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Transcriptional Repression in ES Cells

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ABSTRACT

The dynamics of embryonic stem cell pluripotency is orchestrated by an interplay of transcriptional and epigenetic regulation in a systematic and modular manner. While the ES cell stage is marked by multiple loci with bivalent chromatin marks that prepare genes for imminent activation on differentiation, this open chromatin conformation is tempered by repressive machinery that prevent premature expression of key developmental genes. This review serves to highlight key ES transcription factors and their known links to the epigenetic machinery via known protein complexes. J. Cell. Biochem. 110: 288–293, 2010.

KEY WORDS: STEM CELLS; EPIGENETICS; TRANSCRIPTIONAL REPRESSION; METHYLATION; HISTONE MODIFICATION

The biology of embryonic stem (ES) cells has captured the imagination of both the scientific and general community alike, and the promise it holds for our understanding of early development and future medicine lies in its pluripotent capacity to self-renew or differentiate into almost any cell type. ES cells are derived from the inner cell mass (ICM) of the pre-implantation embryo at the blastocyst stage, therefore represent an in vitro culture equivalent of the developmental processes at this embryonic stage. Importantly, when introduced into a similarly staged blastocyst, mouse ES cells can contribute to the development of all tissues of the resultant animal, demonstrating the relevance of ES cells to our understanding of early development and is the basis for their usefulness in regenerative medicine [Boiani and Scholer, 2005].

The maintenance of ES cell pluripotency engages multiple levels of cellular machinery, and highlights a conundrum faced in ES cell regulation. While self-renewal requires the suppression of genes involved at later developmental time points, the pluripotency stage in vivo is a short-lived one, and differentiation to specific lineages of endoderm, mesoderm, and ectoderm requires a rapid but tightly controlled regulation of gene expression.

Our understanding of these processes in recent years has been buffeted by the emerging role of epigenetics in influencing the regulation of gene expression through processes such as DNA methylation, histone and chromatin modification [Farthing et al., 2008]. The specific application of these processes to relevant genes is likely to lay with core transcription factors active in ES cells that are known to bind to many sites across the genome. Furthermore, these binding sites are often found in proximity to each other especially at key developmental regulators [Chen et al., 2008; Kim et al., 2008], and may suggest a modular approach to transcriptional control by multiple transcription factors that also serve as recruitment platforms for a secondary level of control through epigenetic modifiers.

Indeed, the role for epigenetics in ES cells is potentially large—unlike somatic cells, ES cells are known to hold a greater proportion of their genome as euchromatin with significant nucleosome-free regions and trimethylated histone 3 lysine 4 (H3K4me3) and acetylated histone 4 (H4Ac) marks typically associated with transcriptionally active regions [Lee et al., 2004; Azuara et al., 2006; Yaragatti et al., 2008]. As such, the extent of regulation necessary to prevent untimely differentiation of ES cells is dependent upon both epigenetic mechanisms and transcription factors alike. Trimethylated Histone 3 Lysine 27 (H3K27me3), unlike H3K4me3, is a transcriptionally repressive histone mark and both typically occur in mutually exclusive domains [Cao and Zhang, 2004]. In ES cells however, the presence of bivalent chromatin domains serves to balance the high degree of transcriptional activity in an ES cell, and to prime the cell for its imminent role in a differentiated lineage by the confluence of the activating H3K4me3 and repressive H3K27me3 marks along regions typically associated with highly conserved regions of developmentally important factors [Bernstein et al., 2006; Mikkelsen et al., 2007].

In this review, we highlight key transcription factors and their links with various protein complexes that enable the recruitment of epigenetic modifying proteins for the silencing of genes not involved in pluripotency.

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TRANSCRIPTION FACTORS AND ASSOCIATED REPRESSIVE COMPLEXES

TRANSCRIPTION FACTORS AND THE NANOG AND OCT4-ASSOCIATED DEACETYLASE COMPLEX (NODE)

Oct4 and Nanog, together with Sox2, are key transcription factors necessary for the pluripotency of ES cells [Nichols et al., 1998; Mitsui et al., 2003], but the centrality of pluripotency appears to rest upon Oct4. ES cells with altered Oct4 expression are directed towards the primitive endoderm and trophectoderm when Oct4 levels are increased or decreased, respectively [Niwa et al., 2000], suggesting that Oct4 is involved in the regulation of multiple developmental pathways. Genome-wide analyses of Oct4 by chromatin immunoprecipitation (ChIP) technologies have identified thousands of potential binding sites [Boyer et al., 2005; Loh et al., 2006]. Interestingly, Nanog and Oct4 binding sites were found to co-occur on 345 targets which included genes now identified to be part of the transcriptional network of ES cells such as *Esrrb*, *Rif1*, *Rcor2*, and *Phc1* [Loh et al., 2006]. At other loci encoding differentiation-specific genes such as *Dkk1* and *Foxh1*, Oct4, and Nanog serve as repressors, although specific mechanisms were not explored in that study.

