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Accessing naïve human pluripotency

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Abstract

Pluripotency manifests during mammalian development through formation of the epiblast, founder tissue of the embryo proper. Rodent pluripotent stem cells can be considered as two distinct states: naïve and primed. Naïve pluripotent stem cell lines are distinguished from primed cells by self-renewal in response to LIF signaling and MEK/GSK3 inhibition (LIF/2i conditions) and two active X chromosomes in female cells. In rodent cells, the naïve pluripotent state may be accessed through at least three routes: explantation of the inner cell mass, somatic cell reprogramming by ectopic Oct4, Sox2, Klf4, and C-myc, and direct reversion of primed post-implantation-associated epiblast stem cells (EpiSCs). In contrast to their rodent counterparts, human embryonic stem cells and induced pluripotent stem cells more closely resemble rodent primed EpiSCs. A critical question is whether naïve human pluripotent stem cells with bona fide features of both a pluripotent state and naïve-specific features can be obtained. In this review, we outline current understanding of the differences between these pluripotent states in mice, new perspectives on the origins of naïve pluripotency in rodents, and recent attempts to apply the rodent paradigm to capture naïve pluripotency in human cells. Unraveling how to stably induce naïve pluripotency in human cells will influence the full realization of human pluripotent stem cell biology and medicine.

Introduction

Pluripotency is defined as the capacity of a single cell to generate all cell lineages of the developing and adult organism. This is a property of a transient population of unrestricted cells known as the epiblast, which forms the embryo proper *in vivo*. Epiblast cells from pre-implantation rodent embryos can be perpetually expanded in culture as embryonic stem cell (ESC) lines [1,2]. *In vitro*, the 'naïve' ESCs self-renew indefinitely without genetic

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transformation, can be expanded clonally, and retain pluripotency. Furthermore, naïve ESCs are amenable to homologous recombination, which has allowed for extensive genetic dissection of the mouse genome.

The recent generation of primed epiblast stem cells (EpiSCs) evolved the view that pluripotent stem cell lines may exist as two distinct, stable pluripotent states: naïve and primed [3-5]. Both cell states exhibit features of bona fide pluripotent cell lines, including indefinite self-renewal, tri-germ layer potential and reliance on core transcription factors OCT4, SOX2, and NANOG. Human ESCs and iPSCs share defining features with primed mouse EpiSCs and not naïve mESCs, therefore embodying the primed state. Definitive evidence for a non-rodent naïve pluripotent state is lacking, which suggests that the naïve pluripotent state *in vitro* may be a rodent-specific phenomenon. However, there is growing interest in deriving naïve pluripotent stem cell lines from humans. Although recent advances in gene editing technology have improved the accessibility of primed hESCs/iPSCs for genetic intervention, naïve human pluripotent stem cells would further accelerate dissection of the human genome by permitting translation of gene targeting technologies previously limited to the mouse. In this review, we highlight advances in understanding rodent naïve and primed pluripotent stem cells and recent attempts to stabilize naïve human pluripotency via three routes: directly from pre-implantation embryos, through reprogramming of somatic cells, and through reversion of primed pluripotent cells.

Current ‘primed’ human ES and iPS cells

Naïve pluripotency is represented by the newly segregated pre-implantation epiblast and rodent naïve ESCs [6,7•]. The attainment of naïve pluripotency *in vivo* and *ex vivo* is demarcated by two active female X chromosomes and co-expression of pluripotent-associated transcription factors OCT4, SOX2, and NANOG. Following implantation, extraembryonic and autoinductive signaling prime the epiblast for differentiation and one female X chromosome is inactivated, but expression of OCT4, SOX2, and NANOG is retained. Primed pluripotency is exhibited *in vitro* by epiblast stem cell lines (EpiSCs) initially derived from post-implantation epiblasts and more recently, preimplantation blastocysts [4,5,8•]. Although human embryonic stem cells are derived from preimplantation blastocysts, they more closely resemble post-implantation EpiSCs and preimplantation blastocyst-derived EpiSCs [4,5,8•,9]. The derivation of EpiSCs from murine preimplantation blastocysts suggests human ICM outgrowth cells may progress to an EpiSC-like state during conventional hESC derivation protocols.

Naïve and primed pluripotent stem cells respond to different signaling pathways to sustain and exit the self-renewing state. Unlike mESCs, hESCs do not respond to LIF/STAT3 or 2i, cannot be efficiently propagated clonally, and respond to cooperative signaling between FGF and ACTIVIN/NODAL [4,10,11]. In both hESCs and mEpiSCs, NANOG expression depends on SMAD2/3 signaling. The state-specific divergence in self-renewal mechanism extends to differential response to differentiation-inducing cues. While FGF inhibition promotes mESC self-renewal, FGF/ERK inhibition promotes neuroectoderm commitment of hESCs, hiPSCs and EpiSCs [12•,13••]. Additionally, BMP4 cooperates with LIF to facilitate mESC self-renewal but induces extraembryonic phenotypes in mEpiSCs and hESCs/iPSCs

[14,15]. The shared signaling responsiveness of mouse EpiSCs and human ESCs/iPSCs suggests that these cell lines represent mouse and human orthologs of a primed pluripotent cell state.

