

Human Stem Cell Derivatives Retain More Open Epigenomic Landscape When Derived from Pluripotent Cells than from Tissues

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Abstract

The growing number of identified stem cell derivatives and escalating concerns for safety and efficacy of these cells towards clinical applications have made it increasingly crucial to be able to assess the relative risk-benefit ratio of a given stem cell from a given source for a particular disease. Discerning the intrinsic plasticity and regenerative potential of human stem cell populations might reside in chromatin modifications that shape the respective epigenomes of their derivation routes. Previously, we have generated engraftable human neuronal progenitors direct from pluripotent human embryonic stem cells (hESCs) by small molecule induction (hESC-I hNuPs). Unlike the prototypical neuroepithelial-like nestin-positive human neural stem cells (hNSCs), these *in vitro* neuroectoderm-derived Nurr1-positive hESC-I hNuPs are a more neuronal lineage-specific and plastic hESC derivative. In this study, the global chromatin landscape changes in pluripotent hESCs and their neuronal lineage-specific derivative hESC-I hNuPs were profiled using genome-wide mapping and compared to CNS tissue-derived hNSCs. This study found that the broad potential of pluripotent hESCs is defined by an epigenome constituted of open conformation of chromatin mediated by a pattern of Oct-4 global distribution that corresponds closely with those of acetylated nucleosomes genome-wide. The epigenomic transition from pluripotency to restriction in lineage choices is characterized by genome-wide increases in histone H3K9 methylation that mediates global chromatin-silencing and somatic identity. Tissue-resident CNS-derived hNSCs have acquired a substantial number of additional histone H3K9 methylation, therefore, more silenced chromatin. These data suggest that the intrinsic plasticity and regenerative potential of human stem cell derivatives can be differentiated by their epigenomic landscape features, and that human stem cell derivatives retain more open epigenomic landscape, therefore, more developmental potential for scale-up regeneration, when derived from the hESCs *in vitro* than from the CNS tissue *in vivo*.

Keywords: Human embryonic stem cells; Human pluripotent stem cells; Human neural stem cells; Human neuronal progenitors; Neurons; Human stem cell derivative; Epigenome; Acetylation; Methylation; ChIP-on-Chip; Chromatin; Oct-4; Differentiation; Lineage-specific; Development; Cell therapy; Regenerative medicine

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Introduction

The ability of a stem cell both to self-renew and differentiate into desired phenotypes makes it a potentially inexhaustible cell source for tissue and functional restoration. Discerning the complex identities of human stem cell derivatives from various developmental stages and sources is essential for selection of an optimally lineage-committed human stem cell with sufficient plasticity along the fate restriction continuum to address a particular disease. Genomic and proteomic profiling in isolation has not provided such a tool. Gene expression analysis has indicated that stem cell derivatives do not seem to have a common core transcription profile that dictates the undifferentiated self-renewing state [1-6], which suggests that gene expression alone is not sufficient to define either intrinsic plasticity or developmental potential of a given stem cell. A search for a common set of transcribed genes that defines the characters of all stem cell derivatives, known as stemness, has been unsuccessful; there is virtually no overlap in the gene expression profiles of various types or derivations of stem cells, in spite of their apparent phenotypic similarity [1-6]. Even the expression of a lineage-defining gene within stem cells seems to require additional epigenetic cues [6,7]. It is clear that epigenetic processes are providing additional regulatory dimensions to stem cell behavior.

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. Packaging of eukaryotic genome into chromatin confers higher order structural control over the lineage programming processes [7-14]. Regulation of chromatin structure by covalent modification of DNA and histones, by ATP-driven chromatin remodeling, and by incorporation of alternative histone variants can influence a broad range of cellular processes that include transcription, replication, recombination and DNA repair; therefore, chromatin modifications have been implicated in regulation of developmental processes [7-14]. Chromatin modifications create molecular landmarks that establish and maintain stage-specific gene expression patterns and global gene silencing during mammalian development. Chromatin modifications serve as important epigenetic marks for active and inactive chromatin states, and have emerged as the principal epigenetic mechanism in early embryogenesis. Growing evidences indicate that epigenetic controls in stem cell fate decisions may hold the key to some of the pressing questions regarding the underlying mechanisms of their intrinsic developmental potential. However, these processes, which may involve dynamic equilibrium between active and inactive chromatin states and establishment of chromatin codes by covalent modifications on histones and DNA, remain to be understood.

Pluripotent human embryonic stem cells (hESCs) proffer cures for a wide range of neurological disorders by supplying the diversity of human neuronal cell types in the developing CNS for repair [15,16]. However, realizing the therapeutic potential of hESC derivatives has been hindered by generating functional cells from pluripotent cells through uncontrollable and inefficient multi-lineage differentiation [15,16]. Without a practical strategy to convert pluripotent cells direct into a specific lineage, previous studies and profiling of pluripotent

hESCs and their differentiating multi-lineage aggregates have not been able to resolve the epigenomic landscape features of human stem cell derivations that might be used to predict their intrinsic plasticity and regenerative potential, hence, safety and efficacy, when deriving the optimal human stem cell preparations for clinical indications [7-14]. In previous reports, we found that pluripotent hESCs maintained under the defined culture conditions can be uniformly converted into a specific neural or cardiac lineage by small molecule induction [16-20]. Retinoic acid (RA) was found to induce the specification of neuroectoderm direct from the pluripotent state of hESCs and trigger uniform progression to human neuronal progenitors (hESC-I hNuPs) and neurons (hESC-I hNus) in the developing CNS efficiently by promoting nuclear translocation of the neuronal-specific transcription factor Nurr-1 [16,17,20,21]. Similarly, Nicotinamide (NAM) was found to induce the specification of cardiomesoderm direct from the pluripotent state of hESCs and trigger uniform progression to human cardiac precursors and beating cardiomyocytes efficiently by promoting the expression of the earliest cardiac-specific transcription factor Csx/Nkx2.5 [16,18-20]. This technology breakthrough enables well-controlled generation of a large supply of neuronal or cardiomyocyte lineage-specific derivatives across the spectrum of developmental stages direct from the pluripotent state of hESCs with small molecule induction. Genome-scale microRNA (miRNA) profiling identified that a unique set of pluripotency-associated miRNAs was down-regulated, while novel sets of distinct cardiac- and neural-driving miRNAs were up-regulated upon the induction of lineage-specification direct from the pluripotent state of hESCs, including silencing of pluripotency-associated hsa-miR-302 family and drastic up-regulation of neuroectodermal Hox miRNA hsa-miR-10 family upon hESC neural fate switch [20]. Further profiling of miRNA differential expression patterns during hESC neuronal lineage-specific progression identified novel sets of stage-specific human embryonic neurogenic miRNAs, including silencing of the prominent pluripotency-associated hsa-miR-302 family and drastic expression increases of Hox hsa-miR-10 and the let-7 miRNAs [20,21]. Our findings suggest that these hESC neuronal derivatives have acquired a neuronal lineage-specific identity by silencing pluripotency-associated miRNAs and inducing the expression of miRNAs linked to regulating human CNS development to high levels, therefore, highly neurogenic *in vitro* and *in vivo* [14,17,20,21]. Unlike the prototypical neuroepithelial-like nestin-positive human neural stem cells (hNSCs), these *in vitro* neuroectoderm-derived Nurr1-positive hESC-I hNuPs are a more neuronal lineage-specific and plastic human stem cell derivative, retaining an embryonic acetylated globally active chromatin state [13,14,17,20,21].

