Dynamic stem cell states: naive to primed pluripotency in rodents and humans

Leehee Weinberger, Muneef Ayyash, Noa Novershtern and Jacob H. Hanna

Abstract | The molecular mechanisms and signalling pathways that regulate the *in vitro* preservation of distinct pluripotent stem cell configurations, and their induction in somatic cells by direct reprogramming, constitute a highly exciting area of research. In this Review, we integrate recent discoveries related to isolating unique naive and primed pluripotent stem cell states with altered functional and molecular characteristics, and from different species. We provide an overview of the pathways underlying pluripotent state transitions and interconversion *in vitro* and *in vivo*. We conclude by highlighting unresolved key questions, future directions and potential novel applications of such dynamic pluripotent cell states.

Primordial germ cells
(PGCs). Embryonic progenitor cells that give rise to germ cells in the gonads (sperm and oocytes).

Embryonic stem cells
(ES cells). *In vitro*-expanded pluripotent cells that originate from the inner cell mass.

Inner cell mass
(ICM). The mass of cells inside the pre-implantation blastocyst that will subsequently give rise to the definitive structures of the fetus.

Epiblast stem cells
(EpiSCs). *In vitro*-expanded pluripotent cells that originate from the post-implantation epiblast.

The Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 7610001, Israel. Correspondence to N.N. and J.H.H. noa.novershtern@weizmann.ac.il; Jacob.hanna@weizmann.ac.il

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Pluripotency describes cells that have the potential to give rise to cells from all three embryonic germ layers and possibly primordial germ cells (PGCs), but not extra-embryonic tissues[1]. Although pluripotency is a transient state *in vivo*, pluripotent cells can be derived from different stages of early embryonic development and maintained indefinitely in an artificially induced self-renewal state *in vitro* by supplementing exogenous cues[2]. Thus, it is important to stress that self-renewal is not a defining feature of pluripotency and is only a transient feature during early development. Pluripotency is highly dynamic and evolves at different stages of pre- and post-implantation development[3]. However, the self-renewal aspect is a highly useful *in vitro* 'engineering trick' (REF. 4) that has brought pluripotent cells to the front of the stage as a tool for tissue replacement, disease modelling and animal engineering[5].

There are multiple types of pluripotent stem cell that can be isolated from vertebrates, including rodents and humans, which are typically annotated on the basis of their donor cell of origin (FIG. 1). Embryonic stem cells (ES cells) are isolated from the inner cell mass (ICM) of developing pre-implantation mouse or human blastocysts[6–8]. Epiblast stem cells (EpiSCs) are isolated from mouse post-implantation epiblasts[9,10]; for ethical reasons, no equivalent derivations have been attempted with human embryos. Early migrating rodent PGCs can be converted *in vitro* into pluripotent ES cell-like cells, termed embryonic germ cells[11,12]. Mouse neonatal and adult spermatogonial stem cells can be reverted towards pluripotency and generate male germ stem cells (GSCs)[13–15]. However, GSCs have the disadvantage of retaining only the male imprint signature, which can increase their tumorigenic potential[15]. Intriguingly, no stable and validated embryonic germ cells or GSCs have been isolated from primates thus far[16,17] (FIG. 1).

Somatic cell reprogramming provides alternative routes for isolating pluripotent cell types. Human and rodent somatic cells can be artificially reprogrammed into ES cell-like cells by nuclear transfer, generating NT-ES cells[18–20]. Ten years ago, the direct *in vitro* reprogramming of somatic cells to pluripotency by ectopic expression of defined factors was established[21], yielding induced pluripotent stem cells (iPSCs) without the need for oocytes or embryos[22–25] (FIG. 1). NT-ES cells and iPSCs offer the advantage of being able to generate patient-specific pluripotent cells with nuclear DNA that is identical to that of the donor somatic cell; however, mitochondrial DNA in NT-ES cells is non-isogenic and is provided by the anucleated donor oocytes[26]. This can be an advantage in applications that are aimed at correcting maternally inherited mitochondrial diseases[27–29].

Whereas the above overview pertains to the classification of different pluripotent cell types on the basis of their tissue derivation source, the growth conditions that are used to expand such cells *in vitro* determine the pluripotent state that they attain (for example, an ICM-like, ES cell-like or EpiSC-like state)[30,31]. iPSCs generated in classical mouse ES cell growth conditions yield ES cell-like iPSCs, whereas those reprogrammed in EpiSC growth conditions yield EpiSC-like iPSCs[30,32]. The same analogy applies to explanting rodent ICM

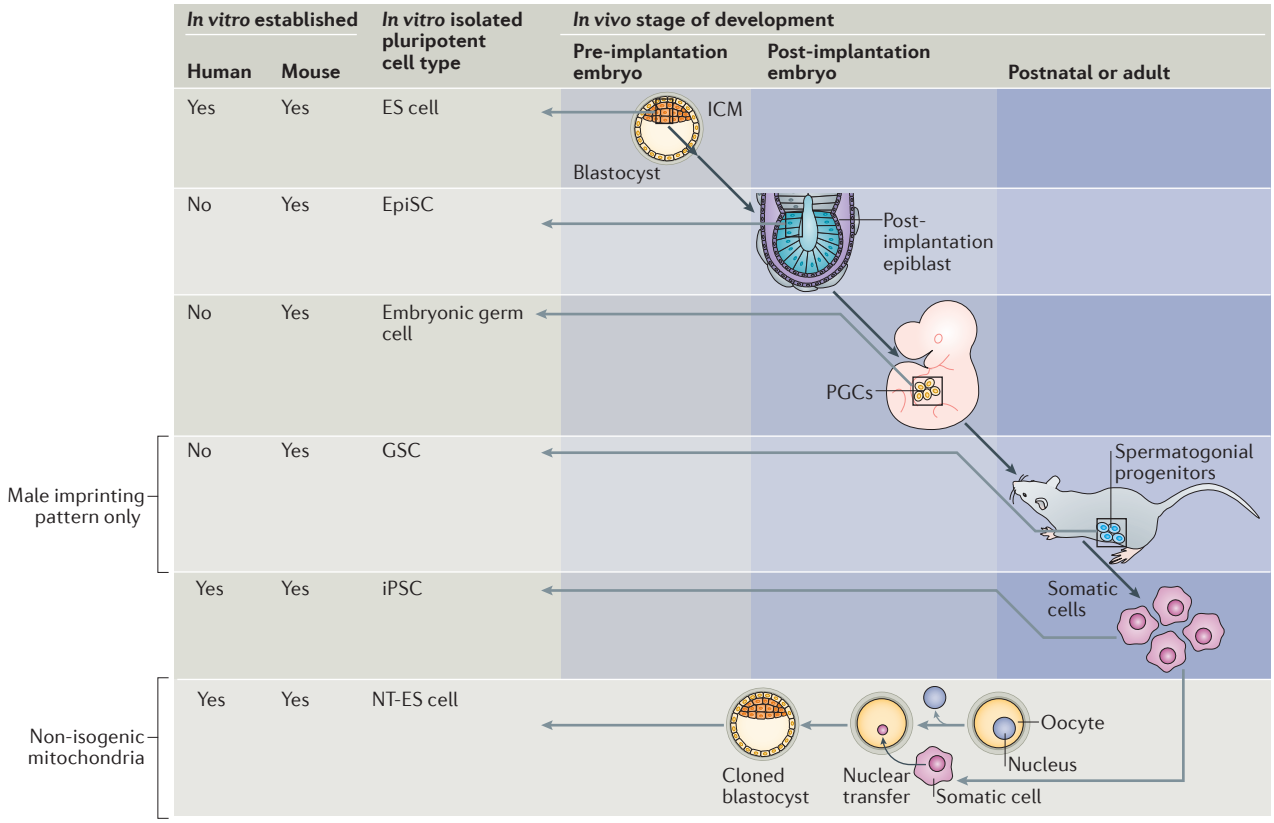


Figure 1 | **Deriving different types of pluripotent stem cell in mouse and human.** Various pluripotent cell types can be derived from different types of embryonic cell harvested at various stages of mouse or human development. Alternatively, somatic cells can be reprogrammed by somatic cell nuclear transfer (producing nuclear transfer embryonic stem (ES) cells (NT-ES cells)) or by the expression of exogenous transcription factors (generating induced pluripotent stem cells (iPSCs)). Pluripotent cells derived from post-implantation epiblast (epiblast stem cells (EpiSCs)) or from the germ cell lineage (embryonic germ cells and spermatogonial germ stem cells (GSCs)) have not yet been stably derived in humans or other primates. For therapeutic purposes, iPSCs and NT-ES cells have the advantage that their nuclear DNA is genetically identical to that of the somatic cells of the donor patient. However, NT-ES cells will retain mitochondrial DNA of the anucleated female oocyte into which the donor somatic cell nucleus is transferred. Spermatogonial GSCs are generated from spermatogonial stem cells that have already established an exclusive male (paternal) imprinting pattern; thus, the GSCs that are derived from them have the same imprinting pattern. If spermatogonial GSCs can be established from adult human males in the future, the fact that these cells lack the maternal imprint will probably limit their therapeutic potential. ICM, inner cell mass; PGCs, primordial germ cells.

Embryonic germ cells
In vitro-expanded pluripotent cells that are derived from embryonic primordial germ cells (PGCs).

Germ stem cells
(GSCs). *In vitro*-expanded pluripotent stem cells that originate from neonatal or adult testis-derived spermatogonial stem cells.

Nuclear transfer
The cloning of a somatic cell-derived nucleus and its introduction into an anucleated host oocyte.

Induced pluripotent stem cells
(iPSCs). *In vitro*-generated pluripotent cells derived by the ectopic expression of defined exogenous factors in somatic cells.

X inactivation
Dosage compensation of the X chromosome in females, whereby one of the X chromosomes is epigenetically silenced.

cells in growth conditions for ES cells or EpiSCs[30,33]. In comparison to developmentally restricted mouse EpiSCs, ES cells are highly competent in generating high-contribution chimeric mice after microinjection into host blastocysts, retain a pre-X inactivation state in female cell lines and have reduced expression of lineage commitment factors[30,31]. Such attributes influence the use of pluripotent cells in cell-differentiation assays and in animal transgenics. Thus, it is of importance to understand and define different pluripotent states and configurations across different species[4].

In this Review, we provide an integrated perspective on recent breakthroughs in our understanding of the diversity and complexity of the regulation of the pluripotent state *in vitro*. This includes advances in preserving naive pluripotency from non-rodent species and alternative pluripotent states. We highlight unresolved issues, key questions and future directions in this exciting area of stem cell research.

Murine pluripotent states

Mouse ES cells were shown to reside in an ICM-like state[30], referred to as naive pluripotency[31], as they retain several molecular characteristics of the ICM. A novel type of pluripotent cell, EpiSCs, were later derived from post-implantation rodent epiblasts[9,10]. In comparison to naive ES cells, EpiSCs retain an alternative pluripotency configuration, which is referred to as primed pluripotency[31]. There are marked molecular and functional differences between pluripotent cell types, which subsequently influence their characteristics, function and safety.

