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Current Opinion in Genetics & Development

Metabolic regulation in pluripotent stem cells

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Pluripotent stem cells (PSCs) have the capacity to give rise to all cell types of the adult body and to expand rapidly while retaining genome integrity, representing a perfect tool for regenerative medicine. PSCs are obtained from preimplantation embryos as embryonic stem cells (ESCs), or by reprogramming of somatic cells as induced pluripotent stem cells (iPSCs). Understanding the metabolic requirements of PSCs is instrumental for their efficient generation, expansion and differentiation. PSCs reshape their metabolic profile during developmental progression. Fatty acid oxidation is strictly required for energy production in naive PSCs, but becomes dispensable in more advanced, or primed, PSCs. Other metabolites directly affect proliferation, differentiation or the epigenetic profile of PSCs, showing how metabolism plays an instructive role on PSC behaviour. Developmental progression of pluripotent cells can be paused, both *in vitro* and *in vivo*, in response to hormonal and metabolic alterations. Such reversible pausing has been recently linked to mammalian target of rapamycin activity, lipid metabolism and mitochondrial activity. Finally, metabolism is not simply regulated by exogenous stimuli or nutrient availability in PSCs, as key pluripotency regulators, such as Oct4, Stat3 and Tfcp2l1, actively shape the metabolic profile of PSCs.

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Current Opinion in Genetics & Development 2022, **75**:101923

This review comes from a themed issue on **Cell Reprogramming, regeneration and repair**

Edited by **Ophir Klein** and **Ryuichi Nishinakamura**

For complete overview of the section, please refer to the article collection, "Cell Reprogramming, regeneration and repair"

Available online 9th June 2022

https://doi.org/10.1016/j.gde.2022.101923

Introduction

Pluripotency is the ability to give rise to all cell types of the adult body. In mammals, pluripotent cells are first found in the inner cell mass and the epiblast at the blastocyst stage. These naive pluripotent cells have been expanded *in vitro* from mouse blastocysts as murine embryonic stem cells (mESCs). Naive mESCs are characterised by efficient oxidative phosphorylation (OXPHOS) and glycolysis [1–4], a global genome hypomethylation and the expression of pluripotency factors such as Oct4/Pou5f1, Nanog, Klf4 and Tfcp2l1 [5].

Initial attempts at obtaining pluripotent stem cells (PSCs) from human embryos led to the derivation of human embryonic stem cells (hESCs), that are mainly glycolytic and display high levels of DNA methylation, while retaining pluripotency [5]. Conventional hESCs have been shown to correspond to a developmental state more advanced than naive pluripotency, matching the epiblast of the post-implantation, peri-gastrulation embryo, described as 'primed pluripotency'.

The differences between murine and human ESCs could exist due to a different developmental stage. Indeed, naive mESCs shift their metabolism towards glycolysis and increase their genome methylation upon exit from naive pluripotency, becoming more similar to hESCs [1]. Furthermore, human PSCs in a naive state were more recently derived from embryos or by reprogramming of somatic cells [6,7] and display bivalent metabolism and genome hypomethylation, as naive mESCs [3,6,8]. Still, species-specific differences in PSC metabolism might exist.

Recently, an intermediate state of pluripotency, between naive and primed, has been described and named formative pluripotency [9–11]. Formative pluripotent cells share molecular features with the peri-implantation embryo, such as the loss of naive markers, intermediate DNA methylation levels, a strong reduction in mitochondrial respiration and increased expression of lipid-metabolism enzymes [11,12].

In general, PSCs display an elevated proliferation rate, requiring elevated ATP (adenosine triphosphate) production and anabolism. We will discuss what metabolites are used to this end, based on recent findings. Proliferation and developmental progression can also be reversibly paused, in a phenomenon known as diapause, in which metabolism seems to have a key role. Finally, it is becoming increasingly clear that pluripotency factors are not only regulated by, but also regulators of PSC metabolism.

Nomenclature

PSCs	pluripotent stem cells
mESCs	mouse embryonic stem cells
hESCs	human embryonic stem cells
hiPSCs	human-induced pluripotent stem cells
OXPHOS	oxidative phosphorylation
TCA	tricarboxylic acid
FA	fatty acids
FAO	fatty acid oxidation
MUFA	monounsaturated fatty acids
FASN	fatty acid synthase
aKG	alpha-ketoglutarate
LPA	lysophosphatidic acid

Metabolic requirements for proliferation, differentiation and epigenetics of pluripotent stem cells

Lipids

Lipid supplements are often used in the culture of human PSCs (hPSCs). Multiple studies recently reported fatty acids (FAs) as key metabolites promoting proliferation of conventional hPSCs, serving either as components of plasma and organelle membranes or as an important source of energy [13,14]; moreover, lipids were also reported to contribute to pluripotency regulation, through mechanisms involving acylation of key regulatory proteins and modulation of the epigenetic landscape [15•] (Figure 1).

