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REVIEW

Metabolic remodeling during the loss and acquisition of pluripotency

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ABSTRACT

Pluripotent cells from the early stages of embryonic development have the unlimited capacity to self-renew and undergo differentiation into all of the cell types of the adult organism. These properties are regulated by tightly controlled networks of gene expression, which in turn are governed by the availability of transcription factors and their interaction with the underlying epigenetic landscape. Recent data suggest that, perhaps unexpectedly, some key epigenetic marks, and thereby gene expression, are regulated by the levels of specific metabolites. Hence, cellular metabolism plays a vital role beyond simply the production of energy, and may be involved in the regulation of cell fate. In this Review, we discuss the metabolic changes that occur during the transitions between different pluripotent states both *in vitro* and *in vivo*, including during reprogramming to pluripotency and the onset of differentiation, and we discuss the extent to which distinct metabolites might regulate these transitions.

KEY WORDS: Epigenetics, Metabolic remodeling, Stem cell

Introduction

Among the various cell types known in animals, only pluripotent stem cells have the capacity to self-renew indefinitely, while still retaining the ability to differentiate into all the other cells that make up an entire animal. This powerful cell type and the mechanisms that govern it must therefore be tightly regulated. For some time now we have had the capacity to derive pluripotent stem cells from the developing embryo (Evans and Kaufman, 1981; Martin, 1981; Brook and Gardner, 1997; Thomson et al., 1998; Boroviak et al., 2014). In particular, at least two states have been stabilized *in vitro* in mouse and human cell lines: the naïve state, which corresponds to the pre-implantation stage of embryo development; and the primed state, which corresponds to the post-implantation stage (Brons et al., 2007; Tesar et al., 2007; Nichols and Smith, 2009; Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Ware et al., 2014; Wu et al., 2015). These states display distinct features in terms of gene expression, epigenetic modifications and developmental capacity. It has also been reported that these two states differ dramatically with regard to their metabolic profile and mitochondrial function (Zhou et al., 2012; Takashima et al., 2014; Sperber et al., 2015). This raises the issue of whether such metabolic differences can instruct transitions between pluripotent states, or whether they are simply the result of them.

Cellular metabolism is the set of chemical reactions that occur in a cell to keep it alive. Metabolic processes can be divided into anabolism and catabolism. Anabolism is the biosynthesis of new biomolecules, for example fatty acids, nucleotides and amino acids, and usually requires energy. Catabolism is the breaking down of molecules into smaller units to generate energy. Traditionally, cellular metabolism has been studied for its crucial role in providing energy to the cell and thereby helping to maintain its function. More recently, however, metabolism has been implicated in cell-fate determination and stem cell activity in a variety of different contexts (Buck et al., 2016; Gascón et al., 2016; Zhang et al., 2016a; Zheng et al., 2016). Mitochondria are the organelles in which a great deal of metabolic activity occurs, generating most of the cell's supply of adenosine triphosphate (ATP). Not surprisingly then, mitochondria have also been implicated in the regulation of stem cell activity and fate (Buck et al., 2016; Khacho et al., 2016; Lee et al., 2016; Zhang et al., 2016a). Furthermore, work in *C. elegans* has revealed surprising beneficial effects of reduced mitochondrial function in cellular states and aging (reviewed by Wang and Hekimi, 2015), further supporting the idea that metabolic pathways regulate cellular processes that go beyond ATP production. The mechanism by which cellular metabolism can influence stem cell fate has only recently begun to be explored; however, it is clear that it does so, at least in part, by influencing the epigenetic landscape, which in turn affects gene expression (reviewed by Harvey et al., 2016). This is a logical explanation in the context of cell fate determination, where it is known that key batteries of gene expression drive the specification of the lineages and determine cell identity.

Pluripotent stem cells possess a very specific metabolic profile that likely reflects their rapid proliferation and the specific microenvironment from which they are derived. As the epiblast transitions from the pre-implantation to the post-implantation stage, its external environment changes dramatically, and so it follows that the availability of certain metabolites may also change (Gardner, 2015). One example of this could be a drop in the level of available oxygen as the blastocyst implants into the uterine wall, which may be hypoxic compared with the uterine cavity. Such a change in the availability of a key metabolite such as oxygen would necessitate significant metabolic remodeling in the implanted blastocyst and the pluripotent cells within it. Similarly, leaving the pluripotent stage is accompanied by significant metabolic remodeling events. Metabolic changes during cellular differentiation and maturation include alterations in the preferred substrate choice for energy production, as well as mitochondrial use for ATP production versus production of intermediates for anabolic pathways (Zhang et al., 2011; Diano and Horvath, 2012). The reverse process, when cells enter a pluripotent state through reprogramming, also requires an early metabolic switch to take place, as the metabolic requirements of differentiated cells are different from highly proliferative pluripotent stem cells. In this Review, we discuss the metabolic changes that occur during the transitions between different pluripotent states, both *in vitro* and *in vivo*. We also discuss the metabolic changes that are observed during reprogramming. In particular, we discuss the extent to which changes in cell fate are

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determined by metabolic remodeling, as opposed to being merely associated with them. Finally, we discuss how energy substrate availability influences the stem cell metabolism and, in turn, stem cell fate, and conclude by summarizing the current challenges and future directions for this field.

The metabolic needs of stem cells

Glycolysis is one of the major energy-producing pathways in living cells, converting carbohydrates to ATP, NADH and pyruvate. When oxygen is available, pyruvate is transported to mitochondria and converted into acetyl-CoA. In addition, many cells also use other metabolic pathways, such as β-oxidation, which is the breakdown of fatty acid molecules into acetyl-CoA. Fatty acids are first activated to acyl-CoA and then through β-oxidation to acetyl-CoA. Acetyl-CoA generated from either glycolysis or fatty acid β-oxidation is further oxidized in the tricarboxylic acid (TCA) cycle (or Krebs cycle), generating the electrons carriers NADH and FADH2 to deliver the electrons to the electron transport chain (ETC). The flow of electrons through ETC results in the pumping of protons from mitochondria inner matrix to outer matrix. ATP is synthesized though ATP synthase when protons flow back to the mitochondria matrix. This process that produces most of cellular ATP is called oxidative phosphorylation (OxPHOS) (see Fig. 1). Although β oxidation generates a much higher net amount of ATP, some cells prefer to use glucose for their energy production. The advantages of using glucose for energy production include the possibility of producing ATP without mitochondrial activity and using the products of glycolysis immediately as building blocks for anabolic pathways (Fig. 1). The potential benefit of eliminating the need for mitochondrial activity is that this period can be used to carry out mitochondrial quality control, allowing damaged mitochondria to be eliminated without causing a bottleneck effect for ATP production (Khacho et al., 2016; Sieber et al., 2016).

