Chapter 3

States of Pluripotency: Naïve and Primed Pluripotent Stem Cells

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Abstract

Pluripotent stem cells are classified into naïve and primed based on their growth characteristics **in vitro** and their potential to give rise to all somatic lineages and the germ line in chimeras. In this chapter, I describe the similarities and differences between the naïve and primed pluripotent states as exemplified by mouse embryonic stem cells (mESCs), mouse epiblast stem cells (mEpiSCs), human embryonic stem cells (hESCs), and human induced pluripotent stem cells (hiPSCs). I also review the efforts for derivation of naïve human pluripotent stem cells by manipulating culture conditions during reprogramming of somatic cells and attempts to revert primed hESCs to the naïve state. Understanding the requirements for induction and maintenance of the naïve pluripotent state will facilitate studies on early human embryonic development and understanding the mechanisms involved in X inactivation **in vitro**. In addition, the development of naïve hiPSCs will improve the efficiency of gene targeting for the purpose of modeling human diseases as well as for generating gene-corrected autologous pluripotent stem cells for regenerative medicine.

Keywords: naïve, primed, pluripotent stem cells, epigenetic, fragile X syndrome

1. Introduction

Two distinct pluripotent states are observed in embryonic stem cells from mice: the ground or naïve state, exemplified by the mouse embryonic stem cells (mESCs) [1], and the primed pluripotent state represented by mouse epiblast stem cells (mEpiSCs) [2,3]. The clearest difference between the two states is colony morphology, growth factor requirement for maintenance of the pluripotent state, and X inactivation status in female cells. Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) identify

more closely with mEpiSCs than mESCs [4]. Understanding the requirements for induction and maintenance of the naïve pluripotent state for human stem cells will facilitate their application for studying early embryonic development, disease modeling, drug screening, and cell-based therapies.

In this chapter, I mention the similarities and differences between naïve and primed pluripotent stem cells (PSCs) and describe various strategies that have been utilized for the derivation of naïve human PSCs and/or reversion of primed state stem cells to the naïve-like ground state. I also discuss how the availability of naïve hESCs and hiPSCs will further the mechanistic studies of various biological phenomena and facilitate genetic manipulations of hPSCs for disease modeling as well as regenerative medicine.

2. Characteristic features of the naïve and primed pluripotent stem cell states

Embryonic stem cells in mice exist in two functionally distinct pluripotent stem cell states. Specifically, mESCs that are derived from the inner cell mass (ICM) of preimplantation embryos represent naïve pluripotent stem cells (PSCs). These cells have an unlimited self-renewal capacity when grown under appropriate conditions and are able to differentiate into tissues of all three germ layers **in vitro**. In addition, when injected back into the early embryos, naïve stem cells contribute to all somatic lineages including the germline. This ability to generate chimeras is indicative of their pluripotency **in vivo**. On the other hand, mEpiSCs that are derived from the epiblast of the post-implantation embryo typify the primed state. Also included in this category are hESCs and hiPSCs that resemble closely the mEpiSCs, even though they are isolated from preimplantation embryos. Like the naïve PSCs, the primed PSCs also have unlimited potential to self-renew and differentiate into three germ layers **in vitro**, but are limited in their pluripotency **in vivo**, as they cannot give rise to germline chimeras [5]. The naïve mESCs typically grow as small, compact, domed colonies, whereas the primed mEpiSC colonies are large and grow as monolayer similar to hESCs. In addition, naïve cells survive better than their primed counterparts when passaged as single cells and have a shorter doubling time [3]. The naïve and primed PSCs also use different modes of respiration for generating energy. While metabolism in naïve stem cells utilizes both oxidative phosphorylation (mitochondrial respiration) and glycolysis, primed cells preferentially generate energy through the glycolytic pathway [6].

Another key feature of the naïve state in mESCs is that both X chromosomes are active in female cells and undergo random X chromosome inactivation (XCI) upon differentiation **in vitro**. In contrast, XCI has already been established in primed mEpiSCs and this feature can thus be used as a reliable marker to distinguish between the two pluripotent states in female stem cells. The XCI status can also be used to identify the optimal culture conditions for maintenance of the naïve stem cell state and for monitoring the epigenetic stability of human PSCs [4]. Other epigenetic differences between the two murine pluripotent states include global DNA hypomethylation [7, 8], reduced prevalence of the repressive histone mark H3K27me3 at promoters, and fewer bivalent domains in naïve ESCs [9]. The key features of naïve and primed PSCs are summarized in **Table 1**.

