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Embryonic stem cell differentiation: A chromatin perspective

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Abstract

Embryonic stem (ES) cells hold immense promise for the treatment of human degenerative disease. Because ES cells are pluripotent, they can be directed to differentiate into a number of alternative cell-types with potential therapeutic value. Such attempts at "rationally-directed ES cell differentiation" constitute attempts to recapitulate aspects of normal development *in vitro*. All differentiated cells retain identical DNA content, yet gene expression varies widely from cell-type to cell-type. Therefore, a potent epigenetic system has evolved to coordinate and maintain tissue-specific patterns of gene expression. Recent advances show that mechanisms that govern epigenetic regulation of gene expression are rooted in the details of chromatin dynamics. As embryonic cells differentiate, certain genes are activated while others are silenced. These activation and silencing events are exquisitely coordinated with the allocation of cell lineages. Remodeling of the chromatin of developmentally-regulated genes occurs in conjunction with lineage commitment. Oocytes, early embryos, and ES cells contain potent chromatin-remodeling activities, an observation that suggests that chromatin dynamics may be especially important for early lineage decisions. Chromatin dynamics are also involved in the differentiation of adult stem cells, where the assembly of specialized chromatin upon tissue-specific genes has been studied in fine detail. The next few years will likely yield striking advances in the understanding of stem cell differentiation and developmental biology from the perspective of chromatin dynamics.

Embryonic stem cells: promise and challenge

Recent interest in both scientific and lay communities has centered on the potential use of embryonic stem (ES) cells as therapeutic agents for the treatment of degenerative human diseases. This excitement stems from the pluripotent nature of ES cells, which allows them to differentiate into a broad spectrum of cell-types that may one day be used for transplantation purposes (Fig. 1). ES cell lines are derived from explanted culture of the inner cell mass (ICM) of blastocyst-stage embryos. In normal development, the ICM is the primordial source of the entire embryo proper, while the trophectoderm and maternal cells contribute to the placenta. ES cells can be maintained in an undifferentiated (and pluripotent) state by culture in the presence of the cytokine LIF (leukaemia inhibitory factor).

A great variety of cell-types have been generated by the differentiation of ES cells *in vitro*. Such cell-types resemble neuronal cells, pancreatic cells, muscle cells and fibroblasts, hematopoietic cells, and many others, but it is unclear at present how closely these cells mimic their nor-

