activation of Akt signaling in NSCs. LDs were also observed to accumulate specifically in glial cells of the *Drosophila* larval central nervous system, which serve as niche cells for neuroblasts (Bailey et al., 2015). The number of LDs in glial cells increases under hypoxic conditions and in the presence of high levels of reactive oxygen species (ROS) (Bailey et al., 2015; Liu et al., 2015). Both FA synthesis and dietary FAs contribute to the formation of these LDs (Bailey et al., 2015; Liu et al., 2015). It was further shown that in response to high ROS levels, lipid biosynthesis increased in neurons, leading to increased transport of lipids from neurons to glia, where they are stored as LDs (Liu et al., 2017). Strikingly, glia-specific disruption of LD synthesis negatively affected neuroblast divisions during hypoxia, but not during normoxia (Bailey et al., 2015). Therefore, in this stem cell niche, LDs in adjacent glial cells are required to support stem cell divisions under adverse conditions, although the underlying mechanisms are still to be identified.

In other models, lipids and associated metabolic pathways have been shown to be required specifically for maintenance of niche cells and, in this way, affect stem cell maintenance in a non-autonomous manner. For example, secreted phospholipase A2, which produces lipid mediators such as lysophosphatidic acid and arachidonic acid, is specifically required for the differentiation and maturation of Paneth cells, which constitute a niche critical for ISC maintenance (Schewe et al., 2016). It is interesting to note that upon a HFD, or treatment with FAs or PPARδ agonists, ISCs show an increased capacity to form organoids *in vitro*, even in the absence of Paneth cells (Beyaz et al., 2016). One of the explanations proposed by the authors of this study is that activation of PPARδ upon excess FAs could induce the expression, in ISCs, of growth factors that are normally secreted and provided by the niche.

Lipid Rafts and Signaling

Lipid rafts are dynamic membrane microdomains that are enriched in cholesterol and sphingolipids and act as platforms for signal transduction and membrane trafficking (Simons and Ehehalt, 2002). As differences in acyl chain composition of membrane phospholipids mediate their inclusion or exclusion from lipid rafts, this could be a mechanism to explain how different forms of phospholipids may have very specific effects on signaling (Imae et al., 2010). Therefore, lipid rafts dynamics are likely to contribute to the regulation of signaling pathways that are required for stem cell functions.

For example, lipid raft clustering is crucial for the regulation of HSC quiescence (Yamazaki et al., 2006). Re-entry of quiescent HSCs into the cell cycle ("activation") requires the clustering of lipid rafts, which is mediated by cytokines, and inhibition of this process is sufficient to induce HSC quiescence. In this context, data suggest that lipid rafts regulate the PI3K–Akt–FOXO pathway in HSCs (Yamazaki et al., 2006). As most HSCs are quiescent in the bone marrow niche, lipid raft reorganization must be strictly regulated, likely by specific niche signals.

Also in the hematopoietic system, Hermetet et al. (2019) recently observed that lipid rafts are enriched in mouse HSCs, relative to more mature progenitor cells. Interestingly, a HFD leads to clustering and enlargement of lipid rafts, which affects the distribution of the TGFβ receptor within the lipid rafts. In this case, the clustering of the lipid rafts results in a decrease in TGFβ signaling, as it becomes concentrated in a smaller percentage of the membrane. As TGFβ signaling is essential for maintaining HSCs in a quiescent state, HFD leads to abnormal re-entry of HSCs into the cell cycle and ultimately causes their exhaustion (Hermetet et al., 2019).

Finally, a recent study reported that lipid rafts are present between niche cells and NSCs and suggested that the integrity of these lipid rafts was required for efficient niche-stem cell communication and NSC differentiation into neurons (Deng et al., 2019).

Lipids and Regulation of the Transcriptome and Epigenome

At the molecular level, cell identity is controlled by a specific gene expression program, such that the transcriptome can be used to track cell fate decisions (Efroni et al., 2015). Activation and repression of gene expression are largely controlled by transcription factors and their interaction with the underlying epigenetic landscape. Stem cells express genes associated with self-renewal and potency (pluri-, multi-, uni-) and maintain a certain degree of plasticity in their epigenome to allow for differentiation (Bernstein et al., 2006; Voigt et al., 2013). In recent years, cellular metabolism has emerged as an essential regulator of the transcriptome and epigenome (Lu and Thompson, 2012; Etchegaray and Mostoslavsky, 2016), as specific metabolites can act as substrates or co-factors for epigenetic modifiers. In stem cells, the inter-relationship between metabolism and epigenetics plays important roles in the maintenance of potency and differentiation (Harvey et al., 2016; Atlasi and Stunnenberg, 2017; Mathieu and Ruohola-Baker, 2017; Fawal and Davy, 2018). Consequently, lipids and lipid metabolic pathways have the potential to regulate stem cell behavior by affecting the expression

of stem cell-specific genes, through either a direct effect on transcription or modulation of epigenetic marks.

There are different mechanisms through which lipids can regulate gene expression (Zhdanov et al., 2016; Fernandes et al., 2018). First, *in vitro* experiments suggested that some lipid species, such as oleic acid, may directly bind to DNA (Zhdanov et al., 2002), which could have significant impacts on gene expression but is not currently supported by data obtained under physiological conditions (Fernandes et al., 2018). There is also some evidence supporting an effect of specific lipid species on DNA polymerase activity (Novello et al., 1975; Kamath-Loeb et al., 2014). Interestingly, although the mechanism remains unclear, changes in palmitate concentration *in vitro* appear to regulate the expression of neuronal genes and increased intracellular palmitate results in increased differentiation of neural precursor cells into neurons (Ardah et al., 2018). However, lipids primarily regulate gene expression through transcription factors, such as nuclear receptors, for which ligands include FAs and cholesterol metabolites. Nuclear receptors are activated by their ligands, bind to promoter/enhancer region of target genes and recruit co-regulators to activate or repress transcription. One of the main families of lipid-activated nuclear receptors is the PPARs. PPAR-regulated genes are mostly genes involved in lipid and carbohydrate metabolism (Barrera et al., 2008). Interestingly, signaling through receptors of the PPAR family was shown to be required for the maintenance of several types of adult stem cells. In the nervous system, this could involve direct regulation of genes associated with "stemness." Specifically, PPARs are expressed in NSPCs of the SVZ and are required *in vitro* for proliferation and maintenance of the undifferentiated state (Bernal et al., 2015). Indeed, in NSPCs, PPARs appear to regulate the expression of the self-renewal gene *Sox2*, likely at the transcriptional level, given that the *Sox2* locus contains PPAR responsive elements (Bernal et al., 2015). This suggests that lipids and lipid-bound nuclear receptors may directly regulate the expression of stem cell specific genes. It would be interesting to assess whether PPAR responsive elements are enriched in the promoter regions of genes involved in the maintenance of self-renewal and multipotency in other adult stem cell models as well.

Similarly, the cholesterol-regulated transcription factor Sterol Regulatory Elements Binding Protein 2 (SREBP-2), which regulates the expression of cholesterol biosynthetic enzymes, was recently found to bind to the promoter regions of *notch1b* and other Notch target genes in *Danio rerio* (zebrafish) (Gu et al., 2019). In this study, transcriptional activation of the Notch pathway by SREBP-2 in conditions that promote hypercholesterolemia was shown to be essential for the mobilization of HSCs. Furthermore, SREBP-1, which regulates the expression of lipogenic genes, is expressed in human muscle cells in an insulin and growth factors dependent manner (Boonsong et al., 2007), despite a low lipogenesis rate in this tissue. Transcriptomics analyses revealed that SREBP-1 regulates the differentiation of satellite stem cells into myotubes by regulating the expression of two transcriptional repressors whose target genes include multiple myogenic genes (Lecomte et al., 2010). Therefore, transcription factors whose activity is modulated by lipids and/or lipid metabolism can contribute to the regulation of stem cell function via the control of target genes involved in establishing or maintaining specific cell identities.

Another crucial mechanism through which lipids can regulate gene expression is by mediating changes in the epigenome. One substrate used for histone acetylation is acetyl-CoA. While acetyl-CoA can be generated by various metabolic pathways, including glycolysis, amino-acid breakdown and FAO, a combination of proteomics and isotope tracing demonstrated that lipid metabolism is a major source of acetyl-CoA used for histone acetylation (McDonnell et al., 2016). Histone H3 acetylation correlates with "open chromatin" and was shown to be important for the maintenance of pluripotency and self-renewal of PSCs (Azuara et al., 2006; reviewed in Gaspar-Maia et al., 2011). In a recent study, the activation of lipid biogenesis pathways was identified as a conserved signature of naïve pluripotency in cultured hPSCs, mPSCs and in the human pre-implantation epiblast (Cornacchia et al., 2019). The maintenance in a naïve pluripotent state by lipid anabolism correlated with higher acetyl-CoA levels and increased histone acetylation, when compared to levels found in hPSCs in a primed pluripotent state, which do not show active lipid biogenesis (Cornacchia et al., 2019). Interestingly, however, examples of regulation of histone acetylation by lipids in adult stem cells were rare. As previously mentioned, activation of quiescent satellite cells is accompanied by a decrease in FAO, which leads to a reduction in intracellular NAD+ levels. This causes a decrease in SIRT1 activity and a subsequent increase in H4K16 acetylation, promoting the expression of muscle differentiation genes (Ryall et al., 2015). In addition, *in vitro* treatment of NSCs with palmitate resulted in a global increase in histone H3 acetylation, with specific enrichment at the promoter regions of some upregulated genes (Ardah et al., 2018). Notably, acetyl-CoA can also be reduced to produce the ketone body beta-hydroxybutyrate, which is an endogenous inhibitor of histone deacetylases (Shimazu et al., 2013). In NSCs, pharmacological inhibition of histone deacetylation promotes neuronal differentiation, while decreasing astrocyte differentiation *in vitro* (Fawal and Davy, 2018).

Although there is currently no direct evidence that lipids may bind to DNA to regulate gene expression, it is conceivable that lipids may bind to chromatin. For instance, cholesterol appears able to bind chromatin fibers, either directly or through proteins, and this interaction would promote the compaction of chromatin fibers, a state generally associated with transcriptional repression (Silva et al., 2017). A structural characterization, at the atomic level, of the lipids binding mode to nucleosomes would provide more insights into this issue (Fernandes et al., 2018).

Finally, LDs may also have the potential to affect gene expression via sequestration of proteins such as histones (Li Z. et al., 2012; Welte and Gould, 2017), which could ultimately cause changes in chromatin composition and underlie variations in gene expression. However, there is little evidence of a variation in LD accumulation during differentiation of adult stem cells that could be implicated in changes in gene expression. In *Drosophila* male GSCs, an abnormal increase of LDs resulting

from perturbation of mitochondrial function appears to correlate with precocious differentiation (Sênos Demarco et al., 2019). A more detailed analysis and profile of LDs and their contents in stem cells and differentiating progeny could provide additional insights into a potential role of LDs in regulating stem cell function under homeostatic conditions.

DYSREGULATION OF LIPIDS AND EFFECTS ON STEM CELLS AND TISSUE HOMEOSTASIS

Diet, aging or disease, including cancer, can cause changes in lipid homeostasis. Accordingly, changes in the lipidome can be used to help in the diagnosis of a disease and/or provide potential therapeutic targets. In this section, we discuss how changes in the lipidome may affect endogenous stem cells. A better understanding of this relationship can provide insight into the pathophysiology of lipid imbalance disorders and improve therapeutic approaches on one hand and, on the other hand, could allow to potentially use lipid metabolites as therapeutic tools in order to modulate stem cell behavior in patients.

Diet

Many studies have investigated how diet impacts the behavior of adult stem cells (Mana et al., 2017). HFD models in animals have been used to better understand the interplay between Western diets, which contain an excess of saturated fats, and the development of obesity and metabolic disorders (Wang and Liao, 2012).

Long term HFD (over 6 months) in mice triggers many of the metabolic phenotypes associated with obesity. A seminal study demonstrated that long term HFD reversibly increases the number and self-renewal capacity of mouse ISCs, at the expense of differentiation, in a cell-autonomous manner (Beyaz et al., 2016). Mechanistically, HFD leads to sustained activity of the PPARδ pathway and increased activity of the Wnt-β catenin pathway, which is required for stem cell maintenance. Treatment of gut organoids with FAs present in the HFD, *in vitro*, was sufficient to increase ISC number and self-renewal capacity, indicating that the effect of HFD on stem cells is unlikely to be an indirect consequence of obesity. As stem cells are considered to be more prone to acquire oncogenic mutations, this increase in "stemness" is proposed to be a mechanism contributing to the increase of tumor incidence observed upon HFD (Beyaz et al., 2016). Moreover, it was recently shown that HFD alters bile acids, which also contribute to drive malignant transformation of ISCs with dysregulated Wnt signaling (Fu et al., 2019). In *Drosophila*, increased dietary cholesterol influences the differentiation of ISCs by modulating Notch signaling, which leads to an increase of secretory entero-endocrine cells in the posterior midgut (Obniski et al., 2018).

In the hematopoietic system, it was suggested that long-term HFD (approximately 6 months) also increases the number and function of HSCs and skews differentiation toward the myeloid lineage, at the expense of the lymphoid lineage (Singer et al., 2014), contributing to inflammation and metabolic disease. In contrast, HFD administered for a short time (usually 4–6 weeks) appeared to cause a decrease in long-term repopulating HSCs, coupled with an increase in HSC proliferation and myeloid differentiation potential (Adler et al., 2014; Luo et al., 2015; Van Den Berg et al., 2016; Hermetet et al., 2019). However, the proposed underlying mechanisms differ among studies. Hermetet et al. (2019) observed a cell-intrinsic effect of HFD on HSCs: HFD promoted the clustering of lipid rafts at the surface of HSCs, which negatively affected TGF-β signaling and induced exit from quiescence and re-entry into the cell cycle, leading ultimately to exhaustion of the HSC pool. On the other hand, data from Luo et al. (2015) suggest that the effect of short-term HFD on HSCs is non-autonomous resulting from PPARγ-dependent changes in the bone marrow niche. Indeed, both short and long-term HFDs cause MSCs to differentiate primarily into adipocytes (Luo et al., 2015; Ambrosi et al., 2017; Tencerova et al., 2018). If this were to occur at the expense of osteoblasts, this could explain the increased risk of bone fracture in obese patients (Tencerova et al., 2018). Interestingly, contribution of the gut microbiome has also been proposed (Luo et al., 2015). These mechanisms are not necessarily incompatible; yet, it is unclear why long and short-term HFD would cause opposite effects on HSC number.

The increased consumption of saturated fats also contributes to neurodegenerative diseases, long-term memory loss and cognitive impairment (Park H. R. et al., 2010), which could be due, in part, to an effect on NSCs. For instance, the proliferation of NSPCs in the SVZ of the hippocampus is impaired upon HFD, without affecting neuronal and glial differentiation (Park H. R. et al., 2010). The decrease in proliferation appeared to involve increased lipid peroxidation and decreased secretion of a brain-derived growth factor. It was recently shown that in the SVZ, HFD decreases the proportion of NSPCs, at least in part, through the abnormal accumulation of senescent cells in the niche (Ogrodnik et al., 2019). Although it is not clear how these lipid-containing senescent cells alter the maintenance of stem cells, the systemic administration of senolytic drugs that eliminate senescent cells rescues, at least partially, the loss of NSPCs. When fed a HFD, mouse hypothalamic NSCs are also depleted, through ectopic activation of the IKKβ/NF-κB pathway (Li J. et al., 2012). In addition, differentiation of these multipotent cells into anorexigenic neurons expressing proopiomelanocortin (POMC neurons) is impaired and, because these neurons control satiety and insulin resistance, their loss upon HFD contributes to the development of obesity and pre-diabetes (Li J. et al., 2012).

Although the effects of HFD on adult stem cells have been investigated primarily in the gut, hematopoietic system and brain, there are examples of HFD-induced obesity also affecting other types of adult stem cells. For instance, HFD-induced obesity affects muscle regeneration in mice by inhibiting AMPK activity and preventing satellite cell activation (Fu et al., 2016). Given that AMPK can be activated by drugs, if this effect is conserved in humans, this finding raises the possibility of enhancing muscle regeneration in obese patients. However, it is unclear whether or not changes in lipid homeostasis are directly involved in this mechanism. In addition, although an increase in proliferation of stem cells in the lung was observed in response to short-term

HFD, it was suggested that this may occur via an indirect mechanism. Indeed, supplementation of a regular diet with a lipid mix or individual lipids had no obvious effects (Hegab et al., 2018). Finally, epidermal stem cells undergo transcriptional changes in mice fed a HFD (Solanas et al., 2017; Lu Y. et al., 2019). Among the differentially expressed genes, genes regulating the extracellular matrix and the PI3K pathway may contribute to changes in niche signaling and altered stem cell function (Lu Y. et al., 2019). Yet, the detailed impacts of HFD on skin homeostasis remain to be investigated.

Overall, these studies suggest that short and long term HFD can impact the behavior of adult stem cells in a range of tissues; however, the mechanisms appear to differ. It will be interesting to determine whether the differences are associated with distinct changes in lipid homeostasis occurring in response to HFD and whether changes in stem cell behavior are a direct consequence of changes in lipid homeostasis upon HFD. In some cases, as for ISCs, treatment with FAs *in vitro* reproduced the effects of HFD (Beyaz et al., 2016), whereas is other cases, such as lung stem cells, this was not the case (Hegab et al., 2018). Importantly, in all cases in which it was assessed, the effects of HFD on stem cells appear to be reversible and could, therefore, potentially be corrected simply by changes in diet. Interestingly, while HFD seems to impair neurogenesis (Park H. R. et al., 2010; Li J. et al., 2012; Ogrodnik et al., 2019), specific types of FAs, including omega-3 FAs appear to increase neurogenesis (Kang et al., 2014; Nascimento et al., 2016; Lo Van et al., 2019), suggesting that diet could be used to rescue neuronal loss in diseases and aging.

Inborn Errors of Metabolism

"Inborn errors of metabolism" encompass a large number of heterogeneous genetic diseases caused by rare mutations that affect the function of individual proteins, generally enzymes, involved in metabolic reactions. Among the inborn errors of metabolism affecting lipid metabolism, deficiencies in FAO have been shown to affect adult stem cells. Mutations in TMLHE, an enzyme catalyzing the first step of carnitine biosynthesis, are relatively frequent among this class of diseases and are associated with developmental neuropsychiatric disorders, including increased risk of autism. To better understand how a mutation that systemically affects lipid metabolism is linked with autism, a group of researchers specifically inactivated TMLHE in the mouse embryonic neocortex and observed a loss of NSCs (Xie et al., 2016). Strikingly, exogenous carnitine rescued at least partially this reduction of NSCs, whereas it had no effect in control animals. Thus, carnitine supplementation could represent a potential therapeutic approach to minimize the developmental brain deficits associated with inborn deficiencies in FAO (Xie et al., 2016).

Aging and Degenerative Diseases

One striking hallmark of aging is altered stem cell behavior and, in some cases, stem cell exhaustion (López-Otín et al., 2013). In addition, there is increasing evidence that age-related changes in metabolism contribute to the age-associated decline in stem cell function (Ren et al., 2017). Lipid homeostasis is one of the metabolic parameters that are affected by aging. In many organisms, including in humans, aging is associated with increased fat storage and an altered membrane lipid composition that tends to decrease membrane fluidity (Gonzalez-Covarrubias, 2013). Moreover, membrane sphingolipids and the ratio of their metabolites sphingosine 1-phosphate (S1P) and ceramides change with age (Montoliu et al., 2014). These two signaling lipids mediate opposite cellular effects, S1P promoting proliferation and cell survival and ceramides promoting apoptosis. Interestingly, higher ceramides levels are associated with shorter lifespan in worms, and a decrease in S1P is often seen in age-related diseases such as Alzheimer's disease (Papsdorf and Brunet, 2018). There is also evidence that the degree of unsaturation of membrane phospholipids gets higher with age, increasing lipid peroxidation products that may lead to more cellular damage (Papsdorf and Brunet, 2018).

However, the mechanism(s) by which changes in lipid metabolism could contribute to age-related changes in stem cell behavior remains largely unexplored. As mentioned above, lipidomics analyses of human MSCs identified changes in lipid profiles that could underlie the age-associated changes in MSC differentiation (an increase of adipocytes at the expense of osteoblasts), which contribute to the increase in osteoporosis in older individuals (Kilpinen et al., 2013; Lu X. et al., 2019). In these studies, lipid profiles were compared at different times across *in vitro* passaging of MSCs, as a way to model aging-related variations. Although this approach identified changes in lipids, the findings should be verified *in vivo*. Importantly, when comparing MSCs isolated from young and old donors, Kilpinen et al. (2013) did not describe significant differences. In addition, changes in lipid profiles with age appear to differ among tissues (Papsdorf and Brunet, 2018) potentially having different effects on the resident stem cells.

Aging is a primary factor for the development and progression of neuro-degenerative diseases (Hou et al., 2019), and alterations in lipid metabolism that impact NSC function have been shown to play a role in this process. For instance, in Alzheimer's disease patients, oleic acid-enriched LDs accumulate in the forebrain, which correlate with a decrease in proliferation of NSCs (Hamilton et al., 2015). In a mouse Alzheimer's model, pharmacological inhibition of the oleic-acid producing enzyme, stearoyl-CoA desaturase, was able to rescue the proliferation of NSCs (Hamilton et al., 2015). Although more investigation is required, this may offer a promising therapeutic approach to prevent cognitive decline and improve stem cell-mediated brain repair in Alzheimer's disease.

Altogether, a better understanding of the relationships between altered lipid homeostasis and stem cell function during aging will contribute to the identification of new therapeutic targets for age-associated pathologies that arise due to changes in stem cell behavior.

SUMMARY AND PERSPECTIVES

An increasing number of studies have implicated lipid metabolism, as well as individual lipid species, in the regulation of adult stem cell behavior. Advances in lipidomics have opened

up new approaches to identify stem cell-specific signatures and novel lipid biomarkers that can be used to identify and sort stem cells. Yet, there are only a few examples of lipidomics in adult stem cells, likely because isolating enough adult stem cells to perform lipidomics remains challenging. Studies in PSCs and MSCs suggest that stem cells may generally contain lipids with a higher degree of unsaturation, when compared to differentiating progeny. Once lipidomics becomes more sensitive and/or stem cell purification strategies are improved, it will be interesting to determine to what extent adult stem cells share a common lipidome. What is clear is that FAO and lipid biogenesis pathways appear to be common regulators of adult stem cell behavior. In various types of adult stem cells, FAO is essential for stem cell maintenance, while *de novo* lipid synthesis promotes stem cell proliferation and differentiation. However, the mechanisms through which these lipid metabolic pathways affect stem cell behavior differ. Lipid catabolism and anabolism modulate the availability of different lipid intermediates and secondary metabolites that can affect stem cells in numerous ways, including regulation of self-renewal and multipotency via changes in signaling and gene expression. As one example, higher FAO activity in stem cells could lead to an increase in acetyl-CoA that can serve as a substrate for histone acetylation and participate in the maintenance of stem cell identity. In addition, *de novo* lipid biogenesis can regulate the pool of signaling lipids, such as PIs, that are involved in cell polarity and asymmetric division. Additional lipidomics and metabolomics analyses on purified adult stem cells should provide further insights into the contribution of specific lipid species to these mechanisms. Such studies will provide a better understanding of how lipid imbalances can affect adult stem cells, thereby contributing to changes in tissue homeostasis and the pathophysiology of diseases.

AUTHOR CONTRIBUTIONS

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

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Energy Metabolism Regulates Stem Cell Pluripotency

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Pluripotent stem cells (PSCs) are characterized by their unique capacity for both unlimited self-renewal and their potential to differentiate to all cell lineages contained within the three primary germ layers. While once considered a distinct cellular state, it is becoming clear that pluripotency is in fact a continuum of cellular states, all capable of self-renewal and differentiation, yet with distinct metabolic, mitochondrial and epigenetic features dependent on gestational stage. In this review we focus on two of the most clearly defined states: "naïve" and "primed" PSCs. Like other rapidly dividing cells, PSCs have a high demand for anabolic precursors necessary to replicate their genome, cytoplasm and organelles, while concurrently consuming energy in the form of ATP. This requirement for both anabolic and catabolic processes sufficient to supply a highly adapted cell cycle in the context of reduced oxygen availability, distinguishes PSCs from their differentiated progeny. During early embryogenesis PSCs adapt their substrate preference to match the bioenergetic requirements of each specific developmental stage. This is reflected in different mitochondrial morphologies, membrane potentials, electron transport chain (ETC) compositions, and utilization of glycolysis. Additionally, metabolites produced in PSCs can directly influence epigenetic and transcriptional programs, which in turn can affect self-renewal characteristics. Thus, our understanding of the role of metabolism in PSC fate has expanded from anabolism and catabolism to include governance of the pluripotent epigenetic landscape. Understanding the roles of metabolism and the factors influencing metabolic pathways in naïve and primed pluripotent states provide a platform for understanding the drivers of cell fate during development. This review highlights the roles of the major metabolic pathways in the acquisition and maintenance of the different states of pluripotency.

Keywords: naïve and primed embryonic stem cells, induced pluripotent stem cells, nuclear reprogramming, glycolysis, oxidative metabolism, oxidative phosphorylation, tricarboxylic acid cycle, amino acids

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PLURIPOTENT STEM CELL FATE *IN UTERO* AND *IN VITRO*

Pluripotency describes the developmental capacity of a cell to form the three primary germ layers, which in turn can give rise to all cell types of the adult body. As such it does not represent a single fixed stage, but rather a gradient of cellular phenotypes from the "naïve" pluripotent stem cells (PSCs) of the inner cell mass (ICM) of the early blastocyst, to the "primed" PSCs of the

early post-implantation epiblast (Weinberger et al., 2016). Upon uterine implantation, the epiblast cells progressively lose expression of core pluripotency genes Oct4 and Nanog as the three germ layers emerge during gastrulation (Weinberger et al., 2016; Mathieu and Ruohola-Baker, 2017). The expression of these transcription factors is spatially restricted and ultimately suppressed by the time of somitogenesis (Osorno et al., 2012).

Although pluripotency exists transiently *in vivo*, several stages can be stably recapitulated *in vitro* (Evans and Kaufman, 1981). Embryonic stem cells (ESCs) derived from the ICM of murine blastocysts are considered the developmental naïve state in terms of their transcriptional activity, epigenetics and metabolic phenotypes (Nichols and Smith, 2009; Weinberger et al., 2016). General characteristics of naïve PSCs include the ability to give rise to all somatic lineages, incorporate into a developing blastocyst generating chimeric embryos, two active X chromosomes in female lines and the utilization of bivalent metabolism [both glycolysis and oxidative phosphorylation (OxPhos)] (Weinberger et al., 2016). While original protocols required leukemia inhibitory factor (LIF) and serum supplementation to maintain this naïve state, serum is dispensable upon the addition of GSK3 and MEK inhibitors (2i) (Ying et al., 2008). In contrast, when cells are derived from the post-implantation epiblast they are termed epiblast stem cells (mEpiSCs) and are considered a primed PSC, representative of a later developmental stage of pluripotency, and as such are functionally different from naïve PSCs (Brons et al., 2007; Tesar et al., 2007). Characteristics of primed PSCs do overlap with those of naïve PSCs, yet there are notable differences: primed PSCs express the core pluripotency genes Oct4, Sox2, and Nanog, however they are not capable of integrating into a developing blastocyst to form chimeric embryos, they are predominantly glycolytic, and inactivation of one X chromosome has been noted in female lines (Weinberger et al., 2016). Interestingly mEpiSCs can be cultured *in vitro* without LIF when in the presence of fibroblast growth factor (FGF) and activin A (Brons et al., 2007; Tesar et al., 2007).

Unlike mESCs, human ESCs (hESCs) derived from the ICM of the human blastocyst resemble a primed rather than naïve state (Thomson et al., 1998) (**Figure 1**). This aligns hESCs more closely with mEpiSCs in terms of their culture requirements, as well as their transcriptional and epigenetic profiles. Reviews by Davidson et al. (2015) and Weinberger et al. (2016) discuss the growing body of literature highlighting the differences between naïve and primed ESCs from murine and human epiblasts in terms of transcriptomic, epigenetic and chromosomal profiles, and postulate these differences are likely the result of species specific developmental programs and requirements. A stable naïve state can be generated by culturing primed hESCs in a cocktail of MEK, RTK, BRAF, ROCK, and GSK3β inhibitors, in addition to LIF and activin A (5i/L/A) or titrated 2i with LIF and PKC inhibitor Gö6983 (t2iLGö) (Takashima et al., 2014; Theunissen et al., 2014). Similar conditions with the addition of ROCK inhibitor and ascorbic acid (t2iLGöY) have also been utilized to derive naïve ESCs from the human ICM (Guo et al., 2016). The resultant naïve hESCs recapitulate the features of mESCs, including X chromosome reactivation in female cell lines. Interestingly, inhibition of Rho kinase with the ROCK inhibitor Y-27632 in single cell hESCs dissociated by enzymatic methods initially results in the suppression of glycolysis, TCA cycle, and glutaminolysis, while promoting cell survival by inhibiting caspase-3 mediated apoptosis (Vernardis et al., 2017). Prolonged culture in ROCK inhibitor (>96 h) results in metabolic adaptation, after which hESC metabolism rebounds with both glycolysis and oxidative metabolism upregulated, an adaptation observed and attributed to the naïve hESC state. Whether the inclusion of ROCK inhibitor in media optimized for the generation of naïve hESCs functions primarily to couple metabolic flux with proliferation, or to inhibit apoptosis in the development of the naïve state is unclear.

Given ethical concerns and restrictions in deriving human ESC lines, combined with the fact that hESCs can only yield allogenic options for cell-based therapies; nuclear reprogramming has paved the way for the generation of patient

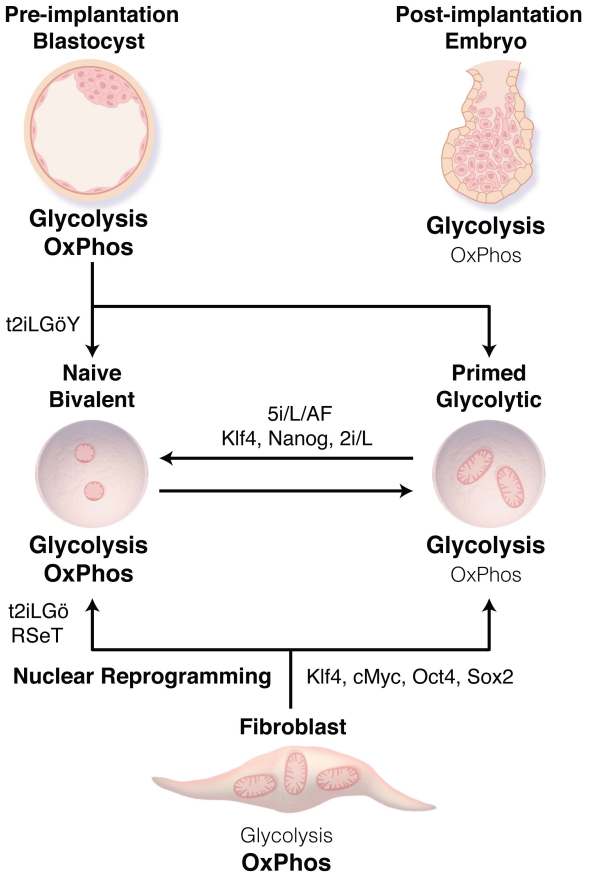

FIGURE 1 | Transitions between human pluripotent stem cell (PSC) states. Human embryonic stem cells derived from the inner cell mass of the blastocyst or through nuclear reprogramming traditionally display a primed state associated with bivalent metabolism using both glycolysis and oxidative phosphorylation (OxPhos). Methods have now been developed to derive naïve PSCs (t2iLGö ± Y, RSeT) or to transition cells from the primed to naïve state (5i/L/AF, or Klf4, Nanog, 2i/L), resulting in a transition from bivalent metabolism to nearly exclusively glycolysis. These metabolic preferences reflect those of the related stage during embryonic development.

derived induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). This technology utilizes the exogenous expression of core pluripotency transcription factors OCT4, SOX2, KLF4, and c-MYC to reprogram somatic cells to a pluripotent state, producing human iPSCs comparable to primed human ESCs. In contrast, nuclear reprogramming of somatic murine cells results in pluripotency more reminiscent of naïve mESCs, which may be due to a better understanding of the mediators of the murine naïve pluripotent state, as it has recently been demonstrated that parallel isogenic primed and naïve iPSCs can be derived by using media supporting the naïve state, RSeT and T2iLGö (Kilens et al., 2018).

Distinct developmental stages are associated with specific patterns of metabolic activity, and growing evidence supports the concept that early metabolic changes are critical for regulating epigenetics and establishing cellular identity. Cells constantly modulate their metabolic state in response to nutrient availability, extracellular signals and reprogramming/differentiation cues, which induces changes in the concentrations of key intermediary metabolites that can serve as cofactors for the epigenetic regulation of gene transcription, including histone methylation and acetylation. This crosstalk between intermediary metabolism and epigenetics may in part account for how metabolic pathways can contribute to stem cell fate determination. In the context of pluripotency, studies have focused on several key metabolites, including S-adenosylmethionine (SAM), alpha-ketoglutarate (αKG) and acetyl-CoA, that sit at the nexus between intermediary metabolism and epigenetics. For example, methylation of discrete residues on histones play a fundamental role in regulating ESCs differentiation and pluripotent state. PSCs have chromatin characterized by "bivalent" domains, which are regulatory regions containing both an activating histone modification, histone H3 lysine 4 trimethylation (H3K4me3), and a repressive modification, histone H3 lysine 27 trimethylation (H3K27me3), which poise developmental genes to maintain their repression in the absence of differentiation signals, yet allow timely activation (Bernstein et al., 2006). Indeed, these critical histone marks for regulation of pluripotency and differentiation are in part regulated through metabolism (Kaelin and McKnight, 2013). H3K4me3 is dependent on SAM levels derived through one-carbon metabolism from exogenous or glucose-derived serine, threonine or methionine metabolism (Shyh-Chang et al., 2013b; Shiraki et al., 2014). Repressive H3K9me3 and H3K27me3 marks are regulated in an αKG-dependent manner through demethylation by JmjC-domain containing histone demethylases (JHDMs) and ten-eleven translocation (TET) enzymes (Kaelin and McKnight, 2013). H3K27 can also be acetylated, which marks enhancer regions and areas of elevated transcriptional activity. Acetylation is dependent on acetyl-CoA derived from glycolysis, which is critical for the regulation of pluripotency versus differentiation (Carey et al., 2015; TeSlaa et al., 2016; Tischler et al., 2019; Vardhana et al., 2019). The mechanisms by which metabolism regulates epigenetics have been reviewed extensively elsewhere (Ryall et al., 2015; Harvey et al., 2016a, 2019). This review focuses on the major metabolic pathways (**Figure 2**) that influence PSC identity and how they change, or can be modulated, to support acquisition of pluripotency through nuclear reprogramming and transition between the distinct stages of pluripotency (**Figure 3**).

PLURIPOTENT STEM CELL METABOLISM

Glycolysis

High glycolytic flux is common to various stem cell populations and is critical for the acquisition and maintenance of pluripotency (Ezashi et al., 2005; Cho et al., 2006; Kondoh et al., 2007; Varum et al., 2009, 2011; Simsek et al., 2010; Zhu et al., 2010; Folmes et al., 2011, 2012; Panopoulos et al., 2012; Takubo et al., 2013). Glycolysis is a sequence of cytosolic redox reactions that convert a single glucose molecule into two pyruvate molecules coupled with the generation of two net ATP and two reduced NADH molecules. While glycolysis is less energetically efficient compared to the complete oxidation of glucose in the tricarboxylic acid (TCA) cycle and OxPhos, this pathway can occur in the absence of oxygen and enables a fast rate of ATP production that can surpass that of oxygen-dependent respiration when glucose is abundant, as is the case in cell culture. In fact, ATP/ADP ratios seem to be unaffected by this metabolic preference in these cell types (Guppy et al., 1993). Indeed, highly proliferating cell types typically utilize glycolysis despite sufficient oxygen availability to support oxidative metabolism (aerobic glycolysis), as incomplete oxidation of glucose enables the conservation of carbon biomass required for biosynthesis of cellular components needed for daughter cell generation (Vander Heiden et al., 2009; Lunt and Vander Heiden, 2011). Glycolytic intermediates can also be shunted into different pathways to meet the high anabolic demands of proliferating cells: lipid synthesis via dihydroxyacetone phosphate and acetyl-CoA, and nucleotide and NADPH synthesis through glucose-6-phosphate and the Pentose Phosphate Pathway (PPP). The importance of the anabolic utilization of glycolytic intermediates is highlighted by ^{13}C-labeling studies that confirm the utilization of glucose-derived carbons in the manufacturing of nucleotides and amino acids such as serine (Gu et al., 2016; Lees et al., 2019). The preferential utilization of glycolysis over mitochondrial oxidative metabolism may also represent a mechanism to preserve the genomic integrity of PSCs by reducing reactive oxygen species (ROS) produced by OxPhos and limiting subsequent damage of nuclear and mitochondrial DNA as well as reducing oxidation of proteins and lipids (Perales-Clemente et al., 2014).

Although glycolytic flux is common across all stages of pluripotency, the relative contributions of glycolysis versus OxPhos do vary. The primed pluripotent state of hPSCs and mEpiSCs is almost exclusively glycolytic (Tesar et al., 2007; Nichols and Smith, 2009; Takashima et al., 2014; Theunissen et al., 2014), whereas naïve hPSCs and mESCs utilize a bivalent metabolic system with a greater reliance on oxidative metabolism (Lee et al., 2012; Zhou et al., 2012; Si et al., 2013; Mu et al., 2015; Cha et al., 2017). Indeed, early preparatory phase glycolytic metabolites such as fructose 1, 6-bisphosphate are enriched in primed PSCs without accumulation of downstream metabolites, suggesting

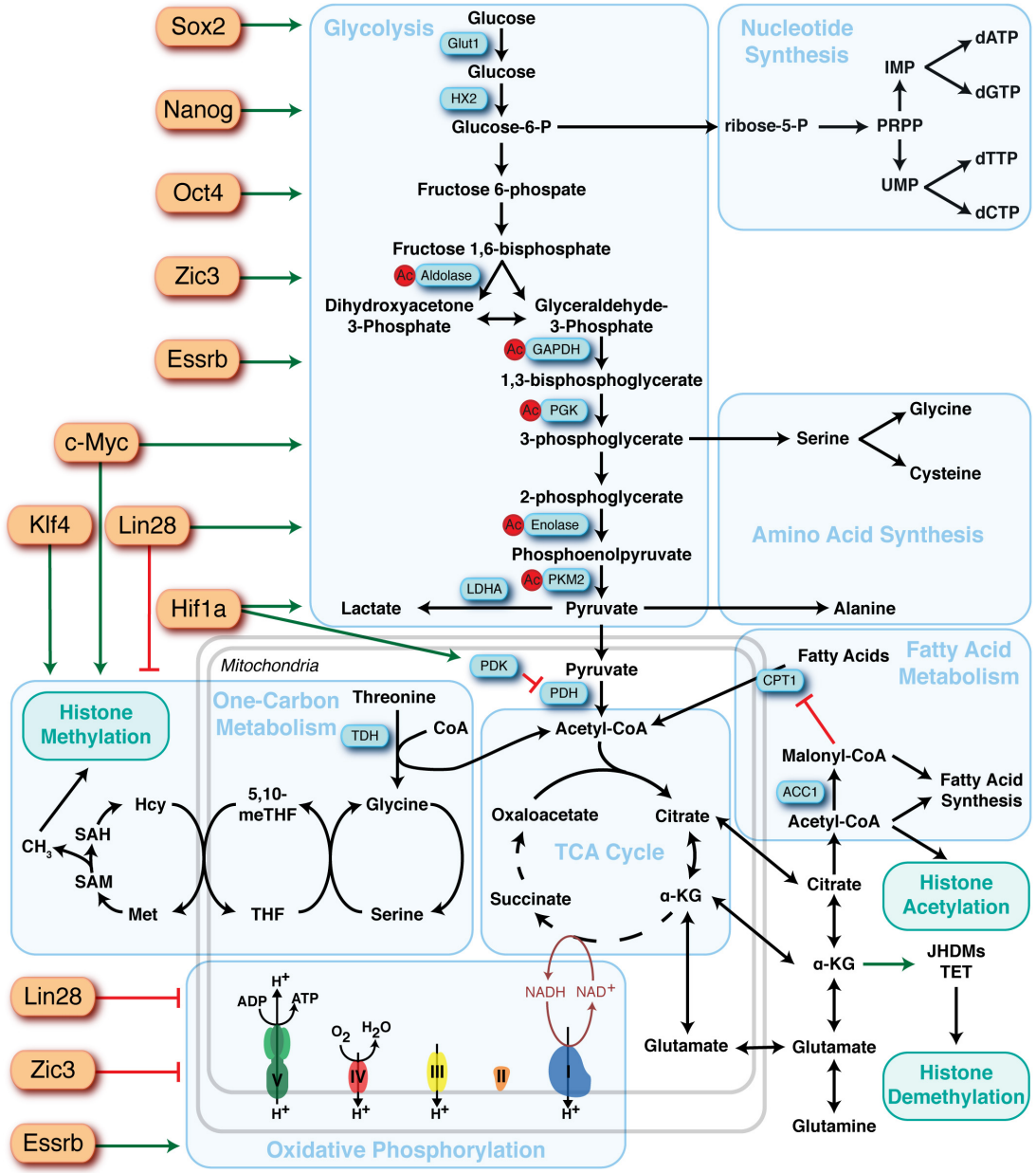

FIGURE 2 | Overview of the major metabolic pathways in pluripotent stem cells and their regulators. Major metabolic pathways are found in blue boxes and key sites of regulation are highlighted in blue rectangles. Identified upstream regulators of metabolic pathways are depicted as orange rectangles and the directionality of regulation represented by red bars (suppression) or green arrows (activation).

that these glycolytic intermediates may be consumed for anabolism or one-carbon metabolism (Tesar et al., 2007; Zhou et al., 2012; Gafni et al., 2013; Takashima et al., 2014; Sperber et al., 2015). In contrast, naïve hESCs exhibited both higher glucose and oxygen consumption, coupled with elevated lactate production compared to their primed counterparts (Gu et al., 2016). This suggests that naïve pluripotent cells may reside in a hypermetabolic state, however, further investigation is needed to understand the exact mechanisms and purpose behind this high energetic requirement in the naïve state (Gu et al., 2016). These metabolic preferences may reflect substrate availability and metabolic regulation during early embryogenesis, which has been reviewed extensively elsewhere (Gardner, 1998; Folmes et al., 2012; Shyh-Chang et al., 2013a; Folmes and Terzic, 2014; Gardner and Harvey, 2015; Zhang et al., 2018). The early embryo displays low glycolytic rates due the inhibition of hexokinase and phosphofructokinase 1 and is dependent on pyruvate supplied by ovarian follicle cells to support oxidative metabolism, with glycolysis progressively increasing during development due to higher glucose availability

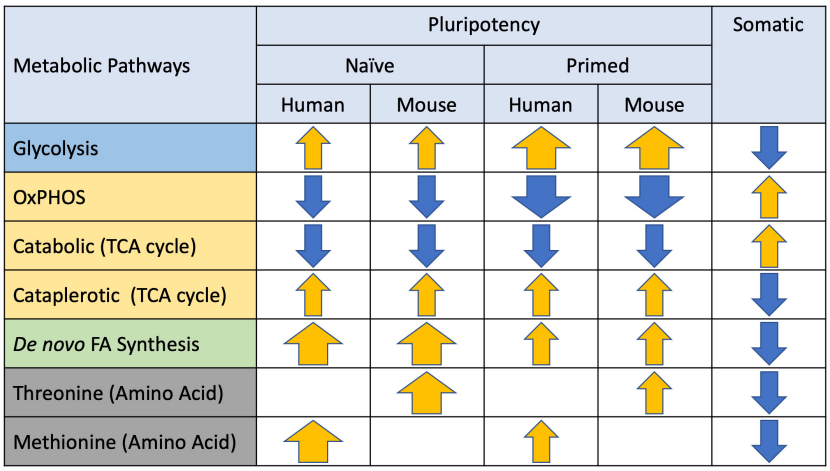

FIGURE 3 | Metabolic preferences of naïve and primed pluripotent stem cells (PSCs). Major metabolic pathways identified in PSCs are listed on the left and arrows depicting directionality (yellow = elevated, blue = suppressed) and magnitude (thickness) of differences relative to somatic cells.

and glucose transporter expression (Barbehenn et al., 1978; Pantaleon and Kaye, 1998).

Core pluripotency factors have been demonstrated to directly regulate glycolysis in PSCs (Folmes et al., 2012; Zhang et al., 2012). OCT4 directly regulates transcription of hexokinase 2 and pyruvate kinase M2 (Kim et al., 2015), consistent with the preference of these isoforms in iPSCs (Folmes et al., 2011; Prigione et al., 2014; Qin et al., 2017). Recently a SOX2, OCT4, and NANOG binding site has been described at the GLUT1 enhancer, which appears to increase GLUT1 expression and downstream glycolytic flux in ESCs (Yu et al., 2019). Additionally, c-MYC has been identified as a strong driver of glycolysis in both the naïve and primed pluripotent states (Folmes et al., 2013b; Gu et al., 2016). Myc expression is regulated through a miR-290/371-MBD2 pathway, with miR-290/371 repressing MBD2 and leading to increased expression of Myc and its downstream transcriptional targets PKM2 and LDHA, supporting elevated glycolysis (Cao et al., 2015). The RNA binding protein LIN28a/b, which plays critical roles in embryonic development, acquisition, and maintenance of pluripotency, and the transition between naïve and primed pluripotency has been demonstrated to regulate glycolysis in addition to a number of metabolic pathways, including maintaining low mitochondrial function, one-carbon metabolism, and nucleotide metabolism (Shyh-Chang et al., 2013c; Zhang et al., 2016b).

A number of upstream regulators have been implicated in regulating the metabolic phenotype during the transition between the naïve and primed state. Early work demonstrated that HIF1α is sufficient to drive the glycolytic phenotype in the primed state through activin/nodal signaling (Zhou et al., 2012). More recently Ras has been implicated in controlling a number of events that are critical for the transition from naïve to primed pluripotency, including stimulation of glycolysis, epithelial-mesenchymal transition (marked by an increase in N-Cadherin) and an increase in H3K27me3. Interestingly, blocking glycolysis reversed the effect of Ras on N-Cadherin and H3K27me3, suggesting these effects are downstream of the metabolic remodeling (Altshuler et al., 2018). Recent work has demonstrated that downregulation of SIRT2 and upregulation of SIRT1 are a molecular signature of primed hPSCs, and that these NAD-dependent deacetylases regulate primed pluripotency through distinct mechanisms (Cha et al., 2017). The authors observed that miR-200c5p, an OCT4 induced miRNA, can directly downregulate SIRT2 mRNA and protein expression leading to hyper-acetylation of glycolytic enzymes (aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, and pyruvate kinases) and acceleration of glycolysis. Knockdown of SIRT2 also suppressed OxPhos in fibroblasts by an unknown mechanism, enabling a higher efficiency of nuclear reprogramming. In contrast, SIRT1 expression is upregulated in primed pluripotency and inhibition of its upstream miRNA-34a, while increased SIRT1 expression promotes nuclear reprogramming (Lee et al., 2012; Cha et al., 2017). However, SIRT1 appears to elicit its downstream effects independent of metabolic regulation, potentially through the deacetylation of SOX2 and p53 leading to increased Nanog expression, and p21 inhibition (Lee et al., 2012; Si et al., 2013; Mu et al., 2015). miRNA-34a deficiency can also endow PSCs with features of totipotent blastomeres, suggesting that these pathways may also play an important role in restricting pluripotent developmental potential (Choi et al., 2017).

Downstream of glycolysis, the fate of pyruvate has been increasingly recognized to regulate stem cell function (Flores et al., 2017; Schell et al., 2017). Pyruvate represents the nexus of multiple metabolic pathways and can be metabolized to: (a) acetyl-CoA by pyruvate dehydrogenase (PDH) to enable entry and subsequent oxidation in the TCA cycle, (b) lactate by lactate dehydrogenase (LDH), in part to enable regeneration of NAD^+ to support high glycolytic rates, and (c) oxaloacetate by pyruvate carboxylase to anapleurotically replenish TCA cycle intermediates that are being utilized for anabolic pathways. In PSCs, pyruvate is largely metabolized to lactate, with the

remaining being catabolized in the TCA cycle, in contrast to differentiated cells where pyruvate is mainly catabolized. Multiple enzymes responsible for pyruvate flux into the TCA cycle are differentially regulated in naïve *versus* primed PSCs, such as pyruvate dehydrogenase kinase (PDK) (Zhou et al., 2012). For example, in primed PSCs, transport of pyruvate to the mitochondria is tightly regulated by HIF1α-induced expression of PDK 1-3, which phosphorylates and inhibits PDH, thus pushing pyruvate to lactate production (Mathieu and Ruohola-Baker, 2017). It was also found that UCP2, an ATP uncoupling protein, blocks the uptake of pyruvate into the TCA cycle (Zhang et al., 2011). Interestingly, a recent study demonstrated that supplementing hESC media with exogenous pyruvate enhanced OxPhos while suppressing glycolysis, but had no impact on expression of pluripotent markers such as *Nanog, Pou5f1*, and *Sox2*, although this metabolic shift supported mesodermal differentiation through activation of the AMPK/mTOR pathway (Song et al., 2019). In summary, regulation of glycolytic flux plays an important role in the acquisition of pluripotency, and more specifically, maintenance and transition between the naïve and primed states.

