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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 trophectodermal 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 trophectoderm [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 at 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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[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, Sall4, Dax1, Esrrb, Nr5a2, Tbx3, Zfx, Ronin, Klf4, Foxd3, Foxo1, Foxp1, Prdm14, Zic3, Nac1 and Zfp281 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, Tcf3, Smad1 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, Foxp1, Prdm14, Nac1 and Zfp281, have been established [25,26,39,45,47,51,56-58]. 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 [25,26,39,45,47,51,56,58]. However, not all the pluripotency-associated factors enhance the stability of the core transcriptional circuitry. For example, Zfp281 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]. Negative regulation of the core transcriptional circuitry might be important for the disruption of the circuitry during ESC differentiation. Consistently, *Zfp281* 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.

Genes	Oct4	Sox2	Nanog
Sall4	+	+	+
Dax1	-	+	+
Esrrb	+	+	+
Nr5a2	+	+	-
Tbx3	+	+	-
Zfx	-	-	-
Ronin	-	-	-
Klf4	+	+	+
Nac1	+	+	+
Zfp281	+	+	+
Zic3	+	+	+
Foxo1	+	-	+
Foxd3	-	+	+
Foxp1	-	+	+
Prdm14	+	+	+
Tcf3	+	+	+
Smad1	-	-	-
Stat3	+	-	+

Data in this table is extracted from [26]

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

TFs	*Oct4*	*Sox2*	*Nanog*	Ref
Sall4	+	+	+	[29, 30]
Dax1	+	+	+	[25]
Esrrb	+	+	+	[26]
Nr5a2	+	-	+	[56]
Tbx3	+	+	-	[58]
Zfx	+	+	+	[26]
Ronin	-	-	-	[57]
Klf4	+	+	+	[25,26,39]
Nac1	+	+	+	[25]
Zfp281	+	+	+	[25,45]
Zic3	ND	ND	ND	-
Foxo1	+	+	ND	[48]
Foxd3	ND	ND	ND	-
Foxp1	+	-	+	[50]
Prdm14	+	+	-	[47,51]
Tcf3	+	+	+	[27,54,55]
Smad1	+	+	+	[26]
Stat3	+	+	+	[26]

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

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 Prdm14 in mouse ESCs. Chia et al [47] identified PRDM14 as a regulator of *OCT4* expression in human ESCs. They also demonstrated that knockdown of *Prdm14* in mouse ESCs has no effect on the expression of *Oct4* and *Sox2*. Moreover, *Prdm14* 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 Prdm14 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 *Prdm14* in mouse ESCs not only reduces the expression of *Nanog* and *Sox2*, but also induces

extraembryonic endodermal differentiation. Notably, in the second study, a substantial fraction of *Prdm14* knockdown cells maintains ESC colony morphology and *Nanog* expression, while other cells differentiate into the extraembryonic endodermal lineage [51]. This phenotype might be explained by either heterogenous knockdown efficiency of *Prdm14* or inherent heterogeneity of mouse ESCs. Given that no phenotype was observed upon *Prdm14* knockdown in mouse ESCs in the first study, it is more likely that the discrepancy between these two studies is due to differential knockdown efficiency of *Prdm14*.

Moreover, many of these pluripotency-associated transcription factors interact with Nanog, Oct4 and/or Sox2 proteins (Table 3) [31,59,60]. It has been demonstrated that Dax1 inhibits the transcriptional activity of Oct4 through its association with Oct4 protein [32]. Together with Nr5a2, Dax1 also activates the expression of *Oct4* gene [34]. It appears that Dax1 plays a dual role in regulating the transcriptional activity of *Oct4*. Consistently, either knockdown or over-expression of *Dax1* causes ESC differentiation [32,33]. Oct4 also interacts with Foxd3, and inhibits Foxd3 to activate endodermal genes *FoxA1* and *FoxA2* [61]. It is not completely understood how other interactions among these pluripotency transcription factors affect their transcriptional activities, and contribute to pluripotency maintenance. Further studies are required to characterize the biological functions of these interactions.

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 (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 (Paf1c), reduces *Oct4* expression level in ESCs and results in ESC differentiation, suggesting an essential role of the whole Paf1c in pluripotency maintenance. Paf1c occupies the promoters of many pluripotency genes, such as *Oct4*, *Sox2* and *Nanog*. Paf1c 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.

TFs	Oct4	Sox2	Nanog	Ref
Sall4	+		+	[31,60]
Dax1	+		+	[31,32,59]
Esrrb	+		+	[36,59]
Klf4	+			[60]
Nac1	+		+	[31,59]
Zfp281	+	+	+	[31,45]
Foxd3	+			[61]

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

Co-operation of Transcriptional Regulation and Epigenetic Regulation

As the substrate of transcription, chromatin is regulated by various epigenetic modifications and high-order chromatin structure. Using chromatin interaction analysis by paired-end tag (ChIA-PET) sequencing, a CTCF-chromatin interactome map in mouse ESCs has been constructed. This map revealed that CTCF-associated interactions facilitate three-dimensional chromatin organization, clustering genes with coordinated expression, promoting communications between regulatory elements over long distances, and demarcating nuclear lamin-chromatin interactions [68]. Using the same technique, widespread promoter-centered chromatin interactions were also detected in human cells [69]. These studies provided a three-dimensional chromatin picture to understand transcriptional regulation in ESCs. It is clear that epigenetic regulation plays critical roles in pluripotency. In this review, we only address the interactions and co-operations between transcription factors and epigenetic regulators in ESCs. More detailed information regarding epigenetic regulators in pluripotency can be found in other reviews [4,5,70].