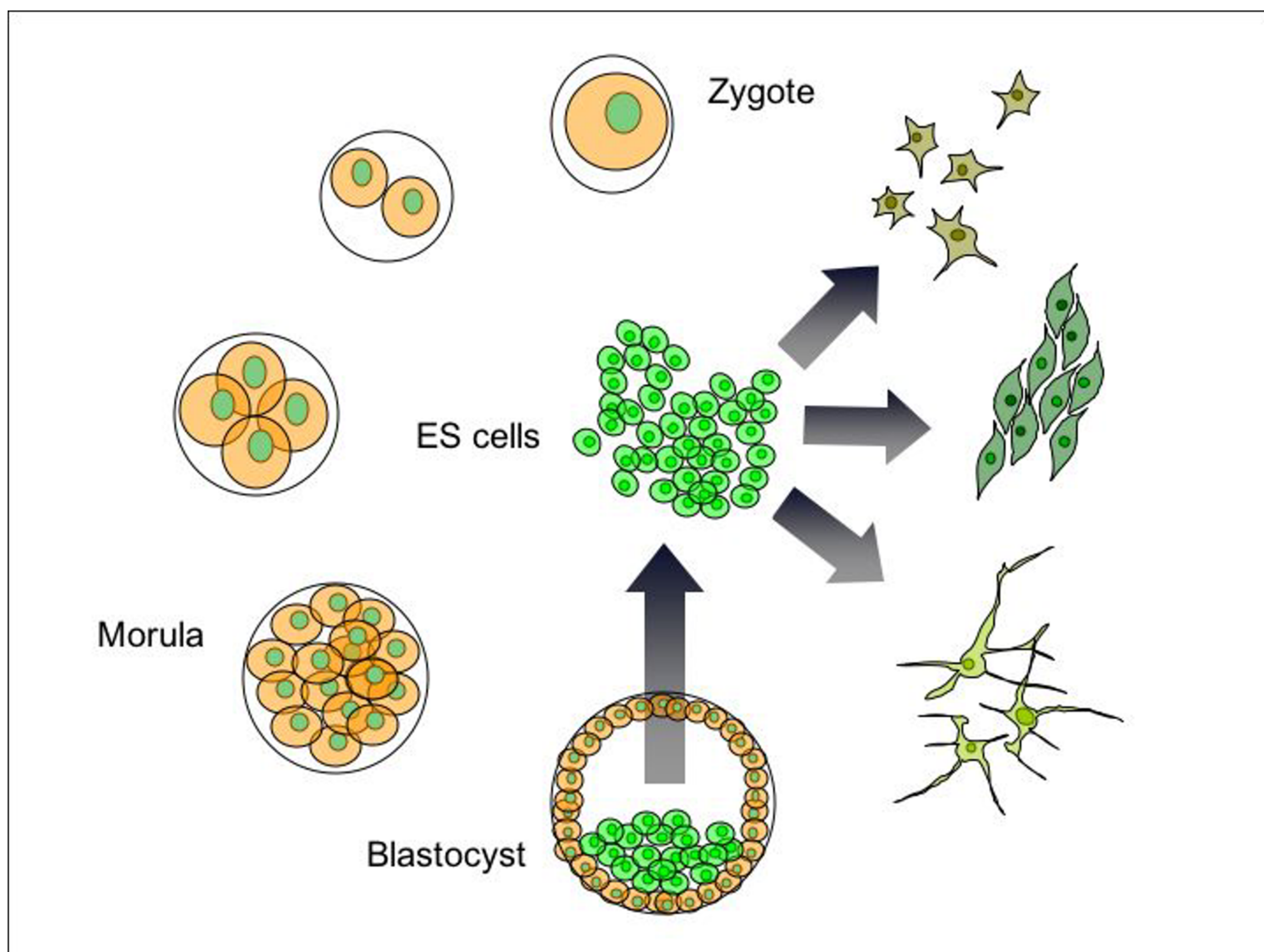

Figure 1
Stem cell therapy viewed from the standpoint of developmental biology. Early mammalian embryonic development involves a series of rapid symmetric cell divisions leading to morula formation. Subsequently, blastocyst-stage embryos form with two cell-types: the trophectoderm (TE), which develops into the embryonic portion of the placenta, and the inner cell mass (ICM), which develops into the embryo proper. Immortal embryonic stem (ES) cells are derived from the ICM, and retain developmental totipotency. In vitro differentiation protocols yield a variety of unique cell-types that are potentially useful as clinical transplantation materials.

mal counterparts created in the course of development *in vivo*. Indeed, many promising derivatives of ES cells have proven to function poorly in animal engraftment models. These difficulties point to the need for a more sophisticated understanding of the differentiation process and the development of new methods to more exhaustively assess the identities of cells generated by *in vitro* differentiation. Future methods that achieve successful directed differentiation will rely heavily on a thorough understanding of the developmental pathways that they attempt to recapitulate. Initial attempts to bring stem cell therapy to a state of effectiveness have been hampered by a paucity of knowledge concerning the underlying mechanisms that govern differentiation.

