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## **Epigenetic Mechanisms Controlling Mesodermal Specification**

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**ABSTRACT:** The pluripotency of embryonic stem (ES) cells is the result of a highly dynamic equilibrium that is controlled by a complex network of transcription factors that confer unique transcriptional properties to ES cells. Regulation of gene expression appears to correlate with the presence of dual chromatin marks called bivalent domains at the promoters of poised developmental genes. These marks keep differentiation genes silenced but poised and ready to be activated or permanently repressed during differentiation. The process of tissue fate specification is initiated by various signalling molecules that directly impact the dynamic equilibrium of ES cells, particularly on the bivalent domains, inclining and predisposing the balance towards a particular lineage. In here we summarized current knowledge on how different transcription factors and signalling molecules impact on the epigenetic status of ES cells and in turn how this guides the process of mesoderm specification.

### **Introduction**

Embryonic stem (ES) cells have unique properties that allow them to proliferate without apparent limit and to give rise to virtually any cell type of an organism. For all these reasons, ES cells hold great promise for regenerative medicine.

Although ES cells can maintain their undifferentiated status indefinitely, they are extremely sensitive to changes in the extra cellular media. This unprecedented capacity to respond to environmental cues is explained by the existence of a highly dynamic equilibrium that can be easily displaced towards the end points. Thus, by affecting this delicate balance, small changes in signalling molecules are able to induce dramatic changes in gene expression that are crucial for the process of specification. The rapid nature of this response suggests that this dynamic equilibrium is controlled by epigenetic mechanisms.

The process of mesodermal specification is controlled by extracellular signalling molecules that mediate the rapid phosphorylation of signal transduction proteins in the cytoplasm as well as the activation of transcription factors which subsequently lead to the activation or repression of their corresponding target genes. These early events highly impact the dynamic equilibrium of undifferentiated cells and finally tip the balance towards mesodermal specification.

### **The dynamic equilibrium of undifferentiated ES cells**

Pluripotency is defined as the ability of cells to activate the expression of lineage specific genes in response to stimuli. However, in order to establish the appropriate program of gene expression, tissue specific genes corresponding to alternative lineages should become permanently silenced during differentiation. Differentiated cells, which have permanently silenced those genes, lose their plasticity and ability to respond to many environmental signals. This permanent silencing is mainly controlled by epigenetic events and its deregulation leads to pathologies like cancer. Two major epigenetic events can control the expression of genes at the transcriptional level; histone modifications and DNA methylation. DNA methylation in mammalian cells is postulated to play multiple roles in cell physiology, including genome stability, genomic imprinting and developmental gene regulation. Regarding its role in gene expression, it is widely accepted that methylation of selected CpG islands located in the regulatory regions of critical genes contributes to their permanent and irreversible silencing.

Genomic DNA in eukaryotic nuclei is packaged into a compact structure known as chromatin. The fundamental unit of chromatin is the nucleosome, which is composed of two copies each of four core histones—H2A, H2B, H3, and H4—wrapped by 146 bp of DNA. The N-terminal tails of histones are subjected to various post-translational modifications that have been demonstrated to control structural chromatin compaction and, in turn, regulate gene transcription.

Histone methylation is probably the most studied histone modification since it is linked to both transcriptional activation and repression. The numerous lysine residues on the histone tails, in conjunction with the various methylation levels (mono-, di- or trimethylation), provide tremendous regulatory potential for chromatin modifications (Martin and Zhang, 2005). Moreover, histone lysine methylation, which was until recently thought to be an irreversible epigenetic mark, can be reversed by histone lysine-specific demethylases, making the histone methylation status extremely dynamic (Shi and Whetstine, 2007). Interestingly, recent studies highlight the importance of histone lysine methylation regarding the predisposition of genes to be activated.

