Review

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Uncovering the true identity of naïve pluripotent stem cells

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Embryonic stem cells (ESCs) are pluripotent stem cells derived from the inner cell mass (ICM) of blastocyst embryos. Although first characterized over 30 years ago, the ontology of these cells remains elusive. Identifying the *in vivo* counterpart of murine ESCs will be essential for the derivation of stable ESC lines from other species. Several hypotheses exist concerning the ontology of murine ESCs. Recent data demonstrate that ESCs emerge from a subpopulation of ICM cells that transit through a Blimp1-positive state, suggesting that perhaps a germ cell developmental program underlies ESC derivation and maintenance. Alternatively, the common dependence of ESCs and diapause embryos on the cytokine LIF (leukemia inhibitory factor) has been thought to signify that murine ESCs employ a diapause-like program for their maintenance of pluripotency. Here we review different hypotheses regarding the nature of murine ESCs and discuss their implications for human pluripotent stem cell biology.

Naïve and primed pluripotent stem cells

ESCs are pluripotent cells that have the capacity to self-renew indefinitely while retaining the ability to differentiate into derivatives of all three somatic germ layers, including the germline. ESCs were first isolated from the inner cell mass (ICM) of preimplantation mouse blastocyst embryos [1,2]. Mouse embryonic stem cells (mESCs) are rapidly proliferating cells that form tight, dome-shaped colonies and require the growth factors LIF and Bmp4 to maintain their pluripotency [3]. Human embryonic stem cells (hESCs) are similarly derived from human blastocysts but, in contrast to murine ESCs, form flat 2D colonies dependent on bFGF and activin/TGFβ signaling [4]. The differences in morphology, growth factor dependency, and epigenetic status between mESCs and hESCs were initially thought to be species-specific variations of comparable pluripotent populations. This idea was challenged by the derivation of epiblast stem cells (EpiSCs) from postimplantation mouse epiblasts, which exhibit strikingly similar morphological, molecular, and epigenetic characteristics to hESCs [5,6]. Based on these findings, it seems that two distinct pluripotent states exist for pluripotent mouse cells: a naïve ICM-like state represented by mESCs and a primed postimplantation epiblast-like state represented by EpiSCs [3]. Despite their blastocyst origin, similarities with EpiSCs suggest that hESCs exist in a primed pluripotent state as well. Several groups have since attempted to derive and maintain naïve human pluripotent stem cells [7–10], but so far stable maintenance of naïve human pluripotent stem cells has been unsuccessful. The question remains whether such a naïve state can exist for human ESCs.

In this review we discuss the current perspectives of the *in vivo* equivalents of naïve ESCs. Because ESCs are derived from the ICM of blastocyst embryos, the most logical assumption is that they reflect the preimplantation epiblast. However, the unique germ cell signature of murine ESCs and the upregulation of germ cell genes during ESC derivation suggest that ESCs have a germ cell origin. By contrast, the common dependency on LIF signaling of ESCs as well as diapause embryos has led to the idea that ESCs utilize a diapause-like program to maintain pluripotency. A better understanding of the nature of naïve pluripotent murine ESCs would provide insight into the biology of naïve pluripotent states in other species, including humans.

A relationship between germ cell development and embryonic stem cells?

Murine ESCs express several genes that are required for germ cell development. Moreover, germ cells are the only postimplantation cell type that continues to express key pluripotency genes including Oct4, Nanog, and Sox2. In fact, prior to ESC derivation from the ICM of preimplantation blastocyst embryos, pluripotent embryonic carcinoma cell lines (EC cells) were derived from teratocarcinomas, a tumor of germ cell origin [11,12]. EC cells, similarly to ESCs, are pluripotent, can generate derivatives of the three embryonic germ layers *in vitro* and *in vivo*, and can contribute to the formation of chimeras [13]. Since then, pluripotent stem cell lines have been derived from explant cultures of primordial germ cells (PGCs) from embryonic (E) day E8.5–E12.5 embryos [14,15] and even from postnatal and adult testes (Figure 1) [16]. These embryonic germ cell (EGC) lines, germline stem cells (GS cells) [16,17], or germline-derived pluripotent stem cells (GPs cells) [18], display striking similarities to ESC morphology, gene expression profile, and functional ability to form chimeras when injected into preimplantation embryos.

Interestingly, the 129 mouse strain displays an elevated incidence of germ cell tumors and is also permissive for the generation of ESC lines. Species where teratocarcinoma formation is rare, such as the rat and non-permissive

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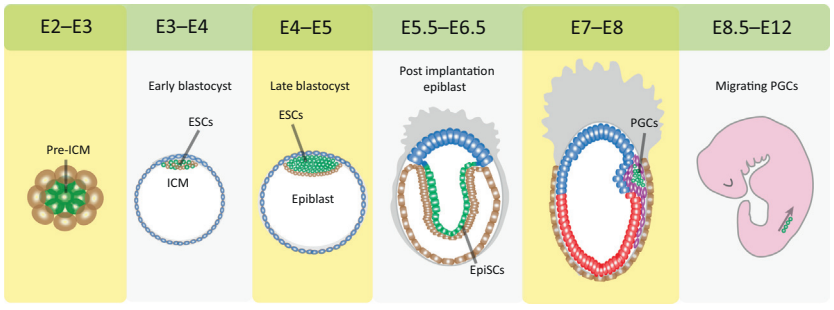

