A Balanced Network: Transcriptional Regulation in Pluripotent Stem Cells

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Abstract

Embryonic stem cells (ESCs) hold great promise for regenerative medicine. It has been an active research field to understand the molecular mechanisms underlying the pluripotency of ESCs. Self-renewal of ESCs relies on maintaining the unique transcriptional profile of ESCs, while differentiation of ESCs requires a flexible transcriptional profile so that it can be altered in different types of cells. Therefore, transcriptional regulation plays important roles in pluripotency. In this review, we summarize recent discoveries on how transcriptional regulation contributes to pluripotency maintenance in ESCs. We emphasize the functions of transcription factors in pluripotency maintenance, as well as in X chromosome inactivation and somatic cell reprogramming.

Keywords: Embryonic stem cells; Pluripotency; Transcriptional regulation; X chromosome inactivation; Nanog; Oct4; Sox2

Introduction

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the blastocyst, and are able to self-renew indefinitely while maintaining the potential to differentiate into all types of cells in the body [1-3]. Thus, ESCs hold great promise for regenerative medicine. The underlying molecular mechanism for pluripotency is a fundamental question being actively investigated. In the past few years, many mechanisms contributed to pluripotency have been revealed, including transcriptional regulation, epigenetic modifications, chromatin dynamics, signaling pathways, and microRNAs [4-6]. ESCs and somatic cells share almost identical genetic information, the genomic DNA. Pluripotency, the unique property of ESCs, is realized through selective gene expression. Therefore, transcriptional regulation plays a pivotal role in pluripotency regulation. Consistently, through ectopic expression of several transcription factors, somatic cells can be reprogrammed back to the pluripotent state [7-9]. In this review, we summarize recent discoveries how transcriptional regulation contributes to pluripotency maintenance in ESCs, with an emphasis on the function of transcription factors. We also discuss the co-operation of transcription factors with epigenetic factors and signaling pathways in pluripotency maintenance, as well as the roles of pluripotency transcription factors in X chromosome inactivation and somatic cell reprogramming.

Core Transcriptional Circuitry for Pluripotency

Early genetic experiments have identified critical pluripotency factors essential for pluripotency establishment in embryos [10-13]. Oct4 was the first identified pluripotency factor. Oct4 null embryos develop to the blastocyst stage. However, the ICM cells of Oct4 null embryos are not pluripotent. Instead, they are diverted to a trophoderm fate. Thus, Oct4 null ICM cells fail to give rise to embryo or ESCs [11]. Another key pluripotency factor is the HMG-box transcription factor Sox2, which heterodimerizes with Oct4 to regulate their downstream target genes [14-16]. Similar to Oct4 depletion, knockout of Sox2 leads to peri-implantation embryonic lethality [13]. When cultured in vitro, ICM cells with Oct4 or Sox2 deficiency differentiate into the trophoblastic lineage, consistent with the transcriptional activity of the Oct4 and Sox2 heterodimer [11,13]. Nanog was first identified as a factor allowing ESC self-renewal independent of leukemia inhibitory factor (LIF) [10,12]. Nanog null embryos die around implantation due to lack of epiblast. Nanog null ICM cells fail to proliferate in vitro. Instead, they differentiate into parietal endoderm-like cells [12].

Consistent with their roles in pluripotency establishment in developing embryos, Oct4, Sox2, and Nanog are important for pluripotency maintenance in ESCs. Knockdown of Oct4, Sox2, or Nanog causes ESC differentiation [17-21]. Interestingly, self-renewal of ESCs requires the precise expression level of Oct4. Enhancing Oct4 expression by less than two-fold induces primitive endodermal and mesodermal differentiation. In contrast, repression of Oct4 results in differentiation to trophoblast [17]. There is some controversy on the essential role of Nanog in pluripotency maintenance. Over-expression of Nanog allows mouse ESC self-renewal in the absence of LIF. No ESCs can be derived from Nanog null embryos [10,12]. These data suggest that Nanog is essential for pluripotency. However, Nanog null ESCs were established by genetic depletion in ESCs. These Nanog null ESCs can self-renew indefinitely in the absence of Nanog, and contribute to fetal and adult chimera. It seems that Nanog is dispensable for the maintenance of ESC pluripotency in culture. Nevertheless, Nanog null ESCs are not fully pluripotent. They are prone to differentiate in culture, and fail to form germ cells in chimera mice [22].

To understand how Oct4, Sox2 and Nanog regulate pluripotency in ESCs, several groups carried out genome wide binding site analyses of these three factors, and found that Oct4, Sox2 and Nanog not only bind to their own promoters, but also occupy one another’s promoter, thus forming a core positive feedback circuitry for pluripotency. These core pluripotency factors activate the expression of protein-coding genes and microRNAs involved in pluripotency maintenance. Meanwhile, Oct4, Sox2 and Nanog repress many key transcription factors for differentiation and development, preventing ESCs from differentiation.