The development of chromatin/nucleosome-immunoprecipitation-coupled DNA microarray analysis (ChIP/NuIP-on-chip) has provided the technology foundation for genome-wide approaches to profile alterations in spatial and temporal patterns of the differentiation-associated epigenetic markers in high-resolution [22,23]. Large-scale profiling of developmental regulators and histone modifications has been used to identify epigenetic patterns, including in hESCs [9-12]. Mapping global patterns of chromatin dynamics in human stem cell derivatives will provide reliably predictive molecular parameters for comparing their intrinsic plasticity dominating stem cell behavior prior to transplantation. In this study, NuIP-on-chip using tiling arrays was used to profile chromatin modifications that make up the epigenome of pluripotent hESCs, in comparison to those of their neuronal lineage-specific derivative hESC-I hNuPs and CNS tissue-derived hNSCs [13,14]. This study found that the broad potential of

pluripotent hESCs is defined by an epigenome constituted of open conformation of chromatin mediated by a pattern of Oct-4 global distribution that corresponds closely with those of acetylated nucleosomes genome-wide. The epigenomic transition from pluripotency to restriction in lineage choices is characterized by genome-wide increases in histone H3K9 methylation that mediates global chromatin-silencing and somatic identity. Tissue-resident CNS-derived hNSCs have acquired a substantial number of additional histone H3K9 methylation, therefore, more silenced chromatin. These data suggest that the intrinsic plasticity and regenerative potential of human stem cell derivatives can be differentiated by their epigenomic landscape features, and that human stem cell derivatives with neural potential retain more open epigenomic landscape, therefore, more developmental potential for scale-up regeneration, when derived from the hESCs *in vitro* than from the CNS tissue *in vivo*.

Materials and Methods

Culture of undifferentiated hESCs under the defined culture

The hESC lines WA01 and WA09 (WiCell Research Institute) and newly-derived biologics-free hESCs (Xcel-hESCs) [19] 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, 100 μ M) (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 μ g/ml) (Sigma), and ascorbic acid (50 μ g/ml) (Sigma), in which activin A (50 ng/ml, Sigma), human albumin (10 mg/ml, Sigma), and human transferrin (8 μ g/ml, Sigma) were added in order to increase cell survival and maintain normal shape and healthy colonies. Long-term stable expansion of pluripotent hESCs maintained under the defined culture (~ 50-150 passages, ~ 12-36 months) has been published in our previous reports [16,19]. These hESCs were stored by cryopreservation over their long-term expansion periods.

Neuronal lineage-specific differentiation direct from the pluripotent state of hESCs

Pluripotent hESCs maintained under the defined culture (~ 50-150 passages, ~ 12-36 months) [16,19] were used to generate hESC derivatives in this study. Undifferentiated hESCs maintained under the defined culture conditions were treated with RA (10 μ M) 3 days after seeding for 4-5 days. These neuroectoderm-differentiated hESCs were transferred to a serum-free suspension culture to allow floating neuroblasts (hESC-I hNuPs) to form in the hESC media lacking bFGF for 4-5 days. To limit the effects of long-term culture on those hESC-I hNuPs, only cells within the first 6 passages were used for the analyses. For further differentiating into a neuronal phenotype, the neuroblasts were then permitted to attach to a tissue culture plate in a defined medium containing DMEM/F-12, N-2 supplement (1%), heparin (8 μ g/ml), VEGF (20 ng/ml), NT-3 (10 ng/ml), and BDNF (10 ng/ml). β -III-tubulin- and Map-2-expressing, extensively neurite-bearing cells (hESC-I hNus) and pigmented cells, typical of those in the ventral mesencephalon, were observed within 2 weeks of continuous cultivation, increased in numbers with time. The

generated hESC neuronal derivatives were stored by cryopreservation over passaging periods.

Generation of CNS-derived hNSCs

The CNS tissue-derived hNSCs were isolated directly from the ventricular zone of the telencephalon (a neuroectoderm-derived structure) of two 11-13 week human fetal cadavers (HFB2030 and HFT13) [14,24]. These hNSCs were propagated without genetic manipulation following a serial growth factor and engraftment selection process, characterized, and maintained in a karyotypically normal state in a defined NSC media containing bFGF (20 ng/ml) and leukemia inhibitory factor (LIF) (10 ng/ml) as previously described [14,24]. Fresh vials of low passage cells from the initial isolation and derivation of these hNSCs were used for these studies. The homogeneity and comparability of hNSCs was verified by insuring that >95% of the cells expressed standard neural stem/progenitor markers, including Nestin, Musashi, and Sox-2, and no longer expressed markers associated with pluripotency, including Oct-4, SSEA-4, Tra-1-60, Tra-1-80, or markers of non-neural lineages.