Compared with differentiated, non-dividing somatic cells, stem cells, particularly those that are highly proliferative, have some unique metabolic requirements (reviewed by Vander Heiden et al., 2009). Proliferation requires energy; however, it also requires significant amounts of nucleotides, amino acids and lipids to assemble the two daughter cells that are produced with every cell division. Although OxPHOS produces far more ATP compared with glycolysis (36 ATP molecules compared with just two ATP molecules for glycolysis), to use all available glucose solely for the purpose of ATP production would be limiting for a proliferating cell. Instead, some glucose must be diverted to generate precursors such as acetyl-CoA for fatty acid synthesis, glycolytic intermediates for nonessential amino acids, and ribose for nucleotides. It is perhaps for this reason that highly proliferative cells, including many stem cell populations, use primarily aerobic glycolysis, glycolysis that results into lactate production, instead of pyruvate oxidation in a mitochondrion, regardless of oxygen availability. Interestingly, this same strategy is also seen in highly proliferative cancer cells, where it is called the Warburg effect (Box 1). Another important metabolic pathway for stem cells is the pentose phosphate pathway (PPP) (see Fig. 1) (Varum et al., 2011; Manganelli et al., 2012), which generates metabolites that fuel nucleotide and lipid biosynthesis. This pathway is particularly active in embryonic stem cells (ESCs) compared with somatic cells, and has been shown to be important in the balance between pluripotency and the onset of differentiation (Varum et al., 2011; Manganelli et al., 2012).

Another key requirement for stem cells is the ability to switch between different metabolic pathways depending on changes in substrate availability. A good example of this is the availability of oxygen, which can determine, at least in part and at least in some contexts, the balance between aerobic glycolysis and OxPHOS. Hematopoietic stem cells, for example, reside in a hypoxic niche, and therefore depend primarily on anaerobic glycolysis rather than

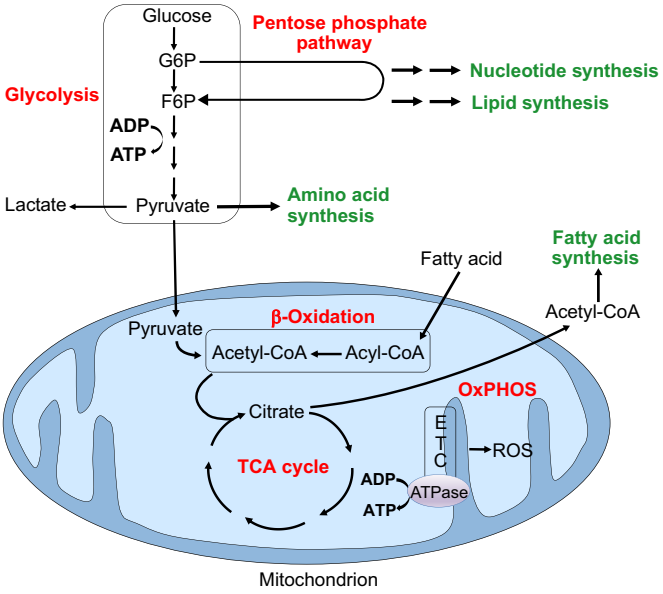

Fig. 1. Overview of cellular metabolism. The major cellular metabolic pathways (indicated in red): glycolysis, pentose phosphate pathway, β-oxidation, TCA cycle and oxidative phosphorylation (OxPHOS). Each of these pathways produces metabolites that are required to fuel cell growth via the production of energy (ATP), nucleotides, lipids, amino acids and fatty acids (indicated in green). The most efficient pathway for the production of energy is OxPHOS, which produces significantly more ATP than glycolysis. Despite this difference, glycolysis is the main energy-producing pathway favored by pluripotent stem cells. ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATPase, ATP synthase; ETC, electron transport chain; F6P, fructose 6 phosphate; G6P, glucose 6 phosphate; ROS, reactive oxygen species; TCA, tricarboxylic acid.

Box 1. The Warburg effect

The Warburg effect, also known as aerobic glycolysis, was first described by Otto Warburg in the 1920s (Warburg et al., 1927). He observed that tumor cells increase their uptake of glucose and that, even in the presence of oxygen, glucose is preferentially fermented into lactate instead of entering the mitochondria to fuel the TCA cycle. He hypothesized that cancer cells rely mainly on glycolysis for the production of energy due to defective mitochondrial oxidative phosphorylation (OxPHOS); however, it was later shown that this phenomenon also occurs in cells with fully functional mitochondria. In addition to malignant cells, some highly proliferative and developing cells, such as embryonic stem cells, also exhibit aerobic glycolysis. The exact function and advantages of the Warburg effect have not yet been fully uncovered. Many cells need to change their metabolism in order to promote proliferation, survival and self-renewal. Even though generation of ATP from lactate is very inefficient compared with mitochondrial respiration, it allows recycling of intermediates for anabolic pathways. It has been proposed that cells might prioritize accumulation of biomass over production of energy to support the metabolic requirements of fast proliferating cells (reviewed by Vander Heiden et al., 2009). Moreover, metabolic switches can affect cell signaling, epigenetics and gene expression. During aerobic glycolysis, the mitochondria generate fewer reactive oxygen species, which affects the regulation of transcription factor activation, as well as numerous signaling pathways, and also modulates apoptosis. The amount of acetyl-CoA, the substrate for histone acetylation, is also affected by the Warburg effect.

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OxPHOS to produce ATP (Simsek et al., 2010). Changes in the oxygen consumption have been detected during the transition from mouse preimplantation to early postimplantation development using a fluorescence-based oxygen sensor (Houghton et al., 1996). Changing levels of oxygen consumption during early embryonic development *in vivo* may therefore reflect the different metabolic pathways that are active in naïve versus primed pluripotent stem cells (Zhou et al., 2012; Takashima et al., 2014; Sperber et al., 2015; Zhang et al., 2016b). Switching between different metabolic pathways has also been shown to be important for the activation of quiescent stem cell populations and for the onset of differentiation (Simsek et al., 2010; Knobloch et al., 2013; Hamilton et al., 2015; Beyaz et al., 2016). In summary, it is clear that a cell's choice of metabolic pathway reflects to some degree the environment in which they find themselves, as well as the function that they must perform. Understanding how metabolic remodeling occurs, whether and how it influences cell fate and the factors that may regulate this is not only fundamental to a better understanding of pluripotency *in vivo*, but will also allow better control over pluripotent stem cells *in vitro*.