Mitochondrial Metabolism

Mitochondria are complex, highly dynamic organelles that are critical for the maintenance of cellular homeostasis, in part due to their canonical role as the major energy generator of the cell. Beyond their role in ATP generation, mitochondria play many other roles within the cell including ROS production, calcium homeostasis, cellular signaling pathways, and synthesis of metabolites, such as fatty acids, amino acids, iron/sulfur clusters, pyrimidines, heme, and steroid hormones (Rizzuto et al., 1993; Dimmer and Scorrano, 2006; de Brito and Scorrano, 2010; Seo et al., 2018). Over the past decade, it has become increasingly recognized that mitochondrial dynamics also significantly impact stem cell function and fate (Liesa and Shirihai, 2013; Labbe et al., 2014; Ma et al., 2015a; Buck et al., 2016; Folmes and Terzic, 2016; Folmes et al., 2016; Khacho et al., 2016; Matilainen et al., 2017). In general, compared to their differentiated counterparts, PSCs have fewer small and globular mitochondria containing poorly developed and immature cristae, that are localized in the perinuclear region (St John et al., 2005; Cho et al., 2006; Lonergan et al., 2006, 2007; Facucho-Oliveira and St John, 2009; Armstrong et al., 2010; Suhr et al., 2010; Folmes et al., 2011; Prigione et al., 2014; St John, 2016). However mitochondrial differences exist between the stages of pluripotency, with primed ESCs containing more elongated mitochondria with better defined cristae compared to their naïve counterparts (Zhou et al., 2012; Ware et al., 2014), despite the observation that primed ESCs have low mitochondrial activity, while naïve ESCs display active mitochondria (Zhou et al., 2012; Takashima et al., 2014; Sperber et al., 2015). Although additional work is required to understand this apparent dichotomy between mitochondrial structure and function, it has been well established that mitochondria and metabolism play a critical role in the naïve-to-primed transition (Zhang et al., 2011; Zhou et al., 2012; Ware et al., 2014; Sperber et al., 2015; Chandel et al., 2016). For instance, a recent study demonstrated that loss of mitochondrial carrier homolog 2 (MTCH2), a direct regulator of mitochondrial fusion/elongation, resulted in less elongated and more fragmented mitochondria in mESCs, leading to delayed naïve-to-primed interconversion. This study indicated that MTCH2 is important for elongated mitochondria which itself was sufficient for driving naïve-to-primed interconversion by altering histone deacetylation and nuclear gene reprogramming (Bahat et al., 2018). In addition, recent work has demonstrated that supplementation with a recombinant truncated human NME7 ($NME7_{AB}$) is sufficient to induce a stable naïve-like state in hPSCs (Carter et al., 2016) associated with reactivation of mitochondrial function and stimulation of ATP production (O'Reilly et al., 2019).

Despite the apparently immature mitochondrial structure in PSCs, they maintain high mitochondrial membrane potential, which helps to define their pluripotency and self-renewal characteristics (Schieke et al., 2008; Armstrong et al., 2010; Mah et al., 2011; Prigione et al., 2011). Indeed, mESCs with high mitochondrial membrane potential have the capacity to differentiate into the three germ layers, while those with low mitochondrial membrane potential differentiated mainly into mesodermal cells (Schieke et al., 2008). Furthermore, fully reprogrammed iPSCs appear to have high mitochondrial membrane potential (Folmes et al., 2011) and PSCs may actively maintain this potential by consuming ATP to enable reverse mode ATP synthase activity. Even though the underlying mechanism for maintaining high mitochondrial potential remains to be elucidated, it can impact stem cell function by: (a) maintaining a network of fragmented mitochondria (Mattenberger et al., 2003; Zhang et al., 2011; Teslaa and Teitell, 2015), (b) maintaining redox potential (Shyh-Chang et al., 2011), and (c) priming the cell to provide energy for differentiation (Folmes et al., 2012). Thus, collectively these findings indicate that mitochondria play functional and developmental roles in metabolism of PSCs.

Tricarboxylic Acid Cycle

The mitochondrial TCA cycle represents a central hub of energy metabolism, where many pathways involved in central carbon metabolism intersect. Canonically, this cycle predominantly oxidizes its major substrate, pyruvate, to CO_2 in order to generate reducing factors and electron donors (NADH and $FADH_2$) to supply the electron transport chain (ETC) and ATP synthesis. Complementing its role in energy generation, the TCA cycle also maintains a balance between anapleurosis, the reactions to replenish TCA intermediates using predominantly pyruvate and glutamine as substrates, and cataplerosis, whereby partially oxidized intermediates can be extracted to serve as building blocks for anabolic processes including lipid, amino acid and nucleotide biosynthesis, as well as post-translational modification of proteins (Wellen et al., 2009; Boroughs and DeBerardinis, 2015). Through balancing energy generation with cataplerosis to supply substrates for anabolism and post-translational protein modification, the TCA cycle is critical for the regulation of stem cell function and fate.

Pluripotent stem cells appear to have reduced reliance on the canonical role of mitochondria for energy generation, which suggests that they repurpose their mitochondria for

other purposes in support of stem cell maintenance. It appears that mitochondrial metabolism in PSCs involves incomplete oxidation of pyruvate, resulting in TCA cycle intermediates that are exported for myriad functions including anabolic reactions and histone modification. In fact, ESCs incompletely oxidize pyruvate through the TCA cycle generating citrate which is then transported and converted to acetyl-CoA through ATP-citrate lyase in the cytoplasm (Wellen et al., 2009; Moussaieff et al., 2015). While fatty acid oxidation (FAO) is known to contribute to the production of mitochondrial acetyl-CoA, its contribution to histone acetylation is limited. Cytosolic acetyl-CoA can serve as a substrate for a number of processes, including acetyl transferases that support the acetylation of many protein classes (Choudhary et al., 2009), as well as a precursor for *de novo* fatty acid synthesis (Wellen and Thompson, 2012; Carey et al., 2015; Moussaieff et al., 2015). It was previously shown that early differentiation of ESCs was correlated to a reduced level of acetyl-CoA production and loss of histone H3 lysine 9 and lysine 27 acetylation, indicating that TCA-derived acetyl-CoA maintains an open chromatin state during pluripotency (Moussaieff et al., 2015). Similarly, it was reported that inhibition of acetyl-CoA resulted in diminished histone acetylation, which in turn stimulated myogenic differentiation (Bracha et al., 2010).

Like other highly proliferative cells, PSCs are dependent on glutamine, which enters the TCA cycle through initial conversion to glutamate by glutaminase, followed by conversion to αKG via glutamate dehydrogenase. αKG is a crucial cofactor for αKG-dependent dioxygenase enzymes, which include JHDMs and TET enzymes (Kaelin and McKnight, 2013). The significance of αKG in regulation of pluripotency through epigenetic modifications has been previously demonstrated by several studies. Addition of cell-permeable dimethyl-αKG (dm-αKG) to culture media was shown to enhance self-renewal, while inhibiting the differentiation of mESCs by promoting histone and DNA demethylation (Carey et al., 2015). The beneficial effect of dm-αKG on pluripotency can be blocked through combined knockdown of the H3K9me2 demethylases, resulting in reduced colony formation. In corroboration with this finding, recent work revealed intracellular αKG can sustain mESCs and hESCs in a glutamine-independent manner (Vardhana et al., 2019). Expression of the pluripotent transcription factors, NANOG or KLF4, in the presence of 2i, resulted in an increased fraction of the TCA cycle intermediates generated from glucose-derived carbon in mESCs and hESCs (Vardhana et al., 2019). Since transient-glutamine depletion eliminated non-pluripotent cells, the authors emphasized that such metabolic rewiring can serve as a selection pressure for pluripotent cells over non-pluripotent cells. A recent study explored a potential functional link between oxidative metabolism, TCA cycle, and αKG on naïve to primed states of mESCs through single-cell analysis (Tischler et al., 2019). The authors demonstrated that isocitrate dehydrogenase 2-mediated production of αKG was critical for sustaining naïve pluripotency in mESCs, even in the absence of 2i. Interestingly, the same study indicates that αKG also contributes to primordial germ cell differentiation (Tischler et al., 2019). This observation is consistent with a previous report that high αKG and αKG-to-succinate ratio promotes differentiation of primed hESCs and mEpiSCs (TeSlaa et al., 2016) through the regulation of histone methylation. This apparent dichotomy between the role of αKG in promoting pluripotency in naïve cells and promoting differentiation in the primed state indicates that metabolite signaling is very dependent on the developmental stage and specific cell type. Overall, the TCA cycle is at the metabolic crossroad contributing to cell fate by coupling its metabolites with both energy production and the chromatin landscape.

Oxidative Phosphorylation

Oxidative phosphorylation is a critical pathway for maintaining bioenergetic homeostasis as it links multiple metabolic pathways including glycolysis, the TCA cycle, and FAO with ATP synthesis. This pathway enables electrons donated from NADH/$FADH_2$ to flow down reduction potential gradients in the ETC and harnesses the energy produced to pump protons across the inner mitochondrial membrane to develop an electrochemical gradient. The transport of protons back across the membrane and into the mitochondrial matrix is performed by ATP synthase, which couples proton transport with ATP synthesis. Compared to glycolysis, OxPhos is a far more efficient pathway for ATP production, producing 36 ATP molecules per glucose, compared to 2 ATP molecules from glycolysis.

Pluripotent stem cells are typically considered to have a lower rate of OxPhos compared to their differentiated counterparts; however, the relative rate of OxPhos is highly dependent on the specific stage of pluripotency. Naïve PSCs display bivalent metabolism, consisting of both glycolysis and OxPhos, in contrast to primed PSCs which have very low rates of oxygen consumption and are almost entirely dependent on glycolysis despite displaying a more mature mitochondrial phenotype (Zhou et al., 2012; Takashima et al., 2014). Although RNA sequencing and microarrays have shown that mitochondrial electron transport genes are down regulated significantly in primed cells, they retain constant levels of mitochondrial DNA and display more elongated mitochondria with better defined cristae compared to their naïve counterparts (Sperber et al., 2015). Indeed, recent work in canine ESCs (cESCs) has demonstrated that inhibition of complex I of the ETC did not alter the proliferation or viability of primed cESCs, while naïve cESCs were sensitive to complex I inhibition, thus supporting the differences in OxPhos dependency of these pluripotent stages. These metabolic preferences may not only be due to cellular ATP and anabolic demand but may also reflect the availability of oxygen and metabolic substrates during these specific stages *in vivo*. Changes in oxygen consumption have been reported during the transition from mouse preimplantation to early post-implantation development, and while these oxygen tensions are typically not maintained during *in vitro* culture, they may be related to the metabolic preferences of naïve vs. primed PSCs. Indeed, the bivalent metabolic phenotype of naïve PSCs may reflect the metabolic preference of the morula and blastocyst, which utilizes a combination of pyruvate oxidation and glycolysis to meet their metabolic demands, while primed PSCs become almost exclusively dependent on glycolysis, reflecting implantation into the hypoxic uterine wall. Indeed, the importance of reduced oxygen tension has been examined across

a number of stem cell populations (Mohyeldin et al., 2010), including a role in improving the acquisition (Yoshida et al., 2009) and maintenance of pluripotency (Ezashi et al., 2005; Forsyth et al., 2006; Prasad et al., 2009; Lengner et al., 2010; Mathieu et al., 2013; Christensen et al., 2015). In part these beneficial effects of physiologically relevant oxygen levels (2–5%) may be due to a reduction in mitochondrial function and oxygen utilization associated with elevated utilization of glucose via glycolysis and amino acid turnover (Forristal et al., 2013; Christensen et al., 2014; Turner et al., 2014; Lees et al., 2015, 2019; Harvey et al., 2016b), although these metabolic changes can occur in the absence of changes in self-renewal (Harvey et al., 2016b). These effects of oxygen may be cell line dependent and may not occur in all iPSC lines, suggesting that metabolic fidelity may represent a marker for PSC and nuclear reprogramming quality (Lees et al., 2015; Harvey et al., 2018; Spyrou et al., 2019). However, the effect of reduced oxygen tension on the transition between naïve and primed pluripotency, as well as the distinct metabolic phenotypes of these states have not been investigated.

Beyond its role in energy generation, the mitochondrial ETC also impacts a number of cellular processes including ROS production (Boveris et al., 1972; Dey et al., 2008), mitochondrial membrane potential (Chen et al., 2014), and mitochondrial protein import (Geissler et al., 2000), which may collectively impact self-renewal and proliferation. While several studies have demonstrated that inhibition of complex I or complex III is associated with impaired cell proliferation (Howell and Sager, 1979; Han et al., 2008; Fendt et al., 2013; Wheaton et al., 2014), the mechanism by which the ETC regulates cell proliferation has only recently been elucidated. Using a CRISPR-based genetic screen Birsoy et al. (2015) revealed that the ETC enables the synthesis of aspartate, which is a precursor for purine and pyrimidine syntheses. To functionally validate the genetic screen, they demonstrated that supplementation with exogenous aspartate or overexpression of aspartate transporter enabled cells without ETC activity to proliferate, while loss of cytosolic aspartate aminotransferase (GOT1), which consumes aspartate to transfer electrons into mitochondria, resulted in cell death upon ETC inhibition. Collectively, this indicates that in addition to the canonical role of mitochondrial ETC in ATP synthesis, it also supports cell proliferation through the generation of aspartate, which functions as an anabolic substrate. In summary, both naïve and primed PSCs have active OxPhos, but the extent to which they rely on OxPhos for energy production and proliferation appears to differ due to the variances in the availability of oxygen and other metabolites that can sustain the high energetic demand of proliferation in PSCs.

Lipid Metabolism

Lipids play vital roles in the maintenance of cellular homeostasis by serving as energy sources, signaling entities and building blocks for membranes. Lipid metabolism represents a carefully regulated balance between catabolism (FAO) and anabolism (*de novo* biosynthesis), which is highly dependent on the metabolic requirements of a specific cell state. FAO consists of active transport of medium and long chain fatty acids into the mitochondria through a regulated carnitine palmitoyl transferase system, with subsequent oxidation by the chain-length specific enzymes of beta-oxidation to generate acetyl-CoA that feeds into the TCA cycle and NADH that donates its electrons to the ETC. In contrast, *de novo* fatty acid biosynthesis requires substrates from multiple metabolic pathways, including acetyl-CoA, reducing factors and ATP, in order to build essential fatty acids. Despite the essential role of lipids in cellular homeostasis, the impact of lipids on PSC maintenance and self-renewal remains relatively unexplored in comparison with other metabolic pathways. Recent studies have begun to examine the impact of lipid availability on hPSCs metabolism and function (Zhang et al., 2016a; Cornacchia et al., 2019) and demonstrated that lipid replete media (E8 and to a lesser extend mTeSR) significantly remodeled the metabolic state in order to sustain lipogenesis (Zhang et al., 2016a). The metabolic reprogramming induced by lipid deficiency significantly increased the oxidative PPP to support NADPH regeneration, increased glutamine consumption and fatty acid biosynthesis, at the expense of oxidative metabolism, which is consistent with the importance of glutaminolysis and the PPP to lipid biosynthesis in PSCs (Varum et al., 2011; Tohyama et al., 2016).

Lipid availability has also recently been shown to regulate the transition between naïve and primed hPSCs, with E8 medium inducing a primed-to-naïve intermediate state of pluripotency associated with increased *de novo* lipogenesis (Cornacchia et al., 2019). The intermediate state in E8-hPSCs recapitulated many of the features of naïve pluripotency, however there were key differences, including a moderate state of DNA hypomethylation, specifically in terms of a global reduction in H3K27me3 and H3K9me3 levels, a known feature of naïve human PSCs. This intermediate state is dependent on lipid-free culture conditions, which promotes active lipid biosynthesis and endogenous ERK inhibition, features which are lost upon lipid supplementation. Interestingly, transcriptional analysis of E8-hPSCs and pre-implantation epiblasts demonstrated that *de novo* lipogenesis is a consistent transcriptional feature across *in vivo* and *in vitro* naïve pluripotency. The lipogenic state also supports the metabolic remodeling of the epigenome, including hyperacetylation of H3K27, H3K9 and H4K8 associated with increased acetyl-CoA metabolism and hypomethylation of DNA due to an increase in αKG to succinate ratio that activates JMJD and TET chromatin demethylases and a reduction in the SAM to S-adenosylhomocysteine (SAH) ratio. Indeed, this metabolic and epigenetic remodeling also increased the propensity for neuroectodermal differentiation, which is consistent with the observation that fatty acid synthase dependent-*de novo* lipogenesis is essential for neural stem cell proliferation and neurogenesis (Knobloch et al., 2013). Therefore, this data supports the concept that baseline pluripotent culture conditions have downstream effects on differentiation capacity.

In addition to their metabolic roles, lipids may also regulate the primed-to-naïve conversion as a signaling molecule (Kime et al., 2016). Lysophosphatidic acid (LPA) lipid signaling and the LPA-producing enzyme autotoxin have been implicated in establishing naïve PSCs in coordination with LIF and bone morphogenetic protein 4 (BMP4) signaling (Kime et al., 2016). These elegant studies support the importance of nutrient

availability for defining stem cell features and suggest that manipulating energy metabolism may be sufficient to promote the transition between stem cell states. These studies indicate that metabolism is intertwined with epigenetics and the transcriptional landscape of PSCs, and additional work is required to determine how these pathways ultimately define the fate of PSCs.

Amino Acid Metabolism

Amino acids are important substrates for the biosynthesis of the basic building blocks of the cell, including proteins, lipids and nucleotides (Locasale, 2013), and as such have been demonstrated to contribute significantly to the maintenance of pluripotency and stem cell fate. Beyond the direct anabolic roles of amino acids, such as *de novo* purine biosynthesis, amino acid metabolism is intimately related to one-carbon metabolism, which consists of the methionine and folate cycles that maintain cellular pools of one-carbon residues associated with S-adenosylmethionine (SAM) and folate (Clare et al., 2019). This one-carbon pool is not only essential for donating methyl groups for the synthesis of amino acids, nucleotides and phospholipids, but SAM also represents the principal substrate for post-translational methylation of RNA, DNA, and proteins, making it a critical connection between changes in metabolism and remodeling of the epigenetic state of the cell.

mESCs are highly dependent on threonine metabolism to maintain their pluripotency and self-renewal (Wang et al., 2009). Threonine dehydrogenase (TDH) and downstream enzymes in threonine metabolism, glycine c-acetyltransferase (GCAT) and glycine decarboxylase (GLDC), are highly expressed in mESCs and are rapidly downregulated during differentiation. Indeed, inhibition of TDH or complete removal of threonine from cell culture medium results in loss of stemness, reduced proliferation, apoptosis, and cell cycle arrest (Wang et al., 2009; Alexander et al., 2011), while L-threonine supplementation and induction of TDH supports induction of pluripotency through nuclear reprogramming (Ryu and Han, 2011; Han et al., 2013; Chen and Wang, 2014). The downstream effects of threonine metabolism on stem cell fate can be contributed to a number of mechanisms, including supplying methyl groups to the one carbon metabolism pool for the biosynthesis of cellular building blocks, as well as maintaining a high ratio of SAM to SAH to promote H3K4me3, which is critical for the maintenance of the pluripotent state (Bernstein et al., 2006; Wang et al., 2009, 2011; Shyh-Chang et al., 2013b). L-threonine may mediate some of its proliferative effects through lipid raft/caveolae-dependent regulation of ERK, p38, JNK/SAPK, and mTORC pathways (Ryu and Han, 2011). In humans, TDH is only expressed as a non-functional pseudogene, thus hESCs rely on methionine metabolism in the same way that mESCs rely on threonine to maintain a high ratio of SAM/SAH (Shiraki et al., 2014). Interestingly, a recent study has also implicated methionine in mESC maintenance downstream of SIRT1 expression (Tang et al., 2017). SIRT1 KO mESCs display an elevated ratio of methionine/SAM due to a reduction in the expression of methionine adenosyltransferase 2a (MAT2a), which catalyzes the conversion of methionine to SAM (Tang et al., 2017). Regulation of methionine metabolism appears to be in part through a SIRT1-dependent protein expression of c-MYC and n-MYC, which bind to the MAT2a promoter and induce its expression. H3k27me3 is also regulated in the naïve state by N-methyltransferase, which consumes SAM, making it unavailable for histone methylation (Sperber et al., 2015).

Proline may also play an important role in regulating PSC identity, as a feedback loop has been identified whereby proline modulates the GCN2-EIF2a-ATF4 amino acid starvation response pathway, which in turn suppresses proline biosynthesis to restrict proliferation and maintain ESC identity (D'Aniello et al., 2015). Supplementation of mESC culture media with proline is sufficient to promote cell proliferation and transition of mESCs to EpiSCs (Washington et al., 2010; Casalino et al., 2011), and induce ESCs into a mesenchymal-like, motile phenotype (Comes et al., 2013; D'Aniello et al., 2015). Interestingly, this effect is completely reversible, as removal of proline results in the restoration of the mESC state. These phenotypic changes may in part be due to an increase in global H3K9 and H3K36 methylation, and can be reversed using vitamin C which promotes the demethylation of these marks (Comes et al., 2013). Ornithine supplementation can also induce a mESC to EpiSC transition, suggesting that this phenotype may be mediated through a common intermediate in the catabolism of proline and ornithine, namely Δ^1-pyrroline-5-carboxylate (Casalino et al., 2011). Collectively, these studies highlight the importance of proline in ESC identity, but further studies are required to decipher the underlying mechanism by which this amino acid impacts stem cell identity.

ENERGY METABOLISM DRIVES ACQUISITION OF PLURIPOTENCY THROUGH NUCLEAR REPROGRAMMING

Multiple studies have now demonstrated that remodeling of energy metabolism plays a critical role early during nuclear reprogramming of somatic cells into iPSCs (Takahashi and Yamanaka, 2006), indicating that metabolic reprogramming in not simply a consequence on cell transition, but a driving force (**Figure 4**). Upregulation of glycolysis has been demonstrated to precede the induction of pluripotency markers and is a critical component for successful nuclear reprogramming (Folmes et al., 2011). Indeed cells that have a glycolytic phenotype reprogram more efficiently than those with greater reliance on OxPhos (Panopoulos et al., 2012) and stimulation of glycolysis enhances reprogramming, while inhibition of glycolytic or stimulation of OxPhos suppresses reprogramming (Yoshida et al., 2009; Esteban et al., 2010; Zhu et al., 2010; Folmes et al., 2011; Prigione et al., 2014). Changes in protein expression of ETC subunits are among the earliest changes that occur during nuclear reprogramming (Hansson et al., 2012), consisting of decreased expression of complex I and IV subunits and increased expression of complex II, III and V subunits. This reorganization of the ETC, particularly the reduced expression of complex I and increased expression of complex II, suggests that $FADH_2$

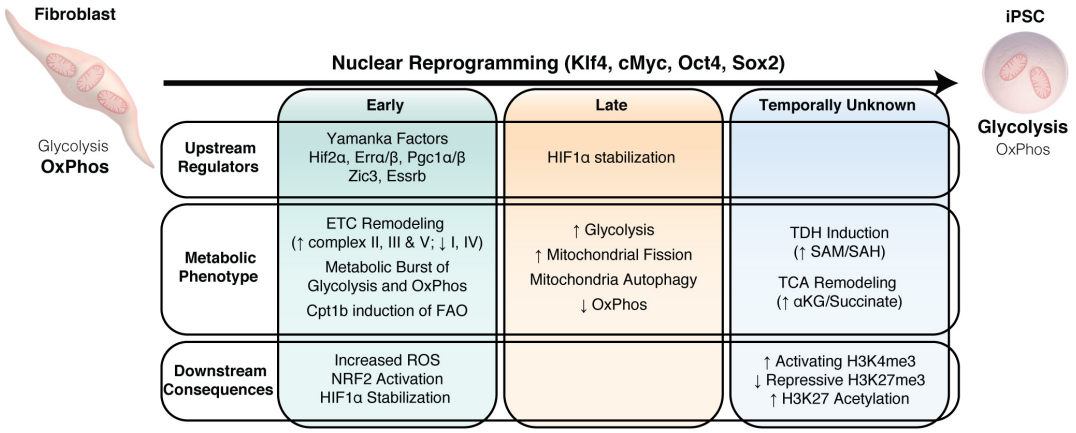

FIGURE 4 | Metabolic remodeling drives nuclear reprogramming. Summary of the temporal changes in upstream regulators, the downstream metabolic changes and the consequences of this metabolic phenotype on nuclear reprogramming.

may be the primary electron donor for ETC function during nuclear reprogramming and in the resultant iPSCs. Functionally, this manifests as a transient burst of OxPhos activity that accompanies the acceleration of glycolysis at the early stage of reprogramming (Prigione et al., 2014; Kida et al., 2015; Hawkins et al., 2016). Indeed, increased ROS generated from OxPhos during this period may be critical for driving the metabolic transition during nuclear reprogramming through modification of cysteine residues on the NRG2 repressor protein KEAP1, leading to NRF2 activation, which subsequently activates HIF1α to support glycolysis (Mathieu et al., 2014; Hawkins et al., 2016). In addition, ZIC3 and ESSRB may act synergistically to promote glycolysis in a HIF-independent mechanism, while offsetting their opposing effects on OxPhos to promote both reprogramming and the conversion of primed PSCs into the naïve state (Sone et al., 2017). As reprogramming progresses, the abundance of mitochondrial DNA and mass gradually decrease as cells undergo autophagic mitochondrial clearance resulting in fewer spherical mitochondria with poorly developed and immature cristae compared to their parental fibroblasts (Folmes et al., 2011; Prigione et al., 2011; Ma et al., 2015b). Mitochondrial fusion/fission dynamics are crucial for somatic cell nuclear reprogramming. Inhibition of the mitochondrial fission protein DRP1 is sufficient to suppress the early stage of reprogramming of somatic cells (Vazquez-Martin et al., 2012), while expression of mitochondrial fusion proteins, such as MFN1 and MFN2, appears to be a barrier for reprogramming (Son et al., 2015). Inhibition of MFN1/MFN2 increases reprogramming efficiency by activating RAS/RAF signaling to enable ROS-mediated HIF1α stabilization to facilitate the transition of OxPhos to glycolytic metabolism (Son et al., 2015). In summary, efficient nuclear reprogramming involves complex and stage specific metabolic changes driven by OxPhos and glycolysis.

Although metabolic remodeling has broadly focused on the transition from predominantly OxPhos to higher glycolytic activity upon nuclear reprogramming (Folmes et al., 2011, 2013a; Prigione et al., 2014; Cao et al., 2015), alternative metabolic pathways, including amino acid and lipid metabolism, are increasingly being identified to play an important role during nuclear reprogramming. As discussed earlier, threonine and methionine are indispensable amino acids to maintain the one-carbon pool that is essential for donating methyl groups for both anabolism and post-translation modifications. Indeed, L-threonine supplementation and induction of TDH supports induction of pluripotency through nuclear reprogramming by promoting H3K4me3 (Ryu and Han, 2011; Han et al., 2013; Chen and Wang, 2014). Recent evidence has also implicated GLDC, a key enzyme in the glycine cleavage, in early metabolic remodeling during nuclear reprogramming and maintenance of the pluripotent state (Kang et al., 2019). GLDC expression occurs early in the reprogramming process and appears to be downstream of the reprogramming factors KLF4 and c-MYC, and reprogramming efficiency can be impaired with GLDC knockdown and enhanced with GLDC overexpression. GLDC knockdown in mESCs leads to a reduction in a number of glycolytic intermediates and the loss of self-renewal, suggesting that GLDC functions in reprogramming by regulating glycolysis (Kang et al., 2019).

De novo fatty acid synthesis also appears critical for acquisition and maintenance of pluripotency. Nuclear reprogramming is accompanied by enhanced lipogenesis due to increased expression of fatty acid synthase (FAS) and acetyl-CoA carboxylase 1 (ACC1) (Wang et al., 2017), and supplementation with exogenous oleic acid can increase reprogramming efficiency. Mechanistically, increased lipogenesis via ACC1 leads to enhanced mitochondrial fission by competing for a limited acetyl-CoA pool, thus blocking acetylation-dependent degradation of the mitochondrial fission 1 protein (FIS1) via the ubiquitin-proteasome pathway. In addition, increased lipid generation also drives mitochondrial dynamics toward a fission phenotype, which ultimately promotes nuclear reprogramming. Interestingly, FAO also appears to regulate nuclear reprogramming, consistent with its crucial role for oocyte and early embryo development (Oey et al., 2005; Dunning et al., 2010; Shyh-Chang et al., 2013a). Long-chain FAO is primarily regulated by the transport of long-chain fatty

acids (LCFA) across the inner mitochondrial membrane by the carnitine palmitoyltransferase (CPT) system, containing CPT1 and CPT2. CPT1b, which catalyzes the rate limiting transfer of the acyl group from coenzyme A to carnitine, was shown to be significantly upregulated at the early stage of reprogramming and overexpression of CPT1b improved reprogramming efficiency (Lin et al., 2018). Addition of palmitoylcarnitine (PC), the product of CPT1, was sufficient to stimulate OxPhos activity and enhance reprogramming only during the first 3 days of reprogramming, after which OxPhos was suppressed with PC supplementation, suggesting that the importance of FAO to reprogram may coincide with the hypermetabolic state observed early in the reprogramming process.

Although significant advances have been made in nuclear reprogramming technology, incomplete reprogramming can generate iPSCs that have transcriptional, epigenetic, and metabolic memory of their parental source (Chan et al., 2009; Kim et al., 2010; Ohi et al., 2011; Lee et al., 2013; Spyrou et al., 2019). While the glycolytic phenotype of iPSCs and ESCs are in general very similar, differences have been identified in specific metabolites in human (polyunsaturated fatty acids, SAM) and mouse (phosphatidylcholine, phosphatidylethanolamine, amino acids and metabolites in polyamine biosynthesis) iPSCs compared to ESCs (Meissen et al., 2012; Panopoulos et al., 2012). Although these differences exist, their functional impact on maintenance of pluripotency or differentiation capacity have not been examined. Partially reprogrammed cells, which are characterized by forming stable ESC-like colonies but not expressing endogenous OCT4, NANOG, SSEA4 and TRA-1-60, appear to have a distinct mitochondria and metabolic profile that are intermediate between fully reprogrammed iPSCs/ESCs and parental fibroblasts in terms of mitochondrial morphology and gene expression, and concentrations of glycolytic and OxPhos intermediates (Lee et al., 2016; Park et al., 2017). This insufficient repression of mitochondrial function and activation of glycolysis in partially reprogrammed cells can be rescued through microRNA 31 overexpression, which suppresses succinate dehydrogenase A activity to promote the transition from OxPhos to glycolysis and enhance reprogramming efficiency (Lee et al., 2016). Interestingly, recent evidence also indicates that dysregulated mitochondria fusion/fission dynamics impairs the ability to achieve full pluripotency and restricts developmental potential of iPSCs (Zhong et al., 2019). Therefore, strategies that optimize mitochondrial and metabolic remodeling during nuclear reprogramming may improve reprogramming fidelity and ultimately their use in downstream applications.

Collectively, these studies demonstrate that complex and coordinated remodeling of energy metabolism is critical to drive efficient nuclear reprogramming, in part thought the resetting of the epigenetic landscape. While this appears to be the case in nuclear reprogramming, early metabolic remodeling may also play an important role in other cell state transitions, including from naive to primed pluripotency and release from pluripotency along lineage specific differentiation.

SUMMARY

Over the past decade, significant advances have been made in our understanding of the metabolic requirements of stem cells and the important role that energy metabolism plays in regulating stem cell function and fate. An emerging concept supports metabolism as not simply a homeostatic system that matches energy supply with energetic demands, but a critical early step during transition between cellular states. This concept is backed by two observations: a) metabolic changes are often some of the earliest to occur during cell fate transitions and b) there is growing appreciation that metabolic pathways directly contribute to the epigenetic remodeling of the cell. This realization has placed emphasis on understanding the microenvironment in which these cells are cultured, as numerous studies have now shown that modifying availability of a single metabolic substrate can dramatically impact stem cell identity. As the field increasingly dissects the finer metabolic distinctions between pluripotent states and discovers how these interact with regulators of stem cell fate, it will be interesting to see if manipulation of substrate supply and energy metabolism may help to enable the derivation and culture of other PSC states, such as totipotent stem cells or formative PSCs. In addition, modulation of energy metabolism may ultimately be harnessed for translational and clinical applications, not only to support manufacturing and lineage specification of PSCs for regenerative therapies, but also to promote innate regenerative capacity and augment current cell-based therapies.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Autophagy and Stem Cells: Self-Eating for Self-Renewal

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Autophagy is a fundamental cell survival mechanism that allows cells to adapt to metabolic stress through the degradation and recycling of intracellular components to generate macromolecular precursors and produce energy. The autophagy pathway is critical for development, maintaining cellular and tissue homeostasis, as well as immunity and prevention of human disease. Defects in autophagy have been attributed to cancer, neurodegeneration, muscle and heart disease, infectious disease, as well as aging. While autophagy has classically been viewed as a passive quality control and general house-keeping mechanism, emerging evidence demonstrates that autophagy is an active process that regulates the metabolic status of the cell. Adult stem cells, which are long-lived cells that possess the unique ability to self-renew and differentiate into specialized cells throughout the body, have distinct metabolic requirements. Research in a variety of stem cell types have established that autophagy plays critical roles in stem cell quiescence, activation, differentiation, and self-renewal. Here, we will review the evidence demonstrating that autophagy is a key regulator of stem cell function and how defective stem cell autophagy contributes to degenerative disease, aging and the generation of cancer stem cells. Moreover, we will discuss the merits of targeting autophagy as a regenerative medicine strategy to promote stem cell function and improve stem cell-based therapies.

Keywords: autophagy, stem cells, self-renewal, quiescence, mitochondria, aging, reprogramming, cancer stem cell

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AUTOPHAGY: A FUNDAMENTAL CELL SURVIVAL MECHANISM

Autophagy is a fundamental cellular process by which cells sequester intracellular constituents, including organelles and proteins, that are delivered to lysosomes for degradation and recycling of macromolecule precursors (Galluzzi et al., 2017). The process of autophagy is evolutionarily conserved from yeast to mammals and serves as an essential adaptation mechanism to provide cells with a source of energy during periods of nutrient deprivation and metabolic stress. Under homeostatic conditions, cells maintain a constitutive basal level of autophagy as a method of turning over cytoplasmic content. Autophagy can also be induced in response to cellular stresses such as nutrient deprivation, oxidative stress, DNA damage, endoplasmic reticulum stress, hypoxia, and infection.

The term autophagy, which literally translates to "self-eating" was invented by Christian de Duve in 1963 to describe the presence of double-membraned vesicles containing cytoplasmic constituents within the cell (Klionsky, 2008; Yang and Klionsky, 2010). These vesicles that encapsulate cytoplasm, organelles and proteins, are known as autophagosomes and are the phenotypic

hallmark of macroautophagy, the most prevalent and extensively characterized form of autophagy. Macroautophagy, which is the focus of this review, will hereby be referred to as autophagy.

Autophagy is a multi-step process of sequential events including induction, nucleation of a phagophore structure, maturation of the autophagosome, autophagosome fusion with the lysosome, and the degradation and recycling of nutrients (**Figure 1**) (Mizushima, 2007). The execution of autophagy is dependent on the formation of several key protein complexes and two ubiquitin-like conjugation steps. Initial studies performed to characterize key players in the autophagy pathway were carried out in yeast and identified a family of autophagy-related genes, referred to as *Atg*, which encode for autophagy effector proteins (Tsukada and Ohsumi, 1993).

Autophagy is negatively regulated by the nutrient-sensing mammalian target of rapamycin (mTOR) kinase, a master regulator of cellular growth and metabolism. Under nutrient-rich conditions, mTOR inhibits autophagy by preventing the formation of the autophagy initiation ULK1 Ser/Thr kinase protein complex. mTOR represses autophagy by direct phosphorylation of ULK1 (the mammalian ortholog of yeast Atg1) at serine residue (Ser) 757. Phosphorylation of ULK1 by mTOR at this site prevents ULK1 from interacting with a second master regulator of metabolism, the energy-sensing AMP-activated protein kinase (AMPK) (Kim et al., 2011). AMPK induces autophagy by alleviating the negative regulation by mTOR through the phosphorylation and activation of TSC2 (Inoki et al., 2003), an upstream negative regulator of mTOR, and via direct phosphorylation of Raptor (Gwinn et al., 2008), a subunit of the mTOR complex. Additionally, AMPK induces the formation of the ULK1 complex through direct phosphorylation of ULK1 at Ser 317 and Ser 777, which result in the activation of ULK1 kinase activity (Kim et al., 2011).

Following activation, ULK1 phosphorylates several downstream targets to initiate the autophagic process. Formation of the ULK1 multi-protein complex requires ULK1-mediated phosphorylation of ATG13 and the scaffold protein FIP200, thus resulting in the assembly of the ULK1-ATG13-FIP200-ATG101 complex (Jung et al., 2009; Zachari and Ganley, 2017). The ULK1 complex translocates to sites of autophagosome initiation, where it is responsible for the activation of a second essential autophagy effector protein complex, the phosphatidylinositol 3-kinase (PI3K) complex.

The PI3K complex is made up of the proteins VPS34, VPS15, Beclin 1, ATG14L, and AMBRA1. VPS34, a class III PI3K, is responsible for producing the phosphatidylinositol 3-phosphate (PI(3)P) that is required for autophagosome formation (Simonsen and Tooze, 2009). Enrichment in PI(3)P at specialized sites form the initiating autophagosome structure, known as the omegasome (Axe et al., 2008). The omegasome serves as a membrane platform, that remains in contact with the endoplasmic reticulum and with vesicles containing the transmembrane autophagy protein ATG9, to recruit the necessary subsequent core autophagy machinery that drive elongation of the autophagosome membrane (Orsi et al., 2012; Karanasios et al., 2016). The haploinsufficient tumor suppressor and critical autophagy effector protein Beclin 1 (the mammalian ortholog of yeast Atg6) is also a key member of the PI3K complex, and can regulate autophagy through its ability to interact with members of the anti-apoptotic BCL-2 family (Yue et al., 2003; Pattingre et al., 2005). BCL-2 directly interacts with Beclin 1 to negatively regulate autophagy, a process that is mediated by the BCL-2 adapter protein CISD2/NAF-1 at the endoplasmic reticulum (Chang et al., 2010).

Elongation of the autophagosome membrane is dependent on two ubiquitin-like conjugation steps (reviewed in Geng and Klionsky, 2008). The first involves the conjugation of the ubiquitin-like protein ATG12 to ATG5, which is catalyzed by ATG7 and ATG10. The ATG12-ATG5 conjugate forms a multi-protein complex with ATG16L1 and functions as an E3-like ligase. The second conjugation step is mediated by ATG7 and ATG3, which together with the ATG5-ATG12:ATG16L1 complex are responsible for conjugating phosphatidyl-ethanolamine to microtubule-associated protein 1 light chain 3 beta (MAP1LC3B, also known as LC3B), which has been proteolytically cleaved by ATG4 (LC3-I). Lipidated LC3B (referred to as LC3-II) is incorporated into the autophagosome membrane during elongation and is commonly used as an experimental marker to detect and quantify autophagosomes within cells (Kabeya et al., 2000).

Mature autophagosomes carrying cytoplasmic cargo are trafficked to lysosomes, and subsequent autophagosome-lysosome fusion is mediated by the Rab family of small GTPases, SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, and membrane tethering proteins (reviewed by Nakamura and Yoshimori, 2017). The contents of the autophagolysosome are degraded by lysosomal acidic hydrolases and are exported back to the cytoplasm to be reused for metabolic processes.

THE PHYSIOLOGICAL IMPORTANCE OF AUTOPHAGY

Autophagy plays critical roles during embryonic development and is essential for maintaining cell survival, tissue homeostasis, and immunity. Importantly, dysfunctional autophagy has been linked to cancer, infectious diseases, neurodegeneration, muscle and heart diseases, as well as aging (Levine and Kroemer, 2008). Accumulating evidence demonstrates that autophagy is also critical for stem cell function. Adult stem cells are long-lived cells that are capable of undergoing differentiation to maintain tissue homeostasis and contribute to tissue repair, while also possessing the capacity for self-renewal to support the life-long maintenance of the stem cell reservoir. This review will focus on the emerging role of autophagy specifically in somatic stem cells (**Figure 2**) and discuss the merits of targeting autophagy as a therapeutic strategy for modulating stem cell function in regenerative medicine (**Figure 3**).

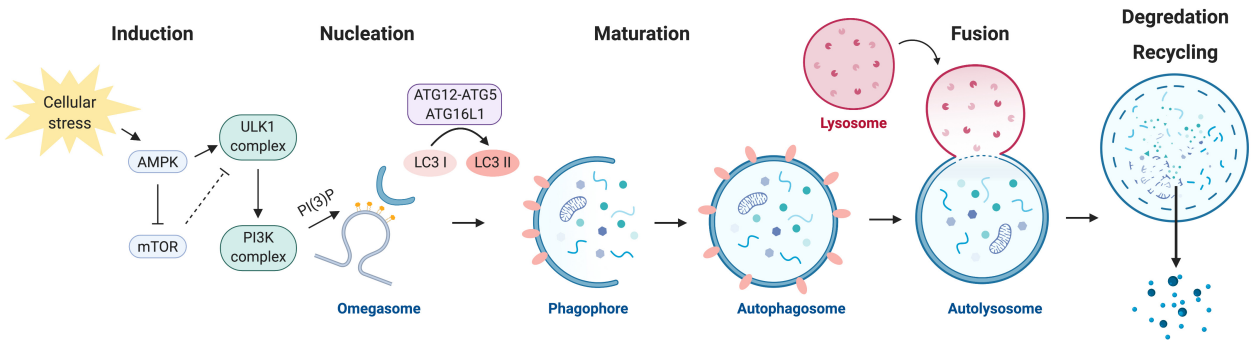

FIGURE 1 | The autophagy program. Autophagy is negatively regulated by mTOR and positively regulated by AMPK. Activation of autophagy involves the formation of the ULK1 and PI3K protein complexes. Nucleation of the autophagosome structure involves the generation of PI(3)P at the omegasome. Maturation and elongation of the autophagosome membrane involves two ubiquitin-like conjugation steps, the conjugation of ATG12 to ATG5 and the conversion of LC3 I to LC3 II. Fully formed autophagosomes fuse with lysosomes to degrade their intracellular content and recycle macromolecule components.

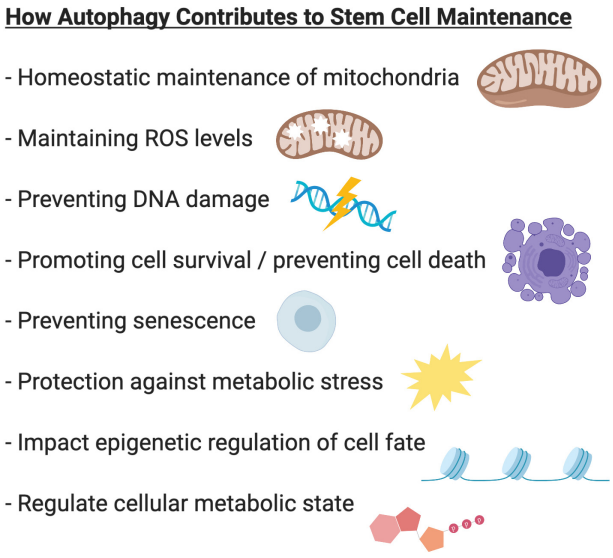

FIGURE 2 | Summary of the role of autophagy in stem cell biology. In various stem cell systems, autophagy plays a critical role in stem cell maintenance. Autophagy regulates mitochondrial content to adapt to the metabolic requirements of the cell. Autophagy prevents accumulation of damaged mitochondria and the production of reactive oxygen species (ROS). Autophagy protects cells against metabolic stress and prevents genome instability, cell death and senescence. Through its ability to regulate epigenetic and metabolic programs, autophagy can also influence cell fate and regulate stem cell quiescence, activation, differentiation, and self-renewal.

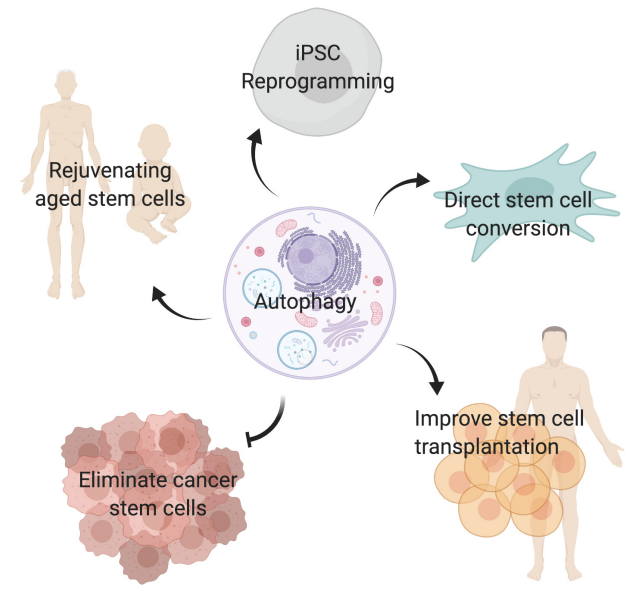

FIGURE 3 | Targeting autophagy in stem cells. Inducing autophagy may improve aged stem cell function, promote reprogramming of somatic cells into induced pluripotent stem cells (iPSC), facilitate direct conversion of somatic cells into tissue specific stem cells, and improve stem cell transplantation therapies. In contrast, inhibiting autophagy in cancer stem cells may be a valid strategy to eliminate tumor initiating cells and prevent cancer treatment resistance.

HEMATOPOIETIC STEM CELLS

Adult hematopoietic stem cells are multipotent stem cells residing in the bone marrow that give rise to both myeloid and lymphoid lineages to generate blood cells, a process termed hematopoiesis. Hematopoiesis supports the life-long daily generation of blood cells within the circulation that are in constant turnover. Thus, hematopoiesis is a highly orchestrated process that is required to maintain a balance between hematopoietic stem cell quiescence, activation, and differentiation. Importantly, perturbations in the regulation of hematopoiesis underlie blood disorders such as anemia (characterized by the loss of red blood cells) and leukemia (blood cancer caused by elevated numbers and defective maturation of leukocytes).

There are multiple lines of evidence supporting an essential role for autophagy in the maintenance and function of hematopoietic stem cells. Deletion of *Atg7* in the hematopoietic system resulted in a significant reduction in hematopoietic stem

cells and progenitors of multiple lineages, indicating a critical role for autophagy in the maintenance of the hematopoietic stem cell compartment. Additionally, *Atg7*-deficient hematopoietic stem cells exhibited an accumulation of damaged mitochondria, increased levels of reactive oxygen species (ROS), and DNA damage (Mortensen et al., 2011).

Quiescent hematopoietic stem cells exhibit low oxidative phosphorylation levels and switch to a high oxidative phosphorylation metabolic state following their activation (Yu et al., 2013). Upon deletion of *Atg12* in hematopoietic stem cells, Ho et al. (2017) observed increased mitochondrial content accompanied by an activated metabolic state and enhanced myeloid differentiation, features that resemble an aging phenotype. Moreover, *Atg12*-deficient hematopoietic stem cells exhibited impaired self-renewal and regenerative potential, as determined by transplantation experiments. These findings indicate that autophagy plays an essential role in removing activated mitochondria as a mechanism for controlling oxidative metabolism to maintain hematopoietic stem cell quiescence and self-renewal. Interestingly, autophagy-dependent metabolic activation was linked to epigenetic reprogramming, as detected by changes in the DNA methylation profile of *Atg12*-deficient hematopoietic stem cells. Thus, basal autophagy is an important mediator of hematopoietic stem cell fate determination by controlling epigenetic-mediated mitochondrial content and metabolism (Ho et al., 2017).

While basal autophagy contributes to stem cell maintenance, hematopoietic stem cells are also capable of inducing the autophagy program in response to metabolic stress as a protective mechanism to prevent cell death. Hematopoietic stem cells express high levels of FOXOA3 (Warr et al., 2013), a transcription factor that targets and activates the transcription of pro-autophagy genes upon induction of the autophagy program (Mammucari et al., 2007). By maintaining high levels of FOXOA3, hematopoietic stem cells are poised for the rapid induction of autophagy genes in response to metabolic stress (Warr et al., 2013). In agreement with these results, *Foxo3a*-null aged mice exhibit a reduced hematopoietic stem cell pool, and loss of FOXOA3 results in impaired self-renewal capacity and defective maintenance of stem cell quiescence (Miyamoto et al., 2007).

In the context of aging, basal autophagy was found to be increased in a subset (around 30%) of aged hematopoietic stem cells compared to their younger counterparts (Ho et al., 2017). Augmented autophagy in a population of aged hematopoietic stem cells is attributed to the need for maintaining life-long survival of old hematopoietic stem cells within the aging bone marrow stem cell niche and conditions of reduced nutrient availability (Warr et al., 2013). It will be interesting to investigate why aged hematopoietic stem cells exhibit heterogeneity with respect to autophagic activity and to understand why hematopoietic stem cells have evolved an augmented autophagy response to aging in contrast to the dampened autophagy observed in other tissues (Rubinsztein et al., 2011).

Additionally, defective autophagy in the hematopoietic stem cell compartment can contribute to blood diseases. Defects in erythropoiesis, the process of generating red blood cells (erythrocytes), result in anemia, a blood condition that is characterized by the reduction of red blood cells. In erythroblasts, the autophagy pathway is critical for the selective clearance of mitochondria during erythroid differentiation. In a hematopoietic specific and inducible *Atg7* knockout mouse model (*Vav-iCre*:*Atg7*$^{fl/fl}$), loss of *Atg7* resulted in severe anemia and eventual lethality at 8–14 weeks of age (Mortensen et al., 2010). Moreover, in an inflammatory cytokine-induced model of anemia in human hematopoietic stem/progenitor cells, it was found that TNFα-induction of anemia occurs via inhibition of autophagy in an mTOR-dependent manner (Orsini et al., 2019). Of note, not all hematopoietic lineages were equally affected by the loss of autophagy, suggesting distinct mechanisms in which autophagy contributes toward hematopoietic differentiation (Mortensen et al., 2010; Rožman et al., 2015).

