REVIEW

The transcriptional regulation of pluripotency

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The defining features of embryonic stem cells (ESCs) are their self-renewing and pluripotent capacities. Indeed, the ability to give rise into all cell types within the organism not only allows ESCs to function as an ideal *in vitro* tool to study embryonic development, but also offers great therapeutic potential within the field of regenerative medicine. However, it is also this same remarkable developmental plasticity that makes the efficient control of ESC differentiation into the desired cell type very difficult. Therefore, in order to harness ESCs for clinical applications, a detailed understanding of the molecular and cellular mechanisms controlling ESC pluripotency and lineage commitment is necessary. In this respect, through a variety of transcriptomic approaches, ESC pluripotency has been found to be regulated by a system of ESC-associated transcription factors; and the external signalling environment also acts as a key factor in modulating the ESC transcriptome. Here in this review, we summarize our current understanding of the transcriptional regulatory network in ESCs, discuss how the control of various signalling pathways could influence pluripotency, and provide a future outlook of ESC research.

Keywords: embryonic stem cells; pluripotency; ESCs; EpiSCs; transcriptional regulation; gene expression; signaling pathways; naïve; primed

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Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of pre-implantation embryos [1–3]; and the two defining features of ESCs are their ability to self-renew, as well as give rise to all cell lineages of the organism. This unique feature of ESCs to form the various tissue types, termed ‘pluripotency’, besides enabling

the *in vitro* study of early mammalian development, has also facilitated the advancement of regenerative medicine, whereby ESCs could be used to create clinically relevant cell types for the replacement of worn-out tissue. However, it is this same remarkable developmental plasticity of ESCs which poses a major challenge towards the efficient control of ESC differentiation into the desired lineage. Therefore, to better understand mammalian development, as well as to exploit the tremendous therapeutic potential of ESCs, it is necessary to identify the molecular mechanisms governing a pluripotent or differentiated ESC fate. Here in this review, we will summarize the current progress towards understanding the ESC transcriptional regulatory network, and also discuss how modulation of the various signalling pathways in ESCs could influence pluripotency.

Dissecting the ESC transcriptional regulatory network

The core ESC pluripotency factors: Oct4, Sox2 and

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Abbreviations: 2C (2-cell); 2i (two inhibitor); BMP4 (bone morphogenic protein 4); ChIP (chromatin immunoprecipitation); ChIP-seq (chromatin immunoprecipitation and sequencing); ESCs (embryonic stem cells); EpiSCs (Epiblast-derived stem cells); Fgf4 (fibroblast growth factor 4); hESCs (human embryonic stem cells); ICM (inner cell mass); iPSCs (induced pluripotent stem cells); LIF (leukemia inhibitory factor); lincRNA (large intergenic non-coding RNA); mESCs (mouse embryonic stem cells); miRNA (micro RNA); ncRNA (non-coding RNA); PcG (Polycomb group proteins); PGCs (primordial germ cells); POU (Pit-Oct-Unc); RNAi (RNA interference); RNA-seq (RNA sequencing); SCF (stem cell factor); siRNA (short interfering RNA); XaXa (double X-chromosome active); XaXi (single X-chromosome inactivated)

Nanog

In ESCs, the pluripotent state is mainly regulated by the core transcription factor trio of Oct4, Sox2 and Nanog [4].

Oct4 (encoded by the *Pou5f1* gene), belongs to the Pit-Oct-Unc (POU) family of homeodomain proteins, and is exclusively expressed within the totipotent mouse blastomeres, pluripotent epiblast as well as primordial germ cells (PGCs) [5, 6]. Importantly, Oct4 plays a critical role in the establishment and maintenance of pluripotency, as *Pou5f1*-null embryos do not form a pluripotent ICM, but rather, differentiate into trophectodermal tissue [7]. Similarly, Oct4 is also critical for maintaining mouse ESCs (mESCs) in an undifferentiated state and has to be tightly regulated. Depletion of Oct4 mRNA by 50% is sufficient to result in the formation of trophectoderm cells, while Oct4 overexpression by 50% will promote mesodermal and endodermal differentiation [8].

Sox2, which contains the high-mobility group box DNA-binding domain, is expressed within the ICM and extraembryonic ectoderm of pre-implantation blastocysts [9]. Like Oct4 knockout mice, *Sox2*-null blastocysts fail to form a pluripotent ICM [9], and mESCs deficient in Sox2 differentiated primarily into trophectoderm [10]. This similarity of phenotypes produced by Sox2 and Oct4 loss is attributed to the synergistic action of Oct4/Sox2 in the regulation of various ESC-specific genes [11–16], including themselves [17–19]. Indeed, ectopic Oct4 expression was found to be sufficient in rescuing the differentiation phenotype of *Sox2*^{-/-} mESCs [10].