Growth conditions for naive pluripotency. To fully understand the biology of mouse naive ES cells and their developmental context, it is of relevance to review the evolution of growth conditions that have been devised to isolate such cells over the past 30 years. ES cells were originally derived from the 129 mouse strain[7,8] by using

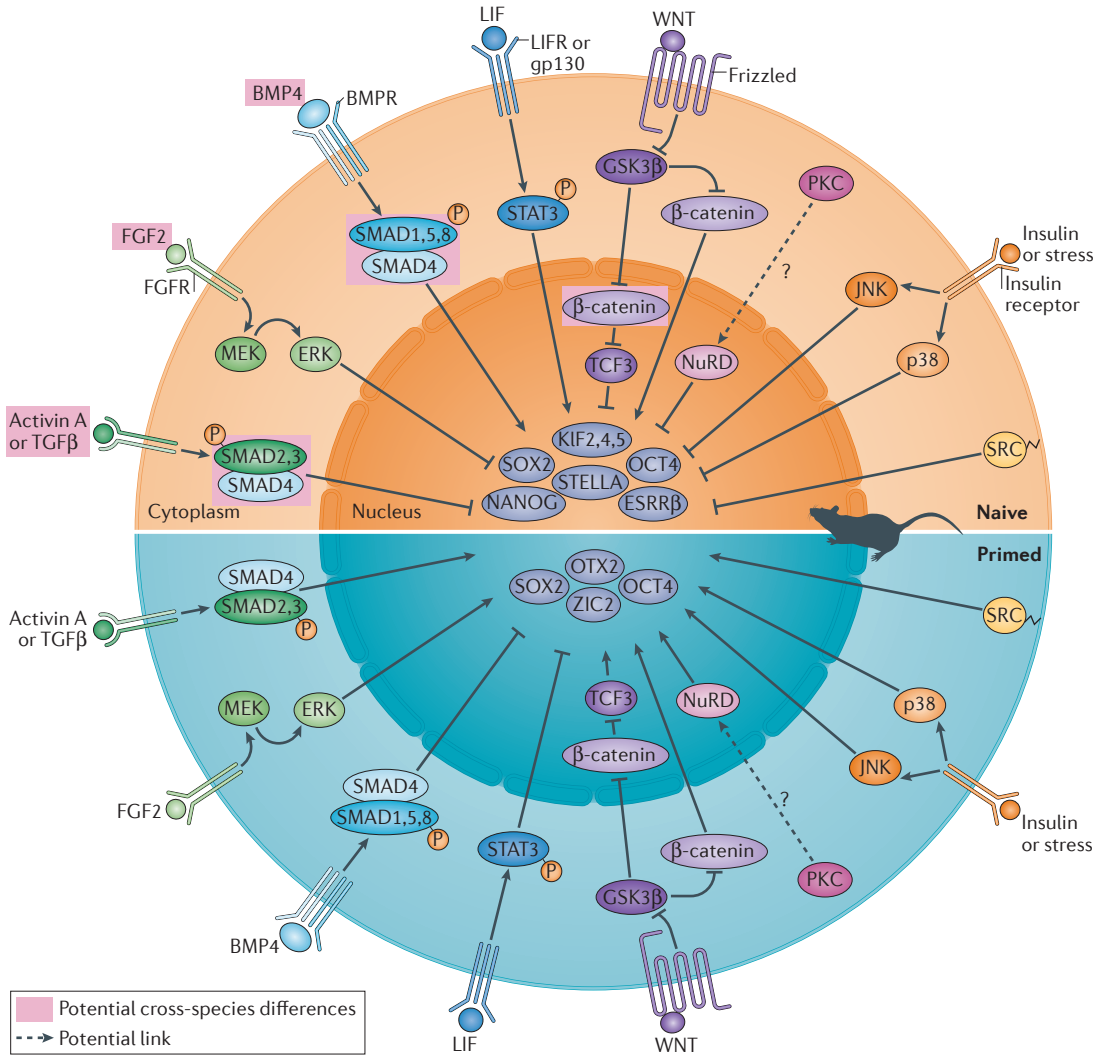


Figure 2 | **Signalling pathways and their influence on naive and primed pluripotent states.** Different signalling pathways can positively or negatively regulate naive and primed murine pluripotent stem cells. Note that the majority of the signalling pathways shown have opposing effects on the naive and primed pluripotent states in mice (for example, the leukaemia inhibitory factor (LIF)–signal transducer and activator of transcription 3 (STAT3) and fibroblast growth factor 2 (FGF2)–ERK signalling pathways). It is important to highlight that other pathways not included in this scheme are likely to also be involved in such regulation and will probably be further characterized in the future. Such pathways may include HIPPO, RHO, NOTCH and nuclear factor-κB signalling. Pink boxes highlight signalling pathways that may function differently in the regulation of mouse and human pluripotent cells. More specifically, it remains to be fully understood whether signalling induced by low doses of transforming growth factor-β (TGFβ), activin–NODAL, nuclear β-catenin or FGF2 (MEK–ERK independent) influences human naive pluripotency in a different manner to that previously observed in rodent naive embryonic stem cells. Dashed arrows indicate potential links that remain to be established. BMP, bone morphogenetic protein; ESRRβ, oestrogen-related receptor-β; GSK3β, glycogen synthase kinase 3β; JNK, Jun-like kinase; NuRD, nucleosome remodelling and deacetylases; OCT4, octamer-binding protein 4; PKC, protein kinase C; TCF3, transcription factor 3. Adapted from Poster http://www.nature.com/nrm/posters/pluripotency/index.html, Nature Publishing Group.

Naive pluripotency
A pluripotent state that resembles the pre-implantation embryonic configuration(s).

Primed pluripotency
A pluripotent state that resembles the post-implantation embryonic configuration(s).

mitotically inactive mouse embryonic fibroblasts (MEFs) as feeder cells and fetal bovine serum (FBS)[34]. Leukaemia inhibitory factor (LIF), which activates the JAK–STAT3 (Janus kinase–signal transducer and activator of transcription 3) pathway, was later identified as a key ingredient that enabled the proliferation of mouse ES cells in FBS/LIF conditions without MEFs[35,36] (FIG. 2). Such naive mouse ES cells express hallmark pluripotency factors (such as octamer-binding protein 4 (OCT4; also known as POU5F1), homeobox protein NANOG and oestrogen-related receptor-β (ESRRβ; also known as ERR2)) and retain a pre-X inactivation state in female cell lines[37] (FIG. 3). Functionally, ES cells can populate the host pre-implantation mouse ICM following micro-injection into blastocysts and generate high-contribution chimeras with colonization of the germ line[37].

The first serum- and feeder-free defined conditions for expanding mouse ES cells were developed by combining low doses of bone morphogenetic protein 4 (BMP4) with LIF[38]. The addition of small-molecule inhibitors for

MEK signalling increased the derivation efficiency of ES cells and their stability[39]. This approach was extended by developing different defined conditions, all involving MEK inhibitors, that can be used to isolate mouse ES cells. A combination of three inhibitors, termed 3i conditions, was shown to stabilize pluripotent cells without LIF, indicating the existence of redundant pathways that can be used to isolate ES cells *in vitro* and can compensate for

			Mouse			Human					
Pluripotent cell property	**Naive pluripotent cell**	**Primed pluripotent cell**	**2i, LIF**	**FBS, LIF**	**FGF2, Activin A or FGF2, TGFβ**	**OCT4, KLF2, KLF4, 2i, LIF**	**2i, LIF, p38i, JNKi, PKCi, ROCKi, FGF2, TGFβ**	**2i, LIF, PKCi, MEF +KLF2, NANOG**	**2i, BRAFi, ROCKi, SRCi, LIF, Activin A, MEF (FGF2, JNKi)**	**FGF2, TGFβ, 2i, BMPi, LIF, MEF**	**FGF2, TGFβ or FGF2, Activin A or FGF2, MEF**
MEK–ERK dependence	No	Yes	●	●	●	●	●	●	●	●	●
Long-term dependence on FGF2 signalling	No	Yes	●	●	●		●			●	●
Long-term dependence on TGFβ or Activin A signalling	No	Yes	●	●	●		●		●	●	●
Dominant *OCT4* enhancer	Distal	Proximal	●	●	●	●	●	●	●		●
H3K27me3 on developmental regulators	Low	High	●	●	●		●		●	●	●
Global DNA hypomethylation	Yes	No	●	●	●		● Mild	● Strong			●
X chromosome inactivation	No	Yes	●	●	●	●	●	●	●	●	●
Dependence on DNMT1, DICER, METTL3, MBD3	No	Yes	●	●	●						●
Priming markers (OTX2, ZIC2)	↓	↑	●	●	●	●	●	●	●	●	●
Pluripotency markers (NANOG, KLFs, ESRRβ)	↑	↓	●	●	●	●*	● Mild*	● Strong*	● Strong*	● Mild*	●*
TFE3 nuclear localization	High	Low	●	●	●		●	●		●	●
CD24/MHC class 1	Low/low	High/mod	●	●	●		●	●	●		●
Expressed adhesion molecules	E-cadherin	N-cadherin	●	●	●		●	●	●	●	●
Promotion of pluripotency maintenance by NANOG or PRDM14	Yes	No	●	●	●		●	●	●	●	●
Metabolism	OxPhos, Glycolytic	Glycolytic	●	●	●		●	●	●		●
Competence as initial starting cells for PGCLC induction	High	Low	●	●	●		●				●
Capacity for colonization of host pre-implantation ICM and contribution to advanced embryonic chimeras	High	Low	●	●	●		● ‡		● ‡		●
Hypomethylation of promoter and enhancer regions	Yes	No	●	●	●			●			●
KIT	Yes	No	●	●	●						●
Tolerance for absence of exogenous L-glutamine	Yes	No	●	●	●						
Mitochondrial membrane activity and depolarization	High	Low	●		●			●			●
Competence as initial starting cell for TSC induction	High	Low	●	●	●						●