Epigenetic management of the genome through chromatin

Adult mammals contain hundreds of cell-types distributed among their organs, each with identical DNA content. Yet each of these cell-types has a unique pattern of gene expression. The field of epigenetics is concerned with influences on gene expression that occur independently of DNA sequence *per se*. In principle, genes behave in three ways during development: Some genes are subject to line-

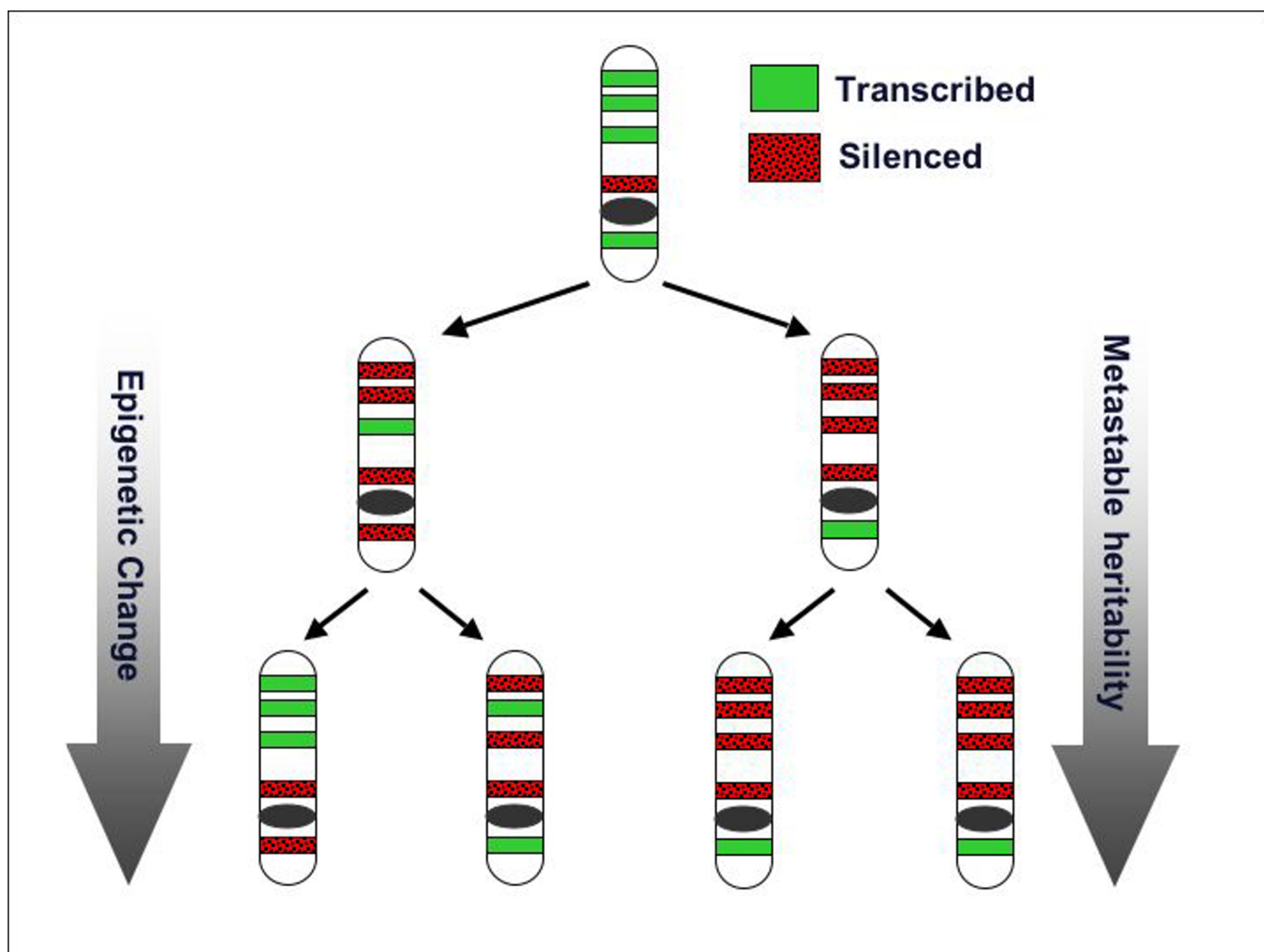

Figure 2
Epigenetic management of the genome. An idealized chromosome is depicted with genes that are either transcribed (green) or silenced (red). As cells undergo developmentally-regulated changes in lineage (in either normal development or in the context of ES cell differentiation), patterns of gene expression change. Genes can be specifically silenced or activated through epigenetic means facilitated by chromatin remodeling (left lineage). Once terminally differentiated states are reached, patterns of gene expression can be maintained in a metastable fashion through maintenance of chromatin configuration (right lineage).