Because the chimera assay cannot be used in humans, recent studies have sought to clarify the relationship between naïve and primed rodent cell lines and hESCs/hiPSCs. A key property that distinguishes rodent naïve and primed pluripotent stem cells is the unique ability of naïve pluripotent stem cells to generate highgrade chimeras upon re-introduction into the pre-implantation blastocyst. Rodent EpiSCs cannot generate highgrade chimeras upon introduction into morula stage embryos or blastocysts, nor efficiently contribute to the germline. More recently, the rodent paradigm was tested in primates. Similarly to mEpiSCs, rhesus monkey ESCs also cannot efficiently home into inner cell mass of preimplantation blastocysts to generate high-grade chimeric monkeys [16••]. Additionally, when rhesus monkey ESCs are introduced into pre-blastocyst stage four-cell stage embryos, intermingling of rhesus monkey ESCs with ICM cells during blastocyst formation can be observed, but differentiation or death is observed in the pre-implantation environment [16••]. These data support the view that current primate ESCs correspond to a rodent primed pluripotent state (Figure 1).

In certain respects regarding transcriptional regulation and marker expression, human ESCs appear to differ from mouse EpiSCs. Whereas disturbance of FGF2/ERK does not influence Nanog in mouse EpiSCs, inhibition of FGF/ERK signaling pathway in human ESCs rapidly downregulates NANOG [12•]. Additionally, in human ESCs, OCT4 binds to the FGF2 promoter establishing an autocrine loop, whereas in mouse EpiSCs, no evidence of regulation of Fgf2 by Oct4 was observed [12•]. Finally, human ESCs share several molecular features associated with mESCs, but not with EpiSCs. For instance, hESCs express the ICM-associated marker REX1, like naïve mESCs, but not EpiSCs; hESCs do not express FGF5, a key EpiSC-associated marker not expressed in mESCs. The tighter rewiring of FGF signaling to the core pluripotent transcription factors OCT4 and NANOG in humans may complicate simple application of the rodent paradigm to humans. An improved classification of the similarities and differences between hESCs and their rodent counterparts, EpiSCs, will sharpen our understanding of what it means to be naïve or primed.

Hence, a body of evidence suggests hESCs/iPSCs functionally resemble a primed EpiSC-like pluripotent state observed in rodents. Given the species-specific differences observed in analogous EpiSCs and hESCs/iPSCs, disentanglement of species-specific and state-specific differences is highly relevant for precisely defining naïve and primed pluripotent states in humans.

Capture of naïve pluripotency from rodent embryos

The relationship between cultured pluripotent stem cell lines and resident cells in the embryo is uncertain [17], but resolving the present gap in knowledge concerning the mechanisms that lead to stable pluripotency *in vitro* may facilitate the derivation of naïve human pluripotent cells.

Historically, naïve pluripotent stem cells are only readily derived from the 129 mouse strain, suggesting intrinsic genetic features in the 129 strain promote entry into or stabilization of naïve pluripotency [18]. It is curious that strain 129 is also predisposed toward testicular germ cell tumors (TGCTs). One study that investigated the genetic basis for 129 permissivity identified individual chromosomes that harbor susceptibility genes for TGCTs [19]. 129-Chr18(MOLF) males are resistant to spontaneous TGCTs, and four regions within chromosome 18 control this susceptibility. When ESC derivation efficiency in LIF/serum was investigated in 129-Chr 18(MOLF), derivation was significantly reduced. Thus, genetic elements contributing to the formation of TGCTs from primordial germ cells contribute to mESC derivation. Intriguingly, when EpiSCs are isolated from preimplantation embryos of 129-Chr18(MOLF) or NOD strains, EpiSCs were obtained at a similar frequencies to strain 129 (~25%) [8•]. Thus, while strain 129 genetic elements modulate entry or stabilization of the naïve pluripotent state, strain-specific genetic elements restricting access to the naïve state do not appear to impact access to the primed state. The generalizability of this principle can be witnessed in the observation that ESCs from other non-rodent mammalian species resemble EpiSCs and human ESCs.

A breakthrough in overcoming the barriers imposed by mouse genetic background was the development of 3i/2i, which involves inhibitors of FGFR, MEK, and GSK3 (3i) or MEK and GSK3 (2i) [20]. To identify Stat3-independent modes of pluripotency, Ying and colleagues applied 3i or 2i to Stat3-KO and recalcitrant mice strains, such as NOD, and successfully stabilized naïve pluripotency in recalcitrant mouse strains. Because chemokine stimulation of Stat3 was not required, Ying and colleagues claimed 2i-cultured mouse ESCs to reside in a novel state of ground state pluripotency, distinct from LIF/serum conditions. Thereafter, LIF/2i stabilized naive rat embryonic stem cells (rESCs) from the SD and DA rat strains [21,22]. These rESCs expressed naïve-associated markers such as Rex1, Klf4, and Tbx3. rESCs exhibited key functional features of naïve pluripotency such as chimerism potential, germ line colonization, and two active X chromosomes in females, a naïve molecular signature. Later, homologous recombination was applied and p53-KO rats were generated. These studies confirm capture of naïve pluripotent cells from a non-murine embryo and suggest the broader utility of naïve pluripotency for transgenesis [23••].