Pluripotency relies on the fact that early developmental genes are not irreversibly silenced in ES cells but remain poised and ready to become active in response to extra cellular signals. Recently, it has been reported that many key developmental genes that are silent in embryonic stem cells display unique histone modification patterns, which make them permissive for activation during differentiation (Azucara et al., 2006; Bernstein et al., 2006; Pan et al., 2007; PMID; Zhao et al., 2007; PMID). This unique histone modification pattern, called “bivalent domains,” consists of large regions of H3K27 trimethylation harbouring smaller regions of H3K4 trimethylation around the transcriptional start site of critical developmental genes. Although initially puzzling, the coexistence of activating (H3K4 methylation) and repressive (H3K27 methylation) marks has been suggested to play a role in silencing developmental genes in ES cells while keeping them poised for activation upon initiation of specific developmental pathways. Moreover, the poised nature of these domains is further reinforced by the presence of poised RNA Polymerase II (Pol II) at the transcription initiation site of the marked genes (Stock et al., 2007). Bivalent genes show absence of DNA methylation despite the presence of numerous CpG islands (Barrero et al., 2012; Fouse et al., 2008; PMID). This might be in part accomplished through the hydroxymethylation of CpGs at these domains mediated by the enzyme Tet1 (Wu et al., 2011). Functionally, the presence of these discrete areas of H3K4 methylation may act as a barrier that prevents the spread of repressive chromatin modifications and perhaps facilitate the binding of lineage-specific transcription factors during differentiation (Szutorisz and Dillon, 2005).

In vertebrates, the polycomb group of proteins (PcGs) play an essential role in maintaining the pluripotent state of ES cells by mediating H3K27 methylation at the bivalent domains. PcG proteins localize at genes encoding developmental regulators and correlate with the presence of H3K27 trimethylation both in mouse and human ES cells (Boyer et al., 2006; Lee et al., 2006). Moreover, mouse ES cells null for specific PcG proteins result in a lack of H3K27 methylation and show aberrantly induced expression of key developmental genes (Azucara et al., 2006; Boyer et al., 2006; Lee et al., 2006). Ubiquitination of histone H2A by the Polycomb complex subunit Ring1B seems to play a role in restraining poised Pol II at bivalent genes (Stock et al., 2007).

On the contrary, the H3K4 methylation marks present at the bivalent domains of silent developmental genes could be catalyzed by several H3K4 methyltransferases that belong to the MLL family (Ruthenburg et al., 2007). The putative contribution of the different members of the MLL family to the establishment of the bivalent domains is mainly unknown, but two recent reports start to unveil details about this fact. Knock down of the newly identified MLL subunit Dpy-30 (Jiang et al., 2011) does not cause self-renewal defects, but rather defects in differentiation. However, knock down of the MLL complex core subunit WDR5 in ES cells has been reported to induce differentiation and loss of self-renewal (Ang et al., 2011).

In order to understand the molecular mechanisms involved in pluripotency, it is fundamental to unravel how the enzymes responsible for H3K27 and H3K4 methylation are specifically recruited to the bivalent domains. Although histone modifying enzymes in mammalian cells are typically recruited to their target regions by transcription factors, a recent report highlights the possibility that the extremely conserved distribution of CpG domains in the regulatory regions of developmental genes plays a role in the recruitment of the Polycomb complex to the bivalent domains (Tanay et al., 2007). Moreover, Tet1 might be facilitating Polycomb recruitment by preserving low levels of DNA methylation at these conserved CpG domains (Wu et al., 2011). Alternatively, the transcription factor JARID2 has been suggested to participate in the recruitment of the Polycomb complex PRC2 to the regulatory regions of developmental regulators in mouse ES cells (Landeira et al., 2010). On the other hand, genome wide correlation studies revealed that the self-renewal transcription factors Oct4/3, Sox2 and Nanog not only occupy gene promoters involved in self-renewal but they are also in high number of developmental genes that contain bivalent domains (Boyer et al., 2005). These findings suggest that these self-renewal factors play a role in keeping developmental genes silenced through the control of the bivalent domains. Although there has been some evidence reported for association of Oct4 with Polycomb components (Wang et al., 2006), nothing has yet been shown for a direct interaction between self-renewal factors and the Polycomb or MLL complexes. Moreover, the association of Nanog and Oct4 with unique transcriptional repression complexes, including subunits of the remodelling complex SWI/SNF, the remodelling and histone deacetylation complex NuRD and the HDAC/Sin3A complex, has been recently reported (Liang et al., 2008). These repressive complexes seem to be involved in the poised silencing of developmental genes mediated by Nanog and Oct4. However, their involvement in the establishment of bivalent domains remains unclear.

