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Signaling pathways in induced naïve pluripotency

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Abstract

Pluripotent stem cells have become powerful tools for both research and regenerative medicine. To date, however, only mouse and rat embryonic stem cells (ESCs)/induced pluripotent stem cells (iPSCs) have the ability to contribute to the formation of germline-competent chimeras. These stem cells are thus considered as “naïve” pluripotent stem cells. Several signaling pathways have been identified to play a critical role in the induction and maintenance of this naïve pluripotent state. Understanding how these pathways induce and maintain naïve pluripotency will likely lead to the generation of germline-competent naïve ESCs/iPSCs from humans and animals phylogenetically close to humans.

Introduction

Pluripotent stem cells can be established from pre-implantation embryos, *e.g.*, embryonic stem cells (ESCs), post-implantation epiblasts, *e.g.*, epiblast stem cells (EpiSCs), and somatic cells through reprogramming, *e.g.*, induced pluripotent stem cells (iPSCs) [1–5]. These pluripotent stem cells can be maintained in culture indefinitely while retaining the ability to differentiate into all cell lineages of the three germ layers. To date, although ESC-like cells from many species have been reported, only ESCs and iPSCs derived from mice and rats possess the unique “naïve” pluripotent state, characterized by the expression of pluripotency markers (*e.g.*, Rex1 and Nr0b1), two active X chromosomes in the female cell, and the ability to generate germline-competent chimeric offspring [1,2,6,7]. In contrast, mouse EpiSCs represent a “primed” pluripotent state, characterized by the low-level expression of the naïve pluripotency genes, a silent X chromosome in the female cell and inability to colonize embryos after being injected into blastocysts [3,4] (Table 1). Interestingly, current available human ESCs/iPSCs are more similar to mouse EpiSCs than to mouse ESCs/iPSCs in their self-renewal requirements, morphology and gene expression

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patterns [8]. Mouse EpiSCs have been successfully reprogrammed to the naïve pluripotent state through genetic manipulation [9•–12•]. More recently, several studies reported that naïve pluripotent human stem cells that resemble mouse ESCs could also be generated from already established human ESCs or human fibroblasts through reprogramming and/or extrinsic stimulation [13–17••]. Current available data suggest that naïve pluripotent stem cells from different species may share a common mechanism that governs their self-renewal. In this review, we will discuss the conservative signaling pathways involved in inducing and maintaining naïve pluripotency.

Self-renewal-promoting pathways in naïve ESCs and iPSCs

Mouse ESCs were first established in 1981 by culturing them on top of a layer of mitotically inactivated fibroblasts (known as feeders) in serum-containing medium [1,2]. The essential cytokine secreted by feeders is leukaemia inhibitory factor (LIF) and the serum can be replaced by bone morphogenetic protein 4 (BMP4) [18]. LIF and BMP4 are dispensable for mouse ESC self-renewal if two small-molecule inhibitors, CHIR99021 and PD0325901 (known as “2i”), are used [19••]. CHIR99021 and PD0325901 inhibit glycogen synthase kinase 3 (GSK3) and mitogen activated kinase kinase (MEK), respectively. LIF/2i has been used to generate germline-competent ESCs and iPSCs from mice and rats [6,7,19••]; and recently, from humans, albeit, with additional cytokines/small molecule inhibitors required [13–17••]. Therefore, it is proposed that the LIF/2i-mediated signaling pathways are likely conserved among naïve pluripotent stem cells derived from different species (Figure 1) In mouse ESCs, Tfcp2l1, Esrrb and Klf2 are the three key downstream targets of LIF/STAT3 and CHIR99021/β-catenin signaling pathways and PD0325901-mediated inhibition of FGF/ERK signaling pathway, respectively, and overexpression of Tfcp2l1, Esrrb and Klf2 can largely replace the effect of LIF, CHIR99021 and PD0325901 in the maintenance of naïve pluripotent state [11•,12•,21,22]. Notably, Tfcp2l1 and Nanog are at the intersection of self-renewal pathways mediated by LIF, CHIR99021 and PD0325901 [12•]. Primed pluripotent state mouse EpiSCs and human ESCs, however, depend on FGF/ERK and Activin A/Smad signaling pathways for self-renewal [3,4]. Recently, it has been shown that the combination of two small molecules, CHIR99021 and IWR1, can also promote mouse EpiSC and human ESC self-renewal through retention of stabilized β-catenin in the cytoplasm [20•]. The primed pluripotent state and somatic cells can both be reprogrammed into the naïve ESC-like state by overexpression of ectopic genes and addition of small molecules [5,9•].