Figure 1. Pluripotent cells in the developing embryo. Schematic representation of the sites of pluripotency in the developing murine embryo. The pluripotent cells are displayed in green. The first pluripotent cells in the embryo appear as pre-inner cell mass (ICM) cells in the morula that will develop into the ICM of the blastocyst by embryonic (E) day E3.5, in which Oct4 expression is sustained. This is followed by ICM segregation into the primitive endoderm and epiblast in the late blastocyst and the appearance of Nanog expression in the pluripotent epiblast cells. Embryonic stem cells (ESCs) can be derived from morula until late blastocyst stage embryos. Epiblast stem cells (EpiSCs) are derived from the postimplantation epiblast at E5.5. After implantation, the pluripotent cells become restricted to the germ line when they develop into primordial germ cells (PGCs) and finally migrate to the gonads to become germ line stem cells.

mouse strains, are remarkably refractory to ESC derivation, but do allow the derivation of EpiSCs [5,6,19]. This has led to the hypothesis that genes responsible for increasing susceptibility to spontaneous germ cell tumors also increase the efficiency of ESC derivation, and prompted the idea that perhaps ESCs have a germ cell origin [12,20].

The unique germ cell signature of mESCs clearly distinguishes these cells from EpiSCs. At the molecular level, the gene expression profile of mESCs reveals the expression of several genes that are specifically expressed in developing germline stem cells. Stella (Dppa3), one of the earliest germ cell markers in the proximal epiblast (E7.5), marks the classic founder PGC population [21]. Oct4 and Nanog, normally expressed throughout the ICM of the early blastocyst, become restricted to the germline between E7.75 and E8.5, once migration of PGCs towards the gonads starts [21]. Finally, ESCs express Dazl, a late germ cell marker that is expressed at the start of PGC migration to the future gonads [22].

Therefore, what is the relationship between the blastocyst ICM, germ cells, and ESCs? Several studies suggest that *in vitro* development of germ cells from a subset of cells in the explanted ICM precedes ESC derivation [12,20]. In other words, ESCs may be derived from very early PGCs. Indeed, during ICM outgrowth *in vitro* expression of Oct4 is only maintained in a subset of cells [23] which could resemble Oct4-expressing germ cell precursors from proximal epiblast cells soon after implantation. PGC-specific genes such as Fragilis, Prdm14, Nanos3, and Blimp1 are upregulated in these Oct4-expressing cells during ESC derivation, suggesting that a germ cell differentiation program is activated during the establishment of ESCs (Figure 2) [24].

A recent study from Chu *et al.* directly addressed ESC ontology using an elegant genetic approach [25]. Upon derivation of ESCs from blastocysts carrying a Blimp1–Cre/lox–stop–lox reporter system, Chu and colleagues demonstrated that >80% of the derived ESCs arise from Blimp1-expressing precursors, demonstrating that ESC derivation involves transition through a Blimp1-positive PGC-like state. Interestingly, the combination of the germ cell factors Prdm14, a family member of Blimp1 (Prdm1), and Klf2 was subsequently shown to accelerate the reprogramming of EpiSCs into ESCs by promoting X-reactivation and demethylation, whereas the expression of germ cell genes is firmly suppressed in EpiSCs [6]. The reactivation of germ cell gene expression, together with epigenetic changes similar to those during PGC development, is a hallmark feature of EpiSC conversion to a naïve pluripotent state (Figure 2) [26–28].

From the studies above it seems that ESC derivation from explanted blastocysts, as well as via EpiSC conversion, transiently activates a transcriptional program that is typically associated with PGC development. The family members Blimp1 and Prdm14 are critical for the early specification of PGCs *in vivo* by initiating epigenetic reprogramming and repression of the somatic program in early germ cells [29,30]. However, Bao and colleagues subsequently demonstrated the derivation of ESCs from Blimp1$^{-/-}$ embryos as well as from Blimp1$^{-/-}$ EpiSCs, indicating that Blimp1 is not required for ESC derivation [27]. It is possible that Prdm14 might have a redundant role during the establishment of ESCs from Blimp1$^{-/-}$ embryos. Indeed, ESCs cannot be derived and maintained in the absence of Prdm14 [31]. Prdm14 inhibits the activation of signaling pathways leading towards differentiation and represses DNA methyl transferases (DNMTs) to keep the epigenome in a naïve state [31,32]. Thus, the upregulation of genes involved in germ cell development may not necessarily indicate that a PGC-like program is activated, but that the activation of these genes is crucial for inhibition of differentiation towards somatic lineages and the maintenance of the pluripotent state. This is supported by recent data demonstrating that Prdm14 is not required when ESCs are derived under conditions of MEK and GSK3 inhibition ('2i' culture conditions), in which somatic differentiation is suppressed [30], which will be discussed later in this review.