De novo FA synthesis sustains proliferation of conventional human-induced PSCs (hiPSCs) by providing building material. Indeed, fatty acid synthase (FASN), the final enzyme in the *de novo* FA-synthesis pathway, is abundantly expressed in conventional hiPSCs but not in hiPSC-derived cardiomyocytes [13].

FASN inhibition via genetic (short-interfering RNAs), pharmaceutical (antiobesity drug) or chemical (FASN inhibitors) tools results in a significant decrease in hiPSC proliferation and an increased cell death. Supplementation with palmitate, the product of FASN, is sufficient to rescue these phenotypes.

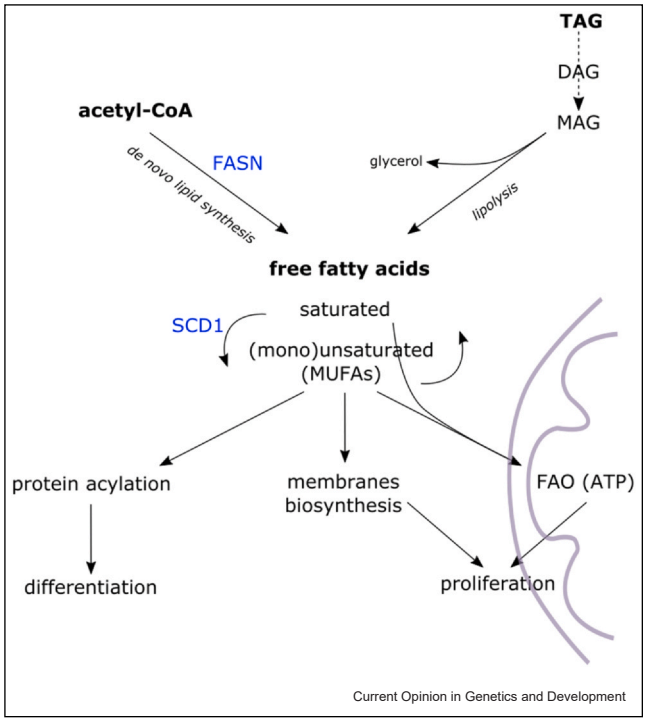
As conventional hiPSCs do not utilise fatty acid oxidation (FAO) for energy production [16], palmitate sustains the proliferation and survival of hiPSCs in a manner independent of its function as a fuel source. Inhibition of FASN causes a reduction in phosphatidylcholine, a glycerophospholipid and a major component of biological membranes, leading to apoptosis only in undifferentiated hiPSCs, while hiPSC-derived differentiated cells are unaffected by FASN inhibition. In sum, in undifferentiated conventional hiPSCs, FA synthesis has mainly an anabolic, pro-survival, function.

FAs have also been reported to serve as substrates for the acylation of different proteins involved in differentiation or in pluripotency maintenance [15•]. A small-molecule inhibitor of the enzyme SCD1 (Stearoyl-coenzyme A desaturase 1), which converts saturated FAs into monounsaturated FAs (MUFAs), can support the maintenance of primed pluripotent state in hiPSCs and hESCs by opposing the initiation of the endodermal differentiation programme. Using oleate (a monounsaturated FA) together with the SCD1 inhibitor in a rescue experiment is sufficient for efficient endodermal marker recovery.

MUFAs play important roles in developmental processes through acylation of the proteins involved in differentiation. Coherently, conventional hiPSCs and hESCs treated with a SCD1 inhibitor show a global decrease in protein acylation during endoderm differentiation. The authors found alterations in the Wnt/β-catenin pathway, which is known to be regulated by lipidation [17], potentially explaining the effects on differentiation.

In line with these findings, Cornacchia and colleagues [18] also linked lipid supplementation with differentiation of hPSC. Conventional hPSCs grown in lipid-free,

Figure 1



Conventional human PSC differentiation and proliferation are controlled by FA metabolism.

chemically defined culture conditions (E8) [19] showed a global epigenetic rearrangement, including DNA hypomethylation and histone hyperacetylation, negligible levels of phosphorylated ERK and an increased expression of pluripotency markers such as NANOG (Nanog homeobox) and KLF4 (Kruppel like factor 4). According to the authors, such changes were triggered by a high requirement for *de novo* lipogenesis in lipid-free E8 medium and they were associated with an intermediate naive-to-primed pluripotent state; such features were all lost upon addition of AlbuMAX, a commercial serum-replacement supplement commonly used for hPSC culture. However, which specific component of AlbuMAX induced the shift was not clear.

