Resetting Human Naïve Pluripotency

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ABSTRACT: The rodent naive pluripotent state is believed to represent the preimplantation inner cell mass state of the developing blastocyst and can derive self-renewing pluripotent embryonic stem cells (ESCs) in vitro. Nevertheless, human ESCs exhibit epigenetic, metabolic, and transcriptomic characteristics more akin to *primed* pluripotent stem cells (PSCs) derived from the postimplantation epiblast. Understanding the genetic and epigenetic mechanisms that constrain human ESCs in the primed state is crucial for the human naive pluripotent state resetting and numerous applications in regenerative medicine. In this review, we begin by defining the naive and primed states in the murine model and compare the epigenetic characteristics of those states to the human PSCs. We also examine the various reprogramming schemes to derive the human naive pluripotent state. Finally, we discuss future perspectives of studying and deriving the human naive PSCs in the context of cellular engineering and regenerative medicine.

KEYWORDS: naive pluripotency, human embryonic stem cell, epigenetic, state change

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Introduction

Pluripotent stem cells (PSCs), capable of indefinite self-renewal in vitro, hold remarkable promise in the era of regenerative medicine. Recently, a spectrum of pluripotency, ranging from the *naive* state to the *primed* state, has been shown to exist in murine PSCs.[1] The establishment of a naive inner cell mass (ICM)-like murine embryonic stem cell (ESC) state and an epigenetically different primed murine epiblast stem cell state (mEpiSC) has provoked investigation toward a similar spectrum in human cells. Compared to the primed state, cells in the naive pluripotent state are more amenable to genome editing, present higher proliferative rate, and have higher chimeric integration potential.[1] Substantial effort has been geared toward reproducing and stabilizing this paradigm in human and other primate PSC systems.

Defining the Murine Paradigm: Naive vs. Primed

Distinct from transient and progressive embryonic development in vivo, in vitro capture of the naive and primed states represents a snapshot of embryonic development. Table 1 provides a brief summary of the common differences between the two states.

Murine ESCs, derived from the preimplantation epiblast of developing blastocysts, indefinitely self-renew in the presence of LIF/Stat3 signaling and serum are generally believed to "represent immortalization of the naive epiblast".[1] Murine ESCs have been shown to integrate into the preimplantation epiblast and effectively contribute to chimera formation. Activation of the autoinductive FGF/MAPK signaling pathway can destabilize the mESC state, restricting lineage commitment abilities.[2] Simultaneous inclusion of the growth factors LIF and Erk/Gsk inhibitors (commonly referred to as LIF/2i) is often used to stabilize the naive-state mESCs and even derive mESCs from genetically nonpermissive mouse strains.[1] KLF2/4 and Esrrb[3] are all further upregulated in the naive state compared to the primed state.

In contrast, mEpiSCs, derived from the postimplantation epiblast, present a *rewired* pluripotency transcriptional network compared to the naive state.[4] Specifically, conventional mEpiSC culture is stabilized by exogenous stimulation of FGF2 and Activin A pathways. EpiSCs do not depend on LIF/Stat3 signaling for self-renewal. In fact, several key pluripotency factors, including KLF4, and Stella are all downregulated in the primed state.[3] The epigenetic differences between the naive and primed states offer a good model for studying cell state changes. Naive mESCs are characterized by diminished H3K27me3 signal at gene promoters, a histone modification linked to gene silencing, relative to their primed counterparts.[5] The naive state also shows that both X chromosomes are active in female cells. Additionally, bivalent domains, the coexistence of both the active H3K4me3 and repressive H3K27me3 marks, are preferentially located at the promoters of lineage-specific genes in the primed state, leaving these genes *poised* for expression. However, the naive state exhibits a decreased amount of H3K27me3 at bivalent domains, as well as fewer

Table 1. Naive vs. primed states.