Are naïve pluripotency and ground state pluripotency fully interchangeable terms? Recent attention has focused on identifying intermediate states leading to stable naïve pluripotency. Using cell-fate mapping strategies and single-cell gene expression profiling to examine ICM outgrowths adaptation to LIF/serum, a germ cell-like precursor state was demonstrated as facultative for mESC generation [24]. ICM outgrowths showed primordial germ cell (PGC)-associated gene enrichment, functional resemblance to PGCs, and a propensity to transition to pluripotency. Interestingly, when 2i was applied to blastocysts carrying Blimp1-Cre and floxed-RFP, the cells were RFP-negative. Thus, classical LIF/serum and 2i may operate through distinct modes to promote entry into naïve pluripotency. Whereas LIF/serum involves transcriptional reprogramming of a few ICM cells with PGC-like potential, 2imay allow ‘direct capture’ of a broader population of epiblast cells with the potential to become naïve ESCs, circumventing the need to pass through a germ-cell state. In contrast to transitioning through a germcell state inLIF/serum conditions, 2i is believed to operate by altering the proportions of epiblast and hypoblast progenitors in favor of epiblast progenitors

in both mouse and rat embryos, a bias that may also be operative during ICM outgrowths when ESCs are derived [25,26•]. The recent observation that mouse ESCs cultured in 2i or in LIF/serum conditions differ with respect to biallelic versus monoallelic expression of Nanog, with 2imESCs corresponding to the state of Nanog transcription in the mature epiblast, supports the view that 2i allows capture of cells that correspond very closely to pluripotent cells of the pre-implantation embryo [27••].

Toward naive human embryonic stem cells

Three studies have described attempts to isolate naïve ESCs from human preimplantation embryos. A recent study reported that female hESCs derived in 5% oxygen retain two active X chromosomes, whereas in atmospheric oxygen, XIST upregulation and an inactive X chromosome are found [28•]. These hESCs were derived in conventional hESC derivation medium containing FGF. These data complement a recent study [7] reporting that female human embryos contain a compartment with two active X chromosomes by demonstrating that ESC lines with two active X chromosomes, a feature of naïve ESCs, can be derived from human preimplantation embryos. However, these cell lines were maintained in conventional human ES conditions containing FGF, were not demonstrated to self-renew in 2i, nor were other features associated with naïve mESCs investigated.

Two additional studies examined whether human preimplantation embryos contain a compartment from which naïve human ES cells could be derived by applying 2i cultural conditions to thawed human embryos and fresh human embryos deemed unsuitable for transfer by preimplantation genetic diagnosis (PGD) analysis [26•,29]. In the first of these studies, the authors first investigated the timing of epiblast segregation in human embryos. The detection of exclusive staining of NANOG (epiblast-specific) and GATA6/GATA4/SOX17 (hypoblast-specific) in day 7 human embryos suggested this stage corresponds to the mouse embryo at E4.5 when all three embryonic lineages – trophectoderm, hypoblast, and epiblast – can be distinguished. The observation of human hypoblast at day 7 prompted various inhibitor treatments initiated at day 3 of development, presumably before ICM has segregated into epiblast and hypoblast. When treated with FGF inhibitors or 2i (in physiological oxygen concentrations), the GATA4-positive human hypoblast forms under conditions where it is blocked in mouse and rat embryos, indicating that human hypoblast specification does not rely on FGF. The second of these studies extended Roode *et al.*'s conclusions to fresh human embryos and the absence of physiological oxygen concentrations [29]. Whether the failure of 2i to suppress hypoblast or additional barriers explain the failure to derive naïveh ESCs remains an area of future investigation.

Nichols and colleagues found when human embryos are cultured in FGF/ERK inhibitors or in 2i, the NANOG-positive epiblast compartment is sustained, which would not be expected If the embryonic NANOG-positive cells corresponded to primed human ES cells. The survival of embryonic NANOG-positive cells provides hope for isolating cells independent of FGF/ERK signaling. Future investigation of whether these ERK-independent, NANOG-positive cells reside in a pre-XCI state would support existence of a transient naïve population in the early human epiblast [7•,26•]. Additionally, whether biallelic expression of NANOG also distinguishes the attainment of ground state pluripotency in the human

epiblast, as has been observed in the mouse, remains to be clarified. Finally, a description of the response of the human ICM outgrowth to the combined effect of hypoxia and 2i could inform strategies to facilitate the derivation of naïve human ES cells from ICM outgrowths. These studies highlight the limitations of the rodent model and suggest how little is known about development of the human blastocyst and in particular, the human epiblast.

Direct reprogramming of somatic cells to naïve pluripotency

Direct reprogramming of human cells provides another platform to investigate derivation of naïve human pluripotent stem cell lines [30,31]. Ectopic expression of Oct4, Sox2, Klf4, and C-myc in LIF generates naïve mouse iPSCs [32], whereas ectopic expression of Oct4, Sox2, Klf4, and C-myc in EpiSC conditions yields iEpiSCs [33•]. These two studies suggest culture conditions dictate the terminal pluripotent state achieved following reprogramming factor induction in mice.

In the first report of human iPS cells, Yamanaka and colleagues stated that retroviral OCT4, SOX2, KLF4, and C-MYC fail to generate naïve hiPSCs in classical mouse ES cell conditions, but their attempts were not specified in detail [30]. Several groups have generated ‘mESC-like’ ‘hiPSCs’ by applying naïve culture conditions following reprogramming factor induction, but the cell lines lack the robust nature that distinguishes bona fide mouse ESCs/iPSCs (Figure 2; Table 1).