A recent study [20••] addressed this question through the analysis of AlbuMAX components, which revealed that free FAs or cholesterol present in the medium relieve the requirement for *de novo* lipogenesis, but they do not induce any additional metabolic shift. Rather, the metabolic and transcriptional landscape of conventional hPSCs cultured in the presence of AlbuMAX is modulated by lysophosphatidic acid (LPA), a hydrophilic lipid with signalling functions. Indeed, LPA is known to promote differentiation of conventional hPSC into neuroepithelial rosettes via SRF (Serum response factor) signalling [21]. LPA treatment induces accumulation of metabolites of glycolysis, pentose phosphate pathway and tricarboxylic acid (TCA) cycle, accompanied by an altered mitochondrial morphology and functionality. LPA causes cell morphology changes, with bigger and flatter colonies and reduced proliferation. LPA also promotes global changes in gene expression, potentially through the modulation of histone protein acetylation, although an involvement of other signalling cascades (e.g. SRF or AP1 - Activator protein 1) cannot be ruled out. Of note, such changes are similar to those induced by AlbuMAX and are all reversible. In contrast with previous findings by Cornacchia and colleagues, the study by Xu excludes the involvement of excessive *de novo* lipogenesis in the cellular state observed in E8, as provision of exogenous fatty acids and cholesterol fails to produce any significant change. Moreover, LPA removal does not recapitulate the naive-to-primed state described by Cornacchia and colleagues, given that ERK is phosphorylated also in the absence of any exogenous lipid supplementation (E8 only) and that the expression of naive markers is unchanged [20••].

In sum, most of the cellular changes occurring between lipid-free E8 and AlbuMAX-containing medium are mediated by LPA, an efficient metabolic modulator inducing an alternative metabolic state in conventional hPSCs, affecting the epigenetic profile and proliferation, while leaving pluripotency unaltered.

Lactate

Glucose is one of the main energy sources in hPSCs *in vitro*. Pyruvate produced from glycolysis can be converted into lactate in the cytosol, which is then released and accumulates in the medium. Lactate is thus considered as a metabolic waste, and the frequent media changes required for hPSC culture are believed to alleviate lactate-induced stress. However, a recent study clarified the role of extracellular lactate in conventional hiPSCs [22].

Odenwelder and colleagues [22] exposed conventional hiPSC to different glucose and lactate concentrations and found that in low glucose and high lactate (i.e. 20 mM), the growth rate and pluripotency of hiPSCs was not altered. Isotopic labelling experiments showed that lactate can efficiently substitute glucose as a carbon source, after conversion to pyruvate and citrate, suggesting that lactate likely supports hiPSC proliferation through *de novo* lipid synthesis. Such behaviour is reminiscent of neurons that efficiently use lactate produced by astrocytes for memory formation [23].

Metabolic network reconstruction also suggested higher production of acetyl-CoA and increased FA synthesis in the presence of high lactate concentrations. Overall, conventional hPSCs display a metabolic flexibility that allows them to adopt different substrate-utilisation strategies to support self-renewal. Thus, lactate accumulation does not negatively impact growth or pluripotency, it can rather support proliferation via *de novo* lipogenesis. It would be interesting to investigate whether such flexibility plays a role in the pre-implantation phase of development, when nutrients are limited. Indeed, in pre-implantation mouse embryo, glucose is not used for ATP production, nor for amino acid and lipid synthesis, while lactate feeds the TCA cycle [24].

Amino acids

Multiple studies indicate that additional nutrients, including the amino acids glutamine, methionine, proline and threonine, have roles in pluripotency maintenance or proliferation of PSCs [25–29]. A recent work includes tryptophan (TRP) to the list, which was shown to regulate proliferation in conventional hiPSCs, without affecting pluripotency [30].

Kynurenine (KYN) is produced from TRP, via an intermediate called N-formylkynurenine (NFK). The KYN pathway leads to the production of nicotinamide adenine dinucleotide (NAD^+). Mass spectrometric analysis of TRP-supplemented medium revealed increased concentrations of NFK and its derivative N-formylanthranilic acid. NFK supplementation promoted proliferation of hPSCs through unexplored molecular mechanisms, whereas KYN treatment had no effects, indicating that the effect of TRP on proliferation is independent of its catabolism into KYN and NAD^+, and thus decoupled from upregulation of glycolysis and oxidative phosphorylation by NAD.

