

# Pluripotent stem cell energy metabolism: an update

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## Abstract

Recent studies link changes in energy metabolism with the fate of pluripotent stem cells (PSCs). Safe use of PSC derivatives in regenerative medicine requires an enhanced understanding and control of factors that optimize *in vitro* reprogramming and differentiation protocols. Relative shifts in metabolism from naïve through “primed” pluripotent states to lineage-directed differentiation place variable demands on mitochondrial biogenesis and function for cell types with distinct energetic and biosynthetic requirements. In this context, mitochondrial respiration, network dynamics, TCA cycle function, and turnover all have the potential to influence reprogramming and differentiation outcomes. Shifts in cellular metabolism affect enzymes that control epigenetic configuration, which impacts chromatin reorganization and gene expression changes during reprogramming and differentiation. Induced PSCs (iPSCs) may have utility for modeling metabolic diseases caused by mutations in mitochondrial DNA, for which few disease models exist. Here, we explore key features of PSC energy metabolism research in mice and man and the impact this work is starting to have on our understanding of early development, disease modeling, and potential therapeutic applications.

**Keywords** differentiation; epigenetics; metabolism; mitochondria; pluripotency

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See the Glossary for abbreviations used in this article.

## Introduction

Energy production in early mammalian development depends upon many factors, including substrate availability, uptake, and O<sub>2</sub> tension. All mammalian cells produce ATP by differing proportions of glycolysis and oxidative phosphorylation (OXPHOS), with the

balance between these processes at specific developmental stages or states of cellular activation controlled by multiple intra- and extra-cellular factors. Glycolysis is the enzymatic conversion of glucose to pyruvate, which generates 2 net ATP molecules per molecule of glucose. Cells that depend mainly on glycolysis for ATP production further convert pyruvate to lactate, which is excreted. By contrast, cells in oxygen-rich environments may prefer OXPHOS for more efficient ATP production, which on average nets 34 additional ATP molecules per glucose by oxidizing pyruvate to acetyl-CoA in the mitochondrial tricarboxylic acid (TCA) cycle. During pre-implantation development of early mouse embryos, ATP is produced mainly by OXPHOS from uptake of pyruvate, lactate, amino acids, and triglyceride-derived fatty acids (Brinster & Troike, 1979; Martin & Leese, 1995; Jansen *et al.*, 2008; Leese, 2012). This is followed by a shift to a more balanced mixture of glycolysis and OXPHOS with increasing glucose uptake in the low O<sub>2</sub> microenvironment of an implanting blastocyst (Leese & Barton, 1984; Houghton *et al.*, 1996; Zhou *et al.*, 2012). *In vitro* studies report a similar increase in glucose uptake in early human embryos advancing to the blastocyst stage in a dish (Gardner *et al.*, 2001). Pyruvate and glucose uptake and amino acid turnover are predictors of human blastocyst quality and enhanced viability for *in vitro* fertilization protocols (Houghton *et al.*, 2002; Brison *et al.*, 2004). In concept, *in vivo* differences in early mammalian embryo energy metabolism should be replicated *in vitro* by cells obtained from distinct stages of embryonic development that are maintained in similar culture conditions.

Human embryonic stem cells (hESCs) originate from the blastocyst inner cell mass and hold great clinical potential for cell replacement therapies because of their high proliferative capacity and their ability to differentiate into any cell type in the body (Thomson *et al.*, 1998). However, the clinical use of differentiated hESCs is limited by ethical concerns regarding the method of hESC acquisition and by potential allogeneic immune rejection (Zhao *et al.*, 2011). To help circumvent these issues, mammalian somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) through ectopic expression of different combinations of transcription factors, such as the “Yamanaka cocktail” of POU5F1, SOX2, KLF4, and MYC (Takahashi & Yamanaka, 2006; Takahashi *et al.*, 2007) or by other

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## Glossary

<b>5hmc</b>	5-hydroxymethylcytosine	<b>MET</b>	mesenchymal-to-epithelial transition
<b>5mC</b>	5-methylcytosine	<b>Mfn1</b>	mitofusin-1
<b>ADP</b>	adenosine diphosphate	<b>Mfn2</b>	mitofusin-2
<b>AMD1</b>	adenosylmethionine decarboxylase 1	<b>miPSCs</b>	mouse-induced pluripotent stem cells
<b>AMP</b>	adenosine monophosphate	<b>MOMP</b>	mitochondrial outer membrane permeabilization
<b>AMPK</b>	AMP-activated protein kinase	<b>mPSCs</b>	mouse pluripotent stem cells
<b>ARNT</b>	aryl hydrocarbon receptor nuclear translocator	<b>mPTP</b>	mitochondrial permeability transition pore
<b>ATP</b>	adenosine triphosphate	<b>mtDNA</b>	mitochondrial DNA
<b>Cited2</b>	CREB-binding protein (CBP)/p300-interacting transactivator with glutamic acid and aspartic acid tail 2	<b>mTOR</b>	mammalian target of rapamycin
<b>DEPTOR</b>	DEP domain-containing mTOR-interacting protein	<b>mTORC1</b>	mammalian target of rapamycin complex 1
<b>Drp1</b>	dynamamin-related protein 1	<b>mTORC2</b>	mammalian target of rapamycin complex 2
<b>EBs</b>	embryoid bodies	<b>NAD</b>	nicotinamide adenine dinucleotide
<b>ETC</b>	electron transport chain	<b>NPCs</b>	neural progenitor cells
<b>FAD</b>	flavin adenosyl dinucleotide	<b>NuRD</b>	nucleosome remodeling and deacetylase
<b>FAO</b>	fatty acid oxidation	<b>OCT4</b>	octamer-binding protein 4
<b>FBS</b>	fetal bovine serum	<b>OPA1</b>	optic atrophy 1
<b>HAT</b>	histone acetyltransferase	<b>OXPHOS</b>	oxidative phosphorylation
<b>hESCs</b>	human embryonic stem cells	<b>PC</b>	pyruvate carboxylase
<b>HIF1<math>\alpha</math></b>	hypoxia-inducible factor 1 $\alpha$	<b>PDH</b>	pyruvate dehydrogenase
<b>HIF1<math>\beta</math></b>	hypoxia-inducible factor 1 $\beta$	<b>PKD1</b>	pyruvate dehydrogenase kinase 1
<b>HIF2<math>\alpha</math></b>	hypoxia-inducible factor 2 $\alpha$	<b>PKD3</b>	pyruvate dehydrogenase kinase 3
<b>hiPSCs</b>	human-induced pluripotent stem cells	<b>Phb2</b>	prohibitin 2
<b>hLIF</b>	human leukemia inhibitory factor	<b>PHD</b>	prolyl hydroxylase
<b>HMT</b>	histone methyltransferase	<b>POU5F1</b>	POU class 5 homeobox 1
<b>hPSCs</b>	human pluripotent stem cells	<b>PSCs</b>	pluripotent stem cells
<b>IMS</b>	intermembrane space	<b>pVHL</b>	von Hippel-Lindau tumor suppressor protein
<b>iPSCs</b>	induced pluripotent stem cells	<b>PYGL</b>	glycogen phosphorylase liver
<b>JmjC</b>	Jumonji domain-containing	<b>REX1</b>	reduced expression 1
<b>Jph2</b>	junctionophilin 2	<b>ROS</b>	reactive oxygen species
<b>KLF4</b>	Kruppel-like factor 4	<b>SAM</b>	s-adenosyl methionine
<b>LKB1</b>	liver kinase B1	<b>SOX2</b>	SRY (sex-determining region Y)-box 2
<b>LSD1</b>	lysine-specific demethylase 1	<b>TCA cycle</b>	tricarboxylic acid cycle
<b>MEFs</b>	mouse embryonic fibroblasts	<b>TDH</b>	threonine dehydrogenase
<b>mEpiSCs</b>	mouse epiblast stem cells	<b>Tet1</b>	ten-eleven translocation
<b>mESCs</b>	mouse embryonic stem cells	<b>Tsc2</b>	tuberous sclerosis 2
		<b>UCP2</b>	uncoupling protein 2

