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Nutrients in the Fate of Pluripotent Stem Cells

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Summary

Pluripotent stem cells model certain features of early mammalian development *ex vivo*. Medium supplied nutrients can influence self-renewal, lineage specification, and earliest differentiation of pluripotent stem cells. However, which specific nutrients support these distinct outcomes, and their mechanisms of action, remain under active investigation. Here, we evaluate the available data on nutrients and their metabolic conversion that influence pluripotent stem cell fates. We also discuss key questions open for investigation in this rapidly expanding area of increasing fundamental and practical importance.

eTOC Blurb

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Author contributions

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Declaration of interests

The authors declare no competing interests.

In this Perspective, Lu and Roy et al. examine the data on specific nutrients and corresponding nutrient-sensitive signal transduction pathways that affect pluripotent stem cell fates. They further consider key open questions in the field, including the implications for metabolic regulation of early mammalian development.

Keywords

nutrients; metabolism; pluripotent stem cell; fate; development; differentiation

Introduction

Pluripotent stem cells (PSCs) self-renew or, with specific cues, can differentiate into the three primary germ lineages *in vitro*. A defining feature of pluripotency is remarkable plasticity in cell identity, leading to potential applications in tissue engineering, regenerative medicine, and studies of early embryonic development (Smith, 2017; Tsogtbaatar et al., 2020). In considering the fate of PSCs, we define pluripotent/self-renewing stem cells as cells that express core pluripotency transcription factors including OCT4, NANOG, SOX2, and others, and differentiation as the silencing of core pluripotency transcription factors coupled with the gain of lineage-specifying biomarkers (Yeo and Ng, 2013). Functionally, two principal hallmarks that define PSCs are blastocyst chimerism and teratoma formation, which test *in vivo* ability to re-enter development in a host embryo and spontaneous generation of the three germ layers, respectively (De Los Angeles et al., 2015). Of particular interest here are studies suggesting that supplied nutrients, with or without intracellular processing, have active, guiding roles in PSC identity and cell fate transitions (Intlekofer and Finley, 2019; Shyh-Chang and Ng, 2017; Tsogtbaatar et al., 2020). For example, the metabolic flux of nutrients through glycolysis, the tricarboxylic acid (TCA) cycle, and one-carbon metabolism generates intermediate metabolites used in reactions to modify the PSC epigenome, chromatin structure, and gene expression (Carey et al., 2015; Moussaieff et al., 2015; Shiraki et al., 2014; Shyh-Chang et al., 2013b; TeSlaa et al., 2016).

In addition to studies linking PSC-produced intermediate metabolites with epigenome modifications, many interesting questions remain regarding the role of nutrients, including sugars, amino acids, lipids, and others in the control of PSC fate. For example, recent results suggest that nutrients can also influence PSC fate through mechanisms that do not include metabolic flux through anabolic or catabolic pathways (Chi et al., 2020; Cornacchia et al., 2019; Na et al., 2020; Song et al., 2019; Vardhana et al., 2019; Zhu et al., 2020). One potential interpretation of these studies is that specific nutrients signal conditions in the microenvironment prior to, or concomitant with, triggers that control PSC fate. In this minireview, we examine the contributions of nutrients in PSC fate through both metabolic conversion and nutrient-triggered signaling.

Nutrients and PSCs in Context

Studies of mammalian pre-implantation embryos grown in chemically-defined media showed that certain nutrients alone, without added growth factors, could induce cell identity transitions (Summers and Biggers, 2003). This result suggested that the

presence of specific niche nutrients could initiate PSC fate and not just support a pre-established PSC fate. Additional studies also showed that specific nutrients reinforced pre-established pluripotency/self-renewal and differentiation fates by influencing fate-specifying transcription programs, enzyme-mediated chromatin changes, and/or nutrient-sensitive signaling (Baksh and Finley, 2021). Together, these studies suggest that the same nutrient(s) can either initiate or support PSC fates, with differential effects that depend on context, such as the state of pluripotency, differentiation cues, culture media conditions, and timed nutrient addition or deprivation (Table 1).

Specific nutrient deprivation or addition does not occur naturally *in vivo*, but *in vitro* nutrient changes provide a valuable experimental tool. For example, the timed withdrawal of glutamine from PSC culture medium shows differential effects on PSC fate. Short-term glutamine withdrawal eliminated cells with a high dependence on glutamine for TCA cycle anaplerosis, enriching for surviving mouse embryonic stem cells (mESCs) and human PSCs (hPSCs) with increased expression of pluripotent transcription factors OCT4 and SOX2 (Vardhana et al., 2019). Other studies conversely showed that high levels of glutamine metabolism prevented the degradation of OCT4 and preserved hPSC self-renewal (Marsboom et al., 2016). Long-term glutamine deprivation also caused more oxidative PSCs to die from a reduction in TCA cycle activity and oxidative phosphorylation (OXPHOS) (Tohyama et al., 2016). In this context, glutamine withdrawal provided a strategy to eliminate PSCs and enrich for hPSC-derived cardiomyocytes (Tohyama et al., 2016) or enhance the angiogenic capacity of endothelial cells (Marsboom et al., 2016). These combined, nuanced results suggest that short-term glutamine withdrawal followed by repletion eliminates more oxidative PSCs (Folmes et al., 2011; Moussaieff et al., 2015; Varum et al., 2011) and yields more homogenous PSCs with higher expression of pluripotency transcription factors. Thus, manipulating the timing of glutamine exposure in PSC culture can control whether PSCs or non-pluripotent cells enrich.