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Property	Naïve state	Primed state
Origin	ICM of early blastocyst	Post-implantation epiblast (Egg cylinder) or embryonic disc
Representative examples	mESCs, miPSCs	mEpiSCs, hESCs, hiPSCs
Expressed genes[1]	High expression of *Oct4*, *Nanog*, *Sox2*, *Klf2*, *Klf4*, *Klf5*, *Zpf42*, *Esrrb*, *Dppa3*, *Tfcp2l1*, *Fgf4*, *Tbx3*, *Cdh1*	*Oct4*, *Sox2*, *Dnmt3b*, *Fgf5*, *Pou3f1*, *Meis1*, *Otx2*, *Sox11*, *Gdf3*
Colony morphology	Compact dome shaped	Flattened
Differentiation bias	None	Variable
Tearatomas	Yes	Yes
Chimeric contribution in rodents[2]	Yes	No
Clonogenicity	High	Low
Single-cell mortality	Low	High
Growth factor dependence	LIF	ACTIVIN, FGF2
Respiration	Oxidative phosphorylation, glycolysis	Glycolysis
XCI status in female cells	XaXa	XaXi
Oct4 enhancer usage	Distal	Proximal
Global DNA methylation	Hypomethylated	Hypermethylated
Response to LIF/stat3	Self-renewal	None
Response to Fgf/Erk	Differentiation	Self-renewal
Response to 2i	Self-renewal	Differentiation
Level of HERVH[3] expression	High	Low
Efficiency of gene targeting	High	Low
H3K27Me3 over developmental regulators	Low	High
TFE3 localization	Nuclear	Cytoplasmic/absent

[1]Gene expression changes are based upon murine PSCs.

[2]Chimera assays are not always possible for hPSCs due to ethical concerns.

[3]HERVH expression is seen only in hPSCs.

Table 1. Characteristic features of naïve and primed pluripotent stem cell states.

Much of what we know about the growth factor dependence of naïve and primed PSCs is based on studies performed with mESCs and mEpiSCs. Mouse ESCs can be maintained long term in the naïve state when cultured in the presence of serum plus leukemia inhibitory factor

(LIF), which signals through a bipartite receptor to activate Janus-associated kinases (JAK) which leads to the activation of signal transducer and activator of transcription 3 (Stat3) [10]. However, in the absence of serum, LIF alone is unable to prevent differentiation of mESCs. This limitation is overcome by the addition of two small molecule kinase inhibitors termed "2i" with LIF. The 2i components include a specific inhibitor of extracellular signal-regulated kinase (ERK1/2)/mitogen-activated protein kinase (MAPK) signal transduction pathway (MEKi, PD0325901) and a specific inhibitor of glycogen synthase kinase 3 beta (GSK3βi, CHIR99021) that protect the ESCs from pro-differentiation stimuli and select against differentiating cells [11]. The most critical effects of GSK3i are mediated via β-catenin, which is the key mediator of Wnt/β-catenin signaling pathway. While fibroblast growth factor (FGF)-mediated activation of MEK signaling drives differentiation of mESCs, primed mEpiSCs require basic fibroblast growth factor (bFGF or FGF2) and are dependent on the activin/nodal pathway for the maintenance of pluripotency [3]. Similar to the mEpiSCs, human PSCs do not require LIF but are dependent on bFGF and activin/transforming growth factor β-1 (TGFβ1) for their long-term maintenance in culture [12, 13]. However, as discussed later, it is possible to revert primed hPSCs to the naïve state using LIF/2i.