methods (Yu *et al*, 2007; Huangfu *et al*, 2008; Lowry *et al*, 2008; Ichida *et al*, 2009; Kim *et al*, 2009; Lin *et al*, 2009; Lyssiotis *et al*, 2009; Zhou *et al*, 2009a; Jia *et al*, 2010; Warren *et al*, 2010; Zhu *et al*, 2010; Anokye-Danso *et al*, 2011; Hu *et al*, 2011; Miyoshi *et al*, 2011; Bayart & Cohen-Haguenaer, 2013; Hou *et al*, 2013; Sommer & Mostoslavsky, 2013). Both hESCs and human iPSCs (hiPSCs) are markedly glycolytic, secreting abundant lactate, in ambient (~160 mm Hg) O<sub>2</sub> (Zhang *et al*, 2011; Zhou *et al*, 2012), which differs substantially from the ~40 mm Hg O<sub>2</sub> partial pressure measured for several mammalian reproductive tracts (Fischer & Bavister, 1993). A similar glycolytic preference in different O<sub>2</sub> environments at first pass suggests a pluripotent stage-specific metabolic program that is relatively insensitive to O<sub>2</sub> levels in chemically defined or undefined culture media. However, hESCs replicate well in 1–5% O<sub>2</sub> and resist spontaneous differentiation compared to culture in 21% O<sub>2</sub>, suggesting that O<sub>2</sub> levels influence the factors that maintain pluripotency (Ezashi *et al*, 2005). Somatic cell reprogramming to hiPSCs or mouse iPSCs (miPSCs) requires a shift from mainly OXPHOS to mainly glycolytic metabolism and high levels of lactate production (Yoshida *et al*, 2009; Zhou *et al*, 2012). iPSC production efficiency is enhanced by performing reprogramming in hypoxia or inducing a shift to glycolysis during this process, indicating a role for metabolism in controlling and not just passively responding to de-differentiation (Yoshida *et al*, 2009; Zhu *et al*,

2010; Jung *et al*, 2013). In fact, a shift to glycolysis may occur early in reprogramming before self-renewal and pluripotent gene expression (Folmes Clifford *et al*, 2011; Mathieu *et al*, 2014; Prigione *et al*, 2014). Interestingly, glycolysis-skewed pluripotent stem cells (PSCs), which include ESCs and iPSCs, resemble many cancer cell types that revert to “Warburg metabolism” (aka “aerobic glycolysis”) upon malignant transformation and coupled cellular de-differentiation (Warburg, 1956; Christofk *et al*, 2008; Figueroa *et al*, 2010; Lu *et al*, 2012; Ward Patrick & Thompson Craig, 2012).

OXPHOS is low in hPSCs, which includes both hESCs and hiPSCs, and the mitochondria are perinuclear and less fused into a filamentous network structure with swollen, less mature appearing inner membrane cristae folds than mitochondria in terminally differentiated cell types (Oh *et al*, 2005; St John *et al*, 2005, 2006; Houghton, 2006; Suhr *et al*, 2010; Zeuschner *et al*, 2010; Folmes Clifford *et al*, 2011; Zhang *et al*, 2011). The perinuclear arrangement of mitochondria has also been noted in cleavage stage embryos of several mammalian species including mice and humans and has been suggested as a “stemness” property (Batten *et al*, 1987; Barnett *et al*, 1996; Wilding *et al*, 2001; Squirrel *et al*, 2003; Lonergan *et al*, 2006, 2007). Mouse ESCs (mESCs), which like hESCs are obtained from the blastocyst inner cell mass, contain mitochondria that display even less mature morphological and ultrastructural features than hPSCs (Folmes Clifford *et al*, 2011; Zhou *et al*, 2012).

However, hPSCs metabolically resemble developmentally more mature, glycolytic mouse epiblast stem cells (mEpiSCs), obtained from the post-implantation epiblast, instead of mESCs, which show a bivalent metabolism that can switch between glycolysis and OXPHOS on demand (Zhou *et al*, 2012). This metabolic comparison is consistent with biomarker and functional features of standard laboratory hPSCs that are “primed”, or more mature, than naïve, or ground state hPSCs. Naïve hPSCs, similar to mESCs that represent the least mature pluripotent stage, have recently been obtained by hPSC exposure to chemical inhibitor and growth factor cocktails or by transient expression of two transcription factors combined with two chemical inhibitors and human leukemia inhibitory factor (Fig 1) (Gafni *et al*, 2013; Takashima *et al*, 2014; Theunissen Thorold *et al*, 2014; Ware *et al*, 2014). hPSCs reset to a naïve state through transient ectopic expression of *NANOG* and *KLF4* respire at a higher level than “primed” hPSCs, similar to pre-implantation mouse embryos and naïve mESCs (Fig 1) (Takashima *et al*, 2014). The regulation of energy metabolism therefore appears intertwined with genetic and epigenetic mechanisms that control PSC maturation state through pathways that require further elucidation.

### Metabolic regulation of self-renewal, reprogramming, and differentiation

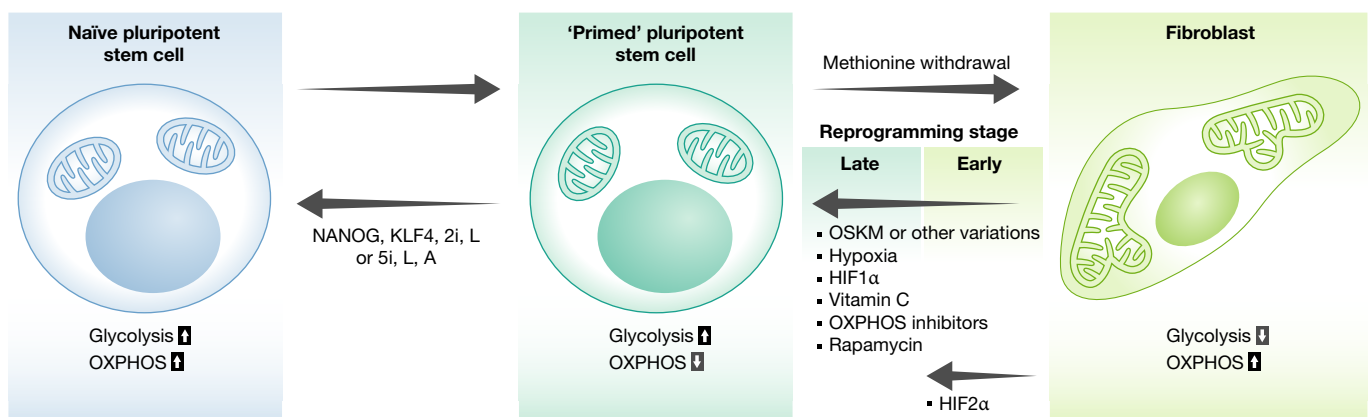
Reprogrammed iPSCs maintain an “epigenetic memory” or chromatin signature of the cells from which they were generated that can impact their re-differentiation potential and function (Kim *et al*, 2010, 2011; Bar-Nur *et al*, 2011). Changes in cellular metabolism can impact the activity of epigenome-modifying enzymes, as discussed below (Kaelin William & McKnight Steven, 2013). Therefore, manipulation of culture conditions could erase or generate new epigenetic marks during iPSC reprogramming, PSC differentiation, or steady-state growth that will affect the functional potential

of the end resulting cell. For therapeutic utility, identifying specific, reproducible, and chemically defined culture conditions to produce safe and functional differentiated cells from hPSCs, or by transdifferentiation protocols, will be required. Several key cell types targeted for cell replacement therapies have high energy demands, such as cardiomyocytes and neurons. Therapeutic applications will therefore require re-establishment of a cell type-specific, fully functional mitochondrial network to support the energy and other mitochondrial supplied factors for these replacement cell types. Importantly, mitochondrial dysfunction due to impaired nucleus and mitochondrial encoded genes has been linked to > 400 named human diseases, including multiple neurodegenerative disorders and cancer (Nunnari & Suomalainen, 2012).