More recently, the role of Oct4 and Nanog in transcriptional repression has been explored in a novel repression complex in ES cells. Using immunoprecipitation, the authors were able to isolate Nanog and Oct4 containing complexes from wild-type ES cells, and identify the components by mass spectrometry [Liang et al., 2008]. The interaction partners identified were associated with multiple repressive complexes, and a unique *N*anog and *O*ct4-associated *D*eactylase (NODE) complex was characterized, containing members such as Gatad2a/b, Hdac1/2, and Mta1/2 in addition to Oct4 and Nanog. Although the interaction partners overlap with members of the better known *N*ucleosome *R*emo*d*eling (NuRD) complexes, key members of the NuRD complexes were not present in the isolated complexes at significant levels, nor were they necessary for the histone deacetylation function of the NODE complex.

TRANSCRIPTION FACTORS AND TIF1B—LINKS TO THE NUCLEOSOME REMODELING COMPLEX (NuRD)

In a separate study, epitope-tagged Oct4 and Nanog were expressed from transgenes in ES cells [Wang et al., 2006]. The protein interaction partners isolated from epitope-tagged Nanog complexes overlapped with the endogenous complexes identified, including Oct4 and Hdac2. In addition, a transcriptional corepressor, Tif1b (Trim28) was also found. Tif1b is part of the transcriptional intermediary factor 1 (TIF1) family [Le Douarin et al., 1996] and contains a bromodomain highly conserved for interactions with acetylated histones 3 and 4 [Winston and Allis, 1999], as well as residues that interact with repressive members of the Krüppel-associated box (KRAB) domain-containing zinc finger proteins [Bellefroid et al., 1991].

Interactions have been established between Tif1b and HP1—the latter protein is a well-known inducer of heterochromatin-mediated silencing via epigenetic mechanisms that include histone deacetylation [Torres-Padilla and Zernicka-Goetz, 2006; Kwon and Workman, 2008]. While detailed insight into the role of Oct4 and Nanog with Tif1b has not yet been established, the loss of Tif1b results in early embryonic lethality just after implantation [Cammas et al., 2000]. In ES cells, Tif1b has been shown to participate in the recruitment of nuclear repression complexes to integrated retroviral promoter sites [Wolf et al., 2008]. These are postulated to be NuRD and ESET repressive complexes, which are known Tif1b associates in alternative cellular contexts [Schultz et al., 2001, 2002].

Two interesting lines emerge—one is the likely recruitment of NuRD complexes by Oct4, Nanog, and Tif1b, supported by the co-identification of Oct4 and Tif1b also in the endogenous Nanog complexes previously described [Liang et al., 2008]. Moreover, Mbd3, a central component of the NuRD complex, was found to be necessary for the maintenance of pluripotency in the ICM, as shown by the failure of *Mbd3−/−* ICMs to expand in vivo [Kaji et al., 2007]. As such, part of proper transcriptional regulation in ES cells may be established by the association of NuRD complexes with sequence-specific transcription factors.

In addition, the recruitment of repressive complexes and Tif1b in ES cells by KRAB-box containing zinc finger proteins has not yet been established. Perhaps not coincidentally, two Krüppel-like transcription factors, Klf4 and Klf5 have recently entered the ES cell transcriptome network, Klf4 for its pivotal role as one of the four factors that could reprogram a somatic cell to an induced pluripotent state with much of the defining characteristics of ES cell identity [Takahashi and Yamanaka, 2006], and more recently, Klf5 as a necessary transcription factor for self-renewal [Parisi et al., 2008].

