PERSPECTIVE

Open conformation chromatin and pluripotency

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Pluripotency, the ability of a cell to give rise to all of the cells of an organism, is a fascinating and mysterious characteristic of embryonic stem (ES) cells. Recent extensive molecular studies of mouse ES cells have revealed the roles played by transcription factor networks and epigenetic processes in the maintenance of ES cell pluripotency (Niwa 2007). However, no direct evidence to connect these two mechanisms had been reported until in the previous issue of *Genes & Development*, in which Loh et al. (2007) presented new evidence concerning this missing link.

Transcription factor network to maintain pluripotency

Analysis of transcription factors expressed in ES cells in an undifferentiated state-specific manner has resulted in the identification of several key transcription factors involved in the maintenance of pluripotency. Of these, the POU family transcription factor Oct3/4 is regarded as pivotal because either its loss of function or its overexpression completely abolishes self-renewal and induces differentiation (Niwa 2007). The Sry-related transcription factor Sox2 was first identified as a partner of Oct3/4 working to activate the transcription of target genes (Yuan et al. 1995). However, a recent report has indicated that the unique function of Sox2 may be the indirect activation of Oct3/4 transcription (Masui et al. 2007). The NK2-class homeobox transcription factor Nanog is also regarded as a component of the core transcription factor network along with Oct3/4 and Sox2 because it is required for the maintenance of pluripotency and has a dominant function in supporting the self-renewal of mouse ES cells in the absence of leukemia inhibitory factor (LIF), the essential cytokine in conventional mouse ES cell cultures (Chambers et al. 2003; Mitsui et al. 2003). Since these three genes possess binding sites for all three of these transcription factors, and the transcriptional activation of these genes by Oct3/4 and Sox2 has been confirmed, it may be that they act as a self-organizing network that sustains the expression levels needed to maintain pluripotency (Niwa 2007).

Epigenetic mechanism to maintain pluripotency

In the past few years, several interesting findings concerning the epigenetic features of ES cells have been reported. The general abundance of transcriptionally active chromatin marks, such as trimethylation of Lys 4 of histone H3 (H3K4me3) and acetylation of histone H4 (H4Ac), has been noted (Azuara et al. 2006). In contrast, the histone marks associated with transcriptionally inactive genes, such as trimethylation of Lys 27 of histone H3 (H3K27me3), are generally more scarce, but are nonetheless enriched in specific regions of the genome (Bernstein et al. 2006). These regions, designated as the bivalent domains, are composed of areas containing short active and long silent histone marks, and frequently overlap the regions in which the binding sites for Oct3/4, Sox2, and Nanog are clustered (Boyer et al. 2005). For this reason, the bivalent domain is generally considered to function as a center that maintains pluripotency in undifferentiated cells by repressing the expression of genes activated immediately after the induction of differentiation. Additionally, polycomb-repressing complex 2 (PRC2) is regarded as a key regulator mediating the gene repression marks recruited to the bivalent domains by pluripotency-associated transcription factors (Lee et al. 2006). However, the functional significance of PRC2 in the maintenance of pluripotency should not be overestimated, because the loss of function of PRC2 components, such as *Eed* and *Suz12*, results in up-regulation of differentiation-associated genes but does not abolish self-renewal in mouse ES cells, indicating that their function is not essential to maintaining pluripotency (Montgomery et al. 2005; Pasini et al. 2007).

There have been no reports to date identifying the mechanisms by which ES cells are able to generate an open chromatin state at the genome-wide level, although there are several possible mechanisms for keeping chromatin in an open configuration. As the acetylation of histone H3 and H4 is important in transcriptional activation, both by promoting open chromatin structural arrangements and by competing with silent histone marks such as di- and trimethylation of Lys 9 of histone H3 (H3K9me2 and H3K9me3), it follows that either the activation of histone acetyltransferases (HATs) or inactivation of histone deacetylases (HDACs) should contribute to the rearrangement of chromatin into an open