NEURAL STEM CELLS

Somatic neural stem cells are multipotent self-renewing stem cells that reside in distinct niches within the subventricular zone of the lateral ventricles and subgranular zone of the hippocampal dentate gyrus of the adult brain. The progeny of neural stem cells, termed neural progenitor cells, can proliferate and differentiate into the three main cell types of the nervous system; neurons, astrocytes, and oligodendrocytes. While the importance of autophagy during embryonic development of the nervous system has been well-documented (reviewed in Boya et al., 2018; Casares-Crespo et al., 2018), the contribution of autophagy in adult neural stem cells and postnatal neurogenesis remain less well-defined. Of note, there is a lack of animal studies that employ genetic deletion of autophagy genes specifically in postnatal neural stem cells. Studies examining the impact of autophagy on the adult neural stem cell population have utilized animal models where the deletion of autophagy genes was performed during development. This makes it difficult to discern the effects of autophagy loss during postnatal neurogenesis that is independent from effects of autophagy loss in the embryo.

Similar to hematopoietic stem cells, transcriptional regulation of the autophagy program in neural stem cells is mediated by the transcription factor FOXO3. In *Foxo3*-deficient neural stem cells, defective autophagy results in the accumulation of protein aggregates (Audesse et al., 2019). Interestingly, another study found that lysosome-associated genes are highly upregulated in quiescent neural stem cells in contrast to activated neural stem cells that express high levels of proteasome-associated genes (Leeman et al., 2018). During quiescence, neural stem cells were found to contain an abundance of large lysosomes that contained insoluble protein aggregates. Inhibiting lysosome function resulted in the accumulation of these aggregates and impaired stem cell activation in response to growth factors. In contrast, activation of the autophagy pathway resulted in the clearance of protein aggregates and enhanced stem cell activation. Aged quiescent neural stem cells exhibited reduced lysosome content, increased amounts of protein aggregates and impaired stem cell activation that was reversible upon induction of lysosome activity. This study highlights the importance of

lysosome-mediated protein degradation mechanisms (such as autophagy) in neural stem cells and also suggests a physiological role for protein aggregates in healthy neural stem cells (Leeman et al., 2018). It will be interesting to see if other stem cell systems exhibit similar protein aggregate content and lysosome dynamics in the transition between quiescence and activated states.

Also similar to the hematopoietic system, there is evidence supporting a role for mitochondria in the regulation of neural stem cell fate. While in hematopoietic stem cells, autophagy is critical for clearing metabolically active mitochondria to maintain quiescence (Ho et al., 2017), the role of mitochondria in neural stem cells is focused on the regulation of ROS-mediated signaling (Khacho et al., 2016). In neural stem cells, elongated mitochondria regulate self-renewal by maintaining low levels of ROS. During stem cell activation, the mitochondria transition to a fragmented state resulting in increased levels of ROS, which subsequently induce a gene expression signature that inhibits self-renewal while promoting commitment and differentiation (Khacho et al., 2016). Further studies are required to determine if autophagy, or even mitophagy (the selective autophagic degradation of mitochondria), plays a role in modulating the switch in mitochondrial dynamics between self-renewing and differentiating neural stem cells.

In another study, FIP200, a component of the ULK1-autophagy activating complex was deleted in neural stem cells in mice using *GFAP-Cre*, where expression of Cre recombinase is under the control of human glial fibrillary acidic protein (GFAP). Developmental deletion of *FIP200* resulted in increased mitochondrial content and ROS levels in postnatal neural stem cells, which lead to progressive depletion of the adult neural stem cell pool (Wang C. et al., 2013). Intriguingly, the same group deleted the autophagy genes *Atg5* and *Atg16L1* using the same *GFAP-Cre* deletion strategy and found no impact on neural stem cell maintenance (Wang et al., 2016).

With respect to differentiation, *in vitro* neurosphere assays with *FIP200-null* neural progenitor cells indicated defects in self-renewal and neural differentiation (Wang C. et al., 2013). Moreover, GFAP-mediated deletion of *FIP200* resulted in increased infiltration of microglia immune cells into the subventricular zone, which inhibited differentiation of neural stem cells. Thus, in addition to a cell autonomous role for FIP200 in neural stem cells, FIP200 also influences neural differentiation via extrinsic mechanisms to restrict microglia infiltration (Wang et al., 2017). Additional *in vitro* studies in primary rat hippocampal neural stem cells have indicated that autophagic flux increases during neural differentiation. Depletion of the autophagy genes *Atg7*, *LC3,* and *p62* using lentiviral shRNA and CRISPR/Cas9 approaches had an inhibitory effect on astrogenesis (Ha et al., 2019). These results collectively demonstrate that autophagy plays a contributing role in neural differentiation.

In addition, autophagy has also been shown to promote survival and prevent cell death in neural stem cells. Adult neural stem cells isolated from *Ambra1* and *Beclin1* heterozygous mice exhibited reduced cell survival and impaired neural differentiation *in vitro* (Yazdankhah et al., 2014). Additional studies using a retroviral strategy to delete *Atg5* in dividing neural progenitor cells in the adult brain found that *Atg5* is critical for the survival of proliferating neural progenitor cells. Loss of *Atg5* in neural progenitor cells resulted in cell death that was dependent on the expression of BAX, a pro-apoptotic member of the BCL-2 family. Interestingly, the subpopulation of *Atg5-null* progenitor cells that survive are able to undergo differentiation and generate functional neurons despite a delay in their maturation (Xi et al., 2016).

Of note, although autophagy is typically viewed as a survival mechanism, induction of autophagy can also result in cell death. Primary rat hippocampal neural stem cells undergo autophagic cell death in response to metabolic stress induced by insulin withdrawal and oxygen-glucose deprivation *in vitro* (Ha et al., 2017; Chung et al., 2018). Additionally, autophagy-mediated cell death programs also occur *in vivo* in mice subjected to chronic restraint stress. Neural stem cells are protected against autophagy-mediated cell death in mice where *Atg7* is conditionally deleted (using a *Nestin-CreERT2:Atg7*$^{fl/fl}$ mouse model) (Jung et al., 2020). Thus, the contribution of autophagy in neural stem cells can be beneficial and detrimental depending on the context in which autophagy is being induced.

Dysfunctional autophagy has been shown to contribute to the development of neurodegenerative diseases, including Alzheimer, Parkinson and Huntington disease (reviewed in Menzies et al., 2015). In the absence of autophagy, persistence and defective clearance of misfolded proteins and protein aggregates result in progressive neurodegeneration. The contribution of autophagy in neural stem cells within the context of neurodegeneration is not well-understood. Loss of either *Atg5* or *Atg7* in neural cells under the control of the Nestin promoter (*Nestin-Cre*) were shown to cause neurodegenerative disease in mice (Hara et al., 2006; Komatsu et al., 2006). As these models perturb the autophagy pathway during embryonic neural development, it will be critical to determine how disrupting autophagy in postnatal neural stem cells contributes to neurodegeneration during adulthood, which would be more relevant to aging-related neurodegeneration. Understanding the role of autophagy in adult neural stem cells is essential for the development of strategies that aim to simulate endogenous repair mechanisms within the neural stem cell and progenitor population for the treatment of neurodegenerative diseases.

MUSCLE STEM CELLS

In skeletal muscle tissue, muscle homeostasis and regeneration are mediated by resident muscle stem cells, which are also known as satellite cells. Short-term caloric restriction in mice was found to increase satellite cell number and improve muscle regeneration and satellite cell transplantation efficiency (Cerletti et al., 2012), suggesting that nutrient deprivation-induced autophagy may enhance muscle stem cell function.

In stark contrast to stem cells in the hematopoietic system, aged muscle cells were reported to have impaired basal autophagy levels. Using GFP-LC3 mice, quiescent satellite cells from young mice were found to exhibit robust levels of autophagic flux which was reduced in an age-dependent manner in satellite cells

from old and geriatric mice (García-Prat et al., 2016). Moreover, stimulating autophagy in old satellite cells improved stem cell function, as determined by transplantation and engraftment assays, and prevented aging-induced entry into senescence. Genetic deletion of *Atg7* in satellite cells resulted in a loss of the satellite cell pool, indicating that autophagy is important for quiescent satellite cell maintenance. Residual satellite cells were found to express markers of senescence and DNA damage, which resembled an aging phenotype, and were unable to participate in muscle regeneration. *Atg7*-deficient satellite cells, similar to old satellite cells, exhibited defective mitophagy and increased levels of ROS. Altogether, these results indicate that autophagy in muscle stem cells is required to prevent stem cell senescence and that inducing the autophagic pathway may improve muscle stem cell function in the context of aging (García-Prat et al., 2016).

Of note, autophagy genes in muscle stem cells were found to be expressed in an oscillatory fashion, expressing higher levels of autophagy and lysosome markers during the day compared to at night. The rhythmic expression of autophagy genes is abrogated in aged muscle stem cells, which can be rescued by subjecting mice to a caloric restriction diet (Solanas et al., 2017). Intriguingly, supplementing nicotinamide adenine dinucleotide (NAD^+) in the diet of aged mice lead to an improvement in aged muscle stem cell function and increased life span. NAD^+ repletion enhanced mitochondrial function and prevented muscle stem cell senescence (Zhang H. et al., 2016). It will be interesting to examine if NAD^+-mediated improvement in muscle stem cell function occurs via stimulation of the autophagy pathway.

On a different note, another study reported that autophagy is induced during satellite cell activation (Tang and Rando, 2014) to help satellite cells adapt to the increased metabolic demands associated with the switch from quiescence to activation (reviewed by Nguyen J.H. et al., 2019). In contrast to the study by García-Prat and colleagues, Tang and colleagues also used GFP-LC3 mice and reported undetectable levels of autophagic flux in quiescence satellite cells and increased autophagic flux in activated satellite cells. Notably, Tang and colleagues also found that inhibition of autophagy lead to a delay in satellite cell activation and did not result in permanent cell cycle arrest. The delay in cell cycle entry was attributed to insufficient ATP levels to support the cellular energy demands associated with stem cell activation (Tang and Rando, 2014). In another study, Fiacco and colleagues performed immunostaining for the autophagy marker LC3 on muscle cross-sections and were unable to detect LC3 expression in resting muscle. Following muscle damage, however, they observed an induction in LC3 expression in regenerating muscle. LC3 immunostaining in combination with myogenic markers for commitment and self-renewal revealed that committed myogenic progenitors (expressing the myogenic commitment factor MYOD) were mostly positive for LC3 expression and not the self-renewing population (PAX7-expressing cells), suggesting that autophagy plays a contributing role during myogenic differentiation (Fiacco et al., 2016). Thus, further investigation is required to resolve these contrasting and potentially overlapping results concerning the role of autophagy in muscle stem cell biology.

Muscle stem cell dysfunction is one of the contributing factors in Duchenne muscular dystrophy (Dumont et al., 2015; Chang et al., 2018), a severely debilitating muscle degenerative disease that results from the genetic loss of the dystrophin gene *Dmd* (reviewed in Chang et al., 2016). Impaired autophagy has been reported in muscles from both dystrophic mouse models and Duchenne muscular dystrophy patients (Spitali et al., 2013; Fiacco et al., 2016). Although there is some indication that the expression of LC3 is reduced in committed myogenic progenitors in muscle biopsies from Duchenne muscular dystrophy patients (Fiacco et al., 2016), it remains unclear how autophagy is impaired specifically in the dystrophic muscle stem cell population and how this may contribute to disease progression.

INDUCED PLURIPOTENT STEM CELLS

Cellular reprogramming of differentiated somatic cells into undifferentiated pluripotent stem cells has been made possible through the pioneering work of Shinya Yamanaka. Ectopic expression of the pluripotency transcription factors Oct4, Sox2, Klf4, and c-Myc permits the resetting of somatic cells into undifferentiated cells that resemble embryonic stem cells, termed induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Interestingly, autophagy has been shown to be required for the quality and efficiency of the somatic reprogramming process.

In particular, autophagy is upregulated during the early stages of reprogramming, which corresponds to an inhibition in mTOR signaling (Wang S. et al., 2013). Inhibitors of mTOR, such as rapamycin, were found to increase the efficiency of the reprogramming process, while activating mTOR impeded reprogramming (Chen et al., 2011; He et al., 2012). Of note, mTOR inhibition at later stages was found to inhibit reprogramming, indicating that early upregulation of autophagy is transient and its downregulation is required to complete the reprogramming process (Wang S. et al., 2013).

It has been proposed that autophagy may promote reprogramming by preventing cell death and cellular senescence, which impede the reprogramming process. However, an alternative explanation for the role of autophagy during reprogramming may be the ability to mediate mitochondrial remodeling. Indeed, stem cells in general have less mitochondria than their differentiated counterparts, in accordance with a heavy reliance on glycolysis rather than oxidative phosphorylation for energy production in stem cells (Ito and Suda, 2014). Metabolic transitions from oxidative phosphorylation to glycolysis during reprogramming have also been observed (Folmes et al., 2011). Several autophagy and mitophagy genes play a critical role in mitochondrial homeostasis and clearance of somatic mitochondria during reprogramming including ATG3, PTEN-induced putative kinase 1 (PINK1), and BCL2-interacting protein 3 like (BNIP3L) (Liu et al., 2016; Vazquez-Martin et al., 2016; Xiang et al., 2017). However, the molecular regulation of autophagy and/or mitophagy-dependent remodeling during somatic reprogramming require further elucidation.

CANCER STEM CELLS

Cancer stem cells, also known as tumor-initiating cells, are a subpopulation of cells within a tumor that are highly tumorigenic and able to hyperactively self-renew and differentiate to generate the different cell types within a heterogenous tumor (Reya et al., 2001). Cancer stem cells were first identified in acute myeloid leukemia (AML) as a population of cells that were able to regenerate AML when transplanted into immune-compromised mice (Bonnet and Dick, 1997). Since the initial discovery of a tumor-initiating cell with self-renewing and differentiating capabilities, cancer stem cells have been identified in many other types of cancers including cancers of the brain, breast, lung, pancreas, colon, ovary, and skin. A key property of cancer stem cells is their ability to maintain a quiescent dormant state and resist anti-cancer treatment, which is a significant contributing factor to tumor recurrence following therapy.

The role of autophagy in cancer is complex as there is evidence demonstrating that autophagy can both promote and inhibit tumorigenesis (White, 2012). On one hand, autophagy has been established as an essential cytoprotective mechanism that safeguards cells against oncogenic stresses such as oxidative stress and DNA damage, thus preventing chromosomal instability and tumorigenesis. Autophagy also protects cells from undergoing necrotic cell death and triggering inflammatory responses, which also contribute to oncogenesis. On the other hand, cancer cells, which are subject to conditions of hypoxia and reduced nutrient availability, upregulate basal autophagy levels in order to promote their own survival. Additionally, induction of autophagy in cancer cells can also prevent the effectiveness of anti-cancer treatment. Altogether, these findings indicate that the contribution of autophagy in cancer is highly complex and context-dependent.

Given the evidence demonstrating that autophagy is essential for stem cell maintenance in multiple stem cell types (**Figure 2**), it is not surprising that autophagy also plays critical roles in cancer stem cell maintenance and function. In breast cancer, cancer stem cells are described as a population of $CD44^{high}/CD24^{-/low}$ expressing cells that express high levels of aldehyde dehydrogenase 1 (ALDH1). Functionally, breast cancer stem cells possess the ability to regenerate breast cancer tumors *in vivo* upon transplantation into the mammary fat pad of immunodeficient mice and can form floating spheres, termed mammospheres, when cultured in serum-free media *in vitro* (Al-Hajj et al., 2003; Dontu et al., 2003; Ginestier et al., 2007). When comparing breast cancer stem cell-enriched mammospheres to heterogenous cultures of adherent breast cancer cells, one study found that autophagic flux and Beclin 1 expression are higher in mammospheres. As well, both proliferation and maintenance of mammospheres were dependent on Beclin 1 expression (Gong et al., 2013). Mechanistically, a subsequent study found that autophagy supports the survival of breast cancer stem cells by mediating the secretion of interleukin-6 (IL6), a cytokine that is important for breast cancer stem cell maintenance (Maycotte et al., 2015). In transplantation assays, depletion of Beclin 1 in breast cancer stem cells inhibited xenograft tumor formation in immunodeficient mice. Interestingly, Beclin1 deletion in adherent breast cancer cells had the opposite effect. Tumor generation was enhanced upon transplantation of Beclin 1-deleted adherent breast cancer cells (Gong et al., 2013), which supports the role of Beclin 1 as a general tumor suppressor (Yue et al., 2003). These distinct differences in the requirement for Beclin1 in tumor formation in differentiated cancer cells versus cancer stem cells may provide an explanation as to why autophagy both promotes and inhibits tumorigenesis in cancer.

In chronic myeloid leukemia (CML), $CD34^{+}$ CML stem and progenitor cells expressed increased levels of autophagy genes. Specifically, ATG4B was highly upregulated at both the transcript and protein levels, indicating that ATG4B may be a potential biomarker and target for CML cancer stem cells. Interestingly, leukemic stem cells that were refractory to treatment with imatinib mesylate, a potent tyrosine kinase inhibitor, expressed higher levels of ATG4B in comparison to cells that responded well to imatinib. Knockdown of ATG4B impaired cell proliferation, colony-formation capacity and cell survival of CML stem cells. Importantly, depletion of ATG4B also sensitized CML cancer stem cells to imatinib treatment, which indicates that inhibiting autophagy in combination with imatinib would have therapeutic potential in the treatment of CML (Rothe et al., 2014).

Similarly, in AML, genetic deletion of *Atg5* and *Atg7*, delayed tumor progression of leukemic mice and reduced the function and survival of AML-initiating stem cells. In the absence of autophagy, AML stem cells exhibited increased mitochondria, enhanced levels of ROS, and a higher frequency of apoptotic cell death. Moreover, in the absence of *Atg7*, *in vivo* treatment of leukemic mice with the chemotherapeutic drug cytarabine, commonly known as AraC, resulted in selective targeting of AML-initiating cells and greatly enhanced the potency of AraC treatment (Sumitomo et al., 2016). These findings demonstrate that autophagy plays a critical role in cancer stem cell survival, which contribute to cancer therapeutic resistance.

TABLE 1 | The role of autophagy in different stem cell types.

Stem cell	Autophagy contribution
Hematopoietic stem cell	Quiescence and maintenance
	Self-renewal
	Metabolism
	Protection against metabolic stress
Neural stem cell	Quiescence and maintenance
	Self-renewal
	Clearance of protein aggregates
	Survival
	Differentiation
Muscle stem cell	Quiescence and maintenance
	Prevent senescence
	Activation
	Metabolism
Induced pluripotent	Reprogramming
stem cell	Cellular remodeling
Cancer stem cell	Self-renewal
	Survival

As well, a recent study found that human AML stem cells exhibited high expression of the mitochondrial fission 1 (FIS1) protein and unique mitochondrial morphology. AML stem cells were highly dependent on FIS1-mediated mitophagy that is induced by constitutively active AMPK. Importantly, disruption of either FIS1 or AMPK in AML stem cells induced myeloid differentiation and cell cycle arrest, and greatly reduced engraftment potential (Pei et al., 2018). Additionally, high expression of the autophagy receptor p62 was associated with poor prognosis in human AML. Specifically in leukemia cells, p62 interacts with damaged mitochondria to mediate their clearance via mitophagy. Loss of p62 impaired leukemia cell proliferation and survival, and delayed leukemia development in an AML mouse model (Nguyen T.D. et al., 2019). These findings indicate a specific role for mitophagy in the self-renewal and maintenance of AML stem cells and provide the rationale for developing compounds that target selective autophagy in leukemia.

MODULATING AUTOPHAGY IN STEM CELLS

While it appears that targeting autophagy to inhibit the autophagy-mediated cell survival properties in cancer stem cells may hold promise for anti-cancer therapy, the importance of autophagy in maintaining normal stem cell function suggest that inducing autophagy may have therapeutic potential for regenerative medicine (**Figure 3**). Certainly within the context of aging, stimulation of autophagy via genetic and pharmacological approaches in aged stem cells have improved their regenerative capacity and function (García-Prat et al., 2016; Leeman et al., 2018).

Intriguingly, few studies have examined the direct impact of autophagy modulation in stem cell transplantation therapy. Stem cell transplantation is an exciting approach in regenerative medicine as it allows delivery of functional stem cells to repair the damaged or degenerative tissue with the potential of repopulating the stem cell niche and providing a long-term means of correcting the degenerative phenotype. However, various limitations have impeded the use of stem cell transplantation within the clinic including difficulty in expanding stem cell cultures *ex vivo*, poor survival of engrafted stem cells, preferred differentiation and limited self-renewing capacity of transplanted cells, as well as the risk of causing graft-versus-host disease. As an example, hematopoietic stem cells isolated from human umbilical cord blood hold tremendous potential for hematopoietic stem cell transplantation therapy. However, widespread clinical use of cord blood as a primary source of stem cells is limited by the low yield of hematopoietic stem and progenitor cells within each cord blood unit. In a recent study, *ex vivo* culturing of human cord blood-derived hematopoietic stem cells in the presence of *N*-(4-hydroxy-phenyl) retinamide (4HPR), an inhibitor of the sphingolipid enzyme DEGS1, enhanced self-renewal of a specialized population of long-term hematopoietic stem cells (Xie et al., 2019). 4HPR treatment increased long-term repopulating cell frequencies following serial xenotransplantation into immune deficient mice compared to untreated cells. Intriguingly, 4HPR-mediated improvement of hematopoietic stem cell function was attributed to activation of quality control proteostasis programs including autophagy and the unfolded protein response. Thus, the identification of compounds that can increase stem cell expansion and simultaneously preserve stem cell self-renewing functions show great promise for the future of stem cell transplantation approaches (Fares et al., 2014; Xie et al., 2019).

Moreover, directed differentiation of iPSCs or direct reprogramming from somatic cells into the various types of tissue-specific stem cells provides the possibility of generating an unlimited supply of stem cells. In addition, correction of genetic defects via CRISPR/Cas9-mediated approaches in patient cells along with reprogramming avoids the problem of transplantation-related immune complications. Interestingly, SMER28, a small molecule enhancer of rapamycin, which enhances autophagy and has been shown to induce clearance of autophagy substrates in fly models of Huntington's disease (Sarkar et al., 2007), was identified in a small molecule screen to improve the efficiency of neural stem cell reprogramming (Zhang M. et al., 2016). It will be interesting to determine the molecular mechanism in which autophagy contributes to reprogramming and examine the benefits of modulating autophagy in reprogramming protocols.

CONCLUDING REMARKS

Historically, autophagy has been viewed as a non-selective housekeeping process that is upregulated during conditions of nutrient deprivation to provide the cell with an alternative source of energy. Our understanding of autophagy has evolved and increasing evidence demonstrates that autophagy is an active program that controls the metabolic status of the cell. In stem cells, autophagy is emerging as an important mechanism for the homeostatic maintenance, function and survival of long-lived stem cells. Autophagy can also influence cell fate decisions through its ability to influence mitochondrial content, energy production, and epigenetic programming (summarized in **Table 1**). Particularly in the context of aging and degenerative conditions that involve the decline of stem cell regenerative capacity, autophagy plays key roles in protecting stem cells against cellular stress and is transpiring as a viable target in regenerative medicine.

AUTHOR CONTRIBUTIONS

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The Role of Metabolic Changes in Shaping the Fate of Cancer-Associated Adipose Stem Cells

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Adipose tissue in physiological and in metabolically altered conditions (obesity, diabetes, metabolic syndrome) strictly interacts with the developing tumors both systemically and locally. In addition to the cancer-associated fibroblasts, adipose cells have also recently been described among the pivotal actors of the tumor microenvironment responsible for sustaining tumor development and progression. In particular, emerging evidence suggests that not only the mature adipocytes but also the adipose stem cells (ASCs) are able to establish a strict crosstalk with the tumour cells, thus resulting in a reciprocal reprogramming of both the tumor and adipose components. This review will focus on the metabolic changes induced by this interaction as a driver of fate determination occurring in cancer-associated ASCs (CA-ASCs) to support the tumor metabolic requirements. We will showcase the major role played by the metabolic changes occurring in the adipose tumor microenvironment that regulates ASC fate and consequently cancer progression. Our new results will also highlight the CA-ASC response *in vitro* by using a coculture system of primary ASCs grown with cancer cells originating from two different types of adrenal cancers [adrenocortical carcinoma (ACC) and pheochromocytoma]. In conclusion, the different factors involved in this crosstalk process will be analyzed and their effects on the adipocyte differentiation potential and functions of CA-ASCs will be discussed.

Keywords: adipose precursors, metabolic reprogramming, Warburg effect, p107, adrenal tumors

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INTRODUCTION: OBESITY-CANCER ASSOCIATION

Obesity is a main risk factor for cancer development in many types of solid tumors, suggesting an association between these two pathologies and a new perspective of cancer as a metabolic pathology, which may open new therapeutic prospects. Though evidence reports obesity as a risk factor for the development of solid tumors (Kyrgiou et al., 2017), it is less clear whether excess body weight is associated with poorer survival in cancer patients. Some studies reported that being overweight or obese, defined by a body mass index (BMI) greater than 25 and 30 kg/m^2, respectively, is associated with improved survival in some types of cancer (Lennon et al., 2016; Park et al., 2018).

Regardless, cancer biology differs between obese and lean patients and this aspect must be taken into consideration when developing personalized therapeutic approaches.

Obesity can affect tumorigenesis and cancer progression at two different levels (**Figure 1**). First, a systemic one, as dysfunctional adipose tissue depots produce an altered profile of adipokines and cytokines affecting the distant tumor mass. Second, obesity also locally alters the adipose microenvironment of the tumor. In general, a crosstalk occurs between the local adipose tissue and the developing tumor mass to sustain tumor progression. In particular, studies focusing on breast cancer have demonstrated that adipose stem cell (ASC) populations have an altered differentiation potential whether derived from obese or lean subjects when cocultured with breast cancer cell lines. Moreover, these changes are associated with an enhanced ability to sustain breast cancer cell proliferation and *in vivo* tumorigenicity (Strong et al., 2013). These effects seem to be mainly mediated by the tumor-induced modulation of the leptin-estrogen axis in ASCs. An increase in leptin, estrogen receptor alpha (ERα) expression, and aromatase activity has been described in obese subjects in coculture with breast cancer, whose specific block reverted tumor progression and metastasis (Sabol et al., 2019).

THE LOCAL ADIPOSE TISSUE MICROENVIRONMENT OF THE TUMOR

The tumor effect in reprogramming the adipose tissue microenvironment can act on differentiated cells (adipocytes), representing the bulk of cells in the fat depot, or on the fewer local ASCs (**Figure 1**). In addition, recruitment of mesenchymal cells at the tumor site from the bone marrow and other sources has been described (Kidd et al., 2012; Berger et al., 2016). Mobilization of ASCs into the peripheral blood has also been reported in obese cancer patients, suggesting an increased ASC recruitment and trafficking to the tumor site is associated with the obese condition (Bellows et al., 2011; Zhang et al., 2016). The majority of studies investigating cancer modulation of adipose precursors at tumor sites have been derived from breast cancers, due to the high content of fat in the mammary gland. However, local effect on adipose tissue has also been extensively studied in prostate, ovarian, adrenal, endometrial, and melanoma cancers.

The ability of the tumor to actively influence ASCs may depend on the direct cell–cell interaction, on the secretion of oncometabolites and signaling proteins, or on the active release of factors encapsulated in "cargoe-like" microvesicles and exosomes (Wu et al., 2019). The latter mechanism might be more effective in cell reprogramming, as these vesicles can contain different types of oncoproteins, miRs, mRNA, and metabolites, which might be selectively integrated in the target cells. Primary breast ASCs treated with exosomes derived from breast or ovarian cancer cells showed a tumor-associated myofibroblast phenotype. This was characterized by increased expression of α-SMA and production of tumor-promoting factors stromal-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), CCL5, and transforming growth factor β (TGFβ), which promote the upregulation of the SMAD signaling (Cho et al., 2011, 2012; Song et al., 2017).

THE ADIPOSE STEM CELL (ASC)

Stem cells play a pivotal role in maintaining the complex tissue architecture in physiology and in cell therapies due to their self-renewal capacity and the ability to differentiate into multiple cell lineages, providing potential therapeutic solutions for different diseases.

Among adult stem cells, the bone-marrow mesenchymal stem cells (BM-MSCs) (Chamberlain et al., 2007; Kokabu et al., 2016), are a multipotent cell type that can give rise not only to osteoblastic cells but also to other cell types, including adipocytes (Kokabu et al., 2016). Other BM-MSCs like adult stem cells can be isolated from almost every tissue including adipose (Mohamed-Ahmed et al., 2018) and interestingly from some tumors (Si et al., 2019). In the adipose tissue, this population represented by the ASCs are one of the most promising adult stem cell populations identified because they are ubiquitous and can be easily recovered and harvested in large quantities with no significant complication to donors (Si et al., 2019). The adipose tissue is a highly heterogeneous tissue for the co-existence of ASCs and different types of adipocytes (white, beige, brown), endothelial cells, pericytes, and immune competent cells. Moreover, differences in the properties and functions of the adipose tissue and its cell components have been identified, which are based on the fat depot location and the metabolic status of the subject.

Human ASCs, first isolated and characterized by Zuk et al. (2001), are not homogenous populations. To be considered ASCs they must display some specific features, such as plastic adherence, positivity for typical BM-MSC markers (CD73, CD90, CD105), and lack of expression of hematopoietic markers such as CD45, CD34, CD14, or CD11b, and of endothelial CD31 and Human Leukocyte Antigen DR (HLA-DR) surface molecules. Several reports show the ASCs can differentiate into multiple lineages both *in vitro* and *in vivo* (Cawthorn et al., 2012). ASC differentiation can be reproduced *in vitro* by selective induction with media containing lineage-specific inductive factors (Zuk et al., 2001, 2002). Although ASCs are of mesodermal origin, controversially they have been shown to differentiate into cells of ectodermal and endodermal origin as well (Zuk et al., 2001, 2002; Baglioni et al., 2009; Si et al., 2019).

ASCs can secrete several growth factors and cytokines critical for tissue repair and remodeling (Pinheiro et al., 2012; Yun et al., 2012) and for the improvement of vascularization and neo-vessel formation (Pinheiro et al., 2012). Moreover, it has been reported that ASC transplants may affect inflammatory processes by displaying immunosuppressive properties (Puissant et al., 2005; Fang et al., 2007; Crop et al., 2010). Unlike BM-MSCs, ASCs do not induce an allogenic lymphocyte response *in vitro*, and prevent lymphocyte proliferation induced by allogenic peripheral blood mononuclear cells or mitogens (Puissant et al., 2005). In two studies, the intravenous infusion of allogenic ASCs was effective

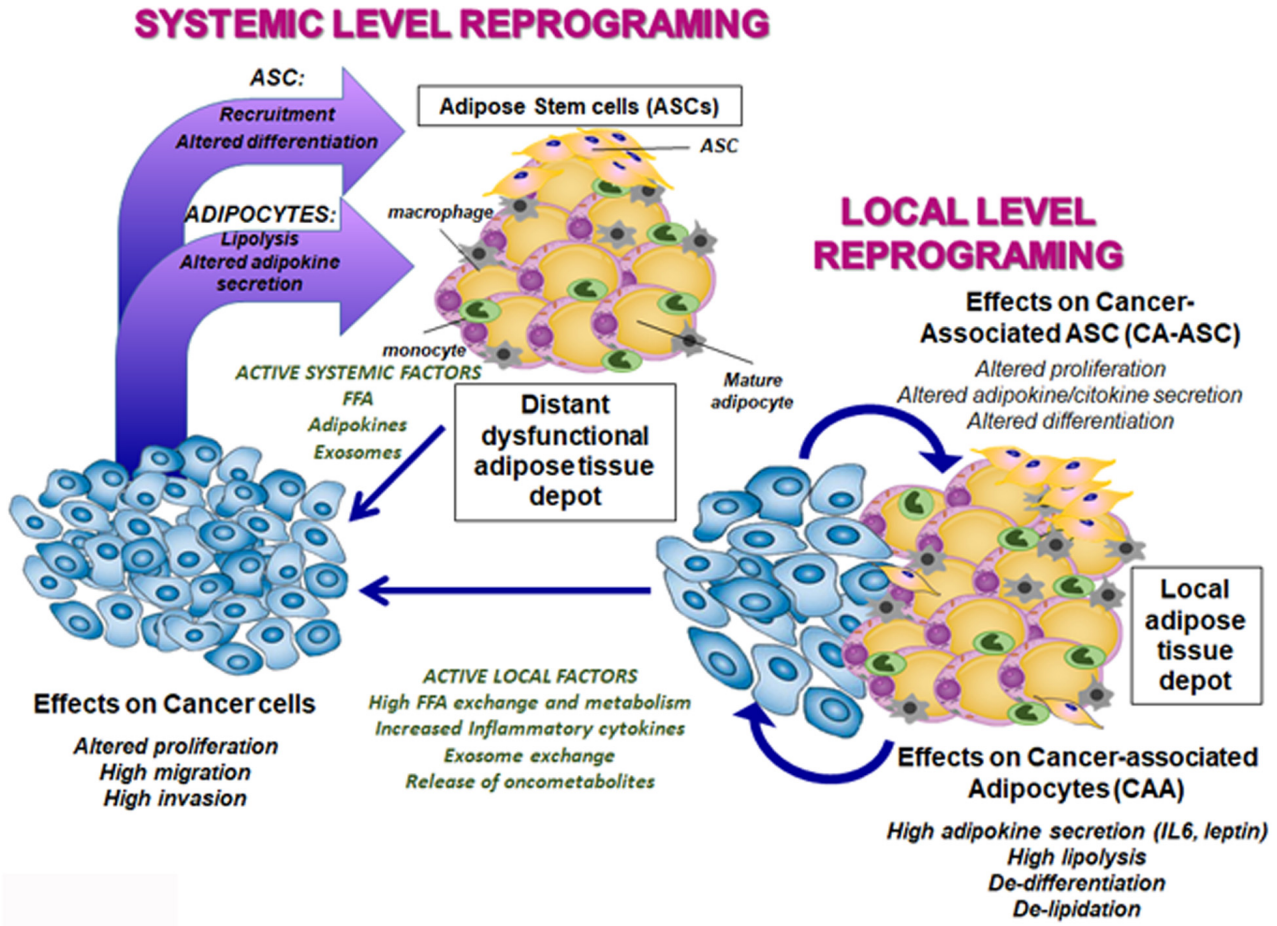

FIGURE 1 | Cartoon of the systemic and local interaction occurring between adipose tissue depots and the developing solid tumor. Interaction between cancer and adipose tissue can occur at systemic levels through factors released by the tumor mass and affecting distant adipose depots both on ASC and mature adipocyte components. Moreover, altered adipose tissue as in obesity can reciprocally affect distant tumor mass by secretion of hormones and adipokines. In addition, a reciprocal interaction between cancer and adipose (ASCs and adipocytes) cells can occur locally on the adipose mass surrounding the developing tumor through exchange of different bioactive factors, which produce effects on cancer cells, adipose precursors and mature adipocytes, as described in the text.

in the treatment of severe acute graft-vs.-host disease, indicating that ASCs may be used for their immunomodulatory effects (Si et al., 2019). However, these ASC immunomodulatory properties might also play a role in contributing to the immunomimetism developed by some tumors to avoid any immune response that might be directed against them.

DEPOT DIFFERENCES FOR WHITE ADIPOSE STEM CELLS

There are intrinsic differences in functional activity, secretion profile, and susceptibility to obesity-induced derangement in the adipose tissue depots according to the subcutaneous (SAT) or visceral (VAT) distribution. Importantly, while SAT expansion can be considered a protective response to the caloric load, excess fat deposition in VAT results in tissue dysfunction and correlates with the development of metabolic diseases. Differences in response to lipid excess are also present in ASCs of the two depots in humans (Baglioni et al., 2012) and mice (Lefevre et al., 2019). In humans, visceral ASCs (V-ASCs) display a reduced ability to differentiate toward functional adipocytes, which produce lower levels of the metabolic protective adipokine adiponectin, and are more prone to lipolysis compared to ASCs from subcutaneous adipose tissue (S-ASCs) obtained from the same subjects (Baglioni et al., 2012). Moreover, the differentiation potential and multipotency toward different cell types, such as chondrogenic, osteoblastogenic, neurogenic, and muscle, are significantly higher in S-ASCs than in V-ASCs (Baglioni et al., 2012), suggesting a strong intrinsic difference in precursor fate potential according to the depot origin. The metabolic pathways predominantly acting in S- and V-ASCs might underlie the differences observed in the precursor fate decisions (Lefevre et al., 2019). In general, the balance between cell proliferation and differentiation is controlled by glycolytic versus. mitochondrial respiration activity (Pattappa et al., 2011). Differentiation often requires a switch from a predominantly

glycolytic metabolic program that sustains cell proliferation, characterizing an undifferentiated status of the cell, to an increase in mitochondrial oxidative phosphorylation (OXPHOS) (Wanet et al., 2015).

Visceral ASCs have a decreased adipogenic fate potential and an inability to differentiate toward the osteogenic lineage compared to S-ASCs. High-resolution nuclear magnetic resonance metabolomic comparisons of murine ASCs revealed that the shortcoming is associated with a more pronounced glycolytic metabolism, with reduced mitochondrial activity, resulting in an increased utilization of pyruvate to produce lactate in V-ASCs. A similar metabolic control exerted by glycolysis might be hypothesized to be acting in CA-ASCs where the crosstalk with the tumor cells results in a restriction of the differentiation potential toward adipocytes to support cancer progression.

Conversely, the tricarboxylic acid (TCA) cycle and mitochondrial respiration are more active in S-ASCs, resulting in a reduced production of lactate and an increased synthesis of citrate (Lefevre et al., 2019). Interestingly, citrate produced in the TCA cycle and transported in cytosol is necessary for fatty acid synthesis in mature adipocytes (Senyilmaz and Teleman, 2015). In addition to glucose, the other metabolite that fuels the TCA cycle is glutamine. Glutamine deprivation induces a Warburg-like effect and completely switches the S-ASC metabolism toward lactate synthesis (Lefevre et al., 2019).

ADIPOSE PRECURSOR METABOLIC ALTERATIONS INDUCED BY THE TUMOR MICROENVIRONMENT

Of note, when positing a potential cross talk between cancer cells and adipose tissue, the main target is represented by mature adipocytes that constitute around the 90% of the fat mass, while resident ASCs represent a limited component.

A shift of white adipocytes toward stem cell like properties has been described for numerous solid tumors (Cao, 2019). These cells are characterized by marked morphological changes such as de-lipidation and de-differentiation, free fatty acid release, and immunomodulatory adipokine secretion profile. In addition, the cells are distinguished by a metabolic shift, induced by the presence of cancer cells, from OXPHOS toward glycolysis, resulting in the so-called reverse-Warburg effect. In different types of cancer, this re-programming of the mature adipose cells is thought to occur to provide the cancer cells with the substrates to fuel β-oxidation and the oncometabolites necessary for growth and invasion (Dirat et al., 2011; Nieman et al., 2011; Romero et al., 2015; Zoico et al., 2018).

The initial concept developed by Otto Warburg was that tumor cells have a defect in the mitochondrial respiration that leads to the increased glycolysis observed in the tumor cells (Warburg, 1925, 1956; Weinhouse, 1956). However, the current "chicken-or-egg" question is if the Warburg effect is just a consequence of cancer due to a defective mitochondrial respiration or does it cause carcinogenesis. The reverse Warburg effect found in ASCs in response to the metabolic reprogramming induced by the tumor is an example of the occurrence of this shift without any mitochondrial defect (Poli et al., 2015; Armignacco et al., 2019). Indeed, the enhanced aerobic glycolysis occurring in the presence of highly active lactate dehydrogenase shifts pyruvate produced from glycolysis toward lactate and not to acyl-CoA to fuel TCA cycle. This induces a reduction in the rate of mitochondrial respiration even in the absence of functional defects of the organelle (Senyilmaz and Teleman, 2015; Devic, 2016).

The tumor interaction with the microenvironment can be described as a "parasitic" energy transfer from adipose cells, where catabolic processes (autophagy, glycolysis, lipolysis) produce energy-rich nutrients that are transferred to the tumor cells to fuel anabolic processes that sustain tumor growth and metastasis (Martinez-Outschoorn et al., 2011; Romero et al., 2015). In such a strict cycle, stromal catabolites (such as lactate, amino acids, and free fatty acids) promote tumor growth by acting as high-energy oncometabolites. Moreover, the tumor cells induce a metabolic shift in ASCs that triggers their fate to be more functional for the tumor growth.

To study the role of the mitochondria in tumor energy metabolism and in tumor-induced reprogramming, a mouse model known as tuning mitochondrial dysfunction (mTUNE) has recently been developed (Gaude et al., 2018). It is applied to investigate the reciprocal relationship occurring between mitochondrial alteration, glycolysis, and reductive carboxylation of glutamine in driving cell reprogramming and migratory activity (Valcarcel-Jimenez et al., 2017).

In addition to lactate secretion, as a consequence of pyruvate redirection during the Warburg effect in the tumor, acidification of the tumor microenvironment is also maintained. During late phases of development, hypoxic conditions occur when the tumor mass interferes with oxygen transport through loss of vascularization. The hypoxic-inducible enzyme, carbonic anhydrase that converts carbon dioxide and water into carbonic acid, protons, and bicarbonate, is upregulated, contributing to the maintenance of the acidic tumor microenvironment. Interestingly, carbonic anhydrase is involved in adipocyte differentiation (Weber, 2016). Thus, its modulation during acidification induced in the tumor microenvironment might be influencing cancer-associated adipocyte differentiation.

We have recently shown the occurrence of an *in vitro* shift in the fate of white adipose precursors, when primary cultures of human ASCs were induced to differentiate in coculture with the adrenocortical carcinoma (ACC) cell line, H295R (Armignacco et al., 2019). The number of mature adipocytes that were formed *in vitro* was severely attenuated and characterized by a reduction of intracellular lipid droplets. In addition, the cells expressed significantly lower levels of proteins associated with functional adipocytes, such as adiponectin, Fatty Acid-Binding Protein 4 (FABP4) and Hormone-Sensitive Lipase (HSL), and had reduced accumulation of intracellular lipid droplets. This shift was accompanied by an increased production of lactate and glucose uptake. Thus, a significant reprogramming of adipose differentiation in the presence of tumor cells was supported by a shift in the intracellular metabolism (Armignacco et al., 2019).

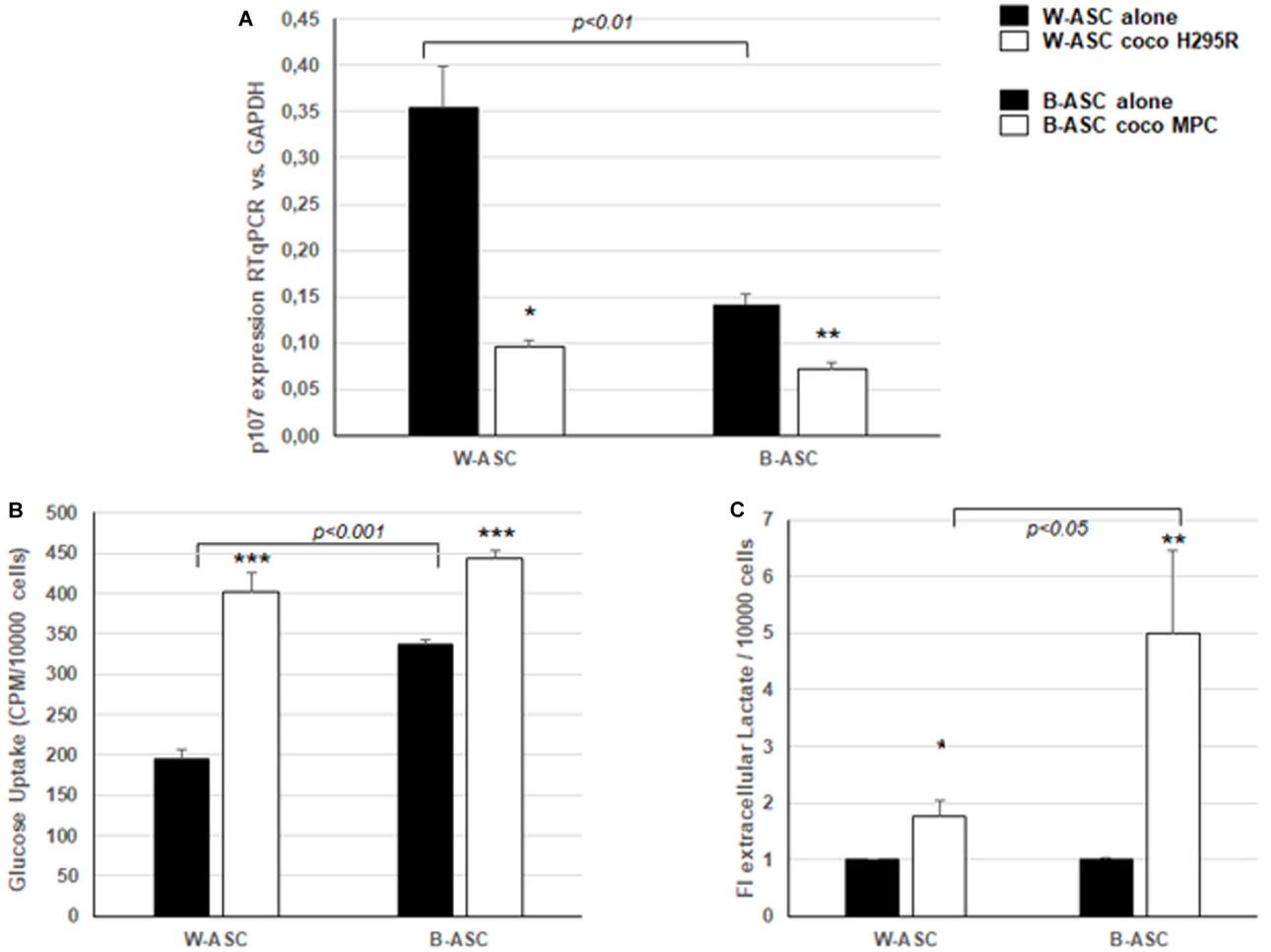

FIGURE 2 | Differences in primary white ASC (W-ASC) and brite ASC (B-ASC) populations in monoculture (alone) or when cocultured (coco) with H295R cells or MPC cells, respectively. **(A)** Gene expression of p107 evaluated by quantitative RT-PCR and normalized for the house keeping gene GAPDH and related to a calibrator was expressed as mean ± SE of $2^{-\Delta\Delta CT}$ in $n = 3$ independent experiments. **(B)** Glucose uptake in W- and B-ASC expressed as mean ± SE of incorporated CPM/10^4 cells after a 10 min pulse of cells with 2-deoxy-[^{3}H]-D-glucose [1 μC/μL]; modified from Armignacco et al. (2019). **(C)** Extracellular lactate concentrations expressed as mean fold increase on cells alone normalized on 10^4 cells ± SE of lactate measured in frozen conditioned media from ASCs cultured alone or in coculture for 7 days (colorimetric method, Siemens Healthcare, Tarrytown, NY, United States). Student's *t*-test: *p* is indicated; and $^*p < 0.01$, $^{**}p < 0.001$; $^{***}p < 0.0001$ alone vs. coco.

Of note, aggressive adrenocortical cancers are often characterized by hyper-activating mutation or upregulation of Wnt/beta catenin pathways (Assié et al., 2014) and hyper-methylation/repression of the G0/G1 switch gene 2 (G0S2) gene (Barreau et al., 2013; Mohan et al., 2019). Interestingly, both Wnt and G0S2 are physiologically implicated in the inhibition of adipogenesis, suggesting that common mechanisms between ACC tumor progression and ASC reprogramming might occur in the tumor microenvironment (Armignacco et al., 2019). Indeed, Wnt5 is upregulated in ASCs and its downregulation is necessary for adipogenesis (Christodoulides et al., 2009). In addition, G0S2 expression is involved in enhancing intracellular triglyceride accumulation and inhibition of lipolysis in adipocytes. This suggests that its repression, as found in the advanced and more aggressive forms of ACC (Mohan et al., 2019), may be associated with a reduced accumulation of lipid droplets not only in the tumor cells but also in the cancer-associated adipocytes. Further studies are needed to clarify if this association really occurs in advanced ACC and if it is limited to adrenocortical cancer.

