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Identifying Human Naïve Pluripotent Stem Cells – Evaluating State-Specific Reporter Lines and Cell-Surface Markers

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Recent reports that human pluripotent stem cells can be captured in a spectrum of states with variable properties has prompted a re-evaluation of how pluripotency is acquired and stabilised. The latest additions to the stem cell hierarchy open up opportunities for understanding human development, reprogramming, and cell state transitions more generally. Many of the new cell lines have been collectively termed 'naïve' human pluripotent stem cells to distinguish them from the conventional 'primed' cells. Here, several transcriptional and epigenetic hallmarks of human pluripotent states in the recently described cell lines are reviewed and evaluated. Methods to derive and identify human naïve pluripotent stem cells are also discussed, with a focus on the uses and future developments of state-specific reporter cell lines and cell-surface proteins. Finally, opportunities and uncertainties in naïve stem cell biology are highlighted, and the current limitations of human naïve pluripotent stem cells considered, particularly in the context of differentiation.

1. Introduction

Pluripotency, the ability to form all cell lineages of the body, can be captured in different states and form phenotypically distinct cell types.[1–4] One of the surprises encountered by Thomson and colleagues when they first derived human and rhesus pluripotent stem cells (PSCs) was that the cells looked nothing like their mouse counterparts – the morphological waymark at the time.[5–7] This unanticipated difference would have misled many researchers involved in the pioneering studies of the 1990s, who perhaps did not realise the cell types they had in their tissue culture dishes. When viewed under the microscope, human PSCs (hPSCs) grow as large, flat colonies of tightly packed cobblestone cells, whereas mouse PSCs (mPSCs) grow in smaller, rounder clumps of cells. Initial characterisation of the newly derived cell lines showed differential cell-surface marker expression when compared to mPSCs, the primate cells more closely mimicking human embryonal carcinoma cells, the haphazard, pluripotent cells that grow from germ cell tumors.[5–9] Further investigations over the following years revealed that molecular, signaling, and metabolic differences are also prevalent between human and mouse PSCs.[10,11] This has an enormous impact on establishing appropriate benchmarks for classifying cells, and also requires the adoption of different protocols as approaches developed for one cell type might not be easily translated to another.

The origins of the divergent mouse and human PSC states are obscure given that the first PSC lines of both species were derived from embryos at equivalent developmental stages and under the same culture conditions.[5,12,13] This observation tells us that embryo outgrowths of mouse and human blastocysts will default to different cell types. We now know that preimplantation-stage epiblast cells can give rise to alternative pluripotent states depending on the culture conditions used. When mPSCs are derived and maintained in the presence of leukaemia inhibitory factor (LIF) and 2i (dual inhibition of MEK and GSK3) the resultant cell lines are in a 'naïve' state of pluripotency and correspond to the preimplantation epiblast.[2,14] Alternatively, when derived in conditions containing FGF and Activin A, epiblast stem cells (EpiSCs) are formed, which are considered to represent a 'primed' state of pluripotency.[15,16] EpiSCs can be obtained from either preimplantation epiblast cells,[17] or more commonly, from the postimplantation epiblast tissue that forms a day or two later in development.[15,16,18] Although both naïve and primed mPSCs can self-renew and are pluripotent, there are substantial transcriptional, epigenetic and metabolic differences that contribute to the classification of pluripotent states.[19–21]

The majority of human PSC lines exhibit properties that resemble primed, mouse EpiSCs, indicating that the conventional culture conditions used to derive hPSCs are unable to hold the cells in a preimplantation state of development. Comparison to transcriptional profiles collected from primate embryos at different developmental stages support the similarity of hPSCs to postimplantation-stage epiblast cells.[22] This observation is reinforced by the considerable gene expression and epigenetic

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differences between hPSCs and preimplantation-stage human epiblast cells.[23–28]

The divergences in PSC states between species, and also between hPSCs and the preimplantation epiblast cells from which they are derived, present an exciting avenue to capture hPSCs in a naïve state of pluripotency.[29–31] Naïve hPSCs offer an important model to study human preimplantation biology including the regulation of lineage decisions, responsiveness to signaling activities, and how developmental progression is compromised by genetic and epigenetic errors. Moreover, naïve pluripotent cells would open up the investigation of how pluripotent states are controlled, and could provide practical advantages such as increased clonal efficiency, growth rates and recombination frequencies compared to primed PSCs. There have been exciting advances towards obtaining naïve hPSCs, which have accelerated over the past 3 years. Here, we aim to review recent progress with a focus on 1) hallmarks of naïve hPSCs; 2) approaches to derive naïve hPSCs; and 3) methods to track human pluripotent states and isolate nascent naïve hPSCs. We then highlight current gaps in our understanding of naïve pluripotency, particularly in the context of uses and limitations of naïve hPSCs, and finally suggest areas for future study.

2. A Molecular Framework to Guide Human Pluripotent State Characterization

Assigning molecular criteria to define human pluripotent states is difficult as there is a shortage of information on human embryos due to the ethical and practical restrictions associated with this line of research. Current efforts to derive and characterise naïve hPSCs are based on knowledge gained from mouse studies and this will often be inaccurate due to the differences between species. Recent molecular and phenotypic investigations of human and primate embryos are beginning to provide an important set of waymarks. Based on these initial studies, several characteristics of naïve human pluripotency have been proposed and collectively provide a helpful framework with which to evaluate human cells (**Figure 1** and **Table 1**).[26,32]

2.1. Transcriptional Signatures of Naïve hPSCs Align to Human Preimplantation Epiblast

Global transcriptome analyses of human embryos have delivered a rich resource to extract lineage–specific and developmental stage–specific gene expression profiles, and provide biologically relevant benchmarks for assessing hPSCs.[23,24,28,33] A cohort of genes enriched in human preimplantation epiblast cells is highly expressed in naïve but not in primed hPSCs (Figure 1).[28] There is a considerable range in the expression levels of naïve-associated genes when comparing between various naïve hPSCs lines derived in different conditions, indicating that some conditions are better than others at recapitulating the transcriptional programmes of human epiblast cells.[25–28,34] Additionally, genes such as *KLF4* and *TFCP2L1* are sensitive to perturbation only in naïve hPSCs, suggesting a rewiring in gene regulatory circuitries and dependencies has occurred between naïve and primed pluripotent states.[35] This is further supported by evidence of cell-type-specific enhancer activity and connectivity at genes that include the transcription factors *POU5F1* (encoding OCT4) and *KLF4*; regulatory switches that can be used to readout pluripotent state when hitched to a reporter gene.[36,37]