Investigators subdivide PSCs into a continuum of states that resemble different gestational stages of blastocyst development, with each state showing specific nutrient requirements and activities (Hackett and Surani, 2014; Smith, 2017). A hypothesized, and recently captured, intermediate “formative” pluripotent state has expanded the dynamic nature of pluripotency (Hoogland and Marks, 2021; Kinoshita et al., 2021; Wang et al., 2021b; Yu et al., 2021). Thus far, only one study investigated the metabolism of formative state PSCs, which upregulate glycolysis and downregulate mitochondrial respiration enzymes (Yu et al., 2021). Reports have also consistently shown that naïve PSCs display bivalent glycolytic and mitochondrial respiration compared to primed PSCs, which are exclusively glycolytic (Hackett and Surani, 2014; Hoogland and Marks, 2021). In usual culture conditions, hPSCs resemble more developmentally advanced, or ‘primed’, PSCs, corresponding to post-implantation epiblast, whereas mESCs resemble less advanced, or ‘naïve’, PSCs, corresponding to pre-implantation epiblast (Hackett and Surani, 2014). Interestingly, differentiation potential varies with glutamine withdrawal for naïve compared to primed PSCs. Maintenance of naïve mESCs requires 2i (MEK and GSK3β inhibitors) plus leukemia inhibitory factor (LIF) media supplementation, which promotes independence from added glutamine (Carey et al., 2015). By contrast, mESCs grown in LIF-containing medium without 2i supplementation are more advanced, with glutamine deprivation causing

increased spontaneous differentiation, mainly into trophectoderm (TE) and mesoderm, and decreased pluripotency transcription factor expression (Carey et al., 2015; Ryu et al., 2015). In primed hPSCs, spontaneous differentiation upon glutamine deprivation inhibits mesoderm and promotes ectoderm lineage development, suggesting a lineage-specific requirement for glutamine (Lu et al., 2019). Together, these reports suggest a context-specific glutamine requirement with different PSC differentiation outcomes based on PSC status. Further studies should reveal whether the species of origin, mouse or human, also contributes to differences in differentiation potential for naïve or primed PSCs with glutamine withdrawal.

Glucose, another key nutrient for mammalian cells, also appears to control PSC outcomes. *Ex vivo* culture with pyruvate, lactate, and glucose as nutrients, without added growth factors or cytokines, initiates pre-implantation mouse embryos through 5 days of development, suggesting embryo self-sufficiency in cell specification with specific nutrients only (Biggers et al., 1997; Nagaraj et al., 2017; Summers and Biggers, 2003). In early mouse embryogenesis, at the compacted 8-cell morula stage, glycolysis is dispensable although glucose is essential (Chi et al., 2020). During the transition from morula to TE and the blastocyst inner cell mass, which is the first fate specification, glucose-dependent signaling directs the formation of extra-embryonic TE, but not the ICM (Chi et al., 2020). These *ex vivo* mouse studies showed glucose import support of pentose phosphate pathway (PPP) nucleotide production and hexosamine biosynthesis pathway (HBP) signaling that activated the TE-specifying transcription factor, CDX2. Culture in glucose-deficient medium showed decreased expression of TE biomarkers with unaffected ICM-specifying OCT4 and NANOG expression (Chi et al., 2020). Thus, earliest cell fate specification shows developmental cues controlled, at least partly, by nutrient availability and nutrient-sensitive signaling.

Mammalian zygotic genome activation (ZGA) triggers increased gene transcription, with metabolism shifting from maternal to embryonic control. During this period, glycolytic flux increases as protein synthesis and metabolic shuttles activate (Gardner, 1998; Gardner and Harvey, 2015; Leese and Barton, 1984; Schulz and Harrison, 2019; Zhang et al., 2018a). These changes add flexibility in nutrient dependence because the developing embryo increases enzymes capable of interconverting nutrients (Sharpley et al., 2020). Later stage development, modeled *in vitro* by PSC-derived tri-lineage germ layer differentiation, loses a requirement for glucose (Chi et al., 2020; Cliff et al., 2017). Initial ectoderm differentiation shifts to glycolysis-dependent, rather than glucose-dependent, metabolism. Inhibition of ectoderm differentiation by blocking upstream steps in glycolysis can be overcome by added galactose or fructose, which enter glycolysis downstream of hexokinase at glucose-6-phosphate or fructose-1-phosphate steps, respectively (Cliff et al., 2017). Glucose oxidation is also not required to sustain pluripotency, as a culture surface embedded with non-degradable glucose enriched for mESCs with elevated pluripotent and low differentiation biomarker expression (Mashayekhan et al., 2008).

Studies indicate that additional nutrients, such as ascorbic acid and amino acids proline and methionine, also regulate pluripotency. Reprogramming of mouse and human somatic cells into iPSCs accelerates with the addition of ascorbic acid by reducing p53 levels to impede cellular senescence (Esteban et al., 2010), by reducing H3K36me3/2 levels through Jhdm1a1/b demethylases (Wang et al., 2011), and by increasing STAT2 phosphorylation,

which increases binding to and activation of the *Nanog* promoter (Wu et al., 2014). In human iPSC colonies, ascorbic acid actively promotes pluripotency and inhibits spontaneous differentiation through enhancement of histone demethylase JARID1 expression (Eid and Abdel-Rehim, 2016). A vast body of work, described elsewhere, provides the unique and varied roles of ascorbic acid in stem cell differentiation (D'Aniello et al., 2017). For example, under cardiac differentiation cues, ascorbic acid promotes cardiac differentiation (Sato et al., 2006; Takahashi et al., 2003) by increasing the proliferation of cardiac progenitor cells through increased collagen synthesis, but only when supplemented to the culture medium during an early time window of differentiation (Cao et al., 2012). While it is clear that ascorbic acid plays a significant role in promoting pluripotency or differentiation depending on different contextual cues, it will be interesting to see whether there are additional ascorbic acid context dependencies, for example, during naïve or primed PSC stages.

Added proline promotes mESC adoption of a primitive ectoderm-like morphology and elevates the expression of genes associated with primitive ectoderm even in culture medium designed to sustain mESCs (Washington et al., 2010). Proline import is through the amino acid transporter SNAT2. Inhibition of SNAT2 with competitive substrates blocks proline transport, maintains mESC pluripotency, and impairs spontaneous embryoid body (EB) tri-lineage differentiation (Tan et al., 2011). Concordantly, the level of SNAT2 increases in mESCs prior to primitive ectoderm development (Tan et al., 2016), suggesting a role for proline in pluripotency exit. Proline supplementation also promotes the differentiation of naïve mESCs into more mature epiblast-like stem cells (EpiSCs) (Casalino et al., 2011). This proline-dependent induction of mESCs into EpiSCs also shows features of the blastocyst to epiblast transition during mouse embryo implantation (Casalino et al., 2011). Implantation involves the degradation of a proline-rich extracellular matrix, raising the possibility that this process makes proline available to support an epiblast transition (McEwan et al., 2009).