In ES cells, bivalent domains are tightly regulated by the balance of activating and repressing activities. The Polycomb complex can mediate the recruitment of the H3K4 demethylase RBP2 to the bivalent domains to maintain the proper balance between H3K4 and H3K27 methylation in mouse ES cells (Pasini et al., 2008). Similarly, the H3K4 demethylase LSD1 is recruited to bivalent domains to regulate the levels of H3K4 methylation in human ES cells (Adamo et al., 2011; Adamo et al., 2011). Moreover, subunits from ATP-remodelling complexes can antagonistically control nucleosome occupancy at bivalent genes (Yildirim et al., 2011). Overall, bivalent domains appear governed by a highly dynamic network of epigenetic activators and repressors that likely makes them extremely sensitive to differentiation signals.

Thus, changes in the balance of transcription and epigenetic factors during specification, including rapid down regulation of self renewal transcription factors and activation of germ layer specific transcription factors, will affect the balance of marks at the bivalent domains and determine the process of specification.

### **Mesodermal specification in stem cells**

ES can be maintained in an undifferentiated state in the presence of feeders and LIF or FGF in the case of mouse or human ES cells, respectively. When cells are grown in suspension and in the presence of fetal calf serum, they form three-dimensional aggregates called embryoid bodies (EBs) that recapitulate the early stages of development.

During ES cell differentiation, expression of the T-box transcription factor Brachyury marks the formation of a primitive streak-like cell population that corresponds to early mesoderm. Experiments carried out in differentiating mouse ES cells suggest that, as is the case during embryo development, commitment to blood lineage occurs in mesoderm cells prior to cardiovascular commitment. Two populations positive for fetal liver kinase-1 (Flk-1 or VEGFR2) seem to emerge at different times from the

Brachyury positive population. The earliest one corresponds to the hemangioblast, a progenitor that has both hematopoietic and endothelial potential and co-expresses Flk-1 and Brachyury (Choi et al., 1998). These cells undergo a yolk-sac-like hematopoietic program that generates primitive erithroid progenitors. After extended periods of time, hematopoietic populations that express CD34 can be detected (Vodyanik et al., 2005).

The second Flk-1 positive population that emerges from the Brachyury positive one, consists of cardiovascular progenitors able to generate cardiac, endotelial and vascular smooth muscle cells (Kattman et al., 2006). Cells undergoing cardiac specification express the cardiac specific transcription factors Nkx2.5, the GATA family members GATA-4, 5 and 6 and the members of the T-box family Tbx 5 and 20 (reviewed in (Menard et al., 2004).

The transforming growth factor (TGF)  $\beta$  superfamily, which includes TGF $\beta$ , nodal, and bone morphogenetic proteins (BMPs), the fibroblast growth factor (FGF) and the Wnt families are typical mesodermal modulators. The binding of agonists to the TGF $\beta$  receptors leads to the activation of Smad transcription factors that in turn activate the expression of genes like Nkx2.5. The activation of the TGF $\beta$  and FGF receptors also activates the MAP kinases pathway leading to the activation of transcription factors like CREB or ATF2. Wnt signalling involves the stabilization and nuclear localization of the transcription factor  $\beta$ -catenin and the subsequent activation of target genes. The complexity of the action of distinct signalling molecules at different spatial-temporal points is further complicated by the existence of cross talking mechanisms between the different signal transduction pathways. Further work will be needed to elucidate the early transcription factors and events targeted by the mesodermal morphogens.

#### **The resolution of the bivalent domains; a crucial step for specification**

Although the bivalent marks are faithfully transmitted through cell division in self-renewing cells, they should not be regarded as static but as the result of a highly dynamic equilibrium that is controlled by the balance of histone modifying enzymes. This dynamic status is likely to be easily displaced by the action of signalling molecules present in the extra cellular media, providing the basis of ES cell plasticity.