Naïve mESCs and primed mEpiSCs also differ in the expression of pluripotency-associated transcription factors. While the pluripotency factors Oct4/Pou5f1 and Sox2 are expressed in both naïve and primed murine PSCs, factors like Nanog, Klf2, Klf4, Prdm14, Sall4, Tfcp2l1, Esrrb, and Tbx3 are preferentially expressed or upregulated in the naïve mESCs. In addition, Tfe3, which is localized in the nucleus of naïve PSCs, becomes cytoplasmic upon their conversion to the primed state and results in reduced expression of Esrrb [14]. The forced expression of naïvity-associated transcription factors has been shown to convert the primed iPSCs into to the naïve state suggesting their importance for the propagation of the ground state [15]. Although conventional hPSCs functionally resemble the primed pluripotent state of mEpiSCs, they are not identical and show specific differences in transcription regulation and the expression of various markers like FGF5, E-CADHERIN, and NANOG (reviewed in [16]).

3. Strategies for the generation of naïve human pluripotent stem cells

The naïve pluripotent state of mESCs is also observed in PSCs derived from rat embryos [17, 18]. However, naïve PSCs were not observed in other species in this study and it was not clear if this was a feature only of rodent ESCs or if it would be possible to capture it in other species. In recent years, naïve-like stem cells have been isolated from porcine fibroblasts [19], rhesus monkey fibroblasts [20], rabbit embryos, liver and stomach [21], finch embryos [22], and bovine amnion-derived cells [23]. In addition, several reports of the successful derivation of human PSCs resembling naïve mESCs in morphology and gene expression signatures have also been published [24–32]. This confirms that the naïve-like pluripotent state is not species specific as originally assumed to be limited to rodents, but may reflect an early developmental stage conserved across mammalian and possibly vertebrate evolution. The ability to capture human

PSCs in the naïve state opens up a plethora of opportunities for their use in disease modeling and regenerative medicine.

Reference	Method used	Growth factors in medium	XCI status	Chimera assay
Hanna et al. 2010 [24]	Sustained or transient expression of OCT4, SOX2, KLF4 or KLF2	LIF/2i or LIF/2i/FK or LIF/2i	Pre-XCI	ND
Gafni et al. 2013 [26]	Small molecules	LIF/2i/bFGF/TGFβ/JNKi/ p38i/ROCKi	Pre-XCI	Positive
Chan et al. 2013 [25]	Small molecules	LIF/2i/BMPi/ bFGF/TGFβ/ ROCKi	ND	ND
Takashima et al. 2014 [27]	Sustained or transient expression of NANOG and KLF2	LIF/2i/Go¨ 6983	Pre-XCI	ND
Valamehr et al. 2014 [29]	Small molecules	LIF/2i/bFGF	Pre-XCI	ND
Theunissen et al. 2014 [28]	Selection based on OCT4 distal enhancer activity	LIF/5i/bFGF/ ActivinA	XaXi	negative
Wang et al. 2014 [35]	Selection of HERVH expressing cells	LIF/2i/bFGF	Pre-XCI	ND
Ware et al. 2014 [30]	Small molecules	2i/bFGF	ND	ND
Duggal et al. 2015 [31]	Small molecules	LIF/2i/bFGF/FK/AA	ND	ND
Yang et al. 2016 [34]	Small molecules	LIF/5i/bFGF/ ActivinA	ND	Positive
Carter et al. 2016 [32]	Recombinant protein	$NME7_{AB}$	Pre-XCI	ND

FK, forskolin; AA, ascorbic acid; Xa, active X; Xi, inactive X; XCI, X chromosome inactivation; ND, not done.

Table 2. Summary of various protocols used for generating naïve human pluripotent stem cells.

Naïve-like human PSCs have been obtained either by forced expression of pluripotency transcription factors or by using various combinations of small molecules and/or growth factors to improve the culture conditions for inducing and maintaining naïve stem cell characteristics (**Table 2**). Buecker and colleagues described the derivation of hiPSCs in the presence of LIF and five ectopic reprogramming factors, OCT4, SOX2, KLF4, cMYC, and NANOG (called hLR5 iPSCs), that displayed morphological, molecular, and functional properties of mESCs [33]. In another study, the Jaenisch group showed that the ectopic induction of OCT4, KLF4, and KLF2 in hESCs grown in 2i/LIF media resulted in naïve stem cells that resembled mESCs in their gene expression profile and reactivation of both X chromosomes [24]. One limitation of this approach is that continuous expression of the pluripotency factors was required for maintenance of the ground state in the resultant PSCs

