

## Histone acetylation and its role in embryonic stem cell differentiation

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### Abstract

The understanding of mechanisms leading to cellular differentiation is the main aim of numerous studies. Accessibility of DNA to transcription factors depends on local chromatin structure and chromatin compaction inhibits gene transcription. Histone acetylation correlates with an open chromatin structure and increased gene expression. Gene transcription levels are changed in early embryonic stem cells differentiation in a tissue-specific manner and epigenetic marks are modified, including increased global acetylation levels. Manipulation of histone deacetylases activity might be an interesting tool to generate populations of specific cell types for transplantation purposes. Thus, this review aims to show recent findings on histone acetylation, a post translational modification and its manipulation in embryonic stem cells differentiation.

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### EMBRYONIC STEM CELLS

Embryonic stem cells (ES cells) are derived from inner mass cells within a blastocyst and can be maintained and proliferated *in vitro*<sup>[1,2]</sup>. The importance of embryonic stem cells for modern biology rests in two major properties which distinguish them from other cell types. Firstly, they are pluripotent cells, capable of developing into any type of cell from all three embryonic germ layers (endoderm, mesoderm and ectoderm) and, secondly, they have the ability to self-renew, going through numerous cycles of cell division while maintaining the undifferentiated state<sup>[3]</sup>.

ES cells represent a suitable model for study of processes involved in lineage specification during mammalian development<sup>[4]</sup>. Also, ES cells can be genetically manipulated. Embryoid bodies or isolated differentiated cells are shown to be a useful tool to investigate the effects of gene insertion and deletion<sup>[5]</sup>.

The first pluripotent cell line was isolated from a teratocarcinoma. Embryonal carcinoma cells served as a model to study cellular differentiation given their potential to participate in embryonic development<sup>[6]</sup>, although their use in clinical research was not possible given their potential to form tumors. Finally, in 1981, ES cells were isolated from the inner cell mass of murine embryos<sup>[1,2]</sup> and in 1998 the derivation of ES cells from human em-

bryos<sup>[7]</sup> brought the concept of regenerative medicine and tissue engineering as possibilities for the treatment of degenerative diseases.

In the future, degenerative diseases caused by destruction or loss of function of certain cellular type could be treated with transplantation of differentiated ES cells<sup>[8]</sup>. Diabetes *mellitus*, a candidate for cellular therapy, is caused by selective destruction of pancreatic cells. Another candidate is Parkinson's disease in which dopaminergic neurons are destroyed in a particular region of the brain.

Also, the use of adult stem cells for replacing damaged cardiomyocytes is described, although clinical application of these cells may be hindered because of their limited ability to proliferate and differentiate in culture<sup>[9]</sup>. Therefore, considering the pluripotency and self-renewal abilities of ES cells, these cells can be expanded *in vitro*, an attractive source of stem cells for treatment of several cardiac diseases. The capacity of murine ES cells to differentiate into cardiac myocytes has been investigated intensively<sup>[10-13]</sup> and important findings were described, including the report of improvement of left ventricular function after myocardial infarction by transplanting murine ES cells in rodents<sup>[14,15]</sup>.

The first step to develop efficient cellular therapies for human diseases is the establishment of *in vitro* differentiation protocols and methods for sorting large amounts of specific cell types from total population without cellular damage. Unfortunately, the heterogeneous nature of cellular differentiation *in vitro* has discouraged the use of ES cells in transplantation studies<sup>[8]</sup>. A better understanding of epigenetic events leading to lineage commitment and differentiation might clarify the pathways of heterogeneous ES cells differentiation and support the development of efficient protocols aiming at the achievement of lineage-restrictive differentiation.

## REGULATION OF GENE EXPRESSION

Mammalian development occurs with the establishment of hundreds of cellular types, all of them derived from the same totipotent cell. Each differentiated cell relies on the same genetic material, although showing specific gene expression patterns, achieved by silencing and activation of tissue-specific genes<sup>[16]</sup>. The mechanisms of gene expression diversification are regulated by epigenetics. These heritable changes based in chromatin structure and not in DNA sequence, permit modulation of gene expression activities in response to external signs<sup>[17]</sup>.