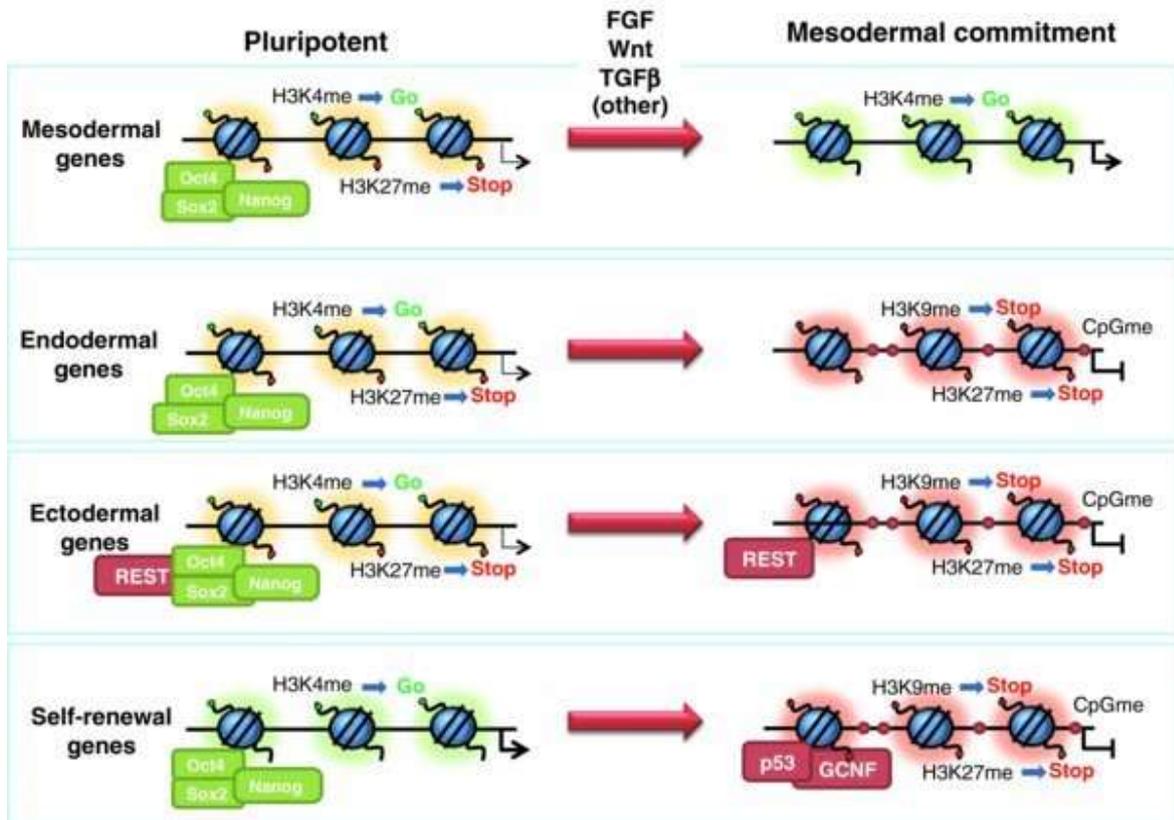
The process of mesodermal specification involves crucial decisions in gene expression that involve both permanent repression and activation of critical genes and that can be summarized in three different events (Figure 1): (i) the activation of early responsive genes involved in mesodermal specification, (ii) the permanent silencing of genes involved in other germ layer specification and (iii) the permanent silencing of self-renewal genes.

In ES cells many early genes involved in mesodermal specification like some of the GATA and Tbx family members, Mixl1 and brachyury contain bivalent domains, reinforcing the idea that these are critical early factors involved in specification. Many of these genes are also occupied by the transcription factors Oct4, Sox2 and Nanog in mouse and human ES cells (Table 1), suggesting that they may play a potential role in controlling their expression by controlling the marks at the bivalent domains.