Like proline, methionine has a role in pluripotency and differentiation. In hPSCs, SAM-supported histone methylation relies on methionine and not threonine metabolism because TDH is a nonfunctional pseudogene. (Shiraki et al., 2014). Short-term methionine deprivation in hPSCs decreases both intracellular S-methyl-5'-thioadenosine (MTA), a methionine precursor, and SAM (Shiraki et al., 2014), which is generated from methionine (Figures 1). This short-term manipulation causes transient cell cycle arrest, whereas long-term methionine deprivation results in cell death. Supplementation with either MTA or SAM during long-term methionine deprivation rescues hPSC proliferation (Shiraki et al., 2014), indicating that methionine-derived metabolites are essential for sustained hPSC self-renewal. Short-term methionine deprivation also can trigger loss of pluripotency and differentiation of hPSCs into the three germ lineages. These changes are similar to mESCs with threonine deprivation and associate with a rapid decrease in global DNA methylation and H3K4me3 (Shiraki et al., 2014). How proline and methionine impact pluripotency status at different developmental stages would be an interesting subject of future investigation.

Together, these studies suggest that the availability of certain nutrients, such as glutamine, glucose, and ascorbic acid, proline, and methionine can elicit differing, and at times

opposing, PSC fate outcomes (Table 1). An open question is whether certain nutrients provide sufficient instructive signals to control specific developmental stages or transitions. For example, can a specific nutrient(s) substitute for certain signaling cytokines, growth factors, hormones, or reprogramming factors to achieve or sustain pluripotency, or to promote lineage specific differentiation?

Intracellular Nutrient Routing Influences PSC Identity

Controlled oxidation and intracellular routing of nutrients by specific enzyme activities and transporters can induce or maintain pluripotency (Fathi et al., 2014; Konze et al., 2017; Shyh-Chang et al., 2013a), suggesting that nutrient handling pathway configurations regulate PSC fate. Lipid metabolism, such as fatty acid oxidation, is essential for early mouse and human embryonic development (Dunning et al., 2010; Dunning et al., 2014; Oey et al., 2005). Indeed, lipid signaling has been implicated in pluripotency, with lysophosphatidic acid (LPA) supplementation promoting conversion from the primed to naïve state transcription factor program in mouse PSCs (Kime et al., 2016). However, *in vitro* studies of PSC fate by addition of lipid-rich bovine serum albumin, or AlbuMAX, yielded differential results. One study showed that added AlbuMAX decreased fatty acid (FA) biosynthesis and NADPH regeneration without affecting *OCT4* expression in hPSCs (Zhang et al., 2016). Separately, AlbuMAX addition stimulated hPSC self-renewal (Garcia-Gonzalo and Izpisua Belmonte, 2008) and porcine induced PSC (iPSC) reprogramming by activating protein kinase A signaling (Zhang et al., 2018b). A potential explanation for these differential results could relate to differences in lipid exposure time, which occurs for other nutrients, such as glutamine (discussed above), as well. In studies reporting hPSCs without changes in *OCT4* expression, cell assays followed short-term AlbuMAX addition (Zhang et al., 2016). Another study also found that an AlbuMAX effect on hPSC colony shape was reversed following short-term addition and withdrawal (Cornacchia et al., 2019), indicating short-term AlbuMAX supplementation has minimal effect on hPSC identity. In contrast, in studies reporting hPSCs with increased self-renewal, cells were adapted to AlbuMAX addition for > 5 passages in culture, followed by spontaneous differentiation for 26 days (Garcia-Gonzalo and Izpisua Belmonte, 2008). Additional studies could clarify the effect(s) of lipid supplementation on PSC fate by systematically testing different lipid compositions and exposure times on PSCs in different states.

Using lipid deprivation as a tool, mouse and human PSC studies showed that *de novo* FA biosynthesis promotes PSC self-renewal, somatic cell reprogramming to pluripotency, and inhibits spontaneous PSC differentiation. *De novo* FA biosynthesis, triggered by exogenous lipid deprivation with added glucose as the major substrate, increased the utilization of acetyl-CoA for protein acetylation and enhanced mitochondrial fission (Vazquez-Martin et al., 2013; Wang et al., 2017; Zhong et al., 2019). Lipid deprivation induced primed hPSCs to a less advanced “naïve-to-primed” intermediate PSC stage of development, even with growth factors that promote the primed stage (Cornacchia et al., 2019). This finding suggests that lipid availability outweighed growth factor signaling at this PSC stage. PSCs express *de novo* FA biosynthesis enzymes acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FASN), which converts glucose-derived acetyl-CoA to FAs, which could siphon substrates for membrane production during self-renewal. Indeed, hPSCs contain more FASN compared

to differentiated cells, and inhibition of FASN caused mitochondria-triggered apoptosis, loss of OCT4, and failure to form teratomas, a measure of pluripotency, in mice (Tanosaki et al., 2020). We posit that PSCs potentially elevate glucose-supplied FA biosynthesis as a mechanism to increase glycolytic flux and route carbons away from OXPHOS consumption in mitochondria, increasing substrates available for acetylation and/or cell replication (Moussaieff et al., 2015). It is unknown whether enforced expression and activation of ACC1 and FASN is sufficient to promote PSC self-renewal. Together, these findings relating lipid availability to PSC fate suggest the absence of lipids triggers elevated glucose routing through FA biosynthesis, which supports pluripotency.