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Received April 12, 2012; Accepted May 07, 2012; Published May 09, 2012


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[23-27]. Moreover, Oct4 and Sox2 appear to participate in cell fate determination upon differentiation. Oct4 promotes mesendodermal differentiation, while Sox2 facilitates neural ectodermal differentiation [28].

Other Pluripotency-associated Transcription Factors

In addition to the core transcriptional circuitry, many other transcriptional factors, including Tcf3, Smad1, Stat3, Sal4, Dax1, Esrrb, Nr5a2, Tbx3, Zfx, Ronin, Klf4, Foxd3, Foxo1, Pdmd14, Zic3, Nac1 and Zip281 have been demonstrated to be involved in pluripotency maintenance [29-55]. Consistent with their role in pluripotency maintenance, the expression of majority of these pluripotency-associated transcription factors are directly regulated by Oct4, Sox2 and/or Nanog (Table 1) [26]. Among these pluripotency-associated transcription factors, Tc3, Sma1 and Stat3 are transcription factor associated with signaling pathways. We will discuss their roles in the section of integration of external signals into transcriptional activity.

The genome wide binding profiles of pluripotency-associated transcription factors in ESCs, including Dax1, Esrrb, Nr5a2, Tbx3, Zfx, Ronin, Klf4, Foxo1, Pdmd14, Nac1 and Zip281, have been established [25,26,39,45,51,56-58]. These pluripotency-associated transcription factors, except for Ronin, bind to the promoters of Nanog, Oct4 and/or Sox2 (Table 2). Most of them contribute to pluripotency maintenance by stabilizing the core transcriptional circuitry. In addition, these pluripotency-associated transcription factors co-occupy many target genes of Nanog, Oct4 and Sox2, suggesting that they might also facilitate activating genes involved in pluripotency maintenance and repressing genes required for differentiation [25,26,39,45,51,56-58]. However, not all the pluripotency-associated factors enhance the stability of the core transcriptional circuitry. For example, Zip281 binds to the promoters of Nanog, Oct4 and Sox2, but acts as a repressor for the key pluripotency genes [25,44,45]. The interaction between Dax1 and Oct4 suppresses the transcriptional activity of Oct4 protein [32].

<table>
<thead>
<tr>
<th>Genes</th>
<th>Oct4</th>
<th>Sox2</th>
<th>Nanog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dax1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Esrrb</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nr5a2</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tbx3</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Zfx</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ronin</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Klf4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nac1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Zip281</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Zic3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Foxo1</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Foxo3</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Foxo1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pdmd14</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tc3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sma1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stat3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Data in this table is extracted from [26]

Table 1: Pluripotency-associated genes regulated by Oct4, Sox2, and Nanog.

<table>
<thead>
<tr>
<th>TFs</th>
<th>Oct4</th>
<th>Sox2</th>
<th>Nanog</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[29,30]</td>
</tr>
<tr>
<td>Dax1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[25]</td>
</tr>
<tr>
<td>Esrrb</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[26]</td>
</tr>
<tr>
<td>Nr5a2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>[56]</td>
</tr>
<tr>
<td>Tbx3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>[58]</td>
</tr>
<tr>
<td>Zfx</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>[26]</td>
</tr>
<tr>
<td>Ronin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>[57]</td>
</tr>
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<td>+</td>
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<td>+</td>
<td>[25,26,39]</td>
</tr>
<tr>
<td>Nac1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[25]</td>
</tr>
<tr>
<td>Zip281</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[25,45]</td>
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<td>Zic3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
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<td>Foxo1</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>[48]</td>
</tr>
<tr>
<td>Foxo3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>Foxo1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[50]</td>
</tr>
<tr>
<td>Pdmd14</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>[47,51]</td>
</tr>
<tr>
<td>Tc3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[27,54,55]</td>
</tr>
<tr>
<td>Sma1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[26]</td>
</tr>
<tr>
<td>Stat3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[26]</td>
</tr>
</tbody>
</table>

Table 2: Pluripotency-associated transcription factors regulating Oct4, Sox2, and Nanog genes.

regulation of the core transcriptional circuitry might be important for the disruption of the circuitry during ESC differentiation. Consistently, Zip281 is dispensable for ESC self-renewal, but is required for proper differentiation of ESCs [44]. Therefore, the expanded transcriptional regulatory network of pluripotency, formed by the core regulatory circuitry and pluripotency-associated transcription factors, ensures the transcriptional profile of ESCs stable enough for self-renewal, but still plastic enough to allow ESC differentiation.

Chromatin immunoprecipitation combined with high-throughput DNA sequencing (ChIP-seq) analysis did not detect Ronin occupancy at the promoters of Nanog, Oct4 and Sox2. Instead, many genes regulated by Ronin are involved in cell metabolism. Therefore, it has been suggested that Ronin contributes to the fast and unimpeded growth of ESCs [57].