age-dependent activation events, while others undergo lineage-dependent silencing events, such as X chromosome inactivation and silencing of embryonic genes such as *Oct 4* [1]. Lastly, the expression of housekeeping genes is maintained constitutively (Fig. 2). Though each cell has identical DNA content, the way in which it is *packaged* with chromosomal proteins (i.e. chromatin) differs greatly from cell to cell. Indeed, recent findings from chromatin research and animal cloning (nuclear transfer) studies suggest that much of the molecular basis of tissue-specific gene expression is rooted in the details of chromatin structure.

The basic subunit of all chromatin is the nucleosome, which consists of a histone octomer containing a pair of each of the standard histones H2A, H2B, H3, and H4 and 146 base pairs of DNA. Chromatin can be broadly divided into two fractions: euchromatin, which is permissive for transcription, and heterochromatin, which is repressive. Heterochromatin itself occurs in two varieties, constitutive and facultative. DNA within constitutive heterochromatin is obligately silenced. Examples include centromeric regions and inactivated repetitive elements such as Alu, LINE, and SINE elements, and inactivated retroviruses. In contrast, facultative heterochromatin is

silenced only in certain contexts. Examples of facultative heterochromatin include tissue-specific genes that are silenced in all but the appropriate tissue, and X chromosomes in female eutherian mammals, which can be either active or silenced for reasons of dosage compensation. Heterochromatin seems to have much more molecular complexity than euchromatin. Most euchromatin consists of standard nucleosomes, but heterochromatin often contains modified nucleosomes in which histone variants substitute for standard histones. Examples include the histone H2A variants macroH2A1 [2] and macroH2A2 [3,4], the histone H3 variant CENP-A [5,6] which associates with centromeric heterochromatin, and histones H2A.X and H2A.Z. Histone H2A.X becomes phosphorylated when DNA is damaged and marks sites of double-stranded breaks in DNA [7], while H2A.Z protects euchromatin from becoming transcriptionally inactive [8].

Chromatin is further modified by the addition of post-translational modifications upon histones such as methyl-, acetyl-, phosphoryl-, and ADP ribosyl-groups (see reviews: [9-11]). Histone modifications can serve as cis-acting binding sites for auxiliary factors such as heterochromatin protein 1 (HP1), which binds to histone H3 upon methylation at lysine 9 [12,13], a key bimolecular interaction in heterochromatin that is controlled by *Suv39h1* and *Suv39h2* histone methyltransferases [14-16]. Histone methylation also occurs upon histone H3 at the lysine 4 position, but in this case, methylation correlates with transcriptional activity [17]. DNA itself can also be methylated, primarily upon cytosines within CpG dinucleotides. Here too, methylation serves to foster bimolecular interactions between heterochromatin and auxiliary proteins such as MeCP2, and MBD1, 2, and 3. On a molecular level, close connections exist between chromatin and the transcriptional apparatus. The importance of transcription factors for gene activation is indisputable, but a remarkable number of transcription factors are now known to interact with transcription-promoting histone acetyltransferase (HAT) proteins [18]. An emerging view is that histone modifications may serve as "switches" to activate or repress gene expression, in much the same way that phosphates toggle the activation or repression of intracellular signal transduction pathways.