The first successful attempt to obtain hiPSCs that resemble mESCs used a combination of lentiviral OCT4, SOX2, NANOG and LIN28 and transfer into LIF/2i and a pan-ALK4/5/7-inhibitor A-83-01 [34]. The obtained cells were maintained for over 20 passages, exhibited reactivation of endogenous OCT4, SOX2 and NANOG, and generated teratomas. LIF-dependency and resistance to MEK inhibition and pan-ALK4/5/7- inhibition suggested a naïve signaling dependency but less efficient silencing of pSIN-EF1-alpha lentiviruses compared with the MMLV-based retroviruses confounds claims of transgene-independence [31]. Indeed, when OCT4 or NANOG is overexpressed in hESCs, the hESCs do not ‘default’ into neuroectoderm when challenged with FGF/ERK inhibition [13••]. Another study employed pSIN-EF1-alpha lentiviral delivery of OSNL, but used drug selection to select for high levels of OSNL transgenes, suggesting the unstable nature of these cell lines [35].

Jaenisch and colleagues reported the envisioned naïve hiPSCs derived from secondary human fibroblasts and hESCs [36,37]. The authors obtained transgene-dependent (OCT4/SOX2/KLF4) cells that required continuous transgene induction in combination with LIF/2i. The transgene-dependent cell lines expressed a hESC-like surface marker profile (SSEA3/SSEA4/TRA-1-60/TRA- 1-81-positive and SSEA1-negative), generated teratomas and exhibited extinction of XIST transcription, a feature associated with two female X chromosomes. To replace transgenes, the Protein Kinase A agonist Forskolin transiently replaced doxycycline and ‘transgene-independent’ cell lines were maintained in LIF/2i/Forskolin for about 15 passages.

The findings in Hanna *et al*., 2010 contrast with the findings of Tada and colleagues [38]. When retroviral OCT4, SOX2, KLF4 and C-MYC are delivered into primary human

fibroblasts and cultured in LIF/2i, the obtained cell lines resemble primitive neural stem cells that retain low levels of OCT4 and NANOG, but high levels of SOX2 expression. Domed-shaped cell lines from integration-free human iPS cells but not human ES cells were obtained upon selection of domed-shaped colonies in LIF/2i. These cells could be mechanically passaged over 50 times. The neural observation resembles another group's attempt to revert human ES cells to a naïve state [39]. The authors speculate that mouse and human cells differentially respond to LIF/2i and this could account for the failure to derive naïve human iPS cells.

Another study produced mESC-like human 'iPSCs' using DOX-inducible lentiviruses for OCT4, SOX2, KLF4, CMYC, and NANOG in LIF [40]. However, the hLR5 state relied on continuous expression of reprogramming factors and expressed high levels of SSEA-1, a mESC marker absent in hESCs. The cell lines generated possessed some mESC-like features such as high clonogenicity and amenability to homologous recombination. Critically, the endogenous OCT4 and NANOG regulatory regions were not reactivated (i.e. H3K4me3 mark), but were bivalent (i.e. H3K4me3 and H3K27me3 marks), suggesting these loci were 'poised' for activation. The bivalent status of OCT4 and NANOG prompted the authors to investigate conversion of hLR5 cells into primed human iPSCs. The resulting primed hLR5-iPSCs generated teratomas and expressed markers associated with bona fide primed human iPS cells.

A more recent report described the generation of naïve hiPSCs by inducible PiggyBac delivery of RARG and LRH1 in combination with OCT4, SOX2, KLF4, and CMYC [41]. Similar to Hanna *et al*., 2010, their cells could be propagated in the absence of doxycycline, grew in LIF/2i, and generated teratomas. However, PiggyBac transposons are not subjected to the same natural silencing process that diminishes retroviral (and less extensively lentiviral) expression. Like previous studies, their study failed to definitively demonstrate a transgene-independent state.

Quasi-pluripotent human cells that resemble mouse ES/iPSCs have been generated by combining canonical reprogramming factor overexpression with LIF and variations on the 2i cocktail. However, the resulting cells are unstable and probably nonequivalent across protocols. The cells described are transgene-dependent, lack stable endogenous expression of OCT4 and NANOG, or resemble primitive neural stem cells. Future attempts to generate naïve hiPSCs will require definitively ruling out the contribution of transgenic expression because stable transgene expression is not desirable for generating clinically relevant cell types.

Direct reversion of EpiSCs to naïve pluripotency

The murine naïve and primed states are readily inter-convertible. When naïve mESCs are cultured in FGF/Activin A, they adopt a primed state [42]. Conversely, gain-of-function studies with EpiSCs identified transcription that can reset primed pluripotency to naïve pluripotency (Table 2). Overexpression of Nanog, Klf4, Klf2, Stat3, Nr5a1, Nr5a2, C-Myc or continuous culture in LIF/serum, LIF/2i reverts 129 EpiSCs to naïve pluripotency [42-48]. Recalcitrant NOD EpiSCs can be reverted by Klf4 or Cmyc and/or culture in 2i or

KP/CH culture [47]. The mechanism of the drug kenpaullone's function remains unknown [49].