Figure 3 | **Naive and primed pluripotent cell properties in mouse and human isolated pluripotent stem cells (PSCs).** The first column on the left lists the properties that can be used to distinguish between murine embryonic stem cells expanded in 2i/leukaemia inhibitory factor (LIF) conditions (naive pluripotent cells) and murine epiblast stem cells expanded in fibroblast growth factor 2 (FGF2)/Activin A conditions (primed pluripotent cells). These two reference states are used to annotate various other growth conditions that have been used for mouse or human pluripotent stem cells. For each growth condition, we indicate whether cells cultured in that condition have naive-like properties (shown in orange) or primed-like properties (shown in blue). Empty boxes indicate lack of characterization. This list of properties is likely to increase with time and can be used to systematically annotate new pluripotent states isolated in unique conditions and from other species; for an extended table including pluripotent cells derived from mouse, rat, human and rhesus macaque, see Supplementary information S1 (figure). BMPi, bone morphogenetic protein inhibitor; DNMT1, DNA methyltransferase 1; FBS, fetal bovine serum; H3K27me3, histone H3 Lys27 trimethylation; ICM, inner cell mass; JNKi, Jun-like kinase inhibitor; KLF, Krüppel-like factor; MBD3, methyl CpG-binding domain protein 3; MEF, mouse embryonic fibroblast; METTL3, methyltransferase-like protein 3; OCT4, octamer-binding protein 4; OxPhos, oxidative phosphorylation; PGCLC, primordial germ cell-like cell; PKCi, protein kinase C inhibitor; PRDM14, PR domain zinc-finger protein 14; ROCKi, RHO-associated protein kinase 1 inhibitor; TFE3, transcription factor E3; TGFβ, transforming growth factor-β; TSC, trophoblast stem cell. *No oestrogen-related receptor-β (ESRRβ). ‡Mouse host embryos. Adapted from Poster http://www.nature.com/nrm/posters/pluripotency/index.html, Nature Publishing Group.

a lack of LIF–STAT3 signalling[40]. Notably, this cell configuration was labelled as ground state pluripotency, as the cells cultured in 3i conditions were reported to grow independently of any exogenous signalling stimuli. However, the use of this term is challenged by the fact that growth in these conditions relies heavily on the inhibition of glycogen synthase kinase 3 (GSK3), which mimics stimulation of the WNT signalling pathway, and on exogenous insulin, which activates PI3K–AKT signalling[40]. Furthermore, 2i/LIF conditions were adopted as an enhanced means to expand mouse ES cell populations, and the reduced proliferation in 3i conditions compared with 2i/LIF conditions indicates a role for autocrine-secreted fibroblast growth factor 4 (FGF4) in promoting the growth of naive ES cells independently of MEK–ERK signalling[30,41,42] (FIG. 2). Notably, complete and combined genetic ablation of *Erk1* and *Erk2* is detrimental to maintaining rodent naive ES cell survival and does not yield an identical phenotype to that resulting from MEK inhibition, which indicates that MEK inhibition has additional roles in maintaining ES cell stability that are independent of ERK[43].

Alternative 2i conditions, involving small-molecule inhibitors for GSK3 and SRC pathways, yield germline-competent ES cells[44] (FIG. 2). Go6983, which is a small-molecule inhibitor of atypical protein kinase C (aPKCi), was identified as another stimulator for isolating mouse ES cells, together with LIF and/or inhibitors of MEK[45]. Single-cell RNA sequencing (RNA-seq) analysis has shown that there is equivalent global heterogeneity between different growth conditions for naive pluripotency; however, differences exist in the identity of the genes that underlie heterogeneity in each condition[46].

Enriched conditions have been important for deriving ES cells from mouse strains that were, until recently, considered to be non-permissive for deriving naive ES cells. Whereas ES cells derived from the 129 mouse strain can be expanded in FBS/LIF conditions, for other mouse strains, such as non-obese diabetic (NOD) mice, supplementation with 2i conditions or with GSK3 inhibitor (GSKi) is essential for both the derivation and maintenance of naive pluripotent cells[30,42]. 3i and 2i/LIF conditions have been used to yield rat ES cells, although these conditions are suboptimal[47,48]. LIF/MEKi/aPKCi conditions are a more robust method of supporting rat ES cells[49] (FIGS 2,3).

The above findings underscore the relevance of analysing rodent ES cells expanded in different naive growth conditions and from different genetic backgrounds. Furthermore, they emphasize the importance of other signalling pathways that remain to be more extensively characterized in the context of pluripotency (FIG. 2). SRC functions as a downstream target of MEK–ERK and calcineurin–nuclear factor of activated T cells (NFAT) signalling to promote ES cell differentiation[50], and its inhibition promotes naive pluripotency[44,50]. Nuclear factor-κB (NF-κB) inhibition has been identified as a downstream effector that mediates naive pluripotency supported by aPKC inhibition[45]; however, other pathways, such as the methyl CpG-binding domain protein 3 (MBD3)–nucleosome remodelling and deacetylases (NuRD) repressor complex, can also be neutralized by aPKCi (J.H.H., unpublished observations).

The HIPPO signalling pathway regulates epiblast versus trophoblast segregation in late mouse morulas and is highly active in pluripotent epiblast cells, leading to the exclusion of YES-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) effectors from the nucleus[51,52]. Depletion of YAP and TAZ in mouse naive ES cells expanded in 2i/LIF conditions further enhances their resistance to differentiation[52], whereas another study indicated that YAP and TAZ are essential regulators of the stability of naive ES cells expanded in FBS/LIF[53]. Reanalysis of these findings in different conditions might resolve these seemingly opposing results (FIG. 3).

It should be noted that signalling pathways are often pleiotropic and may simultaneously have positive and negative effects on naive pluripotency. For example, stabilization of nuclear β-catenin following GSK3 inhibition promotes naive pluripotency by neutralizing the repressive activity of transcription factor 3 (TCF3) on its target genes in the nucleus[54]. Cytoplasmic β-catenin promotes naive pluripotency by increasing E-cadherin membrane stability[55] (FIG. 2). However, nuclear β-catenin can induce mesodermal gene expression through its LEF co-effectors[47], although such differentiation-priming effects are outweighed by the naive pluripotency-promoting functions of β-catenin under optimized conditions. LIF has also been shown to promote primitive endoderm specification in naive pluripotency growth conditions[56]. Such 'non-purist' effects should be kept in mind when dissecting the role of signalling pathways on pluripotency.

The large number of conditions in which naive murine ES cells can be grown have been important in better understanding and revisiting the roles of several classical pluripotency regulators. Whereas NANOG was first purported to be absolutely essential for establishing naive pluripotency through iPSC reprogramming, cell fusion or EpiSC reversion[57], various conditions have since enabled the reprogramming of NANOG-null donor cells *in vitro*[58–60]. However, NANOG-null ES cells cannot be derived from mouse ICM, which indicates that although NANOG is indispensable for establishing pluripotency *in vivo*, it is dispensable during *in vitro* induction[61]. This example highlights that *in vitro* maintenance of pluripotency cannot be considered to be 'authentic', as some *in vitro* conditions can potentiate the robustness of the naive pluripotency programme and compensate for deficiencies that are not sustainable *in vivo*. Similarly, Krüppel-like factor 2 (*Klf2*)-knockout embryos do not present lethality at the pre-implantation stage, and naive ES cells in 2i/LIF or FBS/LIF conditions can tolerate ablation of *Klf2* (REF. 62). However, ES cells in 2i-only conditions cannot sustain loss of KLF2 (REF. 62), as LIF is required to compensate for the lack of KLF2.

Another emerging regulatory principle is that not all factors that are expressed in the ICM or in ES cells necessarily promote naive pluripotency; some of them, in fact, promote its dissolution. However, these factors are tolerated by ES cells *in vitro*, owing to the optimized and enriched growth conditions that are used. For example, binding of TCF3 to its naive pluripotency-promoting

3i conditions
Defined naive pluripotency growth conditions combining three inhibitors (i) for MEK, fibroblast growth factor (FGF) and glycogen synthase kinase 3 (GSK3) signalling.

Ground state pluripotency
Originally described as a state of pluripotency that is independent of exogenous activator signalling input or stimulation.

2i/LIF conditions
Defined naive pluripotency growth conditions containing two inhibitors (i) for MEK and GSK3, together with LIF cytokine.

Alternative 2i conditions
Defined naive pluripotency growth conditions containing two inhibitors (i) for the glycogen synthase kinase 3 (GSK3) and SRC pathways.

LIF/MEKi/aPKCi conditions
Defined naive pluripotency growth conditions containing two inhibitors (i) for MEK and atypical protein kinase C (aPKC) signalling, together with the leukaemia inhibitory factor (LIF) cytokine.

target genes leads to their partial repression but is tolerated in FBS/LIF growth conditions. However, neutralization of TCF3 by adding GSK3i boosts naive pluripotency[54]. In a similar manner, mouse ES cells tolerate expression of the MBD3–NuRD complex, despite the fact that it partially represses naive pluripotency targets[63]. However, genetic ablation of *Mbd3* leads to upregulated expression of master regulators of naive pluripotency and enables LIF-independent cell growth[63]. Consistently, the derivation of *Mbd3*-knockout ES cells from the ICM is not compromised in 2i/LIF conditions[64]. In summary, both TCF3 and MBD3 are expressed in the ICM and in ES cells, where they are likely to set the stage for terminating naive pluripotency. Thus, the molecular characteristics of a pluripotent state in a certain growth condition represent the net outcome of conflicting stabilizing and destabilizing factors that simultaneously reside in that state[65].

Collectively, when analysing the function of pluripotency regulators, it is important to systematically compare different naive growth conditions, genetic backgrounds and *in vivo* contexts[66]. Such integrated analysis is likely to unravel additional layers of underappreciated complexity and may resolve some conflicting results[52,53].

Growth conditions for primed pluripotency. Primed EpiSCs were derived from post-implantation epiblasts of rodents in FGF2/Activin A conditions[9,10] (FIG. 1). EpiSCs are capable of differentiating into cells of all three germ layers *in vitro* or in a teratoma assay, and thus they are pluripotent. However, they are inefficient in yielding chimeric animals once injected in pre-implantation epiblasts (FIG. 3), probably because they have altered molecular characteristics and correspond to a more advanced developmental stage in comparison to the host pre-implantation environment[9,10]. EpiSCs can, however, form low-contribution chimeric embryos when injected into host post-implantation embryos *ex vivo*[67].

EpiSCs maintain OCT4 and SOX2 expression, but they downregulate expression of most of the other pluripotency factors, including NANOG, ESRRβ, KLF2 and KLF4 (REF. 3). EpiSCs have not undergone differentiation, but they upregulate lineage commitment factors such as homeobox protein OTX2, Brachyury and zinc-finger protein ZIC2 (REF. 68). Epigenetically, EpiSCs have distinct characteristics from naive ES cells: they inactivate the X chromosome in females, upregulate global DNA methylation levels and acquire histone H3 Lys27 trimethylation (H3K27me3) at developmental regulators[69,70]. The enhancer landscape is rewired between naive and primed pluripotent states[68], and developmental regulator gene-associated seed enhancers convert from a dormant to an active state in EpiSCs, thus pre-marking the lineage differentiation bias of primed PSCs[71]. FIGURES 2,3 summarize the divergent signalling and molecular characteristics of murine primed and naive pluripotent cells.

At the regulatory level, naive and primed pluripotent cells have been shown to have opposing dependence on epigenetic repressors[66] (FIG. 4). Naive ES cells expanded in FBS/LIF or 2i/LIF conditions tolerate loss of epigenetic repressors such as DNA methyltransferase 1 (DNMT1), DICER, Polycomb protein EED, MBD3 and methyltransferase-like protein 3 (METTL3)[66], which renders these cells 'hyper-naive' and resistant to differentiation[66,72] (FIG. 4). Conversely, the maintenance and viability of murine primed pluripotent cells depend on these regulators, and their ablation destabilizes the murine primed pluripotent state[66] (FIG. 4). Defining precisely how the depletion of each of these repressors destabilizes the primed configuration is of future interest.