PROPERTY	NAÏVE STATE	PRIMED STATE
Model cell system	mESC	mEpiSC
Embryonic origin	Pre-implantation epiblast	Postimplantation epiblast
Morphology in vitro	Dome-shaped	Flattened
Lineage markers	Absent or low	FGF5/T
Growth factors in media	LIF/BMPs	FGF2/Activin A
XX chromosome status	Both active	One inactive
DNA methylation	Global DNA hypomethylation	Lack of DNA hypomethylation
H3K27me3*	Relatively lower	Relatively higher
Klf2/4, Esrrb†	Relatively higher	Relatively lower
Chimerism	Efficient	Inefficient

Notes: *H3K27me3 levels at the promotor for 2i treated cells, relative to the other state. †Relative to the other state.

bivalent domains overall compared to the primed state, suggesting that bivalent domains in naive cells become resolved as the cells partially differentiate to the primed EpiSCs.[5] The naive state is also characterized by global DNA hypomethylation compared to the hypermethylated primed state. Through whole-genome bisulfite sequencing, both Ficz et al[6] and Habibi et al[7] observed rapid and genome-wide hypomethylation for both 5mC and 5hmC in mouse ESCs grown in 2i conditions compared to their primed counterparts. In addition, von Meyenn et al[8] provided mechanistic insight by attributing the global DNA hypomethylation to the synergistic effect from a combined reduction of UHFR1/H3K9me2 and impaired recruitment of the DNA methylation maintenance machinery.

Where Do hESCs Lie?

Established, conventional hESCs closely cluster to the murine primed state. Conventional hESC culture relies upon FGF/Activin signaling pathways.[9] hESCs often express high levels of NANOG; subsequent inhibition of NANOG results in a collapse of pluripotency in the primed state.[10] Additionally, primed hESCs present an increase in DNA methylation and X chromosome inactivation.[11] Naive-state hESCs, on the other hand, may express downregulated XIST transcription and a nearly complete lack of H3K27me3 nuclear foci. Human ESCs grown in reprogramming conditions have previously demonstrated significant reduction in CpG methylation, as well as downregulation of de novo DNA methyltransferase enzymes DNMT3A, DNMT3B, and DNMT3L.[12] Additionally, naive hESCs may present a significant reduction of H3K27me3 in promoter and gene body regions over developmental genes when compared to the human primed state, as well as fewer bivalent domains overall.[13] Global gene expression profiles have determined that naive hESCs had a significant downregulation of lineage commitment genes and grouped closer to human ICM than to primed cells. Furthermore, while hESCs express E-cadherin, a more prominent surface expression of E-cadherin is present in the naive state compared to the primed state, which is consistent with the enhanced single-cell survival of the naive state.[11]

Stabilizing the Dichotomy

Initial attempts at deriving naive hESCs utilizing transgene expression and/or exogenous signaling pathway modulators were largely met with low efficiency and instability. Hanna et al[11] initially reprogrammed secondary human fibroblasts using doxycycline (DOX)-inducible lentiviral vectors encoding OCT4, SOX2, and KLF4 in 2i/hLIF growth conditions. Subsequently, constitutive expression of transgenic OCT4/KLF4 or KLF4/KLF2 was capable of generating DOX-independent lines. Derived lines exhibited epigenetic reversion similar to mESCs, including downregulated XIST transcription. Wang et al[14] also reported successful reprogramming of human dermal fibroblasts. Notably, the expression of RARG/LRH-1 in addition to OCT4, SOX2, KLF4, and C-MYC greatly increased reversion efficiency. Subsequently, Chan et al[15] reported exogenous inclusion of 2i/hLIF, dorsomorphin (a BMP inhibitor), and high doses of FGF2 and TGFβ1 successfully reprogrammed multiple primed hESC lines. While the *3iL hESCs* displayed upregulation of Stella, NANOG, and Pou5F1 as early as four days after treatment, X chromosome reactivation did not occur. In another variation presented by Theunissen et al,[16] a 5i/LIF/FGF/Activin A system composed of additional BRAF, ROCK, and SRC inhibitors reset human fibroblasts screened via OCT4 distal enhancer activity, a trademark of the naive state. The cocktail of inhibitors resulted in cells with higher NANOG mRNA, as well as a globally lower H3K27me3 signal than their primed counterparts. DNA sequencing analysis of the converted cells was marked by a reduced H3K27me3 signal at bivalent domains, consistent with the naive state. However, these 5i ground-state cells displayed an upregulation of XIST, indicating incomplete reactivation of the chromosome, as well as abnormal karyotyping upon conversion.

