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The Dynamics of Global Chromatin Remodeling are Pivotal for Tracking the Normal Pluripotency of Human Embryonic Stem Cells

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Abstract

Pluripotent Human Embryonic Stem Cells (hESCs) have the unconstrained capacity for long-term stable undifferentiated growth in culture and unrestricted developmental capacity. Packaging of the eukaryotic genome into chromatin confers higher order structural control over maintaining stem cell plasticity and directing differentiation. We recently reported the establishment of a defined culture system for sustaining the epiblast pluripotence of hESCs, serving as a platform for *de novo* derivation of clinically-suitable hESCs and effectively directing such hESCs uniformly towards functional lineages. To unveil the epigenetic mechanism in maintaining the epiblast pluripotence of hESCs, in this study, the global chromatin dynamics in the pluripotent hESCs maintained under the defined culture were examined. This study shows that the genomic plasticity of pluripotent hESCs is enabled by an acetylated globally active chromatin maintained by Oct-4. The pluripotency of hESCs that display normal stable expansion is associated with high levels of expression and nuclear localization of active chromatin remodeling factors that include acetylated histone H3 and H4, Brg-1, hSNF2H, HAT p300, and HDAC1; weak expression or cytoplasmic localization of repressive chromatin remodeling factors that are implicated in transcriptional silencing; and residual H3 K9 methylation. A dynamic progression from acetylated to transient hyperacetylated to hypoacetylated chromatin states correlates with loss-of-Oct4-associated hESC differentiation. RNA interference directed against Oct-4 and HDAC inhibitor analysis support this pivotal link between chromatin dynamics and hESC differentiation. These findings reveal an epigenetic mechanism for placing global chromatin dynamics as central to tracking the normal pluripotency and lineage progression of hESCs.

Keywords: Human embryonic stem cells; Pluripotency; Epigenetic; Chromatin; Histone modification; Histone acetylation; Histone deacetylation; Histone methylation; Histone acetyltransferase; Histone deacetylase; Histone methytransferase; Chromatin remodeling; Oct-4; Differentiation; Defined culture system

Abbreviations: acH3: acetylated histones H3; acH4: acetylated histones H4; ESC: Embryonic Stem Cell; HAT: Histone Acetyltransferase; HDAC: Histone Deacetylase; HMT: Histone Methyltransferase; hESC: Human Embryonic Stem Cell; ICM: Inner Cell Mass; meH3K4/9/27: H3K4/9/27 methylated histones; NURD: Nucleosome Remodeling And Deacetylation Complex; RNAi: RNA interference

Introduction

Undifferentiated human embryonic stem cells (hESCs), as essentially the *in vitro* equivalent of the inner cell mass (ICM) of the blastocyst, have the potential for differentiation into any somatic cell type. This pluripotent state of hESCs is associated with the expression of a unique group of genes, including Oct-4, alkaline phosphatase, SSEA-4, Tra-1-60, Tra-1-80, though none of these markers, in isolation, is exclusively expressed by undifferentiated hESCs [1]. Rather, their presence as a group is associated with the undifferentiated state of hESCs. The unrestricted plasticity and undifferentiated state of pluripotent hESCs remain poorly understood at the molecular level. For mouse ESCs, the cytokine-dependent LIF/Stat3 pathway and the cytokine-independent Nanog pathway are involved in the maintenance of pluripotency [2-4]. Both pathways require the sustained expression of Oct-4 in mouse ESCs [2-4]. In embryogenesis, only cells in the ICM express Oct-4. Loss of Oct-4 expression at the blastocyst stage causes these cells to differentiate into extraembryonic lineages, while Oct-4 expression insures embryonic germ layer assignment and lineage differentiation [2-4]. However, human and mouse embryonic stem cells (ESCs) actually express opposite markers and require distinct conditions for maintenance and differentiation [5,6]. Unlike mouse ESCs, the maintenance of undifferentiated hESCs does not require LIF and the LIF/Stat3 signaling pathway, suggesting that an entirely different regulatory system might be employed in human [7,8].

The eukaryotic genome is packaged into a nucleoprotein complex known as chromatin, in which the DNA helix is wrapped around an octamer of core histone proteins to form a nucleosomal DNA structure [9,10]. Packaging of the eukaryotic genome into chromatin confers a higher order of epigenomic structure and control over the unfolding of developmental process, which goes beyond what might be predicted based solely on profiling a cell's genomic or proteomic patterns [9,10]. Recent studies have begun to resolve the interface between chromatin and transcription regulation in ESC maintenance [11-19]. Chromatin is a highly dynamic structure regulated by chromatin remodeling processes that include: 1) covalent modification of histones and DNA by enzymatic activities and 2) ATP-driven DNA/nucleosome translocation by chromatin remodeling factors, and 3) incorporation of alternative histone variants [9-16,20]. In general, histone acetylation and histone H3 K4 methylation correlate with a transcriptionally active (open) chromatin state, whereas histone deacetylation and histone H3

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K9 methylation correlate with a transcriptionally repressed (closed) chromatin state [9-16]. Chromatin modifications serve as important epigenetic marks for active and inactive structures, and have emerged as the principal epigenetic mechanism in embryonic development [11-16]. However, the global chromatin dynamics in maintaining the pluripotency of hESCs that display normal stable expansion remain to be understood.