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 NuRD, the polycomb repression complex 1 (PRC1), the Lsd1 histone demethylase complex, Wdr5 and the chromatin remodeling complexes Chd1, ISWI, SWI/SNF, INO80 and Trrap/p400, as well as the DNA methyltransferases Dnmt3a and Dnmt3l [31,59,60,71]. 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 Trrap/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 Rybp) 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 Jmjd2c, whose expression is positively regulated by Oct4, catalyzes the demethylation of H3K9Me3 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 appears 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, knockout 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 Klf4, thus suppressing the transcriptional activity of Klf4 [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 have 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 *Lrh-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 imprinted 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

[96]. 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 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 *Tsix* 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 *Tsix* and pluripotency factors work synergistically to repress *Xist* expression. Double deletion of the intron 1 binding site and *Tsix* 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 and 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/Tsix* genes but is located far away from the *Xist/Tsix* 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 ESCs.

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 ESCs 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 iPS 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 hESC 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 naïve 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 hESC 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 circuitry for pluripotency, as well as the expanded transcriptional regulatory network of pluripotency, eventually remodeling the transcriptional profiles. Comparing the expression profiles of partially reprogrammed cells and iPS/ES 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 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, iPS cells can be derived from mouse embryonic fibroblasts (MEFs) with Esrrb, 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 iPS cells with improved quality. iPS cells reprogrammed with Tbx3 contribute to germ cells in the gonad more efficiently than iPS cells derived without *Tbx3*. Consequently, iPS cells reprogrammed with *Tbx3* show higher germline 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 iPS cells, which resemble mouse ESCs [131].

Summary

It becomes clear that transcriptional regulation plays a pivot 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 Sall4, 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 Zfp281, 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

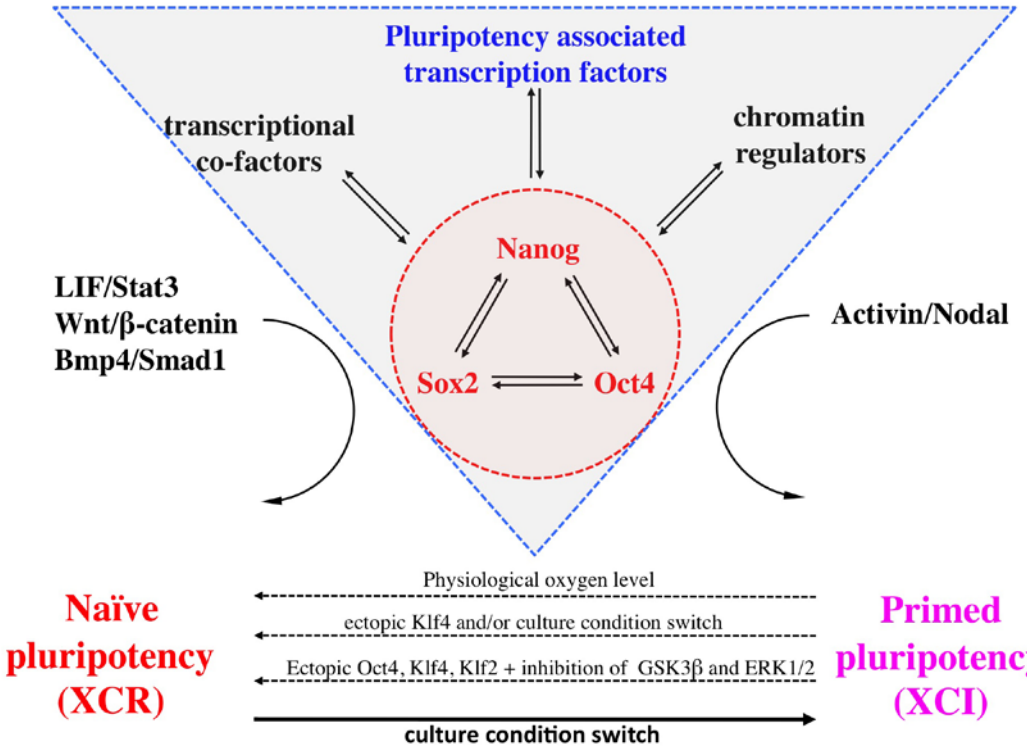

Figure 1: Schematic illustration of pluripotency maintenance by transcriptional regulation, epigenetic regulation, and signaling pathways. Oct4, Sox2 and Nanog form the core transcriptional regulatory circuitry for pluripotency (highlighted with a red circle). Pluripotency-associated transcription factors, together with the core transcriptional regulatory circuitry, form the expanded transcriptional regulatory network of pluripotency. Transcriptional co-factors and chromatin regulators collaborate with transcription factors to maintain pluripotency. Different signaling events facilitate the maintenance of the naïve or primed pluripotency.

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.

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