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Concise Review: Energy Metabolites: Key Mediators of the Epigenetic State of Pluripotency

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ABSTRACT

Recent studies suggest that the metabolic network is an important part of the molecular circuitry that underlies pluripotency. Of the metabolic pathways that were implicated in the pluripotency balance, "energy" metabolism is particularly notable. Its mechanism of action on pluripotency-regulating genes has been partially elucidated when three metabolites, namely acetate, *S*-adenosylmethionine, and *O*-linked β-*N*-acetylglucosamine were recently shown to link cytosolic signals to pluripotent gene expression. The cytosolic levels of these metabolites are the result of environmental perturbations, making them sensitive messengers, which are assumed to diffuse through the nuclear pores, being small molecules. Recent work also suggests that the modulation of the levels of these metabolites in pluripotent cells controls the balance between pluripotency and early commitment via epigenetic modifications. Here, we review recent studies that link metabolism and pluripotency via epigenetic modifications that occur through these three metabolites. STEM CELLS *2015;33:2374—2380*

INTRODUCTION

The metabolome (from the Greek *metabolē*: for "change") consists of small molecules tightly related by a net of complex biological pathways. As metabolic processes take part of every biological event, the metabolome has provided a means for readout of the state of a cell. Characterization of metabolic events that regulate stem cell pluripotency has important implications on our understanding of early embryo development and consequently congenital pathologies. In fact, the optimization of the metabolic environment to be favorable for early embryo development is currently at the core of assisted conception programs [1]. Embryo implantation potential correlates well with blastocyst gene expression profile [2–4], suggesting that embryonic molecular circuitry is critical for successful implantation. Pluripotent stem cells (PSC), either embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC), constitute a prominent model for cell self-renewal and differentiation and for the study of cellular events that are involved in early differentiation. The roles of specific metabolic processes, especially aerobic glycolysis, in stem cell plasticity have been suggested by several recent studies [5–13]. Despite the profound insights provided by recent studies and extensive efforts in the field, the role of cellular metabolism in regulating cell proliferation and differentiation remains poorly understood [11, 14]. Recently, however, we and others demonstrated that the metabolic profile of a cell is not only an end-product of cellular circuitry but also a driving force for differentiation [13]. Accumulating evidence also points to shifts in the epigenetic landscape of PSC as mediators between metabolic signals and differentiation. Here, we will focus on the link between metabolism and epigenetic modifications in PSC that regulate differentiation, while underscoring the main metabolic junctions in the pluripotent-specific metabolic network. Given the potential of nongenetic chemical manipulations of PSC specification, identification of metabolic junctions and metabolic pathways may be of great value in future regenerative medicine. The physiological aspects of mitochondria metabolism will not be addressed, as these have been extensively discussed by others elsewhere [6–8, 15–17].

GLYCOLYSIS

Somatic cells normally metabolize glucose by further oxidation of glycolytic pyruvate in the mitochondrial tricarboxylic acid cycle and the electron transport chain to synthesize ATP, a process known as oxidative phosphorylation (OxPhos). In the absence of oxygen, pyruvate may be converted into lactate for anaerobic glycolysis. A similar shift occurs in PSC, which