THE RISK OF TUMORIGENICITY OF ASCS IN ADIPOSE GRAFTS USED FOR REGENERATIVE MEDICINE

For many years, autologous adipose grafts used in medical procedures, such as lipofilling of healing wounds, scar remodeling, and after surgery removal of tumor masses, have been studied for their regenerative potential. Indeed, the presence of ASCs has been demonstrated to significantly improve the engraft and the reconstruction of the tissue architecture, due to the paracrine effects of ASC secretion

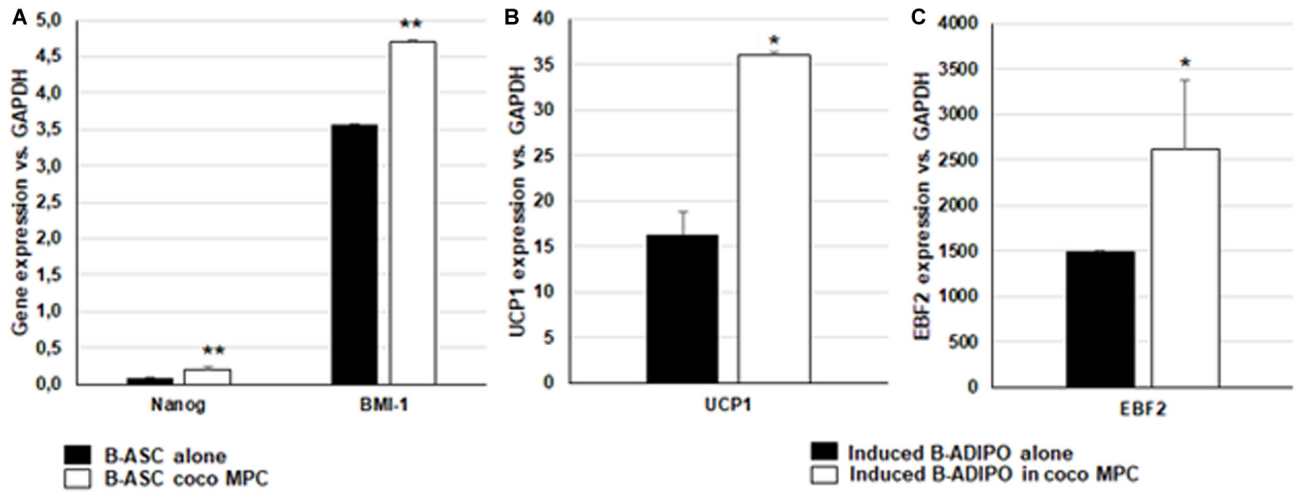

FIGURE 3 | B-ASCs in coculture with MPC cells retain higher levels of stemness and higher levels of brite markers when adipogensis is induced *in vitro*. Gene expression of the stem genes Nanog and BMI-1 **(A)** or of brown markers UCP1 **(B)** and EBF2 **(C)** evaluated by quantitative RT-PCR, normalized for the house keeping gene GAPDH and related to a calibrator, was expressed as mean ± SE of $2^{-\Delta\Delta CT}$ in $n = 3$ independent experiments in B-ASC growth in monoculture or in coculture with MPC cells **(A)**, or when B-ASCs were induced to differentiate toward adipocytes with DIM cocktail in monocolture (ADIPO alone) or in the presence of MPC cells (ADIPO coco) **(B,C)**. Student's *t*-test: $^{*}p < 0.01$ and $^{**}p < 0.001$ alone vs. coco.

and stimulation of adipocyte differentiation (Mashiko and Yoshimura, 2015). Interestingly, *in vitro* studies showed that the presence of scaffolding biomaterials introduced in the graft has the potential to stimulate ASC differentiation and enhancing adipose replenishment (Stellavato et al., 2017). Autologous fat graft procedures are of particular importance to reconstruction of resected tissue rich in adipose mass, such as breast cancer surgery. A recent meta-analysis excluded any increased risk of recurrence after fat graft reconstruction in breast cancer surgery (Agha et al., 2015). However, the safety of this approach in patients with cancer is currently debated due to the potential risk of ASCs to influence tumor recurrence (Tan and Loh, 2016; Mazur et al., 2018). Indeed, residual post-surgery cancer cells may influence ASCs present in the fat graft inducing proliferation and maintenance of a a stem-like state that may promote cancer restart. A predicament similar to what already has been demonstrated for the crosstalk occurring between the primary tumor mass and the ASCs present in the surrounding microenvironment for different solid tumors (Paino et al., 2017; Armignacco et al., 2019). Moreover, both resident and engrafted ASCs can stimulate angiogenesis (Paino et al., 2017). This may contribute to tumor recurrence by secretion of specific adipokines and factors, such as collagen and matrix components, that make the microenvironment more prone to tumor growth (Iyengar et al., 2003). The crosstalk between residual tumor and adipose microenvironment cells may be particularly important in driving the reciprocal fate of ASCs and tumor stem cells (Papaccio et al., 2017). Indeed, ASCs may contribute to the maintenance of a stem-like state and immunomodulatory properties of the cancer stem cells (Si et al., 2019), maintaining them quiescent and masked to the immune response for long time (Paino et al., 2017).

THE p107 CHECK POINT IN CONTROLLING ASC METABOLISM

Tumor-induced systemic wasting and cancer cachexia, characterized by a decrease in muscle mass and white fat accumulation, have been associated with increased thermogenic activity and browning of adipose tissue (Kir and Spiegelman, 2016). Factors produced by the tumor reprogram a shift in the adipose tissue inducing the "browning process." Among these factors, the lipid mobilizing factor zinc-α2-glycoprotein (ZAG), highly expressed in breast, prostate, lung, and bladder tumors (Díez-Itza et al., 1993; Hale et al., 2001) has been demonstrated to promote lipolysis and inhibit lipogenesis in WAT (Taylor et al., 1992), while stimulating white adipose tissue browning and cachexia (Elattar et al., 2018). In a Lewis lung carcinoma mouse model of cancer cachexia, this reprogramming of white to brown differentiation fate is triggered locally and systemically by tumor-produced parathyroid-hormone-related protein (PTHrP), which upregulates the expression of those genes involved in thermogenesis in the adipose tissue (Kir et al., 2014). The causal effect is demonstrated by reversion of browning and muscle wasting cachectic effects through neutralization of PTHrP secretion (Kir et al., 2014) or genetic deletion of the PTH receptor (Kir et al., 2016). In parallel with an effect on "browning" of differentiated adipocytes there is an ASC effect of reduced adipocyte differentiation rate and increased differentiation into brite adipocytes. In some solid tumors that produce PTHrP, the tumor mass can reprogram adipose precursor fate not only by limiting adipogenesis, but also by shifting the differentiation fate toward thermogenic adipocytes. Both consequences appear to be more useful than having only white adipocytes to support tumor metabolic and proliferative requirements.

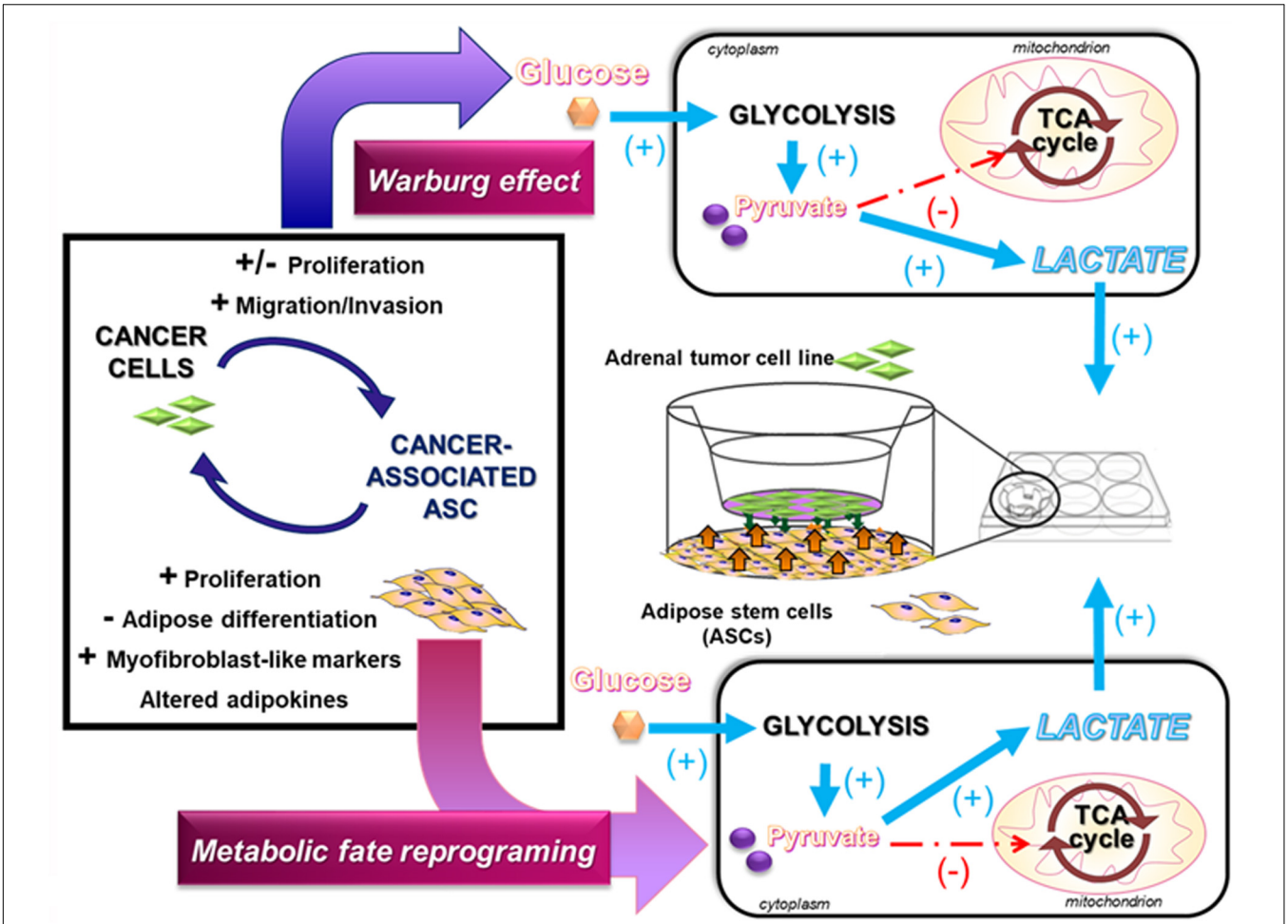

FIGURE 4 | Metabolic shift affecting stem fate in ASCs in cocolture with adrenal tumor cells. Metabolic fate reprograming occurs between adrenal cancer cells and ASCs cocultured using ThinCert^{TM} tissue culture inserts for six-well plates with 0.4 μm pore size (Greiner Bio-One, Kremsmünster, Austria). In particular, an increased uptake of glucose is associated with a shift from mitochondrial OXPHOS to aerobic glycolysis, resulting in production of lactate and reduced utilization in the TCA cycle. This metabolic shift contributes to the reprograming in ASC fate (proliferation, differentiation, morphological changes, altered adipokine secretion, observed when they are grown in coculture with adrenal tumor cells).

A member of the retinoblastoma susceptibility protein (Rb) gene family, Rbl1 (p107), might act as a CA-ASC metabolic checkpoint factor in this development. p107, a co-transcriptional repressor, is involved in cell cycle progression, as its over-expression is known to block cell cycle of many cancer cell lines and its loss associated with proliferative effects (Wirt and Sage, 2010). It has also been shown to determine ASC fate by regulating adipocyte differentiation through modulation of the glycolytic pathway. Adipocyte precursors depleted of p107 display an increased glycolytic flux associated with a reduction in the white adipocyte differentiation rate and a switch toward thermogenesis and "browning processes" (De Sousa et al., 2014; Porras et al., 2017). Importantly, lactate, the end-product of aerobic glycolysis, drives the commitment of unspecified adipocyte progenitors to brite fat *in vivo* (Porras et al., 2017). According to these findings, a suggestive role of p107 in driving CA-ASC fate following ASC interaction with tumor cells can be theorized. In this case, the cancer cell crosstalk with ASCs results in constraining white adipocyte differentiation potential and stimulating proliferation (Park et al., 2011; Koellensperger et al., 2014; Freese et al., 2015; Armignacco et al., 2019), similar to the process observed following p107 suppression in fat precursors (De Sousa et al., 2014). Intriguingly, coculture with ASCs reciprocally induces a reprogramming in the tumor cells, enhancing proliferation and invasion, as demonstrated for several solid tumors (Park et al., 2011; Cho et al., 2012; Klopp et al., 2012; Koellensperger et al., 2014; Armignacco et al., 2019; Xu et al., 2019).

Of note, p107 functions as a switch that regulates adipogenic fate into white versus. brown fat through repression of Peroxisome proliferator-activated receptor Gamma Coactivator 1-alpha (PGC-1α) and PR-DoMain containing 16 (PRDM16) genes (Scimè et al., 2005; De Sousa et al., 2014). In rodents, non-committed embryonic adipogenic progenitors have a metabolic state resembling aerobic glycolysis that is necessary for driving their pro-thermogenic fate. In the absence of p107, however, disruption of the glycolytic capacity reverts their differentiation

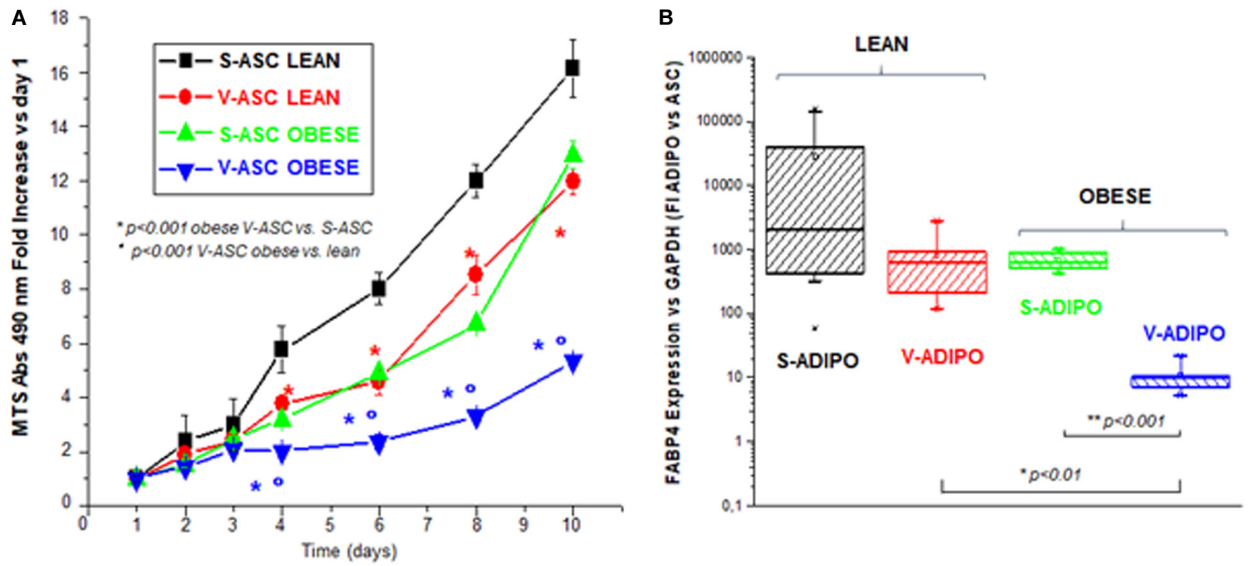

FIGURE 5 | Differences in ASCs according to their fat origin. Primary ASC populations were obtained from subcutaneous (S-) or visceral (V-) adipose tissue (AT) of $n = 4$ and $n = 5$ independent lean or obese subjects, respectively. **(A)** Cell proliferation was assessed by MTS assay for the indicated time intervals in plated cells grown in parallel and expressed as mean fold increase ± SE of MTS absorbance vs. day 1. **(B)** Box charts of FABP4 gene expression, normalized for the housekeeping gene GAPDH and related to a calibrator, assessed as a marker of differentiation by quantitative real time RT-PCR in adipocytes (ADIPO) obtained *in vitro* after the parallel induction with DIM adipogenic medium (Baglioni et al., 2012) of ASC derived from SAT and VAT of $n = 4$ and $n = 5$ independent lean or obese subjects. Student's *t*-test: *p*-values are indicated.

fate from brown to white adipocytes, further confirming the role of p107 as a master switch controlling the adipose fate as well as the necessity of the aerobic glycolytic process to sustain the brown differentiation fate (Porras et al., 2017).

A p107 CHECK POINT IN THE METABOLIC SHIFT OF ASCS IN COCULTURE WITH ADRENAL TUMOR CELLS

We have recently developed a system of *in vitro* coculture between ASCs and two tumors of the adrenal affecting the steroid-secreting component of the gland, the ACC, and pheochromocytoma, which affects the catecholamine-secreting medullary adrenal part. One system makes use of ASCs derived from white adipose tissue (W-ASC) and a cancer cell line of ACC, H295R cells (Armignacco et al., 2019). The other is made up of cancer cells of pheochromocytoma (MPCs) and primary ASCs that exhibit brite cell characteristics (B-ASCs), since they are derived from the fat surrounding the pheochromocytoma lesion (unpublished results). Interestingly, we have previously demonstrated that this peri-adrenal visceral white fat has a great potential of generating brite adipocytes under the control of factors, which include catecholamines, produced at high levels by the pheochromocytoma mass (Di Franco et al., 2014).

p107 expression levels were significantly lower in B-ASCs compared with W-ASCs (**Figure 2A**), confirming that p107 might also interfere with the thermogenic program in human adipocyte precursors, and is not only restricted to mouse (Porras et al., 2017). Moreover, when in coculture with tumor cells, both types of ASCs displayed lower levels of p107, consistent with an altered differentiation potential. The decreased levels of p107 are also associated with a metabolic shift toward aerobic glycolysis. This is supported by the significant increase in ASC glucose uptake (**Figure 2B**), which was significantly higher in B-ASCs compared to ASCs in monoculture, suggesting a higher basal metabolism in the latter type of cells. Consistently, lactate production suggestive of aerobic glycolysis was increased in coculture in both systems, being significantly higher in B-ASCs (**Figure 2C**).

In white ASC cocultured with H295R cells for 7 days, Seahorse analysis™ of basal glycolysis (Agilent Seahorse XF Glycolytic Rate Assay Kit #103344-100) showed a shift toward an increase of the aerobic glycolytic metabolic pathway (glycolytic proton efflux rate: cocultured cells vs. alone, 39,901 ± 1062 vs. 47,321 ± 958 pmol/min, $p < 0.0001$). We have already demonstrated that increased glycolysis is associated with a decreased differentiation potential and an increased maintenance of the stem potential and proliferation (Armignacco et al., 2019).

Consistently, also for the B-ASCs in coculture with pheochromocytoma cells, an increased expression of genes associated with stemness (Nanog) and self-renewal (BMI-1) was evident (**Figure 3A**). In addition, when differentiation was induced *in vitro* in the presence of MPCs, B-ASCs the cocultured cells have an increased potential of differentiating toward brite adipocytes, expressing higher levels of Uncoupling Protein 1 (UCP1, **Figure 3B**) and of the transcription factor Early B-cell Factor 2 (EBF2, **Figure 3C**). These findings suggest

how the metabolic pathway of aerobic glycolysis through potential regulation of p107 supports human cell undifferentiated conditions; upon differentiation a shift toward a pro-thermogenic program occurs. **Figure 4** reports how the metabolic shift occurring in ASCs in coculture with adrenal tumor cells might affecting stem fate and functions.

DEPOT DIFFERENCES IN ASC RESPONSE TO TUMOR CROSSTALK

Metabolic circumstances, in particular obesity, can influence ASC characters, shaping the adipose precursors toward a cancer-sustaining phenotype. Differences in ASC response and interaction with the tumor mass, according to the metabolic profile of the patient, are supported by the higher extent of recruitment of circulating ASCs to the tumor mass in obese versus. lean humans and mice (Cozzo et al., 2017). Moreover, V-ASCs in obese males are shown to contribute to prostate cancer progression (Zhang et al., 2016).

The ASCs derived from abdominal VAT compared to SAT of obese versus. lean subjects display a decreased proliferation rate (**Figure 5A**). Additionally, they have a diminished ability to differentiate into functional mature adipocytes, as shown by a decreased gene expression of FBP4 gene, a marker of mature terminally differentiated adipocytes (**Figure 5B**). Consistently with the hypothesis that V-ASCs rather than S-ASC are more susceptible to be reprogrammed by cancer crosstalk, the majority of solid tumors develop in visceral organs in close contact with visceral fat pads, with the exception of tumors of the skin, such as melanoma. V-ASCs rather than S-ASCs have been described to be more prone to sustain endometrial cancer survival and progression of endometrial cancer in a mouse xenograft model (Klopp et al., 2012). Thus, suggesting that ASC origin can drive different responsiveness in tumor induced cell reprogramming.

CONCLUSION

The findings reported in this review reveal that the elevated plasticity of the adipose tissue is not confined to the mature adipocytes but is present and active in the ASCs in different pathophysiological contexts, including obesity and cancer. Such plasticity in reprogramming ASC fate and functions is strictly regulated by the intracellular metabolic equilibrium through the action of different factors. The regulatory pathways and the factors involved therein might represent valuable druggable targets for the development of novel therapeutic approaches to pathologies such as obesity and cancer.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

GC, AS, MMN, and ML contributed to conception and design of the experimental part of the study. GC, AS, MMG, and ML contributed to conception and design of the review structure and interpretation of the literature. GC and AD performed the experiments shown. ML wrote the first draft of the manuscript. GC, AS, and MMG wrote sections of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Reprogramming of Lipid Metabolism as a New Driving Force Behind Tauroursodeoxycholic Acid-Induced Neural Stem Cell Proliferation

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Recent evidence suggests that neural stem cell (NSC) fate is highly dependent on mitochondrial bioenergetics. Tauroursodeoxycholic acid (TUDCA), an endogenous neuroprotective bile acid and a metabolic regulator, stimulates NSC proliferation and enhances adult NSC pool *in vitro* and *in vivo*. In this study, we dissected the mechanism triggered by this proliferation-inducing molecule, namely in mediating metabolic reprogramming. Liquid chromatography coupled with mass spectrometry (LC-MS) based detection of differential proteomics revealed that TUDCA reduces the mitochondrial levels of the long-chain acyl-CoA dehydrogenase (LCAD), an enzyme crucial for β-oxidation of long-chain fatty acids (FA). TUDCA impact on NSC mitochondrial proteome was further confirmed, including in neurogenic regions of adult rats. We show that LCAD raises throughout NSC differentiation, while its silencing promotes NSC proliferation. In contrast, nuclear levels of sterol regulatory element-binding protein (SREBP-1), a major transcription factor of lipid biosynthesis, changes in the opposite manner of LCAD, being upregulated by TUDCA. In addition, alterations in some metabolic intermediates, such as palmitic acid, also supported the TUDCA-induced *de novo* lipogenesis. More interestingly, a metabolic shift from FA to glucose catabolism appears to occur in TUDCA-treated NSCs, since mitochondrial levels of pyruvate dehydrogenase E1-α (PDHE1-α) were significant enhanced by TUDCA. At last, the mitochondria-nucleus translocation of PDHE1-α was potentiated by TUDCA, associated with an increase of H3-histones and acetylated forms. In conclusion, TUDCA-induced proliferation of NSCs involves metabolic plasticity and mitochondria-nucleus crosstalk, in which nuclear PDHE1-α might be required to assure pyruvate-derived acetyl-CoA for histone acetylation and NSC cycle progression.

Keywords: metabolism, mitochondria, neural stem cells, proliferation, tauroursodeoxycholic acid

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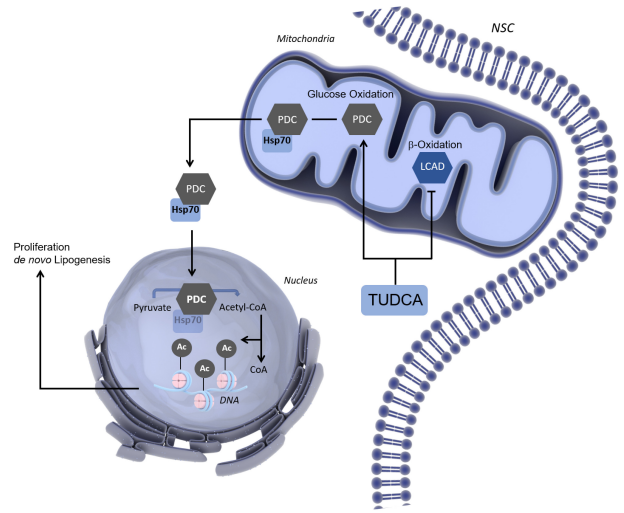
INTRODUCTION

Over the past few years, our perception of neural stem cell (NSC) potential has greatly increased, although we are only beginning to understand their metabolic profile in physiological and pathological context (Ottoboni et al., 2017). A more comprehensive understanding of how adult NSCs rely on different metabolic pathways to keep up with cell-specific bioenergetic demands will



GRAPHICAL ABSTRACT | TUDCA induces a metabolic shift to boost NSC proliferation. The FA and glucose oxidation counterbalance are crucial for NSC fate regulation. In early-differentiating NSCs, TUDCA increases *de novo* lipogenesis and proliferation by inducing a metabolic shift from FA to glucose catabolism that facilitates NSC cell cycle-associated H3 acetylation.

certainly contribute to tune NSCs toward the desired response, including when therapeutically addressing aging and complex metabolic and neurodegenerative diseases (Wallace, 2005; Folmes et al., 2013; Knobloch and Jessberger, 2017).

Mitochondrial dynamics and bioenergetics are closely associated to NSC fate and behavior (Kann and Kovács, 2007; Wanet et al., 2015; Xavier et al., 2015). In this regard, mitochondrial dysfunction can be an underlying problem in the depletion of the stem cell pool and impaired neurogenesis (Wallace, 2005; Khacho et al., 2017). Mitochondria are also responsible for long-term survival, differentiation and synaptic integration of newborn neural cells (Xavier et al., 2015). Therefore, mitochondria and its regulatory network have major implications toward a more efficient use of neural regeneration therapies (Casarosa et al., 2014).

Increasing evidence suggests that metabolic plasticity is crucial to the transition between stemness maintenance and lineage specification (Folmes et al., 2013; Knobloch and Jessberger, 2017). Metabolic changes between stem cells and their progeny also suggest that mitochondrial mass and activity increase with lineage progression to meet the robust energy demands associated with differentiation (Wanet et al., 2015; Hu et al., 2016). Thus, the identity of stage-specific metabolic programs and their impact on adult neurogenesis need to be explored as we are now starting to unravel mitochondria molecular adaptations of metabolic circuits under this scenario. On the road of cellular metabolic pathways, lipid metabolism has also been largely neglected for the role it may play in the neurogenesis process. However, lipids emerge in NSC life as building blocks of membranes, an alternative energy source and as signaling entities (Knobloch, 2016). Indeed, fatty acids (FAs) have been shown to be produced endogenously in adult NSCs and a novel mechanism governing adult neurogenesis has been identified, in which lipogenesis determines the proliferative activity of NSCs (Folmes et al., 2013). Interestingly, during the transition from quiescent to active NSCs, glycolysis and FA oxidation (FAO) gradually decrease, while dependence on glucose to supply oxidative phosphorylation (OXPHOS) for energy generation and lipogenesis for NSC proliferation tend to increase (Shin et al., 2015; Fidaleo et al., 2017).

Apart from signaling pathways responsible for mediating the NSC metabolic state, the redistribution of nuclear or mitochondrial proteins has also emerged as a novel direct way of interorganellar coordination (Lionaki et al., 2016). Surprisingly, one of the largest multiprotein complexes known, the mitochondrial pyruvate dehydrogenase complex (PDC), translocates to the nucleus of mammalian cells. In the nucleus, PDC was shown to be functional and to provide a novel pathway for nuclear acetyl-CoA synthesis in support of histone acetylation and epigenetic regulation (Sutendra et al., 2014). The recent knowledge on the metabolic switches ruling NSC transformation into immature neurons describe fateful metabolic shifts, controlling NSC identity (Knobloch and Jessberger, 2017). Therefore, specific modulation of metabolic pathways might be useful to improve adult neurogenesis.

Ursodeoxycholic acid (UDCA), an endogenous bile acid FDA-approved for the treatment of cholestatic liver diseases is used as a cytoprotective agent that strongly detain programmed cell death (Rodrigues et al., 1998a,b, 1999; Amaral et al., 2009; Vang et al., 2014). Tauroursodeoxycholic acid (TUDCA) is the taurine-conjugated form of UDCA. After conjugation with taurine, TUDCA is orally bioavailable and able to penetrate the CNS (Keene et al., 2002). TUDCA exhibits anti-inflammatory effects and was shown to attenuate neuronal loss in neurodegenerative diseases (Rodrigues et al., 2003; Nunes et al., 2012; Gronbeck et al., 2016). Importantly, gene expression microarray analysis demonstrated that TUDCA specifically modulates several enzymes involved in FA metabolism in primary rat hepatocytes (Castro et al., 2005). TUDCA was also shown to regulate energy metabolism, through the reduction of endoplasmic reticulum stress and improvement of impaired insulin signaling, leading to restored glucose homeostasis in obese and diabetic mice models (Ozcan et al., 2006; Guo et al., 2015). Moreover, it is known that this singular bile acid has an important role in neuroprotection and neurogenesis through mitochondrial regulation (Rodrigues et al., 2000; Xavier et al., 2014). TUDCA prevents early differentiation induced-mitochondrial alterations in a mouse NSC line, eliciting a marked increase in NSC population at S phase with a subsequent reduction at G1 phase, which is typical of cells in expansion. The resulting increase in NSC pool was shown to occur in a mitochondrial redox state and ATP-dependent manner (Xavier et al., 2014). Recently, the potential therapeutic effect of TUDCA in increasing the NSC pool and early choice toward neuronal differentiation *in vivo* was uncovered, reinforcing the proliferative and pro-neurogenic effects of this singular bile acid (Soares et al., 2018).

Here we sought to investigate the molecular mechanisms underlining TUDCA control of NCS proliferation and further exploit its potential effect in enhancing the NSC pool. Our

results deciphered TUDCA-induced changes in mitochondrial proteomic signature of NSCs. Importantly, the downregulation of LCAD *in vitro* and *in vivo* appeared as a major finding, where the fine-tuning of mitochondrial long-chain FA β-oxidation in NSCs leads to important metabolic shifts in subcellular compartments.

MATERIALS AND METHODS

Ethics Statement

The two mouse NSC lines, NS-TGFP and CGR8, used in this study were both obtained from Dr. Smith's Laboratory, University of Cambridge, Cambridge, United Kingdom. The NS-TGFP line was provided by Dr. Henrique, University of Lisbon, Lisbon, Portugal and the CGR8 line was provided by Dr. Margarida Diogo, University of Lisbon, Lisbon, Portugal. The animal experiments were performed in accordance with Portuguese laws (DL 113/2013, 2880/2015, 260/2016) on Animal Care and with the EU Directive (86/609/EEC; 2010/63/EU) on the protection of animals used for experimental and other scientific purposes. In addition, animal welfare fulfilled the recommendation from the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86–23 revised 1985). The Animal Ethical Committee at the Faculty of Pharmacy, University of Lisbon, Portugal waived the need for approval.

Cell Culture

The CGR8 competent cell line was established from the inner cell mass of a 3.5-days male pre-implantation mouse embryo (ECACC 07032901) (Nichols et al., 1990; Smith, 1991; Mountford et al., 1994). Tau-GFP mouse NSC (NS-TGFP) cells were derived from 14.5-dpc mouse fetal forebrain, and constitutively express the fusion protein tau-GFP (Pratt et al., 2000; Silva et al., 2006). Mouse cell lines were established using a method that produces pure cultures of adherent bonafide NSCs, which continuously expand by symmetrical division and are both capable of tripotential differentiation (Conti et al., 2005; Pollard et al., 2006; Glaser et al., 2007). NSCs were grown in monolayer as previously described (Spiliotopoulos et al., 2009) and routinely maintained in undifferentiating medium (self-renewal conditions), Euromed-N medium (EuroClone S.p.A., Pavia, Italy), supplemented with 1% N-2 supplement (Gibco, Thermo Fisher Scientific, Inc., United States), 20 ng/μL epidermal growth factor (EGF; PeproTech EC, United Kingdom), 20 ng/μL basic fibroblast growth factor (bFGF; PeproTech EC) and 1% penicillin-streptomycin (Pen-Strep; Gibco, Thermo Fisher Scientific, Inc.), in 75 cm^2 tissue culture (TC) treated flasks (Falcon, Corning Inc., New York, NY, United States) at 37°C in a humidified atmosphere of 5% CO_2. Neural differentiation was performed by first plating NSCs in undifferentiating medium onto TC-treated cell culture dishes at 5.4×10^6 cells/cm^2 for 24 h. After 24 h of cell stabilization, the medium was changed to optimized neuronal differentiation-inducing medium, Euromed-N medium supplemented with 0.5% N-2 supplement, 1% B-27 supplement (Gibco, Thermo Fisher Scientific, Inc.), 10 ng/μL bFGF, and 1% Pen-Strep. For NSCs that remained undifferentiated, the medium was not changed. Differentiating NS-TGFP cells at 8×10^5 cells/cm^2 were at fixed at 0, 3, 6, 24, 48, and 72 h and processed for immunoblotting. For cellular treatments, cells were maintained in differentiating and undifferentiating medium for 24 h. Then, cells were collected and processed for immunoblotting, differentiated cells were also processed for Oil-Red-O staining, gas chromatography-mass spectrometry (GC-MS) analysis, immunocytochemistry, and quantitative RT-PCR (qRT-PCR).

Animal Models and TUDCA Delivery

Mini-osmotic pumps (Alzet, Cupertino) were implanted in 6-weeks old male Wistar rats for intraventricular infusion of 300 μM TUDCA dissolved in artificial cerebrospinal fluid (aCSF) or aCSF alone, as vehicle, for continuous dosing of unrestrained laboratory animals. Cannulas were inserted in the lateral ventricle of the right hemisphere at the following coordinates: anterior-posterior: –0.4 mm, medial-lateral: 1.2 mm, dorso-ventral: 3.5 mm, having the bregma as a reference. The flow was continuous with a delivery rate was 0.25 μL/h for 28 days. After 28 days of treatment, animals were perfused with saline solution (NaCl 0.9%), and fixed with 4% paraformaldehyde (PFA) in PBS. Animals were sacrificed by decapitation and the brains immediately removed. Contralateral hemisphere was sectioned to perform immunohistochemistry assays.

Cellular Treatments

Cells were treated with 100 μM of TUDCA (T0266; Sigma-Aldrich Corp.) after 24 h of plating and/or upon medium change for differentiation, and then collected after 24 h for protein extraction and other analyses.

Total, Mitochondrial, Cytosolic, and Nuclear Protein Extraction

For isolation of total protein extracts, NSCs were collected and lysed using an ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 1% Nonidet P-40, 2 mM DTT, and protease inhibitors) for 30 min in ice. Samples were then sonicated for 30 s in ultrasounds, the lysate was centrifuged at 10,000 g for 10 min at 4°C, and the supernatant recovered. For isolation of mitochondrial protein extracts, cells were lysed upon incubation in ice for 20 min with an isolation buffer (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM Na_2EDTA, 1 mM EGTA, 250 mM Sucrose), supplemented with 1 mM dithiothreitol (DTT) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.) and, then, disrupted by 40 strokes in a Dounce homogenizer. The homogenates were centrifuged at 2,500 g for 10 min at 4°C, then, the supernatant was saved and a second centrifugation was necessary to increase the product yield, so the pellet obtained from the homogenates was resuspended again in a minimum volume of isolation buffer. The total homogenate recovered was, then, centrifuged at 12,000 g for 30 min at 4°C to remove unbroken cells and nuclei. Finally, the mitochondrial fraction was obtained, the respective pellet resuspended in the isolation buffer and frozen at –80°C. The supernatant was removed and

filtered through 0.2 μm and, then, 0.1 μm Ultrafree MC filters (Merck Millipore Corp., Germany) by centrifugation at 12,000 g for 20 min at 4°C to obtain cytosolic proteins. For nuclear extracts, cells were lysed with hypotonic buffer (10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM potassium acetate, 2 mM DTT, and protease inhibitors), homogenized with 20 strokes in a loose fitting Dounce homogenizer, and centrifuged twice at 500 g for 10 min at 4°C. Cytosolic proteins were recovered in the supernatant and centrifuged at 3,160 g for 10 min at 4°C saving the supernatant again, while the nuclear pellet was washed in a buffer composed of 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.25 M sucrose, 0.5% Triton X-100, and protease inhibitors through centrifugation at 500 g for 5 min at 4°C. Then, the nuclear pellet was resuspended and sonicated for four cycles of 10 s in buffer composed by 10 mM Tris-HCl, pH 7.6, 0.25 M sucrose and protease inhibitors. Finally, the suspension was centrifuged through 0.88 M sucrose at 2,000 g for 20 min at 4°C, and nuclear proteins were recovered in the supernatant. Protein content was measured by the Bradford protein assay kit (Bio-Rad Laboratories, United States), according to the manufacturer's specifications, using bovine serum albumin (BSA) as standard.

Histone Purification

For histone purification cells were lysed with hypotonic lysis buffer (10 mM Tris-HCl, pH 8, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT and protease inhibitors) for 30 min at 4°C. Intact nuclei were recovered by centrifuging at 10,000 g for 10 min at 4°C, and the supernatant discarded. Nuclei were resuspended in 0.4 N H_2SO_4 and incubated on a rotator at 4°C overnight. Nuclear debris were removed by centrifuging at 16,000 g for 10 min at 4°C, and the supernatant containing histones was transferred into a fresh tube. Trichloroacetic acid (100%) was added drop by drop to histone solution, mixed and incubated on ice for 30 min. Histones were recovered after centrifugation at 16,000 g for 10 min at 4°C. The histone pellet was washed twice with ice-cold acetone and air-dried for 20 min at room temperature. Histones were finally dissolved in 100 μL of Milli-Q water and transferred into a new tube. Histone protein content was measured by the Bradford protein assay kit (Bio-Rad Laboratories), according to the manufacturer's specifications, using BSA as standard. Ponceau S (P7170; Sigma-Aldrich Corp.) was used to reversible stain total protein bands and as a loading control of histone extracts, since the total H3 content was also increased in TUDCA-treated NSCs.

Immunoblotting

Protein levels GAPDH, acetyl-H3, histone 3, Hsp70, lamin B1, LCAD, PDHE1-α and SREBP-1 and VDAC were determined by western blot analysis. Briefly, 20 μg of protein from mitochondrial, nuclear, and histone protein extracts and 40 μg of cytoplasm protein fraction and total protein extracts were separated on a 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gel and then subjected to immunoblotting using primary antibodies reactive to the different proteins of interest (**Table 1**). Blots were subsequently incubated with secondary antibodies conjugated with anti-mouse or anti-rabbit immunoglobulin G (IgG), and with horseradish peroxidase (Bio-Rad Laboratories) for 2 h at room temperature. Finally, membranes were processed for protein detection using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore Corp.) or Super Signal™ West Femto substrate (Thermo Fisher Scientific, Inc.) in a ChemiDoc™ MP System (Bio-Rad Laboratories). GAPDH, was used as loading control of total extracts. Ponceau S was used to reversible stain total protein bands. Since the levels of VDAC mitochondrial protein marker and H3 nuclear protein marker are likely to be altered in TUDCA-treated NSCs, Ponceau S was also used as a loading control of mitochondrial and nuclear extracts. Lamin B1, VDAC and GAPDH were used to confirm the purity of the cytosolic, nuclear and mitochondrial fractionation.

TABLE 1 | Primary antibodies used for Western blot.

Antigen	Host	Clonality	Company	Catalog number	Dilution
GAPDH	Mouse	Monoclonal	Santa Cruz Biotec.	sc-32233	1:2500
Acetyl-H3	Rabbit	Polyclonal	Abcam plc	ab47915	1:1000
Total H3	Rabbit	Polyclonal	Millipore Corp.	06-755	1:500
Hsp70	Mouse	Monoclonal	ReD SYSTEMS	242707	1:10000
Lamin B1	Rabbit	Polyclonal	Abcam plc	ab16048	1:10000
LCAD	Rabbit	Monoclonal	Abcam plc	ab196655	1:10000
PDHE1-α	Mouse	Monoclonal	Abcam plc	ab110330	1:1250
SREBP-1	Mouse	Monoclonal	Abcam plc	ab3259	1:400
VDAC	Rabbit	Polyclonal	Cell Signaling Tech.	4866	1:1000

Immunocytochemistry

For visualization of PDHE1-α cellular distribution, cells were incubated with 0.5 μM MitoTracker® Red CMXRos (M-7512; Molecular Probes, Life Technologies Corp.), which preferentially accumulates in mitochondria, for 30 min at 37°C before cell harvesting. Cells were washed twice, fixed with 4% PFA in PBS, washed three times and then blocked for 1 h at room temperature in PBS, containing 0.1% Triton-X-100, 1% FBS, and 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., United States). Cells were incubated with a primary mouse monoclonal antibody reactive to PDHE1-α (ab110330, Abcam®) at a dilution of 1:200, overnight at 4°C. After three washes, the secondary DyLight 488-conjugated anti-mouse (211-482-171; Jackson ImmunoResearch) diluted at 1:200 was added to cells for 2 h at room temperature. Mouse NSC nuclei were stained with Hoechst 33258 (861405; Sigma-Aldrich Corp.) at 5 μg/mL in PBS, for 3 min at RT and, then, washed three times. Samples were mounted using Mowiol® 4-88 (81381; Sigma-Aldrich Corp.) PDHE1-α cellular distribution in NSCs was evaluated using fluorescence microscopy assessments performed with a Zeiss AX10 microscope (Carl Zeiss Corp.), equipped with a 63x/1.4 oil plan-apochromat objective and an AxioCam HRm camera (Carl Zeiss Corp.). Images were processed using ImageJ.

Immunohistochemistry

Brains were postfixed for 1 day in 4% PFA and then cryopreserved in 30% sucrose. For immunohistochemistry of NSC markers, brains were coronally processed in 30-μm-thick cryostat sections

that were collected in a series of 10 slides. Each series contained an anterior-posterior reconstruction of four brain sections separated 300 μm between them. Slides were consecutively washed with 0.1 M phosphate buffer (PB) (0.2 M Na_2HPO_4; 0.2 M NaH_2PO_4; purified water; pH adjust between 7.2 to 7.4 with NaOH). Antigen retrieval was performed by 20 min incubation in 2 N HCl solution at 37°C followed by 10 min incubation in borate solution (pH 8.5). Slides were rinsed in PB solution and blocked for 1 h at RT in block solution (PB + 10% FBS + 0.2% Triton x-100 [10%]). For LCAD detection, slides were incubated overnight with the primary mouse monoclonal antibody anti-LCAD diluted in block solution (1:200) (17526-1-AP, Proteintech). Secondary antibody donkey anti-rabbit Alexa Fluor 568 diluted in block solution (1:400) (A10042; Life Technologies Ltd.) was incubated for 2 h. For DCX detection, slides were incubated overnight with the primary goat polyclonal antibody diluted in block solution (1:200) (sc-8066; Santa Cruz Biotechnology). Subsequently, secondary antibody biotinylated anti-goat IgG diluted in PB (1:200) (BA-9500; Vector Laboratories) was incubated for 30 min. Immunoreactivity was then detected with Cy^{TM}2-conjugated streptavidin-conjugated anti-goat diluted in PB (1:200; Jackson ImmunoResearch Laboratories Inc.) and incubated for 30 min. For negative controls, the primary antibody was omitted during the staining. Nuclei were counterstained with Hoechst 33342 (1:1000 in water) (Invitrogen Corp.). The images were acquired using Leica DMi8 confocal fluorescence microscope and processed using ImageJ.

Oil-Red-O Staining

The intracellular accumulation of lipids in NSCs was evaluated by Oil-Red-O (ORO)/isopropanol method (Fukumoto and Fujimoto, 2002). Free FAs mixture (2:1, oleate: BSA-palmitate) in a concentration of 500 μM was added to NSCs for positive control, also 500 μM of BSA for endogenous control. The work solution of ORO (O-0625; Sigma-Aldrich Corp.) was prepared, ORO was dissolved in ≥99.8% isopropanol (109634; Merck Millipore Corp) and left overnight at room temperature. The solution was filtered with cellulose acetate membrane syringe filter, pore size 0.45 μm (1520014; Frilabo). Three parts of ORO solution were added to 2 parts of bidistilled H_2O. The solution was then filtered, left to stand for 30 min, and filtered again before use. Before staining, the dishes were then gently rinsed with PBS. One milliliter of 4% (w/v) PFA was added to each dish for 20 min to fix the cells at room temperature. Each dish was then rinsed twice with PBS and once with 60% (v/v) of isopropanol. ORO stain was applied for 5 min. The dishes were rinsed with PBS, followed by the addition of Mayer's hematoxylin solution (MHS-32; Sigma-Aldrich Corp.) for 2 min. The dishes were then rinsed with PBS, air dried and mounted under a glass coverslip with 30 μL PBS/glycerol (3:1, v/v). The staining effectiveness was evaluated with Zeiss Axioskop 50 microscope (Carl Zeiss Corp., Germany) inverted research microscope equipped with Axiocam 105 color (Carl Zeiss Corp.). Images (400x) were captured, enhanced and quantified using ImageJ.

Liquid Chromatography Mass Spectrometry-Based Proteomics

To investigate the impact of TUDCA on the mitochondrial proteome in self-renewing and differentiating mouse NSCs, we performed liquid chromatography coupled with mass spectrometry (LC-MS). Briefly, NSC line NS-TGFP was expanded and induced to differentiate for 24 h with or without TUDCA. Mitochondrial protein enriched extracts were obtained by using Qproteome Mitochondria isolation kit (Qiagen) and resuspended in 6 M urea and 50 mM ammoniumbicarbonate. Protein tryptic digestion was performed according to (Llombart et al., 1996). Shortly, disulfide bonds were reduced by DTT and cysteine residues alkylated with iodoacetamide. Samples were digested with trypsin (Promega) and the reaction was stopped by addition of 0.5% formic acid. A total of 2 μg of each digest was first separated by a nano-HPLC system (Proxeon, Denmark) and then the peptide mass spectra acquired using a Maxis Impact Q-TOF spectrometer (Bruker, Bremen, Germany). The peptides were first concentrated on a 100 μm ID 2 cm nano-trapping column (Proxeon) and then loaded onto a 75 μm ID, 25 cm Acclaim PepMap nano-separation column (Thermo). The chromatography run using a 0.1% formic acid – acetonitrile gradient (2–30% in 120 min at a flow rate of 300 nL/min). The column was coupled to the mass spectrometer inlet through a Captive Spray ionization source (Bruker). MS acquisition was set to cycles of MS, each followed by 3 cycles of MS/MS, with an intensity threshold for fragmentation of 2,000 counts, and using a dynamic exclusion time of 2 min, with an automated precursor re-selection when a 3-fold increase in intensity was observed. Spectra were acquired on the range of 150–2,200 Da. LC-MS/MS data were pre-processed using the Data Analysis 4.2 software (Bruker). Data normalization, database searching, and protein identification were performed using MaxQuant software (v1.5.2.8) and protein quantification was determinate by MaxLFQ algorithm. Searches on a mouse database retrieved from Uniprot/SwissProt or NCBI database (downloaded on 2016, containing 166,073 reviewed sequences) included trypsin as digesting enzyme with a maximum of 2 missed cleavages; cysteine carbamidomethylation set as fixed modification and methionine oxidation as variable modification. The peptide mass tolerances of the first search and main search (recalibrated) were <0.07 and 0.006 Da, respectively. The minimum peptide length was seven amino acids, and the maximum peptide mass was 4,600 Da. Both peptides and proteins were filtered with a maximum false discovery rate (FDR) of 0.01. The match between runs feature with a matching window of 0.7 min and an alignment window of 20 min, was activated. Label-free quantitation (LFQ) calculations were performed separately in each parameter group containing similar cell loadings. All peptides were selected for protein quantification. Other unmentioned parameters were the MaxQuant default settings. Potential contaminants and reverse sequences were filtered out. The extracted data were further processed with Metaboanalyst. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD017979. All MS analysis procedures were performed at the Laboratori de Proteoòmica, Vall d'Hebron

Institut d'Oncologia (VHIO), Institut de Recerca Hospital Univ. Vall d'Hebron, Barcelona, Spain. A BLASTp search was performed through the BLAST2GO software (BioBam). This search retrieved the gene ontology (GO) annotations of the identified proteins by using GO categories (cellular location, molecular function and biological process) of the best hit derived from the BLASTp results (BLASTp minimal expectation value set to $<1 \times 10^{-3}$). Assignment of experimental condition-specific pathways and biological processes were performed inSTRING (Search tool for the retrieval of interacting genes/proteins) database. False Discovery Rate of the group of proteins belonging to UniProt Keywords and Biological Process GO was <0.001.

Gas Chromatography–Mass Spectrometry Analysis of Fatty Acids

The qualitative and quantitative study of individual FA was performed using Gas Chromatography-Mass Spectrometry (GC-MS) (Shimadzu QP2010 Plus) according to Costa et al. (1998). Analyses were performed either in scanning or single ion monitoring (SIM) acquisition modes, where the obtained retention parameters and mass spectra were interpreted using an in-house MS library. Internal standard (IS) method was used for quantification (Sigma Chemical Co., United States). For the analysis of metabolites including the most important saturated, unsaturated and hydroxylated short-, medium- and long-chain FA up to C18, cells were collected by scraping in ice-cold 80% (v/v) of methanol (106009; Merck Millipore Corp). The respective homogenate was washed three times and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was evaporated to dryness under a nitrogen stream and derivatized with N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA): methyl-bis(trifluoroacetamide) (MBTFA): Pyridine (4:5:1) (Pierce-Thermo Scientific, United States) at 60°C for 1 h. Samples were injected with an automatic injector (AOC 20i), using the split injection mode (1/50 ratio). Acquisition in SIM mode was based on specific masses (m/z) selected as function of respective FA fragmentation spectrum. The peak area ratios (analyte/IS) were normalized to total protein content (mg). Data are expressed as fold-change relative to untreated cells (control). Total protein extracts were measured by the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's specifications, using BSA as standard.

Transfection Assays

For the RNA interference of LCAD, NS-TGFP were transfected in differentiation induced medium with 60 nM of small interfering RNA (siRNA) specific for LCAD (Acadl Silencer Pre-designed siRNA cat# AM16708 ID162072, Ambion) or the respective negative control (Silencer® Select Negative Control #1 siRNA cat#4390844, Ambion) with Lipofectamine™ 3,000 (Invitrogen Corp.), 24 h after platting. At the end of treatments, cells were detached with accutase and collected for analysis of NSC fate. To assess silencing efficiency, total levels of LCAD were determined by Western blot (**Supplementary Figure S1A**).

TABLE 2 | List of primers used for qPCR.