Interestingly, the role of FOXP1 in pluripotency maintenance is associated with its alternative splicing. The ESC specific isoform of FOXP1 (FOXP1-ES) differs from other isoforms at the exon 18 (human) or the exon 16 (mouse), which changes the DNA-binding preferences of FOXP1. Therefore, only FOXP1-ES activates pluripotency genes, including OCT4, NANOG and NR5A2, and represses genes involved in differentiation. Consistently, FOXP1-ES, but not FOXP1, promotes ESC self-renewal [50].

There are some conflicting data regarding Pdmd14 in mouse ESCs. Chia et al [47] identified PRDM14 as a regulator of OCT4 expression in human ESCs. They also demonstrated that knockdown of Pdmd14 in mouse ESCs has no effect on the expression of Oct4 and Sox2. Moreover, Pdmd14 expression level is extremely low in mouse epiblast stem cells (epiSCs), which is considered as the mouse counterpart of human ESCs. These data suggested that Pdmd14 is essential for the maintenance of human ESCs, but not for mouse ESCs or epiSCs [47]. In contrast, Ma et al showed that knockdown of Pdmd14 in mouse ESCs not only reduces the expression of Nanog and Sox2, but also induces
Pluripotency-associated Transcriptional Co-factors

To regulate gene expression, transcription factors usually recruit other protein factors, which do not bind to DNA by themselves. These factors are named transcriptional co-factors. According to their effects on gene expression, they are categorized into two groups, co-activators and co-repressors. So far, some transcriptional co-factors, such as mediator, cohesin, Cnot, Trim28, Paf1 and the XPC-RAD23B-CETN2 and co-repressors. So far, some transcriptional co-factors, such as mediator, cohesin, Cnot, Trim28, Paf1 and the XPC-RAD23B-CETN2 and co-repressors. So far, some transcriptional co-factors, such as mediator, cohesin, Cnot, Trim28, Paf1 and the XPC-RAD23B-CETN2 (XPC) nucleotide excision repair complex, have been implicated in ESC self-renewal and pluripotency maintenance [62-65].

Mediator and cohesin were identified in an shRNA screen for factors regulating Oct4 expression in ESCs. Further investigation revealed that mediator and cohesin, as well as the cohesin loading factor Nipbl, interact with each other, and co-occupy the enhancer and core promoter regions of actively transcribed genes, including Oct4 and Nanog. Moreover, mediator and cohesin promote the formation of enhancer-promoter DNA looping, which is required for gene activation [62]. Cnot and Trim28 are transcriptional co-repressors, whose down-regulation leads to ESC differentiation [65,66]. Cnot and Trim28 share many downstream target genes with c-Myc and Zfx, indicating that these four factors cooperate together to regulate a unique transcriptional module in ESC self-renewal. Trim28 also binds to the promoters of Nanog and Sox2, and interacts with Nanog, Rex1 and Dax1 proteins [31,65]. Knockdown of individual component of the Pol II-associating factor 1 complex (Paflc), reduces Oct4 expression level in ESCs and results in ESC differentiation, suggesting an essential role of the whole Paflc in pluripotency maintenance. Paflc occupies the promoters of many pluripotency genes, such as Oct4, Sox2 and Nanog. Paflc might collaborate with the Set1 complex to methylate H3K4 at the promoters of pluripotency genes and maintain their expression [64].

Recently, through an in vitro transcription assay, the XPC nucleotide excision repair complex was identified as a co-factor for Oct4 and Sox2 to activate Nanog transcription. Moreover, in ESCs, around 70% of XPC-targeted genes are also bound by Oct4 and Sox2. Given the tight association of XPC with Oct4 and Sox2, it is not surprising that the XPC complex is required for ESC self-renewal and somatic cell reprogramming [63]. In this case, XPC, the protein complex involved in DNA repair, functions as a transcription co-factor for pluripotency maintenance. It is not clear yet whether the DNA repair activity of XPC is also important for pluripotency. Interestingly, an RNA polymerase III subunit POLR3G has been shown to be required for the maintenance of pluripotency in human ESCs [67]. However, it remains unclear how POLR3G contributes to pluripotency maintenance.

Table 3: Interactions between pluripotency-associated transcription factors and Oct4, Sox2, and Nanog.