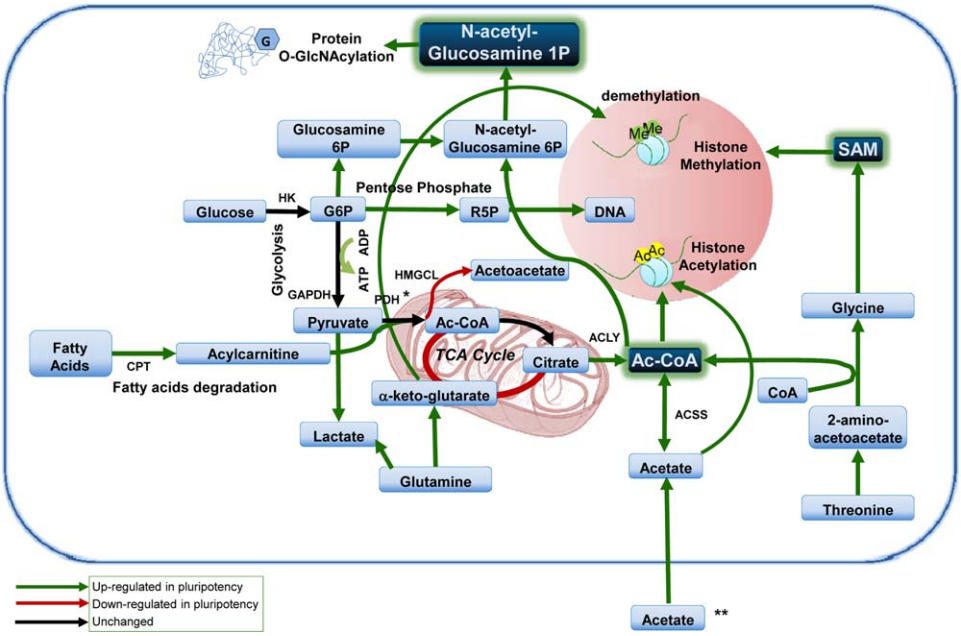

Figure 1. Scheme of metabolic pathways that regulate pluripotency via epigenetic modifications. * The data on PDH is controversial. While in some previous works blockage of PDH retained pluripotency, in our recent study, we observed a twofold lower expression of PDH after 48 hours of differentiation. ** Many metabolites are present in PSC extracellular medium (such as sugars, amino acids, fatty acids, peptides, and other small molecules). Extracellular acetate is representative of such metabolites. Abbreviations: ACLY, ATP citrate lyase; ACS, acyl CoA synthetase; ACSS, acetyl-coenzyme A synthetase; CAT, carnitine-acylcarnitine translocase; CPT, carnitine palmitoyltransferase; HK, hexokinase; HMGCL, HMG-CoA lyase; HMGCS, HMG-CoA synthase; PDH, pyruvate dehydrogenase; SAM, *S*-adenosyl methionoine.

unlike differentiated cells, rely on aerobic glycolysis for energy, decoupling glycolysis and OxPhos (Fig. 1). Already in the early 1980s, changes in the uptake of pyruvate and glucose by preimplantation embryo were observed [18]. OxPhos metabolism during preimplantation shifts to one dependent on aerobic glycolysis at the blastocyst stage [19]. Indications for differences between the glycolytic fluxes in somatic cells and PSC were then provided by two groups: Kondoh et al. uncovered high activity of different glycolytic enzymes, and low mitochondrial oxygen consumption, while Chung et al. linked mitochondrial oxidative metabolism to cardiac differentiation of ESC [20, 21], as well as in the more studied cancer cells. The changes in glycolytic fluxes were correlated to the proliferative capacity of murine ESC (mESC). Such elevation in glycolytic flux and a shift toward lactate production have been the subject of long lasting intensive scientific effort in cancer cells [22, 23], and are commonly referred to as aerobic glycolysis, or the "Warburg" effect, after Otto Warburg, who described it in the 1920s. Following the switch from OxPhos to aerobic glycolysis, the glycolytic process loses efficiency, yielding 2 ATP molecules instead of 32–34 per one molecule of glucose. The sharp decrease in energy efficiency caused by the glycolytic switch raises the question of the advantages offered by this energetic shift, but as aerobic glycolysis is much faster than OxPhos, it yields more molecules of ATP per minute. A high glycolytic flux will also result in increased biosynthesis of macromolecules, from nucleotides, amino acids, and fatty acids, required for rapidly proliferating cells [22]. It has thus been suggested that the Warburg effect is related to high energy demand due to high rate of cell proliferation [22]. The increase in the production of ATP and macromolecules would be altogether advantageous to highly proliferating cells, such as cancer cells and PSC.