Naive pluripotency and a diapause state

During diapause, late blastocyst embryos are arrested during development and their implantation is prevented. This process occurs naturally in lactating females or can be induced experimentally by removal of the ovaries [33]. Diapause embryos remain in an unimplanted, non-progressive state until activated by estrogen [34]. LIF

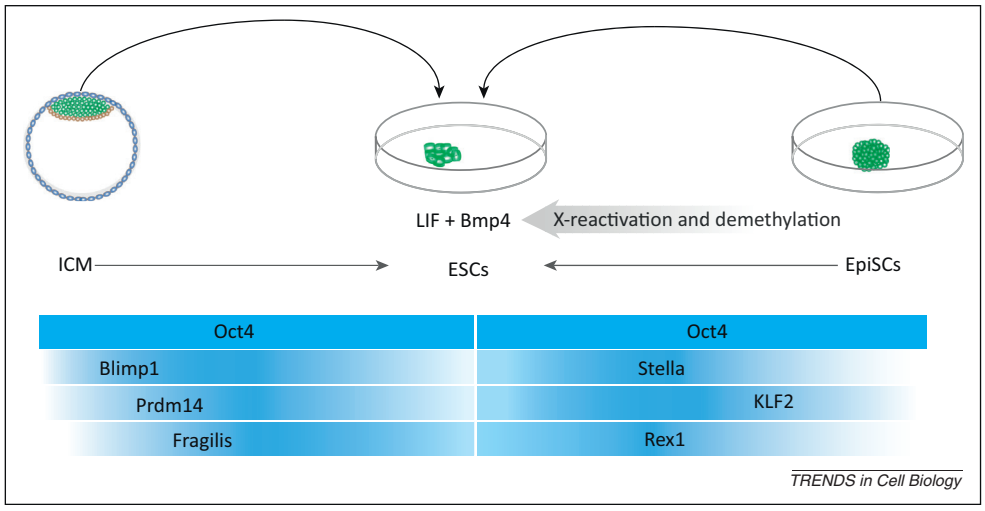

Figure 2. The establishment of ESCs through a transient PGC-like state. The ESC transcription profile is highly similar to that of developing germline stem cells. Oct4 is continuously expressed during the transition of ICM cells to ESCs as well as in EpiSCs. The derivation of ESCs from both ICM and EpiSCs is associated with the upregulation of germ cell genes. The conversion of ICM and EpiSCs to a naïve pluripotent state *in vitro* is accompanied by the induced expression of germ cells markers and epigenetic changes similar to those during PGC development. It appears that a transcriptional program that is typically associated with PGC development is transiently activated during the formation of ESCs from the ICM as well as EpiSCs. Abbreviations: LIF, leukemia inhibitory factor; ICM, inner cell mass; ESCs, embryonic stem cells; EpiSCs, epiblast stem cells; PGC, primordial germ cell.

signaling plays a critical role in the recovery of embryos from diapause, and embryos mutant for the LIF receptor, GP130, display reduced cell numbers in the ICM, and lose their epiblast component after more than 6 days in diapause [35].

Although LIF signaling is dispensable for normal peri-implantation development, it appears to be a unifying property of both ESC maintenance and blastocyst diapause [35,36]. This common LIF-dependence has been a persistent argument for a diapause-like state of naïve ESCs, and this is further supported by the fact that entering diapause increases the efficiency of ESC derivation from blastocyst embryos [37]. Diapause is best characterized in rodents, and it is striking that true naïve pluripotent stem cell lines have only been derived from mice and rats. Despite some contradictory evidence [38], generally it is thought that a diapause-like state does not exist in humans. If the molecular wiring underlying diapause is required for maintaining a naïve pluripotent state, this could mean that the derivation of naïve ESCs from human and other non-diapause species is not possible. Recent evidence suggests however that diapause can be induced in embryos of species that normally do not undergo this phenomenon. A recent paper from Ptak *et al.* reported that sheep blastocysts can enter diapause when transferred into mouse uteri in which diapause conditions were induced [33]. The diapausing ovine blastocysts were able to resume growth *in vitro* and develop into normal lambs after transfer to surrogate ewe recipients. These results indicate that it might be possible to induce diapause in other mammalian species when exposed to adequate uterine conditions, and this creates opportunities for studying the effect of diapause on the efficiency of ESC derivation of non-rodent mammals.

However, vast differences between naïve ESCs and the diapause embryo do not fully support the idea that ESCs resemble a diapause-like state. For one, ESCs proliferate at an astounding rate, with a culture doubling time of approximately 12 h, whereas cells of the ICM of the diapause embryo are arrested at the G0/G1 phase of the cell cycle [34]. In addition, the derivation and maintenance of naïve ESCs from non-permissive mouse strains and rats is inefficient regardless of their ability to undergo diapause, indicating that there are differences in the molecular programs underlying both processes.

A final argument against the diapause hypothesis is that Stat3, the key downstream effector of LIF signaling, is also activated by many other cytokines, which are equally capable of supporting long-term ESC self-renewal [39,40]. Although LIF may be dispensable for early embryonic development, proliferation of the peri-implantation epiblast is severely reduced in Stat3$^{-/-}$ embryos [41]. Thus, although LIF is essential for the recovery from diapause, it is merely one of several redundant factors that lead to Stat3 activation in naïve ESC cultures. A similar redundancy in cytokine signaling may also mediate Stat3 activation during early embryonic development, where perhaps another so far unknown cytokine serves to activate Stat3 and drive the rapid expansion of the pluripotent epiblast. Together, an obligatory relationship between diapause blastocysts and naïve ESCs seems unlikely.