Few attempts to revert primed hESCs to naïve pluripotency are described. Blau and colleagues used histone deacetylase (HDAC) inhibitors such as butyrate to pull hESCs toward an earlier stage [50]. Notably, sodium butyrate maintained hESCs in the absence of FGF. The butyrate-cultured human ES cells lost XIST expression but retained teratoma potential. However, findings were not extended beyond the H9 hESC line and butyrate hESCs did not possess other features of mESCs. Since extinction of XIST expression can be uncoupled from X chromosome status in female hESCs, additional assays to assess X chromosome reactivation status in female butyrate-cultured hESCs are needed [51]. Further, it will be interesting to determine whether sodium butyrate supports hESC derivation from preimplantation embryos. IDPPA5 is described as a key gene induced in butyrate hESCs because Dppa5 overexpression in EpiSCs endows EpiSCs with the potential to incorporate into preimplantation embryos, a feature associated with but not necessarily a definitive feature of naïve mESCs [52•].

Conclusions

The mouse has been the most important model organism for generating hypotheses about maintenance and differentiation of pluripotent stem cells, but the limited applicability of mouse ES cell principles across species, especially humans, challenges the relevance of rodent research to early human development. While naïve ESCs from multiple mouse strains and rats present new opportunities for genetic intervention and have clarified the nature of naïve pluripotency beyond mouse strain 129, the simple application of 2i to direct reprogramming or preimplantation human embryos fails to yield naïve human ES/iPS cells; indeed, definitive evidence for stable naïve human cells is lacking. Additional studies regarding the functional behavior of embryo-derived and reprogrammed-derived pluripotent cells will be needed to identify the best way to induce human pluripotency.

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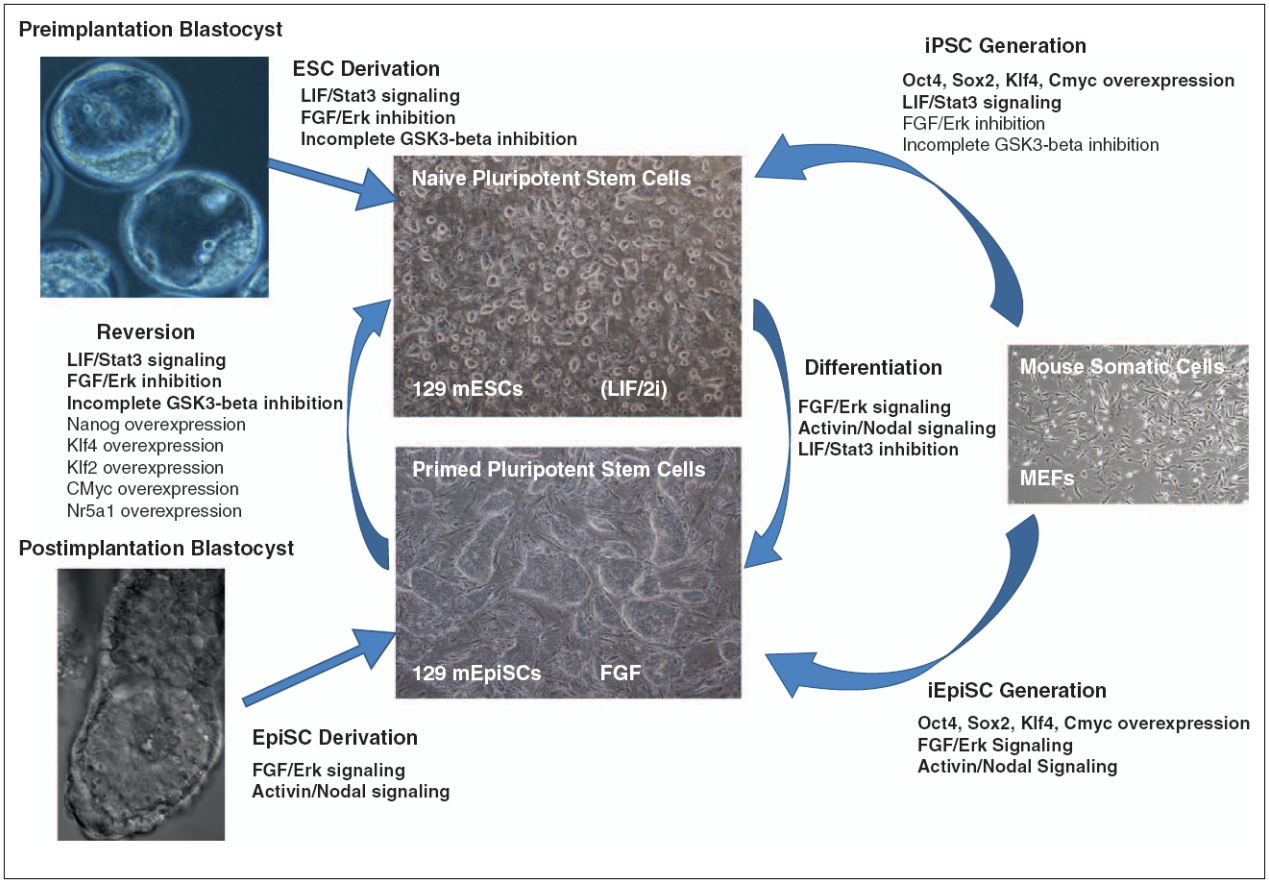

Figure 1.

Accessing rodent naïve pluripotency through three different routes.