Intracellular pyruvate routing can occur to (1) produce lactate via lactate dehydrogenase (LDH) with NAD+ generated to sustain glycolytic flux; (2) produce acetyl-CoA via pyruvate dehydrogenase (PDH) for TCA cycle oxidation; (3) produce oxaloacetate via pyruvate carboxylase (PCB) to replenish TCA cycle intermediates; and (4) localize to the nucleus to regulate the epigenome (Figure 1) (Sutendra et al., 2014). PDH inhibition, which blocks pyruvate entrance into the TCA cycle, supports mouse iPSC reprogramming and viability, whereas increased pyruvate flux into the mitochondria decreases pluripotency biomarker expression, reduces PSC self-renewal, and lowers PSC viability (Rodrigues et al., 2015; Zhang et al., 2017a). Upon differentiation, *PDH* gene expression rapidly decreases, but PDH protein levels stay the same. By contrast, ATP-citrate lyase (*ACYL)* and acetyl-CoA synthetase (*ACCS2*), which provide pyruvate-derived acetyl-CoA for histone acetylation reactions that support pluripotency, rapidly decrease upon differentiation (Moussaieff et al., 2015). This fate-dependent pattern of metabolism enzyme expression suggests that, upon pluripotency exit, the hierarchy of pyruvate routing shifts toward mitochondrial TCA cycle oxidation to support PSC differentiation.

Several studies show that the activation of metabolic enzymes and expression of transporters precedes changes in gene expression and PSC identity, potentially as a mechanism to prepare for cell identity transitions (Folmes et al., 2011; Moussaieff et al., 2015; Zhang et al., 2011). As an example, another regulator of pyruvate fate is uncoupling protein 2 (UCP2), a uniporter that exports TCA cycle four carbon metabolites from the mitochondria to the cytosol, making them unavailable for OXPHOS (Vozza et al., 2014; Zhang et al., 2011). UCP2 repression promotes mitochondrial oxidation of pyruvate and precedes exit from pluripotency with PSC differentiation (Zhang et al., 2011). During differentiation, intracellular pyruvate routing changes from pluripotency to lineage-specific patterns. Accordingly, media supplementation with pyruvate at high concentrations enhances induced mesoderm and endoderm lineage differentiation (Song et al., 2019), whereas pyruvate consumption decreases during ectoderm lineage differentiation of neural progenitor cells (Lees et al., 2018). These data suggest interesting questions regarding the mechanism(s) PSCs primed for directed differentiation use to sense pyruvate concentration, and whether a threshold influences lineage choice. Additionally, the mechanism(s) underlying rapid transcription, translation, localization, turnover, and/or activation of nutrient routing proteins in PSCs preceding a fate transition remains unknown. The fate of pyruvate and its role in PSC outcome is critical during mouse ZGA. Transient nuclear localization of exogenous pyruvate, PDH, PCB, and select TCA cycle enzymes enables chromatin-localized synthesis of metabolites used in epigenome remodeling during ZGA (Nagaraj et al., 2017). How large

enzyme complexes that typically reside within the mitochondrial matrix instead transiently localize within the nucleus of cleavage-division stage mouse embryos requires further study. Nevertheless, the conversion of exogenous pyruvate into both acetyl-CoA (via PDH) and oxaloacetate (via PCB) occurs within the nucleus, thereby providing a “two-for-one” reactant for sustained production of alpha-ketoglutarate (α-KG). Since α-KG is a co-factor for dioxygenases, such as Jumonji-C histone demethylases and ten-eleven translocation (TET) DNA demethylases, and acetyl-CoA is required for histone acetylation reactions, the local generation of α-KG and acetyl-CoA in the nucleus prioritizes essential epigenome modifications during early genome activation to initiate mouse development.

Nutrients and PSC Fate

mTOR in Pluripotency

Mammalian target of rapamycin (mTOR) complex 1 (mTORC1) is a serine/threonine protein kinase whose activation in response to specific nutrient levels phosphorylates downstream substrates that regulate cell growth, proliferation, and survival (Saxton and Sabatini, 2017). An amino acid-mediated interaction between the Rag family of guanosine triphosphatases (GTPases) and mTORC1 causes mTORC1 re-localization from the cytosol to the lysosomal membrane (Kim et al., 2008; Sancak et al., 2008). Specific amino-acid responsive upstream regulators of mTORC1, including lysosomal vacuolar H(+)-adenosine triphosphatase (v-ATPase), S-adenosyl methionine (SAM) sensors, leucine sensor Sestrin2, arginine sensor CASTOR1, SLC38A9, and SAMTOR (Chantranupong et al., 2016; Gu et al., 2017; Wang et al., 2015; Wolfson et al., 2015; Ye et al., 2015) signal to the Rag GTPases to activate or inactivate mTORC1 (Figure 1, Table 2).

Similar to amino acids arginine, leucine, and glutamine, glucose can also directly activate mTORC1. In glucose deprivation studies, mouse embryonic fibroblasts (MEFs) showed a decrease in mTORC1 kinase activation (Kalender et al., 2010). Mechanistically, glucose controls mTORC1 activity by influencing the binding of the v-ATPase to the Ragulator, a complex of five LAMTOR proteins that associates with the Rag GTPases (Figure 1) in order to activate mTORC1 (Efeyan et al., 2012). Additional recent studies showed intracellular glucose detection by mTORC1 occurs through a glucose-derived metabolite, dihydroxyacetone (DHAP). In glucose-starved conditions with decreased mTOR activity, the synthesis of DHAP restored mTOR activity (Orozco et al. 2020). In sum, mTORC1 activation occurs by several distinct amino acid and glucose signaling mechanisms, which aligns with its central role as a nutrient sensor and actuator of cell growth, proliferation, and survival (Figure 1, Table 2)

In conditions of nutrient abundance, mTORC1 binds ULK1 to inhibit autophagy, and conversely, nutrient starvation dissociates mTORC1 from ULK1, triggering autophagosomes to form (Rabinowitz and White, 2010). In hPSCs, nutrient starvation inhibited mTORC1 activation, increased autophagosome formation, and decreased levels of OCT4, SOX2, and NANOG transcription factors (Cho et al., 2014; Zhou et al., 2009). Autophagy inhibition in starvation conditions caused pluripotency transcription factors to accumulate, suggesting a role for nutrients in controlling pluripotency by regulating autophagy (Cho et al., 2014). Immunofluorescence microscopy and immunogold-stained electron microscopy

during nutrient starvation confirmed pluripotency transcription factor interactions with autophagosomes (Cho et al., 2014), suggesting altogether that loss of pluripotency from nutrient starvation occurs through an mTORC1-autophagy response.