<table>
<thead>
<tr>
<th>TFs</th>
<th>Oct4</th>
<th>Sox2</th>
<th>Nanog</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sall4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[31,60]</td>
</tr>
<tr>
<td>Dax1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[31,32,59]</td>
</tr>
<tr>
<td>Esrrb</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[36,59]</td>
</tr>
<tr>
<td>Klf4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[60]</td>
</tr>
<tr>
<td>Nac1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[31,59]</td>
</tr>
<tr>
<td>Zfhx28</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[31,45]</td>
</tr>
<tr>
<td>Foxd3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>[81]</td>
</tr>
</tbody>
</table>

Through affinity purification followed by mass spectrometry, the binding partners of Oct4 and Nanog have been systematically identified. Indeed, Oct4 and Nanog interact with many chromatin regulators, including the histone deacetylase NudR, the polycomb repression complex 1 (PRC1), the Lsd1 histone demethylase complex, Wdr5 and the chromatin remodeling complexes Chd1, ISWI, SWI/SNF, INO80 and Trapp/p400, as well as the DNA methyltransferase Dnmt3a and Dnmt3l [59,71]. Among these binding partners of Oct4 and Nanog, Chd1 has been shown to be essential for maintaining the open chromatin in ESCs [72]. The chromatin remodeling complexes INO80 and Trapp/p400 are also required for pluripotency maintenance [47,66]. Moreover, Oct4 interacts with the histone H3 lysine 9 (H3K9) methyltransferase Setdb1 (also known as Eset) to repress the trophoblast-associated genes Cdx2 and Tcfap2a [73,74]. Wdr5, a core member of the Trithorax (trxG) complex, which catalyzes the methylation of H3K4, is required for ESC self-renewal, as well as efficient somatic cell reprogramming. Through interaction with Oct4, Wdr5 co-occupies many Oct4 target genes. Further analysis suggested that Wdr5 cooperates with Oct4, Nanog and Sox2 to activate genes required for ESC self-renewal [75]. However, the exact role of the
interactions between other chromatin regulators and Oct4/Nanog in pluripotency maintenance remains to be explored.

In addition to the physical interactions between pluripotency transcription factors and chromatin regulators, pluripotency transcription factors also regulate the expression of chromatin regulators. For example, Oct4 binds to genes encoding subunits of the NuRD complex (Mta2, Mbd3, Mta3 and Hdac2), the SWI/SNF complex (Baf155), the PRC1 complex (Phc1and Rbyp) and the LSD1 complex (Rcor2) [25,26,59]. Chd1 gene is occupied by multiple pluripotency transcription factors, including Nanog, Oct4, Sox2, Smad1 and Zfx [26,72]. Conversely, chromatin regulators also participate in regulating the expression of pluripotency genes. The H3K9 demethylase Jmdj2c, whose expression is positively regulated by Oct4, catalyzes the demethylation of H3K9Methyl at the Nanog promoter, and maintains the expression of Nanog [76]. Coactivator-associated arginine methyltransferase 1 (Carm1), an essential factor for the self-renewal and pluripotency of ESCs, binds to the promoter of Oct4 and Sox2, and promotes the methylation of histone H3 arginine 17 and 26 [77].

Non-coding RNAs, including microRNAs and large intergenic non-coding RNAs (lincRNAs), are also important epigenetic regulators for pluripotency maintenance [70,78]. Similar to other genes encoding proteins, many genes encoding microRNAs and lincRNAs are also regulated by Oct4, Sox2 and Nanog in ESCs [27,78]. MicroRNAs appear to be critical to suppress pluripotency genes upon ESC differentiation. It has been demonstrated that miR-134, miR-296 and miR-470 suppress the expression of Nanog, Oct4 and Sox2 [79]. Consistently, knock-out of DGCR8, an RNA-binding protein that assists the RNAse III enzyme Drosha in the processing of miRNA, causes differentiation deficiency of ESCs [80]. The important role of lincRNAs in pluripotent cells has been demonstrated by loss-function assay, as well as somatic cell reprogramming [78,81]. How lincRNAs regulate gene expression is not completely understood. One mechanism is that lincRNAs work in cis to regulate neighboring genes. Alternatively, lincRNA transcripts bind to chromatin regulatory proteins, such as Prc1, Cbx1, Cbx3, Tip60/P400, Prc2, Setd8, Eset, Suv39h1, Jarid1b, Jarid1c and Hdac1, thus regulating gene expression [78].

Integration of External Signals into Transcriptional Activity

In responses to various environmental cues, ESCs either self-renew or differentiate into different cell lineages. Thus, ESCs should be able to sense extracellular signals and transduce the signals into the nucleus to regulate the transcriptional profile accordingly. LIF is widely used in mouse ESC culture medium to maintain ESCs at an undifferentiated state. Bone morphogenetic proteins (BMPs) synergizes with LIF to maintain the pluripotent state of ESCs [52]. In addition, it has been shown that the Wnt pathway also promotes ESC self-renewal [82]. Transcription factors Stat3, Smad proteins and β-catenin/Tcf3, are downstream effectors for the LIF, BMP, and Wnt pathways, respectively. Interestingly, genome-wide binding site mapping of Stat3, Smad1 and Tcf3 revealed that all three factors occupy the promoters of the core pluripotency genes Oct4, Sox2 and Nanog. Furthermore, Stat3, Smad1 and Tcf3 bind to many genes co-occupied by Oct4, Sox2 and Nanog [26,27,54,55]. Therefore, the LIF, BMP and Wnt pathways not only directly regulate the expression of the core pluripotency genes, but also co-operate with these core pluripotency factors to activate genes required for pluripotency maintenance and to repress genes involved in differentiation. Simultaneous inhibition of Mek/Erk and GSK3 can maintain ESCs in the pluripotent state independent of LIF [83].