The coordinated activation of early mesodermal and silencing of non-mesodermal genes during mesodermal specification relies on the impact of signalling molecules in the balance of bivalent domain marks. Although the molecular events leading to the establishment of the mesodermal expression pattern have not been studied in detail, it is likely that the following changes take place. As mesodermal genes become active in response to external cues, they lose the repressive H3K27 methylation mark while keeping the H3K4 trimethylation on their regulatory regions. The change in the balance of marks is expected to be correlated with the presence of elongating forms of Pol II in the coding regions of these genes and activation of transcription. On the contrary, non-mesodermal early genes that are poised in ES cells become irreversibly silenced during mesodermal commitment by losing H3K4 trimethylation, while keeping the H3k27 methylation and accumulating other repressive marks like H3K9 methylation and DNA methylation.

How the resolution of the bivalent domains is controlled at the molecular level is not yet known. The withdrawal of LIF or FGF and the addition of fetal calf serum leads to the differentiation of a percentage of cells into the mesodermal, endodermal and ectodermal lineages. However, why a particular cell commits into any of the different lineages is not fully understood. One can speculate that the three-dimensional organization of the EBs may recapitulate some of the spatial-temporal events of embryonic development

that determine cell fate. On the other hand, it is possible that relative differential expression levels of the self-renewal transcription factors in individual undifferentiated cells at the onset of commitment determines their fate. This different relative expression may affect the balance of marks in the bivalent domains predisposing the cells to activate certain genes upon stimulation. In this regard, it is worth noting that Oct3/4 plays a role in differentiation through a gene dosage effect since an increase in its expression causes differentiation into mesoderm and cardiac lineages (Zeineddine et al., 2006). In contrast, repression of Oct-3/4 induces loss of pluripotency and dedifferentiation to trophectoderm (Niwa et al., 2000).



**Figure 1: Representation of chromatin marks in critical genes during specification.**

In undifferentiated ES cells germ layer specific genes are marked by bivalent domains containing H3K4 and H3K27 trimethylation. These particular modifications play a role in keeping these genes silenced but poised for activation during differentiation. Although the molecular mechanisms responsible for the presence of these marks are not known it is possible that the presence of transcription activators, like the self-renewal factors Oct4, Sox2 and Nanog, and transcriptional repressors like REST at the regulatory regions of the marked genes could explain the simultaneous presence of these unique dual marks. The process of mesodermal specification involves the action of signalling molecules that belong to the TGFβ, Wnt and FGF families, among others. These factors are responsible for the initiation of signalling cascades in the cell cytoplasm and activation of critical transcription factors that turn on the expression of mesodermal specific genes and turn off non-mesodermal genes. How these signals orchestrate the correct pattern of gene expression and repression at the molecular level is not clear. However, these signalling events will ultimately lead to changes in histone modifications and more specifically to the resolution of the bivalent domains at the regulatory regions of differentiation genes, resulting in H3K4 methylation only in active genes and H3K27 methylation only in repressed genes. A critical event for specification is the permanent silencing of self-renewal genes, which involves the removal of the H3K4 trimethylation mark and the acquirement of repressive marks like H3K27 and H3K9 trimethylation and DNA methylation. These changes might be caused by the down regulation of the self-renewal factor network itself during differentiation and the action of differentiation induced repressors like p53 or GCNF.

**Table 1:** List of genes involved in mesodermal differentiation that contain bivalent domains in human ES cells. (according to (Pan et al., 2007; PMID; Zhao et al., 2007; PMID)). Reported occupation by self-renewal transcription factors Oct3/4, Sox2 and Nanog is also shown (according to (Boyer et al., 2005)).

Gene	Oct3/4	Sox2	Nanog
Brachyury	–	–	+
Mixl1	–	–	–
GATA4	+	–	–
GATA5	–	–	–
GATA6	+	–	+
Nkx2.5	+	–	–
Tbx5	+	+	+
Tbx20	+	–	+
Mef2C	–	–	–

A critical event for the resolution of the bivalent domains during specification is the removal of histone methylation marks. In this regard, recent reports point out the importance of the newly described H3K27 demethylases UTX and Jmjd3 in the activation of Hox genes during development (Agger et al., 2007). Both demethylases associate with MLL complexes, suggesting that removal of the H3K27 mark and maintenance of the H3K4 methylation in genes that become activated during differentiation are coordinated events.