2.2. Resetting of DNA Methylation Levels and X-Chromosome Activity are Hallmarks of Naïve hPSCs

The epigenome undergoes global remodeling as cells transition from pre- to postimplantation development, and these changes can be used to define pluripotent state. DNA hypomethylation is a property of human and mouse preimplantation embryos, and provides an important hallmark of naïve pluripotency. Transient loss of DNA methylation during early development resets the epigenome, and might also facilitate developmental plasticity before the onset of cell differentiation.[38,39] As for the transcriptional readouts, the extent of DNA hypomethylation varies between different naïve hPSC lines (Table 1). Cells maintained in some naïve-like formulations have global DNA methylomes close to 70%, which is the same level as in primed hPSCs.[27,40] In contrast, naïve hPSCs maintained in other conditions have a global DNA methylation level of ≈30%, which is broadly similar to the human blastocyst at 20–40%.[26,27,34,38,41] Despite this similarity at the global level, there are substantial differences in the distribution of methylated CpG sites across the genomes of naïve hPSCs and human embryos. For example, imprinted gene control regions are methylated in the embryo but are hypomethylated in naïve hPSCs.[26,27] This difference might be due to the prolonged maintenance of naïve hPSCs in conditions that suppress the DNA methylation machinery, as has recently been suggested in mPSCs.[38] Current models in naïve mPSCs propose that this suppressive effect is mediated by continuous inhibition of MEK,[42,43] although there are MEK inhibitor-containing conditions that can maintain naïve-like hPSCs without the loss of global DNA methylation or imprints (Table 1).[27,40] This observation suggests that either the precise level of MEK suppression is crucial or the presence of other factors are additionally involved in controlling DNA methylation in pluripotent cells.

A further epigenetic hallmark to discriminate between naïve and primed hPSCs is the X-chromosome inactivation status in female cells (Figure 1). Until recently, it was assumed that similar mechanisms might govern X-chromosome dosage compensation in mouse and human, however a number of distinct differences have become apparent that are related to the order, timing of events and molecular control of this process. The first difference is the absence of paternal X-chromosome silencing during human preimplantation development.[44] Instead, there appears to be a gradual dampening of expression from both X-chromosomes to halve the transcriptional output in human embryos.[33] The second difference relates to the control of X-chromosome inactivation. In mouse embryos, induction of the long, non-coding RNA (lncRNA) *Xist* triggers X-chromosome silencing in *cis*. In contrast, *XIST* is expressed from both X-chromosomes in human, female cells without triggering inactivation.[33,45,46] It has been proposed that the presence of

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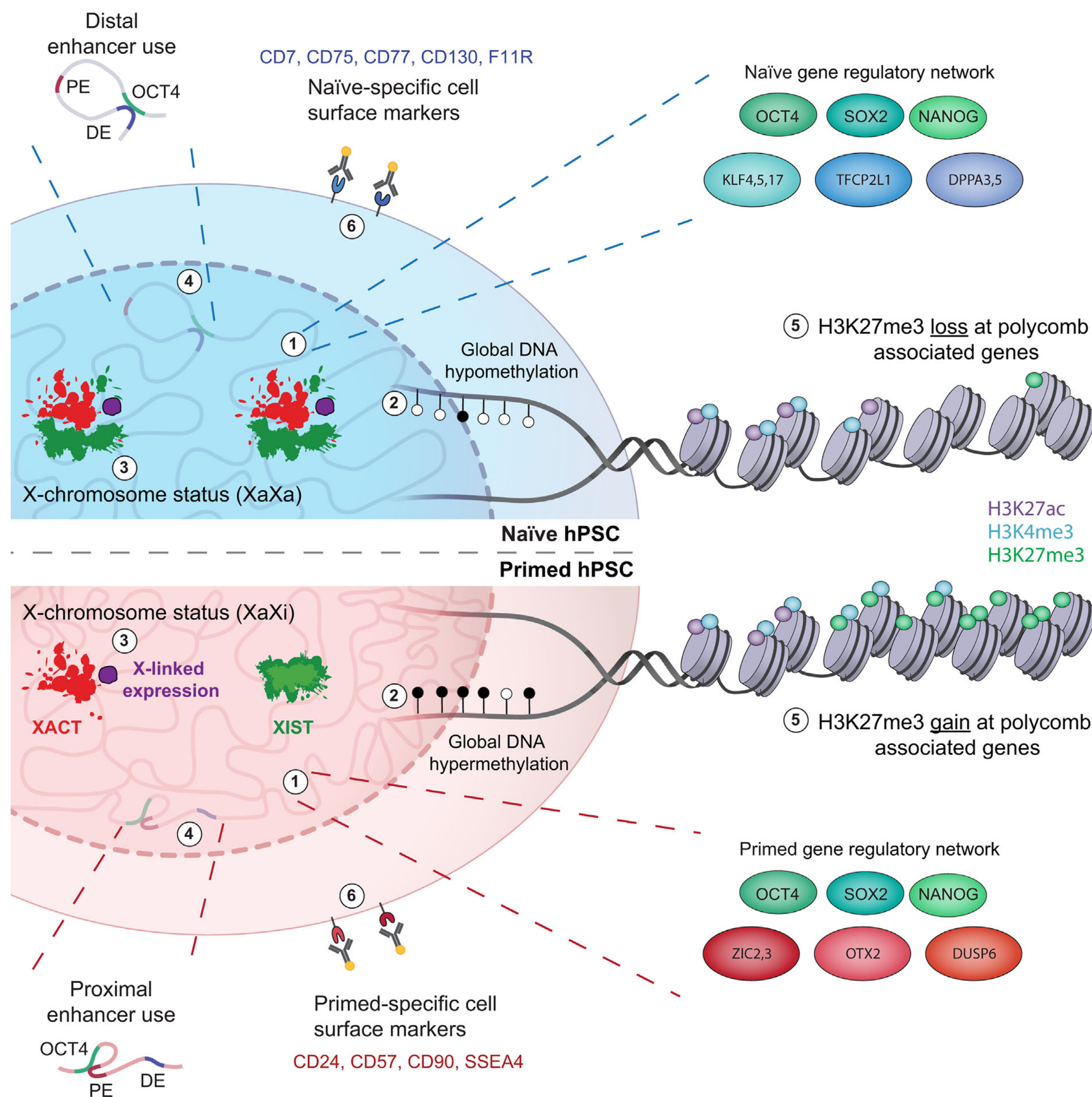