Nanog, the third member of the core ESC transcription factors, was discovered through a screen for pluripotency factors that could sustain mESC self-renewal in the absence of leukemia inhibitor factor (LIF) [20, 21]. Although *Nanog*-null pre-implantation embryos do not possess a pluripotent ICM [21, 22], *Nanog*^{-/-} mESCs could be established through the *in vitro* disruption of both endogenous *Nanog* alleles [21, 23]. Importantly, these *Nanog*-null mESCs although prone to differentiation, could still be kept pluripotent [21, 23]. Therefore, it is believed that Nanog, while necessary for the acquisition of pluripotency, is dispensable once pluripotency is achieved [23].

Given the important roles of the core ESC transcription factors in establishing a pluripotent stem cell fate, chromatin immunoprecipitation (ChIP) technologies have been used to map the genomic-binding sites of these core ESC factors in mESCs and hESCs. Together, these studies found extensive Oct4, Sox2 and Nanog co-binding at numerous active, as well as silent genomic target sites [24, 25]. Together, it is proposed that the core ESC transcription factors serve to establish a pluripotent

state by: (1) activating the expression of other pluripotency-associated factors while simultaneously repressing lineage-specific genes, and (2) by activating their own gene expression and that of each other [4]. Importantly, this model may account for how ESCs can sustain self-renewal and pluripotency, while still remain poised for differentiation.

The expanded ESC pluripotency network

To uncover additional novel ESC regulators, one method is to perform RNA interference (RNAi)-mediated gene knockdown and to observe for any loss of pluripotency. Indeed, large-scale RNAi knockdown studies have led to the discovery of important mESC factors such as *Esrrb*, *Tbx3* and *Tcl1* [26], as well as the chromatin regulators *Tip60*-*p400* [27] and *SetDB1* [28]. Similarly, unbiased genome-wide siRNA screens were able to identify *Cnot3* and *Trim28* [29], *Paf1C* [30] and the mediator and cohesin complex [31] as important mESC transcriptional cofactors. Extending this approach into hESCs, Chia *et al.* [32] used a similar genome-wide siRNA screening to identify components of the INO80 chromatin remodeling complex, the mediator and TAF transcriptional regulatory complexes, and the COP9 signalosomes as important hESC factors. Importantly, the PRDM14 transcription factor was found to be an essential factor for hESCs, but not in mESCs, hence highlighting that critical species-specific differences exist between mouse and human ESCs (hESCs) [32].

Determining the protein-binding partners of known pluripotency factors is another method of identifying novel ESC regulators. Through the coupling of affinity purification methods with mass spectrometry technology, numerous co-binding proteins of the core pluripotency transcription factors have been identified [33–40]. Taken together, these studies reveal an extensive protein-protein interaction network which includes other ESC transcription regulators, chromatin remodeling and modifying factors, DNA methyltransferases and Polycomb group proteins (PcG). This therefore suggests that the core ESC factors may regulate gene expression through the modulation of chromatin states. Importantly, this large and intricate network of protein interactions could suggest how small changes in the levels of core ESC factors, like Oct4 or Sox2, are sufficient to perturb the ESC self-renewal programme to trigger differentiation [8, 41].

Using ChIP with massively parallel DNA sequencing (ChIP-seq) [42], or *in vivo* biotinylated ChIP with DNA microarray [43], two independent groups examined the context in which these additional pluripotency factors could play in the specification of an ESC identity. Together, these studies observed the binding of multiple

transcription factors onto a similar genomic region; and importantly, these factors could also be grouped into either an Oct4- or Myc-centric module based on their genomic targets [43]. These data thus indicate that within ESCs, the Myc-cluster appears to function independently from the core pluripotency network [29, 42, 43].

The role of Myc in ESC transcriptional regulation

The Myc module which consists of c-Myc, n-Myc, Rex1, Zfx and E2f1, is known to be involved in self-renewal and cell metabolism [42-44]. Although approximately one-third of all active ESC genes are bound by both c-Myc and the core ESC pluripotency factors [45], the functions of these two modules in gene regulation appear to differ. For instance, Oct4, Sox2 and Nanog in concert with the Mediator complex are able to recruit RNA polymerase II (RNA Pol II) for gene transcription [31], while c-Myc is believed to control the transcriptional pause release of RNA Poly II via the p-TEFb cyclin-dependant kinase [45]. It is thus believed that the core ESC factors will select ESC genes for expression through the recruitment of RNA Pol II, while c-Myc functions to control gene expression through the release of transcriptional pause [4]. Importantly, it should also be highlighted that initial reports of cancer cells possessing an ESC-like transcriptional program [46, 47] were later attributed to be a consequence of Myc-module activity, as opposed to the core ESC factors [48].