Analysis of nucleoprotein complexes and chromatin fibers

Chromosomal proteins were cross-linked to DNA by adding 1% formaldehyde directly to culture medium and incubating at 37°C for 10 min. Cells were isolated and resuspended in PBS. This cell mixture was loaded into a double-chamber cytofunnel and centrifuged in a Cytospin 3 (Shandon) at 900 rpm for 5 min onto clean glass microscope slides. Immediately after centrifugation, slides were placed in lysis buffer (25 mM Tris [pH 7.5], 500 mM NaCl, and 1% Triton X-100) for 15 min, then processed for immunofluorescence and deconvolution microscopic analysis. Chromatin fibers were prepared similarly except without the cross-linking procedure and processed for immunofluorescence and deconvolution microscopic analysis [25]. Primary antibodies to Oct-4, Brg-1, and hSNF2H were from Santa Cruz Biotechnology, Inc.; antibodies to p300, Tip60, acetylated H3 (K9, 14), acetylated H4 (K5, 8, 12, 16), dimethylated H3 K9, and HDAC1 were from Upstate Biotechnology. Antibodies were used at 1/100-1/50 dilution and the specificity of all antibodies were independently verified on known positive and negative control cells before being used in these studies.

Micrococcal nuclease (MNase) analysis of chromatin accessibility

Cells were isolated and nuclei were prepared and equal amounts of nuclei were digested with increasing concentrations of MNase (Sigma) (0.25, 0.5, 1, and 2 U/ml) [26]. DNA was deproteinized, isolated, electrophoresed on 1.2% agarose gels, and visualized by ethidium bromide staining.

Mononucleosome immunoprecipitation (NuIP) analysis

Undifferentiated hESCs were isolated, dissociated into single cell suspensions with trypsin (0.05%, Invitrogen) digestion, and cross-linked with 1% formaldehyde at 37°C for 10 min. Nuclei were isolated and digested to predominantly mononucleosomes with MNase (Sigma) [26]. Nucleosome particles were extracted and immunoprecipitated with the primary antibodies and analyzed by Western blotting. Primary antibodies to Oct-4, Brg-1, and hSNF2H were from Santa Cruz Biotechnology, Inc.; antibodies to p300, Tip60, acetylated H3 (K9, 14), acetylated H4 (K5, 8, 12, 16), dimethylated H3 K9, and HDAC1 were from Upstate Biotechnology. Antibodies were used at 1/1000-1/500 dilution and the specificity of all antibodies were

independently verified on known positive and negative control cells before being used in these studies.

Chromatin/Nucleosome immunoprecipitation and DNA microarray analysis (NuIP-on-chip)

Cells were isolated, dissociated into a single cell suspension with trypsin (0.05%, Invitrogen) digestion, and cross-linked with 1% formaldehyde at 37°C for 10 min. Nuclei were isolated and digested to mostly mononucleosomes with MNase [22,23,26]. Nucleosome particles were extracted and immunoprecipitated with the Oct-4, acH3, acH4, or meH3K9 antibodies (the specificity of the immunoprecipitation to each antibody was verified before further proceed). DNA was extracted from both input and pull-down samples. Whole genomic DNA and DNA extracted from pull-down samples without using primary antibody were used as controls. The resulting DNA was labeled and amplified by ligation-mediated PCR (LM-PCR) according to the protocol provided by NimbleGen/Roche and further purified with Qiagen quick PCR purification kits. Genomic profiling was done by NimbleGen/Roche Systems as part of a Chromatin Immunoprecipitation Array Service. Arrays were designed to provide 50-bp resolution by tiling 50-mers with 38-bp spacing (12-bp overlap) through the genomic loci of all human ENCODE targeted regions. DNA (3 µg per sample) samples were provided from input and pull-down samples to NimbleGen/Roche System for differential labeling by random priming with Cys3 or Cys5 and hybridization to oligonucleotide arrays. The signal intensities and ratio profiles from each array hybridization were provided by NimbleGen/Roche Systems and analyzed using NimbleGen/Roche SignalMap. The deposition peaks were resolved using NimbleGen/Roche SignalMap software from tiling array profiles from at least two biological replicate sets.

Results

Oct-4 binds globally to the acetylated nucleosomal DNA in preserving the globally open pluripotent chromatin state

To unveil the epigenetic mechanism in maintaining the epiblast pluripotency of hESCs, in previous report, the global chromatin dynamics in the pluripotent hESCs maintained under the defined culture were profiled [13]. To determine the alteration of chromatin structure in association with the transition from pluripotency of the hESC to restriction in lineage choices, in this study, the chromatin accessibility of undifferentiated hESCs versus their neuronal lineage specific derivative (hESC D or hESC-I hNuPs) was subjected to micrococcal nuclease (MNase) digestion analysis [26]. Analysis of the nucleosomal DNA fragments generated from digestion of bulk chromatin with an increasing titration of MNase revealed an increased resistance to MNase digestion -- therefore, decreased chromatin accessibility in hESC lineage specific derivatives as compared to the pluripotent hESC (Figure 1A), suggesting that restriction in lineage choices is associated with less accessible, more compact chromatin structure.

In order to visualize the Oct-4 complexes, chromatin fibers from undifferentiated hESCs that were not treated with a cross-linking reagent were prepared [25]. Immunofluorescence analysis of the non-crosslinked nucleoprotein complexes revealed the co-presence of large Oct-4 complexes with acetylated histone H4 and Brg-1 complexes (Figure 1B). The overlapping patterns of immunostaining suggested that Oct-4 was associated with the chromatin remodeling factor Brg-1 more tightly than with acetylated histones or nucleosomal DNA (Figure 1B). High magnification images of the extended chromatin fibers