	Sequence (5′–3′)
acc1	5′-GGA CAC CAG TTT TGC ATT CA-3′ (fwd)
	5′-AGT TTG GGA GGA CAT CGA AA-3′ (rev)
nestin	5′– CTC AGA TCC TGG AAG GTG GG-3′ (fwd)
	5′-GCA GAG TCC TGT ATG TAG CCA-3′ (rev)
ki67	5′-CCT TTG CTG TCC CCG AAG A-3′ (fwd)
	5′-GGC TTC TCA TCT GTT GCT TCC T-3′ (rev)
map-2	5′-GTT CAG GCC CAC TCT CCT TC-3′ (fwd)
	5′-CTT GCT GCT GTG GTT TTC CG-3′ (rev)
hprt	5′-GGT GAA AAG GAC CTC TCG AAG TG -3′ (fwd)
	5′-ATA GTC AAG GGC ATA TCC AAC AAC A-3′ (rev)

Total RNA Extraction

Total RNA was extracted using the RIBOZOL™ reagent (AMRESCO, LLC, Solon, OH, United States) according to manufacturer's instructions. Each sample was homogenized on 0.5 mL RIBOZOL™ reagent. After mixing with 0.1 mL chloroform, each sample was centrifuged at 12,000 g for 15 min at 4°C and the aqueous phase collected. Total RNA was precipitated by incubation with 0.25 mL isopropyl alcohol at −20°C for 1 h. Samples were centrifuged at 12,000 g for 10 min at 4°C and the RNA pellet was washed with 75% ethanol and centrifuged at 7,500 g for 5 min at 4°C. RNA pellets were air dried and resuspended in 40 μL RNase-free water. The purity of RNA was checked and DNA contaminations were eliminated with DNase I recombinant (04716728001; Roche Applied Science, Mannheim, Germany) following manufacturer's instructions.

Quantitative RT-PCR (qRT-PCR)

cDNA synthesis was performed using the NZY Reverse Transcriptase (NZYTech, Lisbon, Portugal) according to manufacturer's instructions. Real-time RT-PCR was performed using SensiFastTM SYBR® Hi-ROX Kit (Bioline USA Inc., Taunton, MA, United States) in the Applied Biosystems 7300 System (Thermo Fisher Scientific Inc.). Primer sequences can be found in **Table 2**. Relative gene expression was calculated based on the standard curve and normalized to the level of hypoxanthine phosphoribosyltransferase (*HPRT*) housekeeping gene and expressed as fold change from controls.

Densitometry and Statistical Analysis

In proteomics analyses, significance threshold was set to $p < 0.05$, minimum MASCOT ions score of 20. Protein identifications with only one peptide with 95% confidence were performed using two additional quality criteria: sequence coverage of ≥10% and a deviation of predicted mass, RMS90 of ≤50 ppm. The relative intensities of protein bands in immunoblotting were analyzed using the Image Lab Version 5.0 densitometric analysis program (Bio-Rad Laboratories). Results were compared using an unpaired Student's t-test. Values of $p < 0.05$ were considered statistically significant. Statistical analysis was performed with GraphPad Prism 6.1 software (GraphPad Software, Inc., United States).

RESULTS

TUDCA Decreases LCAD Protein Levels in Early Differentiation of NSCs

We have shown that the endogenous bile acid TUDCA is a well-known mitochondrion protecting agent in early stages of neural differentiation (Xavier et al., 2015), while promoting the proliferation of NSCs, both *in vitro* and *in vivo*, to increase NSC pool (Xavier et al., 2014; Soares et al., 2018). However, these intriguing findings await further exploration to better understand how NSC fate is controlled by mitochondria.

Therefore, we investigated the impact of TUDCA on the mitochondrial proteome in mouse NSCs by high-throughput proteomics analysis using liquid chromatography coupled with mass spectrometry (LC-MS). Proteomics data revealed possible novel targets downregulated by TUDCA in NSCs. Surprisingly, most of these mitochondrial proteins were significantly downregulated or even absent in the presence of TUDCA in NSCs (**Figure 1A** and **Supplementary Table S1**). We identified proteins involved in cell metabolism, such as aldehyde dehydrogenase 2 (ALDH2) responsible for converting acetaldehyde metabolized from ethanol to acetate, and the acetyl-CoA acetyltransferase (ACAT1, also known as β-thiolase) that catalyzes the condensation of two acetyl-CoA to acetoacetyl-CoA, as well as the reverse reaction, by breaking down acetoacetyl-CoA (Fukao et al., 1990). Also, the pyruvate carboxylase (PC), which forms oxaloacetate for anaplerotic purposes (Jitrapakdee et al., 2008) was absent in TUDCA-treated NSCs. Curiously, neurons lack PC (Danbolt, 2001; Hertz and Zielke, 2004) and TUDCA-induced PC ablation could represent part of the mechanism by which TUDCA primes NSCs toward neuronal fate (Xavier et al., 2014; Soares et al., 2018). The ornithine aminotransferase (OAT), which participates in amino acid metabolism was also shown to be downregulated by TUDCA. The main function of OAT is to control the production of signaling molecules, such as glutamate (Ginguay et al., 2017).

In relation with FA metabolism, TUDCA was shown to markedly downregulate the long-chain acyl-CoA dehydrogenase (LCAD). In fact, LCAD and ACAT1 can be involved in the β-oxidation of FAs. LCAD catalyzes a key initial step in mitochondrial FAO, acting on C8-C20 FAs (Kompare and Rizzo, 2008), while ACAT1, acts downstream to LCAD (Houten et al., 2016). Interestingly, STRING analysis suggested that all mitochondrial proteins downregulated by TUDCA are somehow involved in acetylation-related processes (UniProt Keywords GO number KW-0007) predicting functional links between proteins, such as between LCAD and ACAT1 (**Figure 1B**). Although LCAD and ACAT1 do not physically interact, the STRING analysis also revealed that they are co-expressed in the same manner, indicating a possible general inhibition of lipid catabolism by TUDCA in NSCs. In mice, LCAD plays an essential role in the degradation of polyunsaturated fatty acids (PUFAs) and of branched-chain FAs. Thus, this protein seems to play a more redundant role in the oxidation of long- and straight-chain saturated FAs (van Vlies et al., 2005; Chegary et al., 2009). To further understand the significance of the β-oxidation

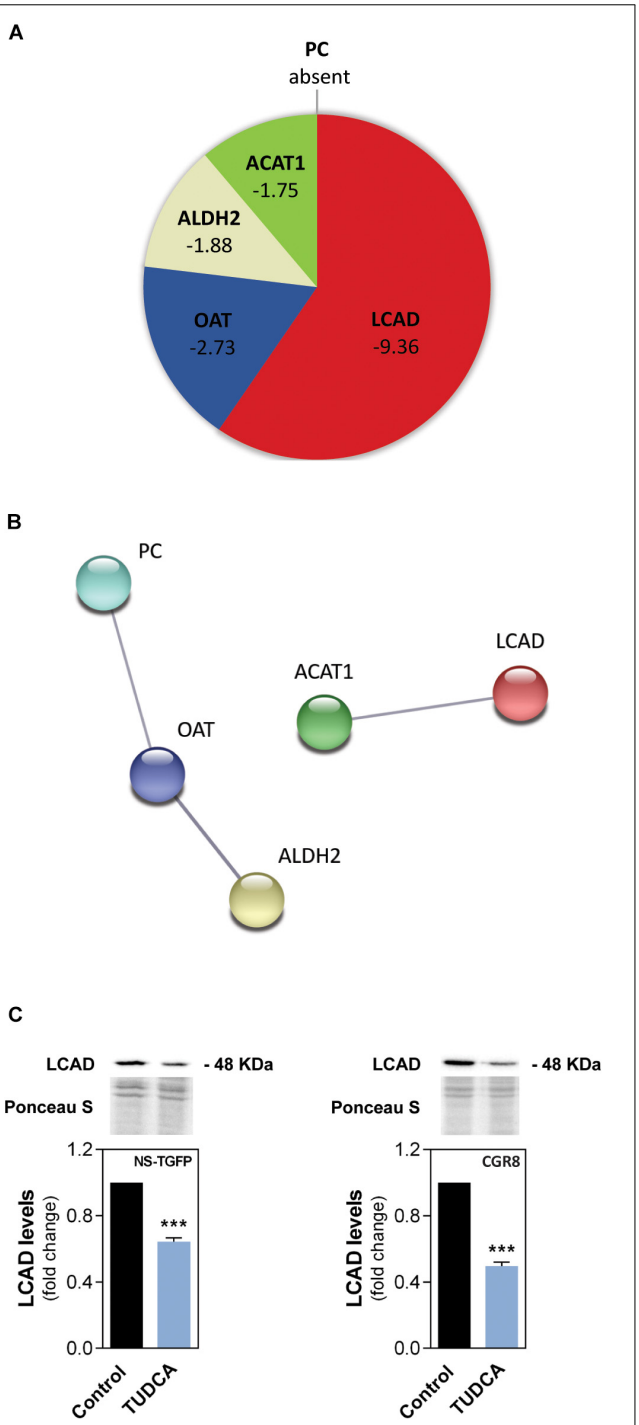


FIGURE 1 | The proliferation-inducing agent TUDCA downregulates a FA oxidation-associated enzyme in NSCs. Early-differentiating mouse NSC lines NS-TGFP and CGR8 were incubated with TUDCA for 24 h. Mitochondrial protein purified extracts were isolated for liquid chromatography mass spectrometry-based proteomics (LC-MS) and/or Western blot analysis, as described in Materials and Methods. **(A)** Pie diagram of the proteomic data showing the mitochondrial proteins significantly regulated by TUDCA in NS-TGFP cell line, and respective variations, when compared with control. The proteins were identified as the long-chain acyl-CoA dehydrogenase

(Continued)

FIGURE 1 | Continued
(LCAD), the ornithine aminotransferase (OAT), the aldehyde dehydrogenase 2 (ALDH2), the acetyl-CoA acetyltransferase (ACAT1) and the pyruvate carboxylase (PC). Results are expressed as mean in fold-change, for at least three independent experiments, when compared with control (untreated cells). Significance threshold was set to $p < 0.05$ from control (untreated cells). **(B)** STRING analysis showing protein-protein network between the most regulated mitochondrial proteins by TUDCA in NSCs. Nodes represent proteins and lines connecting nodes indicate direct, or indirect interactions between proteins. Colorful nodes represent proteins that are downregulated or absent in TUDCA-treated NSCs. Functional protein network database version 10.0 (http://string-db.org, accessed in August 2019).
(C) Representative immunoblots (*top*) of LCAD mitochondrial levels and respective densitometry analysis (*bottom*) in mitochondrial protein extracts from the NSC lines. LCAD levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. $^{***}p < 0.001$ from control (untreated cells).

in NSC fate changes induced by this proliferation-inducing agent we validated the TUDCA-downregulated LCAD from proteomics data. The validation was performed by Western blot analysis in the same mouse NSC line (NS-TGFP) as well as in an additional one (CGR8). Our results showed that TUDCA significantly decreased LCAD protein levels by ∼40 and 50% in NS-TGFP and CGR8 cell lines, respectively, when compared to the respective controls (**Figure 1C**). Therefore, our results revealed that TUDCA markedly reduces LCAD in NSCs, an enzyme pivotal for β-oxidation of long-chain FAs.

LCAD Downregulation Is Associated With Increased NSC Proliferation

How metabolism is reprogrammed during NSC fate remains largely unknown. On the other hand, neural differentiation is a multistep process that shapes and re-shapes NSCs by progressing through several typical stages. Thus, we evaluated the expression of LCAD throughout the early stages of NSC differentiation by immunoblotting analysis, and investigated the impact of LCAD silencing on NSC proliferative and differentiation potential by qRT-PCR.

Our results showed that LCAD protein levels gradually increased up to 2.5-fold (**Figure 2A**), after 3 days of NSC differentiation, when compared to undifferentiated cells. More importantly, we found that 50% of LCAD downregulation (**Supplementary Figure S1A**) almost doubled the proliferative marker ki67 of NSCs and significantly decreased neurogenesis progression. In fact, siRNA-induced silencing of LCAD in NSCs resulted in a marked increase of the undifferentiation marker, Nestin and a significant decrease of the neuronal marker Map-2 (**Figure 2B**). The transiently inhibition of LCAD expression in transfected NSCs was validated by Western blot after 24 h (**Supplementary Figure S1A**).

We then investigated whether LCAD expression was indeed decreased in the neurogenic niches of adult rat brains after TUDCA exposure (**Figure 2C**). Hence, extra brain slices collected from our previous study, revealing the proliferative effects of TUDCA *in vivo* (Soares et al., 2018), allowed the assessment of TUDCA effect on LCAD levels in the same animals through immunohistochemistry staining against LCAD in the subventricular zone (SVZ) neurogenic region. Notably, we found that after 28 days of TUDCA administration in adult rat brains using mini-osmotic pumps induced a significant decrease of this specific FAO-related enzyme in SVZ neurogenic regions, corroborating the negative effect of TUDCA on LCAD in cells. Thus, our results support the idea that TUDCA increases the NSC pool by repressing this specific enzyme suggesting decreased flux of long-chain FAs through mitochondrial β-oxidation.

TUDCA Stimulates *de novo* Lipogenesis in NSCs

To further clarify how TUDCA modulates the balance between FA oxidation and respective biosynthesis in NSCs, we investigated protein levels of the sterol regulatory element-binding protein 1 (SREBP-1) in NSCs, since this protein is a key transcription factor involved in lipogenesis (Eberlé et al., 2004). Curiously, at odds with results previously obtained for LCAD, SREBP-1 protein levels decreased by ∼50% (**Figure 3A**) after 3 days of differentiation, when compared to undifferentiated cells. More importantly, in the same previous conditions, TUDCA significantly increased the levels of nuclear SREBP-1 constitutively active form, when compared to control (**Figure 3B**), suggesting that proliferating NSCs drive cell metabolism to favor lipogenesis rather than lipid oxidation. The TUDCA impact in lipogenesis was corroborated by qRT-PCR experiments in NSCs treated and untreated with TUDCA. Interestingly, our results indicated that TUDCA not only increases SREBP, but also significantly enhances mRNA levels of other lipogenesis-associated genes, such as the acetyl-CoA carboxylase 1 (ACC1) (**Figure 3C**), which in turn connects central energy metabolism to lipid biosynthesis and codes for a protein that is rate-limiting for the *de novo* synthesis of lipids.

To further dissect the metabolic alterations undergoing proliferation, we then compared the intracellular and extracellular FA levels of TUDCA-treated and untreated NSCs. Interestingly, after 24 h of TUDCA treatment, we observed an ∼50% reduction in total levels of dicarboxylic saturated medium chain FA (C6:0; adipic acid) and hexadecanoic acid (C16:0, palmitic acid; PA) (**Figure 3D**), while no significant differences in other relevant saturated or unsaturated long-chain fatty acids (up to C18:0) were observed (data not shown). On extracellular media, milder significant decreases were also found in saturated octanoic (C8:0), decanoic (C10:0), dodecanoic (C12:0) (data not shown). Of note, palmitate serves as building lipid blocks and can be elongated and unsaturated to form more complex lipids who may have critical cell signaling functions (Bieberich, 2012; Knobloch, 2016). It is important to note that, although the marked decrease in palmitic levels may indicate a reduction in lipogenesis rates, it might also indicate a high consumption of this metabolite to assure lipid synthesis progress. Nevertheless, to understand TUDCA impact on NSC lipid

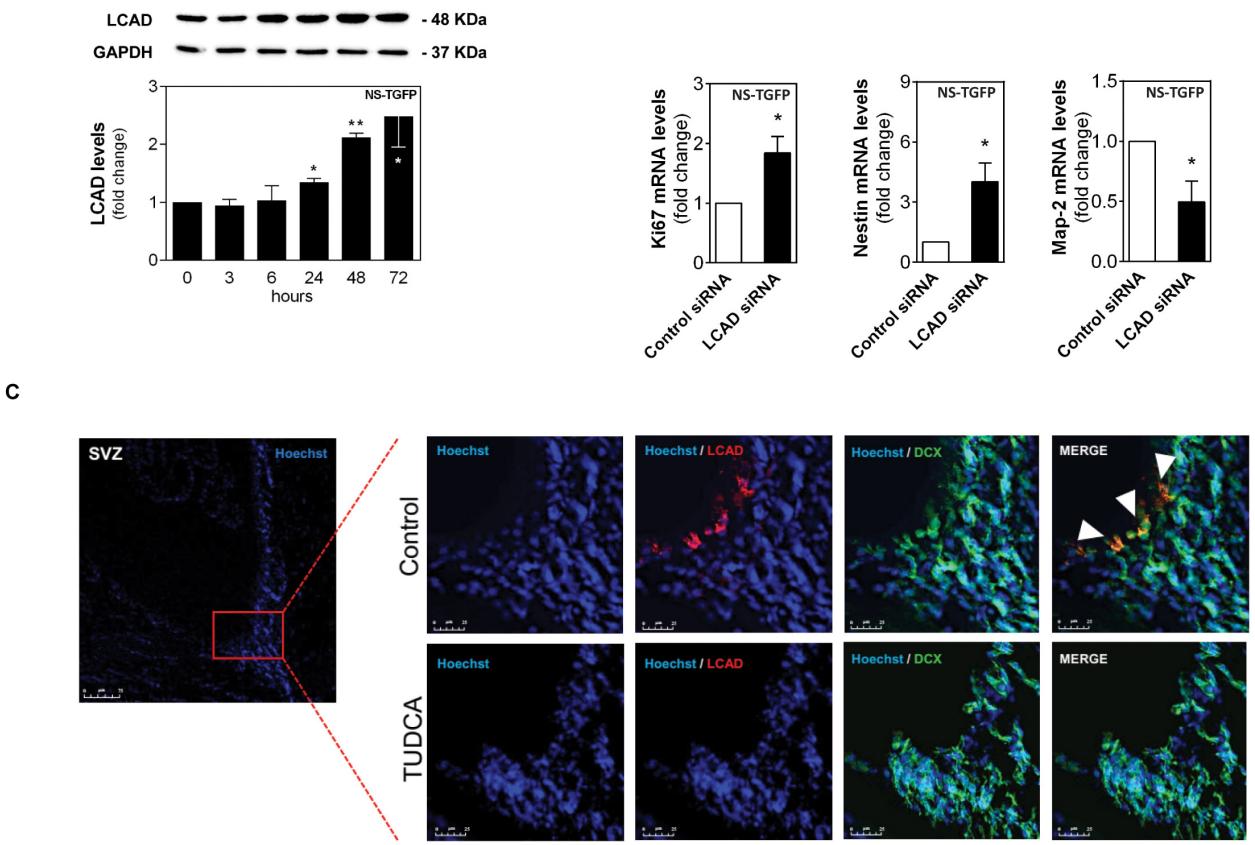


FIGURE 2 | LCAD downregulation is associated with increase of NSC proliferation. The NSC line NS-TGFP was induced to differentiate for up to 72 h and total proteins were collected for immunoblotting analysis. NSCs were also transfected with either control or LCAD siRNAs, and collected for RT-PCR analysis to assess mRNA expression levels of proliferation, stemness and differentiation markers after 24 h. In addition, adult rats were treated with TUDCA or vehicle for 28 days using miniosmotic pumps, as described in Materials and Methods. Immunohistochemistry against LCAD (red) and NSC early differentiation marker DCX (green) was performed. **(A)** Representative immunoblots of total LCAD levels (top) and corresponding densitometry analysis (bottom) in NSCs throughout differentiation. Total LCAD levels were normalized to GAPDH. **(B)** mRNA expression levels of proliferation (ki67), stemness (Nestin) and differentiation (Map-2) markers in NSCs collected 24 h after siRNA transfection. Values were normalized to the internal standard HPRT. Data are expressed as mean ± SEM fold-change for at least three independent experiments. $^{**}p < 0.005$ and $^{*}p < 0.05$ from control (untreated cells). **(C)** Representative confocal images of LCAD- and DCX-positive cells in frontal sections of adult SVZ neurogenic niches in TUDCA-treated and untreated rats ($n = 4$). Nuclei were stained with Hoechst. Scale bars, 75 μm in SVZ image and 25 μm in representative image sections with or without TUDCA treatment.

content we evaluated the intracellular lipid accumulation in NSCs treated and untreated with TUDCA by staining NSCs with Oil-Red-O (ORO). In fact, accumulation of oleic acid was recently shown to decrease NSC proliferation in an Alzheimer's disease mouse model (Hamilton et al., 2015). Interestingly, NSCs did not show any sign of lipid accumulation after 24 h of TUDCA treatment, having a similar appearance compared to control. In contrast, NSCs treated with long-chain FAs, such as oleate: BSA-palmitate, used as a positive control, showed intracellular major lipid droplets around the nucleus whereas the relative control was clear of lipid accumulation (**Figure 3E**). These results show that TUDCA induces a major upstream regulator of lipogenesis, without increasing lipid droplets, which can be important to enable generation of lipid membranes and stimulate the proliferation activity of NSCs.

TUDCA Increases PDHE1-α and Potentiates a Novel Mechanism for Its Mitochondria-Nucleus Translocation in NSCs

Next, we attempted to unravel the possible impact of TUDCA in cellular energy production of NSCs through glucose metabolism and oxidation. In fact, we hypothesized that a decreased β-oxidation of long-chain FAs induced by this bile acid might ignite a metabolic shift in these cells, as a strategy to ensure energy production.

The pyruvate dehydrogenase complex (PDC) provides the primary link between glycolysis and the tricarboxylic acid cycle (TCA) cycle by catalyzing the irreversible conversion of pyruvate into acetyl-CoA (Varum et al., 2011). The pyruvate

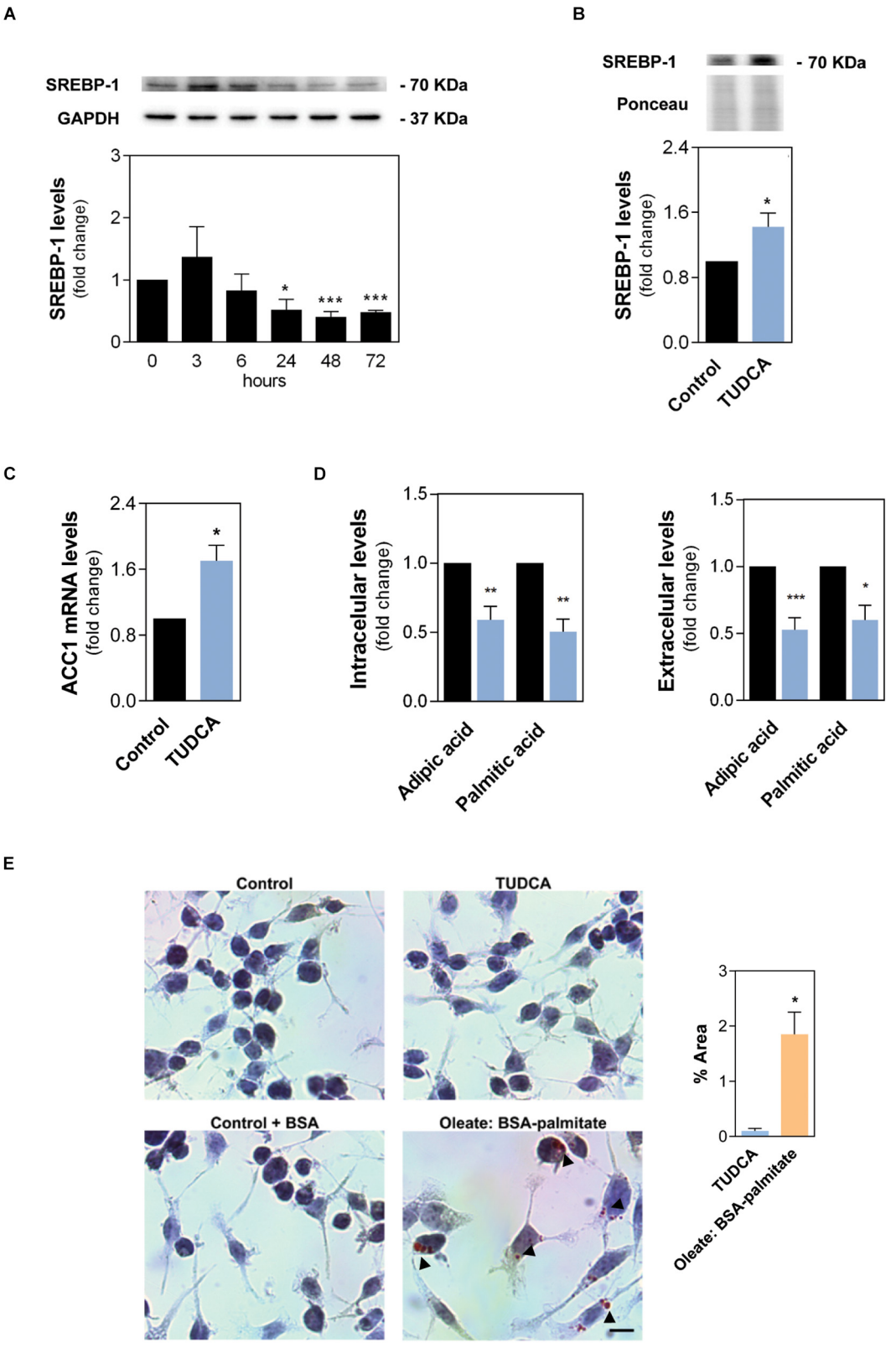


FIGURE 3 | The proliferation-inducing agent TUDCA supports *de novo* lipogenesis in NSCs. The NSC line NS-TGFP was induced to differentiate for up to 72 h and total proteins were collected for immunoblotting analysis. Total and nuclear protein extracts were isolated for Western blot analysis. Early-differentiating mouse NSCs were also incubated with TUDCA for 24 h and collected for Western blot, RT-PCR, GC-MS analysis and Oil-Red-O staining, as described in Materials and Methods.

(Continued)

FIGURE 3 | Continued
(A) Representative immunoblots of total SREBP-1 levels (top) and corresponding densitometry analysis (bottom) in NSCs throughout differentiation. Total SREBP-1 levels were normalized to GAPDH. Data are expressed as mean ± SEM fold-change for at least three independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$ from undifferentiated cells. **(B)** Representative immunoblots of SREBP-1 levels (top) and corresponding densitometry analysis (bottom) in nuclear protein extracts. Nuclear SREBP-1 levels were normalized to Ponceau S. **(C)** mRNA expression levels of ACC1 lipogenesis marker in NSCs collected 24 h after TUDCA exposure. Values were normalized to the internal standard HPRT. **(D)** Cellular amount of palmitic and adipic acid in differentiating NSCs detected by gas chromatography coupled with mass spectrometry (GC-MS) analysis. The peak-area ratios (sample/internal standard) were normalized to total protein content (mg). Data are expressed as mean ± SEM fold-change for at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ from control (untreated cells). **(E)** Representative images of the lipid droplets in red (indicated by arrows) in NSCs and respective quantification. Free FAs mixture (oleate: BSA-palmitate) and BSA were added to NSCs for positive and endogenous control, respectively. Nuclei were stained with hematoxylin. Scale bar, 25 μm. Data are expressed as mean ± SEM fold-change for at least three independent experiments. $^{*}p < 0.05$ from TUDCA-treated cells.

dehydrogenase E1-α (PDHE1-α) contains the E1 active site from PDC, therefore playing a key role in its function. Thus, we investigated possible changes of PDHE1-α subunit during early stage NSC differentiation. Our results showed that, although the total levels of PDHE1-α did not show any substantial differences throughout differentiation induction (**Figure 4A**), TUDCA is capable of significantly increasing mitochondrial levels of PDHE1-α in differentiating NSCs. In NS-TGFP and CGR8 cell lines, PDHE1-α levels increased almost 2-fold, when compared to controls (**Figure 4B**). Upregulation of PDHE1-α by TUDCA appears to indicate that the conversion of pyruvate into acetyl-CoA is favored in mitochondrial matrix.

Nuclear PDHE1-α exists in human normal and cancer cells as a critical step to generation of acetyl-CoA and histone acetylation in mitotic phase (Sutendra et al., 2014). Thus, we decided to also explore this possibility in our context and, surprisingly, our results showed that PDHE1-α was indeed present in the nucleus of NSCs and further accumulated after TUDCA treatment. In fact, TUDCA markedly induced nuclear levels of PDHE1-α, increasing ∼70% when compared to control (**Figure 4C**).

The cellular distribution of PDHE1-α in NSCs was also evaluated by immunocytochemistry, colocalizing with both mitochondria organelles and nuclei. In fact, PDHE1-α was detectable in the nucleus in both untreated and TUDCA-treated NSCs. Corroborating our previous results, TUDCA-induced upregulation of PDHE1-α was observed both in mitochondria and in the nucleus, when compared to control (**Figure 4D**). The purity of mitochondrial and nuclear fractionation was validated by Western blot (**Supplementary Figure S1B**).

The mechanism of PDC mitochondrial-nuclear translocation has been shown to be dependent on the chaperone Hsp70, which facilitates its nuclear import through association with E1 and E2, two of the three enzymes of PDC. In fact, induction of Hsp70 is cell cycle-dependent, with its highest expression also observed during S phase (Sutendra et al., 2014; Tang, 2015). In agreement, immunoblotting analysis demonstrated that Hsp70 is expressed in mitochondria and its levels are increased in NSCs treated with TUDCA (**Figure 4C**).

Acetyl-CoA generated by PDC in the nucleus is apparently important for histone acetylation, as required for S phase entry and cell cycle progression (Tang, 2015). Moreover, TUDCA enhances NSC proliferation by modulating cell cycle progression, decreasing cells in G0-G1 phase and markedly increasing S/G2-M phases 24 h after the induction of NSC differentiation (Xavier et al., 2014). Thus, we finally assessed the acetylation levels of histone 3 (H3) in NSCs. Our results demonstrated that H3 total levels slightly increased in the presence of TUDCA. In addition, the levels of histone 3 acetylation were also similarly increased by TUDCA (**Figure 4E**). This indicates that TUDCA-induced H3 expression is accompanied by an increase in H3 acetylation levels. However, when acetyl-H3 levels were normalized to total levels of H3 (data not shown), we did not detect any significant difference in acetyl-H3 levels by TUDCA treatment, indicating that the increase in the acetylation process is, indeed, dependent on H3 total expression levels. The purity of histone extracts was carefully certified (**Supplementary Figure S1C**). These results strongly support the idea that TUDCA induces glucose oxidation (via pyruvate decarboxylation) and subsequent increases nuclear PDC levels for histone acetylation in proliferating NSC (**Graphical Abstract**).

DISCUSSION

Adult neurogenesis contributes to maintain physiological homeostasis and regeneration of damaged tissue in the brain (Ma et al., 2009). The decrease in the NSC pool is a primary factor for decline of both neurogenesis and its paracrine activity, throughout life (Kuhn et al., 1996). Thus, a better understanding of the mechanisms that regulate adult NSC behavior proves to be pertinent for the discovery of novel efficient strategies that enhance brain regeneration. The present work shows that the endogenous neuroprotective bile acid, TUDCA, a NSC proliferation-inducing agent, drives a metabolic remodeling involving pyruvate dehydrogenase nuclear translocation (Sutendra et al., 2014), previously speculated to also occur in stem cells (Lisowski et al., 2018).

Some of the molecular events involved in NSC differentiation are already well described; however, the bioenergetic demands and how strictly the energy metabolism is ruled to ensure adult NSC life-long activity is still unclear. TUDCA has been described as an antiapoptotic and antioxidant mitochondrial agent in NSCs and in mature neurons (Rodrigues et al., 2000; Xavier et al., 2014). Furthermore, this molecule was shown to enhance NSC proliferation and neuronal differentiation, both *in vitro* and *in vivo* (Xavier et al., 2014; Soares et al., 2018).

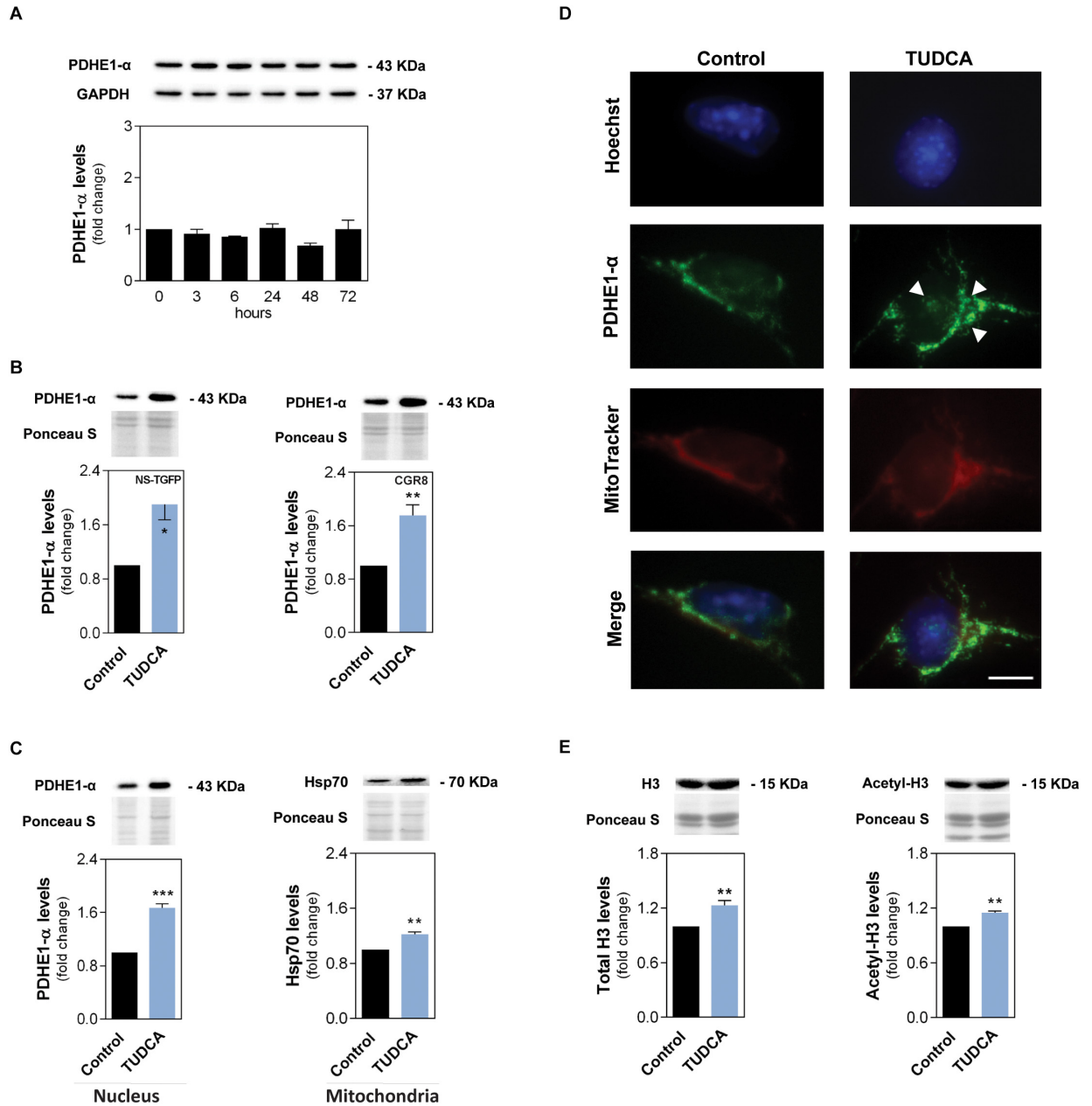


FIGURE 4 | TUDCA increases mitochondrial and nuclear levels of PDHE1-α in NSCs while supporting acetylation of increased H3. The NSC line NS-TGFP was induced to differentiate for up to 72 h and the total protein collected for immunoblotting analysis. Early-differentiating NSCs were also incubated with TUDCA for 24 h and collected for Western blot and immunocytochemistry, as described in Materials and Methods. **(A)** Representative immunoblots of total PDHE-1α levels (top) and corresponding densitometry analysis (bottom) in NSCs throughout differentiation. Total PDHE-1α levels were normalized to GAPDH. Data are expressed as mean ± SEM fold-change for at least three independent experiments. **(B)** Representative immunoblots of PDHE1-α levels (top) and corresponding densitometry analysis (bottom) in mitochondrial protein extracts of NS-TGFP and CGR8 cell lines. **(C)** Representative immunoblots of PDHE1-α (right) and Hsp70 (left) levels (top) and corresponding densitometry analysis (bottom) in nuclear and mitochondrial protein extracts, respectively. **(D)** Representative images of immunofluorescence detection of cells labeled with an anti-PDHE1-α antibody (green). Nuclei were stained with Hoechst 33258 (blue) and mitochondria with Mitotracker (red). Scale bar, 20 μm. **(E)** Representative immunoblots of total H3 (left) and acetyl-H3 (right) levels (top) and corresponding densitometry analysis (bottom) in purified histone extracts from NS-TGFP cells. Specific mitochondrial and histone protein levels were normalized to Ponceau S. Data are expressed as mean ± SEM fold-change for at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ from control (untreated cells).

Here we deciphered the TUDCA mitochondrial proteomic signature in NSCs, demonstrating that this proliferation-inducing molecule also regulates pivotal metabolic-related proteins. Importantly, TUDCA was shown to markedly downregulate the levels of LCAD in early differentiating NSCs. Indeed, LCAD is highly expressed in the fetal brain, having a

relative expression in the CA1 and CA4 areas and granular layer of human hippocampus, regions of adult neurogenesis and with proximal spatial relationship (Sohur et al., 2006; He et al., 2007). Moreover, it was recently demonstrated that LCAD activity is a source of FAO-driven H_2O_2 in mitochondria (Zhang et al., 2019). Accordingly, TUDCA as an antioxidant molecule is known to attenuate H_2O_2-induced reactive oxygen species generation (Xavier et al., 2014), which may indicate that TUDCA-mediated suppression on H_2O_2-induced oxidative stress perhaps happens through LCAD inhibition. Interestingly, hypoxia-inducible factor 1 (HIF-1) was recently shown to suppresses fatty acid β-oxidation in cancer cells through medium- and long-chain acyl-CoA dehydrogenases, hence sustaining proliferation and tumor progression. Again, this effect was revealed to be dependent on reactive oxygen species (ROS) levels controlling process (Zhang, 2015). In fact, it would be interesting, in the future, to clarify whether TUDCA controls LCAD expression by a HIF-1-mediated mechanism.

Our results also showed that the expression profile of LCAD increases during neural differentiation contrasting with the expression profile of the lipid biosynthesis-related SREBP-1 and ACC1. A possible link between FA oxidation and lipid synthesis could also be the cellular levels of palmitate. Indeed, although we detected an increase of LCAD at early stages of NSC differentiation, we did not have any evidence that palmitate levels increase at the same time-points. Since TUDCA exposure downregulates both LCAD and palmitic acid, we do not exclude this potential cross-link. However, in agreement with the increased LCAD levels throughout NSC differentiation, mitochondrial by-product ROS were already shown to be increased during neuronal differentiation in response to higher cellular energetic demands, also promoting transcription of neurogenic genes (Khacho et al., 2016). These results reflect the importance of β-oxidation as source of energy for differentiation. Mature neurons were shown to avoid extensive FAO and favor glucose oxidation in the brain (Schönfeld and Reiser, 2013). Although at the stage of cell differentiation, high rate of oxidative metabolism through oxidation of long-chain FAs no longer represents an alternative energy fuel, it might well support OXPHOS for energy production. In addition, recent findings support our view, indicating that manipulation of FAO is enough to instruct quiescent NSCs to enter the cell cycle and proliferate *in vitro* (Knobloch et al., 2017). In fact, an increase of LCAD expression in this context would likely result in quiescent NSC states or differentiation, negatively impacting the TUDCA-induced NSC proliferation. In addition, while some studies have shown that a FAO-dependent metabolic shift regulates adult NSC activity (Knobloch et al., 2017), others demonstrated that FAO promotes reprogramming by enhancing oxidative phosphorylation and inhibiting protein kinase C (Lin et al., 2018).

TUDCA, in turn, seems to favor NSC proliferation, by causing an inverse shift in the expression of SREBP-1 and LCAD confirming the role of TUDCA driving NSCs into a more proliferative state. Therefore, we conclude that TUDCA is able to trigger a metabolic shift from FA degradation toward lipid synthesis, which has already been associated with the proliferative activity of NSCs (Knobloch et al., 2012). Curiously, insulin may represent a clue for TUDCA induction of SREBP-1 expression, since TUDCA was shown to improve insulin signaling and sensitivity (Ozcan et al., 2006; Guo et al., 2015). Insulin *per se* is able to rapidly modulate SREBP-1c concentration in the nucleus by proteolytic cleavage activation (Eberlé et al., 2004). Further, insulin and the insulin-like growth factors (IGFs) support diverse essential roles in neurogenesis and proliferation of NSCs (Ziegler et al., 2015).

Interestingly, targeted MS-based analysis of FA suggests a significant TUDCA-associated decrease of palmitic acid. This long-chain FA is the major precursor for FA elongation. However, the decreased rate of mitochondrial long-chain FA oxidation due to LCAD repression may not necessarily predict accumulation of PA. In fact, in mice as in humans, the oxidative fate of palmitic acid may be held by other acyl-CoA dehydrogenases. Moreover, analysis of fibroblasts from $LCAD^{(-/-)}$ mice has unequivocally revealed no significant changes in these types of metabolites, as compared with control (Chegary et al., 2009). Thus, our results suggest a highly dynamic control of NSC metabolic fluxes under LCAD downregulation, in which decreased levels of palmitic acid upon TUDCA treatment, might be explained by a rapid consumption of this metabolite, for FA elongation and *de novo* biosynthesis of FAs to enable generation of lipid membranes of high proliferating NSCs. Of note, SREBP-1 promotes the expression of two key enzymes, ACC whose mRNA levels we found to be upregulated in TUDCA presence, and fatty-acid synthase (FASN), for *de novo* FA synthesis. Interestingly, FASN is preferably distributed in major areas of neurogenic niches of the adult murine brain, where expression is high in proliferating NSCs and reduced in differentiated progeny (Knobloch et al., 2012). Importantly, we have shown that TUDCA while increasing lipid synthesis does not generate intracellular lipid accumulation in NSCs as levels of palmitic acid were known to reduce NSC proliferation and cause cell death (Park et al., 2011). Further, the accumulation of lipid droplets has been associated with cognitive disorders, including Alzheimer's disease, and were already shown to directly interfere with NSC behavior, decreasing NSC proliferation (Hamilton et al., 2015).

Nevertheless, we were able to show that NSCs treated with the endogenous metabolic regulator TUDCA assure the energy supply by upregulating the expression of the major pyruvate dehydrogenase subunit, PDHE1-α. In fact, this molecule appears to interfere with the regulation of FA and glucose oxidation counterbalance, safeguarding the amount of substrate entering the TCA cycle through glucose oxidation. Notably, ACAT1 protein, whose mitochondrial levels are downregulated by TUDCA, was recently described to acetylate PDC and inactivate its function in cancer cells (Fan et al., 2016). Other explanation for TUDCA-induced upregulation of pyruvate dehydrogenase could be via regulation of its inhibitor pyruvate dehydrogenase kinase (PDK). Although we were not able to detect significant alterations in PDK levels in NSCs upon 24 h of TUDCA

treatment (data not shown), we cannot exclude that TUDCA might reduce PDK levels at early time-points. On the other hand, PCG1-α, a major player of mitochondria biogenesis, is capable of increasing PDHE1-α protein content (Kiilerich et al., 2010). Thus, it is tempting to speculate that TUDCA-induced upregulation of PDHE1-α could be linked with the downregulation of ACAT1 protein expression and/or its already described effect on PCG1-α, a master regulator of mitochondrial biomass and activity (Fan et al., 2016; Soares et al., 2018).

Finally, we showed that TUDCA enhances a novel signaling pathway of mitochondrial-nuclear communication boosting nuclear PDHE1-α as a mitochondrial expatriate in NSCs. The TUDCA-induced NSC proliferation enhanced H3 supply that might be important for the S phase of cell cycle. Interestingly, histone acetylation may depend on different mechanisms for the generation of acetyl-CoA within the nucleus, such as the nuclear form of ATP citrate lyase using citrate (Wellen et al., 2009). In agreement, TUDCA was also found to up-regulate ATP citrate lyase transcript in primary rat hepatocytes (Castro et al., 2005). Indeed, nuclear PDC ability to generate acetyl-CoA may become important when the citrate pool is shifted toward lipid synthesis (Tang, 2015). Curiously, it was also recently demonstrated that PDC might directly contribute to the production of acetate, providing support for acetyl-CoA pools in all cellular compartments (Liu et al., 2018). Acetyl-CoA, in turn, facilitates lipid synthesis and histone acetylation processes (Shi and Tu, 2015) being also hypothesized that acetyl-CoA generated by nuclear PDC might be useful for lipid synthesis (de Boer and Houten, 2014). In this regard, we might also speculate that, in addition to histone acetylation, it is possible that increased acetyl-CoA generation by TUDCA-induced PDC in the NSC nucleus may also be required for lipid synthesis. Notably, this novel metabolic crosstalk triggered by TUDCA led to the acetylation levels of H3 that accompanied the increase in total H3 levels, possibly due to a nuclear functional PDC, which in turn might assure NSC cycle progression and differentiation-related epigenetic alterations. Finally, it would also be interesting to explore in the future possible changes of histone acetylation in the LCAD gene upon TUDCA treatment.

This study improves our understanding of how NSCs orchestrate adult neurogenesis in a metabolic-dependent manner, suggesting the TUDCA molecule as an efficient metabolic regulator for the maintenance and expansion of neural cells. This information together with the fact that the non-conjugated form of TUDCA is a FDA-approved molecule widely used in liver diseases and is currently in clinical trials for amyotrophic lateral sclerosis (Parry et al., 2010), provides a new framework to keep exploring its use in NSC maintenance and expansion. More importantly, we are now capable of moving toward a more comprehensive understanding of the metabolic machinery linking the processes of NSC proliferation and neuronal differentiation aimed at a more efficient use of neural replacement strategies.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD017979.

ETHICS STATEMENT

Human and Animal Rights Sprague-Dawley rats were acquired from Charles River (France). All experimental procedures were in accordance with current Portuguese laws on Animal Care and with the European Union Directive (86/609/EEC; 2010/63/EU; 2012/707/EU), on the protection of animals used for experimental and other scientific purposes. All efforts were made to minimize animal suffering and reduce numbers.

AUTHOR CONTRIBUTIONS

MF was involved in the majority of the experiments and wrote the first version of the manuscript. MC and MR helped in cell culture and performed several experiments of Western blot and immunohistochemistry. SSi evaluated the ACC1 expression while SSS performed the immunoblotting and immunocytochemistry assays. JM and AC performed the proteomic assays and analyzed the data. MS performed the GC-MS analysis of fatty acids and analyzed the data. CR critically reviewed the manuscript and helped SSo in overseeing aspects related with TU. SSo conceived and designed the study, analyzed the data, and reviewed the manuscript. All authors wrote and approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00335/full#supplementary-material

FIGURE S1 | Early-differentiating NSCs were incubated with TUDCA for 24 h and collected for Western blot, as described in Materials and Methods. **(A)** Total levels of LCAD 24 h after siRNA transfection in NSCs. **(B)** Immunoblots showing the purity of the mitochondria and nuclei extracts of NSCs. **(C)** Total protein profile of histone purified extracts in NSCs.

TABLE S1 | Identification of mitochondrial proteins differently expressed by TUDCA in mouse NSCs using LC-MS.

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REVIEW
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Mitochondrial Function in Muscle Stem Cell Fates

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Mitochondria are crucial organelles that control cellular metabolism through an integrated mechanism of energy generation via oxidative phosphorylation. Apart from this canonical role, it is also integral for ROS production, fatty acid metabolism and epigenetic remodeling. Recently, a role for the mitochondria in effecting stem cell fate decisions has gained considerable interest. This is important for skeletal muscle, which exhibits a remarkable property for regeneration following injury, owing to satellite cells (SCs), the adult myogenic stem cells. Mitochondrial function is associated with maintaining and dictating SC fates, linked to metabolic programming during quiescence, activation, self-renewal, proliferation and differentiation. Notably, mitochondrial adaptation might take place to alter SC fates and function in the presence of different environmental cues. This review dissects the contribution of mitochondria to SC operational outcomes, focusing on how their content, function, dynamics and adaptability work to influence SC fate decisions.

Keywords: mitochondria, satellite cell fates, metabolism, epigenetics, myogenic stem cells

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INTRODUCTION

Skeletal muscle constitutes a significant percentage of total body mass and is indispensable to physical movements, maintaining postures and in vital actions (Diaz-Vegas et al., 2019; Hood et al., 2019). It is also a primary peripheral tissue important for utilizing glucose and fatty acid for energy generation essential for the prevention of obesity and type 2 diabetes (Dumont et al., 2015a; Nguyen et al., 2019). It is made up of thousands of long and cylindrical multinucleated muscle fibers surrounded by the sarcolemma, a lipid bi-layer membrane that attaches a complex extracellular matrix, which is in contact with the basement membrane (Dumont et al., 2015a). The fibers are categorized on the basis of various myosin heavy chain isoforms ranging from oxidative to glycolytic fibers (Schiaffino and Reggiani, 2011).

One of the remarkable properties of skeletal muscle is effective regeneration of muscle fibers to maintain their normal physiology. Myofiber turnover is an ongoing process during the lifetime of an individual to maintain proper muscle tissue viability (Yin et al., 2013; Dumont et al., 2015b). This is especially important during aging, diseases such as Duchenne muscular dystrophy and disuse where skeletal muscle fibers are frequently damaged (Yin et al., 2013; Dumont et al., 2015b). Successful regeneration of skeletal muscle is made possible by the adult myogenic stem cell population known as satellite cells (SCs) (Yin et al., 2013). These are located between the basal lamina and muscle fiber sarcolemma, where they are well positioned to receive signals from the surrounding environment (Dumont et al., 2015b). Inefficient muscle regeneration replaces muscle with fibrotic tissues that cause poor contraction and lead to progressive loss of muscle strength as

observed during aging or muscle wasting diseases (Mann et al., 2011). Thus, an understanding of the control mechanisms that dictate SC fate decisions is crucial to improve skeletal muscle regeneration potential.