Recent data by Lin *et al.* [49] and Nie *et al.* [50] suggest that rather than activating new genes, Myc acts only to amplify the existing transcriptional output of active genes. By increasing the levels of Myc, both groups found the loading of Myc proteins onto the promoters of active genes, but not for promoters of silent genes. This therefore indicates that Myc is unable to initiate *de novo* gene activation. Indeed, higher levels of p-TEFb occupancy with increased levels of elongation-associated RNA Polymerase II phosphorylation were found at Myc-bound sites [49], consistent with the previously proposed idea that c-Myc controls transcriptional pause release [45]. Taken together, this ability to amplify existing active gene transcription may in part suggest how the addition of c-Myc during reprogramming is able to increase the efficacy of induced pluripotent stem cell (iPSC) formation [51].

Transcriptional control of non-coding RNAs in ESCs

MicroRNAs (miRNAs), which post-transcriptionally regulate mRNA levels, are important for proper ESC function [52, 53]. Importantly through ChIP-seq, Marson *et al.* [54] found the binding of Oct4, Sox2, Nanog and Tcf3 transcription factors at ESC-related miRNA genes,

as well as transcriptionally silent PcG-occupied tissue-specific miRNAs, suggesting that the core ESC transcriptional network is able to regulate miRNA expression. A notable example of these inactive miRNAs includes *let-7*, which is known to target c-Myc and the pluripotency factors Sall4 and Lin28 [55]. Interestingly, the ESC-related *miRNA-302/367* cluster which is also regulated by the ESC transcriptional circuit [54], is able to reprogramme fibroblasts into iPSCs without the need for additional protein factors [56].

Large intergenic non-coding RNAs (lincRNAs) expressed in mESCs are also known to be the targets of several pluripotency-associated transcription factors, and have been demonstrated to be essential for maintaining a pluripotent state and suppress lineage specification [57]. Upon knockdown, many of these ESC lincRNAs were shown to induce gene expression changes similar to the depletion of known ESC factors. Importantly, it was found that lincRNAs could associate with multiple chromatin complexes, hence suggesting the possibility that lincRNAs may serve as scaffolds for the recruitment of different protein complexes for specific functions. In that regard, it would be interesting to examine how the ESC-specific lincRNA expression signature, through the assembly of various protein complexes, is able to sustain ESC pluripotency.

Extrinsic signalling and ESC transcriptional regulation

Signalling in mouse ESCs

Traditionally, mESCs were cultured and kept pluripotent on a layer of mitotically inactivated feeder cells in serum-supplemented media [1, 2] (Figure 1). However, the undefined nature of feeder co-culture posed a significant challenge in mapping the specific extrinsic signalling factors, cellular pathways involved and their effects upon gene transcription which ultimately control ESC self-renewal and differentiation.

After the initial discovery of mESCs in 1981, work over the next 20 years then identified the IL-6 family cytokine LIF as the key active component produced by feeder cells which sustains mESC growth [58, 59], as well as Bone morphogenic protein 4 (BMP4) which could substitute for the use of serum in mESC culture [60]. Critically, the combinatorial use of LIF and BMP4 alone in a defined feeder- and serum-free culture was sufficient to derive and maintain germ-line transmittable mESCs [60], hence delineating the signalling pathways controlling mESC self-renewal and pluripotency. At the transcriptional level, LIF and BMP4 signalling is able to induce phosphorylation and activation of their down-

Figure 1 Interconversion between mouse pluripotent states. mESCs are derived from the inner cell mass (ICM) of mouse pre-implantation blastocysts, while EpiSCs are isolated from the epiblast compartment of post-implantation embryos. The conversion between the pluripotent mESC and EpiSC states is possible through cell culture; introduction of transcription factors (TFs), or application of chemical inhibitors. Unipotent PGCs are derived from the embryonic gonad and can also be differentiated from EpiSCs using BMP4; or with BMP4, BMP8b, LIF, and stem cell factor (SCF). Subsequently, PGCs can be restored to pluripotency through the culture with LIF, FGF2 and SCF to form mouse embryonic germ cells (EGCs). mESCs are also known to transiently cycle into a 2-cell (2C)-like state with expanded developmental potential, giving rise to both embryonic and extraembryonic tissues. The process and mechanism by which this 2C transition occurs are still not known, and whether 2C-like cells could be directly isolated from 2C embryos has not been established.

stream transcription factors Stat3 and Smad1 respectively [60, 61]. Importantly, genome-wide mESC transcription factor mapping studies reveal the co-binding of Stat3 and Smad1 transcription factors at Oct4, Sox2 and Nanog regions, thus demonstrating how LIF and BMP4 signalling may sustain the core ESC transcriptional network [42].