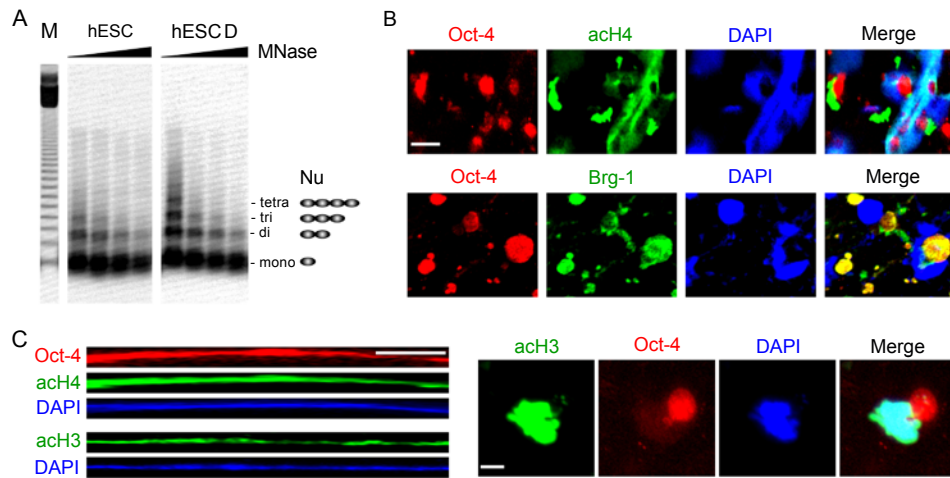


Figure 1: Oct-4 is associated with active chromatin modifiers globally: (A) Analysis of the nucleosomal DNA fragments generated from digestion of chromatin with increasing concentrations of MNase suggests increased resistance in hESC derivatives (hESC D or hESC-I hNuPs) compared to undifferentiated hESCs (hESC) to the same concentration of MNase, indicative of decreased accessibility. M designates the 123 bp DNA ladders. "Mono-", "di-", "tri-", and "tetra-Nu" indicate respective nucleosomal ("Nu") DNA fragments. (B) Immunofluorescence and deconvolution microscopic analysis of the non-crosslinked nucleoprotein complexes reveals the co-presence of large Oct-4 complexes (red) with acetylated histone H4 (acH4, green) or Brg-1 (green) complexes. Chromatin fibers are indicated by DAPI staining (blue). (C) Images of extended chromatin fibers showing the wide distribution patterns of Oct-4 (red), acetylated histone H3 (acH3, green), and acH4 (green) along the nucleosomal DNA (blue). (D) Photomicrographs of nuclei show that Oct-4 is excluded from the condensed mitotic chromosome. Scale bars: 3 μ m.

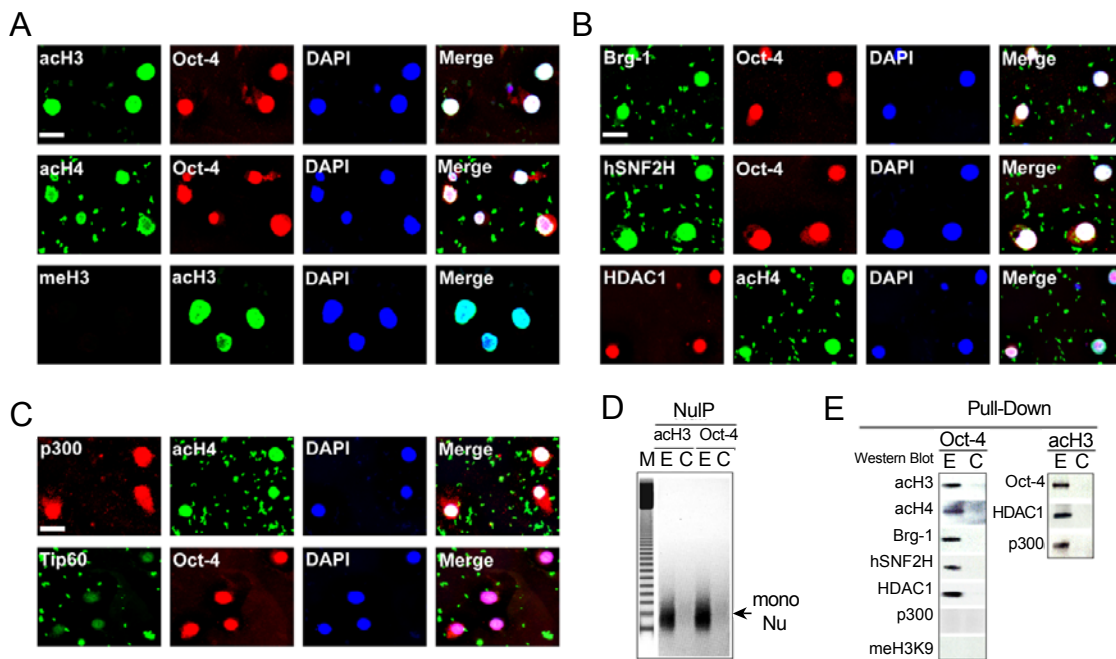


Figure 2: The distribution of Oct-4 on chromatin coincides genome-wide with that of active chromatin modifications: (A-C) Images of cross-linked nucleoprotein complexes showing (A) the large chromatin complexes of Oct-4 co-localizing with acetylated histones H3 and H4 (acH3 and acH4) (green) but not H3 K9 methylated histones (meH3K9) (red) on the nucleosomal DNA (DAPI, blue); (B) the large chromatin complexes of Brg-1 (green) or hSNF2H (green) co-localizing with Oct-4 and the large chromatin complexes of HDAC1 (red) on the acetylated nucleosomal DNA; (C) large chromatin complexes of HAT p300 (red) and free non-chromosomal HAT complexes of Tip60 (green). The particle diameters of the large chromatin complexes range ~ 0.1-3 μ m. Note the presence of additional free non-chromosomal acH4, Brg-1, and hSNF2H protein complexes (particle diameter: ~ 0.01-0.5 μ m). (D) Electrophoresis analysis of the NuIP complexes visualizing the co-precipitated mononucleosomal DNA fragments (arrow) bound to Oct-4 and acH3. (E) Western blot analysis of the NuIP complexes showing HDAC1, acH3, acH4, Brg-1, hSNF2H, but not p300 pulled-down by Oct-4; and Oct-4, HDAC1, and p300 pull-down by acH3 in undifferentiated hESCs ("E"). H3K9 methylation was not detectable. Pull-down from Oct-4-negative differentiated hESCs was used as a control ("C"). Scale bars: 3 μ m.

indicated that Oct-4 was widely distributed along the acetylated nucleosomal DNA marked by either acetylated H3 (acH3) or acetylated H4 (acH4), despite the fact that, without crosslinking, Oct-4 appeared to fall off from the chromatin fiber easily in comparison to acetylated core histones (Figures 1B and 1C). While Oct-4 was associated with open chromatin structures, it appeared to be excluded from condensed mitotic chromatin (Figure 1D).