Previously, we have resolved the elements of a defined culture system necessary and sufficient for sustaining the epiblast pluripotence of hESCs, serving as a platform for *de novo* derivation of clinically-suitable hESCs and effectively directing such hESCs uniformly towards functional lineages with small molecule induction [21-23]. To unveil the epigenetic mechanism in maintaining the epiblast pluripotence of hESCs, in this study, the global chromatin dynamics in the pluripotent hESCs maintained under the defined culture were examined. This study shows that the genomic plasticity of pluripotent hESCs is enabled by an acetylated globally active chromatin maintained by Oct-4. A dynamic progression from acetylated to transient hyperacetylated to hypoacetylated chromatin states correlates with loss-of-Oct4-associated hESC differentiation, as assessed by Oct-4 RNA interference (RNAi) and histone deacetylase (HDAC) inhibitor analysis. These findings reveal an epigenetic mechanism for placing global chromatin dynamics as central to tracking pluripotency and lineage progression of hESCs.

Materials and Methods

Culture of undifferentiated hESCs

The hESC lines WA01 and WA09 (H1 and H9 from WiCell Research Institute, passages 30-50) were used in this study. The defined culture systems consist of DMEM/F-12 or KO-DMEM (knockout-DMEM) (80%), Knockout Serum Replacement (KO) (20%), L-alanyl-L-gln or L-gln (2 mM), MEM nonessential amino acids (MNAA, 1X), β-Mercaptoethanol (β-ME,100μM) (all from Invitrogen), human purified laminin (Sigma) or laminin/collagen (growth factor reduced Matrigel, BD Bioscience) as the matrix protein, and bFGF (basic fibroblast growth factor, 20 ng/ml) (PeproTech Inc). The KO can be replaced with defined essential factors containing MEM essential amino acids (MEAA, 1X), human insulin (20 μg/ml) (Sigma), and ascorbic acid (50 μg/ml) (Sigma), in which activin A (50 ng/ml, Sigma), human albumin (10 mg/ml, Sigma), and human transferrin (8 μg/ml, Sigma) were added in order to increase cell survival and maintain normal shape and healthy colonies.

Immunofluorescence and deconvolution microscopy

The cells were fixed with 4% paraformaldehyde and blocked in PBS containing 0.2% Triton X-100 and 2% BSA. The cells were incubated with the primary antibody in 0.1% Triton X-100 in PBS at 4°C overnight, and then with secondary antibody (Molecular Probe/Invitrogen) in the same buffer at room temperature for 45 min. After staining with DAPI, cells were visualized under an immunofluorescence and deconvolution microscope, and quantified by the image analysis software (Olympus). Primary antibodies to Oct-4, SSEA-3, Brm, Brg-1, hSNF2H, MOZ, HBO1 and PCAF were from Santa Cruz Biotechnology, Inc.; antibodies to p300, Tip60, acetylated H3 (K9, 14), acetylated H4 (K5, 8, 12, 16), methylated H3 (K9), SIRT1, SUV39H1, HDAC1, 3-7, and Nestin were from Millipore/Upstate Biotech.

RNA-mediated interference (RNAi)

The siRNAs directed against two selected Oct-4 targeting sequences were purchased from Qiagen. hESCs maintained in the defined culture were transfected with Oct-4 siRNAs at a final concentration of 300 nM with RNAi HiPerFect transfection reagent (Qiagen) 3 day after seeding. Culture medium was replaced 2 days later and cells were re-transfected with siRNAs (300 nM) once more. Cells were allowed to grow to day 7 and fixed for further analysis.

Manipulation of HDAC activity

Effective histone acetylation can be artificially increased by inhibiting HDAC activity with trichostatin A (TSA) (Sigma). Undifferentiated hESCs were seeded under the defined culture conditions and cultivated for 3-5 days prior to treatment with TSA (100 ng/ml for 24 hours). Controls were similarly treated with DMSO. The cultures were further analyzed by immunocytochemistry and/or Western blotting.

Results and Discussion

The pluripotent state of hESCs maintained under the defined culture is associated with an acetylated globally active chromatin

To profile the epigenetic global chromatin features associated

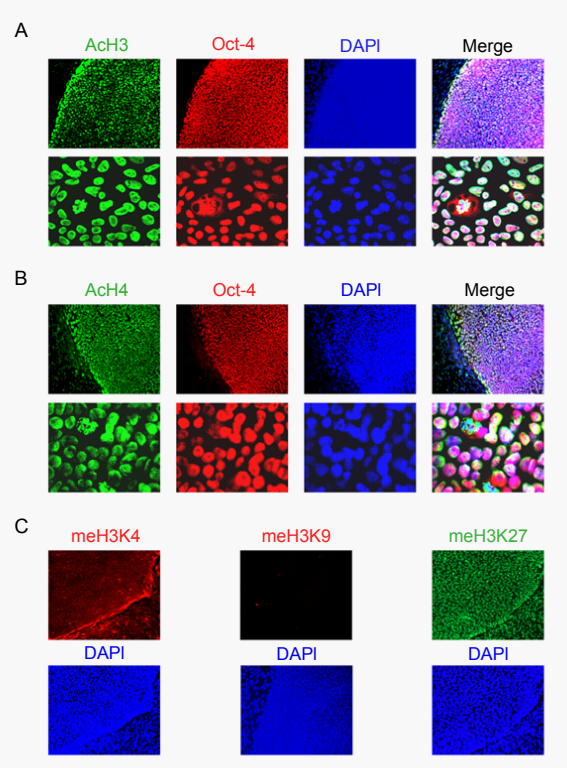

Figure 1: The pluripotent state of hESCs maintained under the defined culture is associated with an acetylated chromatin. (A) High level of expression and nuclear localization of acetylated histone H3 (acH3, green) in undifferentiated hESCs, as indicated by Oct-4 (red) expression, inside the colony, and significantly reduced immune-reactivity of differentiated cells outside the colony. **(B)** High level of expression and nuclear localization of acetylated histone H4 (acH4, green) in undifferentiated hESCs, as indicated by Oct-4 expression, inside the colony, and significantly reduced immune-reactivity of differentiated cells outside the colony. **(C)** Moderate histone H3 K4 methylation (meH3K4, red) and H3 K27 methylation (meH3K27, green), and undetectable histone H3 K9 methylation (meH3K9, red) in hESCs maintained under the defined culture. All cells are shown by DAPI staining (blue). High-resolution microscopic images in the bottom panel reveal cellular localization patterns.