In contrast to the self-renewing effect of LIF and BMP4, mESC autocrine stimulation by fibroblast growth factor 4 (Fgf4) [62, 63], working through the Mek/Erk signalling pathway, is known to induce mESCs to exit self-renewal and initiate differentiation [64, 65]. This pro-differentiation effect of Fgf/Mek/Erk signalling can be further inferred through complementary experiments, whereby chemical inhibition or genetic knockout of Fgf/Mek/Erk signalling cascade components caused impaired mESC differentiation [64–68]. The exact mechanism by which active Mek/Erk signalling induces mESCs to exit pluripotency is still not known. Given that terminal signalling kinases such as Erk have been previously shown in other cell types to regulate gene expression activity via transcription factor phosphorylation [69, 70], phosphorylation of chromatin remodelling complexes [71], and direct binding onto DNA as a transcriptional repressor [72], it would be interesting to determine if Erk may adopt similar mechanisms in regulating mESC pluripotency.

mESCs are also responsive to Wnt-signalling, but whether Wnt signalling promotes self-renewal or causes mESC differentiation is still being actively debated [73,

74]. Active canonical Wnt-signalling leads to the stabilisation of β -catenin, hence allowing for the association of β -catenin with the TCF/LEF family of transcription factors for gene activation [73]. Tcf3, which is the most abundant Tcf protein in mESCs [75], has been previously shown to co-localize with the core Oct4, Sox2 and Nanog-binding sites [54, 76], therefore suggesting that Wnt signalling, like LIF and BMP pathways, is integrated into the ESC transcriptional regulatory core. In mESCs, Tcf3 functions primarily as a transcriptional repressor [75, 77, 78] and *Tcf3*-null mESCs are resistant towards differentiation [75, 79]. However, as the process in which Tcf3 regulates gene expression is still not clear, and with multiple models being proposed to account for the mechanism of how β -catenin and Tcf3 interaction could initiate gene expression [73], additional studies are clearly needed to define the role of Wnt-signalling in ESCs. Additionally, given that Wnt-signalling in hESCs has not been well explored, it would also be interesting to determine if similar Wnt-regulatory pathways are conserved in hESCs.

Signalling in hESCs

Although hESCs may share the same Oct4-Sox2-Nanog core ESC transcriptional regulatory circuit [24, 25]; they differ from mESCs in their extrinsic signalling requirement to maintain self-renewal and pluripotency. Previous studies have shown that LIF is dispensable for

hESC culture [80], while the presence of BMP4 will cause hESC differentiation into trophoblast [81]. Rather, FGF2 and Activin/Nodal signalling is critical for the maintenance of an undifferentiated hESC state [82-84].

The exact mechanism by which FGF2-signalling sustains hESC pluripotency is still not clear and is complicated by the fact that FGF-signalling could activate multiple signalling cascades [85]. Issues pertaining to signalling ‘crosstalk’ by other receptor kinases onto FGF-receptor-associated pathways [86, 87], as well as varying hESC culture conditions employed by different groups, have made the dissection of hESC FGF2-signalling more difficult. However, previous studies have indicated that FGF-signalling through its downstream protein ERK is necessary to prevent extra-embryonic differentiation of hESCs [88, 89], and that FGF2 may cooperate with other growth factors (like Activin signalling, through SMAD2/3 transcription factors), to upregulate the expression of the core pluripotency gene *NANOG* [84, 90].

There have already been several large-scale attempts at profiling the global hESC phosphoproteome via mass spectrometry techniques [91-94], with two studies seeking to specifically address the dynamics of FGF2-dependent tyrosine and serine/threonine phosphorylation [95, 96]. Together, FGF2-signalling in hESCs not only resulted in phosphorylation of proteins of various signalling cascades such as that of PI3K, MAPK, Wnt, but could also lead to phosphorylation of pluripotency-associated transcription regulators like OCT4, SOX2, SALL4 and DPPA4 [95, 96]. These studies while informative in revealing a possible phospho-interactome downstream of FGF2-FGFR, unfortunately do not factor in signalling ‘crosstalk’ by other receptor kinases onto FGFR-associated pathways [86, 87]. Therefore, future attempts at studying the hESC signalling pathways, aside from adopting defined culture conditions, should also seek to utilize specific kinase inhibitors, gene knockdown or gene deletion strategies to more accurately delineate the cell-signalling events.