In PSC, the Warburg effect seems to have further roles, as demonstrated by several groups. Zhu et al. reported that modulation of cell metabolism from mitochondrial oxidation to glycolysis plays an important role in nuclear reprogramming [12]. In agreement with these findings, the metabolic profile of iPSC, similar to that of ESC, shows a decrease in OxPhos and increase in aerobic glycolysis [24–27]. Moreover, stimulation of glycolysis promoted, while blockade of glycolytic enzyme activity blunted, reprogramming efficiency in mouse and human iPSC [12, 28]. By use of pharmacologic inhibitors, these studies point to the involvement of the pyruvate dehydrogenase (PDH) complex in the modulation of pluripotency. Dichloroacetate (DCA), a PDK1 inhibitor, significantly reduced [28], nuclear reprogramming efficiency, suggesting that metabolic intervention may regulate nuclear reprogramming. Indeed, the marked differences in the glycolytic flux between PSC and differentiated cells may provide a golden opportunity for the mass-production of highly purified cell populations. As a matter of fact, when PSC were cultivated in glucose-depleted culture medium containing abundant lactate, only cardiomyocytes efficiently survived [29], suggesting that metabolic redirection could become a powerful tool in the use of PSC for regenerative medicine.

Recently, we demonstrated a metabolic shift from aerobic glycolysis to OxPhos that takes place during the first hours of differentiation [13]. High-resolution NMR analyses of intracellular and extracellular metabolome of undifferentiated

human ESC versus early committed ones pointed to acetate and lactate as discriminatory metabolites. Importantly, supplementation of acetate to cell medium retained cell pluripotency and could even redirect committed PSC to dedifferentiate [13]. These data suggest that the shift from aerobic glycolysis to OxPhos is not an end product, but a driving force of differentiation. Acetate is in equilibrium with acetyl Coenzyme A (acetyl CoA), a product of glucose and lipid metabolism. Inhibition of glycolysis upstream of acetyl CoA drove PSC to differentiate, while inhibition downstream of acetyl CoA (decreasing its consumption) retained pluripotency [13]. Furthermore, specific inhibition of ATP citrate lyase (ACLY) and the biosynthesis of acetyl CoA from citrate in hESC resulted in early differentiation. The increased aerobic glycolysis and low oxidative phosphorylation in PSC were attributed to inactivation of PDH [27] or the expression of uncoupling protein 2 (UCP2) [15]. In line with these studies, our recent results show a similar decrease in glycolysis during the first hours of differentiation. This shift, however, was accompanied by a minor difference in UCP2, and increased PDH beta (PDHB) expression in PSC, suggesting that a different mechanism might be responsible for the lack of oxidative phosphorylation in pluripotent cells.

Inhibition of PDK redirects pyruvate from lactate to acetyl CoA in both cancer and PSC. However, while in PSC, PDK inhibition retained pluripotency, with no effect on cell viability [13], such a shift is lethal for cancer cells [30]. This suggests a metabolic difference between cancer cells and PSC that is yet to be discovered. The seeming contrast between these recent results and a previous study by Folmes et al., that demonstrated that treatment with DCA blocked reprogramming to iPSC [28], may imply an asymmetric effect of PDK inhibition on the axis between differentiation and reprogramming.

The differentiation of naive mESC to primed ones involves a glycolytic switch that resembles the one in early differentiation of human ESC (hESC), and is mediated by HIF1α [31]. In addition, Carey et al. demonstrated a specific metabolic signature to PSC in naive pluripotent state [32]. To mimic naive pluripotency state, they used a medium that contains GSK-3β and MAPK/ERK inhibitors (2i)/leukemia inhibitory factor (LIF), (2i/L). For a more committed ES phenotype, cells were kept in serum/LIF containing medium. The rewiring of cellular metabolism by GSK-3β and MAPK/ERK inhibition resulted in a shift of glucose and glutamine metabolism and the accumulation of αKG, regulating αKG-dependent dioxygenases. These enzymes then favor demethylation of repressive chromatin marks such as DNA methylation and H3K9me3, H3K27me3, and H4K20me3. The authors report a robust proliferation of cells cultured in 2i/L, but not S/L. αKG/succinate ratio also contributed to the ability of ESCs to express NANOG-GFP as a marker for differentiation suppression. These results shed light on an important piece of pluripotent-specific metabolism. One of the questions that still needs clarification is the relationship between the effect of the αKG/succinate ratio on cell proliferation and on differentiation. The effect of metabolic pathways on specific methylation sites is highly intriguing, and has important implications.