Alternative growth conditions to expand murine EpiSCs have begun to emerge. The simultaneous use of a GSK3i (which induces β-catenin stabilization) together with a small-molecule inhibitor of tankyrase, IWR1 (which upregulates levels of axin 1 and axin 2, thus leading to the retention of β-catenin in the cytoplasm) — known as GSK3i/IWR1 conditions — maintains novel primed EpiSCs without exogenous FGF2/Activin A supplementation[73] (FIG. 3). Removal of IWR1 leads to increased nuclear shuttling of β-catenin and EpiSC differentiation[73]. The mechanisms by which cytoplasmic β-catenin prevents EpiSC differentiation remain to be uncovered[73]. It is tempting to speculate that the recently described ability of the cytoplasmic anaphase-promoting complex (APC)–axin–β-catenin destruction complex to function as a sequestration 'sink' for YAP and TAZ and prevent their nuclear shuttling[52] is involved in the ability of GSK3i/IWR1 conditions to maintain EpiSCs. Notably, the latter alternative EpiSC state is different from EpiSCs expanded in classical FGF2/Activin A conditions, in that it retains higher levels of expression of naive markers[73] and is thus relatively less primed (FIG. 3).

Recent studies indicate that different primed conditions can endow EpiSCs with region-specific characteristics of post-implantation epiblasts. EpiSCs expanded in FGF2/Activin A conditions correspond transcriptionally and functionally to anterior late-gastrula primitive streak cells[74]. Alternative FGF2/IWR1 conditions generate murine EpiSCs that correspond to posterior-proximal epiblasts[75]. Furthermore, even in classical FGF2/Activin A conditions, distinct subpopulations of EpiSCs can co-exist, each representing different stages of post-implantation embryonic development[76].

Finally, the length of time for which pluripotent cells are maintained under primed conditions greatly influences their characteristics and functionality[77]. Counter-intuitively, whereas mouse PGCs are specified from the post-implantation epiblast *in vivo*, EpiSCs that are maintained *in vitro* for more than 7 days in FGF2/Activin A conditions lose competence to generate PGCs in response to BMP4 (REF. 77). Starting with naive cells and inducing brief priming for 2–4 days yields distinct primed cells that are highly competent for generating PGC-like cells, termed Epi-like cells[77]. The latter are transcriptionally more similar to *in vivo* post-implantation epiblasts than to EpiSCs[77]. Thus, the above paradigm indicates another aspect of the artificial features that can be acquired by pluripotent cells

FGF2/Activin A conditions
Defined primed pluripotency growth conditions for mouse epiblast stem cells, composed of recombinant fibroblast growth factor 2 (FGF2) and Activin A cytokines.

Seed enhancers
A subgroup of enhancers that are dormant in naive cells but become more active in primed pluripotent and somatic cells.

GSK3i/IWR1 conditions
Defined primed pluripotency growth conditions for mouse epiblast stem cells, containing a glycogen synthase kinase 3 (GSK3) pathway inhibitor and the small-molecule tankyrase inhibitor, IWR1.

FGF2/IWR1 conditions
Defined primed pluripotency growth conditions for mouse epiblast stem cells, containing recombinant fibroblast growth factor 2 (FGF2) and the small-molecule tankyrase inhibitor, IWR1.

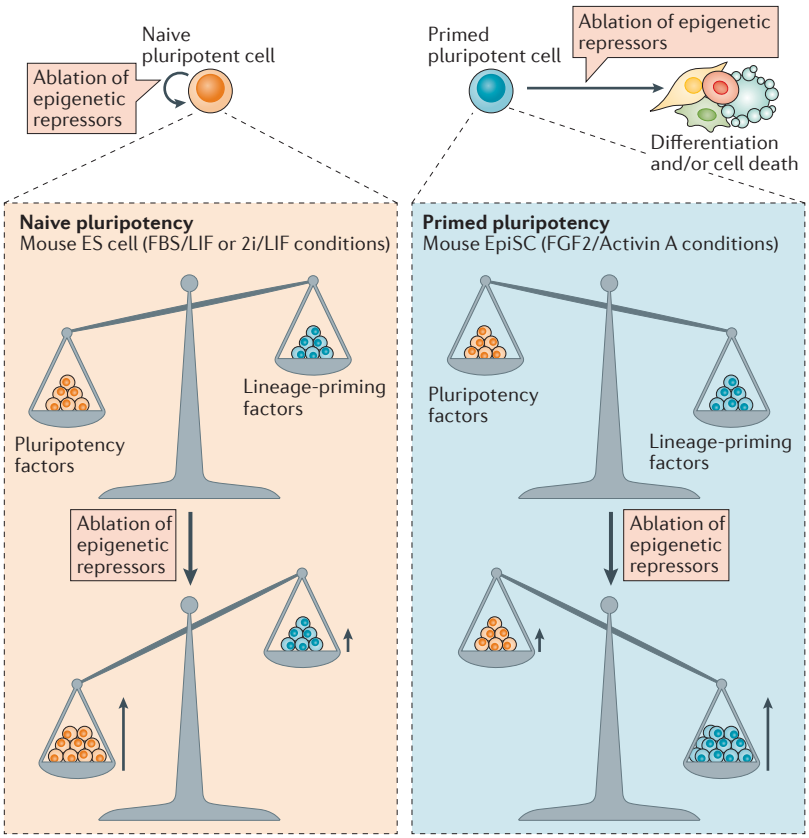


Figure 4 | **The opposing influence of epigenetic repressors on murine naive and primed pluripotent cells.** Murine naive and primed pluripotent cells differ not only in their dependence on distinct signalling pathways and in their epigenetic profiles, but also in their lineage decision-making. Murine naive embryonic stem cells (ES cells) expanded in either 2i/leukaemia inhibitory factor (LIF) or fetal bovine serum (FBS)/LIF conditions tolerate complete loss of epigenetic repressors such as DNA methyltransferase 1 (DNMT1), methyl CpG-binding domain protein 3 (MBD3), DICER, DGCR8, methyltransferase-like protein 3 (METTL3), EED and EZH2. Furthermore, the loss of epigenetic repressors strengthens the equilibrium in favour of pluripotency-promoting factors and generates 'hyper-naive' pluripotent cells that are relatively more resistant to differentiation and can tolerate withdrawal of LIF cytokine. Murine primed epiblast stem cells (EpiSCs) generally respond in the opposite manner to the complete ablation of epigenetic repressors. Established murine primed EpiSCs naturally downregulate pluripotency factors and upregulate lineage-priming factors compared with naive pluripotent cells. Ablation of epigenetic repressors at the primed pluripotency stage tips the balance towards differentiation and/or compromises cell survival. It should be noted that the ablation of various epigenetic repressors (such as METTL3 or DGCR8) overall negatively influences the stability of primed pluripotency; however, the downstream events leading to the state collapse are not necessarily identical in each case and thus should be thoroughly dissected *in vitro* and *in vivo*. FGF2, fibroblast growth factor 2. Adapted with permission from REF. 66, AAAS.

once they are expanded indefinitely *in vitro,* in contrast to their *in vivo* counterparts that exist only transiently during development.

Studies involving clonal lines and single-cell analysis will provide deeper understanding of the features of region-specific EpiSCs and of EpiSCs shortly after their *in vitro* induction from a naive state under different priming conditions[74]. This may help us to understand how lineage priming is established at the single-cell level during these key early developmental transitions[74,75] and may be relevant for optimizing other differentiation protocols and predicting the behaviour of PSCs.

Conversion between naive and primed states. Similar to the reprogramming of somatic cells into a naive ES cell-like state by the combined overexpression of pluripotency factors together with LIF, primed EpiSCs can also be reverted to naive iPSCs. Overexpression of KLF4 or MYC in EpiSCs, under LIF-containing conditions, generates naive ES cells[30,78]. FBS/LIF signalling alone can be sufficient to induce such conversion in cells from permissive mouse genetic backgrounds (such as 129 strains)[79], but not in cells from 'non-permissive' strains such as NOD mice, for which supplementation with small molecules, such as 2i conditions, is necessary[30]. Other factors, such as NANOG, PR domain zinc-finger protein 14 (PRDM14) and ESRRβ, have been shown to synergistically induce and boost the efficiency of this process[80,81]. Explanting post-implantation embryonic day 5.5 (E5.5)–E7.5 epiblasts in naive conditions also reverts them to naive PSCs[30,79]. The opposite conversion can be achieved for *in vitro* and *in vivo* isolated naive cells, as expanding murine naive PSCs or ICM cells under primed conditions leads them to gradually adopt an EpiSC state[30,33,78].

Studies focusing on the molecular changes that accompany the *in vitro* conversion from naive to primed pluripotent states have unravelled key events in the mechanisms of reprogramming[69]. Naive ES cells expanded in 2i/LIF conditions retain global levels of hypomethylation in both promoters and gene bodies, highly similar to those measured in ICM cells[82,83]. When naive ES cells are transferred into FBS/LIF naive conditions, there is an increase in global DNA methylation levels, although promoter and enhancer regulatory regions remain protected from DNA methylation[84]. Only after transfer into primed FGF2/Activin A EpiSC-inducing growth conditions does DNA methylation accumulate over enhancer and promoter regulatory elements[84].

Transitioning naive FBS/LIF-cultured PSCs into 2i/LIF conditions initially leads to changes in the promoter occupancy of the genes encoding the OCT4, SOX2 and NANOG pluripotency factors[85]. Changes in H3K27me3 deposition and the enhancer landscape follow later, probably in response to the rewiring of transcription factor binding[85]. Downregulation of DNA methylation follows next and has mainly been attributed to downregulation in expression of *de novo* DNA methyltransferase enzymes[82]. It should be noted, however, that ablation of DNMT3A and DNMT3B in ES cells in FBS/LIF conditions does not lead to such rapid loss of DNA methylation[86], and other yet-to-be-identified events might be involved in this rapid 2i-induced epigenetic response. MEK–ERK inhibition influences Polycomb interactions and leads to decreased promoter occupancy by Polycomb repressive complex 2 (PRC2) and decreased phosphorylation on the carboxy-terminal domain of RNA polymerase II (Pol II) on lineage-commitment genes[87], leading to loss of H3K27me3 and increased Pol II pausing at bivalent developmental regulatory loci[69]. Analysis of other defined naive pluripotency growth conditions (such as 2i/LIF/PKCi and alternative 2i conditions) and studies in other rodents will be important to discern the redundancies and specificities of different signalling pathways and their crosstalk with chromatin organization.

Human conventional pluripotent cells

The first human ES cells to be isolated from blastocysts[6] were markedly different from murine ES cells in their characteristics and tissue culture requirements. FGF2 and transforming growth factor-β1 (TGFβ1)/Activin A signalling (but not LIF signalling) are the core signalling modules that maintain such conventional human ES cells derived from the ICM, or iPSCs obtained by direct *in vitro* reprogramming[88].