Cellular metabolism at different phases of pluripotency

Metabolic dynamics in the early mammalian blastocyst

The physiology of the embryo dramatically changes during the first steps of mammalian development, and so too does its metabolic activity (see Fig. 2). Measurements in the mouse embryo have shown that oxygen consumption stays steady from the zygote to morula stages, increases at the blastocyst stage, and decreases to pre-blastocyst level by day E6.5 after implantation (Houghton et al., 1996). At the stage of the blastocyst, viable mouse embryos exhibit high glucose uptake (Gardner and Leese, 1987). Glucose is the main substrate consumed by post-implantation embryos (Houghton et al., 1996) and the majority of the glucose is converted into lactate (Clough and Whittingham, 1983; Gott et al., 1990). The metabolic switch that occurs at the time of implantation is extremely important for the developing embryo, as perturbation of metabolic features reduces implantation capacity and embryonic viability (Gardner and Harvey, 2015). The creation of a high lactate and low pH niche around the embryo has been proposed to help implantation by favoring endometrium disaggregation, increasing angiogenesis and modulating the immune response to avoid maternal rejection (Gardner, 2015). These metabolic changes may result, at least in part, from possible changes in the availability of oxygen as a substrate. In hamsters and rabbits, oxygen tension in the uterus has been reported to be 37 mm Hg (5.3% O_2) but decrease significantly at the time of implantation, to 24 mm Hg (3.5% O_2) (Fischer and Bavister, 1993).

Metabolic dynamics of ESCs

After fertilization, the single cell zygote divides into blastomeres, totipotent cells that are capable of generating either trophoectoderm, which gives rise to extra-embryonic tissues, or the inner cell mass (ICM), which gives rise to the embryo. The ICM develops into preimplantation epiblast and primitive endoderm prior to implantation into the mother's uterus (Fig. 2). Following implantation, the epiblast cells lose their pluripotency as the cells

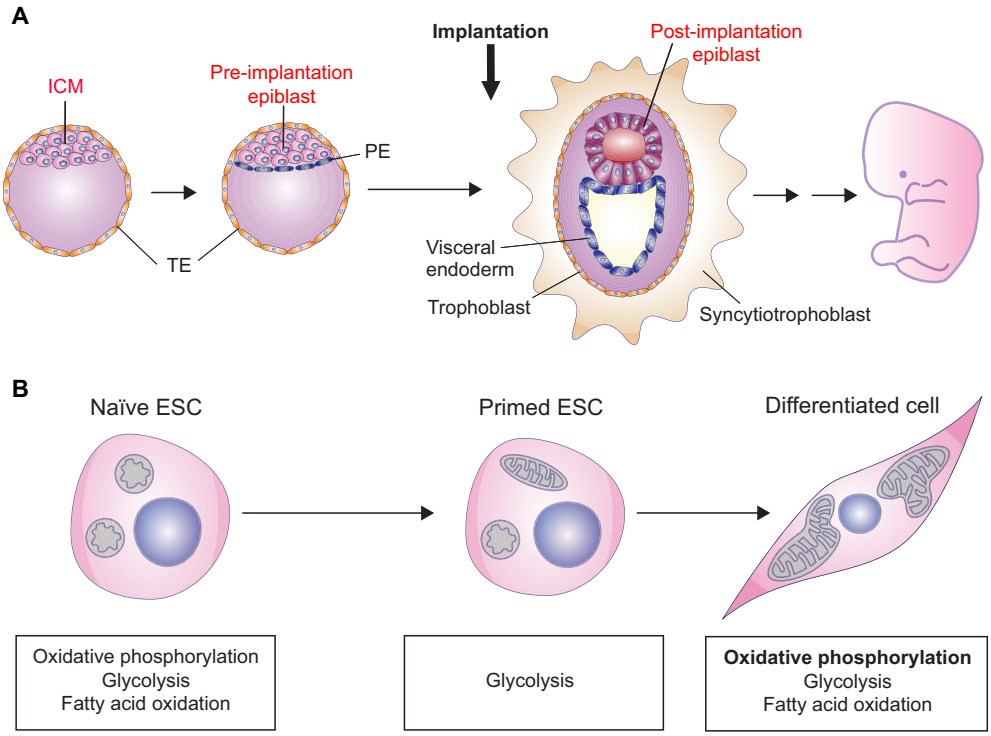

Fig. 2. Dynamic changes in pluripotency *in vivo* and *in vitro*. (A) Pluripotent cells emerge from the inner cell mass (ICM) of the early blastocyst. These cells then segregate to form the primitive endoderm and the pluripotent, naïve epiblast. Following implantation, the epiblast begins to express specification factors, initiates gastrulation and goes on to differentiate into all the cells that will eventually make up the mature organism. (B) Distinct phases of pluripotency can be captured *in vitro* and have been shown to have characteristic metabolic profiles. Naïve embryonic stem cells (ESCs) are able to perform oxidative phosphorylation, glycolysis and fatty acid oxidation, whereas primed ESCs rely almost exclusively on glycolysis to meet their bioenergetic demands. Naïve ESCs contain mitochondria that are more spherical and contain less dense cristae, but as the cells transition to the primed state, a mixture of immature and relatively more mature mitochondria can be seen. Differentiated cells contain mitochondria with fully mature cristae. Differentiated cells rely primarily on oxidative phosphorylation. ICM, inner cell mass; PE, primitive endoderm; TE, trophectoderm.

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commit to the three germ layer fates and gastrulation is initiated. Pluripotency therefore does not represent a fixed stage, but rather a gradient of stages. In both mouse and human, it is possible to stabilize some of these stages *in vitro* (Davidson et al., 2015; Weinberger et al., 2016). Considering the dynamic period in which cells are pluripotent it is perhaps not surprising that the stabilized states can vary in subtle details. Interestingly, the stabilized stages can be divided into two groups based on their metabolism. The stabilized pre-implantation stem cells have wide energy substrate usage, while the cells post-implantation can only use glucose, with a very low mitochondrial ETC activity and low ETC complex IV levels. This highly reduced mitochondrial oxygen consumption rate is also observed *in vivo* in mouse, as ETC complex IV levels are highly reduced in the E6.5 epiblast, which corresponds to the post-implantation embryo *in vivo* (Zhou et al., 2012).