Amino Acid Metabolism

Amino acid turnover is highly correlated to human embryo developmental capacity [33]. Meissen et al. found a sharp decrease in amino acid levels in iPSC following reprogramming, which was not specific to a particular amino acid subclass or biosynthetic pathway [34]. This decrease in the accumulation of amino acids in iPSC may be partially explained by a rapid turnover in PSC, in line with the rapid decrease in the consumption of essential amino acids during differentiation into definitive endoderm [35]. It may be assumed that the low levels of amino acid levels detected in iPSC and the high rate of consumption of these cellular building blocks are due to the high proliferation rate characteristic of these cells. However, the role of the PSC-specific amino acid metabolism is not limited to high proliferation rates, as it provides important support to the pluripotency state. When examining which amino acids are critically required for pluripotency in mESC, threonine was the only amino acid that was found to be essential [26, 36]. The dependence of mESC pluripotency on threonine was attributed to a coupling of the threonine and *S*-adenosylmethionine (SAM) pathways in PSC, resulting in regulation of histone methylation. Threonine catabolism forms glycine, which is then further metabolized into SAM (Fig. 1). The degradation of threonine to glycine is actually a deacetylation, which results in the acetylation of coenzyme A to acetyl CoA. While glycine metabolism was rightfully given a great deal of attention, a complementary mechanism explaining the dependence of pluripotency on threonine could now be provided by increased acetyl CoA levels leading to histone acetylation. This hypothesis, that would agree with our finding on the involvement of acetyl CoA metabolism in pluripotency [13], needs experimental validation.

Lipid Metabolism

In the first comprehensive metabolic profile of ESC, Yanes et al. identified a decrease in the accumulation of unsaturated lipids upon differentiation of mESC to neurons or cardiomyocytes [37]. The authors describe a change in redox state during differentiation, and suggest that lipid oxidation pathways and oxidation of cellular metabolome are driving differentiation to neurons and cardiomyocytes. This hypothesis is consistent with the induction of the expression of fatty acid oxidation genes following cardiomyocyte differentiation [38] and with promotion of cardiomyogenesis by agonists of peroxisome proliferator-activated receptor α [39]. In some contrast to this hypothesis, however, the authors present increased levels of saturated free fatty acids and their acyl-carnitines in mature cell populations [37]. A possible explanation for this seeming discrepancy may be that the products of fatty acid oxidation, especially acetyl CoA, are important for maintaining pluripotency, hence this oxidation is tightly regulated and repressed in PSC. Despite the production of acetyl CoA via fatty acid oxidation, we found no indication of fatty acid oxidation and biosynthesis involvement in the balance between pluripotent and early differentiating hESC. This lack of effect may be a result of the relatively low metabolic flux of fatty acid oxidation in cultivated hESC [13].

Nucleic Acid Metabolism

Meissen et al. reported no difference in pentose phosphate pathway in reprogrammed mouse fibroblasts [34]. In contrast to these findings, many of the metabolites identified by

Panopoulos et al. showing decreased levels upon reprogramming, were nucleotides [25], suggesting that the pentose phosphate pathway metabolism may be involved in pluripotency circuitry and deserves a closer examination in the future.