The essential amino acid methionine also has a well-established role in pluripotency and differentiation, as SAM- (S-adenosylmethionine) dependent histone methylation relies on methionine availability both in hESCs and mESCs [4,29]. New insights about the methionine cycle utilised by naive mESCs to sustain pluripotency were given by Zhu and colleagues [31]. Mouse ESCs exhibit high levels of methionine metabolism, which is reduced during differentiation; depletion via shRNA of the enzyme AHCY (Adenosylhomocysteinase), a key metabolic enzyme in the methionine cycle, causes mESCs to lose pluripotency and self-renewal capacity, activating apoptotic signalling. AHCY inhibition reduced the SAM/SAH (S-Adenosylhomocysteine) ratio and methylation on H3K4me3, a histone mark associated with active transcription. ChIP-qPCR demonstrated that H3K4me3 was reduced at Oct4/Pou5f1 and Nanog loci upon AHCY knockdown, linking AHCY activity with pluripotency maintenance in mESCs.

AHCY activity is positively regulated by O-GlcNAcylation (addition of an O-linked β-N-acetylglucosamine sugar), a nutrient-sensitive post-translational modification. O-GlcNAcylation is rapidly removed upon differentiation to reduce AHCY activity. Site-specific mutagenesis on AHCY on the amino acid modified by O-GlcNAcylation impaired self-renewal and differentiation of mESCs. O-GlcNAcylation thus constitutes a molecular mechanism linking glucose availability with the modulation of the epigenetic landscape and differentiation.

Alpha-ketoglutarate

A recent study by Chen and colleagues [32] links the activity of branched-chain amino acid aminotransferase-1 (Bcat1) enzyme to the epigenetic landscape and pluripotency maintenance in mESCs. Bcat1 is found to be highly expressed in naive mESCs and consistently downregulated upon differentiation. Bcat1-knockout (KO) mESCs proliferate less compared with wild-type and display a partially compromised pluripotency, while overexpression of Bcat1 enhances pluripotency and self-renewal. Bcat1 initiates catabolism of branched-chain amino acids, such as leucine, isoleucine and valine, while converting αKG to glutamate. Therefore, Bcat1 genetic deletion leads to increased αKG levels, causing a global DNA hypomethylation, as previously reported [33,34••].

Alpha-ketoglutarate is also produced via glutamine-reductive pathway, as glutamine is converted in the cytosol into acetyl-CoA and TCA intermediates, directly feeding the TCA. In line with this, we found that intracellular αKG levels in mESCs are dictated by flux of carbon from glutamine-reductive metabolism re-entering the TCA cycle, and that robust αKG production from glutamine is required for genome hypomethylation [34••].

Metabolic regulation of diapause

Pluripotent cells of the blastocyst embryo undergo rapid proliferation and developmental progress, reflected by transcriptional, morphological and metabolic changes. However, both the developmental progression and proliferation of the early blastocyst can be temporarily arrested under unfavourable conditions (e.g. lack of nutrients or decreased levels of oestrogens [35]) in a phenomenon called diapause. Diapause has been identified in over 100 mammalian species and has been thoroughly characterised in rodents. It has also been suggested to occur in primates, although direct evidence is still lacking [35].

A recent study reported that the mammalian target of rapamycin (mTOR) inhibitor INK-128 induces diapause in mouse blastocysts cultured *ex vivo* [36]. Diapaused blastocysts obtained by hormonal treatment of pregnant mice share some features with INK-128-treated blastocysts, such as a reduced phosphorylation of the mTOR targets AKT and 4EBP1, along with a reduction in protein synthesis, global transcription and of H4K16ac, an epigenetic marker of active transcription [36]. Diapause embryos also show increased levels of AMP and increased lipolysis [37•].

Several groups reported different *in vitro* models of diapause, named 'paused pluripotency', 'dormant pluripotency', 'quiescence' or 'diapause-like state' [36–39], in which mESCs are exposed to different genetic or chemical perturbations (Table 1).

Each *in vitro* model shares some similarities with diapause blastocysts (e.g. reduced proliferation or reduced mTOR activity), while keeping pluripotency intact.

In the next section, we will discuss the most recent findings and the open questions.

Paused pluripotency was obtained by reducing the activity of both mTORC1 and mTORC2 complexes, using INK-128 or Rapalink [36]. Xu and colleagues observed that genetic inactivation of the mTORC1-specific protein Raptor is sufficient to strongly reduce proliferation of mESCs without affecting pluripotency, similarly to INK-128 treatment [42•]. Conversely, inactivation of the mTORC2-specific protein Rictor was inconsequential. The effect of mTORC1 on proliferation is mediated by the translation-initiation complex eIF4F. Indeed, the translation of over 3000 mRNAs was impaired upon INK-128 treatment, including cytoplasmic and mitochondrial ribosomal proteins, with a concomitant reduction in mitochondrial mass and activity. Depletion of either the cytoplasmic ribosomal protein Rsp6, or of mitochondrial translation, resulted in reduced proliferation, indicating multiple mechanisms by which mTORC1 regulates proliferation of naive mESCs. Of

Table 1

Perturbation and molecular readouts of diapause.