Figure 1. Hallmarks associated with naïve and primed human pluripotent states. (1) The core pluripotency transcription factors OCT4, SOX2, and NANOG are expressed in naïve and primed hPSCs, whereas several other transcription factors are uniquely detected in each cell type. (2) Naïve hPSCs display global DNA hypomethylation compared to primed hPSCs. (3) Naïve hPSCs have two active X-chromosomes in female hPSCs, indicated by biallelic X-linked gene expression including *XIST* and *XACT*, and localised depletion of the repressive histone mark H3K27me3. (4) Naïve hPSCs exhibit preferential activity of the *OCT4* distal enhancer, whereas primed hPSCs use the proximal enhancer. (5) The distribution of H3K27me3 differs between naïve and primed hPSCs, with a reported reduction of H3K27me3 levels in naïve hPSCs at >3000 Polycomb-associated gene promoters. (6) Identified cell-surface markers can be used to distinguish between naïve and primed hPSCs.

a primate-specific lncRNA, *XACT,* acts as an antagonist of *XIST* by preventing *XIST*-mediated X-chromosome silencing.[45] *XACT* may account for some of the differences observed between mouse and human embryos, where the detection of *XIST* in preimplantation human embryos does not indicate X-chromosome inactivation. Conventional primed hPSCs typically have one inactive and one active X-chromosome in female cells, which infers a progression to the post-implantation stage. Strikingly, cells maintained in several naïve hPSC formulations can recapitulate aspects of the preimplantation X-chromosome status, including the presence of two active X-chromosomes, whereas other conditions do not appear to robustly reactivate the silenced X-chromosome (**Table 2**). In conclusion, the X-chromosome status in female cells can be used as an additional hallmark to assess the relative similarity to the preimplantation embryo.

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Table 1. Characteristics of naïve and primed states of pluripotency.

Attribute	Naïve state	Primed State	Presence of naïve state attributes in studies reporting the conversion of primed to naïve hPSCs									
In vivo terminology	Preimplantation epiblast	Postimplantation epiblast	Hanna[47]	Gafni NHSM[40]	Chan 3iL[48]	Ware[50]	Theunissen 5iLAF[37] 5iLA[26]	Takashima t2iL+PKCi[35]	Chen[49]	Duggal[57]	Qin[58]	Guo t2iL+PKCi[34]
Appearance	Domed (✓)	Flat (X)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Single cell survival	High (✓)	Low (X)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Methylation (global CpG)	Low (~30%)	High (~80%)	–	High	–	–	Low	Low	Med	Med	–	Low
H3K27me3 levels	Low at promoters	High at promoters	–	Low	Low	Low	Low	–	Low	–	–	–
OCT4 enhancer	Distal (✓)	Proximal (X)	✓	✓[40] X[37]	–[48] X[37]	✓[50] X[37]	✓[37]	✓	✓	–	–	✓
Metabolism	Oxidative phosphorylation (✓)	Glycolytic (X)	–	–	–	✓	–	✓	–	–	–	–

✓, presence of naïve state attribute; X, absence of naïve state attribute; –, attribute not examined.

3. Developing Culture Conditions to Sustain hPSCs in a Preimplantation-Like State

By benchmarking to the hallmarks of naïve pluripotency and guided by earlier mouse studies, several groups have reported the development of cell culture conditions that are capable of stabilising hPSCs in a naïve-like state.

3.1. Varying Growth Conditions Induce a Spectrum of Human Pluripotent States

Ectopic expression of the pluripotency transcription factors *OCT4*, *KLF4*, and *KLF2* in combination with 2i/LIF generated human cells with several predicted naïve PSC characteristics.[47] However, the withdrawal of transgene expression induced rapid cell differentiation, providing the key demonstration that 2i/LIF alone is insufficient to maintain hPSCs in a naïve state, unlike in mPSCs.

Since then, stable, transgene-free naïve-like hPSCs have been described in multiple studies.[40,48–50] All culture formulations are based on 2i/LIF supplemented with additional components (**Figure 2** and **Table 3**). The resultant cells displayed changes in some of the predicted properties of naïve hPSCs, including increased single-cell cloning efficiency,[40,48] higher rates of homologous recombination[40] and the upregulation of several naïve-associated genes.[48] Interestingly, impaired self-renewal and morphological changes were reported when the naïve-like hPSCs were grown without LIF or with inhibitors of the LIF pathway, confirming the acquisition of LIF dependency to maintain the cell lines.[48,50] In contrast, exogenous LIF is not

Table 2. X-chromosome status associated with naïve and primed hPSCs.

X-chromosome status	Naïve state	Primed state	Hanna[47]	Gafni NHSM[40] RSeT[45,64]	Ware[50]	Theunissen 5iLAF[37,60] 5iLA[26,45]	Takashima t2iL+PKCi[35,45,60,64]	Chen[49]	Guo t2iL+PKCi[34]
X-linked gene expression	Biallelic	Monoallelic from Xa		[45,64]		[26,45,60]	[64] [45,60,64]		
XIST expression	Biallelic	Monoallelic from Xi		[40,64] [45,64]		[45] [45,60] [26,37,45,60]	[45,60,64] [45,60,64] [45,60,64]		
XACT expression	Biallelic	Monoallelic from Xa		[45,64] [45,64]		[45] [45,60]	[45,64] [45,64]		
H3K27me3 X-chromosome foci	Absence	Enrichment over Xi		[40] [64]		over Xa [60]	[35,45,64] [64]		

Xa, active X-chromosome; Xi, inactive X-chromosome.
Note that Chan et al.,[48] Duggal et al.,[57] and Qin et al.[58] did not report X-chromosome status.