The Epigenetic Landscape: Linking Metabolism to Pluripotency

ESC demonstrate unique epigenetic features, which are involved in the regulation of cell pluripotency. These include decondensed chromatin as well as hyperdynamic association of chromatin proteins with DNA [40], and are mediated by histone acetylation and the methylation of histones or DNA. It is becoming clear that metabolic processes provide substrates for chromatin modifications, thus propelling epigenetic changes in the cell. The link between metabolic shifts and the regulation of epigenetic landscape has drawn great attention in recent years, especially in cancer research [41–50]. The central role this link plays in development and especially in cell differentiation has also been suggested [51], and more work focused on the epigenetic link between metabolism and cell differentiation in PSC has recently been published [11, 13, 26, 52].

Histone Acetylation

In the 1970s, Riggs and colleagues showed that induction of differentiation of Friend erythroleukemic cells into hemoglobin-synthesizing normoblast-like cells by *n*-butyrate was accompanied by histone hyperacetylation [53, 54]. Studies that followed this work suggested that small molecules targeting histone acetylation can regulate cell phenotype and differentiation [55, 56].

Histone acetyltransferases (HATs) transfer the acetyl group of acetyl-CoA to the lysine residues of histones and catalyze histone acetylation. The availability of acetyl-CoA is a major metabolic input into histone acetylation and the activities of HATs are likely sensitive to the fluctuation of intracellular acetyl-CoA levels [41]. In cells where ACLY was knocked down, there was a profound defect in histone acetylation [49]. Further studies suggest that acetyl CoA necessary for histone acetylation is a product of nuclear PDH complex, forming acetyl CoA from pyruvate [57] or the activity of acetyl CoA synthetase (ACS), forming Acetyl CoA from acetate and CoA [49, 58–60]. The presence of endogenous ACLY, ACS, and PDH in the nucleus as well as the cytoplasm suggests that the acetyl CoA used for histone acetylation is synthesized in situ. These findings imply that substrates of acetyl CoA biosynthesis, such as acetate, assumed to diffuse freely through the nuclear pore complex [49, 61], mediate between cytoplasmic signaling pathways and nuclear reprogramming. Free cytoplasmic acetate has been shown to incorporate to histones [60] and was implicated as a critical metabolic hallmark of tumors [62], specifically glioblastoma [63]. The metabolic support of acetate to tumorigenicity was attributed to the regulation of histone acetylation [63]. Our recent analyses identified acetate as a discriminatory metabolite in early differentiation of hESC [13]. We showed that the addition of acetate to hESC medium retained pluripotency in a dose-dependent manner, concomitant to increased histone pan-acetylation. These results suggest that in PSC, the acetate pool provides a cellular reservoir that is regulated by metabolic shifts, especially glycolysis. Our work also suggests that acetate is a paracrine signaling molecule in PSC for histone acetylation and the downstream changes in gene expression. One implication of such insight is that acetate could thus become a cost-effective soluble factor to improve the cultivation of PSC in vitro. Importantly, metabolic signals may also be involved in histone deacetylation [41], and such involvement should be pursued in future studies.

Pluripotency can be defined as two phases: "naïve" and "primed" states, the latter being further advanced on the path of differentiation. Naive and primed ESC will be used as a new and promising cell model of the preimplantation and postimplantation embryo, respectively [64–66]. Moreover, because of their potential in regenerative medicine, reprogramming PSC into a more naive-like state could be of great value. Indeed, the shift from naive to primed pluripotency state in mESC involves changes in glucose and glutamine metabolism that result in a rewiring of histone methylation patterns [11, 31].