The discovery that hypoxia maintains self-renewal and increases the efficiency of reprogramming to pluripotency has stimulated studies to determine the role of oxygen tension in cell fate determination. A striking difference in mitochondrial morphology between PSCs and their differentiated derivatives has similarly spurred studies to decipher the mechanisms that control cell state-specific mitochondrial structure and function. Adding to this complexity are state-specific levels of cellular metabolites, such as the AMP/ATP ratio and amino acid availabilities, which can impact PSC gene expression and cell function. In the first part of this review, the effect that these components of cellular metabolism have on self-renewal and differentiation is explored.

### Oxygen tension and hypoxia-inducible factors (HIFs)

Reduced O<sub>2</sub> (1–5%) can be used for hPSC tissue culture to mimic the hypoxic early embryonic microenvironment *in vivo*. Transcription factors such as hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) and 2 $\alpha$  (HIF2 $\alpha$ ) control the genomic response to low O<sub>2</sub> tension by promoting the expression of genes such as *pyruvate dehydrogenase kinase 1*



**Figure 1. Influence of energy metabolism on pluripotent status.**

Naïve human pluripotent stem cells (hPSCs) show an increase in ATP production through oxidative phosphorylation (OXPHOS) compared to more mature, “primed” hPSCs. Primed hPSCs can be converted to the naïve state through ectopic expression of *NANOG* and *KLF4*, inhibition of the ERK pathway by two inhibitors (2i), and stimulation with human leukemia inhibitory factor (L) (Takashima *et al*, 2014). Alternatively, the naïve state can be induced with a cocktail of five inhibitors and growth factors Activin and hLIF (5i/L/A) (Theunissen Thorold *et al*, 2014). Somatic cells can be reprogrammed with OCT4, SOX2, *KLF4*, and c-MYC (OSKM). Fibroblasts are more oxidative than primed hPSCs. Factors that activate glycolysis and inhibit OXPHOS promote induced PSC (iPSC) reprogramming. Vitamin C enhances iPSC reprogramming as an antioxidant and as a cofactor for epigenetic enzymes. Rapamycin, an inhibitor of the mTOR pathway, also increases the efficiency of iPSC reprogramming. Withdrawal of methionine from hPSC culture, which is required to maintain DNA and histone methylation, promotes differentiation.

(PDK1), lactate dehydrogenase A (LDHA), and glycogen phosphorylase liver (PYGL), which encode for key glycolysis regulating enzymes (Greer *et al*, 2012; Zhou *et al*, 2012). HIF1 $\alpha$  and HIF2 $\alpha$  are degraded in ambient air (~21% O<sub>2</sub> at sea level) by hydroxylation and ubiquitination from O<sub>2</sub>-dependent prolyl hydroxylases (PHDs) and the von Hippel-Lindau tumor suppressor protein (pVHL), respectively (Maxwell *et al*, 1999; Ohh *et al*, 2000; Jaakkola *et al*, 2001). In hypoxia, HIF1 $\alpha$  and HIF2 $\alpha$  are stabilized and form a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT; aka HIF1 $\beta$ ). HIF heterodimers accumulate in the nucleus and bind to promoters of genes that regulate cellular adaptation to hypoxia, stimulating their transcription (Wang *et al*, 1995). As noted above, hypoxia inhibits the spontaneous differentiation of hESCs and also increases the efficiency of iPSC reprogramming (Ezashi *et al*, 2005; Yoshida *et al*, 2009). HIF2 $\alpha$ -dependent transactivation of *Oct4* gene expression promotes self-renewal and the maintenance of pluripotency in hypoxia (Niwa *et al*, 2000; Covelto *et al*, 2006). HIF1 $\alpha$  stabilization promotes a metabolic shift to increased glycolysis and lactate production during the transition from mESCs to mEpiSCs. Ectopic expression of a non-degradable form of HIF1 $\alpha$  in mESCs is sufficient to induce a mEpiSC-like phenotype with a decrease in OXPHOS and an increase in glycolysis, indicating the importance of HIF transcription factors in early embryonic development (Zhou *et al*, 2012).

Hypoxia causes the re-entry of lineage-committed progenitor cells derived from hESCs back into pluripotency. hESCs transiently induced to lineage non-specific differentiation by fetal bovine serum (FBS) addition instead de-differentiate in 2% O<sub>2</sub>, whereas hESCs differentiated with FBS in air (~21% O<sub>2</sub>) proceed ahead. As expected, differentiated hESCs shift their metabolic balance from mainly glycolysis to OXPHOS, whereas de-differentiated hESCs remain glycolytic. De-differentiated hESCs are enriched for HIF1 $\alpha$  and HIF2 $\alpha$  target gene expression, suggesting a role for HIFs in promoting re-entry into the pluripotent state (Mathieu *et al*, 2013).

HIF2 $\alpha$  is required early in iPSC reprogramming for shifting from mainly OXPHOS to lactate-producing glycolysis, but stabilization of HIF2 $\alpha$  beyond day 12 of reprogramming is detrimental (Fig 1). Ectopic expression of sequence-stabilized HIF1 $\alpha$  and/or HIF2 $\alpha$  is sufficient to impair OXPHOS in fibroblasts (Mathieu *et al*, 2014). HIF1 $\alpha$  improves iPSC reprogramming efficiency by increasing glycolysis and lactate production through activation of target genes *PDK1*, *pyruvate dehydrogenase kinase 3 (PDK3)*, and *pyruvate kinase isoform M2 (PKM2)* (Fig 1) (Mathieu *et al*, 2014; Prigione *et al*, 2014). CREB-binding protein (CBP)/p300-interacting transactivator with glutamic acid and aspartic acid tail 2 (Cited2) is a HIF1 $\alpha$  antagonist. Cited2 is expressed and inhibits HIF1 $\alpha$  during lineage non-specific mESC differentiation, with Cited2 knockout mESCs unable to silence *Oct4* or activate differentiation-related genes. shRNA knockdown of *HIF1 $\alpha$*  in *Cited2*-deficient mESCs partially rescues this defect in lineage non-specific differentiation (Li *et al*, 2014).

HIF transactivation also regulates the lineage-specific differentiation of human neural progenitor cells (NPCs) (Xie *et al*, 2014). Neurons are more oxidative than glial cells (Kasischke *et al*, 2004; Bélanger *et al*, 2011). NPCs generated by changing the hPSC culture media to enable the formation of rosette structures can develop into neurons or glia by directed NPC differentiation. NPCs derived from hESC differentiation are mainly glycolytic (Birket *et al*, 2011), and

proteomic comparisons of hESCs at different stages of neural lineage differentiation show differential expression of enzymes that regulate redox homeostasis (Fathi *et al*, 2014). The differentiation of mixed lineage embryoid bodies (EBs) in 2% O<sub>2</sub> also promotes neurogenesis. Most cells differentiated from hPSC-derived NPCs are neurons with a concomitant small number of glial cells. Remarkably, just shifting the O<sub>2</sub> environment during NPC differentiation from 21 to 2% O<sub>2</sub> strongly shifts the culture toward gliogenesis and away from neurogenesis. This effect can be replicated with HIF stabilizing deferoxamine in ambient O<sub>2</sub> as well. HIF1 $\alpha$  promotes gliogenesis through the inhibition of LIN28a by displacement of MYC on the LIN28 promoter. Remarkably, even a transient low O<sub>2</sub> period during NPC differentiation skews the resulting culture strongly toward gliogenesis, suggesting that the O<sub>2</sub>-sensing machinery induces a lasting effect on NPC differentiation potential (Xie *et al*, 2014).