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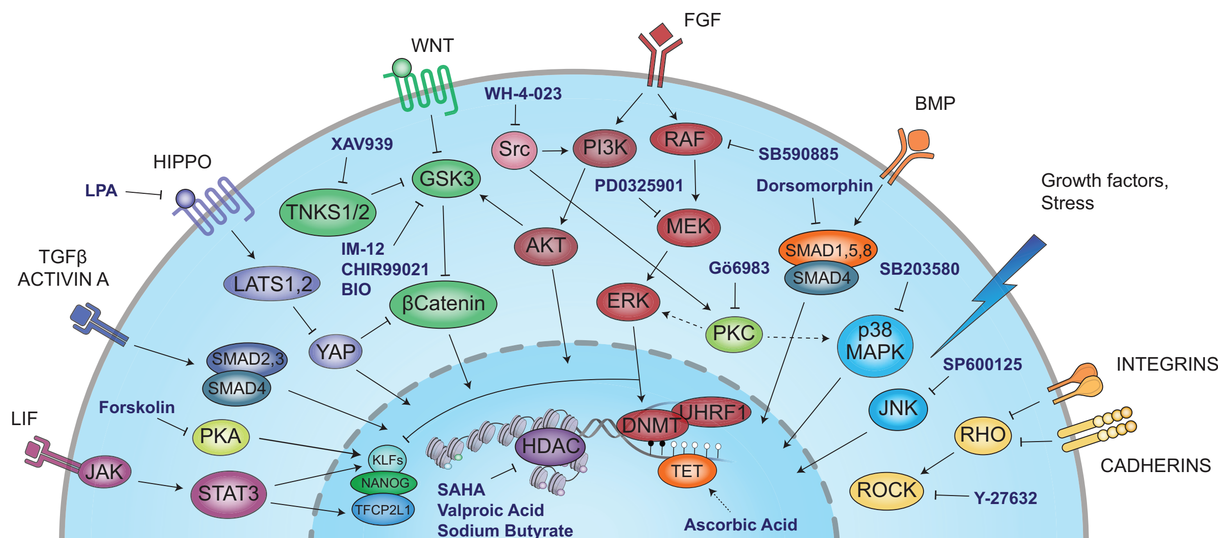

Figure 2. Signaling pathways in naïve and primed human pluripotent states. The different cocktails of inhibitors and growth factors used to induce naïve pluripotency are a likely cause of the reported spectrum of pluripotent states. It is important to note that additional pathways that are not highlighted here are also likely to be involved. Moreover, many of the chemical inhibitors used will non-specifically inhibit additional targets, even extending to components of different signaling pathways. Dashed lines indicate likely indirect effects.

essential for conventional primed hPSC self-renewal.[51,52] Downstream of LIF binding, the activation of STAT3 is both necessary and sufficient for the maintenance of mPSC self-renewal,[53] partly through maintaining *Klf4* and *Tfcp2l1* expression,[54–56] and initial evidence points to a similar role in naïve-like hPSCs.[48] Furthermore, the reinforcement of STAT3 in the presence of 2i/LIF can facilitate primed to naïve-like reprogramming of hPSCs, and in this way, we can begin to understand the crosstalk between different signaling pathways (Figure 2 and Table 3).[49,50] To identify additional factors that might stabilise naïve hPSCs, other media formulations have been developed that include inhibitors of PKA and HIPPO signaling[57–59] (Table 3). These cells have not been fully characterised, particularly for epigenetic hallmarks, and it will be interesting to see how they compare against the others (Table 1).

Gene network analyses and transcriptional comparisons on a subset of cell lines show that there is a substantial variability between the putative naïve hPSC cultures described above, resulting in a spectrum of cell phenotypes that is likely to be driven by their different growth conditions.[25] For many of these cell lines, the shift in transcriptional profiles towards a preimplantation state is rather minor,[25,28,40,48,50] and in some instances might be driven by the induction of a small cohort of metabolic genes rather than robust changes in regulatory circuitry.[25] There is also conflicting data for some of the above growth conditions with evidence that the cells are unable to activate a naïve-specific *OCT4* reporter line, thereby unfulfilling this molecular hallmark (Table 1).[37] Additionally, for some of the conditions, female cells were reported to have reactivated their previously silenced X-chromosome, which would be a feature of naïve pluripotency (Table 2).[40,50] The data presented showed an absence of *XIST* and H3K27me3 foci in the naïve-like hPSCs (Table 2), which at the time was thought to indicate the presence of two active X-chromosomes. However, we now know that *XIST* is expressed from active X-chromosomes in human blastocysts and naïve hPSCs,[45,60] and that the absence of *XIST* and H3K27me3 is probably due to a phenomenon called 'erosion' that occurs in primed cells leading to partial X-chromosome reactivation.[61,62] Based on these recent findings, it is therefore important to define X-chromosome status in hPSCs based on chromosome-specific expression of *XIST*, *XACT* and other genes on the X-chromosome that do not escape silencing by erosion. Taken together, although this suite of cell lines are fascinating additions to the cellular toolbox, they do not align to our current expectations of naïve or primed cells, and future studies are required to further define their cell state and functional properties.