Eukaryotic cells contain approximately 6 million pair bases of DNA corresponding to almost 30 000 different proteins. The main part of the DNA sequence remains silenced in a compact chromatin form which makes transcription difficult or impossible<sup>[18]</sup>. Only a small part of DNA is used for gene expression in each tissue type.

The chromatin structure is established by epigenetic modifications, including DNA methylation, histone post translational modifications, chromatin remodelling and non-coding RNAs<sup>[19]</sup>. Studies have elucidated DNA methylation and histone post translational modifications

as important events that play key roles in mammalian development and lineage specification<sup>[16]</sup>.

Nucleosomes form the fundamental repeating units of eukaryotic chromatin and are composed by 147 DNA base pairs wrapped twice around eight core histone proteins: 2 H2A, 2 H2B, 2 H3 and 2 H4<sup>[20]</sup>. Each core histone protein possesses a globular domain and a long N-terminal tail protruding from the nucleosome which can be covalently modified. Such modifications include acetylation, phosphorylation, methylation and others<sup>[18]</sup>. Histone modifications act in chromatin condensation, replication, DNA repair and transcriptional regulation. Some post translational modifications are associated with euchromatin (histone H3 acetyl-lysine 9, mono-, di- and tri-methyl lysine 4 and histone H4 acetylation) while others are related to heterochromatin (mono-, di-, tri-methyl lysine 9 and histone H3 tri-methyl lysine 27)<sup>[21]</sup>.

Among these post translational modifications, acetylation and methylation have been the most studied. Histone acetylation is related to transcriptionally-active domains and its levels correlate with gene expression<sup>[10]</sup>. Histone methylation can play a different role in gene expression events, depending on which residue is modified<sup>[22]</sup>. *Locus*-specific histone modifications are proposed for description of a code defining the transcriptional potential state of a cell - the histone code<sup>[23]</sup>.

When post translational modifications are blocked, development is affected. Histone-deacetylase 1 deficiency in mice leads to embryonic lethality<sup>[24]</sup>. Depletion of genes responsible for histone H3 lysine 9 methylation, *de novo* methylation and maintenance of methylated status also results in embryonic death<sup>[25-27]</sup>.

The regulatory mechanisms for transcription and chromatin organization involved with histone modifications are not clearly defined. One hypothesis is that epigenetic factors, including modifying enzymes and remodelling factors, are capable of inducing *cis*- and *trans*- chromatin interactions<sup>[28]</sup>. Conformational changes could even be mediated by protein complexes recruited by post translational modifications. These interactions would promote structural changes on chromatin and related DNA, altering their physical properties and affecting higher order structures, leading to consequences in many aspects of genome function<sup>[29]</sup>.

## HISTONE ACETYLATION

Histone acetylation, precisely on lysine residues, promotes neutralization of its positive charge, weakening the interaction between the histone tail and the negatively charged local DNA. This mechanism induces exposure of local chromatin structure<sup>[30]</sup>, permitting the binding of transcription factors and significantly increasing gene expression<sup>[31]</sup>.

Two main enzymes control acetylation. Histone acetyltransferase (HAT) adds acetyl groups to the histone tails, neutralizing them and weakening their nucleosome interactions. Histone deacetylase (HDAC), on the other hand, removes acetyl groups from histones and drives chromatin compaction and gene silencing on the local DNA<sup>[11]</sup>.

Over the past decade, more than a dozen HDAC were identified in mammalian cells. Based on their sequence similarities, HDACs were grouped into four functional classes: class I (HDAC1, HDAC2, HDAC3 and HDAC8), class II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10), class III (SIRT1 and SIRT7) and class IV (HDAC11 and related enzymes)<sup>[32]</sup>.

Studies in mice revealed that HDAC1 gene expression is stimulated by growth factors<sup>[33]</sup> and controlled by negative feedback mechanism of its own product<sup>[34]</sup>. HDAC1 plays a significant role in many biological processes, such as cell cycle progression, cell proliferation and differentiation, being essential for normal embryonic development<sup>[24]</sup>.