Amino acid deprivation is the most studied trigger for mTORC1 inhibition and subsequent induction of autophagy (Saxton and Sabatini, 2017). Amino acids such as glutamine, methionine, and threonine have demonstrated roles in pluripotency maintenance (Carey et al., 2015; Shiraki et al., 2014; Shyh-Chang et al., 2013b; Vardhana et al., 2019) and may promote pluripotency by signaling nutrient abundance to activate mTORC1. For example, threonine is a positive regulator of mESC self-renewal (Han et al., 2013; Shyh-Chang et al., 2013b; Wang et al., 2009). Culture medium deficient in threonine, but not in any other amino acid, caused a decrease in alkaline-phosphatase positive mESC colonies (Wang et al., 2009). Threonine dehydrogenase (TDH), the rate-limiting enzyme in threonine catabolism, supports somatic cell reprogramming into mouse iPSCs, because TDH knockdown decreased reprogramming efficiency (Han et al., 2013). Threonine supports pluripotency by maintaining histone methylation levels (Shyh-Chang et al., 2013b; Wang et al., 2009) and by regulating mTORC1 activity (Ryu and Han, 2011). mESCs incubated with threonine increased expression of pluripotency proteins OCT4, NANOG, and SOX2 (Ryu and Han, 2011). Inhibition of mTORC1 signaling activity with rapamycin abolished this effect, further suggesting a role for nutrient-activated mTORC1 in regulating pluripotency (Ryu and Han, 2011). It is possible that threonine promotes mESC self-renewal by keeping mTORC1 activity below the level that triggers autophagy and degradation of pluripotency transcription factors. Future studies could identify whether other nutrients, such as glucose, or specific amino acids, such as proline, also sustain pluripotency through mTORC1 inhibition of autophagy. Of course, mTOR effects on transcription, translation, and metabolism could also influence pluripotency. For example, nutrient starvation could inhibit mTORC1 activity and reduce translation of pluripotency-related transcripts.

It is important to note that contradicting studies indicate that an autophagy response can promote instead of inhibit pluripotency. For example, FOXO1, a master regulator of core autophagy genes, maintains high autophagic flux to maintain mESC pluripotency, likely by generating substrates that support rapid cell proliferation (Liu et al., 2017). Similarly, PINK1/OPTN-mediated mitophagy is important for clearing depolarized mitochondria in order to maintain mitochondrial homeostasis that appears critical for pluripotency (Wang et al., 2021a). A possible explanation for these contradictory results is that a precise balance of autophagic activities – neither complete activation nor complete inhibition – is required to maintain pluripotency.

mTOR complex activity is also implicated in a process that suspends, or reversibly halts, development, called diapause (Bulut-Karslioglu et al., 2016; Hussein et al., 2020). Preimplantation mammalian embryos can survive *ex vivo* for up to two days, but deprivation of glucose, arginine, and leucine can induce diapause (Gwatkin, 1966; Naeslund, 2010). The Bulut-Karslioglu group hypothesized that inhibiting growth pathways could replicate this nutrient-starved diapause state. Their work identified mTOR as a regulator of developmental timing and pluripotency, determining that mTOR inhibition could induce a diapause state that significantly prolonged pluripotency. Specifically, nutrient starvation activates the

serine-threonine liver kinase B1 (LKB1)-AMPK signal transduction pathway, which inhibits mTORC2 to cause diapause (Hussein et al., 2020). While these studies suggest that nutrient-regulated mTORC2 is a primary player in controlling diapause and prolonging pluripotency, mTORC2 has been documented to additionally regulate mTORC1 activity (Szwed et al., 2021), making it possible that mTORC1 may also play a role in this phenomenon.

A role for nutrients in mTORC1 regulation of pluripotency exit and subsequent differentiation is less clear. Inhibition of mTORC1 during germ lineage non-directed embryoid body formation increased mesoderm and endoderm differentiation and decreased ectoderm differentiation (Jung et al., 2016; Zhou et al., 2009). Chemical screens with additional mTORC1 inhibitors confirmed this result (Nazareth et al., 2016). Furthermore, mouse embryos containing a loss of function mutation in *mTOR* showed malformations in the ectoderm-derived telencephalon with subsequent loss of viability (Hentges et al., 2001). Similarly, a loss of function mutation in *TSC2*, an inhibitor of mTOR, also caused embryonic lethality due to overgrowth of ectoderm-derived neuroepithelium (Rennebeck et al., 1998). These studies combined highlight a role for mTORC1 activity in promoting ectoderm differentiation. Because mTORC1 responds to many different nutrients, it will be interesting to determine whether a particular nutrient can promote lineage differentiation through mTORC1 activated signaling, or whether a combination of nutrients will be necessary for this effect.

AMPK in Pluripotency

AMP-activated protein kinase (AMPK) is a well-characterized energy sensor that responds to levels of AMP, ADP, and ATP, molecules whose ratios indicate cell energy charge and nutrient status. AMPK activity in low energy charge states (high ratios of AMP/ATP) opposes mTOR activity by limiting energy expenditure, thereby slowing or halting cell growth and proliferation. For many years, it was thought that ATP production from glucose oxidation was the only mechanism by which AMPK sensed glucose availability (Salt et al., 1998). However, a second glucose-sensing mechanism for AMPK was identified recently. With glucose starvation, an AXIN/LKB1 complex near the lysosome accesses the v-ATPase-Ragulator complex (Figure 1) to form an AXIN-based, AMPK-activating complex (Zhang et al. 2013). Interaction of these lysosomal components during simultaneous glucose stress causes dissociation and inactivation of mTOR from the lysosome (Zhang et al., 2013). This non-canonical mechanism could also activate autophagy, although this remains unknown. Opposing this activity, fructose 1,6-bisphosphate (FBP), a glycolytic intermediate sensed by aldolase, triggers dissociation of the AXIN/LKB1 complex from the v-ATPase-Ragulator complex, suppressing AMPK activation (Zhang et al., 2017b). In sum, AMPK is an energy charge and nutrient sensor that effects metabolic change through glucose-sensing mechanisms (Figure 1, Table 2).