thereby restricting their potential for downstream applications. To overcome this limitation, several groups have used small molecules to achieve transgene-independent derivation of naïve hPSCs. Using a reporter cell line containing a doxycycline (dox)-inducible OCT4-Green Fluorescent Protein (OCT4-GFP) reporter, Gafni et al. [26] identified a combination of factors that facilitated the derivation of genetically unmodified naïve hESCs from blastocysts, hiPSCs from somatic cells, as well as the reverse toggling and maintenance of primed hESCs into the naïve state. This naïve human stem cell medium (NHSM) included essential components [2i/LIF, TGFβ1, c-Jun N-terminal kinase inhibitor (JNKi, SP600125), p38i (SB203580)] and optimizing components [Rho kinase inhibitor (ROCKi, Y-27632) and protein kinase C inhibitor (PKCi, Go6983)] for supporting the growth of naïve hPSCs [26]. These naïve-like hiPSCs grown in NHSM showed higher integration into chimeras compared to the primed PSCs. In another study, Chan et al. [25] screened 11 small molecules for their ability to increase NANOG expression and identified a combination of three inhibitors [2i plus a bone morphogenetic protein (BMP) inhibitor, dorsomorphin] in TeSR1 base medium and LIF that maintained hESCs in the ground state. The authors observed an upregulation of a number of genes that are expressed in human preimplantation embryos in the hESCs grown in 3i/LIF medium compared to the conventionally cultured hESCs. Valamehr et al. [29] also reported the derivation and maintenance of transgene-free hiPSCs with naïve-like characteristics using fate maintenance medium (FMM) that contains 2i/LIF in conventional hESC medium. These cells showed high survival rates in single-cell dissociation and displayed the characteristics of a pre-XCI state with decreased levels of XIST and H3K27me3. In contrast, Ware et al. [30] were able to reverse toggle primed hESCs by preculture in the histone deacetylase inhibitors, sodium butyrate, and suberoylanilide hydroxamic acid, followed by culture in 2i with bFGF in the absence of LIF. In addition, they reported the derivation of naïve hESCs only in the presence of 2i and bFGF.

To more systematically identify optimal culture conditions for the induction and maintenance of naïve human pluripotency, Theunissen et al. [28] screened a kinase inhibitor library using a specific reporter system based on the activity of the endogenous OCT4 distal enhancer that is exclusively active in the naïve pluripotent state. They described a serum-free N2B27 medium supplemented with five kinase inhibitors (MEKi, GSK3βi, BRAFi, LCK/SRCi, ROCKi), LIF, bFGF, and activin A (5i/L/FA) that was sufficient to maintain naïve hESCs in culture. Cells grown under these conditions showed a dramatic upregulation of transcription factors typically associated with naïve pluripotency and human preimplantation development. However, abnormal karyotype in several converted and newly derived hESC lines in 5i/L/FA was observed leading to the authors' suggestion that naïve hPSCs may be more prone to acquiring chromosomal abnormalities. In addition, in this study, hESCs grown either in the 5i/L/FA medium or as per the Gafni et al. protocol [26] did not form interspecies chimeras efficiently. The authors attributed this discrepancy between the two studies to slight variations in culture conditions or embryo handling. Furthermore, female hESCs grown under 5i/L/FA conditions showed an upregulation of XIST and XCI upon conversion from the primed state. These features illustrate the limitation of hPSCs derived in 5i/L/FA medium or, as suggested by the authors, this may highlight the differences in the naïve pluripotent state between human and mouse. Using this 5i/L/FA medium, naïve iPSCs could be generated from fibroblasts

derived from β-thalassemia patients in whom the genetic mutation was corrected by CRISPR/Cas9 gene editing [34]. More recently, Duggal et al. [31] reported the formulation of yet another culture medium that facilitated the rapid, robust, and efficient induction of naïve pluripotency in primed hESCs. In addition to the 2i/LIF, the authors included ascorbic acid (known to induce DNA demethylation and enhance reprogramming), forskolin (an activator of cAMP, shown to promote naïve pluripotency), and different concentrations of bFGF in the culture medium to identify optimal conditions for converting primed hESC lines toward a naïve state of pluripotency.