Unlike the ambiguity surrounding FGF2 signalling, Smad2/3, the downstream effectors of Activin/Nodal signaling are previously known to directly bind and regulate the expression of *NANOG* [97]. Recent ChIP-seq in hESCs also found the binding of SMAD2/3 at *OCT4*, *TERT*, *MYC* and *DPPA4* genes, with SMAD2/3 sharing approximately one-third overlap with *NANOG* genomic targets [98]. Importantly, the authors found that *NANOG* overexpression could sustain SMAD2/3 target gene expression even in the absence of Activin/Nodal signalling, thus suggesting that *NANOG* may function as a SMAD2/3 transcriptional co-factor during active Activin/Nodal signalling in hESCs [98]. In a separate study,

ChIP-seq revealed extensive co-occupancy of SMAD3 along with the hESC genome with OCT4, although it was not verified if SMAD3 could bind alongside other hESC pluripotency factors [99]. Taken together, these data provide important insight into the potential mechanisms by which Activin/Nodal signalling helps sustain hESC pluripotency.

Transcriptional regulation of ground state mESCs

As previously mentioned, the conventional culture of mESCs requires the presence of LIF and BMP4 [58-60]. However, even in the presence of LIF/BMP4, the autocrine pro-differentiation Fgf4/Mek/Erk signalling in mESCs is still active [60, 100]. This therefore suggests that pluripotency is sustained by LIF and BMP4 acting downstream of the Erk pathway to prevent mESC differentiation.

Expanding upon this observation, Ying *et al.* [100] surprisingly found that the requirement of external LIF/BMP4 in mESC culture could be abrogated via the usage of small molecule kinase inhibitors. This two inhibitor (2i) culture, consisting of PD0325901 and CHIR99021 to respectively target Mek and Gsk3, serves to protect pluripotent mESCs from the pro-differentiation effect of Fgf4 stimulation [64], as well as the transcriptional repressor activity of Tcf3 [101]. More importantly, by using the 2i culture, germ-line transmitting ESCs could be generated from previously recalcitrant mouse strains [100, 102], and from the rat [103, 104].

When mESCs are cultured under conventional LIF conditions, there is a heterogeneous expression of pluripotency transcription factors such as Nanog, Rex1 and Stella [23, 105, 106]. Interestingly, after FACS-purification, these sorted cells rather than maintaining a pure cell population, will revert into a heterogeneous population. These data therefore suggest that gene transcription of mESCs under LIF culture conditions exists in a fluctuating and dynamic state. Upon transfer into 2i conditions, Nanog and Rex1 expression will become homogeneously high [107]. As Nanog-low cells are prone to differentiate, and Rex-low mESCs do not contribute to chimera formation upon blastocyst microinjection [23, 105], the capture of a Nanog/Rex1-high mESC state indicates that pluripotency may have been stabilised under 2i conditions [107]. Interestingly, under 2i or Mek-inhibition, the *Nanog* gene expression in mESCs switches into a biallelic expression mode, as opposed to the monoallelic expression status in conventional LIF/serum culture [108]. With these unique characteristics, 2i-cultured mESCs are said to reside in a novel and distinct ‘ground state’ pluripotency [100].

Although the protein levels of pluripotency regulators like Nanog and Rex1 are known to become uniformly-

high when mESCs are switched from LIF/serum into 2i media [107], Marks *et al.* [109] however found through RNA-sequencing (RNA-seq) that apart from *Tcl1*, the mRNA levels of most other pluripotency genes like *Pou5f1*, *Sox2*, *Nanog*, *Esrrb*, *Klf2*, *Klf4* and *Tbx3* did not change between the two culture conditions. The reason behind this discrepancy is still unclear, and may have arisen due to the presence of LIF with the 2i media used in this study [109]. It is also possible that translational and/or post-translational regulatory mechanisms are involved.