Histone and DNA Methylation

While DNA methylation causes gene repression through its ability to affect factor binding and chromatin structure [67], histone methylation can be associated with either transcriptional repression or activation [68]. In some cases, the methylation of one site of histone impairs the methylation of another one [69]. Histone lysines can be methylated by monomethylation, dimethylation, or trimethylation [70], further extending the indexing potential of this modification. At the time of implantation, almost all methylation is erased and a new pattern is reestablished [67]. Accordingly, extensive DNA methylation characterizes the exit of naive pluripotency state in ESC [64, 71]. These histone modifications are recognized by specific chromatin-binding proteins, which locally orchestrate the gene expression machinery. The cellular methyl donor in mammals is SAM, synthesized from methionine and ATP. SAM is a metabolic junction of the one carbon metabolism pathway, crossing the folate and methionine pathways [72]. Transfer of the methyl unit from SAM results in the production of *S*-adenosylhomocysteine. A groundbreaking study by Shyh-Chang et al. couples the metabolism of threonine and SAM in PSC, resulting in regulation of histone methylation [26]. Threonine is a major substrate of both cellular glycine and acetyl-CoA. The authors demonstrate that threonine levels regulate the trimethylation of histone H3 lysine 4 (H3K4me3) via the formation of SAM, thus controlling growth and differentiation. It would be interesting to see whether one more branch of the threonine catabolism in PSC leads to the formation of acetyl CoA, and increased histone acetylation, providing further epigenetic support to the pluripotency state.

Very recently, another important missing piece of the metabolic puzzle that regulates epigenetic landscape and concomitant pluripotency has been unveiled, as it appears that naive ESC maintain a high level of intracellular α-ketoglutarate (αKG) by degradation of glucose and glutamine. These cells exhibit an elevated αKG to succinate ratio that promotes histone/DNA demethylation and maintains pluripotency. Intracellular αKG/succinate ratios regulate αKG-dependent dioxygenases. Through the regulation of these enzymes, cellular metabolic state influences chromatin methylations, such as H3K27me3

and ten eleven translocation (Tet)-dependent DNA demethylation, which contribute to the regulation of pluripotency-associated gene expression [11]. In line with these findings, supplementation with αKG supports mESC self-renewal while cell-permeable succinate promotes differentiation. In vivo, DNA methylation in mice is affected by nutrition [73], suggesting that metabolites mediate modifications in the DNA methylation pattern by the environment.

O–Linked *N*–Acetylglucosaminylation

Unlike methylation and acetylation that modify Lys and Arg, *O*-GlcNAcylation involves Ser and Thr residues, adding a layer of complex metabolic regulation of epigenetic modifications while not competing for sites. An addition of a sugar moiety to serine/threonine residues of proteins is catalyzed by *O*-GlcNAc-transferase (OGT) in the presence of Uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), removed by *O*-GlcNAcase, and is dependent on glucose concentration. Histones are subjected to *O*-GlcNAc modifications which regulate their function [74, 75]. *O*-GlcNAcylation decreased in differentiating mESC. Increased levels of *O*-GlcNAc restricted the PSC differentiation and attenuated the reprogramming of somatic cells to iPSC [52, 76], while blocking of *O*-GlcNAcylation disrupts self-renewal. The core reprogramming factors Oct4 and Sox2 are also temporarily *O*-GlcNAcylated in ESC. The effect of *O*-GlcNAcylation may be direct on reprogramming factors, as elimination of this *O*-GlcNAc modification reduces the capacity of Oct4 to maintain ESC self-renewal and reprogram somatic cells [77]. However, the *O*-GlcNAcylation of histones is probably also involved in the regulation of pluripotency as inhibition of *O*-GlcNAc hydrolase upregulates genes that are normally epigenetically silenced in ESC, impairing mESC differentiation [52]. Acute activation of AMPK alters the substrate selectivity of OGT and the nuclear localization of OGT, while *O*-GlcNAcylation of the γ1-subunit increases with AMPK activity, and inhibition of *O*-GlcNAc cycling disrupts activation of AMPK, suggesting a crosstalk between these two regulating pathways [56].