### Mitochondria and the electron transport chain

Mammalian cells consume glucose and convert it to pyruvate with ATP production in several enzymatic steps during glycolysis (Teslaa & Teitell, 2014). Pyruvate in turn can be converted to lactate by LDH and will be excreted from the cell. Alternatively, pyruvate can enter mitochondria as acetyl-CoA, through the action of pyruvate dehydrogenase (PDH), or as oxaloacetate, via pyruvate carboxylase (PC), to generate CO<sub>2</sub> and additional ATP through OXPHOS. Pyruvate that enters the TCA cycle regenerates NADH and FADH<sub>2</sub>, which subsequently donate electrons to the electron transport chain (ETC) and establish a hydrogen ion gradient, which is used by the F<sub>0</sub>F<sub>1</sub> ATP synthase to make ATP from ADP plus inorganic phosphate. ETC activity therefore depends on the ADP/ATP ratio as well as the levels of environmental and internal resources that include TCA cycle carbon substrates and electron acceptors. The levels and functional assemblies of nucleus and mitochondrial DNA (mtDNA) encoded ETC subunits that comprise ETC complexes I through V, excluding nucleus-encoded complex II, and their assemblies into higher order supercomplexes, along with mitochondrial network fusion/fission status, further determines the minimal and maximal respiratory potential of most cells, which remains to be established for PSCs. The complexity of ETC complex regulation has been shown in other systems, including the differential expression of subunits in complex IV and the assembly of complexes I, III, and IV into supercomplexes (Fukuda *et al*, 2007; Chen *et al*, 2012b; Ikeda *et al*, 2013; Lapuente-Brun *et al*, 2013).

Low-level respiration and activity of the ETC in primed PSCs, including hPSCs and mEpiSCs, may at least partially result from the hypoxic microenvironment *in vivo*, immediately post-implantation. In addition, the donation of electrons from NADH and FADH<sub>2</sub> to the ETC may result in oxidative stress through formation of reactive oxygen species (ROS). Primed PSCs may limit ROS to prevent damage to proteins, lipids, and importantly DNA within the cell. However, steady-state ROS levels also increase with PSC differentiation and can help drive differentiation at later stages of precursor cell development (Cho *et al*, 2006; Saretzki *et al*, 2008). The addition of antioxidants to the culture medium of hiPSCs enhances their genomic stability, consistent with a benefit for maintaining low ROS in PSCs (Luo *et al*, 2014). Vitamin C, an antioxidant, also enhances the efficiency of iPSC reprogramming (Esteban *et al*, 2010),



although vitamin C may also impact reprogramming efficiency through epigenetic mechanisms described below.

ATP and ROS production by OXPHOS is further limited by several mechanisms in hPSCs, such as by the expression of uncoupling protein 2 (UCP2) (Zhang *et al*, 2011). UCP2 transports four carbon TCA cycle intermediates out of the mitochondria, effectively reducing carbon substrates for use in OXPHOS (Voza *et al*, 2014). Also, nuclear genes encoding multiple subunits of cytochrome C oxidase (complex IV of the ETC), which donates electrons to O<sub>2</sub>, are expressed at a lower level in mEpiSCs compared to mESCs (Zhou *et al*, 2012). DMSO-induced differentiation of mPSCs increases ETC complex I and complex IV activities along with mitochondrial biogenesis to support an increase in mitochondrial ATP production (Han *et al*, 2014). The ETC maintains the mitochondrial inner membrane electrochemical potential,  $\Delta\psi$ , which is required to prevent mitochondrial outer membrane permeabilization (MOMP) and the release of proapoptotic intermembrane space (IMS) proteins, such as cytochrome *c*, that induce apoptosis (Green & Kroemer, 2004). When ETC activity is low,  $\Delta\psi$  can be additionally supported by the hydrolysis of ATP in the complex V ATP synthase, which results in the translocation of protons from the mitochondrial matrix to the IMS to increase  $\Delta\psi$  (Hatefi, 1985). hPSCs have relatively low respiration and ETC activity; therefore, ATP hydrolase activity of the ATP synthase helps to maintain  $\Delta\psi$  and sustain cell viability (Zhang *et al*, 2011). Interestingly, hPSCs maintain a higher  $\Delta\psi$  than their differentiated derivatives (Chung *et al*, 2007; Armstrong *et al*, 2010; Prigione *et al*, 2011), which has been proposed to enable rapid metabolic changes during differentiation (Folmes Clifford *et al*, 2012b; Folmes *et al*, 2012a) and possibly to maintain a fragmented mitochondrial network (Mattenberger *et al*, 2003).

iPSC reprogramming of mouse embryonic fibroblasts (MEFs) causes major changes in the expressed proteome in two stages. ETC complex I and complex IV proteins are reduced early during reprogramming, in contrast to components of ETC complexes II, III, and V, which are transiently induced during a second, intermediate reprogramming phase (Hansson *et al*, 2012). The efficiency and speed of iPSC reprogramming is enhanced when OXPHOS is decreased by inhibition of any of the ETC respiratory complexes, consistent with a required shift toward glycolysis (Fig 1) (Son *et al*, 2013b).

An increase in OXPHOS capacity is required for proper cardiomyocyte lineage-directed differentiation from PSCs. Cardiomyocyte differentiation induces the expression of nucleus-encoded genes for mtDNA transcription factors, mtDNA replication factors, components of the fatty acid oxidation (FAO) machinery, enzymes of the TCA cycle, and ETC subunits (St John *et al*, 2005; Chung *et al*, 2007; Tohyama *et al*, 2013). Cardiomyocyte-directed differentiation is enhanced by the generation of ROS by NADPH oxidase-like enzymes (Sauer *et al*, 2000; Crespo *et al*, 2010). Agonists of peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), a highly expressed nuclear hormone receptor in the heart associated with FAO, promote cardiomyogenesis of mESCs through increasing ROS production (Sharifpanah *et al*, 2008).

Differences in carbon substrate types can be used to purify metabolically mature mouse cardiomyocytes following differentiation from mPSCs because of key differences in metabolite handling capacity between mouse cardiomyocytes and mPSCs. Fetal

cardiomyocytes preferentially consume lactate for the production of ATP (Fisher *et al*, 1981; Werner & Sicard, 1987). Therefore, cardiomyocytes derived *in vitro* from PSCs can utilize lactate in the absence of glucose to produce ATP, whereas mESCs and MEFs are unable to use lactate for ATP production. When cultured in glucose-free media supplemented with lactate, functional mouse cardiomyocytes can be recovered at ~99% purity (Tohyama *et al*, 2013).

## Mitochondrial dynamics

The dynamic fusion and fission/fragmentation of an interlacing mitochondrial network enables mixing of mitochondrial contents and the degradation of damaged mitochondria to maintain robust energy and metabolite production (Twig *et al*, 2008; Westermann, 2012). Mitochondrial network fusion status is a determinant of maximal respiratory capacity (Chen *et al*, 2005; Yu *et al*, 2006). PSCs show a punctate, fragmented mitochondrial network that progressively fuses during differentiation, which increases respiratory capacity (Zhang *et al*, 2011). The GTPase dynamin-related protein 1 (DRP1), which causes mitochondrial fission, can be inhibited to induce a fused mitochondrial network. Pharmacological inhibition of Drp1 to maintain a fused mitochondrial network inhibits iPSC reprogramming (Vazquez-Martin *et al*, 2012a), although shRNA knockdown of Drp1, also resulting in mitochondrial fusion, did not impair iPSC reprogramming of MEFs (Wang *et al*, 2014). These paradoxical results could be reconciled by off target effects of the Drp1 inhibitor, insufficient Drp1 shRNA knockdown, or a combination of these or other confounders. Interestingly, reduced expression 1 (REX1) is a zinc finger-containing protein that is required to maintain PSC self-renewal and is repressed during lineage non-specific retinoic acid-induced differentiation. REX1 expression also increases the expression of cyclin B1, which leads to the phosphorylation and activation of DRP1, fission of the mitochondrial network, and increased glycolytic metabolism that is characteristic of PSCs (Son *et al*, 2013a). The expression pattern of REX1 is concordant with DRP1 activation and mitochondrial fission associated with pluripotency.

Mitochondrial network fusion requires fusion of the outer mitochondrial membrane, mediated by mitofusin-1 and -2 (MFN1 and MFN2), and fusion of the inner mitochondrial membrane, mediated by optic atrophy 1 (OPA1) (Westermann, 2010). Mfn1, Mfn2, and Opa1 are all required for viable embryonic mouse development (Chen *et al*, 2003; Alavi *et al*, 2007). Opa1 also helps to remodel cristae folds of the inner mitochondrial membrane to help mitochondria adapt to changing metabolic demands (Patten *et al*, 2014). At least five different isoforms of the Opa1 protein exist due to differential splicing and proteolytic cleavage. Prohibitin 2 (Phb2), a nucleus-encoded mitochondrial protein, is expressed at high levels in mESCs and promotes expression of the long isoforms of Opa1. Ectopic expression of Phb2 in mESCs inhibits lineage-directed differentiation toward neurons and endoderm and causes mitochondrial swelling (Kowno *et al*, 2014).