	Biological model	Treatment	Proliferation	AMPK AKT mTOR	Global transcription	H4K16ac	DNA synthesis	Protein synthesis	Transcriptome similar to diapause embryo	OXPHOS	Glycolysis	Lipid metabolism	References
In vivo	Blastocyst E3.5	Hormonal treatment (tamoxifen and progesterone)	↓	↓ p-4EBP1 ↓ P-AKT ↑ AMP	↓	↓		↓	N.A.		↓ (gene signature)	↑ lipolisis ↓ FAO genes	[36–40]
Ex vivo	Blastocyst E3.5	INK-128 Rapalink-1 (mTORC1/2 inhibitors)		↓ p-4EBP1 ↓ P-AKT	↓	↓		↓					[36]
	Blastocyst E3.5	ETO (FAO inhibitor)					↓		Yes	↓ (gene signature)			[38••]
	Blastocyst E3.75	MYC inhibitor	↓					↓					[39]
In vitro	mESCs in SL	INK-128	↓ Cell number	↓ p-4EBP1 ↓ P-AKT	↓			↓	Yes		↓ (gene signature)		[36]
	mESCs in 2iL	INK-128		↑ p-AMPK ↓ p-AKT ↓ p-4EBP1 ↓ S6		↓					↑	↑ lipolisis	[37•]
	mESCs in 2iL and SL	INK-128	↓ Cell number	No changes				No changes		↓	↓		[41•]
	mESCs in 2iL	INK-128	↓ Cell number		No changes			↓		↓			[42•]
	mESCs in 2iL	Starvation		↑ p-AMPK ↓ p-AKT ↓ p-mTOR					Yes		↑		[37•]
	mESCs in 2iL	ETO	↓ Cell number	↑ p-AMPK ↓ p-AKT/ p-S6K1						↓		↓ FAO	[38••]
	mESCs in 2iL	Oligomycin Antimycin A Rotenone	↓ Cell number	↑ p-AMPK ↓ p-AKT/ p-S6K1									[38••] [43]
	mESCs in 2iL	CAP (mito protein synth, inhibitor)	↓ Cell number							↓			[42•]

Table 1 (*continued*)

Biological model	Treatment	Proliferation	AMPK AKT mTOR	Global transcription	H4K16ac	DNA synthesis	Protein synthesis	Transcriptome similar to diapause embryo	OXPHOS	Glycolysis	Lipid metabolism	References
mESCs in 2iL	Raptor KO (mTORC1)	↓ Cell number	↓ p-AKT ↓ p-4EBP1 ↓ S6	No changes			↓ (partial)		↓			[42•]
mESCs in 2iL	Rictor KO (mTORC2)	No changes	↓ p-AKT ↓ p-4EBP1 ↓ S6								↑ lipolisis	[37•] [42•]
mESCs in 2iL	MOF KO	↓ Cell number		↓	↓	↓	↓		↓		↓ FAO	[38••]
mESCs in 2iL	MYC dKO or MYCi	↓ Cell number					↓	Yes				[39]

N.A.= not applicable; ETO=etomoxir, an inhibitor fatty acid uptake in mitochondria; MOF= also known as Kat8.

note, although some studies reported a global reduction in transcription in diapause (Table, [36–39]), Xu and colleagues failed to observe it, indicating that such reduction is not causally linked to control of proliferation.

Naive mESCs can be cultured in the presence of faetal bovine serum and the cytokine LIF (Leukemia inhibitory factor) (SL) as a heterogeneous cell population. In contrast, the use of two inhibitors (2i) of the MEK (Mitogen-activated protein kinase kinase) and GSK3 (Glycogen synthase kinase-3) kinases in combination with LIF (2iL) significantly increases homogeneity [5,44]. Paused pluripotency was initially reported in SL [36] and recently multiple groups reported [41•,42] that also mESCs in 2iL respond to INK-128 with a reversible reduction in proliferation, while keeping pluripotency intact. Surprisingly, Sousa and collaborators found that INK-128 did not affect the activity of mTOR, measured by the phosphorylation of its target S6K1 and by protein-synthesis levels. Conversely, mitochondrial respiration was strongly reduced by INK-128, with no changes in the protein levels of respiratory complexes, overall indicating that INK-128 induces a diapause-like state independently from mTOR activity and protein synthesis. The authors observed reduced glycolytic function and uptake of glucose and pyruvate, with decreased production of lactate [41•]. Thus, although genetic data support a role for mTOR in the regulation of proliferation of naive mESCs [42•], INK-128 seems to act also via mTOR-independent pathways, on yet-unidentified targets. Further supporting this hypothesis, INK-128 exerted much more pronounced effects on transcription and translation than genetic mTORC1 inactivation [42•].