Emerging evidence suggests that nutrient-regulation of AMPK may impact pluripotency. In response to glucose starvation, activated AMPK phosphorylates ULK1 at S317 and S777 (Kim et al., 2011) and simultaneously inhibits mTOR, halting mTOR phosphorylation of ULK1 at S757 (Kim et al., 2011). Phosphorylation of ULK1 at S317 and S777, without phosphorylation at S757, supports autophagy, whereas impaired phosphorylation at these

sites blocks autophagy (Kim et al., 2011). A recent study showed that AMPK-dependent phosphorylation of ULK1 is required to maintain mESC pluripotency (Gong et al., 2018). ULK1 knockout mESC lines showed decreased colony formation and pluripotent gene expression, with rescue by re-addition of wild-type ULK1 or mutant S757 ULK1 (Gong et al., 2018). By contrast, ULK1 knockout mESC lines containing expression constructs producing both mutant S317 and S777 ULK1 are unable to restore pluripotency (Gong et al., 2018), indicating an AMPK-specific role in mESC self-renewal (Gong et al., 2018). Compounds such as resveratrol, which increase mESC pluripotent gene expression through activation of the AMPK/ULK1 pathway, support a role for AMPK in PSC maintenance (Suvorova et al., 2019). However, whether glucose through AMPK regulates pluripotency, and if so, whether the mechanism is by AMPK phosphorylation of ULK1 remains unknown.

AMPK may also regulate mESC differentiation through Tfeb, a master transcriptional regulator of lysosomal genes (Young et al., 2016). A genetic gain and loss-of-function study determined that AMPK-regulated Tfeb is required for endoderm differentiation, with Tfeb overexpression in AMPK knockout mESCs restoring lysosomal function and efficient endoderm differentiation (Young et al., 2016). Recent evidence also supports a role for nutrient-regulated AMPK in controlling differentiation from PSCs, as pyruvate activates AMPK in a dose-dependent manner, which in turn promotes mesoderm development, with AMPK inhibition conversely impairing mesoderm differentiation (Song et al., 2019).

Glycosylation in Pluripotency

The hexosamine biosynthesis pathway (HBP) is a nexus of nutrient-responsive signaling that integrates substrates from carbohydrate (glucose), nucleotide (uridine triphosphate; UTP), amino acid (glutamine), and FA (acetyl-CoA) metabolism. The terminal step of the HBP is the production of an acetylated aminosugar nucleotide, uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is the substrate for cycling of O-linked *N*-acetylglucosamine (O-GlcNAc) post-translational modifications (PTM) at serine and threonine residues of nuclear and cytoplasmic proteins (Bond and Hanover, 2015) (Figure 1, Table 2). O-GlcNAc addition, by O-GlcNAc transferase (OGT), and its removal, by O-GlcNAcase (OGA) significantly alters the posttranslational properties and functions of target proteins known to regulate the epigenome (Shi et al., 2013; Vella et al., 2013; Zhu et al., 2020), transcription, proliferation, apoptosis, and proteasome degradation (Love and Hanover, 2005).

The dynamic cycling of O-GlcNAc PTMs regulates PSC maintenance and embryonic development, with knockout of either OGT or OGA causing embryonic lethality in mice (O'Donnell et al., 2004; Shafi et al., 2000; Yang et al., 2012). Pluripotency transcription factors OCT4 and SOX2 contain O-GlcNAcylation motifs that are rapidly removed upon differentiation, and point mutations that block OCT4 and SOX2 Ser/Thr residues normally targeted for O-GlcNAcylation cause a decrease in pluripotency and reprogramming efficiency (Constable et al., 2017; Jang et al., 2012; Myers et al., 2016; Webster et al., 2009). Recently, another pluripotency transcription factor, ESRRB, was identified as an additional O-GlcNAc target. O-GlcNAcylation stabilized ESRRB levels and promoted interactions with OCT4 and NANOG (Hao et al., 2019). Additional evidence that

O-GlcNAcylation is essential for pluripotency maintenance comes from OGT and OGA perturbation studies. Pharmacological or genetic inhibition of OGA during the induction of neuron or cardiomyocyte differentiation caused excessive O-GlcNAcylation of proteins with accompanying suppression of lineage-specific biomarkers and aberrant retention of pluripotency biomarkers (Kim et al., 2009; Maury et al., 2013; Olivier-Van Stichelen et al., 2017; Speakman et al., 2014). Conversely, OGT inhibition resulted in decreased protein O-GlcNAcylation and accelerated PSC differentiation into neuroectoderm lineage cells (Andres et al., 2017).

Recent evidence suggests that nutrient regulation of O-GlcNAcylation may have a role in controlling pluripotency. MEFs reprogrammed in low-glucose culture medium showed reduced cellular O-GlcNAc levels and generated fewer mouse iPSC colonies compared to MEF reprogramming in high-glucose (Jang et al., 2012). Furthermore, during the formation of TE and ICM in early mouse embryos, levels of HBP intermediates glucosamine and UDP-GlcNAc were highly sensitive to glucose deprivation (Chi et al. 2020). Glucose-regulated HBP glycosylation was essential for nuclear localization of YAP1 to activate TE specifying transcription factors, but not for ICM specification (Chi et al. 2020). Combined, these findings indicate that nutrients and their HBP converted products, such as UDP-GlcNAc, are required for nutrient-sensitive O-GlcNAcylation of pluripotency master regulator proteins.

An additional layer of complexity exists for O-GlcNAcylation in regulating PSC fate because a linear relationship between the levels of nutrients and O-GlcNAcylation may not exist. Several studies reported that glucose (Marshall et al., 2004; Swamy et al., 2016; Taylor et al., 2009; Taylor et al., 2008), glutamine (Hamiel et al., 2009; Liu et al., 2007; Swamy et al., 2016), and amino acids (Chaveroux et al., 2016; Zhu et al., 2020) do not show a dose-dependent connection with protein O-GlcNAc levels. This suggests that nutrient regulation of O-GlcNAcylation may be PSC context-dependent, a recurring theme for nutrients controlling PSC fate. For example, whether specific nutrient availability increases or decreases O-GlcNAcylation levels of pluripotency transcription factors may depend on differentiation cues or PSC state, rather than overall HBP flux.