Pluripotent stem cells may be derived from *in vivo* sources such as the preimplantation blastocyst or the postimplantation epiblast, resulting in naïve mouse ESCs or primed EpiSCs respectively. Naïve iPSCs or primed iEpiSCs may be obtained by altering the cultural conditions during somatic cell reprogramming. Finally, naïve and primed pluripotent stem cells are directly interconvertible by differentiation of naïve pluripotent stem cells to primed pluripotent stem cells, or direct reversion of primed pluripotent stem cells to a naïve state. Therefore, naïve pluripotency may be captured *in vitro* in the form of **(a)** embryonic stem cells (ESCs) during ESC derivation, **(b)** induced pluripotent stem cells (iPSCs) during somatic cell reprogramming, or **(c)** Epi-iPSCs during reversion of EpiSCs to a naïve mESC-like state.

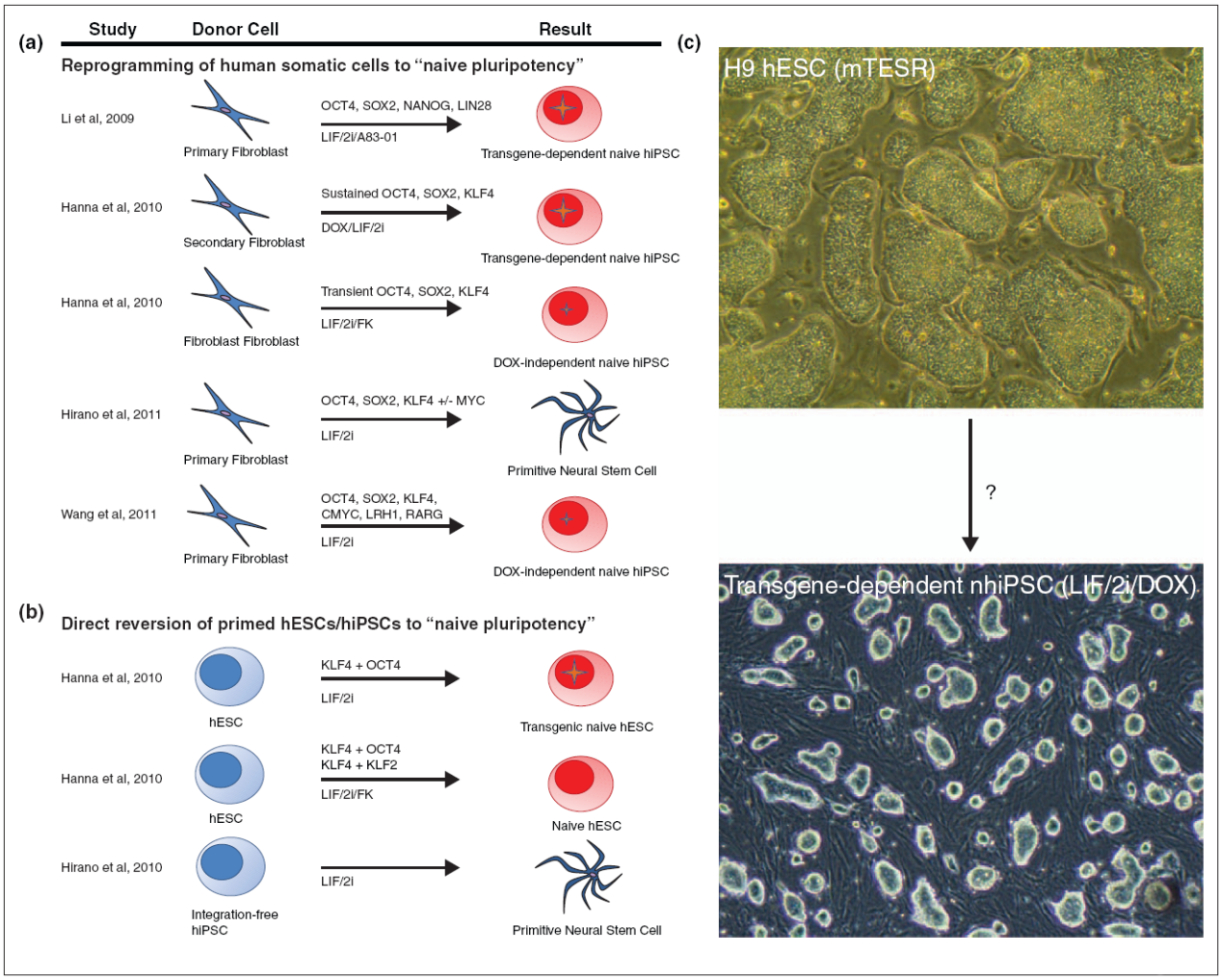

Figure 2.