Hussein and colleagues performed a transcriptional analysis of mouse developing and diapause mouse blastocysts, revealing activation of glycolysis, of cholesterol synthesis and uptake [37•]. The authors developed an *in vitro* model of diapause, by starving (i.e. withdrawal of glucose and serum) naive mESCs in 2iL for 24 h and observing a gene-expression signature similar to a diapause embryo. Starvation induced activation of AMPK (AMP-activated protein kinase), resulting in reduced phosphorylation of both mTOR and H4K16ac. Such changes were reversible, as is diapause *in vivo*.

Starvation or inhibition of mTOR by INK-128 led to higher glycolytic capacity in an AMPK-dependent manner. Such findings were supported by metabolomic analyses on pre-implantation and diapause embryos, revealing increased levels of AMP in diapause. Furthermore, diapause embryos, obtained by hormonal treatment of pregnant mice, showed increased levels of free FAs and phosphatidylcholine in diapause, accompanied by a decrease in tri- and di-acylglycerol (TAG and DAG), which might be interpreted as increased

lipolysis. A similar reduction in mTOR activity and increased lipolysis was observed also in their *in vitro* model upon starvation. Finally, genetic inactivation of Rictor, a key component of mTORC2, resulted in increased lipolysis, placing mTOR upstream of lipolysis. Such results complement the study from Xu and colleagues [42•]: proliferation was not affected by mTORC2 inactivation in both studies, but was under control of mTORC1, while lipolysis was regulated by mTORC2. Thus, the two complexes seem to regulate different processes associated with diapause, possibly explaining why inhibitors of both complexes have stronger effects than the inactivation of single complexes. In sum, Hussein and colleagues proposed that diapause of naive mESCs is associated with increased glycolysis, lipolysis and reduced mTOR activity.

It is worth noting that, despite the use of the same culture conditions (2iL) and the same inhibitor (INK-128), Hussein [37•] and Sousa [41•] reached different conclusions regarding the activity of mTOR and the effects on glycolysis (Table 1). It will be important in the future to study whether the genetic background, the composition of the basal media used or the concentrations of inhibitors might affect proliferation, mTOR activity and other parameters associated with diapause.

Lipid metabolism, such as lipolysis and FAO in mitochondria, is found to be altered in diapause embryos and in *in vitro* models. Khoa and colleagues reported that chemical or genetic inactivation of the histone acetyltransferase MOF/Kat8, the mouse orthologue of Drosophila males absent on the first (MOF), in naive mESCs, resulted in a quiescent state, characterised by a reduction of proliferation, of H4K16ac and of DNA, RNA and protein synthesis [28] This was also associated with reduced FAO, suggesting a role in quiescence. Indeed, blocking the mitochondrial uptake of FA by Etomoxir induced quiescence, while administration of L-Carnitine rescued the proliferation defects observed in MOF-null cells, indicating that naive mESCs rely on FAO for proliferation and energy production via OXPHOS. Furthermore, blocking mitochondrial respiration severely reduced proliferation with no effects on cell death, as previously reported [43]. Strikingly, inhibition of FA uptake induces a diapause-like state in blastocysts *ex vivo*, overall indicating a general role for FAO/OXPHOS in the control of proliferation of naive pluripotent cells. Finally, human naive PSCs, expanded in t2iLGö conditions [45], ceased proliferation in response to FAO inhibition. In contrast, conventional hPSCs that do not utilise FAO for energy production [16], were unaffected by FAO inhibition. These results collectively indicate that naive PSCs specifically rely on FAO for proliferation.

In sum, several metabolic perturbations reduce proliferation of mESCs (Table 1). However, it might be misleading to associate all these perturbations to a diapause-like state, as also nonpluripotent cells (e.g. cancer cell lines) would reduce their proliferation in response to some of them. Thus, a more stringent definition of an *in vitro* diapause-like state is needed, independent of proliferation *per se*, but rather based on the acquisition of a transcriptional and metabolic profile similar to the one of diapause embryos.