A primed pluripotent state. Differences between conventional human and mouse ES cells had initially been attributed to unknown genetic differences between species, as human ES cells were also derived from the ICM and not from post-implantation stages. However, studies of stem cells derived from different mouse strains have discerned a scenario whereby ICM cells can adapt *in vitro* into a primed state if naive conditions are not devised to match the requirements of the particular genetic background of the donor embryos used[30]. Specifically, NOD mice are relatively 'less permissive' than 129 mice to yielding naive ES cells and iPSCs, as LIF alone is not sufficient to maintain naive pluripotency of cells from NOD mice, and 2i/LIF conditions are permanently required to stabilize and maintain naive pluripotency *in vitro* in NOD PSCs[30]. Furthermore, ICM cells from both 129 and NOD mice expanded under primed conditions yield EpiSC-like cells that are indistinguishable from EpiSCs derived from E6.5 embryos[30] or *in vitro* from already established ES cells[30].

The relevance of the latter *in vitro* priming scenario to determining the identity of conventional human ES cells is supported by the fact that human conventional ES cells and iPSCs retain a large number of primed pluripotency features. These include low levels of expression of naive pluripotency markers (such as KLF17 and developmental pluripotency-associated protein 3 (DPPA3)), deposition of H3K27me3 over developmental genes, lack of exclusive nuclear localization of transcription factor E3 (TFE3), loss of pluripotency upon inhibition of MEK–ERK signalling, lack of global hypomethylation as seen in ICM cells, and lack of a pre-X inactivation state in most conventional female PSC lines[70,89,90]. Furthermore, human primed ES cells do not tolerate complete loss of DNMT1 (REF. 86), similar to what has been shown for mouse EpiSCs[66] (FIG. 4). Complete knockout of *DICER*, *MBD3* or *METTL3* has not been achieved so far for human ES cells[64,66] (FIG. 3).

Less primed than murine EpiSCs. Despite the above, it is important to realize that human conventional or primed ES cells are not identical to murine EpiSCs and can be considered to be relatively less primed. For example, human ES cells do not upregulate the expression of FGF5 or N-cadherin (as seen in murine EpiSCs), and human ES cells express high levels of E-cadherin (as detected in mouse naive ES cells)[70]. Human ES cells express high levels of some naive pluripotency markers such as NANOG, PRDM14 and reduced expression protein 1 (REX1; also known as ZFP42) that are not expressed or are residually expressed by mouse EpiSCs[91]. Moreover, human primed ES cells are functionally dependent on NANOG and PRDM14, and ablation of these factors induces the differentiation of human ES cells[91]. The distribution of DNA methylation in human ES cells corresponds to that of murine naive ES cells expanded in FBS/LIF conditions, rather than that of FGF2/Activin A-expanded mouse EpiSCs, as the promoters of human ES cells and mouse naive ES cells are protected from invasion by repressive DNA methylation[84,92]. Furthermore, whereas murine EpiSCs demonstrate exclusive cytoplasmic localization of TFE3, and naive 2i/LIF-cultured ES cells show exclusive nuclear localization of TFE3 (REF. 93), human primed ES cells show an intermediate configuration, in which TFE3 is present in both the cytoplasm and the nucleus[70].

Human naive pluripotent cells

The metastability of naive and primed pluripotent states depending on the growth conditions used[30], and the stringent requirements for exogenous factors to promote the naive pluripotency of isolated naive PSCs from previously 'non-permissive' rodent strains[30,31], have led to a consideration of whether unique and more stringent conditions can be used to isolate previously unidentified alternative naive-like pluripotent states in humans.

Transgene-dependent generation. 2i/LIF conditions are not sufficient to maintain naive human ES cells or iPSCs[94]. However, additional transgene expression can induce an artificial transgene-dependent state that may be of considerable interest. Continued exogenous *OCT4* and *KLF4*, or *KLF2* and *KLF4*, transgene expression can maintain human ES cells and iPSCs in a unique pluripotent state in 2i/LIF conditions[94]. Recently, these observations were extended by optimizing the overexpression of *KLF2* and *NANOG* transgenes, allowing the expansion of human naive iPSCs in 2i/LIF conditions[95]. Overexpression of *KLF2* and *NANOG* transgenes in primed ES cells also enabled their expansion in 2i/LIF/aPKCi conditions[96]. These cells had extensive DNA hypomethylation and marked upregulation of naive pluripotency markers such as transcription factor CP2-like protein 1 (TFCP2L1), KLF2 and KLF4. However, as KLF2 is not expressed in the human ICM[97], as 2i/LIF/aPKCi conditions are insufficient to convert primed ES cells without exogenous transgene induction[96] and as transgene-free cells remain to be validated under 2i/LIF/aPKCi conditions, it is unclear whether this state is indefinitely stable without retaining possibly leaky transgenes or MEFs. Furthermore, independent examination of the DNA methylation landscape in these cells indicates an aberrant global loss of imprinting and excessive hypomethylation of endogenous retroviral genes[89,98]. Finally, although 2i/LIF/aPKCi conditions do not contain exogenous FGF or TGFβ1/Activin A cytokines, short-term inhibition of FGF receptor (FGFR) and TGF receptor (TGFR) signalling is not sufficient evidence to validate the independence of the cells from FGF, TGF and Activin A signalling[96] (FIG. 2). Indeed, unlike in mice, the human pluripotent ICM differentiates following treatment of blastocysts with small-molecule inhibitors for TGF and activin–NODAL signalling[97].

Although the field has shifted to studying transgene-independent conditions, as detailed below, it should be noted that transgene-dependent states may nevertheless be important, as it is possible that the robust naive pluripotency currently obtained in mouse ES cells is a rodent-specific phenomenon. Capturing human naive PSCs identical to those obtained from mice might still involve genetic modifications. Nevertheless, the following studies provide evidence that it is possible to generate alternative pluripotent states in humans and other species[30,94].

Transgene-independent generation. Our team was the first to describe naive pluripotency growth conditions — designated NHSM (naive human stem cell medium) — that involve the complete ablation of MEK–ERK signalling and are compatible with the indefinite expansion of genetically unmodified human PSCs, in both MEF-containing and MEF-free conditions[70]. These naive MEK-independent pluripotent cell lines could be derived from human pre-implantation embryos, through *de novo* iPSC generation, or from previously established primed ES cells and iPSCs[70]. NHSM conditions contain 2i/LIF together with p38 inhibitor (p38i), Jun N-terminal kinase inhibitor (JNKi), aPKCi, RHO-associated protein kinase 1 inhibitor (ROCKi), and low doses of FGF2 and TGFβ1 (or Activin A); they render human PSCs more similar, but not identical, to murine naive PSCs[70] (FIGS 2,3). In fact, human cells cultured in NHSM have features of so-called naive 2i/LIF-induced ground state pluripotency, which are not found even in naive mouse ES cells expanded in FBS/LIF conditions. These features include exclusive nuclear localization of TFE3 and demethylation of H3K27me3 marks over developmental genes[69,70,93]. Transcriptionally, these cells have downregulated expression of lineage-commitment markers such as OTX2, ZIC2 and CD24, and moderately upregulated expression of pluripotency genes (more prominently when cultured on MEFs and when aPKCi is used)[70,99]. Enhancer rewiring has been attained in these human naive PSCs, as seen with mouse cells[71]. The cells had downregulation of expression of DNMT3B[100] and a small global decrease in levels of DNA methylation, while maintaining imprinting integrity and chromosomal stability[70]. Although chimeric analysis with human PSCs and the use of human embryos as hosts are ethically and legally forbidden, these human naive PSCs showed, for the first time, authentic integration into blastocysts following microinjection into host mouse morulas and were able to contribute at low-grade levels in mouse embryos up to E10.5–E17.5 that underwent advanced organogenesis[70] (FIG. 3).

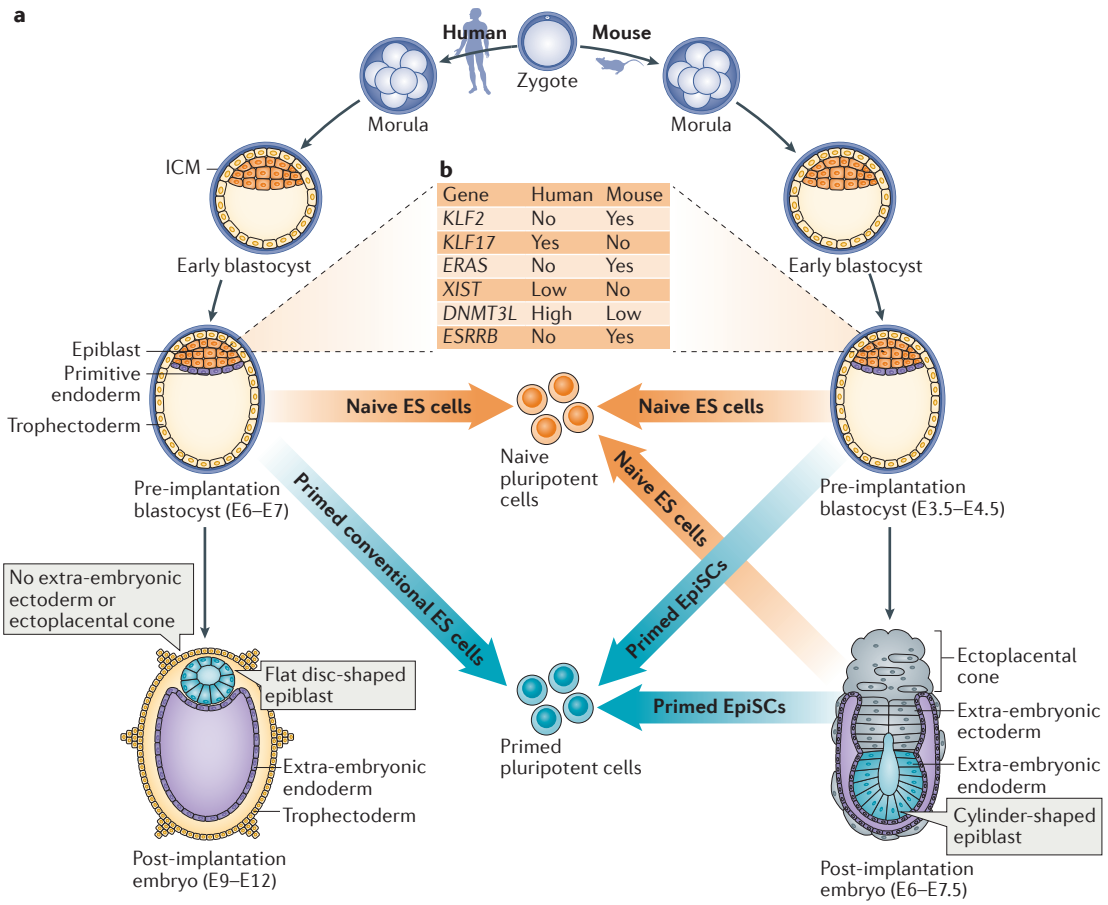
Important publications describing alternative conditions that yield human MEK-independent naive pluripotent cells have emerged since then, each describing the production of cells with different enhanced molecular properties (FIG. 3). A combination of 2i/LIF, ROCKi, BMP receptor inhibitor (BMPRi), and high doses of FGF2 and TGFβ1 could maintain human PSCs only in the presence of MEFs[101]. These PSCs had transcriptional upregulation of pluripotency markers such as STELLA (also known as DPPA3) and KLF5. Another study described culture conditions[95] that used most of the same components found in NHSM[70] (2i/LIF, ROCKi and Activin A (instead of TGFβ1) — with or without FGF2 and JNKi) but were supplemented with inhibitors for the Ser/Thr protein kinase BRAF and SRC pathways (termed 5i/LA-MEF conditions). In comparison to previous studies, cells in 5i/LA-MEF conditions had a more marked upregulation of naive pluripotency markers. However, the cells did not downregulate expression of DNMT3B, maintained an inactive X chromosome state in female cell lines and had an unusual pre-ICM transcriptional signature[95]. Intriguingly, the process of converting primed cells back to a naive state in 5i/LA-MEF conditions is inefficient, taking 2 weeks to isolate initial clones that retain a slow growth rate[95]. Furthermore, these conditions exclusively yield chromosomally abnormal cell lines[95]. Thus, it remains to be determined whether such chromosomal abnormalities are in fact inherent to 5i/LA-MEF-expanded cells and determine the properties described for this state[95], and whether they are being selected for during this inefficient conversion process. Finally, DNA methylation profiling and the epigenetic imprinting integrity of these cells are important aspects that remain to be evaluated.