Advances in the stem cell field have unveiled the importance of mitochondria in controlling stem cell behavior including their fate decisions to self-renew or differentiate (Ryall et al., 2015a; Khacho et al., 2016). Mitochondria are central bioenergetic hubs involved in generating ATP via oxidative phosphorylation (Oxphos) that is associated with their dynamic morphological transformations (Chandel, 2018). Energy output is made possible by housing electron transport chain (ETC) complexes that are supplied by reducing agents made by the tricarboxylic acid (TCA) cycle (Chandel, 2018). Apart from this main role, mitochondria also have other functions such as macromolecule synthesis, apoptosis regulation, production of reactive oxygen species (ROS), calcium homeostasis that might regulate various signaling pathways (Chandel, 2018).

Unlike for terminally differentiated myofibers [see reviews, Diaz-Vegas et al. (2019), Hood et al. (2019)], there is a paucity of data for mitochondrial role in SCs. Despite this shortcoming the past few years have witnessed mitochondrial function linked to dictating SC fate determination, whether to maintain quiescence, become activated, self-renew or commit to differentiate. Importantly, mitochondrial energy output is associated with SC functional outcomes (Lyons et al., 2004; Folmes et al., 2012; Ryall et al., 2015b; Hori et al., 2019). Besides harvesting energy, mitochondria also have other essential functions linked to SC fate decisions. Despite having deleterious effects on SCs, ROS produced by leakage of electrons in the ETC are linked to their fate choices (Le Moal et al., 2017). Moreover, several TCA cycle intermediates are known to act as cofactors for different histone and DNA modifying enzymes leading to epigenetic remodeling required for SC self-renewal, commitment and differentiation (Matilainen et al., 2017). Finally, not much is known regarding mitochondria adaptation to different environmental cues that have a profound impact on SC function predisposing myogenic quality. This review will focus on the contribution of mitochondria to SC operational outcomes, focusing on how their content, function, dynamics and adaptability work to influence SC fate decisions.

SATELLITE CELL DIFFERENTIATION

Over half a century has passed since a population of mononucleated cells was discovered between the basal lamina and plasma membrane of skeletal muscle fibers (Katz, 1961; Mauro, 1961) representing 2–10% of the total myonuclei (White et al., 2010). Due to their peripheral localization they were termed satellite cells (SCs). At that time, their close proximity to the myofiber raised the hypothesis that they might be involved in regeneration and growth. A possibility proved correct with advances over many years that showed SCs as the primary myogenic stem cells necessary for the regeneration and maintenance of skeletal muscle fibers.

As with other stem cells, SCs have the ability to self-renew and give rise to functional progeny. Usually SCs are quiescent within their niche and enter the cell cycle when activated by external cues such as injury or trauma. Their ideal positioning enables them to receive local signals from muscle fibers, fibroblasts, endothelial cells and importantly systemic factors from blood vessels with which they co-localize (Yin et al., 2013). After activation some SCs become committed to enter the myogenic lineage as myogenic precursor cells (MPCs), whereas others self-renew to replenish their pool. In turn the MPCs proliferate and exit the cell cycle differentiating into myocytes that repair damaged muscle by either fusing with pre-existing fibers or together to form entirely new fibers (Yin et al., 2013; Dumont et al., 2015b).

Quiescent SCs are characterized by the expression of paired homeobox transcription factor 7 (Pax7) (Seale et al., 2000). They constitute a heterogeneous population, with most representing a committed population that had expressed myogenic factor 5 (Myf5) and a small uncommitted number (10%) that had never expressed Myf5 (Kuang et al., 2007). The later population can undergo asymmetric division producing progeny with and without Myf5 expression. Apart from asymmetric divisions, both SC populations also maintain and expand their population by symmetric division (Kuang et al., 2007; Yin et al., 2013; Dumont et al., 2015b). The equilibrium between symmetric and asymmetric division to maintain the homeostatic population of SCs is preserved by Wingless-type MMTV integration site 7A (Wnt7A) signaling with its receptor Frizzled 7 (Fzd7) (Le Grand et al., 2009). Their signaling pathway dictates the polarity, parallel or perpendicular, of mitotic division with respect to the basal lamina. Wnt7A by binding Fzd7 induces symmetric division by causing the SC to divide in a parallel orientation producing two identical daughter cells. On the other hand, the absence of Wnt7A favors cellular division perpendicular to the basal lamina resulting in asymmetric division. This produces one daughter cell retaining SC characteristics and another daughter cell that is committed to myogenic program.

During the commitment step into MPCs, SCs likewise with Myf5 also express another muscle regulatory factor known as myogenic determinant factor 1 (MyoD). The proliferating MPCs destined for differentiation downregulate Pax7 and up-regulate muscle specific transcription factor myogenin (Zammit et al., 2006; Olguin et al., 2007; Yin et al., 2013). This marks the entry of MPCs into the differentiation phase accompanied by cessation of proliferation. Differentiating MPCs initiate expression of various muscle specific genes encoding structural proteins resulting in their fusion (Przewozniak et al., 2013).

Alternatively, another model for SC activation and commitment suggest that satellite cells have a more homogeneous character, such that their fate choice to self-renew or differentiate occurs from $MyoD^+$ progeny. In this case, all activated SCs co-express Pax7 and MyoD wherein most will undergo rapid but limited proliferation, eventually down-regulating Pax7 and up-regulating myogenin as they differentiate (Zammit et al., 2006). Moreover, a few $Pax7^+MyoD^+$ SC progeny will lose MyoD but continue to maintain Pax7 expression. Some of these divide slowly to replenish their pool or they directly

differentiate (Zammit et al., 2006). Thus, orchestrated regulation of SC activation, self-renewal, commitment, proliferation and differentiation are necessary for skeletal muscle regeneration, repair and maintenance.

MITOCHONDRIAL FUNCTION AND DYNAMICS

Mitochondria are double membranous organelles that consist of outer and inner membranes, the latter forming numerous folds called cristae (Hood et al., 2019). The area between the outer and inner membranes is called the intermembrane space, whereas the matrix is the space encompassed by the inner membrane. Mitochondria are considered bioenergetic hubs where ATP is produced via Oxphos by the ETC, consisting of five distinct complexes which are located on the inner membrane and cristae (Chandel, 2018). Apart from their canonical function of energy production, mitochondria generate diverse functions such as macromolecule synthesis, apoptosis regulation, redox balance, and calcium homeostasis that might regulate various signaling pathways (Chandel, 2018). Notably, mitochondria matrix house the TCA cycle that performs a series of reactions resulting in the formation of reducing equivalents that include reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$), which are then oxidized by the ETC to drive ATP production (Martinez-Reyes and Chandel, 2020). The TCA cycle can be prompted by pyruvate formed from glycolysis that enters the cycle either as acetyl CoA through the action of pyruvate dehydrogenase (Pdh) or as oxaloacetate via pyruvate carboxylase (Martinez-Reyes and Chandel, 2020). Apart from pyruvate, β-oxidation of fatty acids and glutaminolysis, also drive the production of NADH and $FADH_2$ in the mitochondria (Martinez-Reyes and Chandel, 2020). The electrons formed from oxidation of the reducing equivalents migrate through ETC complexes to the final electron acceptor oxygen that is reduced to water. This passing of electrons through ETC is accompanied by pumping of protons from the mitochondrial matrix to the intermembrane space causing an electrochemical gradient in the inner mitochondrial membrane (Martinez-Reyes and Chandel, 2020). This results in flowback of protons into the matrix through complex V (ATP synthase), which produces ATP from ADP (Martinez-Reyes and Chandel, 2020). During this process, ROS might be generated by leakage of electrons in complexes I and III which might cause oxidative stress to cells (Martinez-Reyes and Chandel, 2020). To protect the cells from the potential harmful effect of the ROS, mitochondria have their own antioxidant defense systems (Oyewole and Birch-Machin, 2015). Notable among them are glutathione peroxidases (GPxs) and superoxide dismutase (SOD) which are able to scavenge ROS to maintain cellular homeostasis (Schieber and Chandel, 2014). Although considered harmful, in some stem cell types an optimal level of ROS has been shown to be important for various signaling pathways involved in proliferation, differentiation and physiological adaptation to stress (Schieber and Chandel, 2014). Depending on their efficiency, antioxidants can keep the level of ROS at an optimum level, so that it can act as a signaling molecule (Snezhkina et al., 2019).

Mitochondria harbor double stranded circular DNA (mtDNA) that generates polycistronic transcripts which encode thirteen mitochondrial genes, which are functional components of four out of five ETC complexes, thus limiting and crucial for ATP synthesis (Mitra et al., 2009). This was evident in diseases and aging where mtDNA mutations caused reduction in mitochondrial function and Oxphos (Carelli and Chan, 2014; Ryzhkova et al., 2018). The significance of mtDNA to ATP generation was underscored by experiments using a high throughput screen to assess real time ATP concentration in live human cells. In this case, a deficiency in mitochondrial encoded genes highlighted a decrease in mitochondria ATP generation (Mendelsohn et al., 2018).

The number of mitochondria within a cell is determined by two opposing forces, which are biogenesis and mitophagy that create and eliminate mitochondria, respectively (Hood et al., 2019). Moreover, mitochondria organization help to maintain metabolic homeostasis by controlling the capacity of ATP generation (Khacho and Slack, 2017). The switch in mitochondrial dynamics caused by fusion or fission are connected to differential energy generation capacities (Khacho et al., 2016). Mitochondrial fusion is a two-stage process where the outer membrane is fused by mitofusin I (MfnI) and II (MfnII), followed by fusion of inner membrane by optic atrophy 1 (OpaI). Mitochondrial fusion produces elongated mitochondria that are associated with increased Oxphos activity mainly through its regulation of mtDNA (Mitra et al., 2009; Mishra and Chan, 2016; Song and Hwang, 2018; Silva Ramos et al., 2019). Deterioration of mitochondrial fusion results in a decrease in mtDNA content, ETC function and mitochondrial membrane potential (Chen et al., 2005, 2010). Moreover, both the fusion factors are crucial for ETC complex assembly and function which indicate that elongation of mitochondria is conducive to energy generation (Cogliati et al., 2013; Mourier et al., 2015). Conversely, fission is associated with division of mitochondria by dynamin related protein 1 (Drp1) and fission protein 1 (Fsn1). Fission results in augmented mitochondrial fragmentation that causes increased oxidative stress and reduced ATP production (Jheng et al., 2012).

MITOCHONDRIAL OXPHOS AND SATELLITE CELL FATES

SCs are characterized by dynamic metabolic reprogramming during different stages of the differentiation process from predominantly Oxphos in quiescence to up-regulation of glycolysis during activation and proliferation, back to reliance on Oxphos during terminal differentiation (**Figure 1**). Quiescent SCs are diminutive with a small layer of cytoplasm surrounding their nuclei. They also have a very few mitochondria, tightly packed around the nucleus, reduced levels of mtDNA and a very low metabolic rate (Latil et al., 2012). This is similar to other adult stem cell populations, such as quiescent long-term hematopoietic stem cells that reside in the bone marrow. These are also characterized by mitochondria with a paucity of

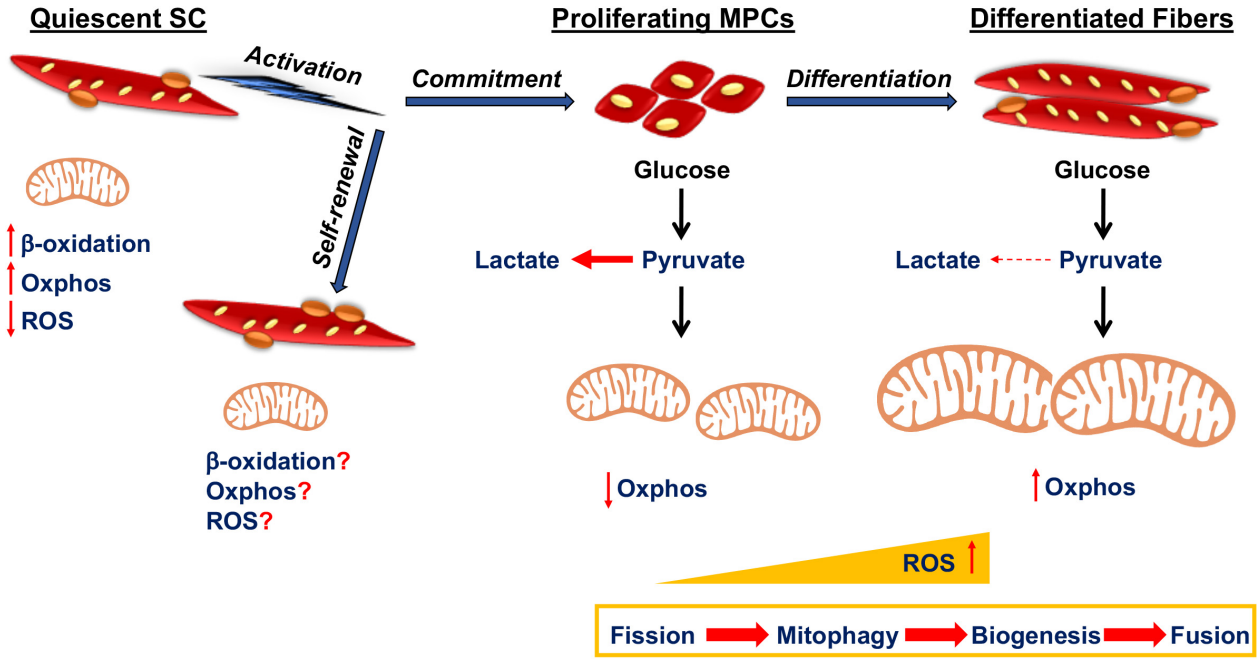

FIGURE 1 | Mitochondrial function in satellite cell fates. Quiescent satellite cells (SCs) utilize fatty acids through β-oxidation for Oxphos in the mitochondria. Activation of quiescent SCs might potentiate their self-renewal or formation of myogenic progenitor cell (MPC) progeny that undergo proliferation and eventually differentiate as part of myofibers. Mitochondrial metabolism is poorly elucidated in self-renewing SCs, whereas MPCs rely on glycolysis to obtain energy for rapid division. As differentiation progresses, there is increased mitochondrial fission, followed by mitophagy, after which the mitochondria is repopulated by mitochondrial biogenesis and fusion. ROS levels increase as MPCs begin to differentiate.

mtDNA, reduced mass and poor development (Simsek et al., 2010). However, unlike long-term hematopoietic stem cells, quiescent SCs barely depend on glycolysis and rely more on the mitochondria to produce ATP through β-oxidation of fatty acids and Oxphos (Ryall et al., 2015b). Quiescent SCs are thought to consist of two distinct populations based on differences in mitochondrial density, which are inversely associated with Pax7 levels (Rocheteau et al., 2012). The SC population with low levels of Pax7 had more mitochondria and mtDNA with greater ATP generation compared to the other resident SCs and expressed higher levels of myogenic commitment markers. Moreover, the quiescent SCs with reduced mitochondrial density and lower mitochondrial activity showed increased markers of stemness and decreased markers of myogenic commitment. This population utilized more time before entering the cell cycle for the first round of division after activation, suggesting that they represented a self-renewal population (Rocheteau et al., 2012).

When they become activated, SCs exhibit a metabolic switch from fatty acid oxidation to higher rates of glycolysis (Ryall et al., 2015b). In muscle regeneration experiments, activated SCs isolated from skeletal muscle had a high level of glycolysis and oxygen consumption rate following 3 days of injury (Pala et al., 2018). The reliance on glycolysis provides the proliferating MPCs with macromolecules to meet their anabolic demands during proliferation and might also act to protect them from ROS produced by mitochondrial Oxphos (Folmes et al., 2012). This is evident in other highly proliferative stem cell types. For the highly proliferative undifferentiated embryonic stem cells, self-renewal is maintained by utilizing a high rate of energy generation and a ready supply of macromolecules for cell division, by relying more on glycolysis producing lactate from pyruvate rather than acetyl CoA for Oxphos (Cliff and Dalton, 2017). Recently, single cell RNA sequencing revealed that expression for mitochondrial genes also increased substantially in activated SCs after injury and in committed MPCs that had been isolated from mouse skeletal muscle and cultured *in vitro* (Dell'Orso et al., 2019). This was also associated with a progressive increase in transcripts associated with TCA cycle, ETC complexes and fatty acid synthesis (Dell'Orso et al., 2019).

To increase their proliferative rate, committed SCs have devised several methods to actively reduce Oxphos production in favor of glycolysis. MPC proliferation is associated with hypoxia inducible factor 1α (Hif1α) activation that promotes glycolysis producing lactate, thereby attenuating Oxphos capacity, which is required for differentiation. Indeed, SCs obtained from genetically deleted Hif1α and Hif2α mice had reduced self-renewal and MPCs underwent precocious differentiation (Yang et al., 2017). When MPCs were pre-conditioned in hypoxic environment and transplanted into *mdx* mice, a mouse model for muscle wasting disease, there was improved regeneration capacity of the pre-conditioned MPCs (Liu et al., 2012). Oppositely, Hif1α has also been shown to inhibit differentiation of MPCs (Majmundar et al., 2015). This was mediated through Hif1α mediated inhibition of Wnt signaling that repressed MPC

differentiation (Majmundar et al., 2015). Thus, the timing of Hif1α activity might be important for SC and MPC fate decisions (Majmundar et al., 2015).

Another potential mechanism that might downregulate Oxphos capacity in favor of glycolysis in MPCs is activation of pyruvate dehydrogenase kinase (Pdk). In the mitochondria Pdk suppresses Pdh that converts pyruvate to acetyl CoA for the TCA cycle, thereby inhibiting mitochondrial oxidative capacity (Zhang et al., 2014). Pluripotent and some adult stem cells have devised this method to inhibit mitochondrial Oxphos and enhance glycolysis to sustain rapid proliferation (Folmes et al., 2011; Takubo et al., 2013). Although not much is known about regulation of MPCs by Pdk, it was shown that in hypoxia or glucose deprivation, MPCs have higher Pdk activity to support enhanced glycolysis (Abbot et al., 2005; Hori et al., 2019).

Reduced Oxphos capacity in human MPCs during proliferation is maintained by low levels of ETC complex III, IV and V subunits, mitochondrial proteins and enzymes compared to differentiating cells (Hoffmann et al., 2018). A similar profile is observed in proliferating pluripotent stem cells, which are characterized by decreased complex I and complex IV ETC nuclear encoded genes and a reduction in mitochondrial biogenesis regulators such as peroxisome proliferator-activated receptor co-activator 1 beta (*Pgc1*β) and estrogen-related receptor gamma (*ERR*γ) (Zhou et al., 2012; Sperber et al., 2015).

As MPCs begin to differentiate, glycolysis subsides in favor of Oxphos, which is essential for terminal differentiation. Recently, a study showed that conditional deletion of Pdh in SCs that resulted in defective MPC proliferation, also caused poor terminal differentiation and inefficient skeletal muscle regeneration upon injury, suggesting the requirement of pyruvate for efficient Oxphos during differentiation (Hori et al., 2019). Indeed, chloramphenicol, an inhibitor of mitochondrial protein synthesis and function, has been shown to inhibit myogenic differentiation (Rochard et al., 2000; Seyer et al., 2006, 2011). Compared to MPCs, differentiated myofibers have a higher mitochondrial mass composed of pronounced levels of mtDNA, ETC complex proteins and TCA cycle enzymes (Lyons et al., 2004; Kraft et al., 2006; Remels et al., 2010; Hoffmann et al., 2018). The terminally differentiated myofibers require functional mitochondria to sustain the high energy demand of skeletal muscles. Impaired mitochondrial function due to mtDNA mutation have been found to cause many diseases of different organs that include skeletal muscle (Chinnery, 2015).

Energy output from mitochondria during MPC differentiation is associated with their dynamic reorganization by fission, mitophagy, biogenesis and fusion. In metabolic complications such as type 2 diabetes, decreased myogenic differentiation is attributed to impaired mitophagy, which suggests the importance of mitochondrial clearance in facilitating differentiation (Henriksen et al., 2019). Importantly, during early myogenic differentiation, there is upregulation of the fission protein Drp1 mediated mitochondrial fragmentation and subsequent mitophagy by sequestosome 1 (Sin et al., 2016). Drp1 inhibition caused a reduction in differentiation of MPCs with reduced mitochondrial elongation, mtDNA content and mitochondrial biogenesis (Kim et al., 2013). However, if Drp1 is not repressed at later stages, myogenic differentiation does not proceed (De Palma et al., 2010).

Following mitophagy, the mitochondrial biogenesis activator, peroxisome proliferator-activated receptor co-activator 1 alpha (Pgc1α) and the fusion protein Mfn2 rebound mitochondrial dynamics. As differentiation progresses, Opa1 mediates mitochondrial fusion and Pgc1α amplifies mitochondrial biogenesis that together form the dense elongated mitochondrial network pertaining to increased Oxphos reliance in differentiated myotubes (Sin et al., 2016). Ectopic overexpression of Pgc1α in an MPC cell line increased mtDNA, mitochondrial encoded *cytochrome c oxidase* gene expression that enhanced mitochondrial respiration and function (Wu et al., 1999; Baldelli et al., 2014). Importantly, overexpressing Pgc1α in human MPCs boosted myofiber formation capacity both *in vitro* and *in vivo* with enhanced metabolic activity (Haralampieva et al., 2017). A key phenotype of Pgc1α genetically deleted mice is a shift from the oxidative type I and IIa toward the glycolytic type IIx and IIb muscle fibers (Handschin et al., 2007). Interestingly, as this is a whole body Pgc1α deficit, it is not clear if SCs had a role in defining the glycolytic phenotype. Nonetheless, oxidative type fibers contain more SCs than the glycolytic fibers (Gibson and Schultz, 1982) that might be due to Pgc1α mediated increase in SC number as well as proliferation (Dinulovic et al., 2016).

Intriguingly, MyoD has recently been shown to control mitochondrial function that might be necessary for efficient differentiation. By genome wide ChIP seq analysis of a MPC line, it was found that the MyoD interacted with several metabolic genes including those involved in mitochondrial biogenesis, fatty acid oxidation and ETC function (Shintaku et al., 2016). Furthermore, MyoD knockdown reduced TCA cycle activity and β-oxidation of fatty acids (Shintaku et al., 2016).

MITOCHONDRIAL OXIDATIVE STRESS AND SATELLITE CELL FATE

In some adult stem cells, ROS signaling is important for fate decisions. For example, in long-term quiescent HSCs barely any ROS is produced, which protects them from precocious differentiation, which is contrary to activated HSCs that require ROS for successful differentiation (Tothova et al., 2007; Owusu-Ansah and Banerjee, 2009; Simsek et al., 2010). For SCs, ROS generation is associated with SC activation and subsequent differentiation, though a functional role for ROS as a signaling factor requires more investigation (Kozakowska et al., 2015; Le Moal et al., 2017). Furthermore, though the contribution of mitochondrial ROS is substantial, it does not preclude the potential ROS effect from other cellular sources that might affect SC fate (Acharya et al., 2013; Le Moal et al., 2017). Despite a reliance on fatty acid metabolism and Oxphos, quiescent SCs have low ROS levels and transcriptomic analysis revealed that quiescent SCs express a greater quantity of antioxidants to protect them from potential harmful effects of ROS (Pallafacchina et al., 2010). SCs obtained from genetically deleted antioxidant superoxide dismutase mice showed lower differentiation ability

(Manzano et al., 2013). Indeed, quiescent SCs had a better survival rate than their activated counterparts after hydrogen peroxide treatment, which causes accumulation of cellular generated ROS (Pallafacchina et al., 2010).

Both mitochondrial and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase induced ROS are increased during the MPC differentiation process (Acharya et al., 2013). NADPH oxidase is thought to instigate more mitochondrial ROS by opening of the mitochondria ATP sensitive potassium channels. This allows the surge of potassium ions in the mitochondrial matrix, thereby reducing the mitochondrial membrane potential (Zhang et al., 2001). Attenuated mitochondrial ROS production by inhibiting NADPH oxidase activity, prevented their dysfunction (Doughan et al., 2008). Although important, excessive ROS is detrimental to MPCs by targeting mtDNA and mitochondrial function (Sestili et al., 2009; Sandiford et al., 2014) that leads to swelling and disruption of mitochondria (Sestili et al., 2009). Moreover, deletion of mitochondrial antioxidant, GPx, in MPCs resulted in lower proliferation and differentiation potential (Lee et al., 2006) and MPCs obtained from GPx null mice poorly differentiated with impaired myotube formation (Lee et al., 2006). Also, upregulation of superoxide dismutase in MPCs increased myotube formation (Hidalgo et al., 2014).

Mechanistically, excessive ROS in MPCs is thought to increase nuclear factor kappa beta (NF-κB) (Ardite et al., 2004; Catani et al., 2004), which reduces MyoD levels, thereby inhibiting differentiation (Guttridge et al., 2000; Sandiford et al., 2014). Also, NF-κB mediated activation of YY1, which is a myogenic transcriptional repressor might be another target of ROS mediated inhibition of myogenic differentiation in MPCs (Wang et al., 2007). Importantly, muscle restricted inhibition of NF-κB in mice enhanced SC activation and muscle regeneration (Mourkioti et al., 2006).

The inhibitory function of NF-κB is thought to occur through its classical signaling pathway, which operates via the p65/p50 heterodimer mediated transcription of target genes (Bakkar and Guttridge, 2010). However, contrary to its role as a negative regulator in undifferentiated MPCs, NF-κB also supports differentiating MPCs. This role is achieved by the use of its alternative signaling pathway, which relies on RelB/p52 mediated transcription (Bakkar et al., 2008; Dahlman et al., 2010). In this case, NF-κB mediates transcription of genes that are known to stimulate Oxphos and mitochondrial biogenesis, which are required for myogenic differentiation. Some reports have shown that NF-κB can also promote myogenic differentiation through insulin like growth factor II or p38 Map kinase, which are both known regulators of myogenic differentiation (Conejo et al., 2001; Baeza-Raja and Munoz-Canoves, 2004; Bakkar et al., 2008; Ji et al., 2010). Interestingly, ROS was required by MPCs to exit cell cycle and initiate the process of differentiation by activating p38α Map kinase (L'Honore et al., 2018). This is similar to neural stem cells where ROS has been shown to act as a signaling molecule to activate a cascade of events that upregulate the transcriptional genes for differentiation (Khacho et al., 2016). Inhibition of ROS by the antioxidant n-acetyl-cysteine or of p38α MAP kinase prevented muscle differentiation, but increased the SC pool, suggesting the importance of both the factors in mediating SC differentiation (Richards et al., 2011; Brien et al., 2013). Thus, the negative and positive effects of ROS on SC function might relate to dose and time dependent considerations. An evaluation of other potential factors that influence ROS during SC engagement is important to unravel its bi-faceted role.

Reactive nitrogen species (RNS) might be made from the superoxide leakage in mitochondria and reactive nitric oxide (NO·) produced from L-arginine by nitric oxide synthase (Le Moal et al., 2017). NO· is important for SC activation, self-renewal and MPC differentiation (Tatsumi et al., 2006; De Palma et al., 2010; Buono et al., 2012; Rigamonti et al., 2013). NO· might be critical for mitochondrial elongation, required for myogenic differentiation. In primary MPCs, inhibition of nitric oxide synthesis, prevented mitochondrial elongation and myogenic differentiation (De Palma et al., 2010).

ENVIRONMENTAL INFLUENCE ON SATELLITE CELL MITOCHONDRIAL ADAPTATION

Many studies have showed that skeletal muscle adaptation through modifiable physiological perturbations can in part be attributed to innate changes that occurred in the quiescent SC pool (Al-Khalili et al., 2003, 2014; Bell et al., 2010; Consitt et al., 2010; Boyle et al., 2012; Bourlier et al., 2013; Bollinger et al., 2015; Maples et al., 2015; Lund et al., 2017). Thus, reflecting a genetic change in specific DNA sequences and/or from changes to the epigenome in quiescent SCs that would influence the transcriptome and the pathways they influence. Caloric availability is one aspect of the external environment that affects mitochondrial function in SCs (**Figure 2**). Caloric restricted post-mortem human and mouse SCs have a remarkable ability to stay dormant owing to their anoxic environment with the ability to become activated up to 17 and 14 days, respectively, post death. They are characterized by reduced mitochondrial mass and density similar to one of the two quiescent SC subpopulations that showed increased stem cell markers and reduced commitment (Latil et al., 2012; Rocheteau et al., 2012). Contrarily, despite a lack of nutrients as found in post mortem SCs, those from caloric restricted mice have a higher mitochondrial activity and Oxphos capacity (Cerutti et al., 2014). Importantly, metformin, a caloric restriction mimicking drug that increases Oxphos, maintained SC quiescence *in vitro* and *in vivo* (Pavlidou et al., 2017). SCs obtained from caloric restricted mice had increased nicotinamide adenine dinucleotide (NAD^+) dependent deacetylase Sirtuin 1 (Sirt1), which had been found to inhibit MPC differentiation and was associated with SC self-renewal (Fulco et al., 2008; Ryall et al., 2015b).

The age-related micro-environment also influences quiescent SCs, as exemplified by their lower numbers and reduced activation potential (Chakkalakal et al., 2012) (**Figure 2**). Among several contributing factors, an alteration in mitochondrial function associated with aging might influence the decreased number and regeneration potential of SCs (Garcia-Prat et al., 2016a; Pala et al., 2018). Importantly, aging is correlated with reduced mitochondrial content, mtDNA and ATP production

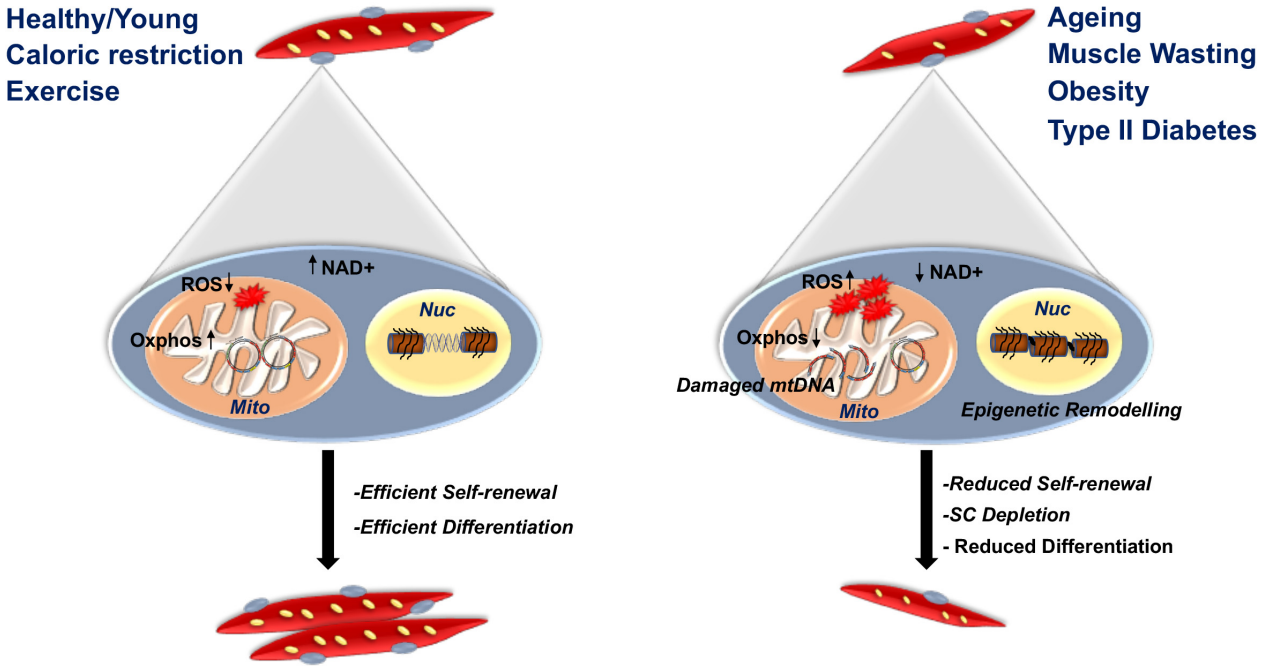

FIGURE 2 | Influence of the micro-environment on mitochondria of quiescent satellite cells. In a healthy, calorie restricted or exercise adapted micro niche, mitochondria from quiescent satellite cells (SCs) operate efficiently, producing Oxphos with very low ROS. This maintains quiescence and effective self-renewal and differentiation. However, mitochondrial adaptations due to metabolic complications as observed in aging, muscle wasting diseases, obesity and type 2 diabetes, lead to fragmented mtDNA, impairment of Oxphos producing capacity, epigenetic alterations and overproduction of ROS that reduce SC self-renewal and differentiation capacity.

(Short et al., 2005). SCs obtained from aged mice showed enhanced ROS mediated irreversible senescence owing to their inability to remove damaged organelles that also include mitochondria (Cosgrove et al., 2014; Garcia-Prat et al., 2016b). In addition, SCs from aged human skeletal muscle displayed decreased expression of genes required for ETC function (Bortoli et al., 2005). Similarly, primary MPCs obtained from the aged skeletal muscles showed impaired mitochondrial Oxphos that attenuated myogenic differentiation (Paasuke et al., 2016).

Severe and progressive skeletal muscle degeneration observed in muscle wasting diseases and complications such as muscular dystrophies and sepsis are also thought to influence SC mitochondria function. Impaired mitochondrial function was revealed in dystrophic mouse models including, the muscular dystrophy (*mdx)* and the double knockout dystrophin strains (Pant et al., 2015; Vila et al., 2017). In addition, absence of dystrophin in SCs reduced the commitment of MPCs by reducing asymmetric division which is thought to exacerbate impaired regeneration in muscular dystrophies (Dumont et al., 2015c; Chang et al., 2018). MPCs obtained from dystrophic mice displayed attenuated oxygen consumption, mitochondrial membrane potential and elevated ROS (Onopiuk et al., 2009). The importance of dystrophin to mitochondria and MPC function was highlighted by restoration of dystrophin in *mdx* MPCs (Matre et al., 2019). This enhanced Oxphos potential to near normal levels, which was associated with improved MPC proliferation and differentiation. Furthermore, transplantation of these intrinsically modified MPCs in dystrophic mice reinstated their regenerative ability (Matre et al., 2019).

Health complications due to sepsis, which might cause long term myopathy is also associated with reduced SC activation, proliferation and regenerative capacity (Rocheteau et al., 2015). Skeletal muscle that had confronted sepsis was characterized by reduced SC numbers, concomitant with defective mitochondria, degraded mtDNA, and increased antioxidant levels to combat excessive ROS (Rocheteau et al., 2015). Normally, on activation, SCs switch from Oxphos to glycolysis that provides macromolecules required for proliferation. However, in sepsis, SC activation was associated with a higher level of Oxphos that was not conducive for division, hence limiting their regenerative capacity (Rocheteau et al., 2015).

Metabolic complications as observed in obesity and type 2 diabetes are also accredited to dysfunctional mitochondria (**Figure 2**). Notably, many reports have showed that cultured MPCs from obese individuals differentiate into myotubes retaining the metabolic character evident in their skeletal muscles of origin. Biopsy derived SCs obtained from musculus vastus lateralis of exercised human subjects committed and differentiated into myotubes with the same enhanced metabolic benefits that were provided *in vivo* by exercise (Lund et al., 2017). Importantly, SCs cultured *in vitro* to form myotubes had enhanced glucose and lipid oxidation, which suggests that the external cue of exercise is capable of altering mitochondrial function directed by changes in quiescent SCs (Lund et al.,

2017). If SCs obtained from lean and obese human subjects were cultured, the latter showed reduced mtDNA copy number and function and altered metabolic derangements (Boyle et al., 2012; Bollinger et al., 2015). Furthermore, the metabolic improvements to skeletal muscle of obese subjects after an exercise training program were also manifested *in vitro*. Indeed, in response to exercise training, cultured primary myotubes from obese donors were characterized by enhanced glucose metabolism (Bourlier et al., 2013).

Similar to obesity, SCs from streptozotocin induced diabetic mice showed impaired myotube formation and regeneration following cardiotoxin induced muscle damage (Jeong et al., 2013). SCs obtained from the mouse model of obesity and diabetes (ob/ob and db/db) showed impaired MPC proliferation and differentiation (Nguyen et al., 2011). In addition, cultured myotubes from diabetic human skeletal muscle showed mitochondrial dysfunction with reduced mitochondrial content and Oxphos capacity (Minet and Gaster, 2011). Moreover, the SCs from human diabetic skeletal muscles lack metabolic flexibility to adapt to the micro-environment (Aguer et al., 2011). When grown in galactose media, which is known to induce oxidative metabolism, SCs from healthy human skeletal muscles acclimatized by enhancing their mitochondrial content and Oxphos capacity, unlike the SCs from diabetic subjects, suggesting that they are obstinate to the changed micro-environment (Aguer et al., 2011).

Intriguingly, environmental cues that can potentially influence mitochondrial function in SCs are also highlighted by a change in Oxphos capacity of uninjured muscle that is distant from a site of a muscle injury (Rodgers et al., 2014). Despite originating from uninjured muscle these SCs had higher mitochondrial activity, mtDNA content, transcriptional activity and regeneration potential compared to SCs from a mouse where no muscle injury was induced. Moreover, the SCs obtained from the uninjured muscle where a muscle injury was generated at a distant site were more primed to become activated (Rodgers et al., 2014). The factor(s) that cause the environmental changes to enhance this phenomenon are unknown.

MITOCHONDRIAL INFLUENCE ON EPIGENETICS OF SC FATES

Mitochondria is a source of metabolic intermediates which controls various key enzymes and key metabolites involved in epigenetic regulation (**Figure 3**) (Matilainen et al., 2017). A crucial facet of epigenetics is histone acetylation, characterized by a dynamic process regulated by opposing effects of enzyme families histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Dutta et al., 2016). Active transcription is associated with HATs that utilize acetyl CoA to transfer acetyl to lysine residues on histones. The HDACs remove the acetyl group from the lysine residues inducing chromatin condensation, which is associated with suppression of gene expression. The availability of acetyl CoA sourced from the mitochondria is crucial to maintain histone acetylation and is strictly dependent on the energy status of the cell (Wellen et al., 2009; Evertts et al., 2013). In the TCA cycle, acetyl CoA and oxaloacetate form citrate that must be exported to the cytoplasm to be reconverted into acetyl CoA by utilizing ATP citrate lyase. During glycolytic energy production, as is the case in activated SCs and proliferating MPCs, high levels of acetyl CoA is produced that leads to histone acetylation (Yucel et al., 2019). A similar phenomenon is observed in self-renewing pluripotent stem cells which have a high rate of glycolysis coupled to high levels of histone acetylation (Xie et al., 2009; Tan et al., 2013).

The HAT, p300 is essential for MyoD activation and its knockdown impaired MPC differentiation (Roth et al., 2003; Fauquier et al., 2018). Furthermore, *MyoD* gene expression is prevented by the myogenic transcriptional repressor Snail1/2 along with histone deacetylases (HDACs) that keep the chromatin in hypoacetylated states, thereby preventing differentiation (Soleimani et al., 2012; Cho et al., 2015). Snail1/2 null myoblasts showed precocious differentiation due to the absence of repression on *MyoD* promoter (Soleimani et al., 2012). This regulatory paradigm that controls the switch in *MyoD* gene expression is important to maintain MPC proliferation and direct differentiation. In contrast to this, in low metabolic states, as with quiescent SCs, acetyl CoA is less available, contributing to less histone acetylation (Yucel et al., 2019). During MPC differentiation, glycolysis derived acetyl CoA is utilized by the TCA cycle to produce more reducing agents because of the elevated energy demand, thus decreasing the availability of acetyl CoA for histone acetylation (Moussaieff et al., 2015; Yucel et al., 2019).

Moreover, in SCs, the energy status determined by the level of NAD^+/NADH connects the metabolic status of the cells to epigenetic modification. In quiescent SCs, where glycolysis is negligible, high NAD^+/NADH activates Sirt1, which mediates deacetylation of histones, in particular reduced acetylation of H4K16. However, activated SCs associated with glycolysis decreases NAD^+/NADH levels, resulting in reduced Sirt1 engagement, which enhances acetylation that increases transcriptional activation of myogenic regulatory factors (Ryall et al., 2015b).

Histone methylation can either repress or activate gene expression by modifying a single lysine or arginine residue on histone proteins (Ducker and Rabinowitz, 2017). The enzymes responsible for methyl group transfer are histone methyl transferases (HMTs) that add a methyl group and demethylases (HDMs) that remove it. Mitochondrial function is crucial for histone methylation, as both HMTs and HDMs require S-adenosyl methionine (SAM) which is produced by the reaction between methionine and ATP (Teperino et al., 2010). Also, the lysine specific demethylase (LSD) is dependent on flavin adenine dinucleotide (FAD), a cofactor of TCA cycle, and Jumonji C domain demethylase (JMJD) is regulated by the TCA cycle intermediate α ketoglutarate (Teperino et al., 2010). Global and site specific histone methylation has revealed that quiescent SCs have high levels of H3K4me3 associated with active transcription of Pax7 and undetectable level of H3K27me3 (Liu et al., 2013; Machado et al., 2017). Activated SCs have the repressive mark, H3K27me3, on the myogenin promoter, thought to prevent premature differentiation (Juan et al., 2011; Liu et al., 2013).

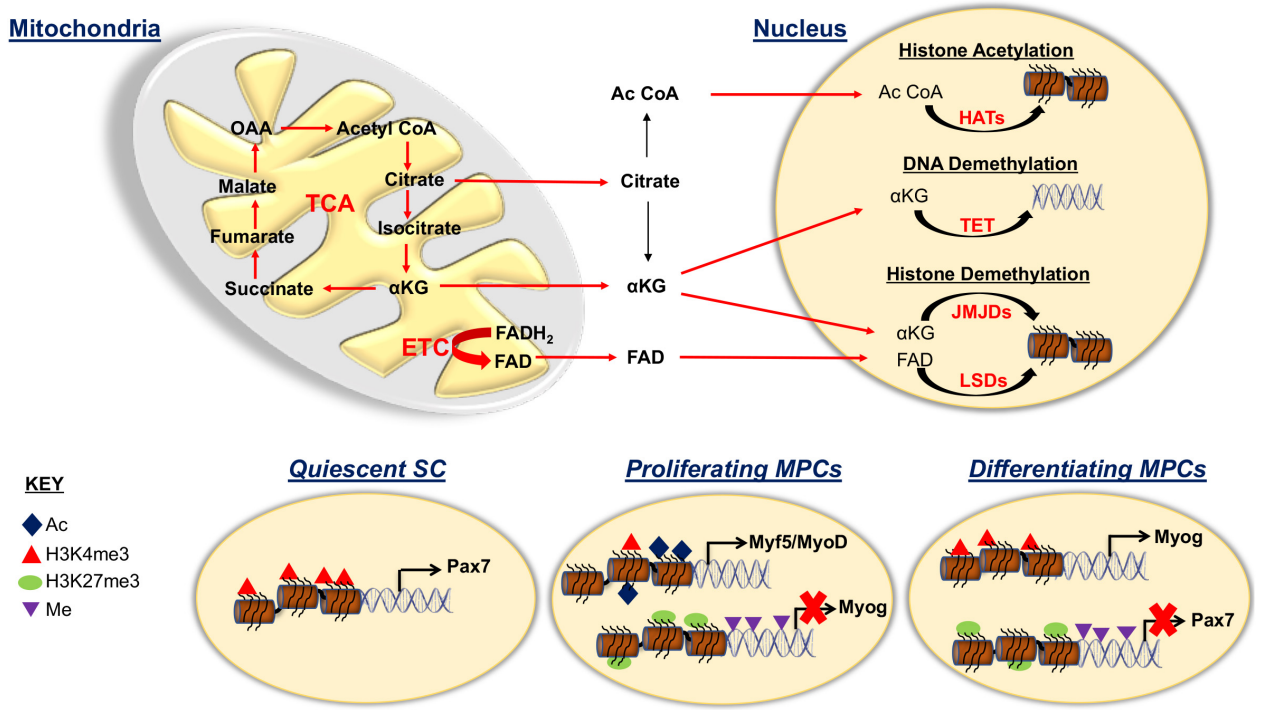

FIGURE 3 | Mitochondrial role for epigenetic regulation of satellite cell fates. Representation illustrating the involvement of the mitochondria for histone acetylation (Ac) and methylation and of DNA methylation (Me) in the maintenance and progression of SC fates. The tricarboxylic acid (TCA) cycle intermediates citrate, acetyl CoA (Ac CoA) and α ketoglutarate (αKG) as well as FAD oxidized from $FADH_2$ in the electron transport chain (ETC), are exported from the mitochondria to be used for epigenetic modification of DNA and histones. Acetyl CoA is a substrate of histone acetyltransferases (HATs), αKG is a cofactor for both histone demethylases; Jumonji C domain demethylases (JMJDs) and DNA demethylases ten eleven translocases (TETs) and FAD is a cofactor for lysine specific demethylases (LSDs) that are associated with active transcription. During quiescence, SCs exhibit activated Pax7 due to H3K4me3 modification on the Pax7 promoter. Proliferative myogenic precursor cells (MPCs) contain the repressive histone modification H3K27me3 and DNA methylation at muscle specific gene promoters such as myogenin (Myog). MPCs also contain activation associated histone modifications characterized by augmented acetylation and H3K4me3 on the Myf5 and MyoD promoters. During differentiation, activation and repression associated histone modifications take place for Myog and Pax7 promoters, respectively.

Intriguingly, a potential cause of functional decline with aging is linked to an accumulation of H3K27me3 in quiescent SCs (Liu et al., 2013). Moreover, cultured SCs from type 2 diabetic and obese human skeletal muscle also showed increased H3K27me3 histone marks that was correlated with dysregulated muscle differentiation (Varemo et al., 2017).

Histone methyl transferase Suv4-20H1 maintains SC quiescence by recruiting the repressive marker H4K20me2 on the MyoD promoter, thereby preventing its activation (Boonsanay et al., 2016). When muscle stem cells differentiate, there is an upregulation of H3K27me3 near promoters of cell cycle genes and H3K4me3 on the myogenin promoter (Blais and Dynlacht, 2007; McKinnell et al., 2008; Sebastian et al., 2009). In addition, there is an increase of the demethylase LSD activity on histones near myogenic transcription factors during differentiation (Choi et al., 2014, 2015; Scionti et al., 2017). Indeed, LSD genetically deleted MPCs and mice were impaired for differentiation and regeneration capacity, respectively (Choi et al., 2010; Tosic et al., 2018). Also, isoforms of JMJD, DN-JMJD2A and JMJD2C are recruited to Myogenin and MyoD promoters respectively, which help to propagate MPC differentiation by removing the repressive histone marks from the promoter (Verrier et al., 2011; Jung et al., 2015). In parallel to this, the activity of the key lysine methyltransferases such as G9a and enhancer of zeste homolog 2 (Ezh2), which are myogenic transcriptional repressors are dampened to propel the differentiation process (Asp et al., 2011; Ling et al., 2012).

DNA methylation generally is linked to silencing genes by the DNA methyltransferase (DNMT) enzymes that methylate cytosine on promoters at the 5′ position, whereas its demethylation occurs by ten eleven translocase (TET) enzymes. Deregulation of TCA cycle affects DNA methylation, indicating the importance of mitochondria in this process (Pastor et al., 2013). Similar to JMJDs, α ketoglutarate is required for TET activity. DNA methylation has been shown to be decreased when MPCs differentiate evidenced by increased demethylation on the myogenin promoter (Palacios et al., 2010). Additionally, DNA methylation prevented *MyoD* gene expression thereby preventing muscle stem cell commitment and 5-aza-2′-deoxycytidine, an anti-demethylating agent, promoted MPC differentiation (Montesano et al., 2013; Laker and Ryall, 2016). Knockdown of TETs lead to a decrease in muscle specific differentiation factors in MPCs (Zhong et al., 2017). Importantly, an altered micro niche associated with aging might

deregulate myogenic differentiation through an increase in DNA methylation (Bigot et al., 2015). Similarly, analysis of genome wide DNA methylation during differentiation of MPCs obtained from obese human subjects showed more methylation compared to non-obese subjects that were associated with increased DNMT expression (Davegardh et al., 2017).

PERSPECTIVES

Widely disparate patterns of mitochondria function are thought to influence SC fates including self-renewal, commitment and differentiation. However, many questions remain unanswered. When activated, the quiescent SCs, which rely on the mitochondria for fatty acid oxidation, switch to glycolysis that is necessary for cell division. Though cell division is required for both self-renewal and commitment, the potential role for mitochondrial function, specifically fatty acid oxidation and Oxphos, in symmetrical versus asymmetrical division has not been elucidated. Other issues that remain unresolved pertain to retrograde signaling from the mitochondria to the nucleus that affect SC function. Crucially, epigenetics regulates chromatin structure that is needed to orchestrate SC quiescence and differentiation. Though it is known that TCA cycle metabolites control epigenetic reprogramming, a paucity of data exits for the pathways that coordinate the specific changes in SC mitochondria. Moreover, the exact role for mitochondrial generated ROS on SC nuclear regulation remains obscure, unlike other adult stem cells where downstream mechanisms have been discovered. Indeed, adipogenic differentiation of human adult mesenchymal stem cells is coupled to mitochondrial ROS production that controls the mammalian target of rapamycin (mTorc) pathway that activates peroxisome proliferator-activated receptor gamma (Pparγ) the master regulator of adipocyte differentiation (Tormos et al., 2011). It will be of interest to discover the downstream pathways and genes that might be directly impacted by mitochondrial ROS in SCs.