This study by Marks *et al.* [109] also found that lineage genes were suppressed under 2i/LIF conditions, and that 2i/LIF mESCs had higher expression of metabolic genes. Taken together, these data argue against the hypothesis proposed by Efroni *et al.* [110] that an innate global transcriptional hyperactivity results in pluripotent developmental plasticity. Rather, it is believed that the presence of lineage gene expression in conventional mESC culture may be the result of serum stimulation [109]. Additionally, unlike cells cultured under LIF/serum conditions, 2i/LIF mESCs were found to contain more proximal-promoter pausing by RNA Polymerase II (RNA Pol II), especially at many lineage-specific genes. It was also proposed that this RNA Pol II pausing could be important towards establishing the ground state pluripotency.

Interconversion between alternative pluripotent states

Mouse epiblast stem cells

While the presence of LIF is necessary for mESCs to maintain pluripotency, the discovery of a novel LIF-independent pluripotent stem cell population derived from E5.5 to E7.5 post-implantation mouse embryos [111, 112], suggests that other states of pluripotency may exist (Figure 1). As these cells were isolated from the post-implantation epiblast tissue of the developing embryo, they were hence termed mouse epiblast stem cells (EpiSCs). While EpiSCs can self-renew and were demonstrated to be pluripotent through teratoma assays and through *in vitro* differentiation into germ cells [111–113], it should be noted that EpiSCs exhibit limited developmental potential and are generally considered inefficient in the formation of chimeras [111, 112, 114].

Similarly, while EpiSCs do express the core Oct4, Sox2 and Nanog pluripotency factors, they differ markedly from mESCs with regards to their gene expression profile, epigenetic status and usage of signalling pathways to maintain a stem cell identity [106, 111, 112, 115, 116]. Notably, EpiSC cultures like that of hESCs, require

the addition of Activin and Fgf2, but not LIF or BMP4 for self-renewal [117]. The expression levels of pluripotency markers such as Rex1, Stella, Klf2, Klf4 are lower in EpiSCs, with a concomitant higher expression of differentiation markers [112]. It should also be highlighted that while female mESCs are in the pre-inactivation state with two active X-chromosomes (XaXa), EpiSCs have already undergone X-inactivation (XaXi), consistent with a more developmentally advanced stage [117]. Therefore, these findings indicate that EpiSCs have already been ‘primed’ for differentiation, as opposed to mESCs which exist in a more developmentally ‘naive’ state.

Overall, EpiSCs appear to partially resemble hESCs, and may potentially be the counterpart of hESCs. Therefore, EpiSCs could serve as a more relevant mouse model for the study of early human embryonic development, as compared with mESCs. However, despite the aforementioned similarities with hESCs, inhibition of Fgf2/Erk signalling in EpiSCs does not result in loss of Nanog expression, and no evidence of Oct4-mediated transcriptional regulation at *Fgf2* promoters was observed for EpiSCs [118]. Similarly, certain key features of EpiSCs like the absence of Rex1, a mESC pluripotency marker, and the expression of the Fgf5 epiblast marker, are not shared by hESCs [118]. Further research is thus needed to establish whether these dissimilarities are due to species- or developmental-specific differences.

The transition between naive and primed pluripotency

As mESCs are derived from a developmentally earlier timepoint (pre-implantation embryos) as compared to EpiSCs (post-implantation embryos), the conversion of mESCs into EpiSCs would therefore correspond to a differentiation step along the normal developmental pathway (Figure 1). In this regard, culturing mESCs with FGF2 and Activin A readily results in the formation of EpiSCs [114]. Similarly, FGF treatment together with LIF/Stat3 blockade could also convert mESCs into EpiSCs [118].

In contrast, the reverse transition of EpiSCs into mESCs is more difficult, occurring at an extremely low frequency through culture with LIF [102, 114]. The reversion rates may be improved by 10^4 – 10^5 fold if the pluripotency factors Klf2, Klf4, Nanog, Nr5a2 or Esrrb were to be ectopically expressed [22, 102, 114, 119, 120]. Similarly, a chemical approach can also be adopted to convert EpiSCs into naive mESCs using a combination of LSD1, ALK5, MEK, FGFR and GSK3 inhibitors [121] (Figure 1).

Recently, Prdm14 and Klf2 were found to synergize with each other to rapidly induce the reprogramming of EpiSCs into mESCs within 3–4 days [122], in contrast to

the usual process of 8-10 days. By itself, the introduction of Prdm14 does not have much effect on the reversion process. However, transcriptomic analysis revealed that Prdm14 actually serves to prime EpiSCs for conversion through the simultaneous repression of lineage-associated genes and activation of early epiblast genes. The synergistic effect of Prdm14-Klf2 can also be attributed to the ability of Prdm14 to enhance Klf2 recruitment onto key mESC pluripotency gene loci such as the *Nr5a2* promoter and the *Oct4* distal enhancer [122].