It is clear from the above summaries that none of the many published conditions generates human naive PSCs that are identical to mouse ES cells or human ICM[97,102,103]. However, these studies implicate new signalling pathways as being involved in human naive pluripotency and suggest avenues of research for the further optimization and characterization of such novel PSCs (FIG. 2). Mechanistically, it will be interesting to test whether there is a connection between RAF and aPKC inhibition and the influence of modulating WNT signalling by applying GSK3i/IWR1 conditions[73] to human naive PSCs. Furthermore, RHO signalling has been shown to promote the nuclear localization of YAP and TAZ in primed human ES cells and to sustain their pluripotency[104]. Thus, it remains to be defined whether ROCKi influences naive pluripotency characteristics[70] through the modulation of YAP and TAZ.

The role of MEK-independent FGF2 and TGFβ1/Activin A signalling, either in an autocrine manner or exogenously provided at low doses, remains to be understood in human naive PSCs. These cells show upregulation of the activin-like ligand growth-differentiation factor 3 (GDF3)[96], and human (but not mouse) ICM cells abundantly express activin receptors[97]. Thus, it is tempting to speculate that primed human ES cells are relatively less primed than murine EpiSCs, owing to differences in their response to activin-like ligands, which might promote some naive features in human cells[70,95] but not in mouse cells. To conclude, systematic analysis of the response of pluripotent cells of different states from different species to a variety of TGF-ligand family members is of importance (FIG. 2). The possibility of generating human PSCs that are entirely independent of FGF and/or TGF signalling cannot be excluded.

Differences between mouse and human epiblasts

Recent studies focusing on single-cell RNA-seq of human pre-implantation embryos are starting to provide answers to some of the questions highlighted above. Although there are no morphological differences between human and mouse blastocysts, they have marked molecular differences at the cellular level[97] (FIG. 5a,b). Human ICM epiblast cells do not express genes such as *KLF2* and *ESRRB* that are thought to be important pluripotency factors in mice. Instead, KLF17 may have a human-specific role in the ICM[97]. The GTPase ERAS, which is an ES cell-specific form of



RAS, has become a pseudogene in humans[105], whereas ERAS-null mouse ES cells propagate slowly in FBS/LIF conditions[105]. Non-human primate (marmoset) pluripotent epiblasts have a similar transcriptional signature to that of human epiblasts, which is very different to that of mouse epiblasts[106]. The marmoset and human naive ICM signatures include a lack of transcription of *KLF2*, nuclear receptor subfamily 0 group B member 1 (*NR0B1*) and *BMP4*, with high levels of expression of NODAL and its downstream signalling mediators[106].

At the post-implantation stage, there are major differences between rodent and human embryos (FIG. 5a,b). Rodents are unusual in that their post-implantation epiblast assumes an egg-like cylinder shape, whereas in humans, the post-implantation epiblast assumes a flat disc shape, similar to that of most other mammals. Although it is not possible to carry out single-cell analysis on early human post-implantation epiblasts, the study of non-human primates may provide some relevant insights. Collectively, these differences between species may directly influence the distinct pluripotent characteristics that are observed in PSCs from different species *in vitro* and their distinct growth requirements. Furthermore, they are of relevance for understanding the developmental context of human pluripotent cells isolated *in vitro*.

◀ Figure 5 | **A model to classify 'relative naivety' within the spectrum of naive to primed pluripotency. a** | There are important similarities and differences in early pre- and post-implantation *in vivo* development in mice and humans. Although mouse and human embryos are morphologically similar until the blastocyst stage, there are important transcriptional differences, as summarized in part **b**. Furthermore, at the post-implantation stage, morphological differences in embryo shape become apparent between mice and humans, including differences in epiblast shape and in extra-embryonic structures. In mice, naive embryonic stem cells (ES cells) can be derived from the inner cell mass (ICM) of pre-implantation blastocysts or from post-implantation epiblasts when naive growth conditions are applied. Primed epiblast stem cells (EpiSCs) can be derived from post-implantation epiblasts or from the ICM when primed culture conditions are used. Thus, the growth conditions rather than the cell source determine the pluripotent state configuration that is acquired *in vitro*. A similar scenario applies for the derivation of both naive and primed pluripotent cells from human ICM, depending on the growth conditions used. Pluripotent cells cannot be derived from human post-implantation embryos, owing to ethical issues; therefore, a molecular analysis of these cells is lacking. **c** | A model to explain 'relative naivety' within the spectrum of naive to primed pluripotency. One major molecular and functional criterion that, in our opinion, can be used to separate naive and primed pluripotent cells is their ability to maintain and stabilize their pluripotent state upon blockade of MEK activity (dashed black line). Within the naive pluripotent state, it is difficult to describe the pluripotent state of cells in absolute terms, as naive cells can also have, to some extent, features of primed pluripotency. Similarly, within the spectrum of primed pluripotency, primed pluripotent cells cultured in different conditions have different features and varying degrees of naivety (FIG. 3). Finally, it is possible that supplementation of 2i/leukaemia inhibitory factor (LIF) conditions with small molecules such as atypical protein kinase C inhibitor (aPKCi), fibroblast growth factor receptor inhibitor (FGFRi) or NOTCH inhibitor (NOTCHi) can be used to consolidate naive pluripotency features, particularly for other rodents such as rats, for which the stability of naive cells in 2i/LIF feeder-free conditions should be further improved. Full annotation of the different human pluripotent states will enable charting of an equivalent landscape for human and other primate pluripotent stem cells. *DNMT3L*, DNA methyltransferase 3-like; *ERAS*, ES cell-expressed RAS; *ESRRB*, oestrogen-related receptor-β; FBS, fetal bovine serum; GSK3i, glycogen synthase kinase 3 inhibitor; KLF, Krüppel-like factor; TGFRi, transforming growth factor receptor inhibitor; *XIST*, X-inactive specific transcript. Parts **a** and **b** are adapted from Poster http://www.nature.com/nrm/posters/pluripotency/index.html, Nature Publishing Group. Part **c** is adapted with permission from REF. 4, Elsevier.

Classification of pluripotent states

The characterization of different conditions to isolate human naive PSCs with distinct characteristics, and the limitations to conducting chimeric analysis in humans, have stimulated discussions regarding the classification of pluripotent states. It is often claimed that the ability to derive ES cells from human ICM cells in a newly devised growth condition constitutes the 'gold standard' for proving naivety. However, it should be kept in mind that the pluripotent state identity is eventually dictated by the derivation growth conditions, and not by whether the source of the cells was the pre- or post-implantation epiblast[30,33] or PGCs[82]. Use of the *OCT4* distal versus proximal enhancer element as a binary distinguishing marker can also be misinterpreted[95]. Both distal and proximal enhancer elements of *OCT4* are active in naive and primed pluripotent states, both in humans and in mice[107,108]. The difference emerges from their relative activity levels (high versus low) and dominance.

Relying on a single attribute marker or functional test to define the pluripotent state is limiting and must be accompanied by systematic analysis of the ever-increasing number of characteristics that continue to be identified for different pluripotent states (FIGS 3,4). Nevertheless, in our opinion, a molecular and functional characteristic that can be considered to be a major divider between naive and primed pluripotent states is the response of cells to the challenge of blocking MEK signalling (FIG. 5c). Human conventional ES cells and mouse EpiSCs rapidly differentiate following MEK inhibition, whereas naive pluripotent cells tolerate MEK inhibition and consolidate their naivety following this challenge[94]. The significance of this characteristic is supported by the ability of MEK or ERK inhibition to expand murine epiblast in ICMs, and by the fact that it signifies consolidation of naive pluripotency *in vivo*[109].

Within the naive and primed ground states of pluripotency, it is clear that if one considers the many naive and primed pluripotency features that were originally described for mouse naive 2i/LIF-cultured ES cells and primed FGF2/Activin A-cultured EpiSCs, different pluripotency growth conditions can simultaneously endow a mixture of primed and naive properties in the same cell type (FIG. 3). As such, pluripotent states can be classified as 'more naive' or 'more primed' by virtue of having more of such properties (FIG. 5c). Human primed ES cells have several features of naive pluripotency (such as protection of promoter regions from hypermethylation and dependence on NANOG), and recent comparative analysis with single-cell RNA-seq of human blastocysts suggested that some conventional human ES cell lines may be transcriptionally relatively less primed than previously thought[97]. Murine naive ES cells expanded in FBS/LIF conditions can give rise to 'all-ES cell' chimeric embryos and tolerate ablation of *Dnmt1* and *Mettl3*; however, they are globally hypermethylated and acquire H3K27me3 marks over developmental genes, as seen in EpiSCs (FIGS 3,5c). Thus, FBS/LIF conditions seem to endow mouse ES cells with relatively less-naive properties than 2i/LIF conditions.

Box 1 | **A 'dark side' of naive pluripotency?**

With the development of naive pluripotency culture conditions and efforts to endow cells with more features of naivety, it has become relevant to ask how much naivety is needed, and whether there is a 'dark side' to permanently maintaining pluripotent stem cells (PSCs) under certain naive conditions.