Naïve versus primed pluripotency

When mouse ESCs (mESCs) were first derived from the ICM of early blastocyst stage, they were stabilized *in vitro* in presence of LIF (Evans and Kaufman, 1981; Martin, 1981). It was later found that culture of mESC in serum-free media in presence of GSK3 and MEK inhibitors in addition to LIF stabilized the cells in a naïve ground state, corresponding more closely to the pre-implantation epiblast (Ying et al., 2008; Tang et al., 2010; Boroviak et al., 2014). Pluripotent mouse cell lines have also been derived from the post-implantation epiblast, and these are called post-implantation epiblast-derived stem cells (EpiSCs) (Brons et al., 2007; Tesar et al., 2007). These cells represent the primed state of pluripotency that corresponds to the post-implantation embryo and are cultured without LIF, but in the presence of FGF and activin A (Fig. 2). Unlike in the mouse, ESCs derived from the ICM of the early human blastocyst do not appear to be naïve, but instead resemble mEpiSCs in terms of culture requirements, transcriptional profile and epigenetics (Thomson et al., 1998; Brons et al., 2007; Tesar et al., 2007; Rossant, 2008). In the past few years, putative naïve-like cells have been isolated from human embryos (Gafni et al., 2013; Theunissen et al., 2014; Ware et al., 2014; Pastor et al., 2016), or generated by exposing primed cells to various cocktails of chemical inhibitors and cytokines (Chan et al., 2013; Gafni et al., 2013; Takashima et al., 2014; Theunissen et al., 2014; Ware et al., 2014; Qin et al., 2016).

Recent studies have begun to uncover the chromosomal, epigenetic, transcriptomic and metabolic differences between naïve and primed ESCs (reviewed by Davidson et al., 2015; Weinberger et al., 2016). For example, human ESCs (hESCs) are thought to depend upon alternative POU5F1 enhancer usage as cells progress through pluripotency, with naïve cells depending upon the distal enhancer, while primed cells use the proximal enhancer (Theunissen et al., 2014, 2016; Ware et al., 2014). Enhancer usage has also been studied in the mouse naïve-to-primed transition (Buecker et al., 2014; Factor et al., 2014). In addition, a significant reduction of H3K27me3 in promoter and gene body regions over developmental genes has been observed in naïve hESC lines over primed hESC (Chan et al., 2013; Gafni et al., 2013; Theunissen et al., 2014; Ware et al., 2014; Sperber et al., 2015). Perhaps some of the most surprising differences between naïve and primed pluripotent states are those that relate to metabolism. Although glycolysis is active in both naïve and primed stages, naïve ESC have active mitochondria, whereas the primed ESC stage shows low mitochondrial oxidative activity (Zhou et al., 2012; Takashima et al., 2014; Sperber et al., 2015). It should be noted, however, that activin A, which is used to culture some primed ESC, has been shown to maintain mitochondria in a condensed state in germ cells (Meinhardt et al., 2000). More work is needed to understand the mechanism and biological relevance of mitochondrial changes in these states. Although mouse naïve-to-primed ESC transition accompanies an increase in glycolytic activity, in human this increase is not observed (Zhou et al., 2012; Takashima et al., 2014; Sperber et al., 2015; Gu et al., 2016). Despite having a relatively more developed and expanded mitochondrial content compared with mESC and naïve-like hESC, EpiSC and hESC have low mitochondrial respiratory capacity that correlates with low cytochrome c oxidase expression (Zhou et al., 2012; Sperber et al., 2015). Whereas hESC can change their fatty acid metabolism based on culture conditions, the mitochondrial change between naïve and primed hESC lines are observed even in the same basic media (Sperber et al., 2015; Zhang et al., 2016b). It is worth emphasizing that the same two metabolic phenomena – active versus inactive mitochondria – have been observed in stabilized pluripotent stages in both species, mouse and human. This is interesting given the temporal differences in gene expression that have been observed between mouse and human pluripotency (Nakamura et al., 2016). It will be also very informative to analyze the metabolic signatures of the newly derived cynomolgus monkey ESCs (Nakamura et al., 2016).

Metabolic remodeling during the acquisition of pluripotency

Metabolic changes in reprogramming

Metabolic remodeling is an important part of the acquisition of pluripotency during somatic cell reprogramming. Somatic cells mainly use OxPHOS for energy production, but upon reprogramming, the transition to pluripotency is accompanied with a shift to a glycolytic metabolism (Folmes et al., 2011; Panopoulos et al., 2012; Prigione et al., 2010; Varum et al., 2011). This metabolic switch during reprogramming occurs early in the process (Folmes et al., 2011; Mathieu et al., 2014; Prigione et al., 2014). The first wave of activated genes during reprogramming is enriched for genes important for increased proliferation and metabolic change (Cacchiarelli et al., 2015). Metabolic remodeling is not only associated with reprogramming, but also appears to mediate the process. It has been shown that metabolic perturbations can affect reprogramming efficiency (Fig. 3A). Stimulation of glycolytic activity with fructose-6-phosphate or PDK1 enhances reprogramming efficiency, whereas inhibition of glycolysis using 2 deoxyglucose (2DG), hexokinase 2 (HK2) inhibition or dichloroacetate (DCA) reduces induced pluripotent stem cell (iPSC) generation (Yoshida et al., 2009; Esteban et al., 2010; Zhu et al., 2010; Folmes et al., 2011; Panopoulos et al., 2012).

Curiously, it has been recently reported that at a very early stage of reprogramming, the increase in glycolysis is accompanied by a transient burst of OxPHOS activity (Kida et al., 2015; Hawkins et al., 2016). These findings could explain the temporal elevation of mitochondrial proteins in cells undergoing reprogramming, which has previously been observed via proteomic analysis (Hansson et al., 2012). This transient 'hyper-energetic' state seems to be required for the reprogramming of fibroblasts into pluripotent cells, and it has been proposed that such a state may be induced by the expression of estrogen-related nuclear receptors (ERRα and ERRγ) (Kida et al., 2015). This could increase reactive oxygen species (ROS) production, leading to activation of HIF1 and enhancement of the glycolytic rate (Hawkins et al., 2016). However, although a transient increase of mitochondrial DNA (mtDNA) was observed very early during the reprogramming of mouse embryonic and tail tip fibroblasts, such an increase was not observed during the