To view the genome-wide localization patterns of these abundant chromatin-associated nucleoproteins, immunofluorescence and deconvolution microscopic analysis of the formaldehyde cross-linked nucleoprotein complexes in the nuclei of pluripotent hESCs was carried out. The active chromatin remodeling factors, including acH3, acH4, ATP-dependent chromatin remodeling factor Brg-1 and hSNF2H, p300, and HDAC1 [13], formed large chromatin-localized nucleoprotein complexes, as indicated by their overlapping with DAPI staining (Figures 2A-2C). In contrast, Tip60 was mostly present as free non-chromosomal histone acetyltransferase (HAT) complexes (Figure 2C), although it was described previously as regulating embryonic stem cell gene expression via Nanog and H3K4 methylation [27]. No histone H3 K9 methylated (meH3K9) nucleoprotein complex was detected in pluripotent hESCs (Figure 2A). AcH4, Brg-1, and hSNF2H formed additional free non-chromosomal complexes (Figures 2A and 2B). These free non-chromosomal complexes might rapidly exchange with chromosomal protein complexes, suggesting the presence of a highly dynamic equilibrium for sustaining the globally active chromatin state in pluripotent hESCs. These observations suggest that Oct-4 appears to be associated with nucleosomes in non-condensed acetylated chromatin and with factors involved in active chromatin remodeling in order to maintain a globally open accessible chromatin state throughout the pluripotent genome.

Oct-4 binding corresponds to the patterns of active chromatin modification genome-wide

Our observations suggested that Oct-4 might play an additional chromatin-remodeling role in maintaining the globally open chromatin state in pluripotent hESCs (Figures 1, 2A-2C), and that changes in Oct-4 expression appeared to promote differentiation by allowing alterations in chromatin state [13]. To support that Oct-4 recruits active chromatin remodeling factors to acetylated nucleosomes in undifferentiated hESCs, nucleosome immunoprecipitation analysis (NuIP) was performed [26]. Undifferentiated hESCs were isolated and formaldehyde crosslinked. Nuclei were prepared and digested to predominantly mononucleosomes with MNase (Figure 2D). Chromatin was extracted and immunoprecipitated with Oct-4 or with acetylated histone H3. Western blot analysis of the immunoprecipitated complexes revealed that Oct-4 pull-downed acH3 and acH4, as well as such chromatin remodeling factors as HDAC1, Brg-1, and hSNF2H, but not p300 (Figure 2E). No H3 K9 methylation was found in the Oct-4 immunoprecipitated complexes (Figure 2E). Complementary to this observation, Oct-4, HDAC1, and p300 were pull-downed by acetylated H3 (Figure 2E).

To resolve the genome-wide inter- and intra-genic localization patterns of Oct-4 as correlated with nucleosomal DNA, NuIP-on-chip analysis using tiling arrays was carried out for pluripotent hESCs and compared to those of hESC neuronal lineage-specific derivative hESC-I hNuPs and CNS tissue-resident hNSCs (Figures 3-6). In general, histone acetylation is associated with a globally active open chromatin state, while histone deacetylation and histone

H3 K9 methylation are associated with a globally repressed closed chromatin state [7-14,26,28]. Representative maps of Oct-4, acH4, acH3, and meH3K9 densities in pluripotent hESCs for typical gene-rich regions covering the annotated sequences showed that Oct-4 distributes widely on the pluripotent genome that is associated with genome-wide acetylated nucleosomes, as marked by either acH3 or acH4, and localized residual H3 K9 methylation (Figure 3). It appears that the distribution patterns of Oct-4 for typical gene-rich regions correspond more closely with those of acH4 than with those of acH3 in general (Figure 3). These data by high-resolution mapping further supported that Oct-4 might play an active chromatin-remodeling role in maintaining the globally acetylated open chromatin state in pluripotent hESCs. The deposition peaks of Oct-4 and acetylated histones relative to the transcription start sites (n=438) were further resolved. The overall pattern of deposition peaks of Oct-4 spanning from -10 kb to +10 kb of the transcription start sites shares close similarity with those of active chromatin modifications marked by either acH4 or acH3, with enrichment from -1 kb to +1 kb of the transcribed regions (Figure 4). It appears that the overall pattern of deposition peaks of Oct-4 spanning from -10 kb to +10 kb of the transcription start sites corresponds more closely with that of acH4 than with that of acH3 in general (Figure 4). These observations suggest that Oct-4 is distributed to widespread inter- and intra-genic regions genome-wide in order to maintain the active pluripotent chromatin state in pluripotent hESCs.

Epigenomic progression from pluripotency to lineage restriction is associated with global increases in chromatin-silencing mediated by H3 K9 methylation

In previous report, we observed that these *in vitro* neuroectoderm-derived Nurr1-positive hESC-I hNuPs expressed high levels of active chromatin modifiers, including acH3, acH4, HDAC1, Brg-1, and hSNF2H, suggesting that hESC-I hNuPs retain an embryonic acetylated globally active chromatin state [14]. The repressive chromatin remodeling factors SIRT1, SUV39H1, Brm, and, consequently, meH3K9 remained weakly expressed in hESC-I hNuPs [14]. In contrast, the prototypical neuroepithelial-like nestin-positive hNSCs derived from the fetal CNS tissue displayed significantly decreased histone acetylation and increased histone H3 K9 methylation [14]. To probe epigenomic landscape changes in the progression from pluripotency to lineage-specific identity in high resolution, in this study, the global patterns of chromatin dynamics of hESC-I hNuPs were profiled using NuIP-on-chip tiling array analysis and compared to the epigenome of CNS tissue-resident hNSCs. The hNSCs isolated directly from the fetal CNS (CNS-derived hNSCs), which have acquired their neuroectodermal identity *in vivo* through normal developmental processes, are among the best characterized of multipotent tissue-resident stem/progenitor cells and have been used in a range of animal models of disease and injury [24,29]. Representative maps of acH4, acH3, and meH3K9 densities for the same typical gene-rich regions covering the annotated sequences of pluripotent hESCs were showed for comparison. Although hESC-I hNuPs began to display overall increases in H3 K9 methylated regions, they retained the pattern of genome-wide acetylated nucleosomes, as marked by either acH3 or acH4, of pluripotent hESCs (Figure 5). This observation suggested that hESC-I hNuPs maintain the globally acetylated open chromatin state, consistent with our previous report [14]. By contrast, the CNS tissue derived hNSCs displayed a pattern of genome-wide nucleosomal H3 K9 methylation, accompanied by global increases in nucleosomal deacetylation, as marked by genome-



Figure 3: Oct-4 distributes widely on the pluripotent genome that is associated with genome-wide acetylated nucleosomes: Representative maps of Oct-4, acetylated histone H4 (acH4), acetylated histone H3 (acH3), and K9 methylated histone H3 (meH3K9) densities were generated by NuIP-on-chip analysis using tiling arrays. Representative tiling arrays for typical gene-rich regions in pluripotent hESCs covering the annotated sequences show that Oct-4 distributes widely on the pluripotent genome that is associated with genome-wide acetylated nucleosomes, as marked by either acH3 or acH4, and localized residual H3 K9 methylation. The extent of each gene oriented 5' to 3' from left to right is indicated by a box above the line and that of each gene orientated 5' to 3' from right to left by a box below the line.