3.2. Identified Culture Conditions Capture Naïve hPSCs With Preimplantation Embryo Hallmarks

Naïve hPSCs maintained in two further media formulations are classified as being similar to human blastocysts based on several molecular hallmarks. In the first, a screening approach identified combinations of growth factors and small molecules (termed 5i/L/A) that can induce and maintain naïve hPSCs (Table 3).[26,37] Naïve hPSCs in these conditions exhibit one of the closest similarities to the transcriptional and epigenetic profiles of the human and primate preimplantation embryos.[22,25–28,60] There are reported drawbacks associated with this method, however, as long-term propagation in this formulation leads to the irreversible loss of DNA methylation at the majority of imprinted loci and to the misexpression of a subset of imprinted genes.[26,27] Chromosomal instability is also frequently reported.[26,63] In the second culture formulation, naïve hPSCs are stabilised and propagated in a cocktail of low-dose 2i, LIF and

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Table 3. Media compositions associated with the induction of naïve hPSCs.

Chemical inhibitor, growth factor	Target effect	Hanna[47]	Gafni NHSM[40]	Chan 3iL[48]	Ware[50]	Theunissen 5iL/A(F)[26,37]	Takashima t2iL+PKCi[35]	Chen TL2i[49]	Duggal[57]	Qin[58]	Guo t2iL+PKCi[34]
LIF	LIF signaling	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
PD0325901	MEK inhibition	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CHIR99021	GSK3 inhibition	✓	✓		✓		✓	✓	✓	✓	✓
IM-12	GSK3 inhibition					✓					
BIO	GSK3 inhibition			✓							
Gö6983	PKC inhibition		✓				✓				✓
Y-27632	ROCK inhibition		✓			✓					✓
WH-4-023	SRC inhibition					✓					
SB590885	RAF inhibition					✓					
SP600125	JNK inhibition		✓								
SB203580	p38/MAPK inhibition		✓								
TGFβ	TGFβ signaling			✓							
Activin A	TGFβ signaling					✓					
FGF	FGF signaling			✓	✓	✓/X			✓		
SAHA	HDAC inhibition				✓*						
Sodium Butyrate	HDAC inhibition				✓*						✓*¥
Valproic Acid	HDAC inhibition										✓*¥
Dorsomorphin	BMP inhibition			✓							
Forskolin	PKA inhibition								✓	✓	
Ascorbic Acid	Demethylation								✓		
Lysophosphatidic acid	HIPPO inhibition									✓	
Base media		(1)	(2)	(3)	(4)	(1)	(1)	(1) (4)¥	(4)	(1) (3)	(1)
O_2 level		20%	20 or 5%	–	5%	5%	–	5%	5%	–	5%
Transgenes		*OCT4* *KLF2* *KLF4*					*NANOG** *KLF2**	*STAT3**			

✓, addition to media; *, short-term induction or addition to the culture media; ¥, choice between two components; ✓/X, optional addition of a component; –, not documented.
Base media: (1) DMEM/F12: Neurobasal, N2B27; (2) KnockOut DMEM, N2B27; (3) TeSR1 (4) DMEM, 20% KSR.

a PKC inhibitor (t2iL + PKCi) (Table 3).[35] This set of factors can maintain naïve hPSCs that have been generated by *NANOG* and *KLF2*–induced, *KLF4*–induced, or *OCT4*, *KLF4*, *SOX2* and *MYC*-induced reprogramming,[35,63,64] by transgene-free chemical resetting of primed hPSCs[34] and captured directly from preimplantation human blastocysts.[41] Naïve hPSCs maintained in t2iL + PKCi exhibit many of the similarities to 5i/L/A naïve hPSCs including a globally hypomethylated genome (Table 1),[27,35,41,64] a close transcriptional alignment with preimplantation human and primate epiblast cells,[22,25,28,64] and, in a proportion of cells, *XIST*-positive active X-chromosomes (Table 2).[45,64] Many of the drawbacks are also shared with the 5i/L/A cells.[27,41] Curiously, a subset of naïve hPSC lines grown in RSeT media, a commercial derivative of 'naive human stem cell medium',[40] showed transcriptional similarly to t2iL + PKCi cells, whereas other lines in the same RSeT conditions aligned more closely to primed hPSCs.[64]

Taken together, recent reports on naïve hPSCs have made great advances in capturing cells that mimic many aspects of human preimplantation epiblast cells. The resultant cell lines provide a spectrum of cell states, with their associated advantages and disadvantages, and further progress in optimising growth conditions is expected to deliver more robust cell types.

4. Developing Methods to Identify and Isolate Naïve hPSCs

Monitoring the changes in cell state and the emergence of naïve PSC populations are essential to optimise current protocols, and will also provide valuable insights into the underlying molecular mechanisms of human pluripotency. One of the predominant approaches to generate naïve hPSCs is by reprogramming primed hPSCs. However, the efficiency of this method is low and variable between protocols, and often generates a heterogeneous mixture of cells. Several of the early reports that document deriving naïve hPSCs relied on obtaining homogeneous populations through the continued passaging of cells under

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naïve hPSC culture conditions. This bulk population approach is dependent on the fully reprogrammed naïve PSCs having a survival advantage when cultured in naïve conditions. Furthermore, this method may not be applicable where culture conditions permit the survival of primed and differentiated cell types, nor does it easily allow characterisation of the process due to the high heterogeneity at intermediate stages of reprogramming. Since then, several alternative approaches have been devised to enrich for naïve PSC populations (**Figure 3**).

4.1. Reporter Cell Lines can Cut Through Reprogramming Heterogeneity

A key feature of naïve mPSCs and preimplantation mouse epiblast cells is the preferential use of the *Oct4* distal enhancer, whereas primed EpiSCs and postimplantation epiblast cells utilise a proximal enhancer.[16,65,66] To capitalise on this regulatory switch defined in the mouse, several groups have developed a reporter system to read out the activity of endogenous human *OCT4* expression coupled to *GFP* in cell lines containing a deletion of the proximal enhancer (OCT4-ΔPE-GFP). In a naive state, hPSCs activate alternative enhancers, presumably including the conserved distal enhancer, to drive *OCT4–GFP* expression,[37,40] which can be detected using flow cytometry. An alternative reporter system termed EOS-*GFP* has also been used to identify naïve hPSCs.[35] EOS-*GFP* expression is driven from a mouse Early Transposon promoter combined with mouse *Oct4* enhancer elements that contain OCT4 and SOX2 binding sites.[67] EOS-*GFP* expression is gradually silenced in primed hPSCs, but reactivated upon reprogramming to a naïve state, thereby providing a straightforward indication of pluripotent cell type. Adopting a similar strategy, naïve-like hPSCs can be identified using a reporter for the expression of the retrovirus *HERVH*, linked to a *GFP* transgene.[68] Primed hPSCs with high *HERVH-GFP* expression exhibit a reduction in gene expression of primed-associated genes. When isolated using cell sorting, *HERVH-GFP*-high cells adopt a naïve-like morphology compared to *HERVH-GFP*-low/negative cells. Nonetheless, repeated re-sorting is required to maintain a homogeneous population of naïve-like hPSCs.[69] The cell phenotype is also unclear given that endogenous *HERVH* transcription is associated with primed, and not with naïve, hPSCs.[26]