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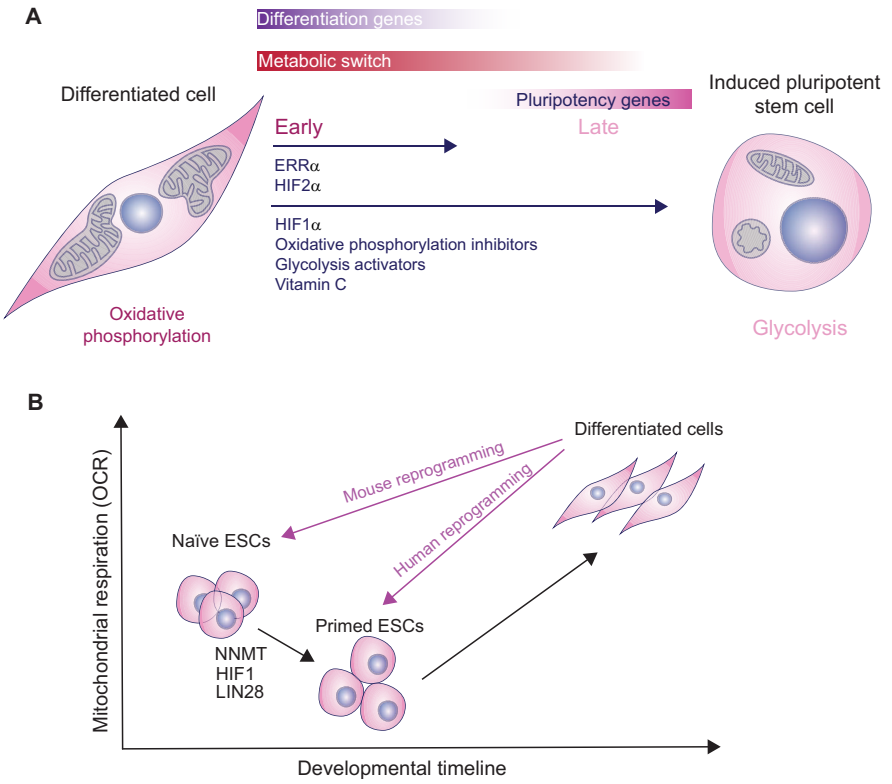

Fig. 3. Metabolic and transcriptional changes during entry into and exit from pluripotency. (A) A metabolic switch from mitochondrial oxidative phosphorylation to glycolysis takes place early during the acquisition of human pluripotency. The resulting human induced pluripotent stem cells (iPSCs) contain a mixture of immature and relatively more mature mitochondria, compared with the fully mature mitochondria of the original differentiated cell. (B) Changes in mitochondrial oxidative phosphorylation upon developmental progression and the re-acquisition of pluripotency during reprogramming. Mitochondrial respiration is significantly reduced during the transition from naïve to primed embryonic stem cells (ESCs) and increases during differentiation. Reprogramming of human differentiated cells leads to iPSCs that resemble primed ESCs, whereas mouse reprogramming leads to iPSCs in a naïve state. ERRα, estrogen-related receptor α; HIF, hypoxia inducible factor; NNMT, N-methyltransferase; OCR, oxygen consumption rate.

reprogramming of pre-adipocytes (Ma et al., 2015), raising the issue of whether the existence of a transient hyperenergetic state is dependent on the cell type. Regardless of starting cell type, the resulting iPSCs appear to consistently have less mitochondrial mass and maturity than the starting population. mtDNA gradually decreases during the course of reprogramming (Ma et al., 2015), while proteins of the complexes I and IV of the electron transport system are downregulated (Hansson et al., 2012) and mitochondria revert to a more immature ESC-like state in terms of morphology, cellular distribution and efficiency of oxidative phosphorylation (Prigione et al., 2011). Mature mitochondria are cleared by Atg5-independent autophagy and new immature mitochondria are generated (Ma et al., 2015).

Molecular pathways implicated in metabolic remodeling

New studies have shed light on the molecular mechanisms that regulate the acquisition of this unique metabolic state during reprogramming (Mathieu et al., 2014; Prigione et al., 2014; Ma et al., 2015; Son et al., 2015; Hawkins et al., 2016; Zhang et al., 2016b). Understanding how this metabolic shift is regulated is of particular importance because it is reminiscent of the change in energy metabolism that has been shown to be a hallmark of cancer, known as the Warburg effect (Box 1). Hypoxia inducible factors (HIFs) are key transcription factors that are activated in response to hypoxia, and their target genes overlap with genes involved in tumor metabolism (Masson and Ratcliffe, 2014). The emerging role of HIFs in the maintenance and acquisition of stem cell properties emphasizes the importance of the metabolic context in cell fate (Yoshida et al., 2009; Mathieu et al., 2011, 2013, 2014; Prigione et al., 2014). Reprogramming cells in 5% oxygen compared with the standard 20% can enhance the efficiency of iPSC formation (Yoshida et al., 2009), and HIFs have also been shown to be important for the metabolism of primed stem cell state (Prigione et al., 2011; Zhou et al., 2012; Mathieu et al., 2013, 2014; Sperber et al., 2015; Hawkins et al., 2016). The switch from oxidative to glycolytic metabolism requires HIFs, as knockdown of HIFs in human fibroblasts prevents reprogramming (Mathieu et al., 2014; Prigione et al., 2014). Interestingly, although both HIF1α and HIF2α were essential for this early step of reprogramming, prolonged stabilization of HIF2α significantly repressed iPSC formation and induced expression of TNF-related apoptosis-inducing ligand (TRAIL) (Mathieu et al., 2014). Thus, HIF1α and HIF2α have distinctive stage-specific roles for during reprogramming. Other transcription factors and pathways are also implicated in the upregulation of glycolysis that occurs during the reprogramming process. These include NRF2 (Hawkins et al., 2016), Akt (Zhu et al., 2010; Khaw et al., 2015) and the reprogramming factors Myc (Folmes et al., 2013) and Oct4 (Kim et al., 2015).

Dynamic mitochondrial activity during the entry into and exit from pluripotency

Structural and functional mitochondrial remodeling

Mitochondria remodeling also takes place during reprogramming of somatic cells into both naïve and primed pluripotent stem cells. Pluripotent stem cells and somatic cells exhibit very different mitochondrial morphology, structure, localization and function (St John et al., 2005; Cho et al., 2006; Varum et al., 2011). Transmission electron microscopy has revealed that mitochondria of naïve ESC are very immature, both in mouse and human. They are globular in shape and contain poorly developed cristae structure (Baharvand and Matthaei, 2003; Facucho-Oliveira et al., 2007; Zhou et al., 2012; Ware et al., 2014). Mitochondria in primed mESCs and hESCs begin to show morphological elongation with relatively more developed cristae than naïve mESC and hESC (Sathananthan et al., 2002; St John et al., 2005; Cho et al., 2006; Zhou et al., 2012; Ware et al., 2014).