TRANSCRIPTION FACTORS, VIRAL ONCOPROTEIN E1A, AND LINKS TO THE CELL CYCLE

The adenoviral protein E1A is known to interact with Oct4 via two binding sites at the POU domain of Oct4, and can act both an enhancer of the transactivation activity of Oct4, or as a repressor to non-DNA-bound Oct4 by a squelching mechanism [Scholer et al., 1991; Brehm et al., 1999; Butteroni et al., 2000]. Evidence has pointed to the recruitment of epigenetic modifiers of expression such as histone deacetylases by E1A and other viral proteins [Horwitz et al., 2008]. A recent study in human embryonic lung fibroblast cell lines suggests that the co-binding of E1A and p107 to the promoters of developmental and differentiation genes results in transcriptional repression via histone deacetylation, and points towards the role of E1A in preventing differentiation through epigenetics and cell-cycle regulation [Ferrari et al., 2008]. The interaction of Oct4 with E1A in ES cells could serve as a recruitment platform for E1A to specific transcriptional start sites. While the role of p107 in ES cells has not been fully explored, early evidence points to the differential expression of p107 in human ES cells at an increased level compared to somatic cells [Becker et al., 2007], and may serve as a connective link between transcription factor control and cell-cycle regulation in pluripotency.

Intriguingly, Sall4 was shown to interact with Cyclin D1, a multifunctional protein involved in cell-cycle progression from the G to S phase through its interaction with transcription factors and chromatin modifiers including histone acetylases, deacetylases, and chromatin remodeling proteins [Bohm et al., 2007]. In the cell lines tested (HeLa, COS-7, MCF-7), the Sall4/Cyclin D1 interaction resulted in the transcriptional repression of reporter gene

activity, and provides the first evidence of a Sall4 involvement in transcriptional repression. Sall1 also shows similar repressive activity in the amino and carboxy domains of the protein [Netzer et al., 2006]. While transcriptional repression by Sall1 or Sall4 has not presently been demonstrated in ES cells, the co-occurrence of Sall1 and Sall4 with other ES-specific transcription factors and cell-cycle proteins by co-immunoprecipitation suggests that this is a possibility [Wang et al., 2006].

TRANSCRIPTION FACTORS AND POLYCOMB GROUP COMPLEXES (PcG)

Most often, the role of transcriptional repression in ES cells are attributed to the Polycomb group (PcG) proteins. In ES cells, the primary Polycomb Repressive Complexes (PRC), PRC1, and PRC2 are present, and the transcriptional locations of some PRC members have been mapped in mouse and human ES cells [Lee et al., 2006; Squazzo et al., 2006]. In support of the essential role of PRCs for pluripotency, knockouts of Ring1b (Rnf2) or Ezh2 are early embryonic lethal [O'Carroll et al., 2001; Voncken et al., 2003], and the binding sites of core components such as Suz12 and Eed have been established by ChIP [Boyer et al., 2006; Lee et al., 2006]. Through these studies, it was found that a large subset of genes affected by PRC1 and PRC2 promoter binding include factors involved in transcriptional regulation and development. In the absence of PcG proteins Suz12 or Eed, or on ES cell differentiation; these factors were selectively de-repressed, pointing to a role for PRC1 and PRC2 in direct transcriptional repression. Direct links between ES transcription factors Rex1 (Zfp42) and Oct4 have been made with PcG proteins Ring1b and RYBP, a known Ring1b-binding protein, although the interaction of Suz12 with core ES transcriptional regulators has not been fully established [Endoh et al., 2008]. Also, genome-wide studies have demonstrated the co-localization of PcG proteins with these transcription factors at promoter regions for developmental genes marked by bivalent chromatin domains. These genes include *Sox1*, *Pax3* (with Oct4), *Msx2*, *Pax6*, and *Hoxd11* (with Rex1), suggesting that the recruitment of PcG complexes by sequence-specific transcription factors could generate the bivalent marks necessary to prepare these genes for active expression on differentiation [Boyer et al., 2006; Lee et al., 2006; Loh et al., 2006; Mikkelsen et al., 2007; Kim et al., 2008]. Future work in this area is likely to highlight the close relationships between multiple ES cell transcription factors and PRC factors.

NEW KIDS ON THE BLOCK

The recent discovery of Ronin ushered a new entrant into the links between transcription factors and epigenetic silencing in ES cells. Ronin knockout mice are embryonic lethal at the peri-implantation stage, and ES cell colonies cannot be derived from *Ronin*−/− mice, suggesting the essential nature of Ronin in the maintenance of pluripotency. Like with Nanog, forced overexpression of Ronin is sufficient for preventing differentiation of ES cells, and is postulated to occur through transcriptional repression. Ronin contains a DNA-binding THAP domain frequently associated with epigenetic silencing when found in other proteins [Dejosez et al., 2008]. Isolation of epitope-tagged Ronin complexes identified HCF1 as a direct interactor of Ronin, with other associated partners THAP7, Sin3a, and Hdac3 typically involved in histone modification and transcriptional repression. While the exact repressive complex(es) recruited by Ronin are not fully understood at present, the association with epigenetic modifiers points to avenues for further exploration.