Although reporter systems can provide a robust and simple readout of cell state, they all have several caveats. First, there is a requirement to genetically modify each cell line. Second, the timing of reporter gene induction and the fidelity of the expression are not always clear, and may not necessarily be linked to cell state. Third, although it is assumed that the human *OCT4* enhancers are functionally similar to the mouse, there has not been an assessment of which regulatory elements are

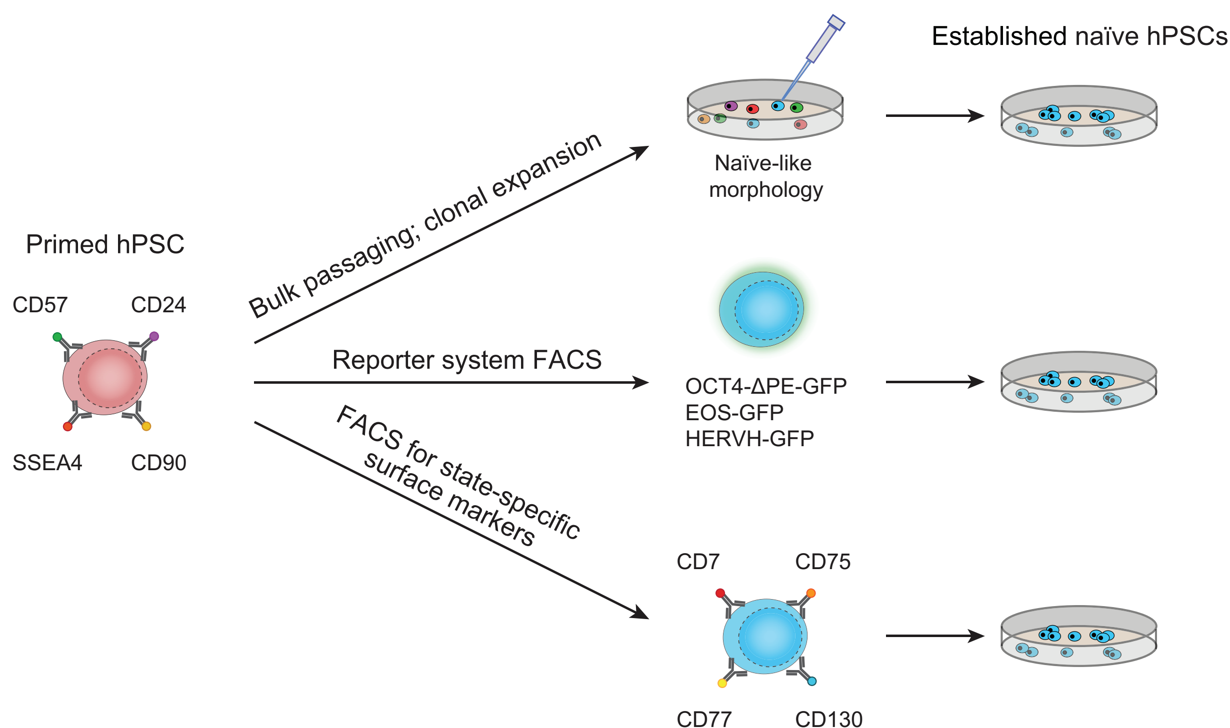

Figure 3. Approaches to isolate naïve hPSCs during primed to naïve reprogramming. Primed to naïve reprogramming can be inefficient, and often generate substantial cell heterogeneity during the process. Strategies to isolate and derive defined populations of naïve hPSCs include: (1) Bulk passaging and progressive selection, or clonal expansion of individual colonies that exhibit a domed naïve-like morphology. (2) Reporter systems coupled to the expression of fluorescent proteins provide a read out of characteristics that are associated with naïve pluripotency. (3) Characterisation of the cell-surface proteins expressed on naïve and primed hPSCs has identified state-specific markers. Using antibodies specific to these markers enables the isolation of naïve hPSCs using fluorescence-activated cell sorting (FACS).

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preferentially used to drive *OCT4* expression in naïve hPSCs and human blastocysts. New reporter-based designs will require a better understanding of transcriptional dynamics and cell-type-specific enhancer activity and interactivity in human development.[70] Promising candidates could include a *KLF17* reporter line as this gene is activated at a late stage of primed to naïve hPSC reprogramming,[71] or strategies to readout biallelic *XIST* expression as this property is present in all preimplantation human epiblast cells and in a subset of naïve hPSCs.[45,46] The recent genetic modification of human preimplantation embryos also opens up the possibility of experimentally testing the fidelity of reporter constructs in human blastocysts.[72–76]