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Mitochondrial maturation continues throughout differentiation, resulting in highly mature mitochondria in terminally differentiated cells, such as fibroblasts or cardiomyocytes, that are elongated, branched and tubular, with numerus well-developed cristae (St John et al., 2005; Varum et al., 2011) (Fig. 2). Therefore, during reprogramming of both human and mouse somatic cells into iPSCs, mitochondria need to undergo significant remodeling, a process known as mitochondria rejuvenation (Prigione et al., 2010; Suhr et al., 2010; Folmes et al., 2011, 2012; Varum et al., 2011) (Fig. 3A). Metabolic reprogramming encompasses not only morphological changes in mitochondria, but also their functional role (Fig. 3B). Most differentiated cells depend on OxPHOS, whereas primed pluripotent stem cells rely mainly on glycolysis, even in presence of oxygen (Prigione et al., 2010; Zhou et al., 2012). Although naïve ESC are also capable of using OxPHOS and fatty acids, their mitochondrial activity is lower than most differentiated cells due to their relative immaturity (Zhou et al., 2012; Gu et al., 2016).

Molecular pathways implicated in mitochondrial remodeling

The oocyte-enriched factor Tcl1 has been shown to inhibit mitochondrial biogenesis and OxPHOS by suppressing the mitochondrial localization of the polynucleotide phosphorylase PnPase, which in turn induces a remodeling of the metabolome of actively reprogramming mouse cells (Khaw et al., 2015). Tcl1 has been implicated in the activation of Akt and appears to be a direct target of Oct4 (Matoba et al., 2006). Its expression is rapidly downregulated during differentiation of mESCs (Glover et al., 2006), although it is not required for blastocyst formation or postimplantation development (Narducci et al., 2002). The role of Tcl1 in mitochondrial remodeling during pluripotency therefore remains unclear. In a recent study, it was reported that the reprogramming factor Lin28 represses expression of OxPHOS genes by binding to their mRNA (Zhang et al., 2016b). The AMPK-mTor signaling axis also seems to play an essential role in iPSC formation by regulating the mitochondria clearance through autophagy (Ma et al., 2015).

A gene expression signature indicative of a metabolic switch has been observed in mouse cells directly derived from *in vivo* ICM and post-implantation embryos (Zhou et al., 2012). It is reasonable to speculate that such a metabolic shift would also occur in the corresponding cells *in vivo* and, if so, that it must confer some kind of benefit to the pluripotent cells. Although reduction of mitochondrial ETC complex IV activity has previously been shown to associate with pathological cases, the developing pluripotent stem cell may be able to harness this reduction to its benefit as a way to protect against oxidative stress (Greer et al., 2012). Interestingly, work in *C. elegans* and more recently in mouse has revealed beneficial effects of low ETC complex IV levels at the cellular, as well as organismal, level (reviewed by Wang and Hekimi, 2015). Although it is well established that the relatively inert mitochondria observed in primed cells rapidly change to highly respiring mitochondria as development proceeds, it is not yet understood how and why the primed, postimplantation stage pluripotent cells reduce their mitochondrial activity, and how, in turn, the mitochondrial activity is increased again as these cells differentiate (Fig. 3B).

Mitochondria also generate ROS, which can act as a signaling molecule to control processes such as proliferation, differentiation and adaptation to stress through HIF1 activation (Schieber and Chandel, 2014; Yun and Finkel, 2014). Interestingly Martínez-Reyes et al. (2016) have shown that mitochondrial membrane potential, through the production of ROS, is essential for HIF1 stabilization. This is a potentially interesting link to pluripotency because, during early reprogramming, ROS levels increase and HIF1 stabilization is observed. Similarly, as HIF1 is stabilized in primed, but not in naïve, ESC stages, it would be interesting to test any potential differences in ROS levels between these pluripotent stages. These data reiterate the complexity of the system.

Metabolites regulate the epigenetic landscape of pluripotency

One-carbon metabolism and acetyl-CoA

Metabolites may play a bigger role in regulating cell fate and development than previously appreciated. The primary mode through which this occurs appears to be via the modification of epigenetic marks that, in turn, modify gene expression (see Fig. 4 and Table 1) (reviewed by Harvey et al., 2016). In mESCs, threonine and S-adenosyl methionine (SAM) metabolism are coupled, resulting in regulation of histone methylation marks due to the action of SAM as a substrate for methyl transferases (Shyh-Chang et al., 2013). Methionine and SAM are also required for the self-renewal of hESCs, because depletion of SAM leads to reduced H3K4me3 marks and defects in the maintenance of the hESC state (Shiraki et al., 2014). SAM, the substrate for the methyl transferases, is therefore a key regulator for maintaining the undifferentiated state of ESCs and for regulating their differentiation. SAM is also present at high levels in iPSCs (Panopoulos et al., 2012).

SAM levels, which can be controlled by the metabolic enzyme nicotinamide N-methyltransferase (NNMT), are crucial during the naïve-to-primed hESC transition, where the epigenetic landscape changes through increased H3K27me3 repressive marks (Sperber et al., 2015). H3K27me3 marks are generated by polycomb repressive complex 2 (PRC2), which consists of many proteins, including Suz12, EED and the methyl transferase EZH2 (Vizan et al., 2015). NNMT consumes SAM in naïve cells, making it unavailable for histone methylation. Histone methylation, specifically H3K27me3, further regulates the key signaling pathways that are important for the metabolic changes necessary for early human development (Sperber et al., 2015; Xu et al., 2016). However, whereas NNMT is known to regulate the substrate levels for the PRC2, the factors that regulate the positional control of this methylation and its function in pluripotency have not yet been identified.

In hESCs, glycolysis has been shown to produce, in addition to lactate, acetyl-CoA. Blocking acetyl-CoA production has been shown to cause a loss of pluripotency, while preventing acetyl-CoA consumption causes delays in differentiation (Moussaieff et al., 2015; Shyh-Chang and Daley, 2015). As histone deacetylase inhibition has previously been shown to elicit an evolutionarily conserved self-renewal program in ESCs (Ware et al., 2009), these findings suggest that at least one of the reasons for the strict requirement of glycolysis in primed hESC may be to the need to generate acetyl-CoA, which in turn can regulate ESC histone acetylation (Fig. 4), thereby regulating chromatin structure. In support of this hypothesis, Moussaieff et al. (2015) have shown that during the first 24 h of ESC differentiation, a metabolic switch takes place: pyruvate becomes fully oxidized in mitochondria, leading to acetyl-CoA deprivation, loss of histone acetylation and subsequent loss of pluripotency marker expression. The activity of the histone deacetylase sirtuin 1 has also been shown to be crucial for reprogramming (Lee et al., 2012) and regulates mESC pluripotency and embryogenesis (Calvanese et al., 2010; Tang et al., 2014; Zhang et al., 2014) (see Fig. 4).