Finally, an overlooked approach to enhance muscle wellbeing is to increase the pre-existing population of inactivated SCs for a greater innate capacity to augment self-renewal and muscle formation. In this case, there is data lacking for how the mitochondria might be manipulated. One approach would be to understand how environmental stressors modify quiescent SCs at the level of global gene expression and epigenetic changes that had occurred during their adaptation. In this way we can learn how the mitochondria are influenced by pathways and genes in quiescent SCs, which will regulate their metabolic behavior in the future. To this end, single cell RNA and ChIP seq on quiescent SCs under different environmental stressors are required to find out a potential effect on mitochondrial and SC adaptation. Further studies, dissecting the consequence of the altered mitochondrial activity on SC fate associated with metabolic diseases is critical. This will further help in ameliorating decreased regeneration efficacy in aging and other muscle wasting diseases.

SUMMARY

The transformative events on mitochondrial structure, number and activity in SCs, the predominant myogenic stem cell, shape how muscle regeneration will proceed. Moreover, mitochondrial function and dynamics wrought through environmental cues affect Oxphos, ROS production and TCA cycle intermediates that directly impact SC plasticity and differentiation. Thus, evaluation of mitochondrial function in SCs will provide new avenues to target for improvements against myogenic impairments and diseases.

AUTHOR CONTRIBUTIONS

DB and AS contributed to the conception, design, acquisition, analysis, interpretation of the work, drafting, illustrating, and revising the work.

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REVIEW
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Organelle Cooperation in Stem Cell Fate: Lysosomes as Emerging Regulators of Cell Identity

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Regulation of stem cell fate is best understood at the level of gene and protein regulatory networks, though it is now clear that multiple cellular organelles also have critical impacts. A growing appreciation for the functional interconnectedness of organelles suggests that an orchestration of integrated biological networks functions to drive stem cell fate decisions and regulate metabolism. Metabolic signaling itself has emerged as an integral regulator of cell fate including the determination of identity, activation state, survival, and differentiation potential of many developmental, adult, disease, and cancer-associated stem cell populations and their progeny. As the primary adenosine triphosphate-generating organelles, mitochondria are well-known regulators of stem cell fate decisions, yet it is now becoming apparent that additional organelles such as the lysosome are important players in mediating these dynamic decisions. In this review, we will focus on the emerging role of organelles, in particular lysosomes, in the reprogramming of both metabolic networks and stem cell fate decisions, especially those that impact the determination of cell identity. We will discuss the inter-organelle interactions, cell signaling pathways, and transcriptional regulatory mechanisms with which lysosomes engage and how these activities impact metabolic signaling. We will further review recent data that position lysosomes as critical regulators of cell identity determination programs and discuss the known or putative biological mechanisms. Finally, we will briefly highlight the potential impact of elucidating mechanisms by which lysosomes regulate stem cell identity on our understanding of disease pathogenesis, as well as the development of refined regenerative medicine, biomarker, and therapeutic strategies.

Keywords: lysosomes, stem cell identity and fate, metabolism, neural stem cell (NSC), pluripotent stem cell (PSC), neural crest (NC), cancer stem cell (CSC)

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METABOLIC ORGANELLE NETWORKS REGULATE STEM CELL FATE

Stem cell fate decisions, including the determination of cellular identity, are intimately linked to gene expression networks with each decision driven by wide-spread transcriptional changes (Walker et al., 2007; Julian et al., 2013, 2017b; Julian and Blais, 2015; Pinto et al., 2018; Abdolhosseini et al., 2019; Wells and Choi, 2019). Transcriptional regulation does not function in

isolation, however. Cell fate decisions are heavily influenced by dynamic communication between the nucleus with multiple biological processes and signaling cascades involving macromolecule interactions at the cell membrane, throughout the cytoplasm, and within or on the surface of organelles (Shah et al., 1996; Zhou et al., 2009; Julian et al., 2013; Julian and Blais, 2015; Singh et al., 2015; Chen et al., 2016; Young et al., 2016; Khacho and Slack, 2017b; Chang et al., 2018; Obernier et al., 2018; Bahat and Gross, 2019; Jaiswal and Kimmel, 2019; Kinney et al., 2019; Shlyakhtina et al., 2019; Chang, 2020). The successful development, long-term homeostasis, and post-injury repair of organs and tissues is dependent on the pliability in cell fate decisions that these integrated biological networks permit. Yet, this flexibility can also give rise to the aberrant stem cell populations that underlie pathology in many diseases including tumor syndromes. This double-edged sword of cell fate pliancy is paralleled by dynamics in metabolic signaling pathways. It is therefore crucial to understand the breadth of regulatory processes and factors that contribute to the biological networks underlying metabolic control and to elucidate their impacts on cell fate.

Metabolic Signaling Impacts Cell Identity

Cellular metabolism is broadly defined by the numerous biochemical pathways that participate in the processing of nutrients including amino acids, carbohydrates, and lipids, into their primary molecular building blocks along with energy in the form of adenosine triphosphate (ATP). The ATP produced through metabolic pathways is necessary to fuel the myriad of biological processes that take place within a cell to support its growth and viability. Metabolic bioenergetic signaling pathways are closely integrated with organelle-based processes, which together focus on balancing anabolic and catabolic cellular activities (Thelen and Zoncu, 2017; Todkar et al., 2017; Gordaliza-Alaguero et al., 2019; Xia et al., 2019). Respectively, anabolism and catabolism function to drive the production versus degradation of biomass, which is necessary to balance cell and tissue growth, homeostasis, and survival. Metabolic pathways are highly interconnected and dynamic, focused on managing supply and demand of available energy and nutrient substrates in the face of ever-changing environmental conditions. While many bioenergetic pathways exist that contribute to ATP production, a cell can be classified based on its relative reliance on two over-arching processes: oxidative phosphorylation (OxPhos) and glycolysis. These processes drive differential rates of ATP production and a unique complement of metabolite by-products (Folmes et al., 2012; Zhang et al., 2018; Gordaliza-Alaguero et al., 2019; Intlekofer and Finley, 2019). Oxidative phosphorylation is linked to the mitochondrial electron transport chain (ETC) and fueled by energy precursors generated through the tricarboxylic acid cycle (TCA). Alternatively, glycolysis takes place in the cytoplasm and can provide pyruvate as a substrate to feed into the TCA or can permit fully anaerobic macromolecule metabolism.

In addition to the production of ATP, metabolic signaling is now known to exert wide-ranging effects within a cell and has in fact emerged as a primary biological process underlying the regulation of cell fate decisions in a wide range of cell types, including stem cell populations. These impacts are achieved through mechanisms that include regulation of transcription factor expression and localization, integration of metabolic networks with growth factor and developmental signaling pathways, and the production of cell type and context-specific metabolites that permit chemical modification of signaling cascades and epigenetic regulation of DNA and histone proteins (Chung et al., 2007; Khacho et al., 2016, 2019; TeSlaa et al., 2016; Young et al., 2016; Cliff et al., 2017; Khacho and Slack, 2017a,b; Zhang et al., 2018; Jaiswal and Kimmel, 2019). Specific patterns of bioenergetic pathway activation influence cell fate decisions including the maintenance of self-renewal, induction of differentiation, homeostasis, activation state, and regenerative potential of distinct stem cell populations. It is now an established concept that cell identity itself, the outcome of multiple fate decisions during lineage development, is closely connected with particular metabolic states.

The connection between metabolic state and cell identity is well appreciated in the context of *in vitro* pluripotent stem cells (PSCs), including maintenance of their self-renewing state and the initial establishment of induced PSCs (iPSCs) from somatic cells such as fibroblasts. Transition to a glycolytic state from an oxidative one typical of somatic cells is universally required for both the acquisition and maintenance of pluripotency, critical for activation of self-renewal programs and inhibition of differentiation (reviewed in Zhang et al., 2018; **Figure 1**). There are subtleties to this rule however, as "naïve" PSCs, which functionally resemble the inner cell mass of the pre-implantation blastocyst (**Figure 1**), employ both OxPhos and glycolysis. This is in contrast to "primed" PSCs, a more developmentally mature population resembling cells within the post-implantation epiblast, which are predominantly glycolytic (Folmes et al., 2011; Zhang et al., 2018). This shift in metabolic preference between naïve and primed PSCs is accompanied by distinct changes in the expression profiles of developmental genes, which actively regulate glycolytic and OxPhos signaling patterns (Zhang et al., 2018). Subsequently, differentiation of PSCs into downstream lineages that parallel the mammalian germ layers is typically associated with transition from a glycolytic state to a preferential reliance on OxPhos (**Figure 1**). The metabolite a-ketoglutarate (aKG), generated through the mitochondrial OxPhos-dependent TCA cycle, functions in naïve PSCs to drive self-renewal but in primed PSCs to accelerate differentiation, a biphasic role attributed to the direct effect of aKG on stage-specific histone and DNA demethylation patterns and consequently epigenetic gene regulation (Carey et al., 2015; TeSlaa et al., 2016). Thus, a differential preference for OxPhos versus glycolysis signaling, reflecting unique needs for exogenous nutrients, regulatory co-factors, and oxygen and energy demands, parallels and can actively drive transitions between distinct stem cell identities.

The specification of ectodermal neural stem cells (NSCs) represents an exception to the paradigm that exit from pluripotency requires reprogramming to an OxPhos-dependent state (**Figure 1**), as a preference for glycolytic metabolism is maintained. Unlike in PSCs, however, glycolytic metabolism in the developing ectoderm appears to be driven by a reliance on glutamine as a primary metabolic substrate (Lees et al., 2018;

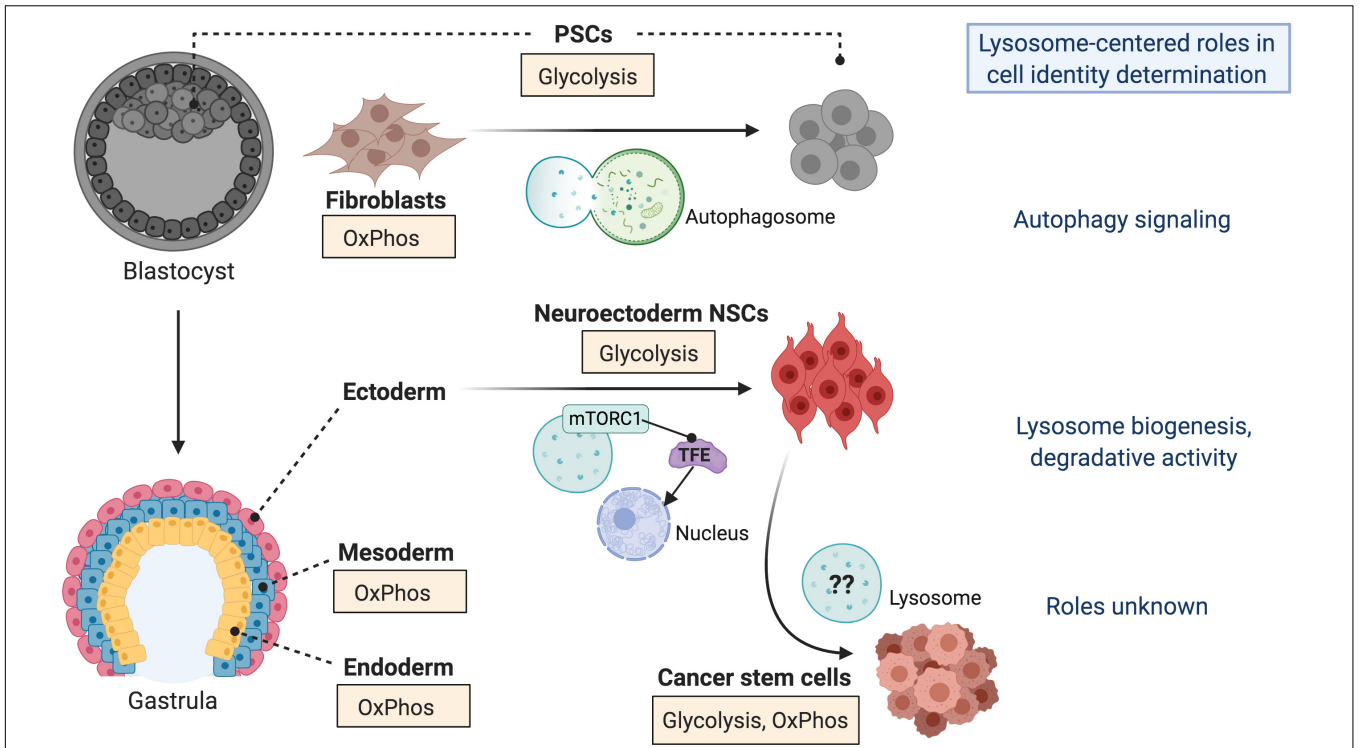

FIGURE 1 | Lysosomes regulate stem cell identity transitions. Precise control of lysosome-autophagy signaling is required at an early stage during the reprogramming of somatic cells (i.e., fibroblasts) to induced pluripotent stem cells (PSCs) *in vitro*. *In vivo*, PSCs are located in the inner cell mass of the blastocyst during embryonic development, which gives rise to the gastrula from which the three primary germ layers (ectoderm, mesoderm, and endoderm) are born. Induction of ectodermal cells, specifically neural stem cells (NSCs) of the neuroectoderm, is accompanied by increased biogenesis and activity of lysosomes through mTORC1-regulated Micropthalmia/Transcription Factor E (TFE) nuclear organization. Alternatively, lysosome activation is associated with reduced endoderm and mesoderm differentiation. Given the prevalence of lysosome activity in various types of cancer, including those affecting the brain, it is possible that lysosomes drive the acquisition of cancer stem cell identity. The potential mechanisms are unknown. These stem cell identity transitions are also associated with metabolic reprogramming, whereby some cell types preferentially activate pathways requiring oxidative phosphorylation (OxPhos) whereas others rely on glycolysis. Cancer stem cells exhibit multiple strategies of metabolic reprogramming.

Harvey et al., 2019; Vardhana et al., 2019). Furthermore, a proper balance of NSC self-renewal versus differentiation following their initial specification, as well as homeostasis of adult NSC populations, depends on a shift toward the oxidative fatty acid metabolic pathway. In mature populations this occurs specifically in quiescent NSCs, which are critical for the long-term maintenance of the stem cell pool and for neurogenic capacity in the brain, but not "activated" proliferative populations (Knobloch et al., 2017; Leeman et al., 2018; Bankaitis and Xie, 2019). Strikingly, inhibition of a single metabolite malonyl-CoA, a factor that drives activation of the fatty acid oxidation pathway, is sufficient to force quiescent NSCs into a proliferative state in a manner dependent on activation of the bone morphogenetic protein (BMP) signaling pathway (Knobloch et al., 2017). This again demonstrates a direct effect of metabolic state on cell fate regulation and underscores the integration of metabolic networks with developmental signaling pathways.

Organelle Cooperation in Cell Metabolism

Metabolic pathways and the biological processes that fuel anabolic and catabolic activities are partitioned among distinct subcellular locations. Over the past decade, however, it has become increasingly clear that communication between organelles and cytoplasmic signaling pathways is critical for the dynamic regulation of bioenergetic networks that underlies cell and tissue homeostasis (reviewed in Schrader et al., 2015). The nucleus, mitochondria, endoplasmic reticulum (ER), and cellular vesicles (including lysosomes, autophagosomes, and peroxisomes) are historically defined, respectively, as regulators of gene expression, ATP production, protein and lipid production/export, and macromolecule degradation. Yet we now know that these organelles in fact exhibit multi-faceted roles within the cell, functionally and physically interacting with one another to cooperatively regulate processes that parallel or can directly impact metabolic control such as cell signaling, survival, immunity, and fate decisions.

Electron microscopy, immunofluorescence and time-lapse imaging analyses have revealed many organelles to be highly interconnected and dynamic structures (reviewed in Schrader et al., 2015; Lim and Zoncu, 2016). Regular fission and fusion events of mitochondria function to remodel their cristae and allow an exchange of intra-organelle and membrane components including ETC channel proteins, metabolites, calcium (Ca^{2+}),

and other ions. These interactions are essential for bioenergetic integrity, and consequently for the regulation of multiple facets of tissue development and maintenance (Khacho et al., 2016, 2017; Baker et al., 2019). Likewise, lysosomes move between the peri-nuclear region and the cell membrane in response to energy signaling demands through the mTORC1 signaling pathway (Starling et al., 2016; Hao et al., 2018), and they undergo regular fission and fusion events with one another (Ballabio and Bonifacino, 2020). This leads to changes in organelle size, vesicle tubulation and subsequent reformation of lysosomes from these hybrid organelles, presumably mediating transfer of membrane and lumenal contents among the broader lysosome population. Though lysosome dynamics appear to be important for bioenergetics and cell homeostasis, the mechanisms and direct consequences of these activities are not well understood (Saffi and Botelho, 2019; Ballabio and Bonifacino, 2020). Lysosomes have also long been known to fuse with autophagosome vesicles which carry excess and damaged cellular materials to the lysosome's acidic lumen for degradation. Thus, lysosomes constitute the end point of the autophagy pathway, a ubiquitous cellular process critical for survival in the face of changing environments that impacts development, cell and tissue homeostasis, immunity, and disease predisposition (Chang, 2020).

In addition to "self-interactions," multiple organelles in fact cooperate with one another in ways that can impact metabolic circuits. An emerging field has focused on the effects that by-products of mitochondrial metabolism can impart within the nucleus, including reactive oxygen species and multiple metabolites generated through the series of redox reactions that fuel oxidative metabolism. Many such metabolites, including αKG, NADH, and acetyl-coA, are produced through the TCA or in parallel pathways and subsequently function in the nucleus as co-factors for enzymes that regulate methylation and acetylation of DNA and histones. Mitochondrial metabolites can therefore significantly impact transcriptional regulation at an epigenetic level (Khacho et al., 2016; Harvey et al., 2019). This functional inter-organelle connection is a critical mechanism to control mitochondrial homeostasis and stability, as it permits activation of nuclear genes required for mitochondrial biogenesis. Disruption of mitochondria-nuclear connections can lead to DNA damage, calcium overload, developmental dysfunction, and disease including tumorigenesis (Guha and Avadhani, 2013; Cantó et al., 2015; Tu et al., 2018; Xia et al., 2019).

More recently, the lysosome has emerged as an organelle that also has direct functional connections to the nucleus. Dynamics of nutrient-sensing signaling complexes on the lysosome surface control the sub-cellular localization of the Micropthalmia/Transcription Factor E (MiT/TFE) family of transcription factors (herein referred to as 'TFE'), critical regulators of genes that drive lysosome biogenesis and metabolic reprogramming (Settembre et al., 2011, 2012; Young et al., 2016; **Figure 2**). Additionally, the lipase LIPL-4 promotes longevity in *Caenorhabditis elegans* by harnessing its degradative activity within the lysosome to establish a lipid–protein chaperone complex that translocates to the nucleus and promotes the transcriptional activity of NHR-49 and NHR-80 nuclear hormone receptors (Folick et al., 2015). Nuclear hormone receptors regulate the expression of genes related to mitochondrial metabolism and the oxidative stress response, a mechanism linked to organismal longevity due to downstream activation of mitochondrial ß-oxidation and ETC activity (Folick et al., 2015; Ramachandran et al., 2019). Biochemical and high throughput metabolomic analyses revealed that the nuclear complex generated by LIPL-4 in *C. elegans* is comprised of the lipid species oleoylethanolamide (OEA) and the lipid chaperone LBP-8. All three elements of this signaling pathway are structurally or functionally conserved in mammals (LIPL-4 and NHR-80 are homologous to mammalian LIPA and HNF4, respectively; the mammalian lipid metabolism transcription factor PPARα is activated by OEA) (Fu et al., 2003; Folick et al., 2015; Ramachandran et al., 2019), thus similar lysosome-nuclear signaling mechanisms are likely to impact cell fate determinant transcriptional programs in mammalian systems.

Physical connections between organelles can take place through either direct membrane fusions or membrane contact sites (MCSs) mediated through tethering proteins. These connections permit the transport of metabolites and signaling molecules, lipid moieties, and ions like Ca^{2+} and iron that function as cofactors for biochemical reactions (Sheftel et al., 2007; Schrader et al., 2015; Todkar et al., 2017; Xia et al., 2019). They also participate in mediating and stabilizing structural processes, such as fission–fusion dynamics of mitochondria and lysosomes and the maturation of endosomes, including their trafficking along microtubules and ultimate fusion with lysosomes (Friedman et al., 2013). Membrane contact sites were in fact first observed, between the ER and mitochondria, by electron microscopy half a century ago (Copeland and Dalton, 1959). Despite this long history, we are only recently beginning to understand the molecular mechanisms that regulate these connections and the specificity of their biological effects.

Recent work demonstrated that in order to promote vascular modeling in the brain, reactive astrocytes develop clusters of mitochondrial–ER contacts to drive Ca^{2+} uptake from the ER. This was shown to be dependent on the mitochondrial fusion protein Mitofusin 2 (Mfn2), and thus is linked to dynamics in mitochondrial structure (Göbel et al., 2020). We now know that multiple organelles establish MCSs with other membrane-bound organelles or cell structures. This includes between the plasma membrane and the ER, which regulates Ca^{2+} dynamics between the extracellular space and the ER, the dominant Ca^{2+} storage site in the cell. Lysosomes have been observed to form MCSs with multiple organelles, including the ER, peroxisomes, Golgi apparatus, and mitochondria (Schrader et al., 2015; Todkar et al., 2017; Xia et al., 2019; **Figure 2**). A tethering complex that mediates mitochondrial–lysosome MCSs has been identified in yeast, where it is an important regulator of cell growth and is activated and maintained in response to metabolic activity (Hönscher et al., 2014). Alterations in the abundance of organelle MCSs are observed in cases of aberrant metabolic regulation and metabolic disorders, and gene knockout studies of proteins that comprise MCSs have begun to reveal that the integrity of at least some of these contacts is indeed crucial

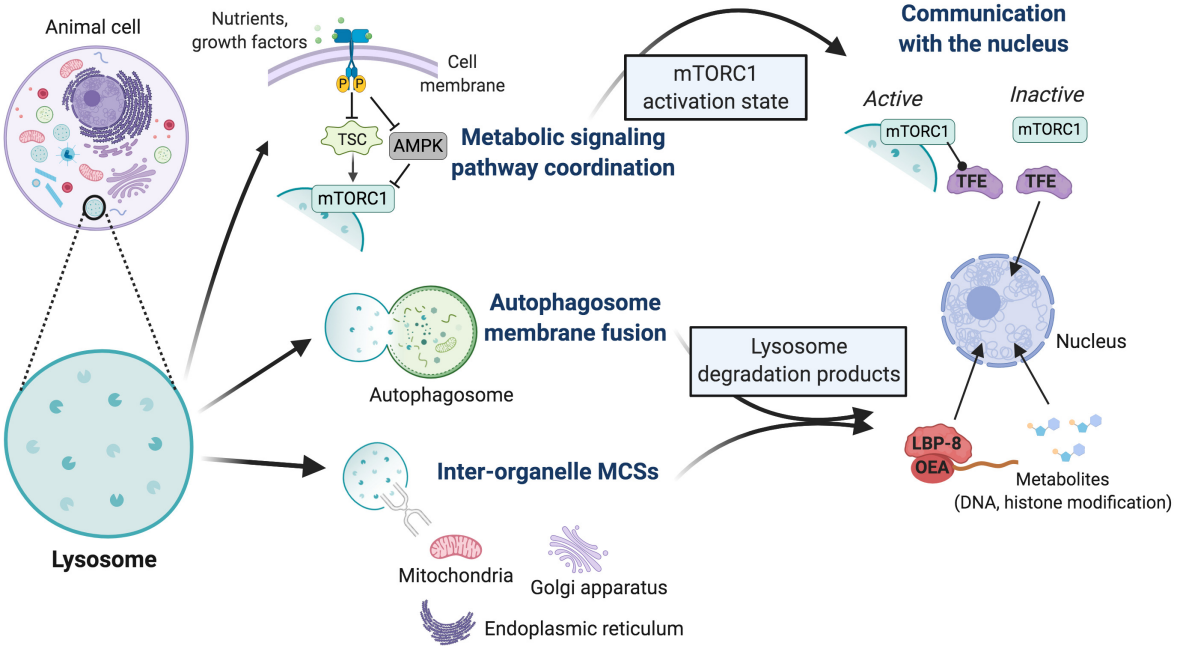

FIGURE 2 | Overview of mechanisms by which lysosomes may impact cell fate decisions. Lysosomes interact physically with cell signaling pathways and other organelles; these interactions exhibit known or putative secondary impacts on transcriptional activity within the nucleus. The activation state of the mTORC1 and AMPK metabolic signaling pathways is coordinately regulated at the lysosome surface, with mTORC1 physically bound to the lysosome in its activated state. TSC is a potent mTORC1 inhibitor complex, whose inactivation underlies the multi-system tumor syndrome tuberous sclerosis. Lysosomes also mediate physical interactions with themselves or autophagosomes through membrane fusions, or with mitochondria, the endoplasmic reticulum, and Golgi apparatus through tethering proteins that establish inter-organelle membrane contact sites (MCSs). These contacts regulate fission-fusion events and inter-organelle transport of ions, lipids, and metabolites, which ultimately impacts the profile of lysosome degradation products. These direct physical interactions of the lysosome have secondary impacts on the nucleus. Currently, lysosomes are known to coordinate the nuclear activity of two classes of transcription factors. The subcellular localization of TFE family members, critical activators of lysosome biogenesis genes, is controlled by the activation state of mTORC1. In its active state, lysosome-bound mTORC1 physically retains TFE in the cytoplasm, while its dissociation from the lysosome upon inactivation leads to TFE release and nuclear translocation. Demonstrated in *C. elegans*, degradation products of the lysosomal lipase LIPL-4 form a lipid (OEA)-lipid chaperone protein (LBP-8) complex that translocates to the nucleus and promotes the transcriptional activity of nuclear receptor hormone receptors, factors that activate mitochondrial metabolism and oxidative stress response genes. Various metabolites are produced through lysosome-mediated digestion of excess or damaged organelles, proteins, and other cellular materials, and can be transported to the cytoplasm by membrane permeases. Given our insights into mitochondrial metabolites, these likely impact multiple biochemical pathways and nuclear activities through modification of DNA and histone proteins. Many of these mechanisms have been implicated in cell fate regulation; it largely remains to be determined which processes impact stem cell identity.

to proper metabolic regulation (Sebastián et al., 2012, 2016; Gordaliza-Alaguero et al., 2019).

Organelle Inheritance and Cell Fate Determination

Gene and resulting protein expression patterns ultimately define and maintain a cell's identity, and mitochondria are well-established regulators of gene expression programs. Alterations in mitochondrial content, fission and fusion dynamics, and signaling cascades including metabolite production and retrograde signaling to the nucleus has a profound impact on cell fate specification (Khacho et al., 2016; Khacho and Slack, 2017a). This encompasses broad populations including pluripotent, somatic and cancer stem cells (CSCs), from development through long-term homeostasis and aging. A compelling indicator that mitochondria play an instructive role in cell identity specification is that their segregation into the progeny of dividing stem cells has been observed to occur in a biased manner. For instance, during meiosis I of mammalian oocyte development mitochondria aggregate around the spindle pole, which will determine the plane of cell division. Upon division mitochondria are preferentially retained in the newly formed oocyte at the expense of the polar body, in a manner dependent on cell cycle progression and mitochondrial transport by the actin cytoskeleton (Dalton and Carroll, 2013). As the polar body is destined for degradation, this mechanism is thought to ensure the inheritance of mitochondria, and the consequent production of ATP and proper cell function, in cells destined to drive early embryo development.

Similarly, in budding yeast, mitochondria are partitioned in a biased manner at cell division such that the daughter bud cell receives a higher proportion of mitochondrial content at a cost to the mother cell (Böckler et al., 2017). As in mammalian oogenesis, this partitioning is dependent on fusion and transport of mitochondria on actin networks and is crucial for the maintenance of proper replicative lifespan of yeast bud cells. An elegant study that employed photoactivatable fluorescence tags

as a relative measure of organelle age revealed that in actively expanding human mammary epithelial cultures, mitochondria exhibit biased distribution such that daughter stem cells with the highest potential for stemness receive predominantly young mitochondria (Katajisto et al., 2015). Genetic knock-down analyses targeting the structural fission proteins Parkin and Drp1 demonstrated that this regulated segregation also exploits fission–fusion dynamics.

As stem cells divide, molecular mechanisms converge to dictate whether their progeny will give rise to new-born stem cells through self-renewal, or instead to specialized differentiated cell types. One mechanism by which this is determined is regulation of the pattern in which cell fate determinant factors, including transcription factors, RNA molecules, signaling proteins, and organelles, are distributed among daughter cells (Venkei and Yamashita, 2018; Shlyakhtina et al., 2019). It is well established that stem cells divide with a spindle pole orientation that is either perpendicular to the surface of a defined stem cell niche or is at an altered angle relative to the niche. Though DNA is equally distributed between both daughter cells, the distribution of many cell fate determinants is polarized relative to the niche instead of the mitotic spindle pole. Therefore, perpendicular spindle orientations result in symmetric divisions in which both daughter cells receive a relatively equal complement of cytoplasmic material and consequently self-renew as clonal stem cells. Reduced angle divisions, however, are asymmetric, whereby cellular components are unevenly divided among daughter cells thus yielding one new stem cell and one differentiated progeny (Venkei and Yamashita, 2018).

The balance between symmetric self-renewing and asymmetric differentiative divisions is essential to tissue development and homeostasis, and misbalances can lead to diseases such as cytopenias or cancer. During early brain development, for example, a stem cell population expands first through symmetric divisions and subsequently through asymmetric divisions, which generate one daughter stem cell and one differentiated progeny (Julian et al., 2013; Matsuzaki and Shitamukai, 2015). In contrast, tissue homeostasis in the adult brain is maintained through a balance of symmetric self-renewing and asymmetric neurogenic divisions (Obernier et al., 2018). Ultimately, the pattern by which cell fate determinants are distributed among daughter cells is a critical factor directing cell identity.

In addition to mitochondria, other organelles exhibit biased segregation among progeny of dividing stem cells, suggesting that they may directly impact stem cell identity determination in their own right. Unequal inheritance of ER content has been observed in asymmetrically dividing stem cell populations in a number of model systems including *Drosophila melanogaster*, *Caenorhabditis elegans*, *Saccharomyces cerevisiae*, the ascidian embryo, and cultured human cells (Estrada et al., 2003; Poteryaev et al., 2005; Sardet et al., 2007; Smyth et al., 2015; Eritano et al., 2017). Asymmetric distribution of the ER has also recently been observed in symmetrically expanding epithelial stem cells very early stages of Drosophila development, through a mechanism dependent on directed positioning of the ER by the highly conserved ER membrane protein Jagunal (Eritano et al., 2017). Strikingly, this biased ER inheritance is observed immediately prior to the delamination event that precedes the transition to asymmetric neurogenic divisions and is restricted to a population of cells destined for neurogenesis. This strongly implies a direct role for the ER in stem cell fate determination; it remains to be determined, however, if asymmetric ER inheritance contributes actively to cell fate determination in mammalian systems.

Asymmetric inheritance of lysosomes and other cellular vesicles has also recently been causally linked to cell fate determination. Peroxisomes, vesicles carrying degradative enzymes involved in fatty acid and energy metabolism, have been observed in both yeast and human cells to exhibit asymmetric segregation during mitosis (Asare et al., 2017; Kumar et al., 2018). Using an unbiased RNA-sequencing and a directed RNA interference (RNAi) approach to profile epidermal stem cells and their newly differentiated progeny in developing mouse embryos, peroxisomes were shown to be critical for epidermal development through regulation of asymmetric divisions (Asare et al., 2017). These vesicles localize to spindle poles and are in fact necessary to control their alignment and the subsequent orientation of cell division relative to the stem cell niche. RNAi-mediated knockdown of the peroxisome membrane protein Pex11b completely disrupted epidermal development by altering cell cycle progression, symmetric and asymmetric division ratios and ultimately fate determination of the stem cell pool and the resulting tissue architecture.

Finally, in the first explicit demonstration that mammalian hematopoietic stem cells (HSCs) divide asymmetrically to control their fate, Loeffler et al. (2019) demonstrated that this asymmetry is paralleled by an uneven distribution of the cell's degradative machinery, specifically lysosomes, autophagosomes, and mitophagososmes. Preferential inheritance of these vesicles into daughter cells destined for differentiation was a defining feature dictating the identity of HSC progeny, and was linked to the regulation of mitochondrial clearance, protein translation capacity, and signaling through the Notch–Numb pathway. Thus, compelling evidence from recent discoveries implicates multiple organelles, focused heavily on cellular metabolic vesicles such as lysosomes, as active regulators of stem cell divisions that dictate cell identity decisions.

LYSOSOMES AS REGULATORS OF STEM CELL IDENTITY

Lysosomes have long been viewed as the primary effectors of the cellular waste disposal system, functioning to degrade excess or damaged organelles and macromolecules by the greater than 60 hydrolytic enzymes present within their acidic lumen (Settembre et al., 2013; Perera and Zoncu, 2016). Lysosomes are formed by the fusion of secretory vesicles from the trans Golgi network with endocytic vesicles established by plasma membrane endocytosis. Lysosomes receive and digest intracellular components including excess proteins, lipids, carbohydrates, nucleic acids, and damaged organelles by autophagosomes, and thus represent a critical end point in the autophagy system (Chang, 2020; **Figure 2**). Lysosomes also digest

extracellular macromolecules and materials such as aging cells, cell debris, bacteria, and viruses delivered through endocytosis and phagocytosis.

Lysosomal degradation reduces its substrates to fundamental building blocks of macromolecules including amino acids, sugars, lipids, and nucleic acids. These metabolites are either retained in the lysosome lumen to buffer their cytoplasmic concentrations, or are actively secreted into the cytoplasm by membrane permeases where they are reused by the cell to fuel biochemical and bioenergetic pathways, or exported out of the cell (**Figure 2**; Sagné et al., 2001; Rong et al., 2011; Jézégou et al., 2012; Liu et al., 2012; Verdon et al., 2017; Wyant et al., 2017). It is becoming increasingly clear that the processing and transport of lysosome-generated metabolites is highly regulated, and that mechanisms permitting contact with other organelles, such as MCS interaction with mitochondria, allow for bi-directional transport of metabolites and lipids that likely fuel metabolic and other biochemical pathways. Lysosomes also function as central players in coordinating supply and demand of critical molecules involved in bioenergetic signaling (Zhang et al., 2011b; Lim and Zoncu, 2016; Savini et al., 2019; Ballabio and Bonifacino, 2020). The signaling pathways and mechanisms that impact lysosome biogenesis and function are linked to many activities that implicate them as regulators of stem cell biology, including self-renewal and differentiation decisions, cell death, and organismal aging and longevity (Ramachandran et al., 2019; Savini et al., 2019; Ballabio and Bonifacino, 2020). An important role for lysosomes in cell identity determination is emerging, yet the mechanisms by which they may drive metabolic reprogramming and impact cell fate specification, and if these activities are indeed linked, are unclear.

Metabolic Signaling Pathways Converge at the Lysosome

Metabolic pathways and the cell biological processes with which they integrate are dynamic. Given that cells are regularly exposed to changing environmental conditions, mechanisms to finely regulate the balance between anabolic and catabolic activities are critical for cell viability, proper function, and homeostasis. This coordination is achieved by hard-wired signaling mechanisms that sense the level of available nutrients and other environmental signals like oxygen, energy status, stress markers, and growth factors, and accordingly relay downstream signals through phosphorylation events. The mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated kinase (AMPK) are the primary mammalian nutrient-sensing kinases, and they function in opposition to one another to control the catabolic–anabolic balance. A major breakthrough in our understanding of metabolic coordination, and one that underscored a profound physical connection between metabolic signaling and the lysosome, is that the activity status of these kinases is in fact regulated on the lysosome surface (Sancak et al., 2010; Zhang et al., 2014; Savini et al., 2019). In the decade since this discovery, we now understand that mTORC1 is in fact recruited to and activated at the lysosome in a two-step process, requiring both sufficient nutrient and growth factor signaling. Nutrient signaling, translated through amino acid availability, activates heterodimeric Rag GTPases which physically recruit mTORC1 to the lysosome (Yang et al., 2017; Lawrence et al., 2018). Subsequently, mTORC1 interacts with the small GTPase Rheb already located on the lysosome surface. If growth factor signaling permits, the GTPase-activating protein "tuberous sclerosis complex" (TSC) will be inhibited, thus promoting activation of Rheb and consequently of mTORC1 (Tee et al., 2003; Menon et al., 2014; Savini et al., 2019).

Hyper-active mTORC1 functions at the lysosome to phosphorylate downstream targets that promote anabolic cellular processes including protein translation, ribosome and lipid biogenesis, cell growth, and mitochondrial metabolism (Delaney et al., 2014; Lawrence and Zoncu, 2019; Savini et al., 2019; Ballabio and Bonifacino, 2020). Conversely, mTORC1 inhibits catabolic activities, predominantly lysosome biogenesis, and autophagy signaling. The primary catabolic targets include the autophagosome assembly protein ULK-1 and members of the TFE transcription factor family, key factors in the activation of lysosome biogenesis genes (Sardiello et al., 2009; Palmieri et al., 2011; Settembre et al., 2011; Martina et al., 2014; Annunziata et al., 2019; Savini et al., 2019).

AMP-activated kinase is activated in response to low nutrient levels, when intracellular ATP levels are low and AMP is high, and functions to promote catabolism and inhibit anabolism largely by countering mTORC1 activities (**Figure 2**). mTORC1 and AMPK are regulated reciprocally by the lysosome-bound v-ATPase that permits nutrient sensing of amino acid levels inside the lysosome in addition to cytoplasmic sensing (Zhang et al., 2014). Likewise, the cytoplasmic/lysosomal and nuclear localization of TFE transcription factors is controlled in opposing ways by mTORC1 and AMPK (Martina et al., 2012, 2014; Roczniak-Ferguson et al., 2012; Settembre et al., 2012; Young et al., 2016; Saxton and Sabatini, 2017; Napolitano et al., 2018; Asrani et al., 2019). Corroborating a coordinated mechanism that impacts the dynamics of lysosome biogenesis as well as cell fate determination, it was recently shown in the amoeba Dictyostelium that activated mTORC1 and AMPK reciprocally regulate an overlapping set of genes that dictate a fate switch between progenitor cell growth and differentiation (Jaiswal and Kimmel, 2019).

Metabolic signaling pathways centered at the lysosome are in fact important regulators of cell fate transitions. The ability to balance mTORC1 activity is critical for proper stem cell fate decisions between self-renewal and differentiation in development and aging in a number of somatic cell types, with mTORC1 hyper-activation typically driving aberrant lineage differentiation (Delaney et al., 2014; Meng et al., 2018). Loss of the TSC complex members hamartin (TSC1) or tuberin (TSC2), which drives constitutive mTORC1 activation, leads to the development of a multi-system low-grade tumor and neoplastic disorder called tuberous sclerosis. In this condition, lesions are characterized by enlarged cells expressing an atypical mixture of lineage-specific stem cell and immature differentiation markers (Delaney et al., 2014).

We and others have additionally found that the capacity to repress the mTORC1 pathway is essential for the reprogramming

of both mouse and human somatic cells to iPSCs (He et al., 2012; Wang et al., 2013; Wu et al., 2015; Armstrong et al., 2017; Julian et al., 2017a). This requirement has been linked to both p53-dependent cell death regulation and an intriguing mechanism whereby the critical cell fate determinant SOX2 transcriptionally suppresses mTORC1, at a very early stage of iPSC reprogramming, to permit transient activation of autophagy at this critical time-point of cell fate specification (Wang et al., 2013; Armstrong et al., 2017). mTORC1 inhibition by the core pluripotency factors during early reprogramming is also required to permit the reduction in mitochondrial mass (Wang et al., 2017, 2013) that coincides with the lower OxPhos activity typical of PSCs (Folmes et al., 2011). Demonstrating that lysosomes and other organelles can affect cell identity determination in distinct ways, this mitochondrial remodeling was strikingly independent of autophagy, evidenced by findings that mitochondria-lysosome co-localization could not be observed and lysosom inhibition with bafilomycin or shRNA targeted knock-down of autophagy regulators (Atg5, Beclin or Vps34) did not affect mitochondrial mass (Wang et al., 2013). The requirement for mTORC1 inhibition in regulating pluripotency appears to be limited to the initial stage of cell identity determination, as we and others have demonstrated that the loss or reduction of TSC2, limiting the capacity to inhibit mTORC1, does not affect the maintenance of a pluripotent state in established human PSCs (Julian et al., 2017a; Blair et al., 2018; Delaney et al., 2019). Thus, mTORC1 inhibition and consequent autophagy activation appears to be critical specifically at a very early stage of cell fate transition (**Figure 1**).

Emerging Evidence for Lysosomes in Lineage-Specific Fate Determination

Though lysosome biogenesis and activity have been linked to stem cell fate decisions from development through aging, it has been unclear if lysosomes are drivers of these events or instead, passengers in the process. The acquisition and maintenance of pluripotency, for instance, requires metabolic reprogramming to a highly glycolytic state (Zhu et al., 2010; Folmes et al., 2011; Zhang et al., 2011a; Hansson et al., 2012; Panopoulos et al., 2012; Wang et al., 2013; Prigione et al., 2014) and *in vitro* differentiation of PSCs to downstream lineages correlates with further metabolic changes (Chung et al., 2007; TeSlaa et al., 2016; Betschinger, 2017; Cliff et al., 2017; Lees et al., 2018; Zhang et al., 2018). Our current understanding of the metabolic mechanisms driving these fate transitions is focused on the mitochondria with limited insight regarding the potential impact of other organelles. Landmark studies over the past few years have provided strong evidence that lysosomes can indeed function as drivers of both metabolic network reprogramming and stem cell fate decisions that affect cell identity determination.

It is now clear that lysosomes function as active drivers of PSC differentiation through their physical and functional interactions with the nutrient-sensing mTORC1, Rag GTPase, and AMPK complexes (Young et al., 2016; Villegas et al., 2019; **Figure 2**). Villegas et al. (2019) performed a genome-wide CRISPR/Cas9 screen in mouse embryonic stem cells (ESCs) under culture conditions that promoted either self-renewal or differentiation, to identify factors whose loss would impede exit from the pluripotent state. This unbiased approach identified a number of factors related to lysosome biogenesis and function as critical regulators of PSC differentiation (Villegas et al., 2019). These include genes that ensure lysosome degradative activity, as well as the Rag proteins and components of the Ragulator complex that recruits Rag proteins to the lysosome surface, necessary for control of mTORC1 activity. Also identified as a critical regulator was the tumor suppressor Folliculin, which normally translates amino acid levels to Ragulator/Rag permitting non-nuclear recruitment and sequestration of Tfe3. The authors discovered that loss of these factors leads to constitutive localization of Tfe3 in the nucleus, which is known to directly drive gene expression programs related to lysosome biogenesis and metabolic signaling. This was confirmed in the study using RNA-sequencing, a strategy that revealed a secondary transcriptional response of Tfe3 that enforces a sustained pluripotency program and represses transcriptional programs associated with peri-implantation development and neural lineage differentiation.

Similarly, mouse ESCs lacking functional AMPK, which antagonizes mTORC1 activation at the lysosome and thereby inhibits its phosphorylation-induced retention of TFE proteins in the cytoplasm, exhibit profound differentiation defects. $AMPK^{-/-}$ ESCs maintain pluripotency but fail to generate chimeric embryos and exhibit a preference for ectodermal compared to endodermal differentiation, due to hypo-phosphorylation of Tfeb and its reduced nuclear localization (Young et al., 2016). This study and others position coordinated regulation of AMPK and mTORC1 signaling to be an essential regulatory node in PSCs that determines an ectodermal or endodermal cell fate, with hyper-active mTORC1 signaling consistently leading to preferential ectodermal differentiation (Zhou et al., 2009; Young et al., 2016; Delaney et al., 2019; Jaiswal and Kimmel, 2019).

Underscoring an essential role for lysosomes in cell fate decisions particularly in NSC pools, differences in proteasome and lysosome-autophagy activity have recently been revealed as defining factors between proliferative "activated" NSC populations versus aged quiescent NSCs, whose accumulation is strongly associated with age-related cognitive decline (Leeman et al., 2018). The authors employed RNA-sequencing, again an unbiased approach, to profile transcriptional signatures of activated and quiescent cell populations within the NSC pool isolated from young and aged mice. Differences in metabolic profiles and active components of the proteostasis system, specifically the proteasome and lysosome machinery, were the most profound signatures discerning proliferative from quiescent NSCs. Transcriptional signatures and functional experiments revealed that the proteostasis machinery is highly functional in activated NSCs, whereas activity is low in quiescent cells. As quiescent NSCs age, their lysosomes become significantly enlarged and accumulate increasing amounts of insoluble aggregated proteins, which is due to a lack of efficient clearance by the lysosome. Activation of lysosome activity by TFEB expression in quiescent NSCs permitted clearance of protein aggregates and, strikingly, altered the fate of these cells to an activated state.

Our recent work further implicates lysosome activation as a critical requirement for NSC fate regulation, in the initial determination of neural lineage identity (Delaney et al., 2019; **Figure 1**). We developed a modeling system of the multi-system tumor disorder tuberous sclerosis by engineering inactivating *TSC2* mutations in human PSCs, thereby inducing mTORC1 hyperactivation, and subsequently differentiating them in a directed manner into NSCs and the developmentally related neural crest (NC). These two lineages are the presumed derivative cell types of the diverse lesions and tumors in tuberous sclerosis patients (Delaney et al., 2014). Seeking to determine the mechanisms by which normal (WT) and $TSC2^{-/-}$ PSCs commit to an NSC or NC fate, we employed RNA-sequencing and functional analysis approaches to identify the primary biological processes at play during the transition from pluripotency to the new cellular identity. An increase in lysosome content, driven by an acute activation of a proteostasis stress response, was evident at very early stages of PSC–NSC induction, but not during NC specification. This was observed in WT cells and was further exacerbated in cells lacking TSC2. Of note, a recent study revealed that cells undergoing NSC specification undergo a reprogramming of protein chaperone networks, which are intimately connected with proteostatic mechanisms; this finding underscores the importance of proteostasis signaling integrity during early NSC development (Vonk et al., 2020). Furthermore, lysosome content changed dynamically throughout NSC induction in $TSC2^{-/-}$ cells, and these patterns correlated with alterations in the expression of cell fate markers (Delaney et al., 2019).

The aberrant lysosome activation driven by TSC2-deficiency was transient during neural development but became reactivated with time as NSC cultures were aged, in a manner that paralleled altered expression of cell fate markers (Delaney et al., 2019). These findings suggest a close association of lysosome biogenesis with cell fate in NSC populations, both during their initial specification and long-term maintenance. Our findings that early induced NSCs lacking TSC2 activate lysosome biogenesis, while maintaining degradative activity through autophagy, in parallel with hyperactive mTORC1 signaling reveals the existence of early adaptive mechanisms in metabolic circuits to ensure proper signaling for lineage development. A recent study suggests that autophagy-dependent lipid metabolism is an important aspect of these adaptations (Wang et al., 2019). We also found that increased lysosome biogenesis preceded later changes in mitochondrial content, which correlated with aberrant activation of oxidative metabolism uniquely in $TSC2^{-/-}$ NSCs (Delaney et al., 2019). This demonstrates that lysosome activation can be a driver of metabolic and cell fate changes, at least in the neural lineage, and is not simply a downstream consequence of mitochondrial alterations (**Figure 1**).

Implications for Disease Mechanisms and Treatment

Many human disorders, ranging from developmental to aging-induced conditions and various forms of cancer, exhibit aberrant lysosome activity as a known or predicted factor contributing to pathogenic phenotypes. The over 50 rare genetic metabolic conditions under the umbrella of "lysosomal storage diseases" typically present early in life, yet we now know that they share many clinical, pathological and genetic features with age-related neurological conditions, including frontotemporal dementia, Alzheimer's and Parkinson's disease (Fraldi et al., 2016). Shared pathological phenotypes are typified by metabolic dysfunction and an accumulation of aggregated proteins as a consequence of insufficient proteostasis mechanisms that include altered lysosome activity. Retrospective studies and PSC-based disease modeling approaches, which permit detailed analysis of the biological effects of allele variants starting from the earliest stages of cell lineage development, have begun to reveal that gene mutations associated with adult onset degenerative conditions can confer measurable phenotypes during development or at much earlier stages than previously considered (Delaney et al., 2019; Smits et al., 2019; Zhu et al., 2019; Johnson et al., 2020). Examples include altered stem cell proliferation, differentiation decisions, metabolic and proteostasis signaling in studies of tuberous sclerosis, Alzheimer's and Parkinson's disease (Delaney et al., 2019; Smits et al., 2019; Zhu et al., 2019). Lysosomal dysfunction and metabolic reprogramming have also been implicated in neurological conditions not directly associated with degeneration, including psychiatric and seizure disorders; likewise, many lysosomal storage diseases and other conditions, such as inherited tumor syndromes, present with psychiatric disease and often neurodegeneration (Staretz-Chacham et al., 2010; Marques and Saftig, 2019).

It is therefore clear that the brain, and the neural cell lineage that builds this organ, are highly dependent on the lysosome for proteostasis regulation and is particularly sensitive to developing disease when faced with lysosome dysfunction. This neural lineage proteostasis sensitivity is often attributed to the fact that neurons are highly active biologically but unable to divide and thereby have no progeny through which to dilute protein aggregates and aberrant regulatory machinery. Our recent work and that of others discussed in this review (Zhou et al., 2009; Young et al., 2016; Leeman et al., 2018; Delaney et al., 2019; Jaiswal and Kimmel, 2019) suggest that unique proteostatic mechanisms with a particular dependency on lysosome-mediated degradation are in fact active at the earliest stages of neural lineage development. Moreover, these findings imply that lysosome activation is critical for NSC fate determination, suggesting a hard-wired integration of lysosomal signaling mechanisms with metabolic and transcriptional programs that coordinate NSC identity.