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conformation. Maintenance of H3K4me3 by activation of a histone methyltransferase (HMTase) and/or inactivation of a histone demethylase (HDMase) specific to this modification would presumably result in keeping chromatin open. It was recently reported that unmethylated H3K4 is specifically recognized by a member of the DNA methyltransferase (Dnmt) family, Dnmt3l (Ooi et al. 2007). Dnmt3l lacks the activity of other Dnmts, but it recruits Dnmt3a, which functions to activate de novo methylation of DNA. DNA methylation is recognized by methyl DNA-binding proteins, such as MeCP2 and MBD1, and triggers the formation of heterochromatin by recruiting heterochromatin proteins (HP). Since ES cells express high levels of both Dnmt3l and Dnmt3a, this mechanism should be countered by proteins that would maintain the methylation of H3K4 in order to keep chromatin in its open configuration. Conversely, exclusion of repressive histone marks—such as H3K9me2, H3K9me3, and H3K27me3, which recruit HPs to form heterochromatin by activating HDM and/or inactivating HMTs specific for these modifications—represents a possible mechanism for generating open chromatin in ES cells.

Until recently, the enzymatic activity of a HDMase in the demethylation of a methylated histone lysine was only identified in the LSD protein, which removes H3K4me1 and H3K4me2 (Klose and Zhang 2007). However, in a breakthrough finding reported in 2006, Tsukada et al. (2006) used a biochemical approach to purify a robust HDMase activity with specificity toward Lys 36 of histone H3 from mammalian cells, and identified Jumonji-C (JmjC) domain-containing histone demethylase 1A (JHDM1A) as the molecule responsible for this HDMase activity. Using the same strategy, a novel H3K9-specific HDMase was identified as JHDM2A (Yamane et al. 2006). The JmjC domain was then to be the catalytic domain of HDMase (Klose and Zhang 2007). Jumonji (abbreviated Jmj) means “cruciform” in Japanese, in reference to the morphological phenotype of Jmj mutant mice, in which the normal neural groove is crossed by abnormal grooves on the neural plates (Takeuchi et al. 1995). There are multiple genes containing JmjC domains in the mouse genome, and JHDM3/JHDM2 enzymes have been identified as HDMases that preferentially demethylate H3K9me3, H3K9me2, H3K36me3, and H3K36me2 (Klose et al. 2006), although their functions in ES cells have not yet been assessed.

JmjC members cross genetic and epigenetic mechanisms to maintain pluripotency

Some researchers have emphasized the importance of genetic mechanisms in maintaining pluripotency, while others have suggested that epigenetic control plays the dominant role, creating something of a chicken-and-egg impasse. However, a recent discovery of great importance has provided a clue to solve this puzzle. Takahashi and Yamanaka (2006) found that transfection of four transcription factor genes (*Oct3/4*, *Sox2*, *Klf4*, and *Myc*) allows the establishment of pluripotency in mouse fibro-

blast cells. In the resulting cells, which Takahashi and Yamanaka (2006) designated induced pluripotent stem (iPS) cells, the global pattern of histone marks is switched from the somatic cell pattern to the pluripotent cell pattern (Maherali et al. 2007), indicating that the transduction of the four transcription factors is able to change both the genetic and the epigenetic states, and that the genetic mechanism takes precedence in this context. Of these four transcription factors, *Myc* is known to be directly susceptible to epigenetic change (Yamanaka 2007). *Myc* is a well-known oncogene that binds to a specific DNA sequence as a transcriptional activator of specific target genes, and also functions as a mediator of DNA replication. It has been reported that one of the target genes of *Myc* is *Gcn5*, which encodes a HAT, and that its transcriptional activation is critical to mediate the function of *Myc* in promoting cell proliferation (Knoepfler et al. 2006). This is in accordance with a mechanism for promoting the open chromatin structure needed to establish and maintain pluripotency, proposed above. However, *Gcn5* function is not essential for early development, and *Gcn5*-null ES cells can be established (Lin et al. 2007), suggesting that it does not provide the sole mechanism at work in keeping pluripotent chromatin in an open conformation.