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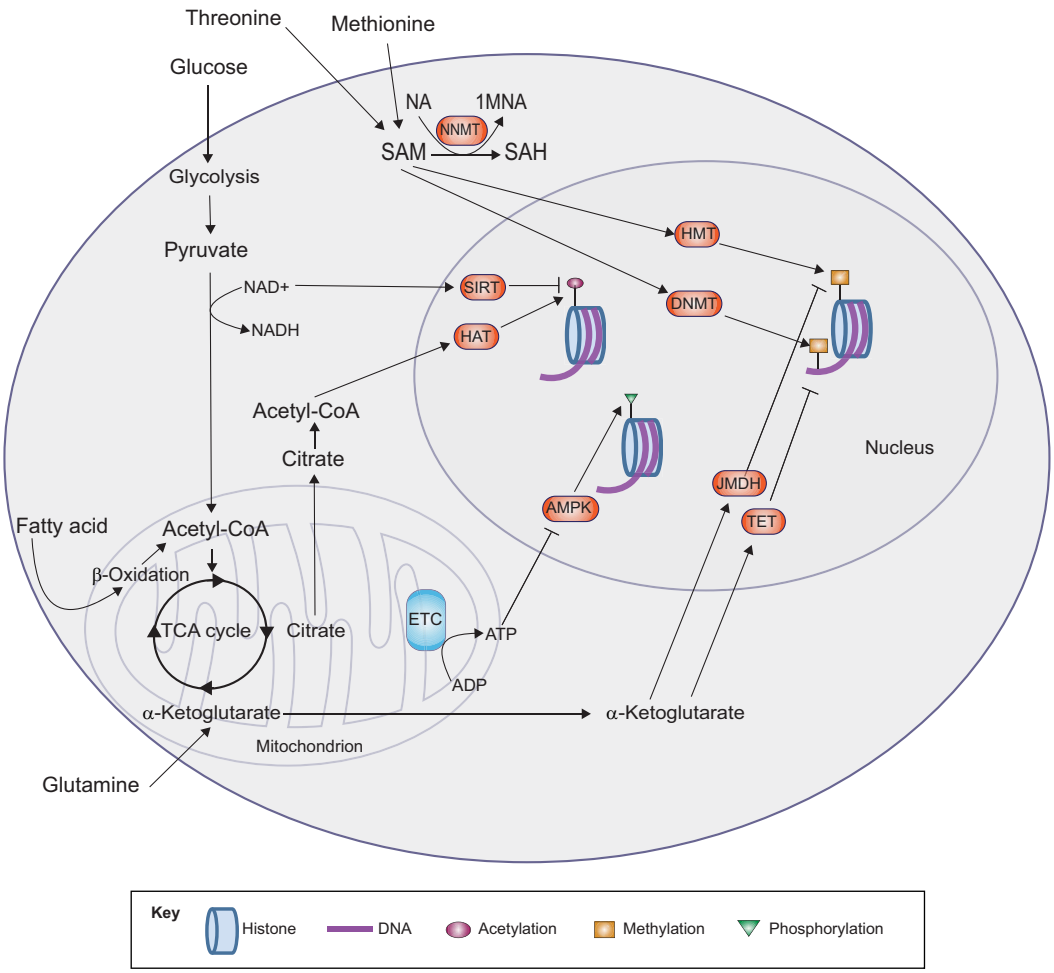

Fig. 4. Metabolite levels influence the epigenetic landscape of ESCs. Cytoplasmic acetyl-CoA acetylates histones via histone acetyltransferase (HAT) activation. NAD+ inhibits DNA acetylation by activating sirtuin 1 (SIRT1). α-Ketoglutarate inhibits histone and DNA methylation by upregulating Jumonji-c domain histone demethylase (JMDH) and Tet methylcytosine dioxygenase (TET). High levels of nicotinamide N-methyltransferase (NNMT) activity prevent histone and DNA methylation by sequestering methyl groups from S-adenosyl methionine (SAM), forming 1-methylnicotinamide (1MNA), which acts as a powerful methyl sink. A depleted pool of methyl groups prevents DNA methyltransferase (DNMT) and histone methyltransferase (HMT) from methylating DNA and histones, respectively. High ATP levels block histone phosphorylation by inhibiting AMP-activated protein kinase (AMPK) activity (Bungard et al., 2010). ADP, adenosine diphosphate; ATP, adenosine triphosphate; ETC, electron transport chain; NA, nicotinamide; NAD+, oxidized nicotinamide adenine dinucleotide; NADH, reduced NAD; SAH, S-adenosyl-L-homocysteine; TCA, tricarboxylic acid.

mESCs also require threonine for growth (Wang et al., 2009; Alexander et al., 2011; Han et al., 2013; Shyh-Chang et al., 2013). This is crucial to supply single carbon equivalents to the folate pool. The folate pool is required for anabolic pathways and for the maintenance of SAM. Furthermore, SAM is an essential substrate for histone methyltransferases (Table 1). In humans, the folate pool is maintained by methionine catabolism to support SAM production (Shyh-Chang et al., 2013). In hESCs, SAM levels are further regulated at an earlier developmental stage, in naïve hESC. Here, SAM levels are reduced by NNMT to maintain low levels of H3K27me3 marks (Sperber et al., 2015).

TCA cycle intermediates regulate the epigenome

Pyruvate is a key metabolite at the junction between cytosolic glycolysis and the mitochondrial TCA cycle (Fig. 4). It has recently been shown that yeast cells regulate pyruvate uptake into mitochondria, and thus its metabolic fate, by expressing alternative pyruvate carrier complexes with different activities (Bender et al., 2015; Rampelt and van der Laan, 2015). In primed pluripotent stem cells, as well as in cancerous cells, pyruvate transfer to the mitochondria is tightly regulated by HIF1α-induced expression of PDK1-3 (Masson and Ratcliffe, 2014; Prigione et al., 2014). When pyruvate enters the mitochondria, it can be fully oxidized in the TCA cycle, or it can be used to produce intermediates, such as oxaloacetate, citrate to generate cytosolic aspartate, and acetyl-CoA, which are then further used to generate pyrimidine and fatty acids (Boroughs and DeBerardinis, 2015). Based on recent findings (Wellen et al., 2009; Moussaieff et al., 2015), it has been suggested that the decision of whether to undergo pyruvate catabolism through an incomplete TCA cycle (cataplerosis, generating cytoplasmic acetyl-CoA) versus through full mitochondrial-based oxidation is a tightly regulated process (Martínez-Reyes et al., 2016). Cytosolic acetyl-CoA can also be used as a substrate for protein acetylation by histone acetyltransferases that regulate histone acetylation (Wellen et al., 2009; Carey et al., 2015), and is thus an important mediator of the epigenetic landscape and, in turn, gene expression and cell fate. In ESCs, pyruvate is incompletely oxidized in the TCA cycle to citrate, which is then transported to the cytoplasm and converted to acetyl-CoA (Wellen et al., 2009; Moussaieff et al., 2015). Acetyl-CoA is further used for H3K9 and H3K27 acetyl mark deposition.