ESCs and the peri-implantation epiblast

Are naïve ESCs therefore purely an *in vitro* artifact, or do they reflect the biology of a specific stage in embryonic development? ESCs are typically derived from the epiblasts of early blastocyst-stage embryos, but can also be derived earlier from the morula stage. ESCs are able to reintegrate with the embryo at all the above stages and contribute to chimera formation. Does this mean that the *in vivo* equivalent of ESCs exists at any of these stages, or is it merely an indication of the flexibility of ESCs to adapt to different stages of embryo development?

Tang and colleagues recently analyzed the changes in gene expression that occur during the conversion of ICM cells into ESCs [24]. As expected, they found a high degree of similarity between ICM and ESCs at the level of pluripotency gene expression and epigenetic status, with two

active X chromosomes and an open chromatin structure [42]. Remarkably, ESCs display higher expression levels of genes involved in epigenetic inactivation and differentiation, such as DNMTs and histone deacetylases (HDACs), and downregulation of epigenetic modifiers that support an active change in epigenetic status, such as histone acetyltransferases and histone demethylases. This may indicate that ESCs are preparing to start a specific developmental program and perhaps represent a slightly more progressed developmental stage than cells in the preimplantation ICM. Nevertheless, at the same time, genes associated with development and lineage specification are repressed in ESCs compared to the ICM. This may be due to the specific conditions of ESC culture, or may be a consequence of greater heterogeneity of cells in the preimplantation ICM. Another profound transcriptional difference observed between ICM and ESCs is in genes associated with metabolism. This is perhaps expected because cells in the preimplantation ICM proliferate slowly, if at all, whereas ESCs display a high proliferation rate. Although technically quite challenging, it would be interesting to compare the transcriptional program of ESC with that of peri-implantation epiblast, between the late blastocyst stage and before E5.5, when cells transition into a primed pluripotent state.

Transcription factors associated with pluripotency are expressed heterogeneously in both the ICM and ESCs. In the late ICM, a mutually exclusive pattern of Nanog- and GATA6-expressing cells emerges, giving rise to the epiblast and primitive endoderm, respectively [43,44]. Similarly, ESCs display a heterogeneous expression pattern of key regulators of pluripotency and differentiation, with single-cell gene expression patterns similar to the ICM, epiblast, or postimplantation epiblast (Figure 3). For example, approximately 80% of ESCs express the pluripotency factor Nanog, whereas Gata6 is exclusively expressed in a fraction of the Nanog-negative cells [45,46]. The Gata6-positive cells are prone to differentiation, and indeed some are lost from the ESC culture by a failure to maintain their pluripotency whereas others recover by re-expressing Nanog [46]. Thus, ESC cultures continually fluctuate between a naïve Nanog$^+$ ICM-like state biased towards self-renewal and an epiblast-like state that is biased towards differentiation.

Similar heterogeneous gene expression in ESCs has also been demonstrated for Rex1, Pecam1, and Stella [22,47,48]. It appears therefore that ESCs teeter on the brink between the preimplantation and the postimplantation epiblast. Indeed, many key properties of the peri-implantation epiblast are preserved in ESCs, including the reactivation of the silenced X-chromosome [42,49] and proliferation rate (8–12 h in ESC cultures and 11.5 h in the blastocyst ICM) [50].

The heterogeneity of ESCs could be the answer to the initial question of whether ESCs reflect any of the stages between morula and late blastocyst; the fluctuating expression of genes associated with early or late preimplantation development may allow subpopulations of the ESC culture to integrate at different stages upon transfer to embryos. For example, the rare contribution of ESCs to extraembryonic tissues can be attributed to a small fraction of ESCs that appear to exist in a transcriptional state akin to the two-cell stage embryo [21]. Despite culture heterogeneity, most ESCs appear to exist in a pluripotent state that is most similar to the peri-implantation blastocyst. The significance of a germ cell signature in ESCs, with expression of late germ cell markers such as Stella and Dazl, remains to be determined but could result from