But what of the roles of the other three transcription factors in modulating the epigenetics of pluripotent cells? Since *Sox2* and *Klf4* function cooperatively with *Oct3/4* (Nakatake et al. 2006), it seems evident that research should focus on elucidating the function of *Oct3/4*, the pivotal gene in this particular network. Lists of the target genes of *Oct3/4* have been developed using a variety of approaches (Boyer et al. 2005; Loh et al. 2006; Matoba et al. 2006). The comparison of global gene expression profiling with cDNA microarrays of ES cells after repression of *Oct3/4* and chromatin immunoprecipitation (ChIP), in combination with a genome-wide location technique for *Oct3/4*, yielded 372 putative primary target genes, which contain several genes encoding epigenetic modifiers (Matoba et al. 2006). From these data, in the previous issue of *Genes & Development*, Loh et al. (2007) identified the two JmjC family members encoding HDMases, *Jmjd1a* and *Jmjd2c*, as *Oct3/4* targets. They first identified clusters of *Oct3/4*-binding sites by ChIP-paired end ditag analysis (ChIP-PET) and confirmed their functional significance for transcriptional activation by reporter assay. Depletion of *Jmjd1a* by small interfering RNA (siRNA) in ES cells resulted in the induction of differentiation with a global increase in H3K9me2, whereas depletion of *Jmjd2c* induced differentiation with a global increase in H3K9me3, confirming that their HDMase activity is linked to the maintenance of pluripotency. They next identified the genes whose expression was affected by depletion of these JmjC genes by cDNA microarray analyses. One of the genes down-regulated after depletion of *Jmjd1a* is *Tcl1*, which encodes the adaptor protein of Akt (Thymoma viral proto-oncogene 1) that enhances its enzymatic activity (Teitell 2005). Akt1 is a key kinase in the Ras/PI3K/Akt1 signaling pathway that is important for propagation of mouse

ES cells. It has been reported that *Tcl1* is expressed in ES cells in a stem cell-specific manner, and that its promoter activity is positively regulated by Oct3/4 (Matoba et al. 2006). Moreover, knockout of *Tcl1* in mouse embryos leads to preimplantation lethality (Narducci et al. 2002), and knockdown of *Tcl1* in ES cells results in either the induction of differentiation or the repression of proliferation (Ivanova et al. 2006; Matoba et al. 2006). Loh et al. (2007) found that the level of H3K9me2 and H3K9me3 in the *Tcl1* promoter increased in *Jmjd1a* knockdown ES cells with a reduction in Oct3/4 recruitment, and that *Jmjd1a* proteins are recruited to this promoter, indicating that removal of H3K9me2 from the promoter by *Jmjd1a* is required for proper Oct3/4-dependent transcriptional activation of *Tcl1* in ES cells. Interestingly, *Tcl1* is functionally significant as the target of *Jmjd1a*, since the ectopic expression of *Tcl1* prevents the differentiation of ES cells induced by the depletion of *Jmjd1a*. They identified *Nanog* as the *Jmjd2c* target and confirmed the recruitment of *Jmjd2c* to the *Nanog* promoter. Depletion of *Jmjd2c* resulted in an increase of H3K9me3 with enrichment of HP1 β , but left recruitment of Oct3/4 unaffected. *Nanog* also has functional significance as the target of *Jmjd2c*, because the ectopic expression of *Nanog* prevents differentiation of ES cells induced by depletion of *Jmjd2c*. These findings show that Oct3/4 activates transcription of target genes either directly as a transcriptional activator or indirectly by transcriptional activation of chromatin modifiers that keep target sites in an accessible chromatin conformation.

Is pluripotency the default state?

This important result provides the missing link between genetic and epigenetic mechanisms in the establishment and maintenance of pluripotency. As a genetic mechanism, a self-organizing network of transcription factors prevents differentiation and promotes proliferation, enabling the continuous self-renewal of ES cells. The epigenetic processes should be under the control of the pluripotent transcription factor network, functioning to keep the target genes of these transcription factors accessible and thereby allowing the self-organizing network to be maintained. The existence of such a positive feedback loop involving genetic and epigenetic systems has been hypothesized but never conclusively shown. The direct transcriptional regulation of *Jmjd1a* and *Jmjd2c* by Oct3/4 is the first evidence to support this model. Oct3/4, a key component of the pluripotent transcription factor network, activates transcription of HDMase genes, and these gene products are recruited to the target genes of Oct3/4 to maintain accessibility. Since one of the target genes of *Jmjd2c* is *Nanog*, which is also an important component of the pluripotent transcription factor network and is supposed to contribute to the transcriptional activation of Oct3/4, here the positive feedback loop is closed. During the establishment of iPS cells, *Myc* might enhance the accessibility of target genes for the other three transcription factors by stimulating DNA replication and/or transcriptional activation of *Gcn5*-