4.2. Cell-Surface Proteins can Distinguish Between Human Pluripotent States

Transgene-free methods to isolate naïve PSCs from a heterogeneous population would offer advantages over reporter-based systems and manual isolation approaches. Until recently, there were no known cell-surface markers that could define naïve hPSCs. Three reports have each identified individual cell-surface markers that are detected on primed, but not on naïve, hPSCs. The first study showed that CD24 expression is higher in primed hPSCs compared to naïve-like cells. Naïve-like hPSCs were isolated by flow sorting using the pan-hPSC antigen TRA-1-60 in combination with low expression of CD24.[77] Notably, greater than ten passages under naïve culture conditions were required before the CD24-low population arose for prospective cell isolation, thereby limiting the ability to interrogate cells during the reprogramming process. A second study examined the expression of SSEA-4, a well-characterised marker of primed hPSCs.[78] Pastor et al. reported heterogeneous expression of SSEA-4 on naïve hPSCs cultured using 5i/L/A conditions.[27] When comparing transcriptional profiles of SSEA-4-positive and SSEA-4-negative populations, preimplantation epiblast-specific genes were more highly expressed in the SSEA-4-negative fraction. In light of this result, it would be interesting to re-evaluate the expression pattern of SSEA-4 in the human blastocyst to see whether epiblast cells show variable levels.[78] A third report identified four antibodies (raised against GPR64, CDH3, NLGN4X and PCDH1) that showed higher reactivity against primed compared to 5i/L/FA naïve hPSCs.[79] Collectively, these studies provide helpful markers to characterise established naïve hPSCs lines. However, the lack of a known positive marker for human naïve cells limited the ability to unambiguously detect naïve hPSCs within a mixed population including during reprogramming.

4.3. A Panel of Cell-Surface Proteins can Detect Nascent Naïve hPSCs During Reprogramming

Given the absence of robust methods to prospectively identify naïve hPSCs without genetic modification, a study screened 486 cell-surface antibodies with the aim of identifying state-specific cell-surface antigens.[71] A set of cell-surface markers were detected uniquely in each cell state, and comprising of 58 primed and 8 naïve-specific markers. A cohort of markers was validated using several primed and naïve hPSCs lines. Moreover, the presence of these markers was examined by immunofluorescent microscopy in human blastocysts. Interestingly, four of the five naïve-specific markers were detected, whereas all primed-specific markers were absent, which is consistent with the expected difference in developmental staging. Notably, however, some of the naïve markers localised to epiblast and extraembryonic cells of the embryo, indicating that the proteins are not necessarily restricted to pluripotent cells at this phase of development.

A multiplexed panel of validated antibodies capable of discriminating between naïve and primed hPSCs was assembled. This panel included four naïve-specific antibodies (CD7, CD77, CD75 and CD130) and three primed-specific antibodies (CD24, CD57 and CD90). The panel could distinguish between naïve and primed cells, track the dynamics of naïve – primed interconversion, and isolate emerging naïve hPSCs from a heterogeneous cell population during reprogramming. Nascent naïve cells that express all four naïve markers and lack all primed markers could be isolated 10 days into the resetting process. This is an earlier time point than permitted by current, alternative isolation strategies, and enabled the first investigation of intermediate cell types during reprogramming. Interestingly, the proportion of nascent hPSCs on day 10 was considerably higher when using 5i/L/A (≈14%), compared to ≈1% seen using t2iL + PKCi conditions, suggesting that the reprogramming process is amenable to further improvements. Flow-sorted hPSCs formed compact and domed colonies upon plating in naïve hPSC conditions, thereby providing a straightforward approach to obtaining naïve hPSC lines.

More recently, a systematic comparison of naïve hPSC lines grown under different conditions revealed a range of cell-surface marker profiles, thereby moving towards developing immunophenotyping as diagnostic for naïve cell types.[63,80] Notably, out of a set of six antibodies used, only one could positively identify naïve hPSCs, and there was increased fluorescent intensity in naïve hPSCs compared with primed cells. The antibody was raised against the cell-adhesion molecule F11R, and it will be interesting to see if this expression difference could underlie the distinct morphological differences between pluripotent cell types.

There are a number of advantages to using cell-surface antibodies compared to alternative isolation strategies. First, live naïve cells can be detected without the need for cell line modification. Second, cell-surface markers provide a quantitative and defined endpoint, allowing the efficiency of different resetting protocols to be compared. Third, naïve cells can be isolated at an earlier time point than permitted by alternative strategies. For the first time, this enables the timing and order of molecular changes that occur during resetting to be examined. Cell-surface markers also have several drawbacks. First, the approach is limited by antibody availability and quality. Second, there are some experimental contexts when cell-surface markers are less applicable, such as during the derivation of naïve hPSCs directly from embryos. Third, the markers themselves do not necessarily have a functional role in human pluripotency. Further work is needed to examine this, although several naïve-specific markers such as CD75 and CD77 are uncharacterised glycoproteins and will not be straightforward to study. Development of neutralising antibodies against their epitopes would provide one approach to investigate

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their function. It may also be possible to use antibody-mediated immunoprecipitation in combination with mass spectrometry to identify proteins that interact with the glycoproteins, which might provide insight into their target pathways. Another line of future research could focus on trying to identify a single, cell-surface marker that can positively discriminate naïve hPSCs. This search might require generating new antibodies potentially guided by proteomic studies to catalogue shared and state-specific cell-surface proteins.

5. Current Applications and Challenges in Naïve hPSC Biology

The capture of naïve hPSCs has opened up new research directions by enabling the investigation and manipulation of human preimplantation-like cells. Exciting examples so far include the functional testing of preimplantation transcription factors,[35] the elucidation of processes that control X-chromosome dosage compensation,[45,60] the connection between transcriptional changes and tissue architecture during pluripotent state transition,[81] and chromosome organisation in the context of gene regulatory interactions.[36] Naïve hPSCs also provide a unique cellular model to examine the regulation of transposable elements, which are highly species- and developmental-stage specific.[26,82] The next few years will continue to yield much needed insights into the regulation of pluripotent states, and naïve hPSCs will provide a valuable and tractable model to interrogate the associated mechanisms.

5.1. Uncertainties About Naïve hPSCs as a Starting Point for Cell Differentiation

It is also important to discuss the limitations of naïve hPSCs. In our opinion, for example, there are few obvious advantages in using naïve hPSCs as the starting point for most cell differentiation experiments, especially when primed hPSCs differentiate effectively and have been tailored by robust protocols. Recent studies have shown that 5i/L/A and t2iL + PKCi naïve hPSCs differentiate poorly into somatic tissues.[63,83] This is perhaps not unexpected, given that naïve hPSCs need to first break the strong signaling inhibition that holds them in a naïve state, before transitioning towards a primed state and further differentiation. Similar events may also occur during embryogenesis, where preimplantation epiblast cells transition to a postimplantation state before the onset of gastrulation.