Rodent embryonic stem cells (ES cells) expanded in 2i/leukaemia inhibitory factor (LIF) conditions have an increased tendency to acquire genomic abnormalities[41], and it remains unclear whether these alterations occur as a by-product of non-specific activity of the small-molecule inhibitors used[95,116] or as a direct result of intrinsic molecular features of naive pluripotency (for example, increased activity of endogenous retroviral elements or a reduction in epigenetic repressive marks). One can envision a scenario in which such features can be tolerated *in vivo* because this configuration exists for only 1–2 days, whereas prolonged *in vitro* expansion of this state might increase the frequency of unwanted damaging events.

This concern may also relate to safeguarding the integrity of DNA methylation and imprinting in naive PSCs expanded *in vitro* over an extended period of time. Studies focusing on the loss of DNA methylation following the transfer of mouse PSCs into 2i/LIF conditions have quantified methylation levels 10–24 days after transfer and have documented a rapid global loss of DNA methylation accompanied by the relative resistance of retrotransposons and imprinting regions to such demethylation[83]. However, it is unclear whether this methylation state represents a final plateau that naive cells achieve, or whether further culture in 2i/LIF conditions would lead to a decrease in the relative resistance of such regions to demethylation. Indeed, methylation over imprinted genes and retrotransposons is partially, yet significantly, reduced in 2i/LIF conditions[83].

If such effects are frequent, researchers will have to re-evaluate how to optimize the application of naive conditions. One scenario might involve decreasing inhibitor levels to avoid excessive hypomethylation or other unwanted effects. An alternative scenario is to maintain cells in primed pluripotency culture conditions and transfer them into naive culture conditions only for a short time before the initiation of differentiation.

Another functional test that can be used to assess the stringency and extent of naivety in different primate naive PSCs is whether the cells can tolerate complete ablation of epigenetic repressors such as *METTL3*, *DNMT1*, *DGCR8* and *MBD3* (REFS 4,66) (FIGS 3,4). Furthermore, such tests might be useful for optimizing conditions that can close the gap between mouse and human naive pluripotent cells (BOX 1; FIG. 4). It will also be informative to annotate different naive and primed states from other species that have been isolated thus far according to such criteria (FIG. 3).

Implications and future directions

The breakthrough discovery that it is possible for somatic cells to be reprogrammed to pluripotency[21] has provided the foundation for a deeper investigation of pluripotent states, and for the understanding that pluripotent configurations can be rewired. The ability to rewire cells for pluripotency has a direct influence on current hurdles and limitations related to the quality and characteristics of human iPSCs (BOXES 1,2).

One of the most fascinating questions related to the naive-to-primed pluripotency continuum is why these divergent pluripotent configurations exist. This is often accompanied by the pragmatic consideration of which cells — naive or primed — are better to work with. In our opinion, as this phenomenon is deeply rooted in early embryonic development *in vivo*, it is likely that both naive and primed configurations constitute essential and integral components of the developmental process, to optimize and maximize the benefits of multipotency and lineage specification simultaneously. We hypothesize that naive pluripotency emerged as an epigenetic erasure state that renders pluripotent cells free of lineage and epigenetic restriction, while simultaneously making these cells relatively less responsive to signalling pathways that might interfere with the establishment of such a lineage-neutral state. The induction of specification by morphogens may not be efficiently enforced during or immediately after establishing naive pluripotency, without a short 'delay period'. As such, the naive pluripotency network is gradually resolved and becomes more receptive to inductive cues at the post-implantation stage, and PSCs are differentially patterned and primed according to their spatial localization before overt somatic differentiation occurs.

At the functional level, it remains to be established whether using human naive PSCs as a starting material, with or without a brief period of priming, would resolve the problems that are currently associated with protocols for the *in vitro* differentiation of human PSCs. Will human naive PSCs yield increased consistency in differentiation between independent iPSC lines[110]? Can naive PSC conditions yield better quality cells in differentiation protocols when used as a starting material? Can human naive PSCs be used in differentiation protocols that have not been successful with human conventional PSCs? Encouraging support for this possibility has recently been provided by results showing the enhanced ability of human PSCs expanded in NHSM conditions (even in the absence of aPKCi) to undergo *in vitro* differentiation into PGCs, which is a protocol that was

Box 2 | **Potential implications of induced pluripotent stem cell (iPSC) reprogramming**

Recent studies have elucidated how certain epigenetic regulators have opposing effects on the maintenance of naive and primed murine PSCs[66] (FIG. 4). These findings may be relevant when comparing the induction of pluripotency mechanisms in humans versus mice, as human iPSCs, but not mouse iPSCs, are typically reprogrammed in conventional (primed) pluripotency conditions[117]. Consequently, some of the differences observed between human and mouse iPSC regulators may be related not to species differences but rather to the fact that distinct pluripotent states are being induced. Therefore, it will be imperative to expand screens of human iPSC reprogramming regulators to include different pluripotency conditions, as these may yield different outcomes.

Another consideration related to the effect of pluripotent state characteristics on reprogramming is whether naive conditions might improve the quality of the iPSCs obtained and facilitate the loss of residual epigenetic memory and heterogeneity[118,119]. Similarly, subtle epigenetic differences in DNA methylation between nuclear transfer embryonic stem cells and iPSCs generated from the same human donor cells[120] might also be neutralized when deriving iPSCs in naive conditions that mimic more closely the epigenetic features of the inner cell mass.

inefficient for primed human PSCs[111]. The molecular rationale for evaluating the potential benefits highlighted above is that naive pluripotency is more associated with the removal of epigenetic repressive marks in regulatory regions, compared with primed pluripotency[70,96]. This might enable more efficient activation of lineage specifiers during differentiation of naive pluripotent cells. Furthermore, lineage biases in human primed PSCs are heavily associated with the localized accumulation of repressive marks such as DNA methylation[112].

The recent advances in generating human naive PSCs will continue to boost attempts to generate naive-like PSCs from other species and to test same-species and inter-species embryo chimerism assays[113]. Cynomolgus monkey naive ES cells derived in NHSM conditions gave rise to the first chimera-competent ES cells from a non-human primate[114]. Developmentally advanced mouse embryos (E10.5–E17.5) with low levels of chimerism were obtained following injection of naive human[70] or monkey iPSCs[115]. These observations raise various questions related to defining the frequency, lineage preference and developmental quality of such integrated primate iPSC-derived cells. Systematic efforts will be key to determining whether humanized animal models[70] might become relevant to disease modelling, the study of human development or the generation of transplantable human organs[113].

Continued breakthroughs in single-cell technologies and their application to different pluripotent cell types and embryonic samples will facilitate identification of the properties that are relevant for adequate functionality of PSCs. This will help to set standards for optimal starting materials for stem cell-based therapeutics and research (BOX 1). It is expected that while this previously under-appreciated complexity of pluripotency is being investigated to enable scientists to better control cell fate, proposed criteria and standards will need to be debated and revised.

Note added in proof

A recent study has reported and confirmed the aberrant and inevitable global loss of DNA imprinting in human naive pluripotent cells generated in 5i/LA or 2i/LIF/aPKCi/NANOG/KLF2 conditions[121].

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Competing interests statement
The authors declare competing interests: see Web version for details.

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SUPPLEMENTARY INFORMATION

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MOLECULAR CELL BIOLOGY

Stem cell states: naive to primed pluripotency

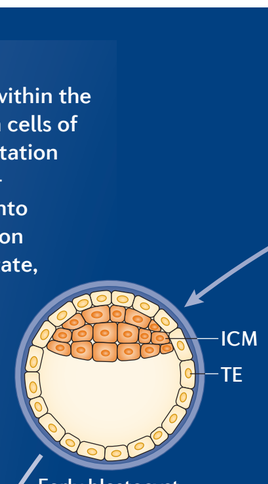
Leehee Weinberger, Yair S. Manor and Jacob H. Hanna

Pluripotency refers to the ability of cells to differentiate into all cell types of the three embryonic germ layers. Deriving and maintaining pluripotent stem cells thus offers the possibility of generating valuable sources of cells for tissue replacement therapies and for developmental studies. Pluripotent cells are found during a short window of time in developing embryos. They progress from a naive ('ground') state to a primed state before lineage commitment. Different culture conditions are being developed to maintain or induce these states *in vitro*.

Pluripotent cells in developing embryos

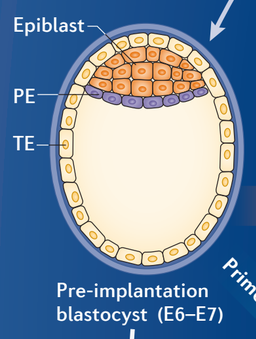
Pluripotency is a transient state *in vivo*. It is acquired within the ICM of developing pre-implantation blastocysts, when cells of the ICM segregate into PE and pluripotent pre-implantation naive epiblasts, and is gradually lost during early post-implantation development, before cells differentiate into somatic lineages. This transition from a pre-implantation pluripotent state to a post-implantation pluripotent state, which are referred to as naive and primed states, respectively, is associated with changes in molecular and functional characteristics.

Differences between human and mouse pre- and post-implantation embryos may be reflected by the different characteristics of naive and primed pluripotent cells *in vitro* and by the different requirements for their maintenance.



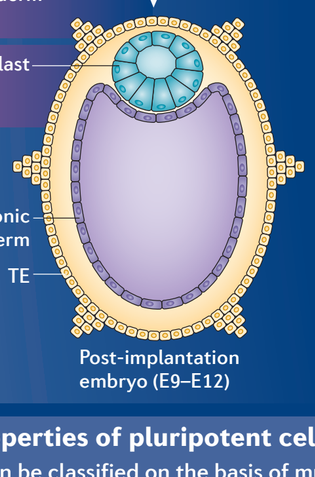
Gene expression in pre-implantation epiblasts