encoding HAT. Once Oct3/4 has the chance to activate *Jmjd1a* and *Jmjd2c*, these gene products manifest their HDMase activity by removing H3K9me2 and H3K9me3 from target genes to allow activation by Oct3/4. Activation of the target genes of Oct3/4, including *Nanog*, promotes the establishment of the pluripotent transcription factor network, ultimately resulting in the establishment of pluripotency in somatic cell nuclei. Of course, to test this model, the role of these JmjC family genes in the generation of iPS cells should be examined.

It is now possible to propose that the pluripotent state is a default state of genetic systems (Fig. 1). The transcription factor network to determine pluripotency might be self-organized by a positive feedback mechanism, and this also might be true for other transcription factor networks that confer differentiated phenotypes. Therefore, if all genetic information is freed from epigenetic repression, all transcription factors begin to establish their preferred network via self-activation. However, in such a scenario, the networks must be mutually exclusive in order to give rise to a single phenotype in any given cell. If exclusion occurs simply by chance, all phenotypes would arise with equal frequency. That said, it remains possible that one transcription factor network is extremely stable due to the extraordinary power of its positive feedback and its ability to repress other networks. In the first differentiation event in mouse development, trophoblast is segregated from the pluripotent cell lineage via competition between two transcription factors—Oct3/4 and *Cdx2* (Niwa et al. 2005). These two transcription factors possess positive feedback loops that activate their own expression, respectively, and a complex formed by the two factors competes against the activation of both. In morula-stage embryos, *Cdx2* might exclude Oct3/4 via a signal from polarized epithelium, while the opposite may occur in the inner cells lacking such a polarity signal, suggesting that pluripotency is dominant in the default state. The dominant character of the pluripotent network has been shown by cell fusion experiments. When pluripotent stem cells are fused to differentiated cells, the resulting tetraploid cells tend to acquire pluripotency (Tada et al. 2001). Therefore, the ability of the pluripotent transcription factor network to promote an epigenetic mechanism to keep chromatin open may serve as an element of the positive feedback mechanism that maintains the network. This may be the reason why pluripotency can be established in somatic cell nuclei more easily than had been expected previously.

Further genetics of the epigenetic mechanism in ES cells

Activation of JmjC by Oct3/4 might not be the sole mechanism linking genetic and epigenetic systems. As mentioned above, there are several mechanisms other than the removal of silent histone marks for maintaining euchromaticity, and the introduction of histone marks for active transcription—e.g., H3K4me3—might be especially important. Methylation of H3K4 is introduced by

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Figure 1. A model of the default pluripotent transcription factor network that arises from open chromatin. The unique gene expression profile specific to a particular differentiation state is thought to be generated by a combination of genetic (the presence of a set of transcription factors) factors, as shown for the differentiated state. In the pluripotent state, most of the epigenetic repression of genetic information is removed, resulting in the allowance of basal transcription for virtually all genes. In this situation, genes exhibiting positive feedback regulation—i.e., those encoding transcription factors that activate their own promoters—become activated. Competition between such genes ensues, resulting in the selection of the most potent and stable transcription factor network. When this transcription factor network has the ability to activate the transcription of genes encoding epigenetic modifiers and thereby to keep chromatin open, the characteristic pluripotent epigenome is stable in conjunction with the pluripotent transcriptome.