with the epiblast pluripotent state of hESCs, we performed immunoanalysis of chromatin modifications on hESC colonies maintained under the defined culture [21-23]. The hESC colonies appear to have a constitutive developmental dynamic. The undifferentiated hESCs maintained under the defined culture form the typical tightly packed colonies of small compact cells that are Oct-4 positive, while large differentiated cells migrate outside the colonies and become Oct-4 negative (Figure 1A, 1B). We observed high levels of expression and nuclear localization of acetylated histone H3 and H4 in Oct-4-positive undifferentiated hESCs inside the colony, suggesting an acetylated active (open) chromatin state in the pluripotent hESCs (Figure 1A, 1B), consistent with previous observation for hESCs maintained on feeders [12,13]. Conversely, in the Oct-4-negative differentiated cells outside the colony, immune-reactivity for acetylated histone H3 and H4 was significantly reduced, suggesting a hypoacetylated repressive (closed) chromatin in the differentiated cells (Figure 1A, 1B). In addition, we detected moderate histone H3 K4 methylation and H3 K27 methylation in hESCs maintained under the defined culture (Figure 1C), consistent with previous observations for their involvement in silencing developmental genes in hESCs [13-15]. However, we did not observe histone H3 K9 methylation in hESCs maintained under the defined culture (Figure 1C), a chromatin modification implicated in transcriptional repression during development [24-26].

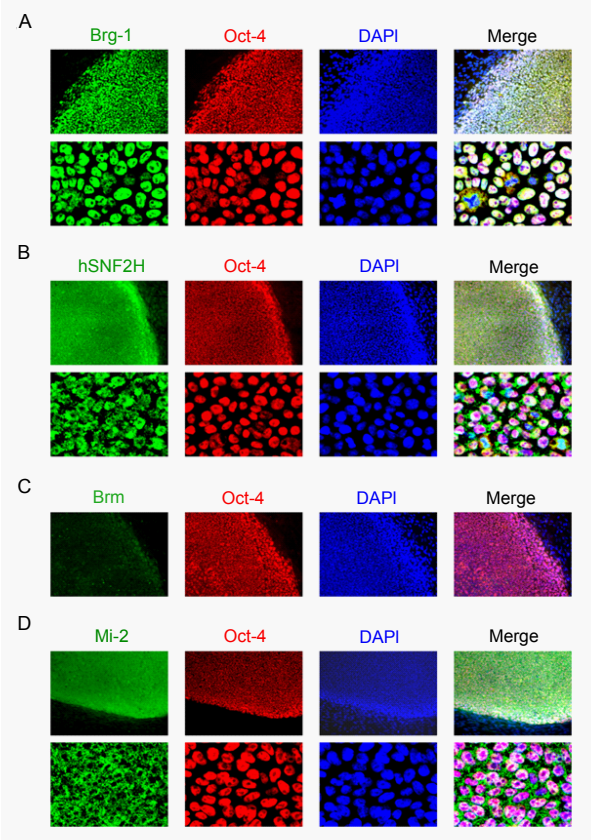

Figure 2: The pluripotent state of hESCs is associated with active chromatin remodeling factor Brg-1 and hSNF2H. (A) High level of expression and nuclear localization of Brg-1 (green) in undifferentiated hESCs, as indicated by Oct-4 (red) expression, inside the colony, and significantly reduced immune-reactivity of differentiated cells outside the colony. **(B)** High level of expression and mostly nuclear localization of hSNF2H (green) in undifferentiated hESCs, as indicated by Oct-4 expression, inside the colony, and significantly reduced immune-reactivity of differentiated cells outside the colony. **(C)** Weak expression of Brm (green) in undifferentiated hESCs, as indicated by Oct-4 expression, inside the colony. **(D)** Mostly cytoplasmic localization of Mi-2 (green) in undifferentiated hESCs, as indicated by Oct-4 expression, inside the colony, and significantly reduced immune-reactivity of differentiated cells outside the colony. All cells are shown by DAPI staining (blue). High-resolution microscopic images in the bottom panel reveal cellular localization patterns.

Further, we observed high levels of expression and nuclear/perinuclear localization of the ATP-dependent chromatin-remodeling factor Brg-1 and hSNF2H in Oct-4-positive undifferentiated hESCs inside the colony, suggesting active chromatin remodeling in the pluripotent hESCs (Figure 2A, 2B). Conversely, in the Oct-4-negative differentiated cells outside the colony, immunoreactivity for these factors was significantly reduced (Figure 2A, 2B). Chromatin remodeling factors are ATP-utilizing motor proteins that mediate the interaction of proteins with nucleosomal DNA by DNA/nucleosome-translocation [27]. Brg-1 is a subunit of the Swi/Snf chromatin remodeling complex implicated in the regulation of cellular proliferation and as a tumor suppressor; while hSNF2H is a human homolog of the ISWI family of chromatin remodeling proteins [28,29]. By contrast, we observed weak expression of chromatin remodeling factor Brahma (Brm) and cytoplasmic localization of chromatin remodeling factor Mi-2 in the pluripotent hESCs (Figure 2C, 2D). Brm is a chromatin-remodeling factor implicated not only in histone H3 K9 methylation and transcriptional silencing, but also in neural differentiation and mouse embryonic development [30-32]. Mi-2 is an integral component of a nucleosome remodeling and deacetylation complex (NURD) [33,34]. Therefore, lack of Brm expression and cytoplasmic localization of Mi-2, suggestive of being inactive, in the pluripotent hESCs are consistent with their acetylated active chromatin state.