LIF/STAT3 pathway: an essential cue for naïve pluripotent state induction

The binding of LIF to LIF/gp130 receptors results in activation of JAK1 and, the latter phosphorylates STAT3 at a single site tyrosine 705. Phosphorylated STAT3 then translocates into the nucleus and induces target gene transcription [23]. Activation of LIF/STAT3 signaling not only promotes, but is also required for the establishment of naïve pluripotency during somatic cell reprogramming [24•], as overexpression of a constitutively active STAT3 mutant (STAT3C) significantly increases the total number of Oct4-GFP-positive colonies in Yamanaka factors-infected mouse embryonic fibroblasts, whereas inhibition of the LIF/STAT3 signaling using JAK1 inhibitor abolishes iPSC generation

[24•]. Furthermore, activated STAT3 can promote partially reprogrammed cells (pre-iPSCs) to become fully reprogrammed iPSCs [25•,26•]. This is in line with the finding that hyperactivation of STAT3 is sufficient to convert mouse EpiSCs into naïve pluripotent states, even in the presence of FGF and Activin A [25•,26•], a condition that instructs and maintains the primed pluripotent state of EpiSCs [3,4]. The reprogramming effect of STAT3 in mouse EpiSCs can be amplified by Nanog, which drives elevation of phosphorylated STAT3 partly through repressing Socs3 expression, a negative regulator of STAT3 signaling [27,28]. Moreover, many STAT3-regulated genes are co-regulated by Nanog [29], therefore, Nanog and phosphorylated STAT3 could synergistically induce activation of many naïve pluripotency genes, such as *Klf4* (A STAT3 target and can reprogram the primed pluripotent state EpiSCs into the naïve pluripotent state iPSCs when overexpressed [9•]), resulting in rapid and efficient EpiSC reprograming [27]. However, Klf4 is not the only factor responsible for STAT3-induced reprogramming, since LIF is also required [9•].

Recently, more STAT3 targets have been identified, such as c-Myc, Pim1, Pim3, Pramel7, Rhox5, Gbx2 and Tfcp2l1 [10–12•]. Forced expression of each of these STAT3 targets can partially recapitulate the self-renewal-promoting effect of LIF. However, knockdown of *Tfcp2l1*, but not other STAT3 targets, impairs STAT3-mediated ESC self-renewal and EpiSC reprogramming [11•,12•]. Tfcp2l1 provides a major connection between extrinsic LIF and intrinsic core pluripotency factors, such as Oct4, Nanog, and Sox2 [11•,12•]. Notably, *Tfcp2l1* is highly expressed in the inner cell mass of human blastocysts, but becomes significantly downregulated during derivation of human ESCs [30], similar to its expression pattern during the transition from mouse ESCs to EpiSCs [12•], suggesting that it may play a role in establishing and maintaining the naïve pluripotent state.

Canonical Wnt/β-catenin pathway: an accelerator of naïve pluripotent state induction