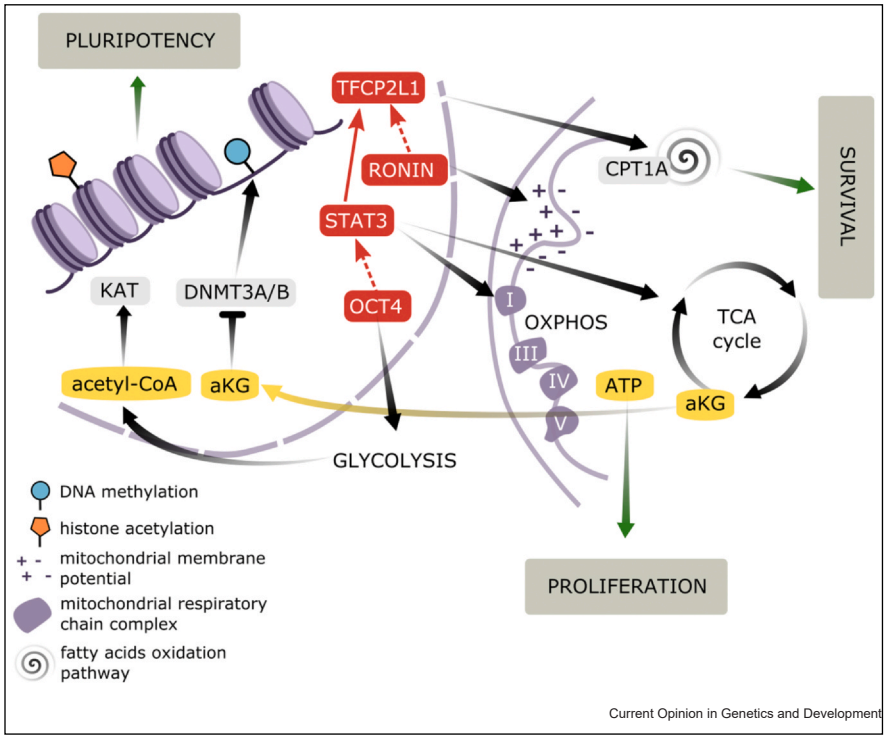
A second issue that should be considered is that diapause is the combination of reduced proliferation and a block in the developmental progression. Naive pluripotent cells of the diapause blastocysts remain in the naive state for several days. Current *in vitro* models show that some perturbations are sufficient to reduce proliferation, without affecting pluripotency, which is similar to diapause *in vivo*. However, in all studies, mESCs are exposed to molecules (LIF and 2i) that are known to maintain the naive state. Thus, we do not know whether the sole inhibition of mTOR, which reduces proliferation, would also be sufficient to maintain naive pluripotency. The presence of LIF in all *in vitro* diapause models might not be accidental, as LIF signalling plays a role in diapause *in vivo*. Indeed, ovariectomy induces diapause in wild-type embryos [35], while embryos with mutated LIF co-receptor gp130 arrest their proliferation, but fail to maintain the naive epiblast, which undergoes cell death and differentiation towards hypoblast derivatives [46]. Moreover, both Stat3-null and Tfcp2l1- null embryos fail to maintain the naive epiblast upon diapause induction [47]. Thus, the developmental arrest observed in diapause depends on the LIF pathway. In the future, it would be interesting to study the interplay between LIF and mTOR in the context of diapause.

Pluripotency factors regulating metabolism

Several metabolic pathways regulate pluripotency and differentiation of PSCs, however, it is becoming increasingly clear how also canonical pluripotency regulators affect, in turn, cell metabolism (Figure 2).

The core pluripotency transcription factor Oct4 has been reported to interact with the promoters of the key metabolic genes encoding for the glycolytic enzymes Hk2 and Pkm2, that sustain the glycolytic flux in highly proliferating naive mESCs [48]. Notably, transcriptional analysis of Oct4-mutant pre-implantation embryos confirmed regulation of glycolysis-related genes by Oct4 [49]. KAT (lysine acetyltransferase) enzymes use acetyl-CoA, a product of glycolysis, as a substrate for the acetylation of histone proteins. A significant down-regulation of several KAT enzymes was observed by Stirparo and colleagues in the absence of Oct4. Thus, Oct4 might promote the open-chromatin state associated with naive pluripotency by providing sufficient acetyl-CoA and boosting the expression of KATs.

Figure 2



Pluripotency factors regulating metabolic processes in naive murine pluripotent cells.

Additionally, downregulation of genes involved in cell respiration was detected in Oct4-mutant embryos; as a reduced Stat3 signalling was concomitantly observed in Oct4 KO embryos, the modulation of oxidative respiration by Oct4 likely constitutes a downstream event driven by Stat3, as also supported by previous evidence on Stat3-mediated metabolic regulation of pluripotency [34••,43].

The LIF/Stat3 pathway boosts OXPHOS and alpha-ketoglutarate levels through transcriptional and non-transcriptional mechanisms [34••,43,50,51], resulting in robust proliferation and hypomethylation of mESCs.

Notably, the Stat3 downstream target Tfcp2l1 [5] has also been involved in metabolic regulation of mESCs, specifically promoting FAO [50•] and generally supporting energy production and survival of mESCs. Tfcp2l1 genetic deletion in mESCs causes broad gene-expression changes, framing Tfcp2l1 as a positive regulator of many metabolic pathways, among which lipid metabolism is significantly enriched; coherently, FAO is decreased in Tfcp2l1 KO ESCs compared with wild-type cells. Among the metabolic genes regulated by Tfcp2l1, Cpt1a (Carnitine palmitoyltransferase 1a) encodes an enzyme that transfers the acyl group of long-chain fatty acids to carnitine, which is a rate-limiting step for FAO. Cpt1a promoter is directly bound by Tfcp2l1 and the overexpression of Cpt1a rescued the FAO defects in Tfcp2l1 KO cells, suggesting that Tfcp2l1 regulation of FAO is mediated by Cpt1a.