Takashima et al also reported that ectopic expression of KLF2/NANOG with 2i/LIF/aPKCi conditions could reset primed PSCs grown on MEFs.[17] The resulting naive cells displayed increased mitochondrial respiration, indicative of cells in the ICM. Furthermore, reset cells underwent epigenetic reorganization. Naive-state cells presented notably lower 5mC and 5-hydroxymethylcytosine signal globally, as well as more than 50% loss of CpG methylation. Consistent with X chromosome activation, H3K27me3 foci were almost entirely lacking in reset female cells. The cells produced by Takashima et al maintained the naive-state transcription circuitry and clustered closely with naive mESCs, characterized by robust NANOG, KLF4, and TFCP2L1 expression. The Smith group further claimed that naive-state hESCs exhibited FGF/Activin A signaling independence. However, the feeder-dependent growth could potentially confound their claims. Valamehr et al[18] reported the reversion of primed

Table 2. Overview of the various reprogramming efforts.

GROUP	SOURCE	REPROGRAMMING			RESULTANT CHARACTERISTICS			
	INITIAL CELL TYPE	GENE DELIVERY METHOD	TRANSGENES	SMALL MOLECULES	CHROMOSOME STATUS	MORPHOLOGY	IMMUNOFLUORESCENCE	TRANSGENE DEPENDENCY
Hanna et al, 2010[11]	C1 Secondary Human Female Fibroblasts	DOX-inducible lentiviral vector	OCT4 SOX2 KLF4	PD03 CHIR LIF	XIST elimination	Dome	SSEA4 TRA-1-60 SSEA3 TRA-1-81 SSEA1	No
	WIBR3 hESC	DOX-inducible lentiviral vector	OCT4 KLF4	PD03 Forskolin CHIR LIF	XIST elimination	Dome	SSEA4 TRA-1-60 SSEA3 TRA-1-81 SSEA1	No
	WIBR3 hESC	DOX-inducible lentiviral vector	KLF4 KLF2	PD03 Forskolin CHIR LIF	XIST elimination	Dome	SSEA4 TRA-1-60 SSEA3 TRA-1-81 SSEA1	No
Wang et al, 2011[14]	Neonatal Foreskin Dermal Fibroblast	PiggyBac	OCT4 C-MYC KLF4 SOX2 RARG LRH-1	PD03 CHIR LIF	Low XIST, upregulation of X-linked Genes	Compact, raised colonies	SSEA3 TRA-1-81 SSEA4 TRA-1-60	Yes
Chan et al, 2013[15]	H1, hES2, hES3	N/A	N/A	PD03 LIF Dorsomorphin BIO	Inconclusive	Small, compact colonies	TRA-1-60 TRA-1-81	N/A
Takashima et al, 2014[17]	H9	N/A	N/A	PD03 LIF CHIR aPKCi	XIST elimination	Dome	OCT3/4 TFCP2L1 NANOG STELLA KLF4 TFE3	No
Theunissen et al, 2014[16]	WIBR3 hESC	DOX-inducible lentiviral vector	KLF2 NANOG	PD03 SB590885 IM12 Y-27632 LIF WH-4-023	XIST upregulation, X-linked gene downregulation	Dome	OCT4	Yes
	WIBR2 hESC	DOX-inducible lentiviral vector	KLF2 NANOG	PD03 SB590885 IM12 Y-27632 LIF WH-4-023 FGF/Activin A	XIST upregulation, X-linked gene downregulation	Dome	OCT4	No
Valamehr et al, 2014[18]	FTC007	Episomal Induction	OCT4 SOX2 SV40LT	PD03 SB431542 LIF CHIR ThiazovivinbFGF	XIST upregulation, X-linked gene downregulation	Dome	SSEA4 TRA-1-81 TRA-1-60	No
Ware et al, 2014[13]	Six-to-Eight cell Embryo	N/A	N/A	PD03 LIF CHIR FGF	XIST elimination	Mounded	SSEA4 TRA-1-60 OCT4 TRA-1-81 NANOG	N/A

hESCs via episomal induction of OCT4/SOX2/SV40LT in combination with stage-specific medium supplementation. Ware et al[13] also reported the maintenance of a directly derived naive hESC from the preimplantation blastocyst that is dependent on the inclusion of an FGF inhibitor and 2i/LIF. While the resulting cell line could be grown for more than 60 passages and was confirmed via increased mitochondrial metabolism, X chromosome activation, microRNA patterns, and complete ERK inhibition seemed to negatively affect line viability.