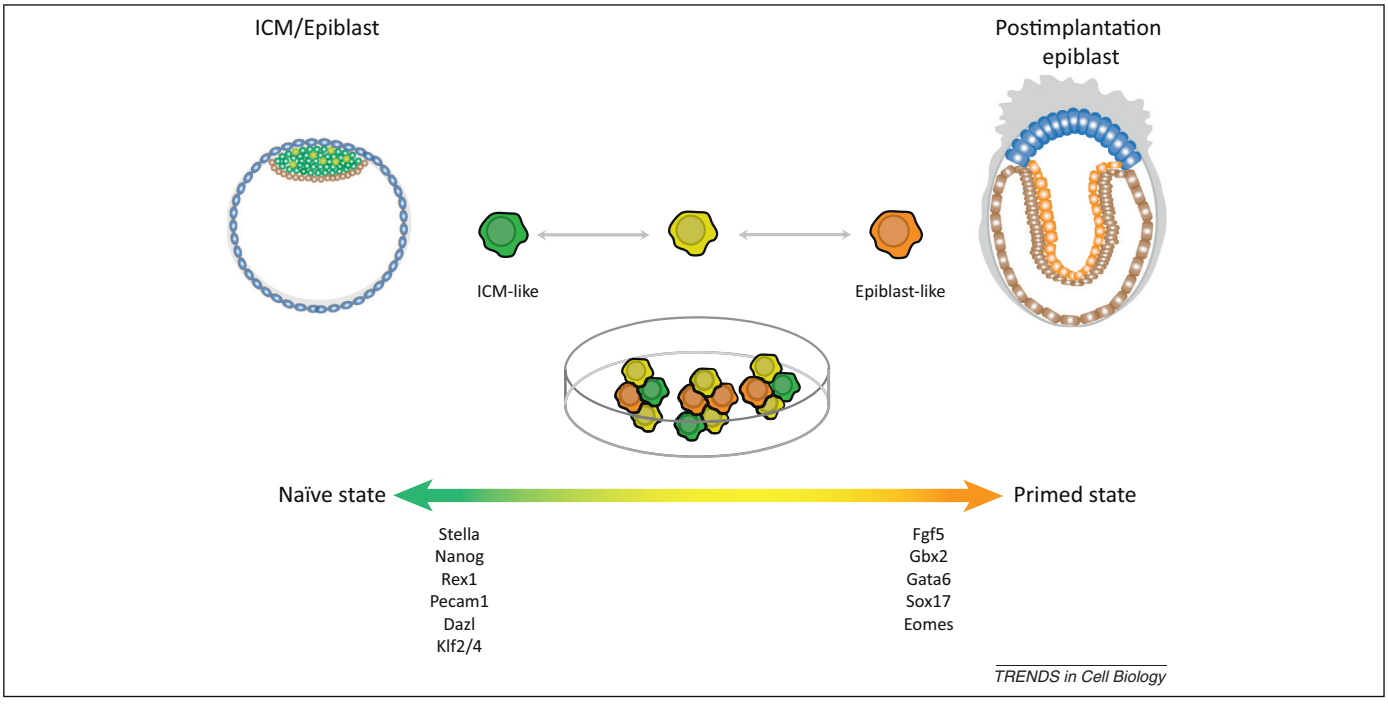

Figure 3. Embryonic stem cells (ESCs) exist in a heterogeneous state. ESC cultures consist of cells with subsets of different gene expression that exist in equilibrium and can convert into each other. These subsets continually fluctuate between a naive state that is equivalent to the Nanog-positive cells in the inner cell mass (ICM) of blastocyst embryos, characterized by the expression of pluripotency and germ cell markers, and a primed state that resembles the postimplantation epiblast, accompanied by the expression of early differentiation markers.

serum culture induced differentiation signals towards to the postimplantation epiblast.

Elimination of differentiation cues by combined MEK/ERK and GSK3 inhibition

In recent years a combination of small-molecule inhibitors has been identified that greatly facilitates murine ESC derivation and maintenance. This so-called 2i inhibitor cocktail consists of a MEK/ERK inhibitor and a GSK3 inhibitor. Furthermore, in the presence of the 2i inhibitors, naïve ESCs can be derived from non-permissive mouse strains such as non-obese diabetic (NOD) mouse [51,52] as well as from the rat [53,54]. 2i culture conditions promote naïve pluripotency [55] by blocking the differentiation-promoting effect of the FGF4–MEK–ERK signaling pathway and simultaneously inhibiting GSK3β, thereby promoting Wnt/β-catenin signaling, which has been shown to promote ESC self-renewal [56]. In contrast to standard culture in serum, factors associated with lineage-specification are repressed under 2i culture conditions [57,58]. At the epigenetic level, 2i culture conditions result in a reduction of bivalent domains at the promoters of many genes [57,59]. Bivalent domains are present at promoters of key developmental regulators and, interestingly, it is the repressive histone mark H3K27me3 that is reduced at these promoters under 2i conditions [57]. Additionally, increased promoter-proximal pausing by RNA polymerase II at these lineage-specifying genes was found in 2i cultured cells [57], and could indicate that this mechanism plays an important role in preventing ESC differentiation and in the maintenance of naïve pluripotency.

In addition to changes in histone modification, 2i culture conditions induce a more homogeneous expression of key pluripotency regulators and, in particular, affect allelic expression of the key pluripotency regulator Nanog [60]. When ESCs are grown in serum, Nanog predominantly displays mono-allelic expression, whereas under 2i culture conditions Nanog is expressed from both alleles [61]. Interestingly, during epiblast development, Nanog expression switches from mono- to bi-allelic expression. By contrast, $Nanog^{+/-}$ embryos, in which bi-allelic expression is impaired, display increased apoptosis in the ICM and delayed primitive endoderm formation, demonstrating that bi-allelic Nanog expression is important for the survival of the peri-implantation ICM.

As outlined above, one of the hallmark characteristics of naïve murine ESCs is the expression of a distinct germ cell signature, and ESCs have been shown to develop from a subpopulation of cells in the epiblast that transiently express the early germ cell marker Blimp-1, suggesting an early germ cell ontology [25]. However, the derivation of ESCs in 2i conditions occurs via a Blimp-1 independent route [25,27]. No major gene expression differences are observed between conventional or 2i-derived ESCs, meaning that the routes of derivation do not influence the characteristics of the established ESCs. Although ESCs derived in 2i conditions do not seem to transit through an early Blimp1-positive PGC-like state, they too were reported to express many genes associated with (late) PGC development, including Dazl, Stella, Rex1, and Nanog [25]. The elimination of differentiation signals during the derivation of ESCs in 2i conditions bypasses the need for Blimp1 and Prdm14 to repress somatic differentiation. Indeed $Prdm14^{-/-}$ ESCs can be successfully grown in 2i culture conditions whereas transfer to serum induces differentiation [31]. These results suggest that there may be two pathways via which the naïve ESC state can be acquired *in vitro:* one that involves transition through an early PGC identity and a second pathway in which 2i inhibition allows direct establishment of naïve ESCs from the pluripotent epiblast.