Hard-wiring of lysosomal signaling in NSCs has implications for understanding susceptibility to neurological disease, as well as the stage-specific drivers of diseases that are associated with changes in markers of cell fate and identity, such as cancer (**Figure 1**). Similar to stem cells transitioning between cell fates, tumorigenesis is associated with dramatic metabolic alterations. These are typically unique to each tumor type but are common conceptually, given that significant metabolic remodeling occurs at each phase from pre-malignant lesion, to aggressive primary tumor, and consequently metastasis (Faubert et al., 2020). These changes occur as tumors, typically thought to be driven by

populations of adaptive CSCs, face ever changing environments that are commonly low in oxygen and standard energy sources (Nazio et al., 2019).

Many new and developing therapeutic strategies for cancer are directed toward targeting metabolic vulnerabilities of the tumor. Inhibition of lysosome activity by targeting autophagy signaling, using lysosomotropic agents like bafilomycin or chloroquine derivatives, or degradative capacity and ion transport are popular among these approaches (Nazio et al., 2019; Méndez-Lucas et al., 2020). Yet, despite the clear vulnerability of metabolic and lysosome-based networks in cancer, current lysosome-targeted drugs have had limited success in clinical trials, often failing to provide adequate tumor-specific toxicity at least as single agents. We posit that a deeper understanding of the organelle-based processes that drive CSC adaptations is one avenue that will lead to more targeted selection of therapeutic agents based on the tumorigenic stage, the lineage identity of resident CSCs, and the driving biological mechanisms (**Figure 1**).

Efforts aimed at elucidating the extent to which lysosome dynamics influence cell fate specification in stem cell populations are likely to yield important therapeutic insights. Using CSCs as an example, an important approach is to discern whether altered lysosome signaling in a given tumor type is an early precipitating event in cell fate and metabolic dysfunction, as we observed in our human stem cell model of tuberous sclerosis (Delaney et al., 2019), or instead a secondary event, potentially consequent to mitochondrial reprogramming. An improved understanding of the driving and secondary adaptive mechanisms that contribute to stem cell-based disease has high potential to reveal effective stage-specific biomarkers and to guide rational development of therapeutic strategies.

Lysosomes are complex organelles, with an ever-growing list of associated biological functions with the potential to impact stem cell fate and identity determination (**Figure 2**). Beyond their canonical role of degrading macromolecules through their acidic environment and portfolio of hydrolytic enzymes, they also act as nutrient sensors, platforms for signaling pathways, regulators of lipid production and transport, and mediators of ion, nucleic acid, amino acid, and metabolite signaling and transport. Lysosomes are also structurally dynamic, undergoing fission and fusion events, relying on their connection to the actin cytoskeleton, and can physically and functionally interact with multiple organelles. Additionally, they have emerged as regulators of transcriptional programs, for instance by modulating TFE sub-cellular localization and engaging lipid metabolites for nuclear signaling.

It is likely that many more functions remain to be uncovered by which the lysosome impacts stem cell fate. For instance, mTORC1 and AMPK are not the only signaling hubs located at the lysosome surface; additionally, there are likely many more mechanisms to be uncovered that contribute to mTORC1-dependent molecular sensing (Young et al., 2016; Savini et al., 2019; Ballabio and Bonifacino, 2020). Moving forward it will be critical to understand the mechanisms by which lysosomes impact cell fate specification, especially their impacts on transcriptional and translational programs. For example, are there additional mechanisms outside of lipid and TFE signaling by which the lysosome affects transcriptional programs? Possibilities include direct regulation of the expression or localization of additional transcription factors, or the production and transport of metabolites that affect epigenetic signatures. Lysosome metabolite profiling represents a particularly exciting and newly developing area of research that will undoubtedly help deepen our understanding of the mechanisms by which this organelle impacts stem cell identity determination, and furthermore how it may be efficiently targeted in cancer and disease states (Abu-Remaileh et al., 2017). Acquiring a deeper understanding of lysosome-based signaling at the metabolite level and beyond has the potential to discover targeted biomarkers and therapeutic approaches for stem cell-based disease, and to improve strategies for tissue regeneration.

CONCLUDING REMARKS

Regulation of stem cell fate decisions is heavily predicated on the integration of transcriptional and metabolic signaling networks. Our understanding of the metabolic impacts on cell fate have been largely limited to the mitochondria, yet it is becoming increasingly evident that multiple organelles interact with one another in diverse ways to regulate cell fate. The lysosome is now well appreciated as not only the waste disposal center of the cell but as an active and multifunctional player in coordinating metabolic networks; exciting work over the past few years has provided compelling evidence that lysosome-based activities are in fact crucial for regulating cell identity determination and other fate decisions. The lysosome is a multi-functional organelle whose activities and regulatory partners are still being uncovered. Thus, focusing efforts on unraveling the biological impacts of lysosome signaling on cell fate regulation has high potential to significantly impact our understanding of tissue development and aging, disease predisposition and pathogenesis, as well as disease treatment and regenerative medicine strategies.

AUTHOR CONTRIBUTIONS

LJ wrote the manuscript and prepared the figures. WS reviewed and edited the manuscript, provided intellectual insights, and acquired funds for publication. Both authors contributed to the article and approved the submitted version.

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Excess Glucose Impedes the Proliferation of Skeletal Muscle Satellite Cells Under Adherent Culture Conditions

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Glucose is a major energy source consumed by proliferating mammalian cells. Therefore, in general, proliferating cells have the preference of high glucose contents in extracellular environment. Here, we showed that high glucose concentrations impede the proliferation of satellite cells, which are muscle-specific stem cells, under adherent culture conditions. We found that the proliferation activity of satellite cells was higher in glucose-free DMEM growth medium (low-glucose medium with a glucose concentration of 2 mM) than in standard glucose DMEM (high-glucose medium with a glucose concentration of 19 mM). Satellite cells cultured in the high-glucose medium showed a decreased population of reserve cells, identified by staining for Pax7 expression, suggesting that glucose concentration affects cell fate determination. In conclusion, glucose is a factor that decides the cell fate of skeletal muscle-specific stem cells. Due to this unique feature of satellite cells, hyperglycemia may negatively affect the regenerative capability of skeletal muscle myofibers and thus facilitate sarcopenia.

Keywords: satellite cell, proliferation, self-renewal, glucose, primary culture

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INTRODUCTION

Skeletal muscle has the ability to regenerate after injury. The main players of muscle regeneration are muscle-specific stem cells, known as satellite cells, which reside between the sarcolemma and basal lamina of myofibers. Satellite cells normally stay quiescent and have a low metabolic rate but initiate the myogenic program in response to muscle injury (Brack and Rando, 2012). Activated satellite cells express myoblast determination protein 1 (MyoD), a key transcription factor in myogenesis, and proliferate as myoblasts before differentiating and fusing to repair damaged muscles (Tajbakhsh, 2009). A portion of satellite cells does not commit to muscle differentiation but remains as stem cells by asymmetric division to maintain the satellite cell pool; this is termed as self-renewal (Collins et al., 2005). The ability of satellite cells to regenerate skeletal muscle is crucial not only for repairing muscle injury but also for maintaining muscle mass (Keefe et al., 2015). Since satellite cells are applicable to regenerative medicine for muscle diseases (Sacco et al., 2008), it is necessary to investigate the mechanism underlying satellite cell behavior.

Glucose is an essential energy substrate and an anabolic precursor for various mammalian cells. In anaerobic glycolysis, two ATP molecules are generated from one glucose molecule, and the

central metabolite pyruvate is used as a substrate in the mitochondrial tricarboxylic acid (TCA) cycle through oxidative phosphorylation. In addition to its catabolic role, glucose is used for the synthesis of nucleotides through the pentose phosphate pathway, which is essential to produce ribose for DNA synthesis during cell division. Proliferating cells, such as cancer cells, prefer glucose as fuel to support their rapid proliferation (Han et al., 2011; Jones and Schulze, 2012; Ito et al., 2017; Luo et al., 2018; Zhou et al., 2018). For these reasons, it is believed that the media of various cultured cells require high concentrations of glucose to improve cell proliferation.

High-glucose media have also widely been used for culturing muscle cells (Shefer and Yablonka-Reuveni, 2005; Pasut et al., 2013). However, here we found that a high-glucose medium was not suitable for culturing satellite cells under adherent conditions. The proliferation activity of satellite cells was higher in a low-glucose medium than in the standard high-glucose medium. Additionally, the population of reserve cells, as indicated by Pax7 expression, was increased by lowering the glucose concentration, suggesting that glucose affects the cell fate determination of satellite cells. High glucose levels disturb important functions of satellite cells, such as cell proliferation and self-renewal. An excessive glucose concentration seems to represent a negative factor for skeletal muscle homeostasis because hyperglycemia is known to induce the impairment of muscle regeneration and atrophy.

Experimental Procedures

Animals and Experimental Design

Adult C57BL/6 male mice (8–12 weeks) were used in this study. Mice were fed normal chow and water under standard lighting conditions (12-h:12-h light–dark cycle).

Myofiber and Satellite-Cell Isolation and Culture

Extensor digitorum longus (EDL) and soleus muscles were isolated and digested using type I collagenase, as described previously (Ono et al., 2012). Collected myofibers were confirmed to be surely clean and not to include other cells such as fibroblasts under the high-magnitude microscope. Single myofibers were incubated with Accutase (Innovative Cell Technologies) for 10 min and cultured on Matrigel-coated dishes. Standard growth medium was composed of high-glucose Dulbecco's modified Eagle's medium (DMEM) containing GlutaMAX (Thermo Fisher Scientific), 30% fetal bovine serum (FBS) (BioWest or Nichirei), 1% chicken-embryo extract (CEE) (USBiological), and 1% penicillin–streptomycin. We prepared the low-glucose growth medium using glucose-free DMEM (Thermo Fisher Scientific) supplemented with 1% GlutaMAX to adjust other components with the standard growth medium. Cells were incubated at 37°C under an atmosphere of 5% CO_2, and media were changed every day, beginning on the third of culture. Myogenic differentiation was induced using the differentiation medium (high-glucose DMEM supplemented with 5% horse serum and 1% penicillin–streptomycin) at 37°C under an atmosphere of 5% CO_2. For floating culture, isolated myofibers were cultured in the plating medium (DMEM supplemented with 10% horse serum, 0.5% CEE, and 1% penicillin-streptomycin) without incubation of Accutase (Ono et al., 2012).

Analysis of Cell Proliferation and Differentiation

For the cell proliferation assays, 20 myofibers were cultured in each well (24-well plate) in duplicate, and stained with DAPI or Ki67, a marker of cell proliferation. To detect EdU incorporation, the Click-iT EdU Imaging Kit (Thermo Fisher Scientific) was used according to the manufacturer's instructions. On day-6 of culture, the cells were treated with 10 μM EdU for 6 h, and fixed in 4%PFA for 15 min.

To analyze the apoptosis of cultured satellite cells, the TUNEL assay was performed using the Click-iT^{TM} Plus TUNEL Assay for in situ Apoptosis Detection, Alexa Fluor^{TM} 594 dye (Thermo Fisher Scientific) according to the manufacturer's instructions.

For evaluation of myoblast differentiation, cells cultured in low-glucose growth medium for 6-days were passaged in 24-well plates to the same density and cultured for 3-days in differentiation medium. Myosin heavy chain (MHC), a contractile protein expressed in differentiated muscle cells, was stained. The fusion index, the number of nuclei inside myotubes as a percentage of the total number of nuclei, was calculated and compared between glucose concentrations.

Degradation of Glucose in FBS Using Immobilized Enzymes

Glucose oxidase (GOD) and catalase were dissolved in phosphate-buffered saline (PBS) at 100 and 3,700 U/mL, respectively. The enzyme solution was mixed with Biosurfine-AWP-MRH (Tokyo Gosei Kogyo Co.) at a ratio of 1:10 (v/v) and pasted on a glass slide (Matsunami). Each side of the glass slide was exposed to UV light for 30 min to immobilize the enzymes on the slide. FBS was incubated with the glass slide by gently shaking in a 50-mL tube for 3 weeks at 6°C. The glucose concentration was measured using the glucose (HK) assay kit (Sigma Aldrich), according to the manufacturer's instructions. Glucose-depleted FBS was filtered through a 0.22 μm polyvinylidene fluoride (PVDF) membrane before use for cell culture.

Immunostaining

Immunocytochemistry of cultured myoblasts and myotubes was performed as described previously (Ono et al., 2012). Samples were fixed with 4% paraformaldehyde, blocked/permeabilized with PBS containing 0.3% Triton X-100 and 5% goat serum for 30 min at room temperature, and incubated overnight with primary antibodies at 4°C. The following antibodies were used: anti-alpha-actinin (Sigma Aldrich, A7811), anti-Ki67 (Cell Signaling Technology, 9129), anti-MHC (R&D Systems, MAB4470), anti-Pax7 (DSHB, PAX7), anti-MyoD (Santa Cruz Biotechnology, sc-760), and anti-myogenin (Santa Cruz Biotechnology, sc-12732). Immunostained samples were visualized using the appropriate species-specific Alexa Fluor 488- and 594 fluorescence-conjugated secondary antibodies

(Thermo Fisher Scientific). The samples were observed and photographed using a Keyence BZ-X800 microscope or a Nikon Ti-U microscope. Images were automatically taken in a scan to cover the entire 24 wells and then tiled. Cell numbers were quantified using the Keyence software BZ-H3C and H3CM.

Immunostaining myofiber-associated satellite cells was performed as described previously (Ono et al., 2012). Briefly, cultured myofibers were fixed with 4% paraformaldehyde, blocked/permeabilized with PBS containing 0.3% Triton X-100 and 10% goat serum for 30 min at room temperature, and incubated overnight with primary antibodies for Ki67 (Cell Signaling Technology, 9129) at 4°C. The primary antibodies were visualized by appropriate species-specific 488 and 594 fluorescence -conjugated secondary antibodies (Thermo Fisher Scientific). Nuclei were stained with VECTASHIELD fluorescent mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, United States).

Western Blotting

Total protein extracts were obtained from homogenized tissues and cultured cells, and lysed with RIPA or lysis buffer (Manabe et al., 2014). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with Tris–buffered saline containing 5% nonfat dry milk and 0.1 % Tween 20. The membranes were then incubated overnight with the following primary antibodies: anti- β-actin (Cell Signaling Technology, 4967), anti-MHC I (Sigma Aldrich, M8421), anti- MHC II (Sigma Aldrich, M4276), anti-GAPDH (Cell Signaling Technology, 2118), and Phospho-AMPK (Thr172) (Cell Signaling Technology, 2531). Subsequently, the membranes were treated with rabbit (GE Healthcare) or goat (Millipore) secondary antibodies conjugated to horseradish peroxidase. The blots were developed using ECL plus (PerkinElmer Life Sciences) and analyzed with a Luminescent Image Analyzer LAS-4000 mini (GE Healthcare). Data were quantified using the ImageJ software.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Two-sided unpaired t-tests were used to compare data between the two groups. N in the figure legends indicates the number of mice used in the experiments. The level of significance was set to $p < 0.05$.

RESULTS

Glucose Limitation Facilitates the Cell Proliferation of Primary Satellite Cells

In general, high-glucose DMEM is the standard medium for culturing primary satellite cells (Ono et al., 2010, 2012). Therefore, we used a high-glucose medium containing 30% FBS and some other cell culture supplements (**Figure 1A**). The final glucose concentration was 19 mM in the high-glucose medium containing 30% FBS. We also prepared a growth medium containing a very low glucose concentration using glucose-free DMEM as a basic medium. The low-glucose medium had a final glucose concentration of 2 mM due to carry-over from 30% FBS. Despite the carry-over from FBS, the total glucose concentration in the low-glucose medium was only 10% of that in the high-glucose medium. We concluded that the glucose concentration in the FBS used was about 1.3 g/L (**Figure 1A**). Detailed information and formulation of the media are cited in **Supplementary Table 1**. The glucose concentration in serum and CEE were cited in **Supplementary Table 2**.

To examine the effect of glucose concentration on satellite cell proliferation, we determined cell growth curves in high- and low-glucose media. We cultured satellite cells obtained from 20 myofibers in each 24-well plate for 3, 4, 5, and 6 days before counting cells visualized by DAPI staining (**Figure 1B**). As shown in **Figure 1C**, cell proliferation was promoted in the low-glucose medium compared to that in the standard high-glucose medium. A statistically significant difference in cell number was observed on the sixth day of culturing between high and low glucose conditions. Ki67 is a routinely used cell proliferation marker. The ratio of Ki67-positive cells to total cells evaluated by immunohistochemical staining (**Figure 1D**) and the total expression level of Ki67 protein quantified by immunoblotting (**Figure 1E**) were significantly elevated for the low-glucose medium compared to the corresponding values for the high-glucose medium after 6 days of culturing. To confirm the change in proliferation due to glucose, the EdU pulse-chase assay was performed under high and low glucose conditions. The satellite cells grown in the low-glucose medium had a higher number of EdU-positive cells compared to that in the high-glucose medium (**Figure 1F**), suggesting that low glucose facilitates cell proliferation of satellite cells. These data provide direct evidence that glucose restriction facilitates the proliferation of satellite cells.

We also examined whether glucose regulated cell proliferation in a dose-dependent manner. Satellite cells derived from 20 myofibers were cultured for 6 days in media with different glucose concentrations, and the ratio of Ki67-positive cells to total cell number was quantified. Cell proliferation was enhanced in media with a glucose concentration of 8 mM or less (**Supplementary Figure 1**). To minimize the effects of the adhesive property of the myofibers on the Matrigel and the myoblast migration rate from the myofibers attached to the Matrigel on cell proliferation, we also analyzed the proliferation capacity after passage. The primary cells were first grown from a single dish, and the same number of cells recovered from the dish was re-cultured in high-glucose and low-glucose media. We observed that the cell number and percentage of Ki67-positive cells tended to be higher in the low-glucose medium (**Supplementary Figure 2**).

Cell number is potentially affected by cell viability and cell death. Therefore, we evaluated the apoptosis of satellite cells in high- and low-glucose media for 6 days using a TUNEL assay. The percentage of apoptotic cells was lower in the low-glucose medium than in the high-glucose medium, indicating that glucose restriction ameliorated cell damage and maintained cell viability (**Supplementary Figure 3**). We analyzed the cell proliferation of satellite cells while they were still attached to myofibers. Isolated myofibers were cultured in high and low

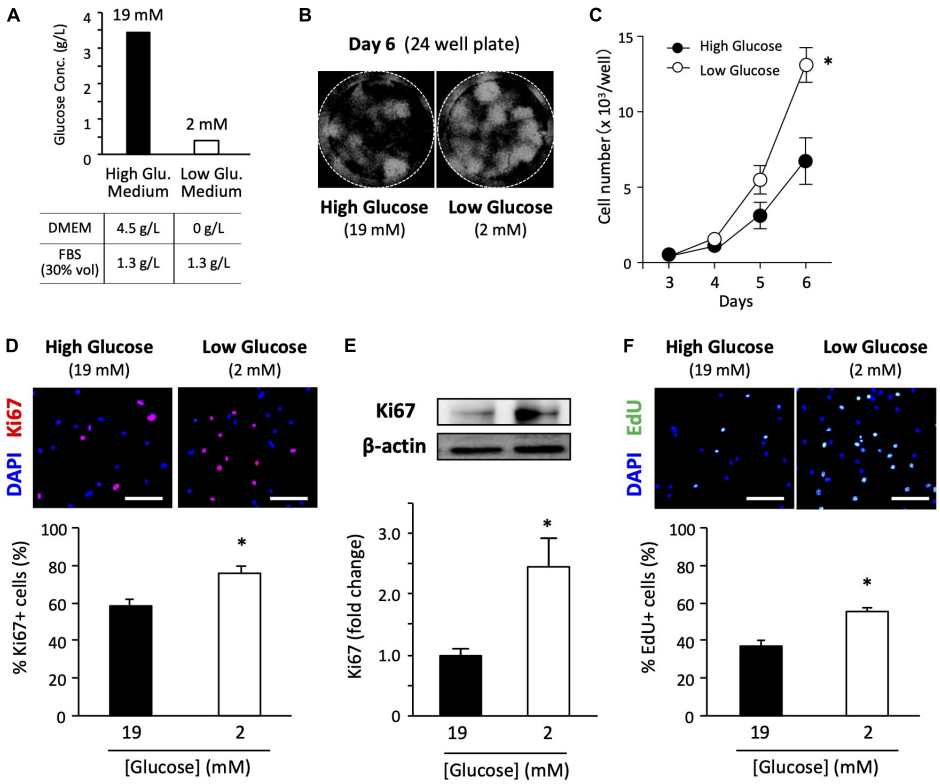

FIGURE 1 | Low-glucose medium increases the proliferation of primary satellite cells. **(A)** Glucose concentration in each growth medium used in this study. **(B)** Proliferation of primary satellite cells in high- and low-glucose media. Satellite cells from 20 myofibers were isolated from EDL and seeded in 24-well plates. Cell nuclei were visualized using DAPI and marked by the Hybrid Cell Count application (Keyence software). All the cells cultured in each well were automatically counted. **(C)** Cell growth curves. Values are presented as the mean ± SEM (n = 7). *$p < 0.05$. **(D)** Immunofluorescence analysis of proliferating cells cultured for 6 days. The population of Ki67-positive cells was quantified in high- and low-glucose media. Scale bars are 100 μm. Values are presented as the mean ± SEM (n = 3). *$p < 0.05$. **(E)** Western blot analysis of Ki67 protein expression in high- and low-glucose media after 6 days of cultivation. Ki67 expression was normalized to that of β-actin. Values are presented as mean ± SEM (n = 13). **(F)** Representative images of EdU+ satellite cells and the quantification of the number of EdU+ cells cultured for 6 days in high- and low-glucose media. Scale bars are 100 μm. Values are presented as the mean ± SEM (n = 4). *$p < 0.05$.

glucose medium for 72 h and then satellite cells were stained for Ki67. The number of proliferating cells, marked as Ki67-positive cells, did not differ between high- and low-glucose conditions in this type of experimental model (**Supplementary Figure 4**).

We also performed experiments to examine whether low-glucose medium maintained the proliferative activity of the satellite cell-derived myoblasts. When high-glucose medium was used, cell proliferation was inhibited in around 1 week. However, we observed that the cells cultured in the low-glucose medium could be passaged, and they maintained their proliferative activity for more than 2 weeks (**Supplementary Figure 5**). The passaged cells had the capacity for differentiation, because they fused and expressed MHC protein after being cultured in the differentiation medium (**Supplementary Figure 5**). We demonstrated that myoblasts cultured in low-glucose medium were able to be frozen, and could proliferate again after thawing. We also observed that cells frozen twice were able to differentiate normally (**Supplementary Figure 6**).

We examined whether glucose regulates myoblast differentiation. Myoblasts grown in low-glucose medium were passaged and cultured in differentiation medium (5% horse serum in DMEM) at different glucose concentrations for 3 days. The myotubes were fixed, and fusion indices were calculated by staining the MHC. Myoblasts were normally differentiated even when cultured in low-glucose medium, and fusion indexes were similar regardless of glucose concentration (**Supplementary Figure 7**).

Glucose Concentration Affects Cell Fate Determination

We determined the myogenic status of the satellite cells using myogenic markers. Pax7 is an important transcription factor for maintaining the stemness of satellite cells, and is a recognized self-renewed cell marker (Halevy et al., 2004; Olguin and Olwin, 2004). MyoD is a master regulator of myogenesis that is expressed at the early stage of muscle differentiation (Bentzinger et al., 2012). Myogenin is a differentiation marker expressed during muscle differentiation. Cultured cells were co-immunostained with Pax7 / MyoD or MyoD / myogenin. The Pax7+/MyoD – phenotype reflects self-renewed cells. The Pax7+/MyoD+ phenotype represents cells that are

activated/proliferating cells, and the Pax7–/MyoD+ phenotype indicates myogenic committed cells (Brun et al., 2014). Further, the MyoD+/ myogenin – phenotype represents activated/proliferating cells, MyoD+/myogenin+ represents myogenic committed cells, and the MyoD–/myogenin+ phenotype represents cells differentiating cells (very minor population). As shown in **Figures 2A,B**, the number of cells of each myogenic status was increased in low-glucose media compared to that in high-glucose media, as was the overall cell number. Noteworthily, the higher percentage of Pax7+/MyoD– cells in the low-glucose medium suggests that the self-renewed satellite cells is better maintained under low glucose conditions in vitro (**Figure 2A**). There were no changes in the populations of activated and committed cells between high and low glucose conditions, as indicated by MyoD and myogenin expression levels (**Figure 2B**). To examine the effect of glucose on the generation of reserve cells, which are undifferentiated but retain their myogenic potential (Yoshida et al., 1998), the cells were stained for Ki67 in addition to Pax7 and MyoD. The frequency of reserve cells, marked as Pax7+/MyoD-/Ki67- cells was greater in low-glucose medium than in high-glucose medium (**Figure 2C**). We performed western blotting to quantify the expression of Pax7, MyoD, and myogenin in the cells, showing that these proteins were increasing under the low glucose condition (**Figure 2D**). The data show that cell proliferation/differentiation was progressing in the experimental situation, while the progression of cell fate was different between cells in 2 vs 19 mM glucose conditions.

The Low Glucose Condition Inhibited the Expansion of Non-Myogenic Cells and Enabled the Culture of High Purity Muscle Cells

Primary culture of satellite cells from single myofibers was performed using EDL muscle because the myofibers can be isolated with a low risk of contamination by non-muscle cells or connective tissues (**Figure 3A**). EDL muscle is mainly composed of type II fibers, which express the MHC II, and shows glycolytic characteristics. Accordingly, myotubes differentiated from satellite cells, which were isolated from EDL fiber, expressed MHC II, which indicates that they are type II myotubes. To obtain slow-type myotubes, we isolated myofibers from soleus, a representative slow-type muscle. It is, however, difficult to isolate clean single myofibers from soleus because most myofibers contain non-muscle cells (**Figure 3A**); therefore, these myofibers must be eliminated to prevent contamination with non-muscle cells as pointed by previous reports (Rosenblatt et al., 1995; Danoviz and Yablonka-Reuveni, 2012). We cultured satellite cells from soleus myofibers and stained α-actinin, a marker of differentiated muscle cells. Staining revealed that most of the cells were non-muscle cells, such as fibroblasts (α-actinin-negative cells). When satellite cells contaminated with fibroblasts are plated on dishes, the fibroblasts expanded rapidly and inhibited the growth of satellite cells, due to their high capacity for cell proliferation (**Figure 3B**).

When satellite cells were cultured in a standard high-glucose medium, fibroblasts preferentially grew, as shown above (**Figure 3C**). Mononuclear cells were observed, but they were not satellite cell-derived myocytes because MyoD, a marker of myogenic cells, was not expressed by them. On the contrary, when satellite cells were cultured in a low-glucose medium, most of them differentiated and expressed α-actinin. This indicated that satellite cells only grew normally in the low-glucose medium in which other cells could not survive (**Figure 3C**). Using this medium, we successfully cultured soleus-derived satellite cells in high purity and obtained myotubes from both EDL and soleus muscles. As expected, soleus-derived myotubes exhibited slow-type characteristics because they predominantly expressed MHC I instead of MHC II. The expression pattern of the MHC isoforms in primary myotubes was similar to that in muscle tissue (**Figure 3D**).

Satellite Cells Survive in Glucose-Depleted Growth Medium

As indicated before, the glucose-free DMEM we used in this study contained a small amount of glucose (2 mM) that originated from the FBS component. To test whether satellite cells can survive under even lower glucose conditions, we degraded the glucose in FBS using the enzymes GOD and catalase. GOD breaks down glucose into gluconic acid and H_2O_2 (1). Since H_2O_2 is harmful to cells, catalase was used to degrade it to H_2O and CO_2 (2).

$$\beta - \mathrm{D} - \text{glucose} + \mathrm{O_2} + \mathrm{H_2O} \rightarrow \mathrm{D} - \text{gluconic acid} + \mathrm{H_2O_2} \quad (1)$$

$$2\mathrm{H_2O_2} \rightarrow 2\mathrm{H_2O} + \mathrm{O_2} \quad (2)$$

To remove the enzymes after the reactions, we immobilized them on a glass slide using a UV curable polymer. FBS was incubated with the enzymes immobilized on the glass slide (**Figure 4A**). After enzyme treatment, the final glucose concentration in the medium was 0.1 mM, which was less than 1% of that in the standard high-glucose medium.

Using the glucose-depleted medium (termed as very-low-glucose medium), we prepared three different media containing the following glucose concentrations: 0.1, 2.0, and 19.0 mM by adding glucose powder. Satellite cells from 20 myofibers were cultured in each medium for 6 days using 24-well plates. The cell numbers were evaluated by microscopy. Surprisingly, the satellite cells proliferated normally in the very-low-glucose medium (**Figure 4B**), and the cell number in this medium was similar to that in the low-glucose medium (2.0 mM) (**Figure 4C**). We also prepared a serum-free medium containing chemically defined supplements instead of FBS and tried to use it to culture satellite cells. However, the cells did not survive in this medium, suggesting that serum factors are essential for the proliferation of satellite cells (data not shown).

AMPK Is Not Involved in Low Glucose-Promoted Cell Proliferation

To explore the molecular mechanism of low glucose-promoted cell proliferation, we studied AMP-activated protein kinase

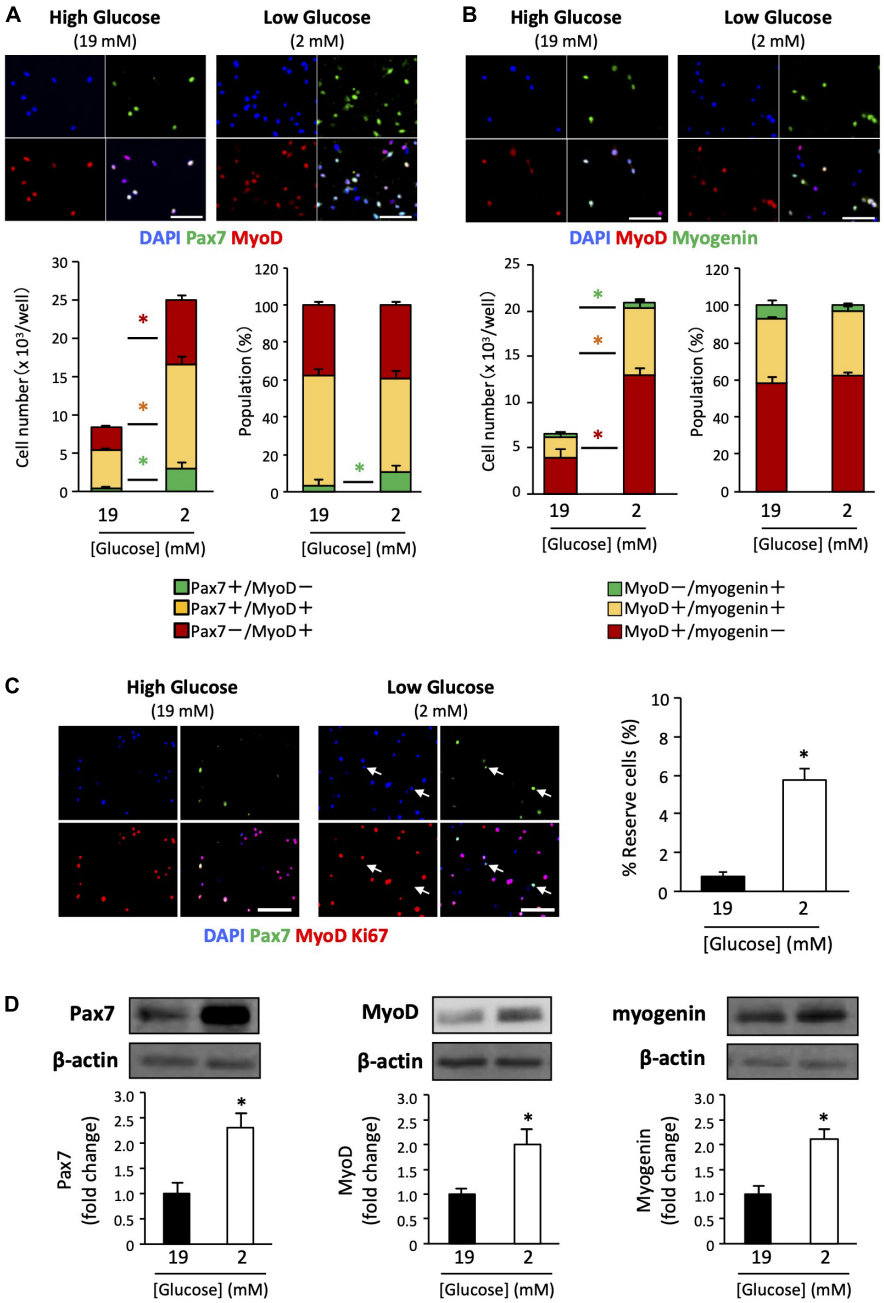

FIGURE 2 | Expression patterns of myogenic factors in satellite cells derived from EDL under low- and high-glucose media. **(A)** Satellite cells stained with Pax7 and MyoD antibodies after 6 days of cultivation. Scale bars are 100 μm. Quantified data are expressed as absolute and relative values. Population of self-renewed cells is indicated by Pax7+/MyoD-. Values are presented as mean ± SEM (n = 6). *p < 0.05. **(B)** Satellite cells stained with MyoD and myogenin antibodies after 6 days of cultivation. Scale bars are 100 μm. Values are presented as mean ± SEM (n = 6). *p < 0.05. **(C)** Reserve cell frequencies of primary myoblasts cultured in high- and low-glucose growth media. The cells were stained with Pax7, MyoD, and Ki67 antibodies after 6 days of cultivation. Reserve cells, identified as Pax7+/MyoD-/Ki67- cells, were indicated by arrows. Scale bars are 100 μm. The percentages of reserve cells were quantified, and higher in the low-glucose condition than in the high glucose condition. Values are presented as mean ± SEM (n = 4). *p < 0.05. **(D)** Western blot analysis of Pax7, MyoD, and myogenin protein expression levels in cells cultured for 6 days in high-and low-glucose media. The expression of all proteins was normalized to that of β-actin. Values are presented as mean ± SEM (n = 6). *p < 0.05.

(AMPK), a serine/threonine protein kinase that plays a role in several signaling pathways by sensing the intracellular energy state. Because low glucose potentially decreases energy level of the cells, AMPK is expected to be involved in acting as a mediator of energy state-induced cell proliferation regulation (Mounier et al., 2015). To examine whether activation of AMPK

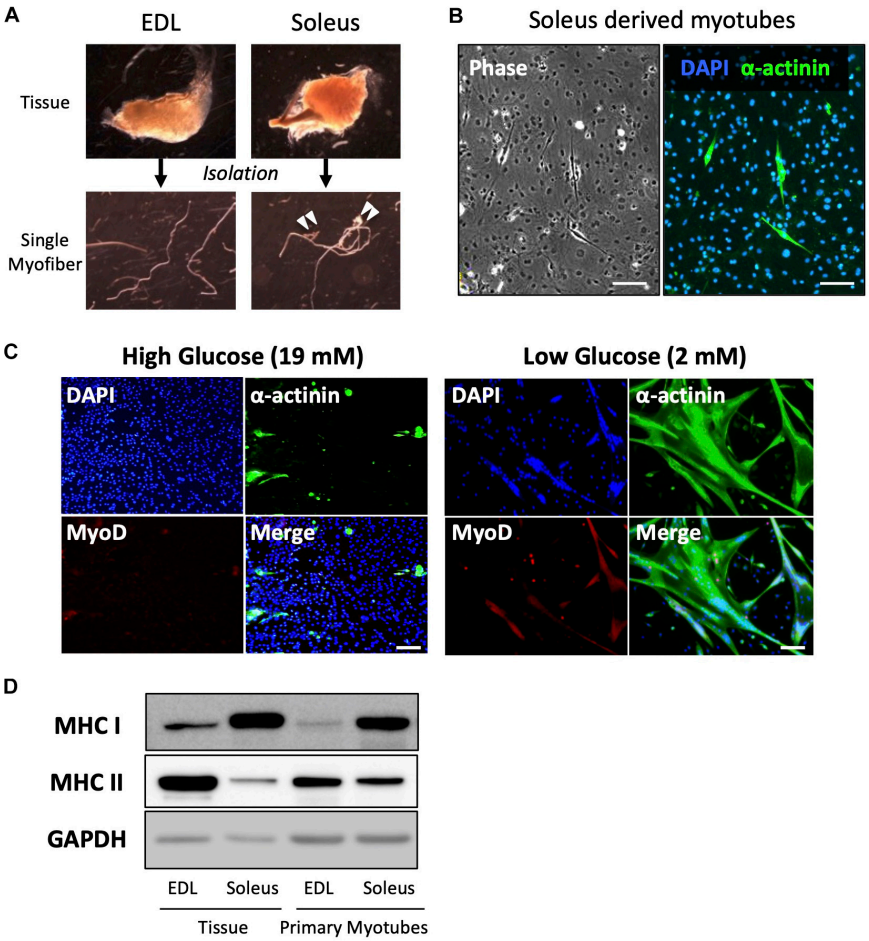

FIGURE 3 | Low-glucose medium enables the culturing of soleus-derived satellite cells in high purity. **(A)** Images of EDL and soleus tissues and single myofibers isolated from each tissue. Soleus muscle contains an Achilles tendon and connective tissues and cells (indicated by white arrowheads). **(B)** Images of phase-contrast (left) and immunofluorescence (right) of α-actinin (green) and DAPI (blue) in cultured satellite cells derived from soleus. Only a few muscle and α-actinin-positive cells were observed, and most of the cells visualized by DAPI were contaminated with non-muscle cells. Scale bars are 100 μm. **(C)** Immunofluorescence analysis of the purity of muscle cells derived from soleus satellite cells. In the standard high-glucose medium, most of the cells were contaminated with non-muscle cells because neither α-actinin nor MyoD (markers of myotubes and myoblasts, respectively) was expressed in the cells. On the contrary, most of the cells in the low-glucose medium expressed α-actinin, suggesting that muscle cells were purified by glucose restriction. Scale bars are 100 μm. **(D)** Western blot analysis of MHC I and II expression in cultured satellite cells isolated from EDL and soleus myofibers. GAPDH was used as house-keeping protein. The cells were differentiated for 5 days before western blotting was performed. The amounts of loaded protein derived from tissues and cell samples were 2.5 μg/lane and 10 μg/lane, respectively.

increases the proliferation of cells cultured in the high-glucose medium, we used the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). Primary cells cultured in the high-glucose medium were co-treated with 200 μM AICAR for 6 days. An increase in AMPK activation was confirmed by immunoblotting of the phosphorylation of threonine-172 in the AMPKα subunit, the major site of phosphorylation and activation of AMPK (**Figure 5A**). However, the cell number was not affected by the increased Phospho-AMPK (P-AMPK) (**Figure 5B**). In accordance with the abovementioned results, the P-AMPK level did not increase in the low-glucose medium (**Figure 5A**), in which cell proliferation was accelerated (**Figure 5B**). Taken together, these findings indicate that AMPK does not contribute to the increased cell proliferation activity under low glucose conditions.

DISCUSSION

Since glucose is a major energy source for cell proliferation, many cell types, such as cancer cells, benefit from using a sufficient amount of glucose (Wolf et al., 1992; Han et al., 2011; Jones and Schulze, 2012). Our finding that glucose restriction increases the proliferation of cultured muscle cells revolutionizes the existing concepts. DMEM containing 5 mM glucose is often used as a basic medium for culturing muscle cells (Elkalaf et al., 2013). However, in this study, we found that glucose-free DMEM (0 mM) improved the proliferation of satellite cells. Furthermore, we prepared glucose-depleted FBS to lower the glucose concentration in the medium, leading to a barely detectable glucose concentration (0.1 mM) in the culture medium. Although experiments to knock out glucose

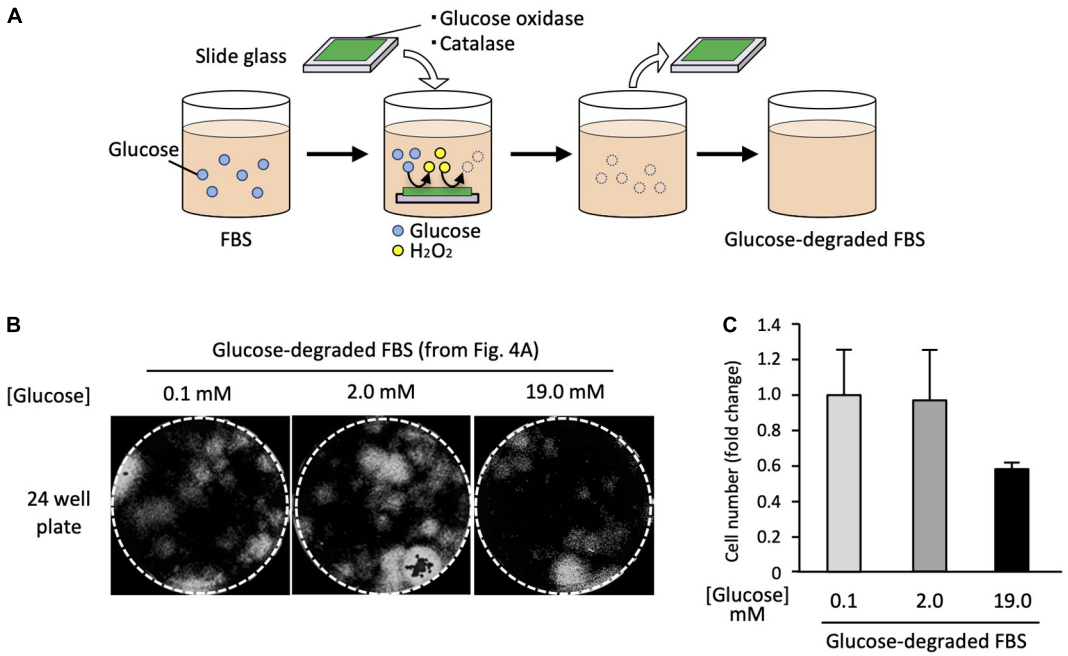

FIGURE 4 | Satellite cells are able to grow in glucose-depleted medium. **(A)** Scheme of the enzymatic glucose degradation in FBS. To deplete the glucose included in FBS, GOD and catalase were immobilized on a glass slide and incubated with FBS. After incubation, enzymes immobilized slide glasses were removed. **(B)** Analysis of cell proliferation at different glucose concentrations. EDL derived from satellite cells were cultured in each different growth medium for 6 days, stained with DAPI, and analyzed by microscopy. **(C)** Cell numbers (derivation shown in **Figure 4B**) at different glucose concentrations. Values are presented as mean ± SEM (n = 2–4).

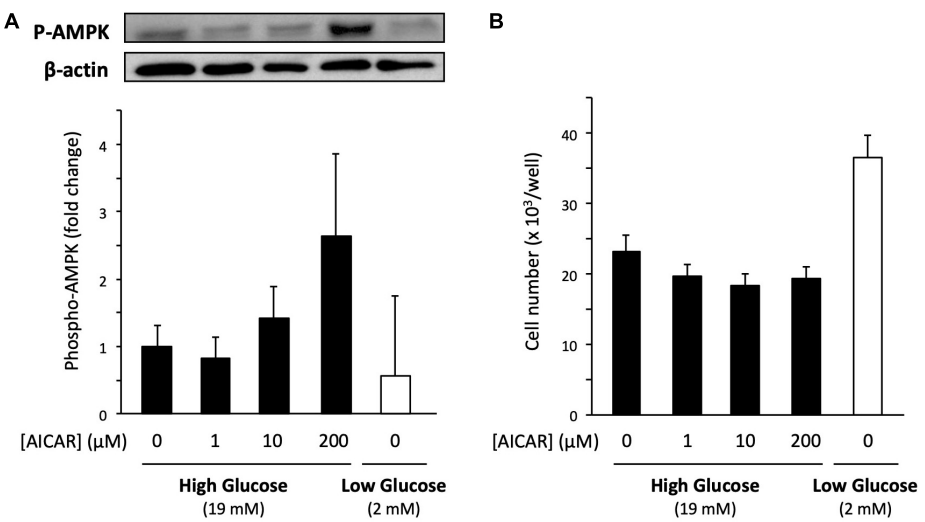

FIGURE 5 | Effect of AMPK on the proliferation of primary satellite cells in low- and high-glucose media. **(A)** Amount of phosphorylated AMPK (P-AMPK) activated by AICAR in primary satellite cells derived from EDL cultured in the high-glucose medium for 6 days. The amount of P-AMPK in cells cultured in the low-glucose medium is also shown. P-AMPK was normalized to the expression of β-actin. Values are presented as mean ± SEM (n = 3). **(B)** Cell numbers in low- and high-glucose media after 6 days of cultivation. The cell number increased in low-glucose medium without AICAR administration. Values are presented as the mean ± SEM (n = 4–5).

transporters (Glut1/4) are necessary to conclude that glucose governs cell proliferation, satellite cells were able to proliferate normally even under very low glucose conditions. Since FBS itself was found to be essential for cell survival, amino acids, lipids, and lactate may be alternative energy sources for cells.

The glucose concentration in standard high-glucose growth media seems to be physiologically too high compared to the blood glucose level in humans. The normal blood sugar level in fasting non-diabetics is under 6.1 mM (110 mg/dL) (American Diabetes Association, 2003), whereas the glucose concentration

in the present high-glucose medium was 19 mM (342 mg/dL) and equivalent to the blood sugar level in patients with severe diabetes. In accordance with the physiological glucose levels, our *in vitro* experiments suggested that satellite cell proliferation was inhibited at glucose concentrations above 8 mM under adherent culture conditions; however, this effect was not observed in suspension culture. Diabetes mellitus is recognized as a risk factor for age-related muscle atrophy (sarcopenia) (Buford et al., 2010). Recent study showed that high glucose induces muscle atrophy via transcription factor KLF15 which act on differentiated myofibers (Hirata et al., 2019). In addition, our finding that high glucose levels inhibited cell proliferation and self-renewal gave us the idea that accelerated muscle atrophy in patients with diabetes is caused by satellite cell dysfunction due to hyperglycemia. Since blood glucose increases with age, independent of the development of diabetes (Basu et al., 2003; Oh et al., 2016), glucose-induced satellite cell impairment possibly causes sarcopenia.

Metabolic disorders, such as obesity and type two diabetes, are associated with reduced AMPK activity in skeletal muscles (Guan et al., 2016). Therefore, AMPK has been focused on as a potential factor to control muscle regeneration and mass in people with metabolic disorders. Previous studies reported that the satellite cell-specific deletion of AMPK reduced the proliferation and myogenic capacity of satellite cells during muscle regeneration (Fu et al., 2016; Theret et al., 2017), leading us to hypothesize that AMPK regulates the proliferation activity of satellite cells in response to glucose concentration. However, AMPK is probably not involved in the mechanism underlying the promotion of cell proliferation under limited glucose conditions because the level of phosphorylation of AMPK did not change and the activation of AMPK by AICAR did not enhance cell proliferation (**Figure 5**).

Although glucose restriction increased the number of cells in every population expressed Pax7, MyoD, and myogenin, the relative percentage of reserve cells, which were identified as Pax7+/MyoD-cells, was higher in low glucose medium. This means that lowering glucose concentration enhanced self-renewal of cultured satellite cells and delayed the differentiation, leading the maintenance of cell proliferation capacity. We also observed that Pax7 expression level was higher under the low glucose condition compared to high glucose (**Figure 2C**). Because the protein amount quantified by western blotting was normalized by β-actin level, this result indicates that the protein expression per a cell was upregulated by glucose restriction. Pax7 expression levels was known to be quickly down-regulated during satellite cell activation (Machado et al., 2017). Enhancements of myogenic factor expressions imply that the specificities of the cultured satellite cells were increased by lowering glucose concentration. High glucose potentially alters the DNA methylation status of several important genes which could regulate the cell proliferation and stemness of neural progenitor cells (Kandilya et al., 2020). Also, the other report suggested that glucose is required for histone acetylation in proliferating satellite cells and the determination of myogenic differentiation potential (Yucel et al., 2019). At present it is not clear whether low glucose medium directly downregulates the histone acetylation level in the cultured cells, or whether other factors such as pyruvate dehydrogenase (PDH) regulates them.

Primary cultures of satellite cells have widely been used to study the myogenesis, metabolism, myokine secretion, and contractile capacity of muscle cells (Lecompte et al., 2017; Sanchez et al., 2018; Chen et al., 2019). However, a disadvantage of satellite cells is their low proliferation activity compared to that of other muscle cell lines, such as C2C12 and L6; therefore, it takes effort to obtain a sufficient number of satellite cells for experimental use. We found that a low glucose concentration promotes the proliferation of satellite cells and may therefore overcome the abovementioned problem. Furthermore, cells cultured in the low-glucose medium could be passaged and frozen, indicating that this medium is valuable to experimenters. In particular, we found that contaminated other cells that extrude satellite cells can be eliminated in low-glucose media to obtain a pure satellite cell population, even from the soleus, a difficulty type of muscle for isolating satellite cells.

In summary, we showed here, that satellite cell proliferation is promoted by glucose restriction under adherent culture conditions. This finding suggests that a supra-physiological concentration of glucose directly inhibits the regeneration of skeletal muscles and facilitates sarcopenia in diabetes mellitus. More research is required to further elucidate the mechanism by which glucose restriction regulates satellite cell proliferation; identification and investigation of molecules that act as glucose sensors may be useful for this purpose.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental Animal Care and Use Committee of Tokyo Metropolitan University.

AUTHOR CONTRIBUTIONS

YF, YK, MA, and YMi performed and analyzed the experiments. YF, YMa, and NF designed the experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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with the study: Effects of glucose concentration on myoblast differentiation (**Supplementary Figure 7**). This work was also supported by the Uehara Memorial Foundation (awarded to YF).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.640399/full#supplementary-material

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