Analysis of histone acetyltransferases (HATs) revealed high level of expression and nuclear localization of p300, cytoplasmic localization of Tip60, PCAF, Moz, and weak expression of HBO-1 in Oct-4-positive undifferentiated hESCs inside the colony (Figure 3A). p300 and PCAF are transcriptional co-activators involved in a variety of signaling pathways, including Notch, one of the highly conserved in development and previously implicated in the self-renewal of haematopoietic stem cells [35]. Tip60, Moz, and HBO-1 are human homologues of MYST family of acetyltransferases implicated in transcriptional silencing [36].

Having assessed the presence of histone modification activities that would promote an acetylated chromatin, we next examined the expression pattern of those that might provide a balance, including histone deacetylase (HDAC) and histone methyltransferase (HMT), in the pluripotent hESCs maintained under the defined culture. Among the class I HDACs tested, we observed strong expression and nuclear localization of HDAC1, and weak expression of HDAC3 in the pluripotent hESCs (Figure 3B). HDAC1 is a general maintenance histone deacetylase that sustains global transcription at a basal level [37]. Among the class II HDACs tested, which contain a group of large molecules homologous to yeast Hda1 and which are tissue-specific [37], we observed mostly cytoplasmic localization of HDAC4, HDAC5, HDAC6, and HDAC7 in the pluripotent hESCs, suggestive of being inactive (Figure 3C). Although the class III NAD-dependent histone deacetylase SIRT1 and the H3 K9 histone methyltransferase (HMT) SUV39H1, which are involved in histone H3 K9 methylation and transcriptional silencing in development [9,24-26], were expressed in hESCs, their cytoplasmic localization patterns indicated that they