A second debutante to the core ES regulatory network is Tcf3, a DNA binding transcriptional repressor that is a downstream effector of Wnt signaling [Tam et al., 2008]. ChIP of Tcf3 in ES cells showed that of genes bound by Tcf3, Oct4, and Nanog, approximately half were also associated with developmental targets occupied by PRC complexes [Cole et al., 2008; Tam et al., 2008]. While this points to a role for PRC recruitment by Tcf3 and transcriptional repression of differentiation-specific genes, an additional means of transcriptional repression can also occur via co-repressor proteins CtBP and Groucho/TLE, for which interactions have previously been established in other cell lines. Groucho/TLE proteins repress by chromatin remodeling events that include the recruitment of histone deacetylases and other co-repressors such as N-CoR and Sin3a [Buscarlet and Stifani, 2007], while CtBP proteins can interact with Groucho/TLE, as well as recruit histone modifiers for silencing by deacetylation and methylation at H3K9, and demethylation at H3K4 [Chinnadurai, 2007]. In functional studies of Tcf3, the deletion of the Groucho/TLE interaction domain resulted in a loss of repressive activity at the *Oct4* promoter [Tam et al., 2008]. Similarly, in *Tcf3*−/− ES cells, expression of Oct4 or Nanog-regulated genes were increased, and the cells remained pluripotent even on removal of LIF from the culture media [Yi et al., 2008]. In light of these observations, Tcf3 is plausibly involved in at least two types of repressive complexes; the first as a repressor of differentiation-specific genes through PcG proteins, and the second as a negative regulator of Oct4 and Nanog-mediated positive regulation on ES cell-specific genes with Groucho/TLE and CtBP proteins.

FUTURE PROSPECTS

The unique open chromatin conformation of ES cells and their continued ability to self-renew suggests that a means of transcriptional control must be present for the complete or partial suppression of genes involved at later stages of development. Through the analysis of histone modifications along the chromatin, it was discovered that bivalent domains exist across the promoter regions of key developmental regulators that are only active upon differentiation of ES cells [Bernstein et al., 2006]. These bivalent regions are held in a state of suspended animation, poised for transcriptional activation with H3K4me3 marks, but silenced by repressive H3K27me3 marks. In addition, ES cell-specific transcription factors such as Oct4 and Nanog have been found to be essential for the maintenance of pluripotency, and alterations to their expression level can result in changes to the differentiation potential of the ES cell. Together, the role of transcription factors and chromatin modifications in regulation of the ES cell state suggests that intermediary roles may be played by transcriptional repression complexes to bridge the gap between the initiator and outcome.

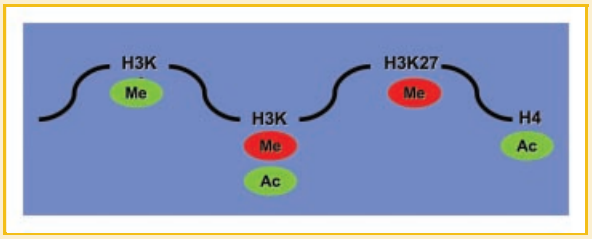

Fig. 1. Key histone modifications in ES cells. Key histone modifications known to be involved in the maintenance of embryonic stem cell pluripotency. Bivalent domains consisting of trimethylated H3K4 and H3K27 are surmised to silence lineage-specific genes in the pluripotent state, but poise them for activation upon differentiation. Other modifications such as acetylation at the marked histones also occur. Green and red ovals represent modifications for and against ES cell renewal.

In this light, studies of protein–protein and protein–DNA interactions in ES cells have been particularly helpful in elucidating the identity, potential localization, and relevance of these repressive complexes at a genome-wide level. In addition to the transcriptional repression complexes highlighted in the earlier sections, other chromatin modifying complexes are also likely to hold key roles, although their links to specific transcription factors are not fully known at present. SWI/SNF complexes are one such example where the composition of specific members is altered on ES cell differentiation. The losses of core members of the SWI/SNF complexes, Smarca4 (Brg1), Snf5, and BAF155, result in embryonic lethality and loss of BAF250A or BAF250B can also compromise the pluripotency of ES cells [Gao et al., 2008]. While BAF155 has been isolated in Nanog, Oct4, Rex1, and Nac1 complexes, and Smarca2, Smarca4, and BAF180 in Nanog-containing complexes, the large number of SWI/SNF members may have overlapping functionalities that complicate the analysis of ES transcription factors with SWI/SNF complexes.