Inhibition of GSK3 stabilizes β-catenin to facilitate the maintenance of pluripotency [37,82]. Recently, it has been demonstrated that Erk1 and Erk2 phosphorylate Ki67, thus suppressing the transcriptional activity of Ki67 [84]. Yet, these data do not exclude the possibility that other downstream targets of the Mek/Erk and GSK3 signaling are involved in pluripotency maintenance. Further studies are necessary to clarify the mechanisms of the Mek/Erk and GSK3 signaling in pluripotency maintenance.

The expanded transcriptional regulatory network of pluripotency and the interaction network of pluripotency factors further facilitate the connection between signaling events and transcriptional regulation of pluripotency. It has been shown that LIF acts through the PI(3) K-Akt and JAK-Stat3 pathways to activate Tbx3 and Klf4, respectively. Subsequently, Tbx3 and Klf4 positively regulate Nanog and Sox2 to sustain the core transcriptional circuitry for pluripotency [40]. In addition, β-catenin and Tcf3, the downstream effectors of the Wnt pathway, promote the expression of Nr5a2 (also known as Lhtr-1), which in turn activates pluripotency genes Nanog, Oct4 and Tbx3 [37].

In contrast to mouse ESCs, human ESCs rely on different signaling pathways to maintain pluripotency. The Activin/Nodal and FGF2 signaling are required for pluripotency maintenance in human ESCs [3,85-87]. Smad2/3 downstream of Activin/Nodal can activate Nanog to maintain pluripotency [88]. However, how the FGF2 signaling pathways connect to the transcriptional regulatory network of pluripotency are not well understood.

Pluripotency and X Chromosome Inactivation

X chromosome inactivation (XCI) is an intriguing topic related with pluripotency. One X chromosome in each female mammalian cell is inactivated for transcription in order to compensate the X-linked gene dosage between males and females. In the mouse, XCI shows a tight correlation with pluripotency during embryonic development. In the early cleavage stage embryos, the paternal X chromosome is non-randomly inactivated (a special form of XCI known as the imprint XCI) [89,90]. When the epiblast lineage is specified in the blastocyst, pluripotency is established; meanwhile, the inactive paternal X chromosome is reactivated. Two active X chromosomes (Xa) can be found in each epiblast cell of a female blastocyst and in the corresponding female ESCs. Shortly after implantation, around E5.5, XCI occurs again; meanwhile, the pluripotent epiblast cells start to differentiate and lose the pluripotency. In the second wave of XCI, each female cell independently and randomly chooses one of the two Xs as the inactive X (Xi). This form of XCI is known as the random XCI. The XCI status of each cell, once established, is clonally maintained in the subsequent cell generations of all but one type of somatic cell, the primordial germ cell (PGC). The specification of PGCs is a process of de-differentiation [91]. The genome in each PGC is epigenetically re-programmed to be prepared for regeneration of a new life cycle. By applying special culture conditions, PGCs can be readily converted to a pluripotent cell type in culture, called the embryonic germ (EG) cells. It is no coincidence that X reactivation (XCR) occurs during PGC specification. Besides all the embryonic developmental events, XCI is also tightly linked to pluripotency in vitro. XCI occurs during in vitro differentiation of ESCs; meanwhile XCR is observed when pluripotency is artificially generated in induced pluripotent stem (iPS) cells [92], by nuclear transfer [93] or cell fusion [94]. Besides the mechanistic connection of the regulatory mechanisms of XCI and pluripotency, dosage compensation is also critical for early embryonic development. Embryos with XCI defects are early embryonic lethal [95]. Mutant ESCs with XCI defects could not survive during in vitro differentiation.

Although the undifferentiated female mouse ESCs, with two active Xs per cell, are pluripotent, these cells are more difficult to be maintained in high quality in culture. The culprit is believed to be the lack of dosage compensation in these cells. Therefore, X-linked gene dosage is essential for pluripotency in females. Furthermore, XCI is an interesting topic of pluripotency also because the inactivation status of the X chromosome is arguably the most stringent test available to access the pluripotency in the current human ES cell lines. We will discuss on this in more details in the following paragraphs.