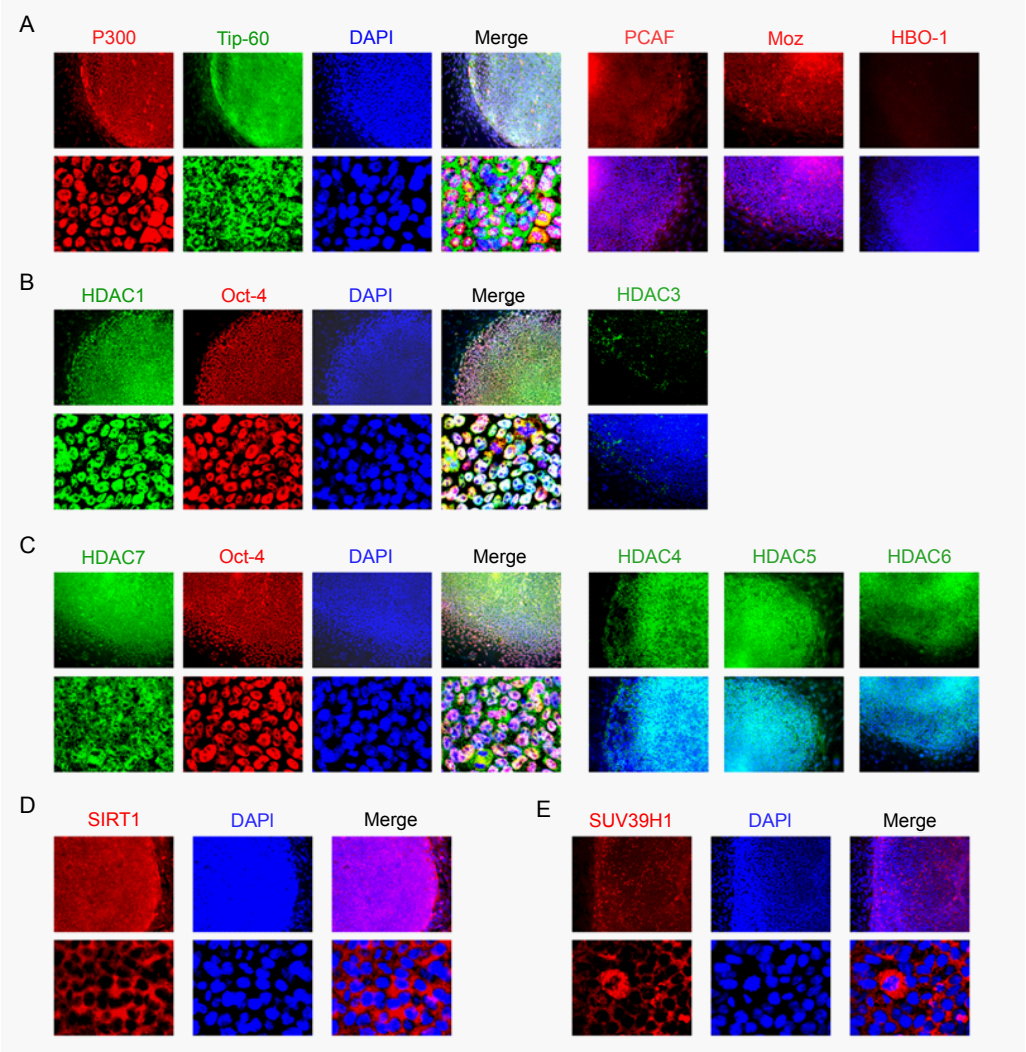

Figure 3: The pluripotent state of hESCs is associated with p300 and HDAC1. (A) High level of expression and nuclear localization of p300 (red), and cytoplasmic localization of Tip60 (green) and Moz (red), and weak expression of HBO-1 (red) in undifferentiated hESCs, as indicated by Oct-4 (red) expression, inside the colony. **(B)** High level of expression and nuclear localization of HDAC1 (green), and weak expression of HDAC3 (green), the class I HDACs, in undifferentiated hESCs, as indicated by Oct-4 expression, inside the colony. **(C)** Mostly cytoplasmic localization of HDAC7 (green), HDAC4 (green), HDAC5 (green), and HDAC6 (green), the class II HDACs, in undifferentiated hESCs, as indicated by Oct-4 expression, inside the colony. **(D)** Cytoplasmic localization of SIRT1 (red), the class III HDAC, in undifferentiated hESCs inside the colony. **(E)** Cytoplasmic localization of SUV39H1 (red) in undifferentiated hESCs inside the colony. All cells are shown by DAPI staining (blue). High-resolution microscopic images in the bottom panel reveal cellular localization patterns.

remained mostly inactive in the pluripotent hESCs as well (Figure 3D, 3E).

The pluripotence of undifferentiated hESCs maintained under defined culture system for a prolonged period (>30 passages) was further affirmed by teratoma formation following injected into SCID mice. Histological analysis of the resulting teratomas confirmed the presence of tissues of all three embryonic germ layers, including pigmented and neural tissue (ectoderm); gut epithelium (endoderm); adipose cells and vascular endothelium, cartilage, smooth muscle and connective tissue (mesoderm) (Figure 4), indicating that the hESCs retain pluripotent under the defined culture system.

Taken together, these observations suggest that the pluripotent state of hESCs is associated with an acetylated globally active chromatin that would maintain their ability to replicate and respond to inductive signals in the milieu and provide the molecular foundation for the normal pluripotency of hESCs.

A dynamic progression from acetylated to transient hyper-acetylated to hypo-acetylated chromatin states correlates with loss-of-Oct4-associated differentiation

The hESC colony is a structure that displays spontaneous early differentiation processes that can be divided into three zones (Figure 5A), hence providing a simple system for assessing the alterations in chromatin state and Oct-4 level that accompany this transition. Zone 1, within the core of the colony, contains small compact undifferentiated hESCs that are Oct-4-positive, but immune-negative for the cell surface marker SSEA-3 and the intermediate filament Nestin (Figure 5A). Initiation of differentiation typically occurs spontaneously in cells towards the periphery, a transitional Zone 2 (those with broader peripheries are shown in Figure 5A for demonstrative purpose). Zone 2

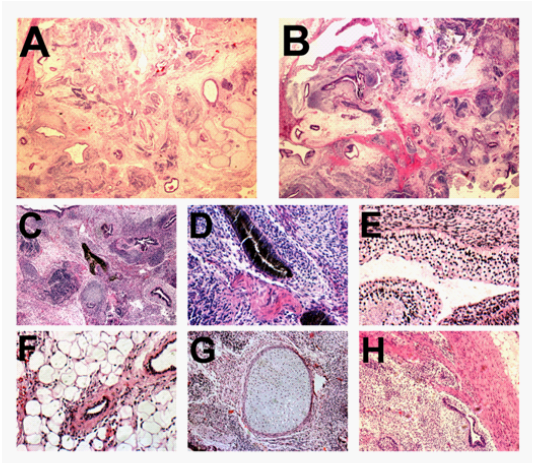

Figure 4: Assessing pluripotency of hESCs maintained under the defined culture with teratoma formation. Undifferentiated hESCs after prolonged propagation in the defined system were injected into SCID mice. Histological analysis of the resulting teratomas confirmed the presence of tissues of all three embryonic germ layers (**A-C**), including pigmented and neural tissue **(D)** (ectoderm); gut epithelium **(E)** (endoderm); adipose cells and vascular endothelium **(F)**, cartilage **(G)**, smooth muscle and connective tissue **(H)** (mesoderm). Magnification: **(A)** and **(B)**, 4X; **(C)**, 10X; **(D-H)**, 20X.

cells become flattened and begin to express SSEA-3 and Nestin (Figure 5A). Further differentiation begins to become stabilized in cells that have migrated beyond the colony -- Zone 3 – composed of large cells that are Oct-4- and SSEA-3-negative but continue to express Nestin robustly (Figure 5A). It is in Zone 3 that differentiation in cells begins to become stabilized.

A decreased level of Oct-4 expression was observed in cells in the transitional Zone 2, and, decreasing expression of Oct-4 was associated with transiently enhanced H3 and H4 acetylation, and hence hyperacetylation (Figure 5A), coincident with the enhanced expression of the HAT p300, as confirmed by quantitative intracellular imaging analysis (Figure 5A). Therefore, decreasing expression of Oct-4 may itself trigger hyperacetylation and initiate differentiation by allowing alterations in chromatin state. Complete suppression of Oct-4 occurred in Zone 3 cells outside the colony, which, in contrast, displayed dramatically reduced H3 and H4 acetylation and p300 expression (Figure 5A), indicating now a hypoacetylated repressive chromatin structure.