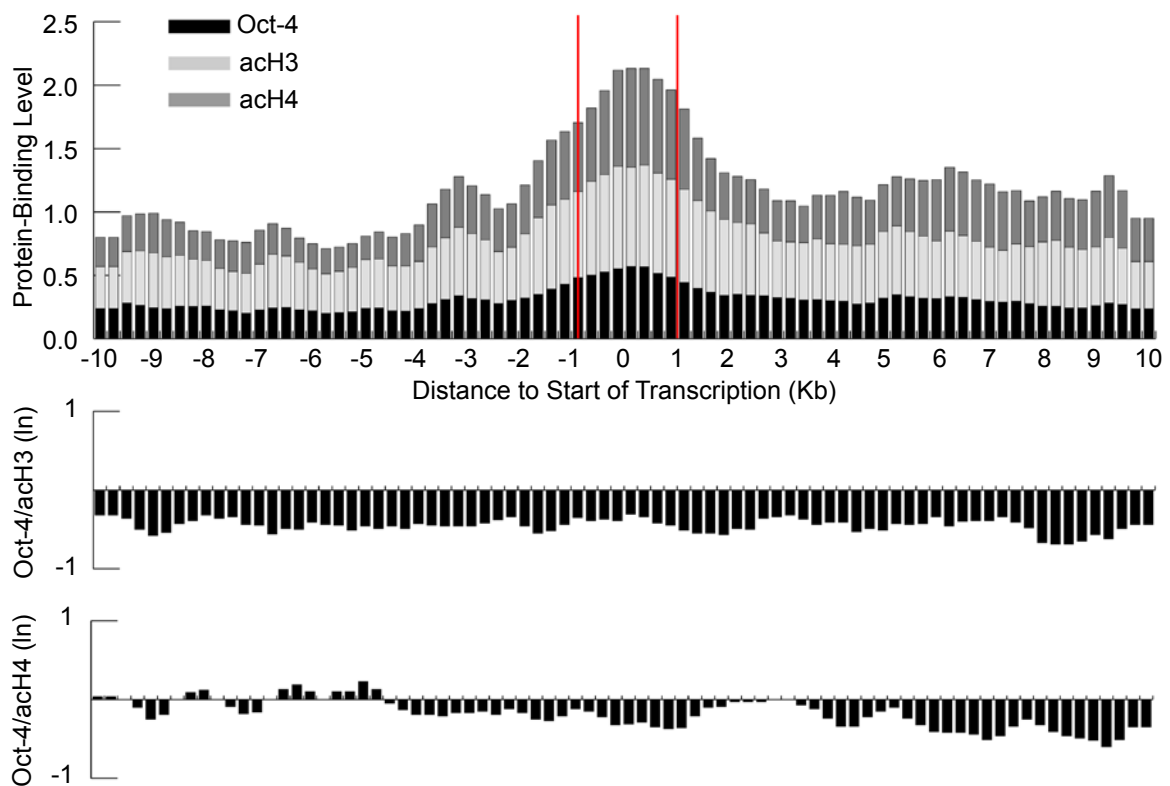


Figure 4: The overall distribution pattern of Oct-4 corresponds closely with that of acetylated nucleosomes: The top panel: The overall pattern of deposition peaks of Oct-4 spanning from -10 kb to +10 kb of the transcription start sites shares close similarity with those of active chromatin modifications marked by either acH4 or acH3. The red lines demarcate the prominent enrichment from -1 kb to +1 kb of the transcribed regions. The 2 lower panels: Ln ratios of Oct-4 binding/acH3 or acH4 binding suggest that the overall pattern of deposition peaks of Oct-4 spanning from -10 kb to +10 kb of the transcription start sites corresponds more closely with that of acH4 than with that of acH3 in general.

wide decreases in either acH3 or acH4 (Figure 6). The global increases in repressive chromatin-remodeling mediated by H3 K9 methylation suggested that the tissue-resident CNS-derived hNSCs had acquired a more silenced chromatin.

The deposition peaks of meH3K9 in both inter- and intra-genic regions from tiling array analysis displayed a distribution pattern of enrichment of global H3K9 methylation to increasing degrees as one progressed from pluripotent hESCs to hESC neuronal derivative hESC-I hNuPs and then to tissue-resident CNS-derived hNSCs (Figure 7A). From a meH3K9 pattern of ~ 0.03% of total arrayed regions in pluripotent hESCs, the overall resolved regions occupied by meH3K9 increased ~ 13.5-fold in the hESC-I hNuPs and ~ 40-fold in the CNS-derived hNSC (Figure 7A). Although the two human stem cell derivatives shared ~ 1/4 of the methylated regions, tissue-resident CNS-derived hNSCs proceeded to acquire a substantial number of additional silenced regions (increased meH3K9), such that ~ 75% of its H3 K9 methylated regions were unique and not present in embryonic derivatives (Figure 7A).

Discussion

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 (acH3 and acH4), Brg-1, hSNF2H, histone acetyltransferase (HAT) p300, and histone deacetylase 1 (HDAC1); weak expression or cytoplasmic localization of repressive chromatin remodeling factors that are implicated in transcriptional silencing; and residual H3 K9 methylation [13]. In embryogenesis, only cells in the inner cell mass (ICM) or epiblast express Oct-4 [30]. Loss of Oct-4 at the blastocyst stage causes these cells to differentiate into extraembryonic lineages, while Oct-4 expression insures embryonic germ layer assignment and lineage differentiation [30,31]. Oct-4, the abundant nuclear protein specifically associated with hESCs in their undifferentiated state, binds to a variety of non-specific AT-rich sequences and its expression pattern appears to be less consistent with that of a specific activator and more consistent with an epigenetic process [28,32]. Previous genome-scale profiling has identified ~ 3% of the promoter regions for known protein-coding genes as associated with Oct-4, forming synergetic regulatory circuitries with other pluripotency-inducing factors, such as Sox-2 and Nanog, in embryonic stem cells [1-4]. These existing literatures have placed Oct4 on a discrete set of pluripotency gene enhancers [1-4]. Our previous report suggested that Oct-4 might play an additional chromatin-remodeling role in preserving the globally active chromatin state in pluripotent hESCs by maintaining a balanced level of histone acetylation and that change in Oct-4 expression appeared to promote hESC differentiation by allowing alterations in chromatin state [13].