XCI occurs in a female-specific and allele-specific manner, which cannot be achieved by the core regulatory circuitry of pluripotency alone. The tight correlation between pluripotency and XCI must be achieved by pluripotency factors controlling key XCI regulators. The search for the direct connections of pluripotency factors with the key regulatory factors of XCI has started. The chromosome-wide gene silencing of XCI is triggered by an ncRNA, called Xist (Xi specific transcript) [97-99]. The X-linked Xist gene is expressed at a low level from both Xs in each undifferentiated female ESC. Upon differentiation, the transcription of Xist is allele-specifically up-regulated along the chosen Xi. The up-regulated Xist RNA transcripts spread and coat the chromosome territory in cis to establish multiple layers of epigenetic modifications along the chromosome. The chromosome-wide gene silencing is then achieved. In RNA FISH, the Xist RNA can be visualized as a cloud signal (the Xist cloud) enveloping the Xi chromosome territory [100]. Interestingly, Nanog expression is correlated with Xist repression during epiblast lineage specification in blastocyst [101]. Over-expression of Nanog accelerates Xist repression during epiblast lineage specification without affecting XCR status [102]. However, Nanog knockout in ESCs only causes minor up-regulation of Xist expression [103]. Different from Nanog, deletion of Oct4 in ESCs caused Xist cloud formation in a small fraction of cells [103,104]. Key pluripotency factors have broad effects on ESCs. Therefore, direct manipulation on pluripotency factors may not reveal their direct relation with XCI. It is important to identify the DNA binding site of pluripotency factors along the key genes involved in XCI. Deletion of these DNA binding sites can help to reveal the direct connection of pluripotency factors and XCI. By chromatin immunoprecipitation, one prominent binding site of Oct4, Nanog and Sox2 was identified within Xist intron 1 [103]. A few other binding sites of Oct4 and Nanog were also identified within or close to the Xist gene body [104,105]. A binding site of Rex1, cMyc and Klf4 was identified at the 5’ region of the Xist gene (an anti-sense RNA of Xist) [106]. Binding sites of Oct4, Sox2 and Nanog can also be found upstream of the RNF12 gene [26], which encodes an E3 ubiquitin ligase involved in sensing X chromosome copy number per cell during the initiation of XCI. The binding sites of pluripotency factors within Xist intron 1 have been investigated by a few studies. Knocking out this DNA region did not cause a significant up-regulation of Xist [107]. It is possible that Tisx and pluripotency factors work synergistically to repress Xist expression. Double deletion of the intron 1 binding site and Tisx confirmed this notion [108]. However the double deletion only caused the up-regulation of the Xist in a small fraction of cells. It should be noted that the double knockout was carried out on a Xist transgene in male cells. It is worth to repeat the knockout on the endogenous DNA locus in female cells. It is also important to re-check the binding pattern of the pluripotency factors along the double knockout DNA allele. In addition, pluripotency factors and co-factors are known to be involved in long distance chromatin interactions, for example the promoter and enhancer interaction [62]. It is important to search for the DNA region, which interacts with the Xist/Tisx genes but is located far away from the Xist/Tisx gene body. Furthermore, Oct4, Nanog and Sox2 are all expressed at a similar level to ESCs in a different cell type, the epiblast stem cells (EpiSCs, see the following paragraph for details) [109,110]. However, in female EpiSCs, XCI has been established. Therefore, the three key pluripotency factors alone cannot fully explain the Xist repression in EpiSCs.

Besides its mechanistic connection with pluripotency, XCI is also a unique epigenetic identity, which distinguishes the primed and naïve pluripotency in mouse. The epiblast cells from a post-implantation embryo can be cultured in vitro to establish a cell line called epiblast stem cells [109,110]. Oct4, Sox2 and Nanog are all expressed in EpiSCs at levels comparable to ESCs. EpiSCs can be differentiated into all three germ layers in vitro and form teratoma in the nude mice. Therefore, EpiSCs are pluripotent. Different from ESCs, EpiSCs show different colony morphology, require different culture conditions (FGF), and rely on different intracellular signaling pathways (Activin/Nodal) to maintain pluripotency. In addition, EpiSCs are unable to pass more stringent tests on their pluripotency, for example EpiSCs cannot give germline transmission. Furthermore, in female EpiSCs, XCI already occurs. It has been proposed the pluripotency carried in EpiSCs is naïve pluripotency, and the pluripotency carried in EpiSCs is primed pluripotency [111]. Since both ESCs and EpiSCs express Oct4, Nanog and Sox2, comparing the gene expression profiles of the two cell types may help to identify new genes critical for naïve pluripotency. It is already known that Klf4 shows a much higher expression level in ESCs than EpiSCs [109]. Forced expression of Klf4 in EpiSCs and switching the culture condition to ESC culture condition could convert the primed pluripotency in EpiSCs to naïve pluripotency, in which the Xi was reactivated [112]. Meanwhile, the same conversion could also be achieved by prolonged culture of post-implantation epiblast cells in ES culture conditions [113]. However both methods showed low conversion efficiency. Comparing the silencing status of Xi in EpiSCs and in somatic cells also generated some interesting findings [114]. The Xi in EpiSCs, which does not carry the enrichment of macroH2A (a histone variant), was still permissive for reactivation after the nucleus of mouse cell was transferred into xenopus germinal vesicles. The macroH2A-enriched Xi from mouse fibroblast was resistant for reactivation in this experimental system. In summary, the similarity and the difference between ESCs and EpiSCs are interesting topics for pluripotency. The XCI status is one epigenetic signature, which shows the difference between the two types of pluripotency.