In addition to the functional relevance of interactions between transcription factors, repressive complexes, and the resultant changes in chromatin structure that controls gene expression, other factors abound. Increasingly, the significance of nuclear localization in transcriptional activation or silencing is being recognized, the best characterized region being the nuclear periphery [Ahmed and Brickner, 2007]. It has been suggested that the recruitment of chromatin to distinct regions of the nucleus can act as a form of transcriptional memory to distinguish short- and long-term repressed regions by interacting with specific proteins at each location [Brickner et al., 2007]. While the majority of these studies are presently done in yeast, it will be unsurprising should these mechanisms be conserved in higher eukaryotes, and will serve to complement our understanding of transcriptional repression in ES cells, especially since ES cells are known to have an atypically large nuclear to cytoplasmic ratio. Also, the emerging role of small non-coding RNAs such as microRNAs (miRNAs) in ES cells may reinforce the transcriptional repression initiated by core ES transcription factors. Because such factors themselves may be post-translationally regulated by miRNAs, it will be interesting to unravel the interdependent relationships between proteins, RNA, and DNA in regulating gene expression and cellular identity.

CONCLUSION

The link between local transcription factor binding and resultant changes in cellular identity has been explored through the examples

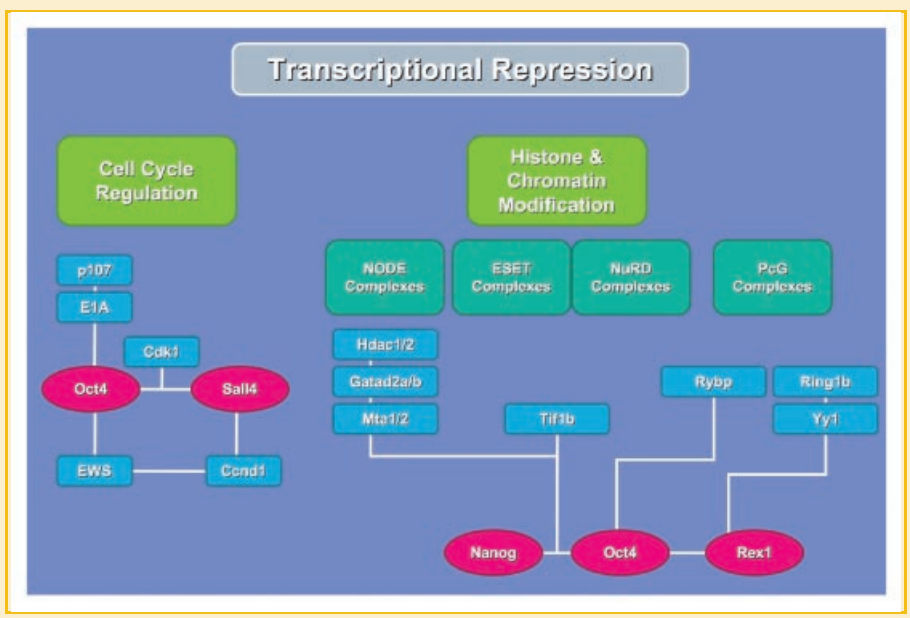

Fig. 2. Links from transcriptional regulation. Demonstrated interactions between key ESC transcription factors to other proteins support a view for the shared role of chromatin remodeling and cell-cycle regulation in transcriptional regulation, as well as the recruitment of specific remodeling complexes dependent upon the combination of transcription factor interactions.

of transcriptional repression complexes that seek to maintain ES cell identity. Clearly, multiple repression complexes are at work, and are likely to address the need for a variety of responses to gene expression in a spatial and temporal fashion in the developing animal.

However, our current knowledge of the specific combination of transcription factors required for recruitment of these repressive complexes is limited at best, and will be enhanced with global studies of transcription factor complexes and their co-localizations. Recently, two large-scale ChIP studies explored the binding sites of multiple ES cell transcription factors, and future developments in this line, along with structural studies of transcription factor binding at the DNA will serve to improve our understanding of the recruitment of such complexes [Chen et al., 2008; Kim et al., 2008].

Additionally, while these studies are carried out in ES cells, a convenient in vitro representation of the corresponding events in the ICM of the early blastocyst, it is salient to note that the cell culture conditions may introduce artifactual differences into our understanding. Indeed, a study has demonstrated that epigenetic silencing marks in the ICM are more extensive than in ES cells [O'Neill et al., 2006]. With improvements in technical protocols for the assessment of small cell numbers, the viability of examining the ICM population directly will allow for further clarification on this front (Figs. 1 and 2).

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