Mfn2 and Opa1 are required for the differentiation of mESCs into beating cardiomyocytes (Kasahara *et al*, 2013), suggesting that shifting to OXPHOS during cardiomyogenesis also requires mitochondrial network fusion. Mfn2 also tethers mitochondria to the sarcoplasmic reticulum, which is required for Ca<sup>2+</sup> signaling and

energy metabolism in cardiomyocytes (Chen *et al*, 2012a). Junctophilin 2 (Jph2), which is also part of the junctional membrane complexes that physically link mitochondria with the sarcoplasmic reticulum, is required for proper mitochondria function,  $\text{Ca}^{2+}$  homeostasis, and the differentiation of mESCs into cardiomyocytes (Liang *et al*, 2012). Opening of the mitochondrial permeability transition pore (PTP) enables macromolecular diffusion across the mitochondrial inner membrane, which inhibits ATP production by OXPHOS (Hunter *et al*, 1976; Kim *et al*, 2003). Inhibition of the PTP promotes cardiomyocyte differentiation of mPSCs through increasing mitochondrial function (Hom Jennifer *et al*, 2011; Cho *et al*, 2014). Interestingly, antioxidant exposure during PTP inhibition synergistically enhances cardiomyogenesis by an unknown mechanism(s) (Cho *et al*, 2014).

### AMPK, mTOR, and autophagy

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a sensor of the AMP/ATP and ADP/ATP energy charge ratio in cells and coordinates the cellular response to changes in energy status. In response to increasing AMP/ATP and ADP/ATP ratios, AMPK becomes phosphorylated by liver kinase B1 (LKB1) to activate catabolic pathways that generate ATP and inhibit anabolic pathways that consume ATP (Hardie *et al*, 2012). Phosphorylated AMPK inhibits protein translation by inactivating the mammalian target of rapamycin (mTOR) signaling pathway. mTOR exists as two distinct protein complexes, complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1 is an amino acid sensor that regulates protein translation and autophagy. Autophagy is a process that degrades cytoplasmic macromolecules and organelles to provide substrates for energy production, or to remodel cellular functions with changes in differentiation or activation state, and provides a rapid cellular response to changing environmental conditions (Mizushima & Levine, 2010). Chemical activation of AMPK in mouse and human fibroblasts decreases iPSC reprogramming efficiency, potentially from a failure to fully induce *Oct4* gene expression (Vazquez-Martin *et al*, 2012b). mTOR activity decreases during iPSC reprogramming whereas rapamycin, an inhibitor of the mTOR pathway, enhances iPSC reprogramming of mouse fibroblasts (Fig 1) (Chen *et al*, 2011; He *et al*, 2012; Morita *et al*, 2013). Concordantly, hyperactivation of mTORC1 by knockout of tuberous sclerosis 2 (*Tsc2*), an upstream kinase inhibitor of mTOR, suppresses iPSC reprogramming (He *et al*, 2012). Inhibition of the mTOR pathway leads to activation of autophagy and enhances iPSC reprogramming efficiency, possibly from assisted cellular remodeling. Another inducer of autophagy, exposure to spermine, also enhances miPSC reprogramming efficiency (Chen *et al*, 2011). Moreover, the reprogramming transcription factor *Sox2* inhibits *mTOR* gene expression, which in turn activates autophagy during iPSC reprogramming. *Sox2*-induced *mTOR* gene repression occurs by recruitment of the nucleosome remodeling and deacetylase (NuRD) repressor complex to the *mTOR* gene promoter (Wang *et al*, 2013a). Although mTOR activation impairs iPSC reprogramming, its inhibition with rapamycin also disrupts expression of *OCT4*, *SOX2*, and *NANOG* genes in hESCs and promotes the expression of endoderm and mesoderm lineage differentiation genes (Zhou *et al*, 2009b). In contrast, DEP domain-containing mTOR (DEPTOR)-interacting protein, a negative

regulator of mTORC1/2, maintains pluripotency for mESCs and hESCs (Agrawal *et al*, 2014).

The AMPK/mTORC1 pathway regulates mitochondrial biogenesis in somatic cells, and therefore, its role in hPSC differentiation merits further consideration as a mechanism to regulate cell type-specific mitochondrial content and function (Zong *et al*, 2002; Reznick & Shulman, 2006; Morita *et al*, 2013). In addition, AMPK can directly regulate gene expression through histone phosphorylation (Bungard *et al*, 2010). Studies to determine the mechanism(s) that regulate energy-sensing pathway activation and deactivation during iPSC reprogramming and PSC differentiation may reveal how these changes occur *in vivo* to control organismal and lineage-specific development.

### Other molecular players

c-Myc is one of the original four reprogramming transcription factors used in iPSC reprogramming of fibroblasts, but it can be removed and/or replaced by *Lin28a* or other transactors (Takahashi *et al*, 2007; Yu *et al*, 2007; Nakagawa *et al*, 2008; Wernig *et al*, 2008). miPSCs reprogrammed with *Oct4*, *Sox2*, *Klf4*, and c-Myc transactors show an increase in glycolytic metabolism compared to miPSC reprogramming that excludes c-Myc (Folmes *et al*, 2013b). While the metabolic influence of c-Myc in PSCs has not been further characterized, c-Myc promotes RNA splicing of PKM2 in cancer cells, which activates biosynthetic anabolic pathways (David *et al*, 2010; Chaneton & Gottlieb, 2012). c-Myc also has a large role in stimulating glutamine metabolism in lymphocytes and cancer cells (Gao *et al*, 2009; Le *et al*, 2012; Liu *et al*, 2012; Murphy *et al*, 2013).

LIN28 is an evolutionarily conserved regulator of microRNAs (miRNAs) that can be used for iPSC reprogramming in combination with *Oct4*, *Sox2*, and *Klf4* (Yu *et al*, 2007; Viswanathan *et al*, 2008). *Lin28* knockout mice have defects in growth and glucose metabolism (Shinoda *et al*, 2013). *LIN28* expression is regulated by *let-7*, a miRNA that is also post-transcriptionally repressed by LIN28a in a feedback regulatory loop. Knockdown of *let-7* in fibroblasts enhances iPSC reprogramming (Melton *et al*, 2010). LIN28 preferentially binds to the mRNAs of metabolic enzymes to control their translation, which influences cell growth and survival. Metabolic enzymes targeted by LIN28a in hPSCs include enzymes involved in glycolysis, cholesterol biosynthesis, and mitochondrial metabolism (Peng *et al*, 2011). *Lin28a* also enhances the translation of enzymes involved in OXPHOS during the repair of damaged tissues (Shyh-Chang *et al*, 2013b).

Adenosylmethionine decarboxylase 1 (*Amd1*) participates in the biosynthesis of polyamines. Polyamines are positively charged metabolites that can bind to acidic sites of macromolecules including nucleic acids, proteins, and phospholipids. Elevated levels of *Amd1* are required for self-renewal of mESCs. Additionally, translational inhibition of *Amd1* by miR-762 is required for the differentiation of NPCs (Zhang *et al*, 2012).

### Metabolic influence on epigenetics

Differentiation from and reprogramming to pluripotency, along with transdifferentiation between lineage-specific cell types, involves

changes in the structure, function, and expression of the nuclear genome. Binding of transcriptional regulators to their target genes before, during, and after these cell fate changing processes depends on dynamic alterations in DNA methylation, histone modifications and variants, chromatin remodeling complex activities, and global and local three-dimensional chromosome topologies (Papp & Plath, 2013). Cellular metabolism directly influences the epigenetic landscape of a cell by modulating the level and activity of metabolite cofactors and substrates for enzymes that control at least DNA and histone modifications (Kaelin William & McKnight Steven, 2013). Therefore, cellular metabolite levels and flux help determine cellular fate during iPSC reprogramming and PSC differentiation.