As a counterpoint, proposals have been made that naïve hPSCs could offer a uniform and less restricted starting point for differentiation experiments, potentially due to the erasure of the lineage-biases or X-chromosome variability that have been detected in primed cells. At the moment, however, there is little evidence to support this proposition. Indeed, the use of current naïve hPSCs as a starting point would come at the cost of misregulated imprinted genes and the increased probably of acquiring genetic changes, which would introduce substantial variability into the differentiation outcomes. Potentially cell states and growth conditions could be identified that reduce the intrinsic biases of primed hPSC lines without transitioning fully to a naïve state and the adoption of associated instabilities. For example, a recent study described the derivation of 'Advanced' mPSCs, which exhibit a combination of properties that is distinct from naïve and primed cells including DNA hypermethylation and high developmental potency in chimeras.[84]

There are two specific examples in which naïve hPSCs might offer an advantageous starting point for differentiation experiments. The first is for germ cell differentiation. Mouse PSCs that have been induced transiently to a naïve state undergo effective germ cell differentiation.[85] Initial indications using hPSCs look promising but are too early to tell.[86,87] The second is whether naïve hPSCs can form extraembryonic cell types. This has not been examined directly, although curiously, the expression of several trophectoderm-associated genes are detected in naïve hPSCs and not in primed hPSCs, and on a global scale some naïve hPSC lines show high transcriptional overlap with morula stages of development.[26] Could naïve hPSCs harbour a transcriptional 'memory' of cells before epiblast segregation and retain some extraembryonic differentiation capacity? As lineage segregation in the human embryo is specified at a relatively late stage of development[33] then this scenario is possible and further experiments are warranted. Particularly useful in this context are on-going efforts to project naïve hPSCs on to a developmental map to see how closely the cells grown in various conditions align to embryo stage and cell type.[28]

5.2. Gene Regulatory Networks and Signaling Pathways are Poorly Understood in Naïve hPSCs

Other major knowledge gaps include the definition of gene regulatory networks that govern naïve pluripotency in humans, and an understanding of how the networks reform upon transition to a primed pluripotent state. Differences in enhancer rewiring have been detected between naïve and primed hPSCs, and often correspond to a transcriptional change for the set of genes examined.[36] Expanding this by integrating with data sets of chromatin accessibility, transcription factor occupancy, and high-resolution DNA interactions will reveal a more precise view of regulatory control in human naïve pluripotency. This information could also feed into the design of a new generation of sensitive and accurate reporter systems for human pluripotent cells.

In addition, little is known about the signaling requirements of naïve hPSCs. The signaling pathways targeted for inhibition and activation, and their downstream effects, are largely unexplored. Naïve hPSC growth conditions will be optimised as signaling pathways are tested. Progress in this area may benefit from moving away from expectations based on mouse, and instead use unbiased approaches, such as chemical and genetic screens in combination with evidence of signal pathway activity from proteomics and phosphoproteomics. Lastly, a consensus on how to phenotypically define pluripotent states needs to be reached, and a recent article has argued that the reliance on a narrow set of readouts, such as the OCT4-ΔPE-GFP reporter, should be expanded to include additional state-specific markers.[21] Collectively, these studies will also raise interesting new hypotheses about how signaling pathways and gene regulatory networks operate in the early stages of human

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embryogenesis, which can be tested experimentally in blastocysts to provide new insights into human development.

6. Conclusions and Outlook

A dozen or so reports have now described growth conditions that can lead to human cells acquiring characteristics of the preimplantation-stage embryo. These publications have resulted in a spectrum of hPSCs with variable properties that have collectively, and perhaps confusingly, been termed 'naïve'. Efforts to reach consensus on how to define bona fide naïve hPSCs using measurable and unambiguous properties are underway and will hopefully be adopted widely by the field.

Carefully defining hPSC states allows the informed selection of the most appropriate set of methods for cell growth and differentiation, and is important for interpreting and replicating experimental outcomes. A range in cell phenotypes could be advantageous to researchers as certain growth conditions might be better at mimicking preimplantation biology, and perhaps others for germ cell differentiation. The composition of each media formulation will be further refined and optimised, and researchers and reagent suppliers will need to be responsive to the changing conditions. In this regard, defining the activity and necessity of signaling pathways in naïve hPSCs is a priority for the field. An emerging picture is that achieving the optimum level of MEK inhibition is critical for propagating stable naïve mPSCs, and it will be interesting to see if there are similar requirements in human cells and how this might crosstalk with other signaling pathways.

Advances made in understanding naïve hPSCs will have important significance in a range of biological contexts. For example, studying how the transitions between naïve and primed pluripotent states are controlled will provide new insights into defining how cells change state in other systems, including concepts such as whether there are multiple routes of cell state change, and exploring the nature of intermediate transition phases. Another example includes characterising the epigenomic events that occur upon human pluripotent state transitions, such as X-chromosome reactivation and global DNA hypomethylation. These major events occur in other cellular transitions, particularly in the onset of disease, and may share similar pathways and targets.

Abbreviations

2i, dual MEK and GSK3 inhibition; 5i/L/A, MEK, GSK3, ROCK, SRC, and RAF inhibitors supplemented with LIF and Activin A; EpiSCs, epiblast stem cells; FGF2, fibroblast growth factor 2; hiPSCs, human induced pluripotent stem cells; HNSM, naïve human stem cell medium; hPSCs, human pluripotent stem cells; LIF, leukaemia inhibitory factor; lncRNA, long non coding RNA; mPSCs, mouse pluripotent stem cells; PSC, pluripotent stem cell; ROCK, Rho-associated coiled-coil kinases; t2iL + PKCi, titrated 2i with LIF and PKC inhibitor; TGF, transforming growth factor.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

development, embryonic stem cells, epigenetics, pluripotency, reprogramming, X-chromosome inactivation

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