the SET domain-containing proteins belonging to the trithorax group, which contains six members (SET1a, SET1b, MLL1–4) in the mouse genome (Glaser et al. 2006). Knockout studies in ES cells have been reported for Mll1 and Mll2, but no obvious phenotype has been found (Ernst et al. 2004; Glaser et al. 2006). Since the global level of H3K4me3 was not altered in these knockout ES cells, it appears that there may be other genes responsible for the trimethylation of H3 Lys 4 in ES cells. It was recently reported that Mll2 interacts with UTX, the JmJc family member that specifically demethylates H3K27me3 (Issaeva et al. 2007). The coupling of H3K4 methylation and H3K27me3 demethylation should have an important role in establishing the bivalent domain as well as its regulation during differentiation. Interestingly, in contrast to *Jmjd1a* and *Jmjd2c*, *Jmjd3* encodes a H3K27me3 HDM (Agger et al. 2007; Lan et al. 2007), and is up-regulated after repression of Oct3/4 in ES cells (Matoba et al. 2006). This suggests that differential HDM activities might be coordinated by the pluripotent transcription factor network.

High levels of chromatin remodeling activity might also contribute to keeping chromatin open. The essential role of *Snf2b* (*Brg1*, *Smarca4*)—encoding a core component of the SWI/SNF and ISWI family remodeling complex—in the self-renewal of F9 embryonal carcinoma cells has been reported (Sumi-Ichinose et al. 1997), and *Smarca4* belongs to the SNF2 family of DEAD/H-box-containing helicase superfamily and was identified as a putative primary target of Oct3/4 (Matoba et al. 2006). The NuRD (nucleosome remodeling and histone deacetylation) complex component Mbd3 is required for the differentiation of ES cells, and it functions in the inner cell mass of the blastocyst to expand into ES cells in vitro (Kaji et al. 2007). However, most of these genes are expressed ubiquitously, and careful analysis will be required to distinguish the functions specific to the maintenance of pluripotency from other housekeeping functions.

The hyperdynamics of architectural chromatin proteins has been pointed out as a unique characteristic of ES cells, and the nucleosome assembly factor HirA was

proposed as one of the factors responsible for this feature, since *HirA*-null ES cells exhibit elevated levels of unbound histones and the formation of embryoid bodies is accelerated in these mutant cells (Meshorer et al. 2006). In contrast to *HirA*, which acts as a chaperone of histone H3.3 in a DNA synthesis-independent manner, CAF1 acts as a chaperone of H3.1 in a DNA synthesis-dependent manner. It was recently reported that *CAF1*-null mouse embryos die at the 16-cell stage, suggesting it has an essential function in pluripotent cell populations (Houlard et al. 2006). In addition, a novel epigenetic feature of ES cells was revealed by Szutorisz et al. (2006), who showed that the proteasome acts on specific regulatory regions in ES cells to prevent incorrect transcriptional initiation to restrict permissive transcriptional activity while keeping the genes in a potentiated state. This system might contribute to keeping the threshold level of the promoter activity high for significant gene expression, thereby allowing the exclusive activation of the pluripotent transcription factor network, a hypothesis that has yet to be functionally confirmed.

For all epigenetic mechanisms, an important unsolved question remains. It has been clearly shown that *Jmjd1a* and *Jmjd2c* do not remove methylation of H3K9 ubiquitously, but that they are instead recruited to specific target genes to modulate local chromatin structure. This target-specific function has been observed in the proteasome. Since proteins there lack the ability to recognize specific DNA sequences, they should be recruited to the target genes by interaction with transcription factors binding a specific sequence. But which sequence-specific transcription factors work to recruit them? One possible and interesting candidate is none other than Oct3/4. Oct3/4 works as a transcriptional activator via interaction with multiple transcription factors in a context-dependent manner. In the case of PRC2, the role of Oct3/4 in recruiting it to target sites was suggested by the overlap of their binding sites in the genome (Lee et al. 2006). Oct3/4 cooperates with Sox2 in several cases, and with both Sox2 and Klf4 on a subset of target genes. In addition, cooperation with factor(s) other than Sox2 is suspected for the activation of the *Zfp42* and *Tcl1* promoters. Oct3/4 may recruit different JmjC family members in a cofactor-dependent manner. If so, the stability of the positive feedback mechanism, including both genetic and epigenetic factors, would be enhanced significantly. The solution of this puzzle will doubtless contribute to improving the efficiency of methods for establishing iPS cells, which may one day be used in the development of patient-specific cell therapies.

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