DNA and histone methylation is a major regulator of gene expression and chromatin remodeling (Cedar & Bergman, 2009). Methyltransferase enzymes that methylate DNA and histones utilize S-adenosyl methionine (SAM) as a methyl donor for transferring methyl groups. Interestingly, SAM levels are elevated in both human and mouse PSCs compared to fibroblasts, but are even higher in hiPSCs when compared to hESCs (Panopoulos *et al*, 2012; Shyh-Chang *et al*, 2013a). Additionally, SAM levels increase in the late stages of iPSC reprogramming, indicating coordinate accumulation with increasing pluripotent potential (Shyh-Chang *et al*, 2013a). Global DNA methylation is also higher in some hiPSC lines when compared to hESCs, suggesting that SAM levels participate in regulating the extent of global DNA methylation (Deng *et al*, 2009). In mESCs, SAM is generated by uptake of extracellular threonine, which is converted to glycine by threonine dehydrogenase (Tdh) (Fig 2). Therefore, threonine uptake and Tdh activity are required to maintain high levels of SAM in mPSCs. Cleavage of glycine, the product of Tdh, produces SAM by fueling methionine production, which leads to the conversion of methionine plus ATP into SAM via methionine adenosyltransferases (Fig 2) (Shyh-Chang *et al*, 2013a). mESCs cannot survive in culture medium lacking threonine (Wang *et al*, 2009) at least partially because histone 3 lysine 4 di- and trimethylation (H3K4me2 and H3K4me3) is lost in mESCs deprived of threonine, whereas MEFs remain unaffected (Shyh-Chang *et al*, 2013a). The human *TDH* gene is a nonfunctional pseudogene due to two splice acceptor mutations and one nonsense mutation. Therefore, threonine cannot be used for SAM production or level regulation in human cells (Wang *et al*, 2009). hESCs instead depend on the uptake of extracellular methionine for SAM production, with methionine deprivation resulting in a loss of H3K4me3 that predisposes hESCs to differentiation into any of the three embryonic germ layers (Figs 1 and 2). Long-term methionine deprivation leads to cell apoptosis through a p53/p38 mitogen-activated protein kinase (MAPK)-mediated stress signaling response (Shiraki *et al*, 2014). Therefore, adequate methionine in culture media is required to maintain SAM levels and global DNA and histone methylation. Short-term removal of methionine from hPSC culture media may be used to inhibit enzymatic methyltransferase reactions.

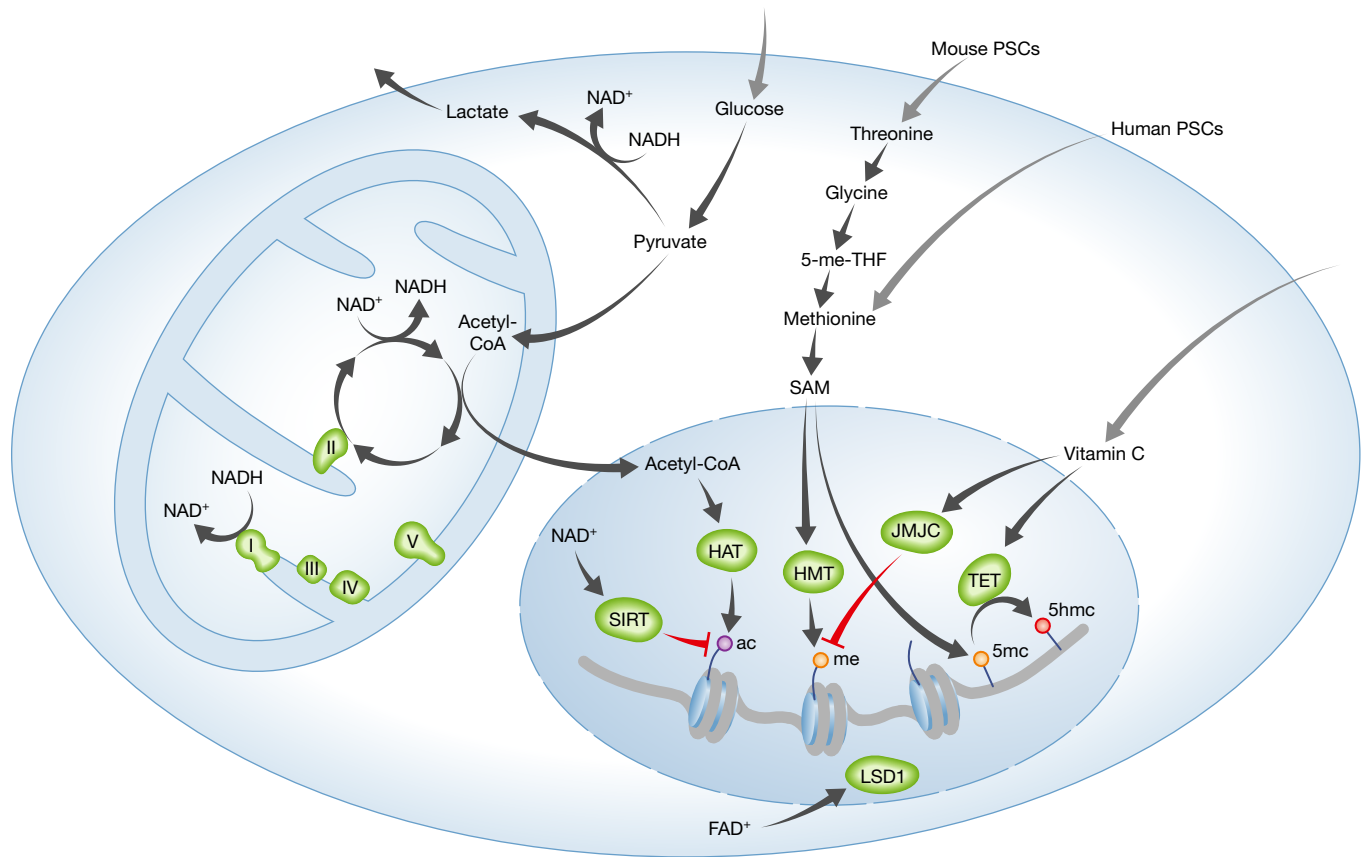
DNA and histone methylations are also susceptible to demethylation reactions. Vitamin C, 2-oxoglutarate ( $\alpha$ -ketoglutarate,  $\alpha$ KG), and Fe(II) act as cofactors for 2-oxoglutarate(2-OG)-dependent dioxygenases, which include ten-eleven translocation (TET) methylcytosine dioxygenases and Jumonji domain-containing (JMJC) histone demethylases (Fig 2). TET methylcytosine dioxygenases oxidize 5-methylcytosine (5mC) in DNA to form 5-hydroxymethylcytosine (5hmc), which is an initial modification

on the pathway to cytosine demethylation (Delatte *et al*, 2014). Vitamin C enhances iPSC reprogramming (Fig 1), and Tet1 can replace Oct4 in the iPSC reprogramming cocktail because its expression activates the transcription of *Oct4* (Esteban *et al*, 2010; Gao *et al*, 2013). Interestingly, the absence of vitamin C impairs hiPSC reprogramming by Tet1, whereas the converse is also observed. With vitamin C, Tet1 inhibits the mesenchymal-to-epithelial transition (MET), but in conditions lacking vitamin C, Tet1 promotes reprogramming without activating the MET (Chen *et al*, 2013). The differences in the role of Tet1 that depend on the level of vitamin C in the culture media highlight the importance of optimizing vitamin C levels during iPSC reprogramming, PSC differentiation, and transdifferentiation protocols. Tet dioxygenases can also promote the MET in combination with thymine DNA glycosylase, which enhances iPSC reprogramming (Hu *et al*, 2014). Moreover, Tet1 can enhance reprogramming by interacting with Nanog to increase expression of key pluripotency-associated target genes (Costa *et al*, 2013).