All the foregoing knowledge about pluripotency and XCI were generated in mouse studies. Human ESCs (hESCs) are studied less extensively, but are more important for future regenerative medicine. Interestingly, hESCs are similar to mouse EpiSCs in colony morphology, culture conditions, and the required internal signaling pathway to maintain pluripotency [109,110]. hESCs can also pass low stringent tests on pluripotency, such as in vitro differentiation and teratoma formation. However, more stringent tests, such as chimera formation, cannot be applied in hESC studies due to the ethical concerns. It is an enormous concern that the current hESC lines may resemble the primed pluripotency in mouse. Indeed, XCI has occurred in many hESC lines [115]. Some of the cell lines even showed an inactive X chromosome missing the Xist RNA coating. hESC lines with two Xa per cell are available, but maintaining such cell lines in culture requires scrupulous care. Similar to hESCs, different XCI status in human iPS cells has been observed. Some groups reported that Xi was retained in female human iPSC cells [116-118], while others reported partial XCR [119,120]. These observations clearly show that the culture conditions need to be further improved to maintain hESCs in culture more stably. Many further believe that naïve hESC lines can be achieved by improved culture conditions.
Indeed, culturing the cells under physiological oxygen level [121], in altered culture medium [122] and by forced gene expression [123] help to establish female hESCs lines with two Xa per cell. Similar to hESCs and human iPS cells, different observations on the XCI status have been made on human pre-implantation embryos. A recent study on the pre-implantation human embryos observed the onset of XCI dramatically different from the one in mouse [124]. Xist cloud formation was observed in the ICM on all the X alleles (the male X, and the two female Xs); and Xist up-regulation did not trigger gene silencing. Interestingly, a different observation on the onset of XCI in human embryos was made in a separate study [125]. One possible explanation on these different observations is that the current techniques of in vitro handling human pre-implantation embryos needs to be further optimized. On the other hand, whether the naive pluripotency can be harvested from human embryos in culture is still a concern [111]. The egg cylinder, an embryonic structure where the EpiSCs are isolated, and the diapause, a phenomenon in which the development of a blastocyst is “paused” in the uterus for a later implantation, may explain why naïve pluripotency can be stably harvested from mouse embryos. However, egg cylinder and diapause are specific to rodents. The embryonic development programs of other mammals, such as the human, may not be permissive for a stable harvest on the naïve pluripotency. In summary, whether hESCs lines with naïve pluripotency can be harvested or stably preserved in culture conditions is a pressing and challenging issue. The functional criteria, such as chimera formation and germ line transmission, which can be used to distinguish the primed and naïve pluripotency in mouse cannot be applied to human cells due to moral concerns. XCI is a unique epigenetic identity, which may help to distinguish the primed and naïve pluripotency in hESCs.

**Transcription Factors in Reprogramming**

By expression of several defined factors, differentiated cells can be reprogrammed into a pluripotent state, namely induced pluripotent stem cells. Many of these reprogramming factors are indeed core pluripotency or pluripotency-associated transcription factors. The most widely used Yamanaka factors are Oct4, Sox2, Klf4 and c-Myc, and Yu et al. [9] used OCT4, SOX2, NANOG and LIN28 to reprogram human fibroblasts [7,9]. To reprogram somatic cells to the pluripotent state, these exogenous reprogramming factors might activate the endogenous core transcriptional regulatory network of pluripotency, as well as transcriptional co-factors, build up associated transcription factors, the physical interactions of these epigenetic regulators cooperate with transcription factors to their transcriptional activity to activate and maintain the transcriptional profiles. Comparing the expression profiles of partially reprogrammed cells and iP/iPS cells, transcriptional regulators are not sufficiently activated in partially reprogrammed cells, further demonstrating the importance of the transcriptional regulatory network in the establishment and maintenance of pluripotency. It have been shown that the genome-wide binding profiles of the four Yamanaka factors have significant overlap in iP/iPS and ES cells, but not in partially reprogrammed cells. In partially reprogrammed cells, the binding of Oct4, Sox2 and Klf4 to their targets is more severely compromised than the binding of c-Myc. It is possible that other factors which are absent in partially reprogrammed cells, such as Nanog, are required for the binding of Oct4, Sox2 and Klf4 to their targets [126].