Figure 5: The hESC-I hNuPs maintain the globally acetylated open chromatin state: Representative maps of acH4, acH3, and meH3K9 densities generated by NulP-on-chip analysis using tiling arrays for typical gene-rich regions in hESC-I hNuPs covering the annotated sequences show that they began to display overall increases in H3 K9 methylated regions, though they retained the pattern of genome-wide acetylated nucleosomes, as marked by either acH3 or acH4. The extent of each gene oriented 5' to 3' from left to right is indicated by a box above the line and that of each gene orientated 5' to 3' from right to left by a box below the line.

This study of profiling of Oct-4 binding by genome-wide approaches suggests that Oct-4 binding is widespread and particularly enriched far upstream and downstream of transcribed regions (Figures 1-4). The wide distribution pattern of Oct-4 coincident with sites of active chromatin modification genome-wide suggested that Oct-4 might play a role in the interface of chromatin

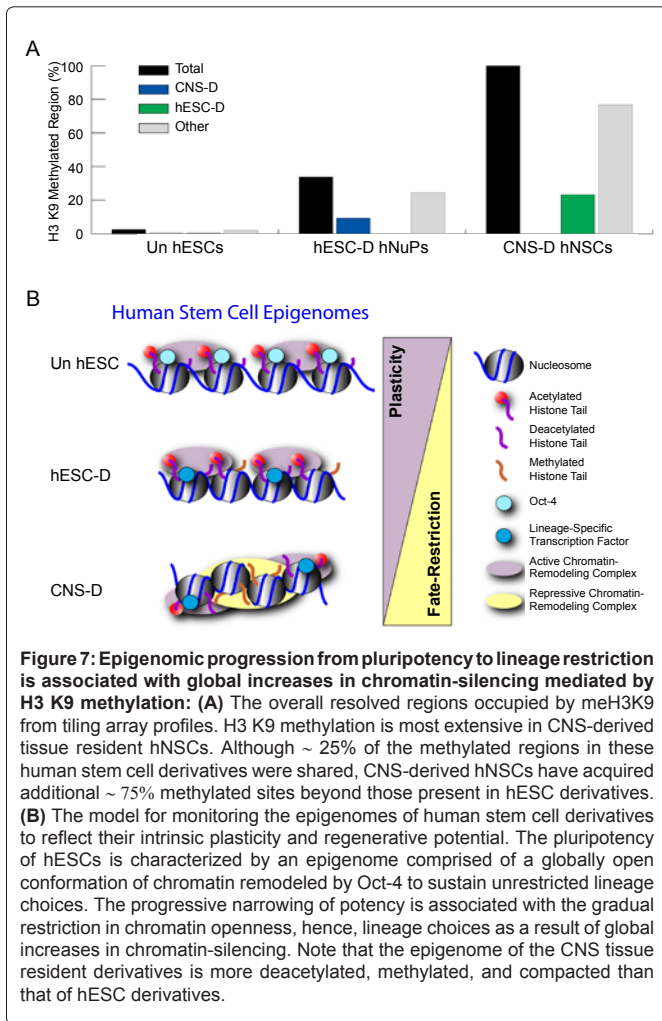
and transcription regulation to maintain a pluripotent epigenome enabled by a globally active open chromatin. It appears that Oct-4 binding is also responsible, in part, for inter-genic transcription, a wide-spread process that has previously been suggested for replication-independent assembly and transcription derepression of developmental regulatory genes [33,34]. Therefore, Oct-4 might

play an additional structural role – as a global chromatin-remodeling factor distributed to widespread inter- and intra-genic regions -- in maintaining the active pluripotent chromatin state in pluripotent hESCs.

It appears that the distribution patterns of Oct-4 for typical gene-rich regions as well as the overall pattern of deposition peaks of Oct-4 spanning from -10 kb to +10 kb of the transcription start sites correspond more closely with those of acH4 than with those of acH3



Figure 6: The tissue-resident CNS-derived hNSCs had acquired a more silenced chromatin: Representative maps of acH4, acH3, and meH3K9 densities generated by NuIP-on-chip analysis using tiling arrays for typical gene-rich regions in CNS-derived hNSCs covering the annotated sequences show that they displayed a pattern of genome-wide nucleosomal H3 K9 methylation, accompanied by global increases in nucleosomal deacetylation, as marked by genome-wide decreases in either acH3 or acH4. The extent of each gene orientated 5' to 3' from left to right is indicated by a box above the line and that of each gene orientated 5' to 3' from right to left by a box below the line.



in general (Figures 3 and 4). In general, acetylation of core histones correlates with transcriptional active ('open') chromatin state, whereas deacetylation of core histones correlates with transcriptional repressed ('closed') chromatin state [26,28]. Acetylation impacts chromatin structure through the neutralization of the charge inherent to the amino group of lysine, thereby weakening intra- and inter-nucleosomal interactions of the chromatin fiber and facilitating its decondensation by increasing accessibility to the nucleosomal DNA [25-28,35]. In addition, acetylation is recognized and targeted by the bromodomain of a variety of chromatin factors that mediates transcription activation and ATP-dependent chromatin remodeling through recruitments of other specific regulators [25-28,35]. A considerable amount of evidence suggests that acetylation of histone H3 and H4 has distinct functional and temporal patterns [14,35]. The H3 modifications seem to be connected to proper control of gene expression, whereas acetylation of H4 seems to be most important in histone deposition and chromatin structure [14,26,35]. Of the four lysine residues in the N-terminal tail of H4 (K5,8,12,16), lysine 16 (K16) is the specific target of the class III NAD-dependent histone deacetylase (HDAC) SIRT1 and plays a unique role in regulating chromatin structure [20,26,35]. Histone H4 K16 acetylation is important in epigenetic regulation as substantiated by its being the only lysine residue among the N-terminal tails of all histones that is targeted by an exclusive category of HATs as well as HDACs, such

as the MYST family of HATs and the class III NAD-dependent HDACs to mediate silencing of chromatin locus during phenotype switch in human development [13,14,20,26,35]. These data by high-resolution mapping in this study further supported that Oct-4 might play an active chromatin-remodeling role in maintaining the globally acetylated open chromatin state in pluripotent hESCs.