Homogeneous naïve pluripotency or heterogeneous totipotency?

A recent study by Macfarlan and colleagues discovered that ESC cultures contain a small subfraction of cells that express high levels of transcripts found in two-cell (2C) stage embryos [46]. Despite the lack of expression of pluripotency genes such as Oct4, Sox2, and Nanog, these 2C-like cells appear to be totipotent because they contribute to the formation of both embryonic and extra-embryonic tissues. ESC cultures depleted of 2C-like ESCs were still capable of self-renewal and contribution to chimaeras, but these cultures displayed a differentiation bias towards mesoderm and ectoderm *in vitro*, indicating that 2C-like cells may be required for the maintenance of a pluripotent ESC culture. 2C cells suggest that factors other than the core pluripotency factors Oct4, Sox2, and Nanog can maintain pluripotency or even totipotency in ESC cultures.

In line with these findings, two recent reports describe changes in gene expression at various stages during the reprogramming process process [62,63]. As it turns out, neither Oct4, Sox2, nor Nanog are good predictors of progression to induced pluripotent stem cells (iPSCs) during early reprogramming phases, and they are also not among the factors that activate the pluripotency circuitry in the late hierarchical phase. Instead, Esrrb, Nr5a2, Utf1, Lin28, and Dppa2 were identified as factors that accurately mark the rare cells that will become iPSCs [62,63]. Although Oct4 is very efficient in the reactivation of the core pluripotency circuitry, reprogramming was shown to be possible by various combinations of factors, and even in the absence of the 'Yamanaka' factors. Interestingly, Glis1, which is enriched in unfertilized oocytes and in one-cell stage embryos, enhances the generation of iPSCs by activating pro-reprogramming pathways including Myc, Nanog, Lin28, and Essrb [64]. In addition, Ronin, which is expressed during the earliest stages of embryonic development, was suggested as a candidate pluripotency factor because it is essential for the maintenance of pluripotent stem cells both *in vitro* and *in vivo*, supports ES cell self-renewal even in the absence of LIF, and can compensate for *Oct4* knockdown in ESCs [65].

It would be interesting to study whether genes such as Lin28, Essrb, Glis1, and Ronin are expressed in 2C-like cells and whether (any combination of) these genes mark a totipotent cell *in vitro* and *in vivo*.

Thus, the capacity of ESCs to form chimeras with embryos at different stages of preimplantation development could be a consequence of different developmental stages that exist within the ESC culture itself. Interestingly, ICM-like 2i-cultured ESCs can also integrate both

morula and blastocyst stage embryos [54]. If 2i culture conditions specifically capture the naive pluripotent cell population, are there 2C-like cells present under these conditions? If so, the truly naïve pluripotent stem cell may be characterized by other factors than the core Oct4, Sox2, Nanog pluripotency team.

Pluripotent states in human ESCs: lessons from mice

Determining the nature of naïve murine ESCs will be essential for our understanding of the properties of naïve pluripotent stem cells from other species, including humans. Although murine and human embryonic stem cells (hESCs) are both derived from the pluripotent ICM cells of the preimplantation blastocyst, they exhibit fundamental differences and it appears that human ESCs exist in a 'primed' postimplantation state [3], akin to murine EpiSCs [5,6]. The question remains whether a naïve pluripotent state exists for human ESCs and, if so, are its properties similar to murine ESCs?

It is possible that human ICM cells develop into hESCs via an epiblast-like state *in vitro*. Indeed, the derivation of pluripotent stem cell lines from murine preimplantation blastocyst embryos under epiblast stem cell conditions has been shown to yield epiblast stem cells [66]. A recent study monitoring the conversion of human ICM to hESCs demonstrates that several molecular and epigenetic changes occur during this transition [67]. This study identified PICMIs (post-ICM intermediates), epiblast-like structures occurring within 7 days after blastocyst plating, as the cells that ultimately give rise to hESCs. The gene expression profile of the PICMI structure displays the expression of both early and late epiblast genes. Similarly to the epiblast, PICMI cells have undergone X-inactivation. However, the absence of E-cadherin, a typical epiblast marker, and the presence of Rex1, Nanog, and Klf4 in PICMI structures and hESCs, suggest that they do differ from murine EpiSCs. Because the PICMI also expressed some PGC markers, the possibility that hESCs are derived from germ cell precursors cannot be eliminated. Together, it is possible that PICMI represents a transient population of early *in vivo* epiblast cells in human blastocysts that convert to late epiblast stage hESCs which can be stably maintained *in vitro*.

Concluding remarks

Recently, several labs have attempted to generate and maintain human pluripotent stem cells in a naïve state [7–9], but stable long-term maintenance of naïve human pluripotent stem cells has not yet been accomplished. It is possible that other, so far unknown signaling pathways are required for stable maintenance of a human naïve pluripotent state. Indeed, cAMP [8] and retinoic acid signaling [9] were shown to enhance the ability to derive and maintain human pluripotent stem cells that display properties of naïve murine ESCs but, even with the addition of these factors, cells could not be maintained long term. This could signify that we simply do not fully understand the culture conditions that allow stable long-term maintenance of naïve human PSCs. Nevertheless, key questions remain – whether a stable naïve pluripotent state of human stem cells is biologically possible, and how to recognize it once it is established.