In search for other reprogramming factors, more pluripotency-associated transcription factors have been shown to have reprogramming activities. For example, iP cells can be derived from mouse embryonic fibroblasts (MEFs) with Esrbr, Oct4 and Sox2 [127]. Nr5a2 can reprogram MEFs with Sox2 and Klf4, without the need of Oct4 or c-Myc [56]. Tbx3, together with Oct4, Sox2 and Klf4, allows the derivation of iP cells with improved quality: iP cells reprogrammed with Tbx3 contribute to germ cells in the gonad more efficiently than iP cells derived without Tbx3. Consequently, iP cells reprogrammed with Tbx3 show higher germine transmission frequency [58]. PRDM14 also enhances the reprogramming efficiency of human fibroblasts by the four Yamanaka factors [47]. Esrrb, Nr5a2, Tbx3 and PRDM14 all bind to at least two of the Oct4, Sox2 and Nanog promoters (Table 2). Moreover, these four reprogramming factors share large amount of common target genes with Oct4, Sox2 and Nanog [26,47,51,56,58]. It has been suggested that occupying by multiple factors is associated with stronger transcriptional activation [126]. Therefore, the reprogramming activities of these pluripotency-associated factors can be explained by their transcriptional activity to activate and maintain the transcriptional regulatory network of pluripotency.

As we discuss in the section of integration of external signals into transcriptional activity, signaling events can be integrated into the transcriptional regulatory network of pluripotency. Thus, through modulating the activities of signaling pathways, the efficiency of somatic cell reprogramming can be improved. Indeed, many chemicals targeting signaling pathways have been demonstrated to promote reprogramming. For example, inhibition of MEK and GSK3 pathways by PD0325901 and CHIR99021 allows completely reprogramming to the ground state of pluripotency from MEF-derived and neural stem cell-derived pre-iPS cell clones [128]. Wnt3a enhances the iPS derivation rate from MEFs by Oct4, Sox2 and Klf4 by around 20-fold [129]. An inhibitor of TGF-β signaling, RepSox (also named E-616452), allows derivation of iPS cells with Oct4, Klf4 and c-Myc, in the absence of Sox2. One possible mechanism of RepSox to facilitate reprogramming is to activate Nanog expression [130]. Moreover, simultaneous inhibition of MEK, GSK3 and TGF-β signaling by PD0325901, CHIR99021 and A-83-01, has been applied in the derivation of rat and human iP/iPS cells, which resemble mouse ESCs [131].

**Summary**

It becomes clear that transcriptional regulation plays a pivotal role in pluripotency maintenance. Illustrated in Figure 1, Oct4, Sox2 and Nanog form the core transcriptional regulatory circuitry for pluripotency [23-27]. Through regulating the expression of Oct4, Sox2 and Nanog, some pluripotency-associated transcription factors, including Sal4, Esrrb, Nr5a2, Tbx3, Zfx, Klf4, Foxo1, Foxp1, Prdm14 and Nac1, stabilize the core transcriptional regulatory circuitry [25,26,39,47,51,56,58]. Other pluripotency-associated transcription factors, such as Dax1 and Zifp281, negatively regulate the expression or the transcriptional activity of core pluripotency factors, thus facilitating the destruction of the core transcriptional regulatory circuitry during differentiation [25,32,44,45]. In addition to the transcriptional regulatory network among the core transcriptional regulatory circuitry and pluripotency-associated transcription factors, the physical interactions of these transcription factors, as well as transcriptional co-factors, build up another regulatory network for pluripotency [31,59,60]. Moreover, epigenetic regulators cooperate with transcription factors to maintain the unique transcriptional profiles of ESCs. Meanwhile, the pluripotency status is connected with and further stabilized by external signals, so the pluripotent cells are able to respond to environmental cues to initiate proper differentiation program [52-55].

Despite the fast accumulating data of transcriptional regulation in ESCs, there are many important questions to be answered. Why do ESCs apply such a complicated network to regulate the expression of Oct4, Sox2 and Nanog? How is the expanded transcriptional regulatory network of pluripotency disrupted during ESC differentiation? How do external signals direct ESC differentiation? Ultimately, our
understanding of transcriptional regulation in ESCs should be applied to further improve derivation of novel pluripotent stem cells, as well as directed differentiation of ESCs into desired cell types.

Acknowledgement

This work was supported by grants from the National Natural Science Foundation of China (No. 90919009), the National Basic Research Program of China (No. 2009CB941000 and No.2010CB833603), the transgenic program, the Ministry of Agriculture of China (No. 2009ZX08006-010B and No. 2009ZX08006-011B) and the Singapore Stem Cell Consortium (SSCC/09/015).

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