Establishing a mESC-like hESC state

In addition to post-implantation embryos, EpiSCs can also be readily isolated from pre-implantation stage mouse blastocysts [123], which have been traditionally used to derive mESCs (Figure 1). This meant that modulation of the signalling environment could influence the formation of naive or primed pluripotency states. Because hESCs resemble EpiSCs more than mESCs, despite also having been derived from pre-implantation blastocysts, it is believed that during the hESC derivation process, pre-hESCs may have progressed into a more developmentally advanced EpiSC-like state [124]. In support of this ‘primed’ pluripotent state of hESCs, non-human primate ESCs from rhesus monkeys, like mouse EpiSCs, are unable to contribute to chimera formation [125].

Currently, hESCs suffer from very poor gene-targeting efficacy unlike mESCs, making the genetic manipulation of hESCs for research or therapeutic purposes extremely difficult [126]. Critically, the developmental stage differences between the two cell types imply that many of the protocols for mESC differentiation into various lineages may not work in a hESC system. In light of these problems, the creation of a mESC-like hESC state would not only make hESCs more amenable to gene targeting, but also enable the transfer of existing mouse differentiation protocols into hESCs.

There have been several attempts by various groups to generate mESC-like human pluripotent stem cells. The methods include either a direct conversion from conventional hESCs [127, 128] or the reprogramming of somatic cells into mESC-like hESCs [128-131] (Figure 2). Unfortunately, these cells either were dependent upon transgene expression for long-term culture, or have not been thoroughly characterised for features such as naive mESC characteristics and complete transgene independence. Taken together, while these studies have demonstrated the feasibility of creating hESCs which exhibit characteristics of naive pluripotency, future efforts should focus on improving the culture conditions to enable transgene-free long-term maintenance of these

A Direct conversion approach

B Reprogramming approach

Figure 2 Methods to create mESC-like hESCs. **(A)** hESCs with mESC-like characteristics can be generated directly from conventional hESCs using OCT4 with SOX2, or, KLF4 with KLF2, in the culture with LIF/PD/CH [128]. Similarly, the generation of hESCs which resemble mESC colonies could be achieved through culture with LIF/PD/SB [127]. **(B)** Human fibroblasts can also be reprogrammed into mESC-like human iPSCs through expression of OCT4, SOX2, NANOG, LIN28 with LIF/PD/CH/A83 [129]; through OCT4, SOX2, KLF4, c-MYC, NANOG with LIF [130]; using OCT4, SOX2, KLF4 with LIF/PD/CH [128]; or OCT4, SOX2, KLF4, c-MYC, NR5A2, RAR-γ with LIF/PD/CH [131]. PD: MEK inhibitor PD0325901. CH: GSK3 inhibitor CHIR99021. SB: p38 inhibitor SB203580. A83: ALK4,5,7 inhibitor A83-01.

mESC-like hESCs.

Several studies have also explored the possibility of generating mESC-like hESCs directly from pre-implantation human embryos. Female hESCs derived from the

culture of human blastocysts in hypoxic (5% O₂) conditions were found to be in an XaXa status, a characteristic of mouse naive pluripotency [132]. However, these cells were maintained in conventional FGF-containing media and were not tested further for features of naive pluripotency.

The generation of naive hESCs from embryos using 2i media was reported to be unsuccessful [133]. In mice, culturing 8-cell stage embryos under MEK inhibition will result in the failure to form the hypoblast compartment with a reciprocal expansion of the pluripotent epiblast [134]. However, culturing early human embryos with MEK inhibitor does not block hypoblast formation, and neither would the development of the NANOG-positive epiblast compartment be affected [133, 135]. Thus taken together, these studies suggest that additional signalling pathways could be involved in the segregation of the human ICM into the epiblast and hypoblast, or that species-specific differences exist between mouse and human embryos in the biological functions of FGF/MEK signalling.

The totipotential of 2C-like mESCs?

Recently, Macfarlan *et al.* [136] reported the discovery of a rare transient population of mESCs, which can give rise to both embryonic and extraembryonic tissues. By comparing the gene expression signature between mouse oocytes and 2-cell (2C)-staged embryos using RNA-seq, the authors found that the 2C transcriptome contained many genes, which were driven by retroviral elements. Among these repeats, the MuERV-L family of retroelements were found to be the most abundant. Indeed, by combining this MuERV-L regulatory sequence with a td-Tomato red fluorescence gene, the authors demonstrated through zygotic injection that strong MuERV-L reporter fluorescence was detected at the 2C stage, which would then gradually decrease and become undetectable by the blastocyst stage.