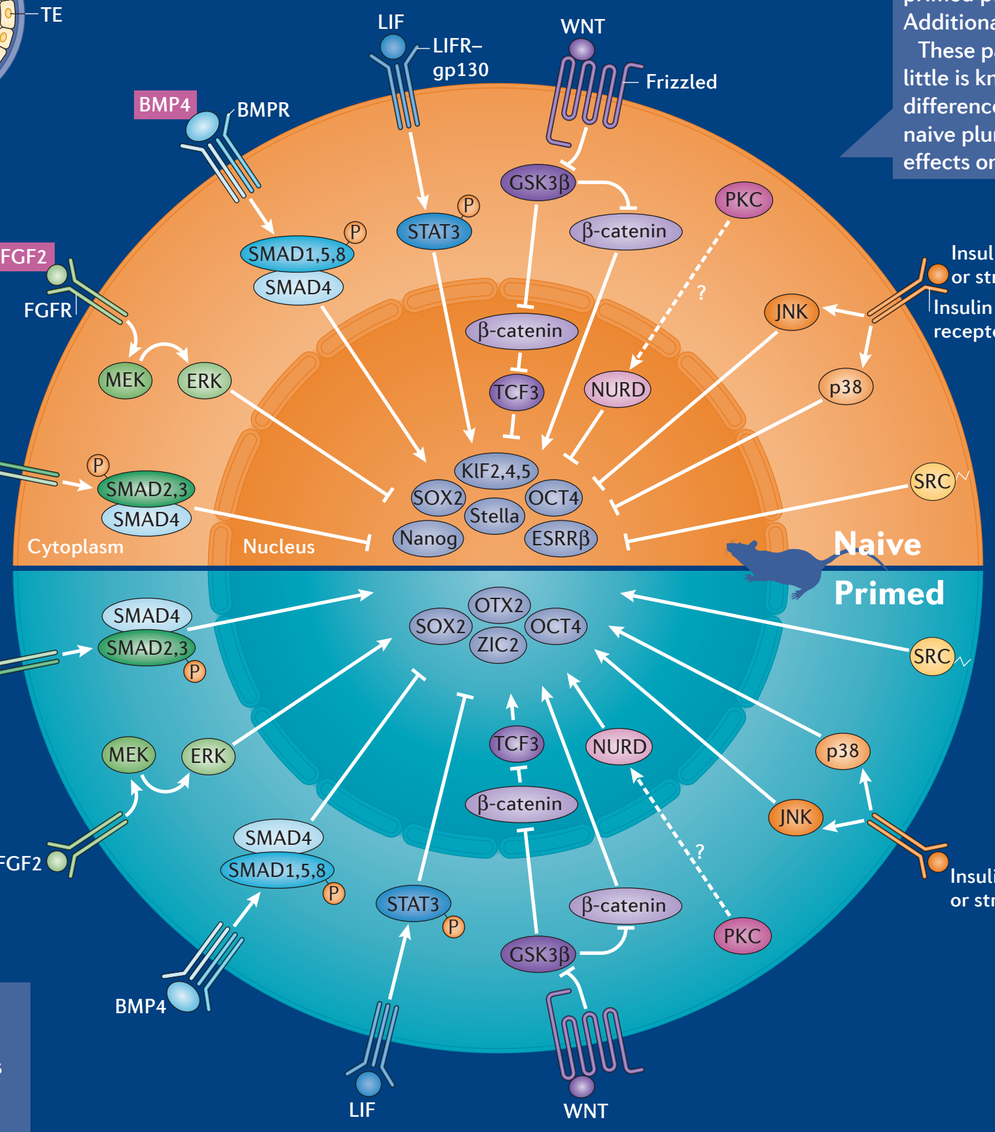
Gene	Human	Mouse
KLF2	No	Yes
KLF17	Yes	No
ERAS	No	Yes
XIST	Low	No
DNMT3L	High	Low



Naive ESCs

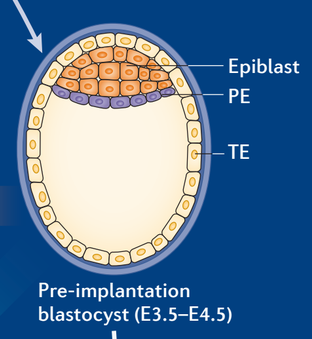
Primed conventional ESCs

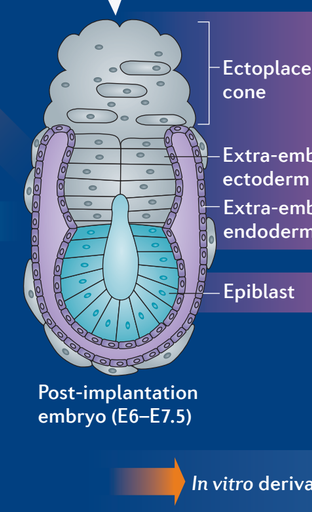




ESCs

EpiSCs





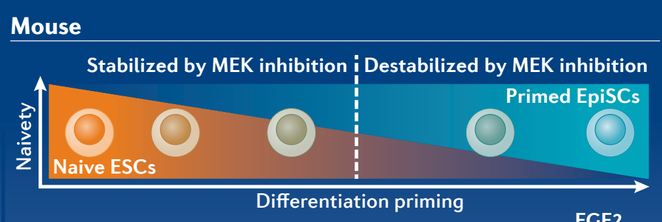
Signalling pathways that affect naive and primed pluripotency in mice

Mouse naive ESCs and primed EpiSCs can be artificially maintained in a self-renewing state *in vitro* by the continuous supplementation of various exogenous cytokines and/or small-molecule inhibitors. These factors regulate signalling pathways that can positively or negatively affect the stability of naive and primed pluripotency, which is regulated by a network of transcription factors. Additional regulatory pathways may yet be revealed.

These pathways affect primed pluripotency similarly in humans and mice, but little is known about their effects on human naive pluripotency. However, some differences have been identified between humans and mice in the regulation of naive pluripotency: low doses of FGF2, activin A and BMP4 may have opposing effects on its stability in different species.

Naive and primed properties of pluripotent cells *in vitro*

Naive and primed states can be classified on the basis of multiple characteristics that each state can retain *in vitro*. Different combinations of exogenous factors confer distinct characteristics to pluripotent stem cells *in vitro*. As a result, cells acquire a distinct set of naive and primed properties. In mice, ESCs cultured in a medium supplemented with 2i (two inhibitors of MEK and GSK3) and LIF, and EpiSCs cultured in a medium containing FGF2 and activin A, constitute the two extremes of the naive and primed pluripotency spectrum; cells maintained in other media are in 'intermediate states' that display a mixture of naive and primed features. Human 'conventional' ESCs, which are considered to be 'primed', are distinct from mouse primed EpiSCs and have various naive features. Optimizing conditions to derive and maintain human naive cells with properties identical to mouse naive pluripotent cells is an ongoing challenge. Moreover, primed cells can be stabilised in a distinct pluripotent state in the presence of FGF2 and WNT inhibitors.



In vitro derivation in naive conditions

In vitro derivation in primed conditions

Potential cross-species differences

Potential link

Pluripotency-associated property	Naive	Primed	Mouse: 2i, LIF	Mouse: FBS, LIF	Mouse: SRCi, GSK3βi	Mouse: MEKi, LIF	Mouse: BMP4, LIF	Mouse: GSK3βi, AXINs	Mouse: FGF2, activin A or FGF2, TGFβ	Rat: 2i, LIF, MEF	Rat: LIF, MEKi, PKCi	Rat: FGF2, activin A or FGF2, TGFβ	Human: OCT4, KLF2, KLF4, 2i, LIF	Human: 2i, LIF, p38i, JNKi, PKCi, ROCKi, FGF2, TGFβ	Human: 2i, LIF, PKCi, MEF (KLF2, NANOG)	Human: 2i, BRAFi, ROCKi, SRCi, LIF, activin A, MEF (FGF2, JNKi)	Human: 2i, BMPi, LIF, MEF	Human: FGF2, TGFβ or FGF2, activin A or FGF2, MEF	Rhesus: 2i, LIF, JNKi, p38i, FGF2, MEF	Rhesus: FGF2, TGFβ or FGF2, activin A or FGF2, MEF
MEK–ERK dependence	No	Yes	●	●	●	●	●	○	○	●	●	○	●	●	●	●	●	○	●	○
Long-term dependence on FGF2 signalling	No	Yes	●	●	●	●	●	●	○	●	●	○		○			○	○	○	○
Long-term dependence on TGFβ–activin A signalling	No	Yes	●	●	●	●	●	●	○	●	●	○		○		○	○	○		○
Dominant OCT4 enhancer	Distal	Proximal	●	●		●		○	○				●	●	●	●		○		
H3K27me3 on developmental regulators	Low	High	●	○					○					●		●	●	○		
Global DNA hypomethylation	Yes	No	●	○					○					● Mild	● Strong			○		○
X chromosome inactivation	No	Yes	●	●		●		○	○				●	●	●	○	○	○	●	○
Dependence on DNMT1, DICER, METTL3, MBD3	No	Yes	●	●					○									○		
Priming markers (OTX2, ZIC2)	↓	↑	●	●		●	○	○	○	○	○	○	●	●	●	●	●	○		○
Pluripotency markers (NANOG, KLFs, ESRRβ)	↑	↓	●	●	●	●	●	○	○	●	●	○	●*	● Mild*	● Strong*	● Strong*	● Mild*	○ *	● Mild*	○ *
TFE3 nuclear localization	High	Low	●	●		●			○					●	●		○	○		
CD24/MHC class 1	Low/low	High/mod	●	●					○					●	●	○		○		
HERV-H and HERV-K expression	High	Low	Primate specific											●	●	●	●	○		
Expressed adhesion molecules	E-cadherin	N-cadherin	●	●	●	●	●	○	○	●	●	○		●	●	●	●	○	●	○
Promotion of pluripotency maintenance via Nanog or Prdm14	Yes	No	●	●	● Nanog only	●	●	○ Nanog only	○					●	●	●	●	○		
Metabolism	OxPhos, glycolytic	Glycolytic	●	●					○							○		○		
Competence as initial starting cells for PGCLC induction	High	Low	●	○					○					●				○		
Capacity of colonization of host pre-implantation ICM and contribution to chimaeras	High	Low	●	●	●	●	●	○	○	●	●	○		●‡	●‡	○‡		○	●‡	○

* No ESRRβ; ‡ Mouse host embryos.

Recommended further reading

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Abbreviations

aPKC, atypical protein kinase C; AXINs, AXIN stabilizer (that is, tankyrase small-molecule inhibitors); BMP, bone morphogenetic protein; BMPR, BMP receptor; DNMT, DNA methyltransferase; E-cadherin; epithelial cadherin; EpiSC, post-implantation epiblast-derived stem cell; ESC, embryonic stem cell; ESRRβ; oestrogen-related receptor-β; FBS, fetal bovine serum; FGF, fibroblast growth factor; gp130, gylcorprotein 130; GSK3β, glycogen synthase kinase 3β; HERV, human endogenous retrovirus; H3K27me3, histone H3 Lys27 trimethylation; ICM, inner cell mass; JNK, JUN amino-terminal kinase; KLF, Krüppel-like factor; LIF, leukaemia inhibitory factor; LIFR, LIF receptor; MBD3, methyl-CpG-binding domain protein 3; MEF, mouse embryonic fibroblast; METTL3, methyltransferase-like 3; MHC, major histocompatibility complex; N-cadherin, neural cadherin; NuRD, nucleosome remodelling and deacetylation; OCT4, octamer-binding protein 4; OTX2, orthodenticle homeobox 2; OxPhos, oxidative phosphorylation; PE, primitive endoderm; PGCLC, primordial germ cell-like cells; PRDM14, PR domain zinc finger protein 14; ROCK, RHO-associated protein kinase; STAT3, signal transducer and activator of transcription 3; TE, trophoectoderm; TFE3, transcription factor E3; TGFβ; transforming growth factor-β; XIST, X-inactive specific transcript; ZIC2, ZIC family member 2.

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Contact information

Leehee Weinberger, Yair S. Manor and Jacob H. Hanna are at the Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel.
Jacob H. Hanna's laboratory homepage: http://hannalabweb.weizmann.ac.il/

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