In addition to the LIF/STAT3 pathway, the canonical Wnt/β-catenin signaling pathway is also beneficial for the establishment of germline-competent pluripotent stem cells [6,7,19••]. β-catenin is the key mediator of the Wnt/β-catenin pathway. Activation of Wnt signaling by Wnt3a or GSK3 inhibitor leads to the stabilization of β-catenin. Stabilized β-catenin enters the nucleus, where it regulates gene transcription through interaction with the Tcf/Lef1 family of transcription factors [31]. Activation of Wnt/β-catenin signaling in mouse ESCs significantly enhances the ability of ESCs to reprogram somatic cells into a naïve pluripotent state during ESC-somatic cell fusion [32]. Activation of Wnt/β-catenin signaling can also dramatically increase iPSC generation efficiency from *Oct4/Sox2/Klf4*-infected somatic cells. This reprogramming-promoting effect is greatly repressed by inhibition of Wnt/β-catenin signaling [33••]. CHIR99021 is sufficient to convert *Oct4/Klf4*-transduced mouse embryonic fibroblasts into chimaera-forming iPSCs if combined with BIX01294, a histone methyltransferase G9a inhibitor [34]. In another approach, *Oct4/Klf4*-transduced neural stem cells treated with CHIR99021 could attain naïve pluripotent state in the presence of PD0325901 and LIF [35]. Furthermore, CHIR99021 is also one of the pivotal factors in inducing fully-competent iPSCs from mouse EpiSCs [9•], human ESCs [13–17••], mouse fibroblasts transfected with Oct4 only [34], and chemically treated non-transgenic mouse

neural stem cells [37]. Wnt/β-catenin signaling-mediated mouse ESC self-renewal has been attributed to the stabilization of the intracellular β-catenin protein [38,39] and the repression of Tcf3 by stabilized β-catenin [39]. Tcf3 localizes to the promoters of many pluripotency genes, including *Nanog*, *Nr0b1*, *Tfcp2l1* and *Esrrb* [21], and acts as a transcriptional repressor. Among these genes, *Esrrb* is required for mediating ESC self-renewal downstream of GSK3 inhibition. Depletion of *Esrrb* results in loss of expression of pluripotency genes in response to CHIR99021, while its overexpression replicates the effect of GSK3 inhibitor or *Tcf3* deletion [21]. Thus either *Esrrb* overexpression or *Tcf3* deletion can enhance the efficiency of neural precursor cell (NPC) reprogramming to iPSCs [40,41]. Notably, there are two phases of Wnt signaling in reprogramming somatic cells [42•]. In the early stage, Tcf/Lef1 family members Tcf1 and Lef1 inhibit while Tcf3 and Tcf4 promote reprogramming. In the late stage, however, deletion of *Tcf3/Tcf4* enhances iPSC generation in a Tcf1- or Lef1-dependent manner [42•]. Accordingly, manipulating the levels of *Tcf3*, from slight early overexpression to late depletion, could enable efficient reprogramming in the absence of ectopic *Sox2* [42•]. The fact that modulation of Wnt signaling activity enables reprogramming of somatic cells to iPSCs in the absence of Sox2 and c-Myc suggests that Wnt signaling pathway is likely important in achieving chemical-defined reprograming in combination with other transient cues that can replace the remaining Yamanaka factors.

FGF signaling pathway: diverse functions in naïve pluripotent state induction

FGF signaling pathway is an evolutionarily conserved pathway that plays an important role in regulating cell fates during development [43]. FGFs bind to FGF receptors (FGFR) and activate multiple signaling cascades, including MEK, JAK/STAT, PI3K and phosphoinositide phospholipase C (PLCγ) pathways [43]. FGF signaling is required for the generation and maintenance of human ESCs and iPSC [8,44–46]. Interestingly, FGF signaling also promotes mouse iPSC generation in the early phase but functions adversely in the late phase of mouse iPSC induction [47•]. In mouse ESCs, however, FGF-mediated activation of MEK signaling drives lineage commitment [48••]. Inhibition of FGF/MEK signaling enables the derivation of germline-competent ESCs from different strains of mice and rats [6,7], and might also facilitate reprogramming to the naïve pluripotent state. Consistently, the medium containing FGF/MEK inhibitor allows the conversion of human ESCs to the naïve pluripotent state through the overexpression of O*ct4*, *Klf4*, and *Klf2* [13]. A chemical-defined medium containing LIF/2i, JNK inhibitor SP600126, p38 inhibitor SB203580, Tgfβ and bFGF has also been used to derive naïve human ESCs from already established human ESCs, the ICM of the human blastocyst and Yamanaka factors-infected somatic cells [16••]. Notably, the expression profile exhibited by these naïve human ESCs does not appear to be fundamentally different from conventional primed human ESCs for key naïve pluripotency marker genes such as *Prdm14*, *Tbx3* and *Klf4*. Additionally, the methylation profile of primed and supposedly naïve human pluripotent state also appears to be relatively similar in contrast to mouse primed and naïve pluripotent state in which a more dramatic difference in the methylation profile is observed [16••]. However, these naïve human ESCs are highly similar to mouse ESCs in their growth properties, gene expression profile, X chromosome activation status and signaling pathway dependence [16••].