Attempts to generate naïve human pluripotent stem cells through direct reprogramming or reversion of hESCs/hiPSCs. Several groups have attempted to access the naïve human pluripotent state through **(a)** direct reprogramming of primary somatic cells or secondary fibroblasts, **(b)** reversion of conventional 'primed' human ESCs, iPSCs, or 'secondary' human iPSCs. **(a)** Direct reprogramming of human cells using constitutive lentiviruses, inducible lentiviruses, retroviruses, or Piggybac Transposon has given rise to transgene-dependent naïve human iPS cells, transgene-independent naïve human iPS cells and primitive neural stem cells. **(b)** Reversion of conventional human ESCs/iPSCs by stable or transfection of OCT4, KLF2, KLF4 and transfer into LIF/2i or LIF/2i/FK yields transgene-dependent or transgene-independent naïve human iPSCs; transfer and selection of integrationfree human iPS cells in LIF/2i yields primitive neural stem cells. **(c)** Can primed hESCs be converted directly into a stable naïve pluripotent state? Primed human ESC line H9 cultured in mTESR medium (top) and transgene-dependent naïve human iPS cell line cultured in LIF/2i/DOX conditions. The transgene-dependent nhiPSC line is dependent on continuous induction of a transgene-cassette encoding the four factors OCT4, SOX2, KLF4, and CMYC and was generated from the BJ fibroblast cell line. 2i = MEK inhibitor

PD0325901 and GSK3-beta inhibitor CHIR99021. FK = Forskolin. rtTA = reverse tetracycline transactivator. Large star indicates self-renewal is sustained by high levels of transgenes. Small star indicates the presence of residual transgenes.

Table 1

Attempts to generate naïve human iPS cells

	Transgene delivery method	Transgene	Donor cell line	Culture conditions	Marker expression	Functional pluripotency	Functionally naïve?	Stability	Transgene independence
Li *et al.*, 2009	pSIN-EF1A lentivirus	OCT4, SOX2, NANOG, LIN28	IMR90	LIF, PD03, CHIR, A-83-01	SSEA3, SSEA4, TRA1-60, TRA1-81	*In vitro* differentiation, Teratomas	Self-renewal in MEK/ALK5 inhibitors	>30 passages (mESC conditions)	Yes
Ware *et al.*, 2009			H9 human ESC	Sodium butyrate	SSEA3, SSEA4, TRA1-60, TRA1-81	*In vitro* differentiation, Teratomas	XIST extinction	Enzymatic	Yes
Lengner *et al.*, 2010			Human blastocyst	FGF, 5% oxygen	SSEA3, SSEA4, TRA1-60, TRA1-81	*In vitro* differentiation	XIST extinction	Sensitivity to cryopreservation and oxidative stress	Yes
Hanna *et al.*, 2010	FUW-tetO lentivirus	OCT4, SOX2, KLF4	C1 secondary fibroblast	LIF, PD03, CHIR, DOX	SSEA3, SSEA4, TRA1-60, TRA1-81	Teratomas	XIST extinction, signaling dependency	>50 passages (Trypsin)	No
Hanna *et al.*, 2010	pCAG	OCT4, KLF4	WIBR3 hESC	LIF, PD03, CHIR	SSEA3, SSEA4, TRA1-60, TRA1-81	Teratomas	XIST extinction, signaling dependency		No
Hanna *et al.*, 2010	pCAG	OCT4, KLF4	WIBR3 hESC	LIF, PD03, CHIR, Forskolin	SSEA3, SSEA4, TRA1-60, TRA1-81	Teratomas	XIST extinction, signaling dependency	~15 passages (Trypsin)	Yes
Hanna *et al.*, 2010	pCAG	KLF4, KLF2	WIBR3 hESC	LIF, PD03, CHIR, Forskolin	SSEA3, SSEA4, TRA1-60, TRA1-81	Teratomas	XIST extinction, signaling dependency	~15 passages (trypsin)	Yes
	Transgene delivery method	Transgene	Donor cell line	Culture conditions	Marker expression	Functional pluripotency	Functionally naïve?	Stability	Transgene independence
Buecker *et al.*, 2010	FUW-tetO	OCT4, SOX2, KLF4, CMYC, NANOG	Secondary fibroblast or primary human fibroblast	LIF, DOX	SSEA1	Not pluripotent	LIF-responsiveness, amenability to homologous recombination		No
Xu *et al.*, 2010			hESC	LIF, PD03, SB	SSEA3, SSEA4, TRA-1-60, TRA-1-81	*In vitro* differentiation	Not determined	>30 passages	Yes
	Transgene delivery metdod	Transgene	Donor cell line	Culture conditions	Marker expression	Functional pluripotency	Functionally naïve?	Stability	Transgene independence
Pompe *et al.*, 2011	pSIN-EF1A lentivirus	OCT4, SOX2, NANOG, LIN28		LIF, PD03, CHIR, Forskolin	Mixed SSEA1+/TRA-1-60+		Growth in 2i, Female H3K27me3 focus not observed		No
Hirano *et al.*, 2011	pMX retrovirus	OCT4, SOX2, KLF4 +/− CMYC	TIG1/TIG3 fetal lung fibroblasts, adult dermal fibroblasts	LIF, PD03, CHIR	Nestin-positive, but SSEA1-, TRA1-60-	Neural (but EB detection of T and GATA4)	No, Female H3K27me3 focus observed	~50 passages (mechanical dissociation)	Transgenes detected
Hirano *et al.*, 2011			Nonintegrated hiPSC	LIF, PD03, CHIR	Nestin-positive, but SSEA1-, TRA1-60-	Neural (but EB detection of T and GATA4)	No, H3K27me3 focus observed	>50 passages (mechanical dissociation)	Transgenes detected
Wang *et al.*, 2011	Inducible PiggyBac	rtTA, OCT4, SOX2, KLF4, CMYC, LRH1, RARG	Neonatal fibroblast, adult fibroblast	LIF, PD03, CHIR	SSEA3, SSEA4, TRA-1-60, TRA-1-81	*In vitro* differentiation, Teratomas	Upregulation of X-linked genes, XIST downregulation	>50 passages (Accutase)	Yes