Human stem cell transplantation represents a promising therapeutic approach closest to provide a cure to restore the lost nerve tissue and function for a wide range of devastating and untreatable neurological disorders. However, to date, lack of a clinically-suitable source of engraftable human stem/progenitor cells with adequate neurogenic potential has been the major setback in developing safe and effective cell-based therapy as a treatment option for restoring the damaged or lost CNS structure and circuitry. The traditional sources of engraftable human stem cells with neural potential for transplantation therapies have been multipotent hNSCs isolated directly from the human fetal CNS [15,24,29]. However, cell therapy based on CNS tissue-derived hNSCs has encountered supply restriction and difficulty to use in the clinical setting due to their limited expansion ability and declining plasticity with aging, potentially restricting the tissue-derived hNSC as an adequate source for graft material. Alternatively, the pluripotent hESCs proffer cures for a wide range of neurological disorders by supplying the diversity of human neuronal cell types in the developing CNS for regeneration and repair. However, realizing the therapeutic potential of hESC derivatives has been hindered by conventional approaches for generating functional cells through multi-lineage differentiation of pluripotent cells, which is uncontrollable, inefficient, instable, highly variable, difficult to reproduce and scale-up, and often causes phenotypic heterogeneity and instability, hence, a high risk of tumorigenicity following transplantation [15,16]. Under protocols presently employed in the field, the prototypical neuroepithelial-like nestin-positive hNSCs, either isolated from CNS *in vivo* or derived from pluripotent cells *in vitro* via conventional multi-lineage differentiation, appear to exert their therapeutic effects primarily by their non-neuronal progenies through producing trophic and/or neuroprotective molecules to rescue endogenous dying host neurons, but not related to regeneration from the graft or host remyelination [14-16,24,29]. We recently reported that pluripotent hESCs maintained under a defined platform can be uniformly converted into a cardiac or neural lineage by small molecule induction [16-21]. This technology breakthrough enables well-controlled generation of a large supply of neuronal lineage-specific derivatives across the spectrum of developmental stages direct from the pluripotent state of hESCs with small molecule induction.

Compared to the prototypical neuroepithelial-like nestin-positive hNSCs, hESC-I hNuPs, which have acquired a neuroectodermal identity through RA induction of pluripotent hESCs *in vitro*, did not express the canonical hNSC markers [e.g., nestin], but assumed uniformly strong expression and nuclear localization of Nurr-1 [14,16,17]. Under neuronal differentiation conditions, hESC-I hNuPs yielded exclusively neurons that expressed neuronal markers with a drastic increase in efficiency (~ 95%) when compared to the yields of β -III-tubulin-positive neurons differentiated under similar conditions from hESC-derived hNSCs (~ 6%) or CNS-derived hNSCs (~ 13%) [14,21]. These *in vitro* neuroectoderm-derived hESC-I hNuPs yielded neurons efficiently and exclusively, as they did not differentiate into other neural cell types such as glial cells, suggesting that they are a novel

more neuronal lineage-specific embryonic neuronal progenitor than the prototypical neuroepithelial-like hNSCs, providing an engraftable human embryonic neuronal progenitor in high purity and large supply with adequate neurogenic potential for scale-up CNS regeneration as stem cell therapy to be translated to patients in clinical trials [14,21]. Under conventional multi-lineage differentiation approaches, hESC-derived cellular products consist of a heterogeneous population of mixed cell types, including fully differentiated cells, high levels of various degrees of partially differentiated or uncommitted cells, and low levels of undifferentiated hESCs, posing a constant safety concern when administered to humans [15,16]. This novel lineage-specific differentiation approach by small molecule induction of pluripotent hESCs not only provides a model system for investigating human embryogenesis, but also dramatically increases the clinical efficacy of graft-dependent repair and safety of hESC-derived cellular products [14,16-21]. This study by high-resolution genome-wide mapping of chromatin modifications in human stem cell derivatives further supported the view that the tissue-resident CNS-derived hNSCs have acquired more silenced chromatin, therefore, they are likely resides at a more advanced stage of development with more limited developmental potential or plasticity for tissue or organ regeneration. Conversely, the hESC derivatives retain more open epigenomic landscapes, therefore, they might start at an earlier embryonic developmental stage with more plasticity for scale-up regeneration, consistent with our previous observations [14].

Conclusion: A Model for Monitoring the Epigenomes of Human Stem Cell Derivatives to Reflect their Intrinsic Plasticity and Regenerative Potential

In summary, this study shows that the intrinsic plasticity and regenerative potential of human stem cell derivatives can be differentiated by their epigenomic features. These data presented here suggest that dynamic changes in epigenomic features parallel the progressive narrowing of potency of human stem cell derivatives as they transit along the developmental continuum from pluripotency to tissue- and organ-resident identity. The pluripotency of hESCs is characterized by an epigenome comprised of a globally open conformation of chromatin remodeled by Oct-4 primed for unrestricted lineage choices (see a model in Figure 7B). The progressive narrowing of potency is associated with the gradual restriction in chromatin openness, hence, lineage choices as a result of global increases in chromatin-silencing (Figure 7B). Note that, along this continuum, the epigenome of the CNS-derived tissue resident hNSC is more deacetylated, methylated, and compacted than that of hESC neuronal derivative hESC-I hNuPs (Figure 7B). The tissue-resident CNS-derived hNSCs have acquired more silenced chromatin, therefore, they are likely resides at a more advanced stage of development with more limited developmental potential or plasticity for tissue or organ regeneration. Conversely, the hESC derivatives retain more open epigenomic landscapes, therefore, they might start at an earlier embryonic developmental stage with more plasticity for scale-up regeneration. Safe and effective clinical translation of stem cell biology requires a better knowledge of the inherent cellular mechanisms maintaining plasticity and then stabilizing lineage commitment. This study suggests that one of these pivotal controls is a chromatin-mediated mechanism governing differential potential along the continuum from pluripotency to somatic identity. Uncovering epigenomic landscape features might

help us to select the human stem cell derivative prospectively that strikes the proper balance to insure the safety and efficacy of human stem cell engraftment for future therapeutic tasks.

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Disclosures

The author declares competing interests. XHP is the founder of Xcelthera and has intellectual properties related to hESCs.

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