The pattern of DNA 5hmc differs between hESCs and hiPSCs at large hotspots where hiPSCs have been incompletely hydroxymethylated (Wang *et al*, 2013b). mESCs lose DNA 5hmc when cultured without vitamin C, and addition of vitamin C leads to a rapid Tet1- and Tet2-dependent increase in DNA 5hmc and DNA demethylation (Blaschke *et al*, 2013; Minor *et al*, 2013). Resulting vitamin C-induced changes in DNA methylation occur in genomic regions that tend to gain DNA methylation after *in vitro* culture in contrast to blastocysts *in vivo* (Blaschke *et al*, 2013). Consequently, vitamin C may be an important component of culture media to use to more closely replicate the *in vivo* environment. Vitamin C levels can also modulate the activity of the JmjC class of 2-oxoglutarate(2-OG)-dependent dioxygenases (Fig 2). JmjC family member proteins Jhdmla/b enhance iPSC reprogramming in a vitamin C-dependent manner (Wang *et al*, 2011). Further investigations on the regulation of other JmjC demethylase family member proteins by vitamin C levels may show similar activities.

Surprisingly, the non-essential amino acid L-proline influences the epigenetic state of PSCs. Culturing of mESCs with L-proline causes a mesenchymal-like invasive phenotype while maintaining expression of pluripotency genes. L-proline-treated cells show augmented levels of H3K36me2 and H3K9me3, which can be reversed by vitamin C exposure (Comes *et al*, 2013). Another potential link between cellular metabolism and histone methylation is lysine-specific demethylase 1 (LSD1), which requires flavin adenosine dinucleotide (FAD) for enzymatic activity. Therefore, FAD levels, which also serve as a concentration-dependent cofactor for FAO and respiration, can control the activity of LSD1. LSD1 is required for the maintenance of hESC pluripotency and occupies gene promoters that also bind OCT4 and NANOG transactors (Adamo *et al*, 2011).

Histone acetylation is generally associated with gene activation and can often block histone methylation. Sodium butyrate, a histone deacetylase inhibitor, enhances iPSC reprogramming efficiency, emphasizing the importance of dynamic regulation of histone acetylation (Mali *et al*, 2010; Hou *et al*, 2013). Sirtuins, a family of NAD-dependent deacetylases, can remove acetyl groups from histones depending on the metabolic state of a cell. The NAD<sup>+</sup>/NADH ratio is significantly higher in hESCs compared to fibroblasts (Salykin *et al*, 2013), and therefore, the sirtuins may



**Figure 2. Influence of metabolites on pluripotent stem cell epigenetics.**

Intermediate metabolism sets and maintains levels of metabolites that serve as substrates or cofactors for epigenetic modifying enzymes. Uptake of threonine and methionine from the culture media is required to maintain S-adenosylmethionine (SAM) levels in mPSCs and hPSCs, respectively. SAM is a methyl donor for histone methyltransferases (HMT) and DNA methyltransferases (DNMTs). Vitamin C is a cofactor for the JMJC family of demethylases and TET methylcytosine dioxygenases (TET). Acetyl-CoA, a TCA cycle intermediate, is an acetyl group donor for histone acetyltransferases (HAT). NAD<sup>+</sup>, generated through glycolysis or by the electron transport chain (ETC), is a cofactor for the sirtuin (SIRT) family of deacetylases.

have differential activity in PSCs versus differentiated cells. Sirt1 is required for genomic stability and telomere elongation of iPSCs (De Bonis Maria *et al*, 2014). SIRT6 can improve the iPSC reprogramming efficiency of fibroblasts obtained from older patients (Sharma *et al*, 2013).

How intermediate metabolites from programmed patterns of metabolism and environmental influences regulate epigenome-modifying enzyme activities, such as the sirtuins, requires further study in early development and PSCs. Many studies, especially in cancer, have investigated the role of specific epigenetic enzymes, and their mutant forms, through genetic manipulation. The link between acetyl-CoA levels and protein acetylation has been studied in cancer, but its role has not been investigated in pluripotency, iPSC reprogramming, or hPSC differentiation (Fig 2) (Wellen *et al*, 2009; Choudhary *et al*, 2014). In addition to vitamin C,  $\alpha$ KG is an important cofactor for dioxygenases.  $\alpha$ KG is a TCA cycle intermediate and can also be produced through conversion from glutamate by aminotransferases involved in other metabolic pathways in the cytosol. In some cancers, the majority of  $\alpha$ KG is produced by phosphoserine aminotransferase 1 (PSAT1) (Possemato *et al*, 2011).

### Mitochondrial disease modeling with hiPSCs

mtDNA is maternally inherited and encodes genes for 13 protein subunits in 4 of 5 ETC complexes, 2 rRNAs, and 22 tRNAs. Disease-causing mutations in these mtDNA genes occur in an estimated 1 in 5,000 children and adults (DiMauro & Schon, 2003; Schaefer *et al*, 2004). Mammalian cells may contain up to ~5–10 mtDNAs with sequence variations, a mixture state that is termed heteroplasmy (Legros *et al*, 2004). The heteroplasmy ratio, or the ratio mtDNA carrying a mutant gene to mtDNA carrying the wild-type (WT) gene, varies in cells of each individual, and afflicted patients with the same mtDNA mutation can exhibit a very different range and severity of symptoms (Pickrell Alicia & Youle Richard, 2013). Cells with the highest energy requirements, such as muscle and brain, are most often affected, but patients may not manifest symptoms until their cells accumulate enough of the disease-causing mutant mtDNA through cell proliferation over time. When the mtDNA mutation burden accumulates to roughly 60–90% of the total mtDNA present in a cell, OXPHOS may become compromised and symptoms ensue (Mishra & Chan, 2014). Accurate mouse models of mtDNA mutations are infrequent, and iPSCs have emerged as a potentially good



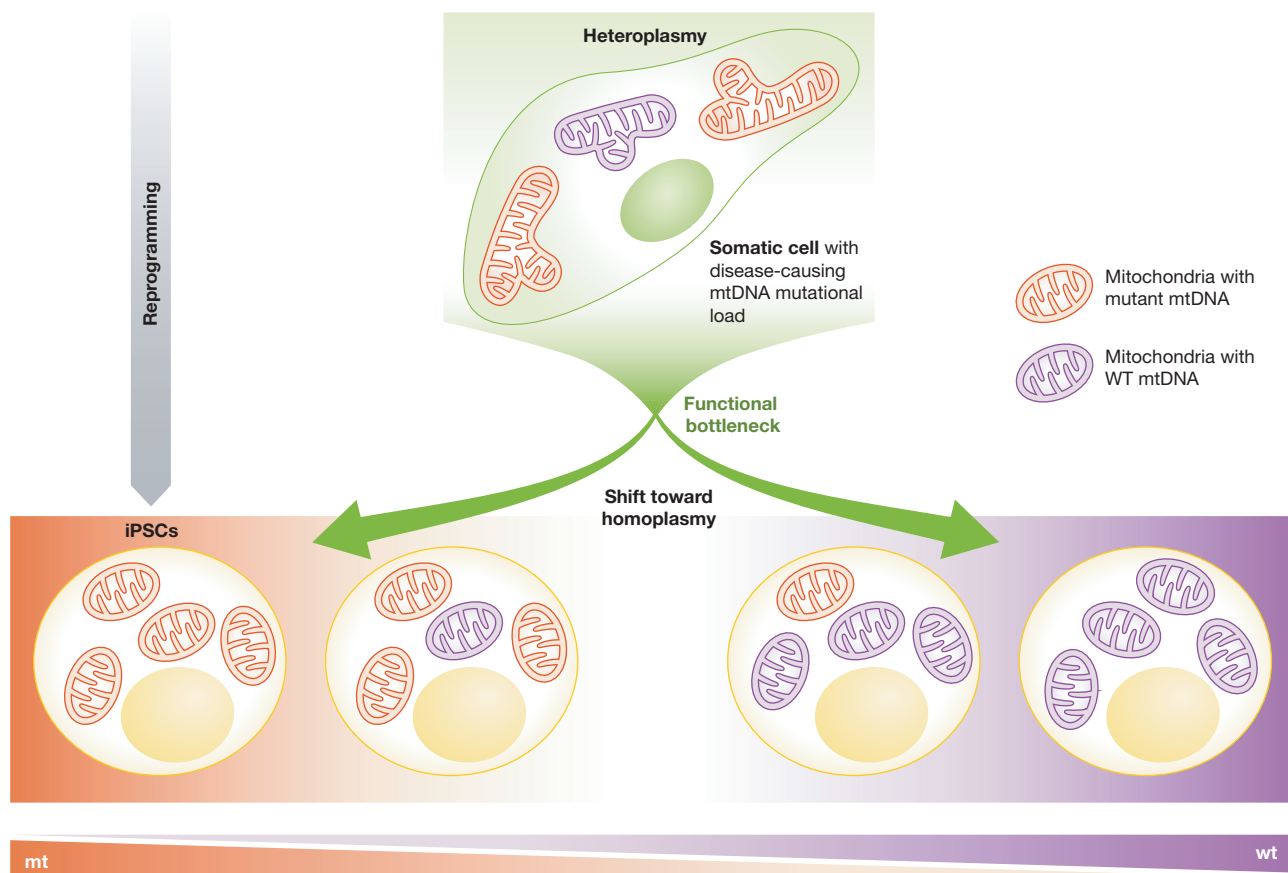
model system to study the different cellular manifestations of mutant mtDNA diseases.