As for the removal of the H3K4 mark in genes that become repressed, several members of the jumomji domain-containing family of demethylases and the enzyme LSD1 can display this enzymatic activity (Klose et al., 2006; Shi et al., 2004). LSD1 plays a role in repressing neural genes during non-neural differentiation by associating with the coREST complex, which in turn associates with the repressor REST. This repressor targets many neural genes and keeps them silenced through the H3K4 demethylation activity of LSD1. This mechanism ensures that neural genes remain silenced during non-neural specification. During neural specification, REST is rapidly degraded allowing transcriptional activation of neural genes (Ballas et al., 2005; Westbrook et al., 2008). LSD1 has also been recently reported to contribute to the downregulation of the expression of genes of the pluripotency network through H3K4 demethylation at their regulatory regions during differentiation (Whyte et al., 2012).

It is possible that not only changes in the level of classical transcription factors contribute to specification. Extra cellular signals may contribute to regulate the levels or the activity of the different histone methyltransferases and demethylases, causing a switch in the balance of histone marks and contributing to the resolution of the bivalent domains. The levels of the subunits of the Polycomb complex Ezh2 and Eed decline during differentiation (de la Cruz et al., 2005) and may contribute in this way to the resolution of the bivalent domains. It is not really known whether the activity of the H3K27 or H3K4 demethylases can be affected by developmental signals. While UTX seems to maintain a constitutive expression, Jmjd3 has been reported to be a target gene of the transcription factor NF- $\kappa$ B, which mediates its induction during inflammatory stimuli in macrophages. Whether Wnt, activin/Nodal, and BMP signalling can modulate the levels of these histone modifying enzymes during mesodermal specification remains to be determined. Changes in subunit composition of chromatin-related complexes might also contribute to establishing the new epigenetic landscapes during differentiation. Such is the case of the Cbx subunits of the Polycomb complex. During the differentiation of mouse ES cells, the expression of Cbx7 is down-regulated, while Cbx2, Cbx4, and Cbx8 are induced, leading to changes in the complex composition and properties (O’Loghlen et al., 2012). In a similar fashion, changes in the expression of histone variants might also be involved in establishing the appropriate patterns of gene expression during differentiation. This is the case of histone linker variant H1.0, which is induced during differentiation and specifically

recruited to the regulatory regions of pluripotency and developmental genes, contributing to their repression (Terme et al., 2011).

The remodelling of the chromatin structure is also likely to be actively involved in the induction of gene expression during differentiation. More specifically, recent data describes that the HMG superfamily member of remodellers HMGA2, is necessary for cardiogenesis. HMGA2 interacts with members of the Smad transcription factor family and synergistically stimulates the transcription of the cardiogenic transcription factor Nkx2.5 (Monzen et al., 2008).

Another important event during specification is the silencing of the self-renewal genes. This silencing is mediated by the methyltransferase G9a, which catalyzes the methylation of histone H3 at residue 9 (H3K9) thereby promoting the binding of the heterochromatin protein 1 (HP1) and DNA methylation (Feldman et al., 2006). Thus, in adult somatic cells, the regulatory regions of self-renewal genes like Oct4, Sox2 or Nanog are marked by these repressive marks, which render these genes refractory to stimulation. This permanent silencing guarantees that these genes remain silent and its deregulation may contribute to adult cell dedifferentiation and cancer. Regarding the differentiation of ES cells, the absence of critical signalling molecules like LIF or FGF in the media leads to the down regulation of the expression of self-renewal factors. Due to the intricacy of the self-renewal factor network and the ability of these transcription factors to regulate each other, it is likely that small changes in gene expression are amplified into a rapid silencing response. However, specific repressors like p53 (Lin et al., 2005), GCNF (Gu et al., 2005) and NR2F2 (Rosa and Brivanlou, 2011) have been described to contribute to the silencing of self-renewal genes.