Importantly, these naïve human ESCs can contribute to the formation of cross-species chimaeras when microinjected into mouse morula-stage embryos [16••]. LIF/2i, when combined with other factors, such as Yamanaka factors/Lrh1/Rarg [14], HDAC inhibitors (HDACi) plus bFGF [17••], Rock inhibitor plus bFGF [49] or BMP4 inhibitor [15], can also induce and maintain naïve human ESCs/iPSCs. Intriguingly, bFGF is included in some of the culture conditions [16••,17••,49], indicating that FGF signaling might have a positive effect on self-renewal of both naïve and primed human pluripotent stem cells, although the underlying mechanism is largely unknown [17••].

How FGF/MEK inhibitor maintains and induces naïve pluripotency is not well understood. A recent study reports that FGF signaling inhibition in ESCs drives rapid genome-wide demethylation via negative regulation of the de novo methyltransferase genes *Dnmt-3a*, *-3b* and *-3l*, a pattern remarkably resembling the epigenomes of ICM cells in the blastocyst [50]. A further study shows that Prdm14 is involved in the FGF/MEK inhibitor-mediated suppression of *Dnmt-3* gene expression. Prdm14 is highly expressed in 2i-treated ESCs. It binds to the promoter region of *Dnmt-3b* gene and the knockout of the *Prdm14* gene leads to significant upregulation of *Dnmt-3b* expression and increased methylation in ESCs cultured in 2i conditions [50]. However, depletion of *Prdm14* has no obvious effect on 2i-mediated ESC self-renewal, therefore, Prdm14 is unlikely to be the primary mediator of the FGF/MEK inhibitor [50,51]. Forced expression of each of the pluripotency factors Tfcp2l1, Nanog and Klf2 can substitute for FGF/MEK inhibitor in promoting ESC self-renewal. However, neither Tfcp2l1 nor Nanog is essential for the maintenance of the naïve pluripotency state by 2i [12•,22,52]. Interestingly, Klf2-null mouse ESCs are not viable under 2i, suggesting that Klf2 has an essential role in 2i-mediated ESC self-renewal [22]. Activation of FGF/MEK signaling in mouse ESCs leads to the phosphodependent degradation of Klf2 protein, and therefore it has been suggested that MEK inhibitor PD0325901 maintains naïve pluripotent state partially through prevention of Klf2 protein phosphodegradation [22]. Recently, Tee *et al* found that loss of FGF signaling results in alterations in chromatin accessibility and profoundly impacts the expression of developmental genes [53]. It will be interesting to further explore the precise molecular mechanisms of the FGF signaling pathway in inducing and maintaining naïve pluripotency.

Conclusion

LIF/2i-mediated activation of LIF/STAT3 and Wnt/β-catenin signaling along with inhibition of FGF/MEK are necessary and sufficient for inducing and maintaining naïve pluripotency in mouse and rat ESCs/iPSCs. Manipulation of other signaling pathways through additional factors might be required for generation of naïve pluripotent stem cells from species other than mice and rats. Understanding how naïve pluripotency is induced and maintained will facilitate the application of pluripotent stem cell-based therapies.