Another method, based on the selection of cells with naïve-like characteristics from a heterogeneous population of naïve and primed PSCs, has been published. Since LTR7/human endogenous retrovirus, HERVH, expression was found to be required for maintaining pluripotency in hPSC, Wang et al. developed an LTR7-driven GFP reporter construct to tag, select, and maintain naïve-like hPSCs [35]. By using this reporter, the authors were able to sort and enrich for LTR7-GFP-expressing cells and thus maintain a homogeneous population of naïve-like hPSCs over the long term. The hPSCs selected based on high expression of HERVH shared many features with naïve mESCs that included 3D rounded colonies, reactivation of the X chromosome in female cells, and uniform expression of core pluripotency transcription factors with downregulation of genes expressed in differentiated cells. Moreover, these cells grew faster and had improved cloning capacity making them suitable for genetic manipulation. However, the level of HERVH expression was found to be an important determinant of the ability of the stem cells to differentiate with higher levels of HERVH interfering with the differentiation potential. The LTR7-GFP reporter could be useful for further optimization of culture conditions for the maintenance of naïve pluripotent state for human stem cells.

Although it is possible to generate human PSCs with features that are characteristic of naïve mESCs by culturing them in the presence of exogenous factors or a cocktail of small molecules, their tendency to develop karyotypic abnormalities in long-term culture [28] raises concerns that these may not be the true, naturally occurring human naïve stem cells. To capture this natural naïve state of hPSCs without additional growth factors or small molecules, Carter et al. [32] developed a novel approach using a naturally occurring human growth factor, NME7, in minimal media without bFGF, LIF, or any other small molecule. NME7 belongs to the NME family of nucleotide phosphate kinases but lacks the kinase activity. It is proposed to act by binding to the MUC1* growth factor receptor that is expressed on the surface of stem cells and results in its dimerization which promotes growth and pluripotency. The hPSCs grown in the presence of $NME7_{AB}$, recombinant human NME7 protein, grew faster, showed resistance to differentiation, demonstrated increased cloning efficiency, had two active X chromosomes in the female cells, and had a normal karyotype after 30 or more passages. However, $NME7_{AB}$-derived cells did not form colonies and grew preferentially as a monolayer. The various strategies used for the derivation of naïve-like hPSCs are summarized in **Table 2**.

While all of the above-mentioned approaches claim to have successfully derived the so-called ground state naïve-like hPSC, the resultant cells do not share the same gene expression patterns, although there is some overlap [32]. Therefore, a set of core genes/markers that clearly define naïve hPSCs is not currently available but is highly desirable.

4. Naïve hPSCs for gene targeting and regenerative medicine

The ability to generate homogeneous populations of stable naïve hPSCs that have the potential to differentiate equally toward all lineages will be critical for their use in disease modeling and regenerative medicine. The faster doubling time of naïve PSCs and their capacity for clonal expansion is important for generating large number of cells required for screening applications. Marked differences in the propensity of hESCs to differentiate into certain lineages or cell types have been reported [36]. Additionally, hiPSCs generated using conventional methods were shown to be limited in their proficiency of lineage-specific differentiation that can be influenced by the donor cell type [37]. However, new data show that the differentiation propensities of hiPSCs are significantly biased by donor-dependent variability and not by cell type of origin [38]. It has been suggested that the conversion of hiPSCs to a more naïve-like state could improve their **in vitro** differentiation potential toward specific cell types by resetting their epigenetic status [39, 40]. However, a systematic study to directly compare the differentiation efficiency of isogenic naïve and primed hPSCs into different cell types has not been done and will be necessary to establish that naïve hiPSCs have a better differentiation ability than their primed counterparts. It has been observed that the efficiency of homologous recombination is significantly higher in mESCs in comparison to hESCs and hiPSCs [41, 26], suggesting that the PSCs in the naïve state might be more amenable to gene editing. In addition, the very low clonal efficiency and slow growth rate of primed hPSCs limit the full potential of gene targeting mediated by site-specific nucleases, thereby supporting the generation of naïve PSCs for efficient gene editing. Hu et al. [42] converted primed state iPSCs from Parkinson's disease patients to a so-called “naivetropic” state by dox-induced expression of transgenes (OCT4, SOX2, KLF4, c-MYC, and NANOG) and the use of LIF/2i. Using these cells, they were able to target GFP to the PITX3 locus by transcription activator-like effector nuclease (TALEN) very efficiently. Similarly, Yang et al. [34] showed significant improvement in the mutation correction efficiency in naïve hiPSCs derived from β-thalassemia patients using the 5i/L/FA system [28].