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Table 1. Different metabolites affect epigenetic enzymes, epigenetic marks and cell fate change in pluripotent stem cells

Metabolic pathway	Metabolite	Metabolite-activated enzyme	Epigenetic consequence	Cell fate change	Species	Reference
1 carbon metabolism	SAM	HMT	Histone methylation (H3K4m3)	Maintenance of pluripotency	Mouse Human	Shyh-Chang et al. (2013) Shiraki et al. (2014)
		DNMT (DNMT1, DNMT3A, DNMT3B)	DNA methylation	Maintenance of pluripotency	Human	Shiraki et al. (2014)
		NNMT	Reduced histone methylation (H3K27me3, H3K9me3)	Transition between pluripotent states	Human	Sperber et al. (2015)
TCA cycle	α-Ketoglutarate	HDM (UTX, JMJD)	Histone demethylation (H3K27me3, H4K20me3)	Maintenance of pluripotency	Mouse	Carey et al. (2015)
		TET (Tet1, Tet2)	DNA demethylation	Maintenance of pluripotency	Mouse	Carey et al. (2015)
TCA cycle	Acetyl-CoA	HAT	Histone acetylation	Maintenance of pluripotency	Mouse Human	Moussaieff et al. (2015)
Glycolysis	NAD+/NADH	HDAC (SIRT1)	Histone deacetylation	Acquisition of pluripotency, maintenance of pluripotency	Mouse Human	Lee et al. (2012) Calvanese et al. (2010) Tang et al. (2014) Zhang et al. (2014)

DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, hstone deacetylase; HDM, histone demethylase; HMT, histone methyltransferase; JMJD, Jumonji-c domain histone demethylase; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NNMT, N-methyltransferase; SIRT1, sirtuin 1; SAM, S-adenosyl methionine; TCA, tricarboxylic acid; TET, Tet methylcytosine dioxygenase; UTX, ubiquitously transcribed tetratricopeptide repeat, X chromosome.

Inhibition of this conversion reduces histone acetylation, reducing stemness and increasing myogenic differentiation (Bracha et al., 2010). By contrast, culturing stem cells in ample amounts of acetone delays differentiation (Moussaieff et al., 2015). Another intermediate of TCA cycle, α-ketoglutarate, that can promote histone and DNA demethylation, has shown to maintain pluripotency in mouse ESCs and accelerate initial differentiation of hESCs (Carey et al., 2015; Hwang et al., 2016; TeSlaa et al., 2016; Zhu et al., 2017) (Fig. 4).

Taken together, these data suggest that differences in the availability of metabolites between pluripotent stages and during the loss of pluripotency may regulate epigenetic dynamics and metabolic signaling (Fig. 4; Table 1). The key goals now are to identify how the level of metabolic flux regulates epigenetic changes and the key target genes that are affected by this process. Dissecting the mechanism of metabolic-epigenetic interactions during the naïve-to-primed hESC transition will provide information regarding the pathways that are affected as stem cells develop, and should help us not only to understand and identify different stem cell stages, but also to control them.

Future perspectives

This review discusses the newly identified functions of metabolites in the epigenetic control of cellular state and fate. In particular, it has become clear that pluripotency is very sensitive to metabolic flux, most likely because different metabolites can regulate the epigenetic landscape, which in turn affects gene expression. The main states of pluripotency that we have discussed throughout this Review are naïve and primed; however, recently an additional state has been hypothesized to exist between naïve and primed, called 'formative' pluripotency (Smith, 2017). In addition, diapause, which is a facultative delay before uterine implantation (Renfree and Shaw, 2000), represents another state of pluripotency that has recently been captured *in vitro* and is referred to as 'paused' pluripotency (Bulut-Karslioglu et al., 2016; Scognamiglio et al., 2016; Boroviak et al., 2015). The metabolic profiles of these states remain to be fully defined, although, interestingly, paused pluripotency is associated with a reduction in mTOR activity, a key regulator of cellular metabolism both *in vivo* and *in vitro* (Bulut-Karslioglu et al., 2016). It will be interesting to see how the metabolic profile of these different pluripotency states will fit into the context of metabolic change during the transition through pluripotency *in vivo*, as well as between pluripotent states *in vitro*.

The challenge now in the field is to understand how metabolic switches are regulated at the right time to initiate the correct epigenetic changes and gene expression required for cell-fate decisions. Interestingly, environmental factors such as nutrient availability may play an important role in this process, giving hope that in the future it may be relatively straightforward to manipulate metabolic flux and thus cell fate in both normal and pathological states. Further metabolic analysis of cells and their environment is essential for such progress. Another challenge in the field is to determine unequivocally whether the leading cause for cell fate change in a given context is metabolic remodeling, rather than simply being associated with it. A recent study from Zhu et al. highlights the intricate interplay between cellular metabolism and epigenetics (Zhu et al., 2017). Here, the authors showed that a histone regulator complex, together with a protein called prohibitin (PHB), control chromatin architecture at the promoters of metabolic genes, and that this function is essential for maintaining pluripotency in hESCs. A key strategy for teasing out the complexity of the interplay between metabolism and epigenetics will be to perform more-detailed, single-cell metabolite analysis together with gene expression and epigenetic analyses

Recent findings have emphasized the importance of metabolic switches in normal cellular differentiation and organismal development (Bracha et al., 2010; Yanes et al., 2010; Folmes et al., 2011; Greer et al., 2012; Panopoulos et al., 2012; Rafalski et al., 2012). But metabolic remodeling is not only a feature of early development, it is also a major hallmark in disease pathology. Understanding how this phenomenon occurs in normal development may help us understand how metabolic remodeling takes place in pathological situations and, in turn, how it can influence disease progression. Although metabolic changes are

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inarguably important during cellular development, more work is still required to resolve the issue of whether metabolic changes are driving the cell-fate changes or vice versa.

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Competing interests

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