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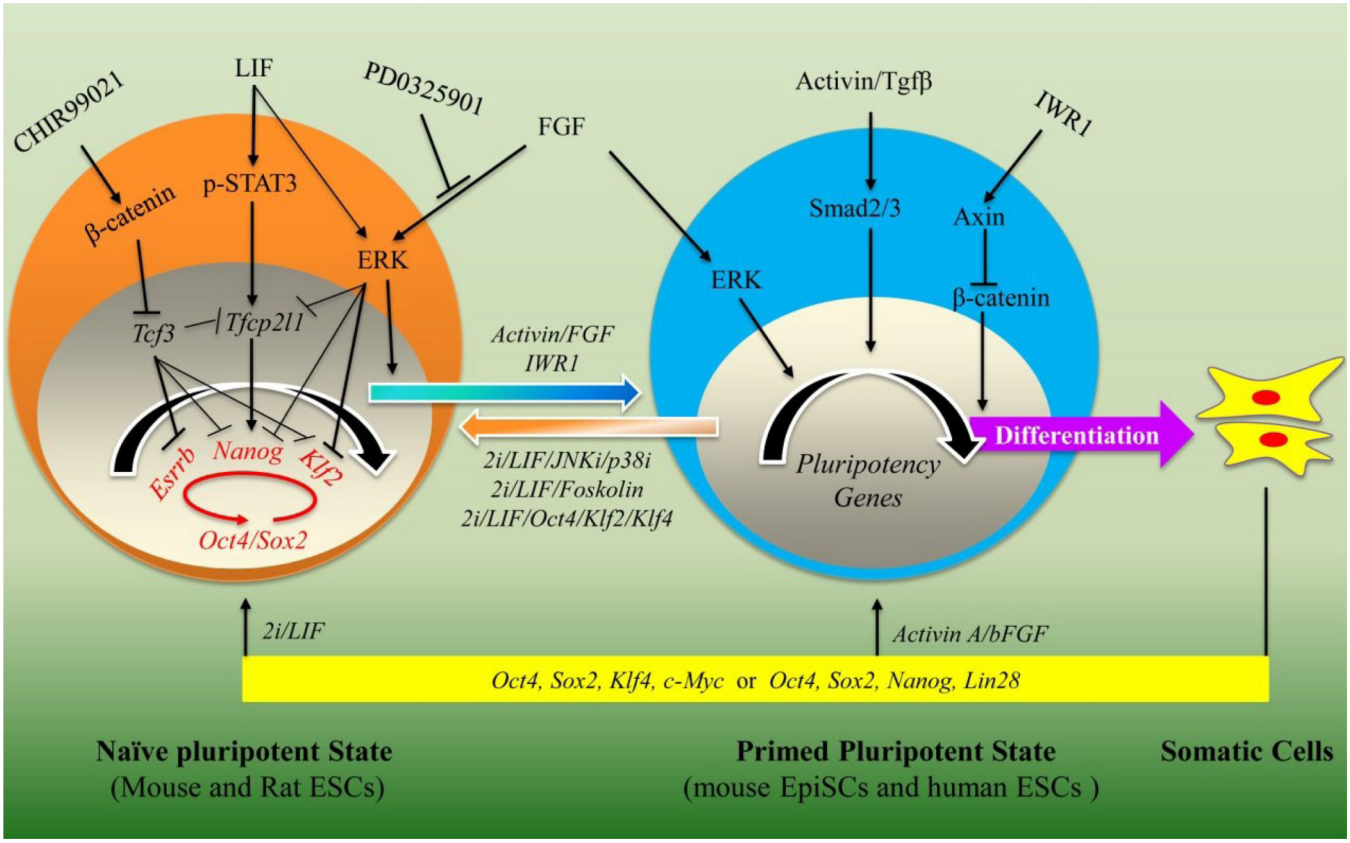

Figure 1.
Signaling pathways involved in the maintenance of naïve and primed pluripotent states.

Table 1

Comparison of naïve and primed pluripotent states

	Naïve	Primed	References
Origin	ICM of blastocyst	Post-implantation epiblast	[1–4,6,7]
Colony Morphology	Dome	Flat	[1–4,6,7]
Single-cell Mortality	Low	High	[1–4,6,7]
XX Chromosomes	Dual Activation	Single Activation	[1–4]
GermlineTransmission	High	Low	[1–4,6,7]
Culture Conditions	LIF+BMP4, 2i	Activin+bFGF, CHIR+IWR1	[3,4,18–20•]