These observations suggest a dynamic whereby hESCs in their undifferentiated state (Zone 1) are associated with an acetylated active chromatin governed by Oct-4; that transient histone hyperacetylation occurs with the decreasing expression of Oct-4 and the initiation of differentiation (Zone 2), which may then trigger Oct-4 independent

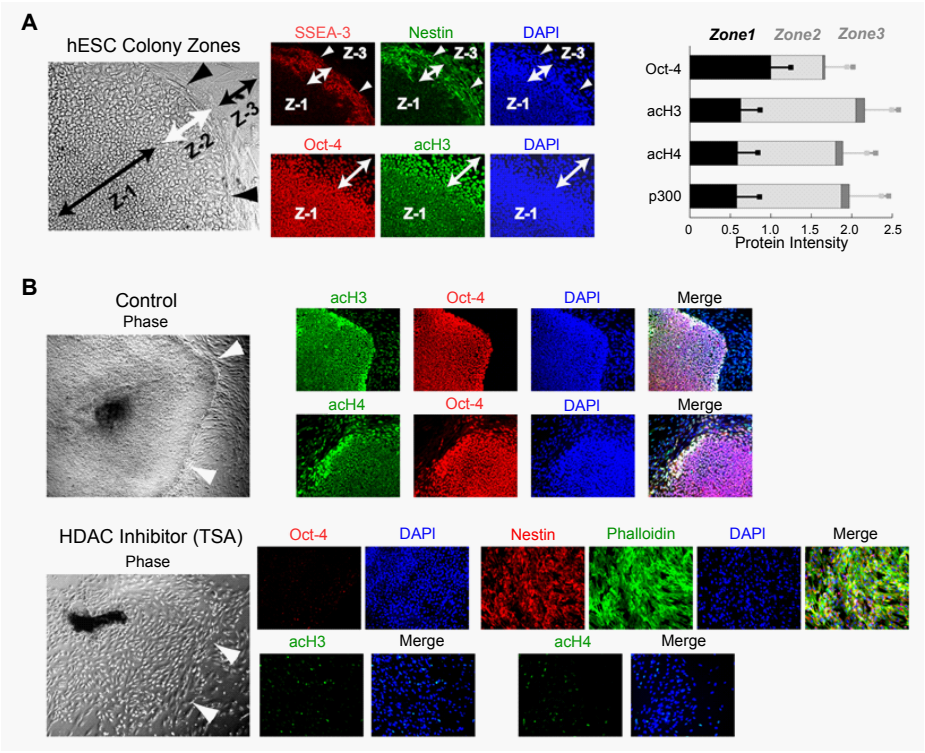

Figure 5: Loss-of-Oct-4 associated hESC differentiation is mirrored by a dynamic progression from acetylated to transient hyperacetylated to hypoacetylated chromatin states. (A) The hESC colony structural zones of spontaneous differentiation. The small compact cells at the core of the colony (Zone 1 **[Z-1])** are SSEA-3 (red) and Nestin (green) negative; cells at the periphery (Zone 2 **[Z-2]**, delineated by double-headed white arrows) begin to express SSEA-3 and Nestin; and cells migrated beyond the colony (Zone 3 **[Z-3]**) continue to express Nestin but lose SSEA-3 expression. Decreased expression of Oct-4 (red) in transitional Zone-2 cells is associated with transiently increased H3 acetylation (acH3, green). Analysis of protein intensities from quantitative intracellular imaging confirms that the decreased expression of Oct-4 in transitional Zone-2 cells is associated with transiently increased histone acetylation, coincident with enhanced expression of p300. **(B)** Inhibition of HDAC activities results in hESC differentiation. The hESCs maintained under the defined culture were treated with specific HDAC inhibitor trichostatin A (TSA); controls were similarly treated with DMSO. Photomicrographs are shown 3 days after removal of the HDAC inhibitor. Inhibition of HDAC activity resulted in the extinguishing of Oct-4 expression and the appearance of large differentiated Oct-4-negative cells throughout the colony that began to express Nestin (red) and phalloidin (green), and show significantly reduced immune-reactivity to acetylated histone H3 (acH3, green) and acetylated histone H4 (acH4, green). All cells are shown by DAPI staining (blue). White arrows indicate the colony edge.

recruitment of HDAC complexes to chromatin and hence deacetylation leading to the compaction of hypoacetylated nucleosomes into a repressive chromatin structure such that progress towards a more differentiated state can proceed in Zone 3.

Altering the balance between acetylation and deacetylation with HDAC inhibitor and Oct-4 RNAi supports the pivotal link between chromatin dynamics and hESC differentiation

The observations above suggested that HDAC activities were required for maintaining the undifferentiated state of hESCs by balancing HAT action and preventing hyperacetylation, a balance possibly mediated by Oct-4. To test this notion, we experimentally manipulated HDAC activities by using the specific HDAC inhibitor (HDACi): trichostatin A [TSA]. Treating undifferentiated hESCs maintained under the defined culture with TSA resulted in dramatic differentiation, indicated by the ectopic appearance throughout the colony (including in Zone 1) of large Oct-4-negative Zone-3-like cells that expressed Nestin and phalloidin and subsequently became dramatically hypoacetylated after withdrawal of the HDACi (Figure 5B). HDAC inhibitors have reportedly distinct stage-specific effects on promoting or inhibiting myogenesis, including enhancing Oct-4 expression in myogenic cells, mirrored by changes in the state of histone acetylation present at a muscle-gene enhancer [38,39].