In studies of chromatin function, including histone acetylation, ES cells differentiation represents a unique model during lineage commitment in DNA regions regulated throughout development<sup>[35]</sup>. Pluripotent ES cells exhibit chromatin domains with both transcriptionally active and silent histone modifications<sup>[36]</sup>. At the onset of differentiation, changes in morphology and gene expression pattern become evident<sup>[37]</sup>. During early differentiation, transcription levels of many genes are strongly altered in a temporal manner. Epigenetic changes involving post translational histone modifications are related to regulation of both local and global gene expression<sup>[38]</sup>. Histone acetyltransferases stimulate transcription through the acetylation of histones, resulting in relaxation of nucleosomes<sup>[39]</sup>.

Histone deacetylases and acetylases transmit differentiation signals to initiate appropriate epigenetic modifications, such as erasure of pre-existing chromatin structure and establishment of new histone modification patterns during *in vitro* differentiation of ES cells<sup>[37]</sup>.

According to Meshorer *et al.*<sup>[40]</sup>, ES cells undergo a wave of global acetylation with the beginning of differentiation process. Histone H3 lysine 9 acetylation is an active euchromatin-related modification. Its level is almost undetectable in pluripotent ES cells and dramatically increases when cells leave the undifferentiated state. Histone H3 lysine 9 trimethylation, a silent heterochromatin functional component, is almost undetectable in pluripotent ES cells and increases on day 7 of differentiation<sup>[40]</sup>. Another study identified higher levels of histone H3 lysine 9 acetylation on day 2 of differentiation which increased two fold on day 4 and was maintained until day 18, the last day evaluated. On the other hand, histone H3 lysine 9 trimethylation levels increased on day 2, corresponding to a deacetylation of this residue<sup>[35]</sup>.

When pluripotent genes were evaluated, including *Oct4*, *Nanog*, *Utf1*, *Foxd3*, *Cripto* and *Rex1*, they revealed an opened chromatin on undifferentiated cells and condensed chromatin after differentiation. In contrast, neural-specific genes including *Pax3*, *Pax6*, *Irx3*, *Nkx2.9* and *Mash1* were revealed as transcriptionally silent on pluripotent cells and active after tissue-specific differentiation<sup>[41]</sup>. All these changes reveal the importance of epigenetic control over ES cells differentiation.

## USE OF HDAC INHIBITORS TO INDUCE ES CELLS DIFFERENTIATION

HDAC inhibitors supplementation aims to evaluate histone acetylation effects on gene expression before and after cellular differentiation and elucidate molecular pathways controlling the loss of undifferentiated state and commitment to a specific cellular lineage<sup>[9,39,42-45]</sup>.

Trichostatin A (TSA) is a reversible HDAC inhibitor that reacts at nanomolar concentrations with most class I and II HDACs<sup>[30]</sup>, promoting histone hyperacetylation and strongly increasing cellular protein synthesis. This event leads to differentiation of tumor cells, preventing them from proliferation<sup>[46]</sup>. In some cases histone hyperacetylation can cause cell cycle arrest or even apoptosis<sup>[18]</sup>. Crystallography studies indicate that TSA blocks the enzyme catalytic site by chelating a zinc ion on the enzyme tubular structure base<sup>[46]</sup>.

TSA treatment promotes histone H3 and H4 hyperacetylation even when performed in the presence of LIF, rapidly leading to morphological and molecular changes resembling those observed in the early phase of ES cells differentiation<sup>[47]</sup>.

The increased acetylation levels caused by TSA induced in pluripotent ES cells the same cellular behaviour as those involved in differentiation processes. The authors also reported decreased levels of histone H3 lysine 9 acetylation in undifferentiated ES cells treated with TSA on pluripotent related genes: OCT3/4, REX1 and FGF4. In differentiation-related genes, histone H3 lysine 9 acetylation was higher in TSA treated cells.

HDAC inhibition is related to neuronal lineage progression<sup>[48-50]</sup>. Histone deacetylation is involved with repression of neuronal genes in non-neuron cells. HDAC 1 and 2 combine with co-repressors CoREST, N-CoR and mSin3A and are recruited by REST (also known as NRSF - *neuron-restrictive silencer factor*)<sup>[51]</sup>. REST blocks transcription of neuronal genes by linking to NRSE - *neuron-restrictive silencer element* - present on regulatory regions of many neuronal genes. HDAC inactivation inhibits REST mechanisms and prevents its role against neuronal differentiation. Studies using rat hippocampal progenitor cells revealed a neurogenic transcription factor (NeuroD - *neurogenic differentiation transcription factor*) increased after HDAC inhibition, leading to neuronal differentiation<sup>[52]</sup>.