Further research into reversion procedures also call upon the use of p38i, JNKi, and FGF2/Tgfβ1 cytokine supplementation as additional positive stabilizers for reprogramming of naive hESCs.[19] Converted naive hESCs subsequently displayed XIST downregulation and X chromosome reactivation, as evidenced by a nearly complete lack of H3K27me3 nuclear foci. Chromatin mapping of reset cells showed a significant decrease of both H3K4me3 and H3K27me3 marks across all gene promoters and bodies, with a notable emphasis over developmental genes. However, separate groups attempting to replicate these reversion procedures discovered abnormally downregulated or abolished expression of key pluripotency factors, suggesting cellular differentiation.[16] As new key pathways are identified, they will have to be worked into existing reprogramming models. Table 2 summarizes the different reprogramming schemes.

What's Next for the Paradigm?

Despite accumulating efforts in generating stable naive hESCs, substantial obstacles remain, preventing the rapid assimilation of the naive state.

It has since become clear that pluripotency lies upon a spectrum, on which many factors coincide and affect cellular characterization (ie, genetic background, epigenetic features, etc.).[1] Lack of a standard for defining the naive state raises concerns regarding the true identity of any resultant cell lines. Notably, many of the aforementioned reversion protocols successfully reproduced some, but not all, of the characteristics of the naive state. How can these cells be proven to accurately capture the corresponding developmental stage? Further elaboration on the role of the inhibitors and activated pathways can present clearer guidelines for the definition of the ground state. It is our belief that any potential naive-state candidate must be subjected to a rigorous and intensive analytical process, combining transcriptome and epigenomic profiling to prove stabilization and pluripotency. Furthermore, future studies should elucidate the underlying mechanisms for in vivo and in vitro distinctions.

The possibility that the murine model is more permissive for stabilizing the pluripotent paradigm compared to other models should also be considered.[20] Knowledge of underlying permissive genes or factors in the murine model could be adapted to mammalian culture to allow for increased efficiency in reversion and stabilization. Additionally, it remains a possibility that mechanical stimulants surrounding the pluripotent cells can play a role in their propagation and stabilization. Further research should seek to elucidate the effect of mechanostimulants on naive-state reversion and propagation.[21] Successful stabilization of the naive state in human ES culture may ultimately be subject to considerable growth condition modification.

Perspective

In combination with recent advances in CRISPR-Cas9 genomic engineering techniques, human naive ESCs sit as a significant gateway to understanding the human embryonic developmental process.[22] In particular, preliminary reports suggest that the preimplantation state is capable of more efficient directed differentiation, opening a gateway toward application in cell therapies and cellular development. Naive-state cells, which exhibit higher global hypomethylation, are potential candidates for resolving the issue of residual memory in human iPSCs.[23] Enhanced chimeric capability in the ground state could alternatively present a promising method for studying embryonic development and lineage specification in vivo.[24] Finally, the successful capture of the naive state paired with the appropriate exogenous and endogenous conditions could prove to be a viable solution to some of the most pressing issues facing regenerative medicine, such as lack of differentiation efficiency and low cellular yields for transplantation.[25] It is our firm belief that further research into the role of critical regulatory factors such as KLF4/TFCP2L1 could identify significant pathways to stabilize the naive PSCs. It is conceivable that the generation of a stable ground state could accelerate development of regenerative therapies targeting a host of illnesses, including bone loss, diabetes, and neurological disorders.

Author Contributions

Compiled and contributed to writing of manuscript: JX, DM, LX. Contributed to figure design: JX, DM, LX. Made critical revisions and approved final version: JX, DM, LX. All authors reviewed and approved of manuscript.

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