Future Perspectives and Conclusions

Studies over the past two decades have convinced most investigators of the active, rather than consequential, role of metabolism in controlling PSC fate, but many questions remain. These include the role for nutrients in PSC fate commitment, before or during cell identity transitions. For example, does specific nutrient availability initiate or merely reinforce a PSC fate ‘decision’? What roles do exogenous nutrients versus cell intrinsic nutrient requirements have in instructing or reinforcing PSC fate? Because of evidence for both instructive and supportive roles, it is likely that nutrient availability plays a synergistic and potentially cyclic role in enabling PSC fates through metabolic conversion and/or nutrient-sensitive signaling. Progress in technology, with detection and sensitivity advances in mass spectrometry for single-cell metabolomics, could uncover which nutrients are consumed, produced, and secreted by different niche cell types *in vivo* and during PSC identity transitions *in vitro* (Duncan et al., 2019).

A practical area for future study is determining threshold concentrations that trigger a nutrient signal that affects PSC fate. Results from such studies could have implications for how maternal diet and/or diabetes affects development. As an example, an exceedingly high concentration of glucose is detrimental to PSC-derived neural lineage generation due to oxidative and endoplasmic reticulum stress, but also promotes PSC-derived cardiomyocyte generation (Chen et al., 2018; Crespo et al., 2010; Yang et al., 2016). In addition, a high concentration of pyruvate promotes mesoderm but not ectoderm differentiation through AMPK activated signaling (Song et al., 2019). PSC culture methods can affect the amount of imported glucose (Gu et al., 2016), raising questions on how nutrient concentrations in culture medium can affect pluripotency/self-renewal and differentiation biases through altered metabolic flux and/or signaling. A potential approach to understanding biologically significant nutrient concentrations is to replicate the *in vivo* growth environment of the developing embryo, similar to an approach with human plasma-like medium used for growing tissue culture cells (Cantor et al., 2017).

Reports on nutrient-sensitive signaling in pluripotency are increasing as interest is growing, and yet studies uniting nutrient availability, nutrient-sensitive signaling, and PSC fate outcome remain limited. The emergence of additional data connecting nutrient availability and signaling in PSC outcomes promises to inform on how maternal diet/malnutrition, metabolic disorders, and/or the embryonic microenvironment affects mammalian development.

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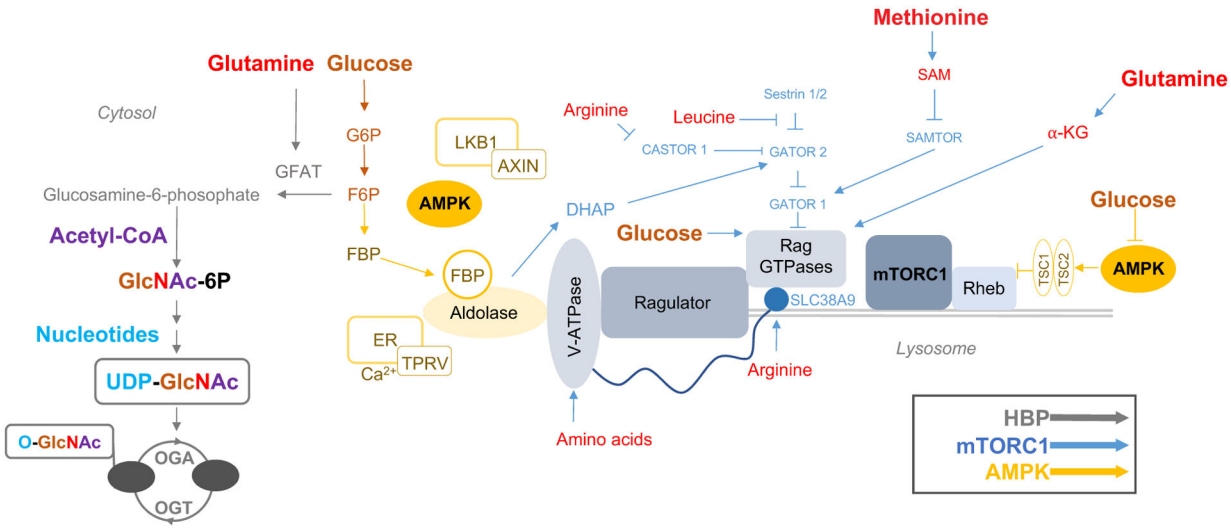

Figure 1. Nutrient Sensing Pathways Impact Pluripotent Stem Cell Fate

Nutrient-specific sensing occurs via the hexosamine biosynthesis pathway (HBP, gray), mTORC1 (blue), and AMPK (yellow). HBP integrates carbohydrates (glucose), amino acids (glutamine), lipids (acetyl-CoA), and nucleotides (UTP), culminating in the generation of UDP-GlcNAC, a substrate for O-GlcNAcylation reactions by OGA/OGT. Signaling by mTORC1 and AMPK are regulated by concentrations of glucose and amino acids at multiple sensors, with activities localized to the lysosomal membrane. These specific nutrients fuel enzyme-based alterations in the epigenome, gene transcription, translation, and PTMs to shape PSC fate (see Table 2).

Key: Glucose-6-phosphate (G6P); fructose-6-phosphate (F6P); glutamine-fructose-6-phosphate transaminase (GFAT); fructose 1,6-bisphosphate (FBP); endoplasmic reticulum (ER); O-GlcNAc transferase (OGT); O-GlcNAcase (OGA); dihydroxyacetone phosphate (DHAP)

Table 1:

Examples of a Nutrient Eliciting Distinct PSC Fates

Nutrient	Cell State	Possible Outcomes *(−) withdrawal (+) supplementation*	Context	Reference
Glutamine	Pluripotency *less committed*	(−) Cell death	Withdrawal in hPSCs (long-term)	Tohyama et al., 2016
		(−) Increased self-renewal	Withdrawal in mESCs and hSPCs (short-term, followed by repletion)	Vardhana et al., 2019
		(−) Maintained pluripotency marker expression	Naïve mESC state with 2i	Carey et al., 2015
		(−) Decreased pluripotency marker expression	Naïve mESC state without 2i	Carey et al., 2015; Ryu et al., 2015
	Differentiation *more committed*	(−) Increased trophectoderm and mesoderm lineage expression	Naïve mESC state without 2i	Ryu et al., 2015
		(−) Inhibited mesoderm expression, promoted ectoderm expression	hPSC spontaneous differentiation (embryoid bodies)	Lu et al., 2019
Glucose	Pluripotency	(−) Inhibited formation of the extra-embryonic TE, but not embryonic ICM	Pre-implantation mouse morula	Chi et al., 2020
	Differentiation	(−) Inhibition of glucose oxidation (2-DG, 3-BrPA, DCA) inhibited ectoderm, but not mesoderm and endoderm, differentiation. Defect can be rescued with glucose-6-phosphate and fructose-1-phosphate, so ectoderm exhibits glycolysis, not glucose, requirement	hPSC spontaneous differentiation	Cliff et al., 2017
		(+) Culturing mESCs in high glucose concentration promotes generation of cardiomyocytes	mESC lineage-directed differentiation	Crespo et al., 2010
		(+) Culturing mESCs in high glucose concentration inhibits generation of neural lineage cells	mESC lineage-directed differentiation	Chen et al., 2018; Yang et al., 2016
Ascorbic Acid	Pluripotency	(+) Accelerated reprogramming of somatic cells into iPSCs	Mouse and human somatic cells	Esteban et al., 2010; Wang et al., 2011; Wu et al. 2013
		(+) Promoted pluripotency	Human iPSC colonies	Eid et al., 2016
	Differentiation	(+) Inhibited spontaneous differentiation	Human iPSC colonies	Eid et al, 2016
		(+) Promoted cardiac differentiation, but only occurs when supplemented during early window of differentiation	Cardiac cell differentiation	Sato et al., 2006; Takahashi et al., 2003, Chan et al. 2009, Cao et al., 2011
Proline	Pluripotency	(−) Blocking proline transport into cell maintained mESC pluripotency	mESC cultured in maintenance media (+LIF)	Tan et al., 2011
	Differentiation	(+) Promoted mESC adoption of primitive endoderm-like morphology and gene expression	mESC cultured in maintenance media (+LIF)	Washington et al., 2010
		(−) Blocking proline transport into cell impaired spontaneous differentiation	hPSC spontaneous differentiation (embryoid bodies)	Tan et al., 2011

Nutrient	Cell State	Possible Outcomes *(−) withdrawal (+) supplementation*	Context	Reference
		(+) Promoted the differentiation of naïve mESCs into more committed epiblast-like stem cells (EpiSCs)	mESC to EpiSC differentiation	Casalino et al., 2011
Methionine	Pluripotency	(−) Transient cell cycle arrest	Withdrawal in hPSCs (short-term)	Shiraki et al., 2014
		(−) Cell death	Withdrawal in hPSCs (long-term)	Shiraki et al., 2014
		(−) Triggered loss of pluripotency	Withdrawal in hPSCs (short-term), parallel findings with threonine deprivation in mESCs (short-term)	Shiraki et al., 2014
	Differentiation	(−) Promoted differentiation into the three germ lineages	Withdrawal in hPSCs (short-term), parallel findings with threonine deprivation in mESCs (short-term)	Shiraki et al., 2014
Lipids	Pluripotency	(+) mRNA expression of *OCT4* is unaffected	hPSCs cultured in E8 minimal media + AlbuMAX (short-term)	Zhang et al., 2016
		(+) Stimulated self-renewal and pluripotency retention	hPSC cultured in differentiation media + AlbuMAX (long-term)	Garcia-Gonzalo and Izpisua Belmonte, 2008
		(+) Favored dedifferentiation/pluripotent state	Porcine iPSC reprogramming + AlbuMAX	Zhang et al., 2018b
		Fatty acid synthesis promoted dedifferentiation/pluripotent state by regulating acetyl-CoA and mitochondrial fission	Mouse and Human iPSC reprogramming	Vazquez-Martin et al., 2013; Wang et al., 2017
		(−) Induced reprogramming from primed to “naïve-to-primed” intermediate PSC state through endogenous ERK inhibition	hPSC cultured in minimal (E8) pluripotency maintenance media	Cornacchia et al., 2019
		FASN inhibition eliminated pluripotent cells and prevents differentiation	hPSC cultured in maintenance media	Tanosaki et al., 2020
	Differentiation	(−) Germ layer differentiation bias, increased ectoderm but reduced mesoderm/endoderm propensities	hPSC lineage-directed differentiation	Cornacchia et al., 2019
		FASN inhibition did not affect committed cell types: hPSC-derived cardiomyocytes (mesoderm), hepatocytes (endoderm), and neurons (ectoderm)	hPSC lineage-directed differentiation	Tanosaki et al., 2020
Pyruvate	Pluripotency	Blocking pyruvate entrance into mitochondria favored pluripotency, increased pyruvate flux opposed pluripotency	mESC and hPSC pluripotency maintenance and iPSC reprogramming	Rodrigues et al., 2015; Zhang et al., 2017a, Vozza et al., 2014; Zhang et al., 2011
	Differentiation	(+) High concentrations of exogenous pyruvate enhanced mesoderm and endoderm lineage differentiation	hPSC lineage-directed differentiation	Song et al., 2019
		Pyruvate consumption decreased during ectoderm lineage neural progenitor differentiation	hPSC lineage-directed differentiation	Lees et al., 2018

Table 2:

Nutrient-sensitive Signal Transduction Pathways Consist of (a) Substrate (Nutrient), (b) Sensor, (c) Transducer, and (d) Effector

Nutrient-Sensing Pathway	Substrates	Sensors	Transducers	Effectors
HBP	glucose, glutamine, glucosamine	GFAT, (OGA & OGT: unclear)	OGA, OGT	O-GlcNAcylation of core pluripotency transcription factors
mTOR	methionine, leucine, arginine, glutamine, asparagine, glucose, SAM, α-KG	Rag GTPases, Arf1 GTPases, V-ATPase, SAMTOR, GATOR, Sestrins, SLC38A9	mTOR complex	4E-BP, S6K
AMPK	glucose	V-ATPase, AMPK	AMPK	ULK1, acetyl-CoA carboxylase 1, SREBP1c