Neuronal differentiation in TSA-treated cultures seemed to be enhanced at the expense of oligodendrocytes which need HDAC activity for the progression of neural progenitors into mature oligodendrocytes<sup>[53]</sup>. Also, even though astrocyte differentiation involves HAT activity for glial fibrillary protein (GFAP) expression<sup>[53,54]</sup>, TSA apparently decreases its proportion in comparison to neuron cells, suggesting that HDAC inhibitors enhances neuronal differentiation also at the expense of astrocytes<sup>[50]</sup>.

Histone acetylation is also involved in striated myocyte differentiation. Class II HDACs are highly expressed in adult cardiomyocytes and skeletal myoblasts<sup>[15]</sup> where they bind and repress myocyte enhancer factors 2 (MEF2)

functions<sup>[55,56]</sup>. HDACs are also involved in the repression of neuronal genes in cells that are not committed to neuronal specification and the HDACs inhibition is related to cellular commitment in many lineages<sup>[51]</sup>.

In this respect, the control of global histone acetylation can be obtained by using HDAC inhibitors. Class II HDAC are highly expressed in skeletal and cardiac muscle<sup>[15]</sup> and interact with myocyte enhancer factors (MEF2) inactivating them. Because of this property, HDAC are used clinically for cardiac hypertrophy treatment. Studies *in vitro* have demonstrated that TSA enhances striated myocyte population in undifferentiated stem cells when applied at day 6 or 7 of differentiation<sup>[9,57,58]</sup>. The last authors<sup>[9]</sup> verified that TSA induces the expression of p300, an intrinsic histone acetyltransferase that is co-activator of GATA-4 gene (a critical cardiac transcription factor), suggesting that mechanisms of TSA-induced cardiac-specific differentiation involve acetylation of specific transcription factors such as GATA-4.

TSA treatment induces entry of mesoderm cells into the cardiac muscle differentiation process in a dose-dependent manner, increasing Nkx-2, MEF2C, GATA4 and cardiac actin transcripts<sup>[58]</sup>. Authors believe that HDAC4 expression inhibits cardiomyogenesis, decreasing cardiac muscle related genes expression, and inhibition of HDAC activity is sufficient for increasing early cardiomyogenesis<sup>[9,57]</sup>.

Nonetheless, there are studies<sup>[39]</sup> that show TSA detrimental effects to striated myogenesis when the HDAC inhibitor is applied at the onset of ES cell differentiation. HDACs have a critical role in cellular biology by controlling the expression of selective cell cycle inhibitors<sup>[24]</sup> and many studies have shown that HDAC inhibition leads to anti-proliferative effects on ES cells<sup>[39,47,59,60]</sup>. Thus, the effects of TSA over the cells appear to be completely dependent on dosage and stage of differentiation.

Also, HDAC inhibition causes an increase in the number of apoptotic cells by inducing the expression of pro-apoptotic genes<sup>[61]</sup> as well as histone hyperacetylation since the relaxed form of DNA is easily catalyzed by endonucleases<sup>[62]</sup>. Therefore, the HDACs inhibitors require attention on its use, aiming at minimum dosages.

## CONCLUSION

Elucidation of mechanisms driving ES cells differentiation and the consequent control of these events are the objective of numerous studies in cellular and molecular biology. Unfortunately, this process is still not clear in many aspects and the same culture system drives cellular commitment in different ways.

During differentiation, levels of histone acetylation are increased, leading to rises in gene expression from all germ layers. But, in this early differentiation phase, cells are not committed yet to specific lineages<sup>[63]</sup>. The understanding of when these chromatin modifications can drive cellular differentiation and how they are achieved is an objective of many studies. For instance, studies inducing

histone hyperacetylation in ES cells in order to establish a desired cellular phenotype are conducted in cardiomyocytes and neuronal cell types.

In ES cells differentiation, even though changes in gene expression patterns can alter phenotype and function of cells, more studies are needed in order to elucidate whether these transient changes in gene expression promoted by HDAC inhibitors can sustain differentiation or only a transitory phenotype. In addition, the establishment of adequate protocols in order to minimize antiproliferative effects and obtain desirable effects are needed, considering the dose and the stage of cellular differentiation.

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