Having observed the link between a change in histone acetylation and hESC differentiation (Figure 5), we next attempted to link loss-of-Oct-4-function itself to the triggering of these chromatin-mediated dynamics. Oct-4 is a DNA-binding factor to a variety of non-specific AT-rich sequences, however, its role in regulating the transcription of specific genes has barely been demonstrated [2,17,40]. Unlike most transiently expressed transcriptional factors, Oct-4 is an abundant nuclear protein associated specifically with hESCs in their undifferentiated state [2,40]. Of new interest, we observed that changes in Oct-4 expression level appeared to promote differentiation by allowing alterations in chromatin state (Figure 5). This evidence suggested that Oct-4 might actually function not as a specific promoter-binding transcription factor – the convention view of Oct-4 that has proven inadequate to interpreting its role in maintaining pluripotency and ensuring lineage differentiation [2,17,40] -- but rather as a global DNA-binding regulator for maintaining the pluripotent chromatin state -- by recruiting other chromatin-remodeling activities. To further decipher the function of Oct-4 in chromatin-mediated dynamics described above, undifferentiated hESCs maintained under the defined culture were transfected with small double-stranded RNAs (siRNA) (the efficacy and specificity of the RNAi having been affirmed by appropriate knock-down of mRNA, based on RT-PCR, and of protein, based on Western and immunocytochemical analysis). The Oct-4 siRNA effectively reduced Oct-4 expression in cells inside > 90% of the hESC colonies, compared to persistent Oct-4-positivity in cells inside > 70% of control colonies (Figure 6). Oct-4 knockdown resulted in a dramatic reduction of HDAC1 but not HAT p300 (Figure 6), a condition that would serve to tip the balance towards hyperacetylation. Indeed, the Oct-4 knocked-down cells displayed strong H3 and H4 acetylation, and strong expression of Brg-1 and hSNF2H, which, in turn, coincided with the induction of differentiation within these colonies as indicated by the appearance of large flattened cells and expression of the differentiation marker Nestin (Figure 6), recapitulating the emergence of hyperacetylated transitional Zone-2-like cells in hESC spontaneous differentiation (Figure 5A). These observations further supported the view that Oct-4 might recruit HDAC1 to preserve a globally active chromatin state and maintain a balanced level of histone acetylation to sustain the pluripotent state in hESCs, and that changes in Oct-4 expression appeared to promote hESC differentiation by allowing alterations in chromatin state.

Taken together, these results support a dynamic linking change in acetylation with loss-of-Oct4-associated hESC differentiation. Although undifferentiated hESCs are associated with an acetylated active chromatin structure, HDAC activities appear to be required for maintaining the pluripotent state by forestalling hyperacetylation and the onset of differentiation (Figures 5, 6). Histone hyperacetylation, coincident with decreasing Oct-4, may trigger the initiation of differentiation (Figures 5, 6). Further Oct-4 independent deacetylation leads to the stabilization of the differentiated state via the compaction of hypoacetylated nucleosomes into a repressive chromatin structure (Figure 5).

An role for Oct-4 in maintaining pluripotence: orchestrating global active chromatin-remodeling

These observations suggest a chromatin remodeling model that

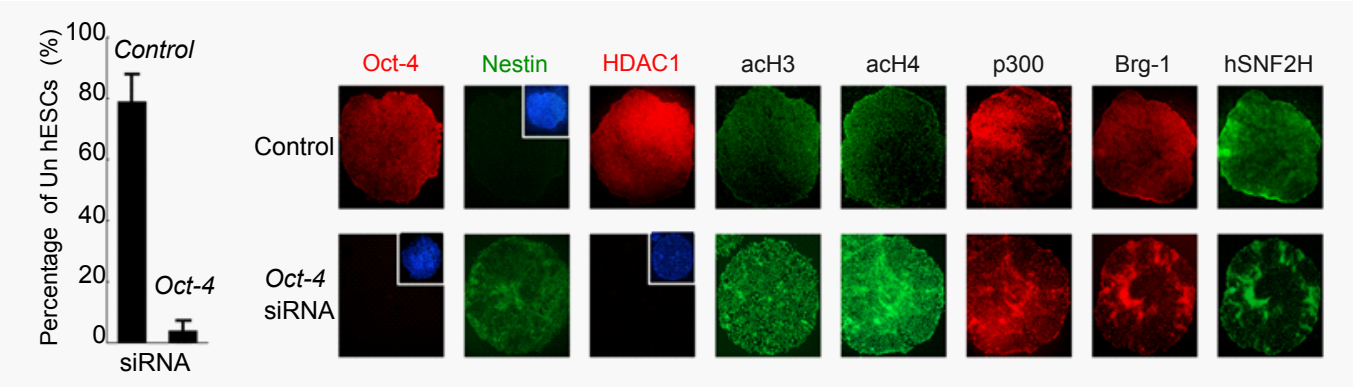

Figure 6: RNAi analysis of Oct-4's role in maintaining pluripotency. siRNAs directed against Oct-4 effectively reduced Oct-4 expression in >90% of cells, as compared to persistent Oct-4-positivity in >70% of control colonies. The Oct-4 siRNA effectively reduces Oct-4 (red) expression in cells inside the colony, and induces concomitant reduction of HDAC1 (red) expression and differentiation, as indicated by the expression of Nestin (green). These Oct-4 knocked-down cells also showed strong histone H3 and H4 acetylation (acH3 and acH4, green) and strong expression of p300 (red), Brg-1 (red), and hSNF2H (green). The Insets are DAPI-stained (blue) colonies shown that cells were present but immuno-negative for the respective antibody.