Conclusion and Perspectives

Cellular metabolism is emerging as an essential sensor of stem cell state. Recent studies have established a pluripotent-specific metabolic state, which shares many characteristics with the cancer cell metabolic profile, such as the Warburg effect. By now it is clear that the metabolic changes during PSC commitment form an important segment of the molecular circuitry that drives differentiation. Given the prominent change in central carbon metabolism, the metabolic shift during differentiation was considered as a change in energy metabolism. Importantly, however, this change in energy metabolism results in the formation of substrates for epigenetic modifications, thus regulating the balance between pluripotency and early differentiation (Fig. 1). Further understanding of the mechanism by which metabolic shifts control the pluripotency state will deepen our understanding of the molecular circuitries that are involved in pluripotency. It now appears that the metabolic changes that were so far identified in fully differentiating PSC are in fact changing at early stages of differentiation and play a critical role in the exit of pluripotency. It would be of great interest to see how these metabolic changes and others affect later stages of cell specification and the final cell fate. Such insights will have far reaching implications on the use of PSC with minimal genetic intervention. The transient state of a stem cell largely depends on its microenvironment. Our observation that exogenous acetate supports pluripotency and even dedifferentiates committed PSC suggests that it is a mediator between the microenvironment and intracellular pluripotency factors [13]. Thus, using exogenous metabolic signals, a cell can adapt rapidly to a novel environment. Thus, a change of extracellular metabolite concentration by a single cell could influence both the metabolism and the epigenetic state of surrounding cells. Such regulation could partly explain non-cell autonomous and bystander effects found in vivo. Of interest, it has been shown recently that acetate is a major metabolic fuel of cancer cells in vivo [63] and that deprivation of acetate could provoke tumor asphyxia.

The construction of the metabolic network that is responsible for epigenetic modifications would greatly benefit our understanding of cellular control of the epigenetic landscape. An important step toward this goal would be the characterization of the metabolic balance between histone methylation and acetylation. Within this metabolic network, the metabolic pathways that generate acetyl CoA, all cause nonspecific histone pan-acetylation, and are in consensus for retaining pluripotency, whereas metabolic processes that propel histone methylation are more ambiguous. Thus, while threonine-dependent histone H3K4 trimethylation leads to increased growth and decreased differentiation [26], whereas accumulation of αKG favors demethylation of repressive DNA methylation such as H3K9me3, H3K27me3, and H4K20me3, leading to the suppression of differentiation [32]. A thorough study of the exact mechanisms leading to specific histone methylation/demethylation, and the resulting shift in the pluripotency/commitment balance should shed important light on the molecular reasoning of cell fate decisions. One more aspect of this network that lacks sufficient understanding is the kinetics and reversibility of the metabolic pathways involved in histone modifications and its synchronization with cell fate decision.

Although useful for most molecular studies on self-renewal and pluripotency, PSC do not reflect perfectly the in vivo context. One recent elegant example is the discrepancy between abnormal methylation in PSC at genomic sites that are critical for proper differentiation and these sites in vivo, that fail to modify [78]. Culture conditions, including defined metabolites and oxygen pressure, are very different from what embryonic cells are exposed to in vivo. Therefore, caution should be applied to all the studies on pluripotency in vitro.

As energy metabolic cues are transferred from the environment to cell nuclei, where they regulate histone modifications and the downstream reprogramming factors that govern cell fate decision, it is plausible that energy metabolites signals that transmit environmental changes to the nuclei. It has become clear that these cues cannot be understood at the level of singular metabolites, but rather as a network of metabolic pathways. A systemic view of the metabolic events that control epigenetic modifications and cell fate should therefore provide basic insights into the regulation of developmental and pathological processes. The identification of key metabolic junctions in this network would also provide a means for controlling these processes. Such key junctions are acetyl CoA, SAM, and *O*-GlcNAc. The pathways that go through these

metabolites converge into acetyl CoA, a product of energy metabolic pathways, including glycolysis and threonine catabolism, leading to direct histone acetylation and upregulation of reprogramming factors, producing *N*-acetyl-Glucosamine 6P (to further participate in *O*-GlcNAcylation), or mitochondrial formation of αKG, a cofactor of histone demethylation. Future studies on the environmental regulation of these three metabolic junctions and acetyl CoA in particular, would be of great value in future academic and commercial use of PSC.

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Author Contributions

A.M., N.M.K., and D.A.: manuscript writing and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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