5. Naïve hPSCs for studying the process of X inactivation during early human embryonic development

In addition to the therapeutic potential of pluripotent stem cells for drug screening and regenerative medicine, their ability to recapitulate early embryonic differentiation is useful for study of the pathways involved in cell commitment and embryonic differentiation. One such process that is developmentally regulated and tightly linked with cell differentiation is the random inactivation of one of the two X chromosomes in all of the cells in female mammals that balances the sex difference in dosage of X-linked genes. This is a highly coordinated and stepwise process involving many noncoding RNAs that affect epigenetic modifications and cause transcriptional silencing of the entire X chromosome barring a few genes that escape silencing (see [43] for a recent review on the subject). Most of what we know about the mechanism of XCI comes from many elegant studies done with mice and has benefited from

the existence of naïve state mESCs with two active X chromosomes that undergo random XCI upon further differentiation. However, there are distinct differences between the XCI process in mice and humans (reviewed in [44]). For example, the imprinted inactivation of the paternal X chromosome seen in the early mouse embryo and maintained in the placenta does not occur in human embryo [45] which only exhibit random XCI. In addition, some of the genes that escape XCI in humans are inactivated in mice [46] and substantial diversity in the timing and regulation of XCI between mammals has been observed [47]. Therefore, having the ability to model XCI using hESCs or hiPSCs is highly desirable and will definitely benefit from optimized methods to derive and maintain female hESCs or hiPSCs in the naïve state with two active X chromosomes.

6. Naïve hPSCs for in vitro modeling of human diseases

Human PSCs can be used to study underlying mechanisms of diseases that cannot be modeled easily in mice. One such example is the repeat-mediated aberrant silencing of the *fragile X mental retardation 1 (FMR1)* gene in fragile X syndrome (FXS). FXS is the most common cause of inherited intellectual disability and autism spectrum disorder [48]. The most common mutation in FXS is the expansion of a CGG-repeat tract in the 5′-untranslated region of the *FMR1* gene that causes its transcriptional silencing by DNA methylation and heterochromatin formation [49, 50]. It is believed that the repeat-mediated *FMR1* gene silencing is developmentally regulated with the gene being active in the early embryo and silenced at a later time during development (see [51] for a recent review). Mouse models for the expanded CGG repeats do not result in *Fmr1* gene silencing [52, 53] and therefore cannot be used for studying the underlying mechanism of gene silencing in FXS or its effect on the developing brain. In the first reported FXS embryonic stem cell line, the *FMR1* gene was active in the ESCs and showed silencing upon differentiation into embryoid bodies [54]. This generated a great interest in developing stem cells as a model to study the mechanisms involved in gene silencing. However, in most of the subsequently generated FXS ESCs, the *FMR1* gene is already silenced [55, 56]. Additionally, iPSCs generated from FXS patient fibroblasts do not show reactivation of the *FMR1* gene [57]. Using a strategy that would favor the generation of naïve iPSCs, Gafni et al. [26] were able to derive naïve state FXS iPSCs that had an active *FMR1* gene. These observations lend support to the idea that the *FMR1* gene is active in the very early embryo but that silencing occurs very soon after that perhaps associated with the transition to the primed state. A better understanding of this process may allow the modeling of developmental silencing of the *FMR1* gene **in vitro**. In addition, these studies will also illuminate the general process by which heterochromatin might be reset during the process of reprogramming cells to pluripotency.

7. Conclusions

Recent research suggests that it might be possible to achieve naïve pluripotency of hESCs and hiPSCs by using specific combinations of small molecule inhibitors and growth factors in the

culture medium during their derivation and growth. The ability to generate and maintain naïve pluripotent stem cells will facilitate studies on early human embryonic development and the mechanisms involved in XCI **in vitro**. This will also improve the odds of genetically correcting mutations that cause human diseases and thus help in dissecting the mechanisms of disease pathogenesis. In addition, the ability to stably maintain the epigenetic state of the pluripotent stem cells will be critical for their use in stem cell-based gene therapies.

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