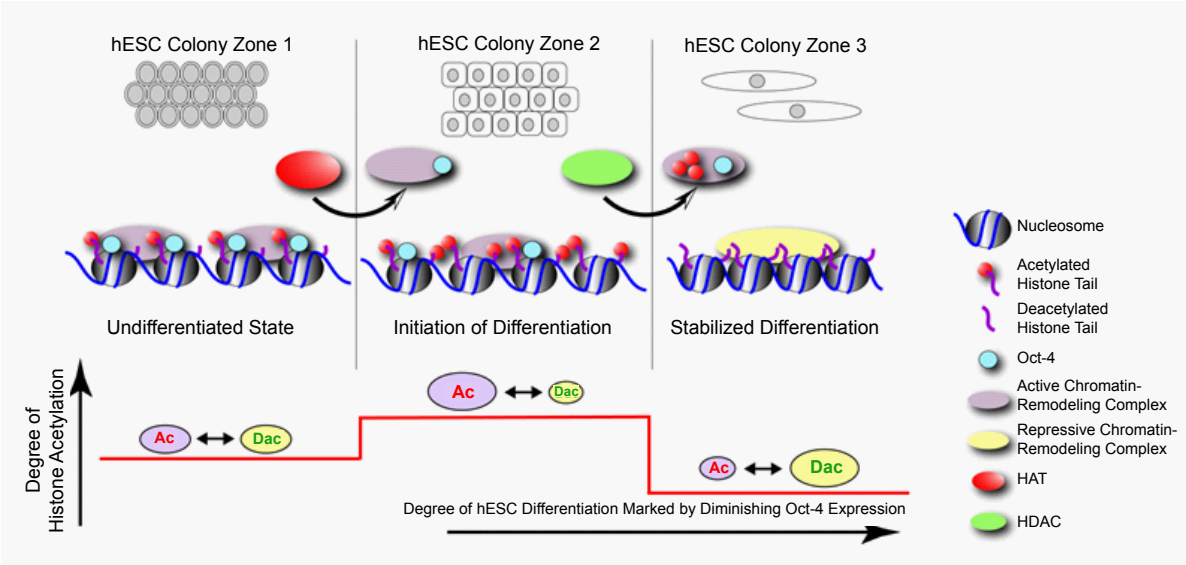

Figure 7: A model for chromatin dynamics in Oct-4 associated hESC pluripotency maintenance and differentiation. The genomic plasticity of the undifferentiated state of hESCs (hESC Colony Zone 1) is enabled by a highly dynamic globally active (open) chromatin which reflects a balance between acetylation and deacetylation. Oct-4 maintains this equilibrium and hence a pluripotent genome by recruiting active chromatin-remodeling-complexes containing HDAC1, Brg-1, and hSNF2H. The loss of this balance, as a result of decreasing in either Oct-4 or HDAC1, induces hyperacetylation and subsequent onset of differentiation in cells at transitional Zone 2. Differentiation is stabilized in cells that have migrated into Zone 3, as an Oct-4 independent deacetylation process promotes the compaction of hypoacetylated nucleosomes into a general repressive (closed) chromatin structure. The stylized graph below this model schematizes the equilibriums between acetylation (Ac) and deacetylation (Dac) as differentiation progresses -- from acetylated to transiently hyperacetylated to hypoacetylated.

Oct-4 might function as a global chromatin-remodeling factor in maintaining the pluripotent chromatin state in undifferentiated hESCs (Figure 7). In this model, the genomic plasticity of pluripotent hESCs is enabled by a highly dynamic, globally-active chromatin that reflects a balance between acetylating and deacetylating forces. Oct-4 governs this equilibrium and, hence, insures an acetylated active chromatin state throughout the entire pluripotent genome by recruiting active chromatin remodeling factors. The loss of this balance – for example, as a result of a decrease in either Oct-4 or HDAC1 -- induces hyperacetylation and the subsequent onset of differentiation. Differentiation is then stabilized as histone hyperacetylation becomes rapidly reversed by an Oct-4-independent deacetylation process that promotes the compaction of hypoacetylated nucleosomes into a general repressive chromatin structure. This model offers an expanded view of Oct-4's role in maintaining pluripotency and then directing differentiation, and is supported by our experimental manipulation of Oct-4 expression (via siRNA) and of the equilibrium between acetylating and deacetylating processes (via HDAC inhibitor) (Figures 5, 6). Indeed, such a view better accommodates previous reports that Oct-4-associated differentiation is dose-dependent and cannot be readily explained as an "on-off" switch [2, 40].

This study suggest that the pluripotency of hESCs that display normal stable expansion is associated with high levels of expression and nuclear localization of acetylated histone H3 and H4, active ATP-dependent chromatin-remodeling factor Brg-1 and hSNF2H, HAT p300, and HDAC1 (Figures 1A, 1B, 2A, 2B, 3A, 3B), suggesting an acetylated globally active chromatin. Consistent with this observation, repressive chromatin remodeling factors that are implicated in transcriptional silencing, including SIRT1, SUV39H1, and Brm that regulate histone H3K9 methylation [9,24-26], Mi-2 of the deacetylation complex (NURD) [33,34], the MYST family of acetyltransferases Tip60, Moz, and HBO-1 [36], and the class II tissue-specific HDACs, were either localized to the cytoplasm, suggesting they were inactive, or were weakly expressed in pluripotent hESCs (Figures 2C, 2D, 3A, 3C-3E). In addition, residual H3 K9 methylation was observed in the pluripotent hESCs that display normal stable expansion (Figure 1C). Residual repressive chromatin remodeling implicated in chromatin silencing, including H3 K9 methylation, might be essential for stabilizing the pluripotent state of hESCs at a normal developmental stage. In fact, aberrant H3 K9 methylation at embryonic stage has been associated with DNA hypermethylation and cell malignant transformation in abnormal pluripotent embryonic carcinoma cells [41,42]. Collectively, these findings reveal an epigenetic mechanism for placing global chromatin dynamics as central to tracking the normal pluripotency and lineage progression of hESCs.

The transitions between distinct chromatin states, from the open acetylated chromatin of the pluripotent hESC to the more compact deacetylated chromatin of the differentiated cells, suggest a self-regulated complex dynamic determined by a progression of global chromatin remodeling as commitment proceeds. Furthermore, an appreciation of various global chromatin modification marks in correlation with these chromatin states might be used to determine the developmental stage of the human stem cell, and help predict the regenerative utility of a plastic human cell along the continuum of a progressive fate restriction for a particular disease.

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