The identification of a naïve pluripotent state in human ESCs requires appropriate markers for this naïve state. The X-inactivation state also appears to fluctuate in normal human ESC cultures, and is therefore not a conclusive marker for the naïve pluripotent state [68,69]. To obtain better insight into the derivation of naïve hESCs it is therefore essential that we better understand the nature of murine ESCs, the significance of the germ cell gene expression signature in these cells, and the significance of their LIF-dependence and relation to the diapause embryo. Only when we determine the *in vivo* equivalent of naïve murine ESCs can we identify these cells in embryos of other species. Its properties will ultimately teach us the characteristics and perhaps culture requirements of the naïve pluripotent stem cells in those species.

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References

1 Evans, M.J. and Kaufman, M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292, 154–156
2 Martin, G.R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. U.S.A.* 78, 7634–7638
3 Nichols, J. and Smith, A. (2009) Naive and primed pluripotent states. *Cell Stem Cell* 4, 487–492
4 Thomson, J.A. *et al.* (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147
5 Brons, I.G. *et al.* (2007) Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 448, 191–195
6 Tesar, P.J. *et al.* (2007) New cell lines from mouse epiblast share defining features with human embryonic stem cells. *Nature* 448, 196–199
7 Buecker, C. *et al.* (2010) A murine ESC-like state facilitates transgenesis and homologous recombination in human pluripotent stem cells. *Cell Stem Cell* 6, 535–546
8 Hanna, J. *et al.* (2010) Human embryonic stem cells with biological and epigenetic characteristics similar to those of mouse ESCs. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9222–9227
9 Wang, W. *et al.* (2011) Rapid and efficient reprogramming of somatic cells to induced pluripotent stem cells by retinoic acid receptor gamma and liver receptor homolog 1. *Proc. Natl. Acad. Sci. U.S.A.* 108, 18283–18288
10 Li, W. *et al.* (2009) Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell* 4, 16–19
11 Hochedlinger, K. (2011) Embryonic stem cells: testing the germ-cell theory. *Curr. Biol.* 21, R850–R852
12 Zwaka, T.P. and Thomson, J.A. (2005) A germ cell origin of embryonic stem cells? *Development* 132, 227–233
13 Brinster, R.L. (1974) The effect of cells transferred into the mouse blastocyst on subsequent development. *J. Exp. Med.* 140, 1049–1056
14 Matsui, Y. *et al.* (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* 70, 841–847
15 Tada, T. *et al.* (1998) Epigenotype switching of imprintable loci in embryonic germ cells. *Dev. Genes Evol.* 207, 551–561
16 Guan, K. *et al.* (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 440, 1199–1203
17 Donovan, P.J. and de Miguel, M.P. (2003) Turning germ cells into stem cells. *Curr. Opin. Genet. Dev.* 13, 463–4671
18 Ko, K. *et al.* (2009) Induction of pluripotency in adult unipotent germline stem cells. *Cell Stem Cell* 5, 87–96
19 Batlle-Morera, L. *et al.* (2008) Parameters influencing derivation of embryonic stem cells from murine embryos. *Genesis* 46, 758–767
20 Nichols, J. and Smith, A. (2011) The origin and identity of embryonic stem cells. *Development* 138, 3–8