Table 2

Proteins implicated in reversion of EpiSCs to a mESC-like state

Protein	Tissue distribution	Gain-of-function or loss-of function phenotype in mice/ ESCs	Reversion mouse background	Reversion culture condition	Reversion tissue origin	Refs
LIF/STAT3		Gp130 knockout eliminates capacity for diapause; Stat3 activation significantly enhances EpiSC reversion to naïve pluripotency	129	LIF/serum	Postimplantation epiblast	Bao *et al*., 2009; Yang *et al*., 2010; All LIF/2i papers
FGF/ERK	Nearly ubiquitous signaling pathway	Fgf4-null or Erk2-null ES cells blocked lineage commitment;	129, NOD	N2B27-LIF/2i; KSR-LIF/2i	*In vitro*-derived EpiSC; Embryo-derived EpiSC	Kunath *et al*., 2007; Stavridis *et al*., 2007; All LIF/2i papers
WNT/GSK3-Beta	Nearly ubiquitous signaling pathway	Promotes self-renewal of mESCs/hESCs; GSK3 inhibition in combination with ERK inhibition allows efficient derivation of naïve mESCs	129, NOD	N2B27-LIF/2i; KSR-LIF/2i		Sato *et al*., 2004; Ying *et al*., 2008; Ten Berge *et al*., 2011; All LIF/2i papers;
Klf4	High expression in gut; Highly expressed in naïve ESCs	Overexpression sustains LIF-independent self-renewal	129, NOD	N2B27-LIF/2i	*In vitro*-derived EpiSC; iEpiSC; NOD ICM outgrowth; NOD EpiSC	Guo *et al*., 2009; Hanna *et al*., 2009; Han *et al*., 2011
C-Myc	Highly expressed in proliferating cells	Overexpression sustains LIF-independent self-renewal	129, NOD	N2B27-LIF/2i	NOD ICM outgrowth; NOD EpiSC	Cartwright *et al*., 2005; Hanna *et al*., 2009
Nanog	Inner cell mass, germ cells; Highly expressed in naïve ESCs	*In vivo* knockout ICM fails to form; conditional knockout increases ES cell propensity for differentiation; Overexpression in mESCs sustains LIF-independent self-renewal; overexpression in EpiSCs reverts mESCs to pluripotency	129	N2B27-LIF/2i	*In vitro*-derived EpiSC	Silva et al, 2009
Klf2	High expression in lung; Highly expressed in naïve ESCs	Overexpression in mESCs sustains LIF-independent self-renewal; Knockdown reduces efficiency of EpiSC reversion to naïve pluripotency; replacement of exogenous Klf4 for somatic cell reprogramming	129	N2B27-LIF/2i	*In vitro*-derived EpiSC	Hall et al, 2009
Nr5a1	High expression in adrenal tissues	Overexpression reverts EpiSCs to naïve pluripotency; replacement of exogenous	129	N2B27-LIF/2i	*In vitro*-derived EpiSC	Guo et al, 2010

Protein	Tissue distribution	Gain-of-function or loss-of function phenotype in mice/ ESCs	Reversion mouse background	Reversion culture condition	Reversion tissue origin	Refs
		Oct4 for somatic cell reprogramming				
		Oct4 for somatic cell reprogramming				
Nr5a2	High expression in liver cells; Expressed in naïve ESCs	Overexpression reverts EpiSCs to naïve pluripotency; replacement of exogenous Oct4 for somatic cell reprogramming	129	N2B27-LIF/2i	*In vitro*-derived EpiSC	Guo et al, 2010; Heng et al, 2010

	Mouse background	Donor cell type	TF transfection?	Transgene delivery method	Transgene	Culture conditions	Naïve pluripotency
Guo *et al*., 2009	129	EpiSC	Yes	PiggyBac	Klf4	N2B27-LIF-PD03/CH	Yes
Hanna *et al*., 2009	NOD	ICM outgrowth	Yes	FUW-Ubc or FUW-tetO	Klf4/Cmyc	Serum + LIF	Yes
Hanna *et al*., 2009	NOD	EpiSC	Yes	FUW-Ubc or FUW-tetO	Klf4/Cmyc	Serum + LIF	Yes
Hanna *et al*., 2009	NOD	ICM outgrowth	No			PD/CH or KP/CH	Yes
Hanna *et al*., 2009	NOD	EpiSC	No			PD/CH or KP/CH	Yes
Silva *et al*., 2009	129	EpiSC	Yes	PiggyBac	Nanog	PD/CH	Yes
Bao *et al*., 2009	129	Postimplantationepiblast	No			LIF/serum	Yes
Hall *et al*., 2009	129	EpiSC	Yes	PiggyBac	Klf2	PD/CH	Yes
Greber *et al*., 2010	129	EpiSC	No			KSR-PD/CH	Yes
Han *et al*., 2011	129	iEpiSC	Yes	Lentivirus	Klf4		Yes
Yang *et al*., 2010	129	EpiSC	Yes	PiggyBac	Stat3	PD/CH	Yes
Guo *et al*., 2010	129	EpiSC	Yes	PiggyBac	Nr5a1	PD/CH	Yes