Despite the classical view that the activation of mesoderm specific genes is the most critical event controlling specification, it is becoming clear that the silencing of non-mesodermal genes is as critical for the correct establishment of cell lineages. Thus, other epigenetic events, like microRNAs, participate in ensuring that non-mesodermal and self-renewal factors are silenced during specification.

### **Control of specification by microRNAs**

MicroRNAs (miRNAs), single-stranded RNAs of 19–23 nucleotides, are known to negatively regulate gene expression by direct mRNA cleavage, mRNA decay by deadenylation, or translational repression. In mammals, miRNAs usually have imperfect complementation to a 3'-untranslated region (UTR) region in their mRNA targets and are primarily believed to attenuate translation of the target mRNA.

Studies to characterize the levels of miRNA expression revealed that a specific set of miRNAs are expressed in undifferentiated cells while others are induced during differentiation, suggesting that miRNAs have a role in these processes (Lakshminpathy et al., 2007). Importantly, recent reports show that is possible to reprogram somatic cells to pluripotency by overexpressing the miR-302/367 cluster (Anokye-Danso et al., 2011; PMID S1934-5909(11)00111-1 DOI - 10.1016/j.stem.2011.03.001). This cluster is highly expressed in ES cells and plays roles both in maintaining pluripotency and regulating differentiation (Lipchina et al., 2011; Rosa and Brivanlou, 2011).

Recent data indicates that de novo DNA methylation during ES cell differentiation is controlled by miRNAs. Loss of the miRNA pathway component Dicer causes the ablation of the ES-specific miR-290 cluster and impairs the differentiation of mouse ES cells due to the absence of DNA methylation in the Oct4 regulatory regions. The methylation defect correlates with down regulation of de novo DNA methyltransferases mediated by transcriptional repressors that are targets of the miR-290 cluster (Sinkkonen et al., 2008). These findings exemplify how microRNAs can affect the chromatin structure by indirectly controlling the expression of chromatin modifying enzymes.

Several miRNAs are involved in the silencing of non-mesodermal genes during mesodermal specification (Ivey et al., 2008). More specifically, it has been shown that muscle-specific miRNAs, miR-1 and miR-133, act to promote mesoderm differentiation by suppressing the expression of genes involved in alternative lineages. Thus, miRNAs can control cell lineage determination from pluripotent ES cells by fine-tuning the transcriptome of differentiating cells during commitment to a particular fate.

How the expression of miRNAs is regulated during differentiation remains to be determined. Some miRNAs induced during differentiation are kept repressed at the transcriptional level by REST in undifferentiated ES cells and are likely to target self-renewal factors during differentiation (Singh et al.,

2008). On the other hand, expression of muscle-specific miRNAs, miR-1 and miR-133, are regulated at the transcriptional level by muscle-specific transcription factors (Rao et al., 2006), suggesting that their function is important during later phases of specification.

### **Future directions**

The highly dynamic status of ES cells and their extreme plasticity suggest that the early specification events are controlled at the epigenetic level. Crucial events for specification are initiated by specific signalling molecules that stimulate the transduction of signals through the cytoplasm and ultimately lead to the activation of transcription factors. Although many reports highlight the importance of different signalling molecules and transcription factors in mesodermal specification, how all these signals are orchestrated and how they function to convey the coordinated expression and repression of genes is not fully understood and remains to be determined.

Although the precise molecular mechanisms are not known, it is expected that the signalling events unchained by the mesodermal morphogens impact the equilibrium of activating and repressing chromatin marks in the regulatory regions of poised developmental genes and tip the balance towards transcriptional activation or repression. Although classical studies have highlighted the importance of gene expression activation for specification, it is now becoming clear that permanent silencing of non-lineage genes is crucial for this process. These silencing events seem to be mainly controlled at the epigenetic level through two different mechanisms. One of them ensures transcriptional silencing through a high level of chromatin compaction. The other entails the silencing of the remaining gene expression through postranscriptional mechanisms and seems to be controlled by microRNAs.

Future challenges involve understanding how signalling molecules affect the highly dynamic equilibrium of ES cells at the molecular level and lead to the proper and coordinated gene activation and repression during mesodermal specification.

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