The most common mtDNA mutation is a heteroplasmic 3243A>G mutation in the *tRNA-Leu<sup>(UUR)</sup>* gene, which can result in two distinct patient phenotypes. Maternally inherited diabetes and deafness (MIDD) is one manifestation of this mutation, whereas the other main manifestation is mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS syndrome) (Goto *et al*, 1990; Chae *et al*, 2004). hiPSCs were successfully generated with heteroplasmic mtDNA 3243A>G of variable mutational loads. While mitochondrial transcripts are unaffected in fibroblasts derived from MELAS patients due to their low energy requirements, there was a decrease in mitochondrial transcripts in hiPSCs with high mutational loads (MELAS-high iPSCs) and in neurons derived from the MELAS-high iPSCs. Respiratory ETC complex activity, however, was decreased in MELAS fibroblasts and not in MELAS-high iPSCs or neurons derived from MELAS-iPSCs (Folmes *et al*, 2013a; Hämäläinen *et al*, 2013). Interestingly, MELAS-mutant mtDNA and WT mtDNA had a bimodal segregation pattern at the end of hiPSC reprogramming, resulting in hiPSCs containing either more WT or more mutant mtDNA (Fig 3). Therefore, there is a mtDNA “bottleneck” during hiPSC reprogramming (Cherry *et al*, 2013),

perhaps similar to the mtDNA “bottleneck” that occurs during activated oocyte cleavage divisions in early mammalian development (Shoubridge, 2000; Smith *et al*, 2002; Cree *et al*, 2008; Carling *et al*, 2011), and there is no selection for or against the MELAS mutation (Fig 3) (Hämäläinen *et al*, 2013). The extent of heteroplasmy in hiPSCs decreases with increasing passage number *in vitro* (Folmes *et al*, 2013a).

Fibroblasts from mtDNA “mutator” mice, which carry a mutation in the polymerase gamma (*Polg*) gene, carry a high mtDNA mutation load due to errors in mtDNA replication (Trifunovic *et al*, 2004). Mutator iPSCs reprogrammed from mutator MEFs with heavy mtDNA mutational loads proliferate at lower rates and reduced ability to form EBs, teratomas, and chimeric mice. EBs generated from mutator iPSCs are more skewed to glycolytic metabolism than are EBs from WT iPSCs, which could account for their decrease in differentiation potential (Wahlestedt *et al*, 2014).

Defects in the function of mitochondria may also be caused by mutations in nuclear-encoded genes with roles in mitochondrial energy metabolism, mitochondrial dynamics, mitochondrial transport, apoptosis, or mitophagy. One disease associated with mitochondrial dysfunction by perhaps one or more of these mechanisms is the neurodegenerative disease, Parkinson’s disease (PD).



**Figure 3. Somatic cell reprogramming to pluripotency causes a mtDNA “bottleneck”.**

mtDNA undergoes a genetic bottleneck, or reduction in copy number, during de-differentiation, similar to the mtDNA bottleneck that occurs during normal female germ line oogenesis. This reduction has an unresolved mechanism that results in a shift from a heteroplasmic toward a homoplasmic state with no clear preference for wild-type or mutant mtDNA. This shift does not occur during the continuous culturing of fibroblasts in which characteristic levels of heteroplasmy are maintained over time.

iPSCs have been generated from fibroblasts of PD patients caused by multiple different mutations including *parkin* (PARK2), *PTEN-induced putative kinase 1* (PINK1), and *leucine-rich kinase 2* (LRRK2) (Seibler *et al*, 2011; Cooper *et al*, 2012; Imaizumi *et al*, 2012). PINK1 and Parkin proteins interact to regulate mitophagy, the process of selectively targeting poorly functioning mitochondria with low  $\Delta\psi$  for engulfment by an autophagosome and eventual degradation (Clark *et al*, 2006; Park *et al*, 2006). PARK2, an E3 ubiquitin ligase, is recruited to damaged mitochondria in a PINK1-dependent manner to polyubiquitinate mitochondrial outer membrane proteins (Narendra *et al*, 2008, 2010; Chan *et al*, 2011). Neurons differentiated from PINK1 mutant iPSCs have abnormalities in mtDNA copy number (Seibler *et al*, 2011). Additionally, neurons differentiated from both mutant PINK1 and LRRK2 hiPSCs are vulnerable to oxidative stress when exposed to PD-associated toxins. Mitochondria in mutant LRRK2 iPSC-differentiated neurons respire less and are more mobile than those from healthy subjects. Sensitivity of PD iPSC-differentiated neurons to PD-associated toxins is rescued by treatment with either an LRRK2 inhibitor, coenzyme Q<sub>10</sub>, or rapamycin (Cooper *et al*, 2012). PARK2 mutant iPSC-differentiated neurons show increased oxidative stress,  $\alpha$ -synuclein accumulation and Lewy body formation, which are clinical manifestations of PD, providing a model for this aspect of PD pathophysiology (Imaizumi *et al*, 2012).

### Concluding remarks

Shifts in cellular metabolism accompany shifts in cell identity and facilitate changes in cell function. Applications in regenerative medicine will likely require a fuller understanding of metabolic mechanisms that can alter cellular identity, function, and longevity. Glycolytic metabolism generally accommodates a high rate of biosynthesis and cell proliferation, whereas OXPHOS generates ATP more efficiently for functioning differentiated cells. While progress has been made in understanding how cellular energy metabolism is correlated with pluripotent and differentiated states, most cause-and-effect features have not yet been determined. Glycolysis is linked to the primed pluripotent state which is favored in hypoxic environments and by HIF transfactor stabilization. Further work is necessary to identify the transcription factors and signaling pathways that regulate glycolytic flux and overall capacity in PSCs and during induced differentiation. Additionally, the mitochondria in PSCs are rudimentary and the mechanism(s) regulating their maturation or return to immaturity are only starting to be discovered. How the mitochondrial fusion/fission machinery is regulated, what causes changes in mitochondrial localization, what senses and instructs lineage-specific differential mitochondrial mass accumulation and maintenance, and what factors facilitate transitions in metabolism and cell fates remains a significant area of ongoing and future investigations.

PSC metabolism regulates the activities of epigenetic modifying enzymes and therefore influences gene expression patterns, differentiation potential, and functional competence. While the influence of metabolism on SAM levels and global methylation patterns is more heavily studied, the regulation of other key metabolites, such as acetyl-CoA, has not been thoroughly investigated in PSCs and iPSCs. As the connections between energy metabolism and cell fate

become unearthed, methods for manipulating PSC metabolism may be harnessed to improve efficiencies and functional outcomes for nuclear reprogramming, PSC differentiation, and transdifferentiation.

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### Conflict of interest

The authors declare that they have no conflict of interest.

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