21 Macfarlan, T.S. *et al.* (2012) Embryonic stem cell potency fluctuates with endogenous retrovirus activity. *Nature* 487, 57–63
22 Hayashi, K. *et al.* (2008) Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states. *Cell Stem Cell* 3, 391–401
23 Buehr, M. *et al.* (2003) Rapid loss of Oct-4 and pluripotency in cultured rodent blastocysts and derivative cell lines. *Biol. Reprod.* 68, 222–229
24 Tang, F. *et al.* (2010) Tracing the derivation of embryonic stem cells from the inner cell mass by single-cell RNA-Seq analysis. *Cell Stem Cell* 6, 468–478
25 Chu, L.F. *et al.* (2011) Blimp1 expression predicts embryonic stem cell development in vitro. *Curr. Biol.* 21, 1759–1765
26 Hajkova, P. *et al.* (2008) Chromatin dynamics during epigenetic reprogramming in the mouse germ line. *Nature* 452, 877–881
27 Bao, S. *et al.* (2012) The germ cell determinant Blimp1 is not required for derivation of pluripotent stem cells. *Cell Stem Cell* 11, 110–117
28 Gillich, A. *et al.* (2012) Epiblast stem cell-based system reveals reprogramming synergy of germline factors. *Cell Stem Cell* 10, 425–439
29 Ohinata, Y. *et al.* (2005) Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature* 436, 207–2013
30 Yamaji, M. *et al.* (2008) Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nat. Genet.* 40, 1016–1022
31 Yamaji, M. *et al.* (2013) PRDM14 Ensures naive pluripotency through dual regulation of signaling and epigenetic pathways in mouse embryonic stem cells. *Cell Stem Cell* 12, 368–382
32 Leitch, H.G. *et al.* (2013) Naive pluripotency is associated with global DNA hypomethylation. *Nat. Struct. Mol. Biol.* 20, 311–316
33 Ptak, G.E. *et al.* (2012) Embryonic diapause is conserved across mammals. *PLoS ONE* 7, e33027
34 Lopes, F.L. *et al.* (2004) Embryonic diapause and its regulation. *Reproduction* 128, 669–678
35 Nichols, J. *et al.* (2001) Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines. *Development* 128, 2333–2339
36 Stewart, C.L. *et al.* (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* 359, 76–79
37 Brook, F.A. and Gardner, R.L. (1997) The origin and efficient derivation of embryonic stem cells in the mouse. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5709–5712
38 Tarin, J.J. and Cano, A. (1999) Do human concepti have the potential to enter into diapause? *Hum. Reprod.* 14, 2434–2436
39 Conover, J.C. *et al.* (1993) Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. *Development* 119, 559–565
40 Rose, T.M. *et al.* (1994) Oncostatin M (OSM) inhibits the differentiation of pluripotent embryonic stem cells in vitro. *Cytokine* 6, 48–54
41 Takeda, K. *et al.* (1997) Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc. Natl. Acad. Sci. U.S.A.* 94, 3801–3804
42 Okamoto, I. *et al.* (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* 303, 644–649
43 Chazaud, C. *et al.* (2006) Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2–MAPK pathway. *Dev. Cell* 10, 615–624
44 Rossant, J. *et al.* (2003) Lineage allocation and asymmetries in the early mouse embryo. *Philos. Trans. R. Soc. Lond. B: Biol. Sci.* 358, 1341–1348
45 Chambers, I. *et al.* (2007) Nanog safeguards pluripotency and mediates germline development. *Nature* 450, 1230–1234
46 Singh, A.M. *et al.* (2007) A heterogeneous expression pattern for Nanog in embryonic stem cells. *Stem Cells* 25, 2534–2542
47 Furusawa, T. *et al.* (2006) Gene expression profiling of mouse embryonic stem cell subpopulations. *Biol. Reprod.* 75, 555–561
48 Toyooka, Y. *et al.* (2008) Identification and characterization of subpopulations in undifferentiated ES cell culture. *Development* 135, 909–918
49 Silva, J. *et al.* (2008) Promotion of reprogramming to ground state pluripotency by signal inhibition. *PLoS Biol.* 6, e253
50 Snow, M.H. and Bennett, D. (1978) Gastrulation in the mouse: assessment of cell populations in the epiblast of tw18/tw18 embryos. *J. Embryol. Exp. Morphol.* 47, 39–52
51 Nichols, J. *et al.* (2009) Validated germline-competent embryonic stem cell lines from nonobese diabetic mice. *Nat. Med.* 15, 814–818
52 Hanna, J. *et al.* (2009) Metastable pluripotent states in NOD-mouse-derived ESCs. *Cell Stem Cell* 4, 513–524
53 Buehr, M. *et al.* (2008) Capture of authentic embryonic stem cells from rat blastocysts. *Cell* 135, 1287–1298
54 Li, P. *et al.* (2008) Germline competent embryonic stem cells derived from rat blastocysts. *Cell* 135, 1299–1310
55 Ying, Q.L. *et al.* (2008) The ground state of embryonic stem cell self-renewal. *Nature* 453, 519–523
56 ten Berge, D. *et al.* (2011) Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. *Nat. Cell Biol.* 13, 1070–1075
57 Marks, H. *et al.* (2012) The transcriptional and epigenomic foundations of ground state pluripotency. *Cell* 149, 590–604
58 Wray, J. *et al.* (2010) The ground state of pluripotency. *Biochem. Soc. Trans.* 38, 1027–1032
59 Bernstein, B.E. *et al.* (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 125, 315–326
60 Silva, J. *et al.* (2009) Nanog is the gateway to the pluripotent ground state. *Cell* 138, 722–737
61 Miyanari, Y. and Torres-Padilla, M.E. (2012) Control of ground-state pluripotency by allelic regulation of Nanog. *Nature* 483, 470–473
62 Buganim, Y. *et al.* (2012) Single-cell expression analyses during cellular reprogramming reveal an early stochastic and a late hierarchic phase. *Cell* 150, 1209–1222
63 Polo, J.M. *et al.* (2012) A molecular roadmap of reprogramming somatic cells into iPS cells. *Cell* 151, 1617–1632
64 Maekawa, M. *et al.* (2011) Direct reprogramming of somatic cells is promoted by maternal transcription factor Glis1. *Nature* 474, 225–229
65 Dejosez, M. *et al.* (2008) Ronin is essential for embryogenesis and the pluripotency of mouse embryonic stem cells. *Cell* 133, 1162–1174
66 Najm, F.J. *et al.* (2011) Isolation of epiblast stem cells from preimplantation mouse embryos. *Cell Stem Cell* 8, 318–325
67 O'Leary, T. *et al.* (2012) Tracking the progression of the human inner cell mass during embryonic stem cell derivation. *Nat. Biotechnol.* 30, 278–282
68 Lengner, C.J. *et al.* (2010) Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations. *Cell* 141, 872–883
69 Tomoda, K. *et al.* (2012) Derivation conditions impact X-inactivation status in female human induced pluripotent stem cells. *Cell Stem Cell* 11, 91–99