Owing to their higher dependency on glucose and glutamine as energy sources, Tfcp2l1 KO ESCs experienced more cell death than wild-type cells in nutrient-deprived conditions, an effect that was partially rescued by Cpt1a overexpression (OE). Since Tfcp2l1–Cpt1a axis protects ESCs from cell death upon glutamine deprivation and glycolysis inhibition, Yan and colleagues hypothesised that mTOR-mediated metabolic stress responses are underlying the protective role of Tfcp2l1 against spontaneous cell death. Similar to nutrient deprivation, mTOR inhibition by INK-128 led to increased cell death in Tfcp2l1 KO 2i-ESCs than in wild-type cells, and this phenotype was rescued by Cpt1a OE. These results were recapitulated using *ex vivo* blastocysts with mTOR inhibition as a diapause-like model, where Tfcp2l1–Cpt1a–FAO axis promoted the survival of paused blastocysts.

Strikingly, a recent study from the Nichols laboratory, further confirmed a key role for Stat3 and Tfcp2l1 in diapause and lipid metabolism. Indeed, both Stat3-null and Tfcp2l1-null embryos failed to maintain the naive

epiblast during diapause induction. Furthermore, transcriptomic profiling revealed a specific alteration of genes involved in lipid metabolism in Stat3-null embryos at implantation [47].

Similar to Oct4 and Tfcp2l1, additional pluripotency regulators have been reported to act via the LIF/Stat3 pathway [49,51], highlighting a central role of LIF/Stat3 as regulators of mESC metabolism. Ronin is a transcription factor controlling mESC proliferation; Ronin conditional KO mESCs show substantially reduced growth with no concomitant increase in cell death, indicative of a 'quiescent state'. Additionally, Ronin depletion triggers a broad transcriptional response, including Tfcp2l1 among the most upregulated markers, together with other LIF/Stat3 target genes such as Gbx2, Klf4 and Tcl1 [52]. Among genes downregulated in Ronin KO mESCs, there are many factors involved in the organisation of the inner mitochondrial membrane; their downregulation has a profound impact on mitochondrial biology, ultimately resulting in structural changes (loss of cristae), reduced mitochondrial transmembrane potential and reduced oxygen consumption rate, which are likely responsible for a global reduction in energy production and a consequently impaired proliferative state.

These findings collectively indicate that the metabolic state of naive mESCs is actively shaped by key pluripotency factors.

Conclusions

Data from pluripotent cells of the embryo, although technically challenging to obtain, are crucial to identify metabolic processes that are truly associated with pluripotency, rather than the culture conditions used for *in vitro* expansion. For example, the use of glucose for ATP production might be an adaptation induced by culture in the presence of glucose — as also observed in cancer cell lines — but we do not know whether the same holds true *in vivo*. For instance, classical studies revealed that glycolysis is inactive in the early embryo [53], while lactate/pyruvate fulfil the bioenergetics needs. Instead, glucose exerts signalling functions, crucial for the specification of the inner cell mass [24]. Thus, the metabolism of the pre-implantation embryos, given the limited availability of nutrients, might be significantly different from the one of PSCs in culture.

Still, studying the metabolism of PSCs *in vitro* is crucial from a practical perspective: the use of hPSCs for cell-replacement therapies requires large-scale expansion and efficient differentiation, two processes under the control of metabolism. The study of PSC metabolism might also reveal molecular pathways at play under pathological conditions. For example, genetic data from acute myeloid leukaemia patients and mouse models support an interplay between Stat3 and Dnmt3a/b, recently described also in mESCs [34••].

The majority of studies on human PSC metabolism are based on conventional PSCs [3,33]. Human naive PSCs display an unprecedented developmental flexibility [54,55], as they give rise to extraembryonic tissues. It will be interesting to understand whether this flexibility is due to a human-specific metabolic state.

CRediT authorship contribution statement

Conceptualisation: **Linda Diamante and Graziano Martello**. Writing – original draught: **Linda Diamante and Graziano Martello**. Visualisation: **Linda Diamante**.

Conflict of interest statement

Nothing declared.

Funding

This work was supported by the European Research Council [ERC Starting Grant — MetEpiStem], and by the Armenise-Harvard Foundation.

Acknowledgements

We apologise to all scientists whose important work could not be cited because of the space limitations. We thank Sirio Dupont and members of the Martello lab for critical reading of the paper.

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