Surprisingly, stable integration of this MuERV-L td-Tomato reporter into mESCs led to the detection of a similar red fluorescence within a very small population of cells (approximately 0.2%-1.5%); and gene expression profiling of these tdTomato+ mESCs revealed that they resembled 2C embryos (Figure 1). Like Nanog, Rex1 and Stella [23, 105, 106], MuERV-L tdTomato expression was also found to exist in a dynamic state. Subsequent genetic labelling experiments suggested that all mESCs within the culture could transiently pass through this 2C-like state. Intriguingly, these 2C-like mESCs do not contain any Oct4, Sox2 and Nanog proteins, even though the transcript levels of these genes were unaffected. Perhaps the most fascinating aspect of this study, is the

demonstration that injection of these 2C-like mESCs into morula stage embryos could result in the contribution of these 2C-like donor cells towards both embryonic and extraembryonic tissues, hence suggesting a totipotent-like capability.

Together, this study raises several new interesting questions. First, what is the significance of this 2C-like stage, and why do mESCs transiently enter this phase? Second, as these 2C-like mESCs do not possess Oct4, Sox2 and Nanog proteins, how would the transcriptional network of these 2C-like mESCs be regulated? Third, as only a very rare population of mESCs are expressing MuERV-L at a given time, what would be the signalling pathways or cellular mechanisms that regulate the entry or exit of this phase? And lastly, do hESCs possess a similar transient 2C-like phase, and if so, would it also be regulated by endogenous retroviral elements?

Future outlook

Deciphering the ESC transcriptional network is essential towards understanding the cellular mechanisms that govern pluripotency. In this regard, the different studies involving various experimental approaches have now enabled researchers to appreciate the processes by which the core ESC transcription factors establish an overall ESC identity.

To bring the potential of hESCs one step closer towards its application in regenerative medicine, future research should likely focus upon dissecting the pathways that regulate lineage commitment. Recently, by probing the temporal gene expression and chromatin changes during the directed differentiation of ESCs into cardiac lineages, several novel regulators of cardiac tissue formation have been identified [137, 138]. This is achieved either by (1) determining the stage-specific activation of gene enhancers, and applying a DNA binding motif search to predict the transcription factors that are involved during cardiac differentiation [137]; or (2) by predicting key regulatory genes based on the induction of RNA expression, loss of repressive H3K27me3 marks and reciprocal increase of active H3K4me3 modification [138]. Therefore, it would be interesting to test if other novel tissue-specific regulators could be identified for different somatic lineages through analysis of temporal chromatin changes. In this regard, the ability to genetically modify and introduce hESCs reporter genes into that specify certain tissue lineages would be of tremendous value for the purpose of studying directed differentiation. For instance, by coupling these lineage-specific hESC reporters with high-throughput genome-wide siRNA screens or miRNA mimic libraries, numerous factors

that regulate differentiation into specific lineages could be identified.

Improving the efficacy of directed ESC differentiation into the desired cell-type, as well as overcoming the immature phenotype of hESC-derived differentiated cells [139] represent key issues to be tackled in the future. Resolution of these problems likely will require the optimization of important cell culture parameters such as the extracellular matrix, growth factor and cytokine-signalling environment, three-dimensional (3D) cell organization and cell culture duration. Similarly, the capture of ESC-differentiated cells at the progenitor stage may enable easier expansion and more efficient differentiation into the desired cell type of choice. This can be achieved either through the co-culture of ESC-differentiated tissue with the appropriate mesenchymal cells [140], or through the isolation of self-renewing progenitors within a heterogeneous cell population [141].

While directed differentiation is useful for the derivation of a single, or a few cell types for tissue replacement, the complete generation of complex organs comprising many cell types that work in a coordinated fashion presents a greater challenge. Most remarkably, it was recently demonstrated that pluripotent stem cells retain a self-organising ability to differentiate into 3D organoids, which resemble optic cups [142, 143] and adenohypophysis tissues [144]. Similarly, intestinal organoids have been reported to develop from hESC-derived posterior endoderm monolayers upon exposure to the appropriate signalling factors [145], and functional thyroid gland tissue has been generated from mESCs [146]. Therefore, if one were to harness this powerful self-developing property of pluripotent stem cells for the generation of novel complex organ types for research or medical purposes, it would be important to first determine the mechanisms underlying these processes. In this regard, studying the temporal transcriptional changes occurring at the whole-organoid, or at the single-cell level, may be a good starting point to dissect the pathways involved.

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