Embryonic cells contain robust chromatin-remodeling and reprogramming activities

As ES cells undergo differentiation, they spontaneously execute developmentally-regulated programs of gene expression and gene silencing that are intimately coordinated with alterations in chromatin structure. Perhaps the best studied of these is X chromosome inactivation (XCI), where a number of step-wise chromatin remodeling events lead to the formation of stably-silenced X chromosomes in differentiating female ES cells. The initiation step of X inactivation is governed by a mutually-antagonistic expression pattern involving the genes *Tsix* and *Xist*, which lie adjacent to one another within the X inactivation center (XCI) of the X chromosome [19]. Tsix and Xist give rise to overlapping untranslated RNA transcripts that are retained upon X chromosomes by an unknown mechanism. In undifferentiated cells, Tsix expression prevents the accumulation of Xist RNA. As differentiation commences, Tsix expression is extinguished from the future inactive X chromosome (Xi), which allows Xist to accumulate, spread, and coat the Xi. Expression of Xist RNA is required for initiation of X inactivation but not its maintenance [20,21]. Shortly after Xist coating, the silencing of X-linked genes can be detected, but this early silencing is unstable and reversible [22]. A number of subsequent (and ordered) chromatin remodeling events then occur to stabilize the inactive state. These include a transient association of the histone methyltransferase complex Eed-Ezh2 which methylates histone H3 at lysine 27 [23,24]. Another early event in X chromosome inactivation is late replication timing of Xi relative to the active X during S phase of the cell cycle [25,26]. Subsequently, histones become hypoacetylated [27,28] upon lysine residues and methylated at lysine 9, an event probably performed by the *Suv39h1* and *h2* histone methyltransferases [29,30]. Later, the histone variant macroH2A is incorporated into inactivated X chromosomes within female nuclei. These local macroH2A concentrations are called macrochromatin bodies (MCBs) [31]. MCB formation occurs relatively late in the X chromosome inactivation process [32,33], and requires prior expression of *Xist* [20,34]. Recent evidence points to a functional role for macroH2A1 in gene silencing since it can cause down-regulation of reporter gene activity [35]. In addition, macroH2A1 can inhibit binding of the transcription factor NF-kappaB and retards the action of SWI/SNF chromatin remodeling complexes [36]. A final step in the X inactivation process is marked by the methylation of the CpG islands of silenced X-linked genes [37]. Interestingly, all of these chromatin-remodelling events (with the exception of the action of the Tsix/Xist RNAs) can also occur on autosomes [38]. Therefore, it seems that all post-RNA events of XCI may have been co-opted from pre-existing autosomal systems during the evolution of XCI in placental mammals.

Interestingly, the mammalian nuclear transfer cloning process can reprogram X chromosome inactivation. In this case, inactive X chromosomes from somatic cells were reactivated in cloned embryos but not trophectoderm [39]. Though cloned embryos transit the blastocyst developmental stage (with concomitant formation of the ICM), it is unclear at which stage the somatic donor XCI status was erased (or reactivated), though failure of erasure to occur in trophectodermal cells suggests the possible involvement of the ICM cells or their progenitors in repro-

gramming events. Another interpretation of these results is that the somatic pattern of XCI is read out in the trophectoderm much like an imprint, a distinct possibility since XCI is imprinted only in the extra-embryonic tissues in the mouse. In either case, it is clear that reprogramming of inactive X chromosomes during the cloning process shows that oocytes or primitive embryonic cells have extensive chromatin remodeling activity.

Mammalian cells contain a variety of chromatin remodeling complexes, which typically are composed of several proteins. Inactivation of chromatin remodeling complexes usually results in developmental arrest at about the blastocyst stage. For instance, loss of *SNF5*, a shared component of two related mammalian SWI/SNF chromatin remodeling complexes causes developmental arrest at about the time of embryo implantation and is required for ES cell viability [40]. Homozygous deletion of *SNF2*β (another component of mammalian SWI/SNF chromatin remodeling complexes), leads to lethality in F9 embryonal carcinoma cells [41]. The polycomb group gene *rae28* is required for efficient renewal of hematopoietic stem cells [42], while the polycomb group gene *Ezh2* (which contains a histone methyltransferase SET domain) is required for post-implantation development and ES cell viability [43].

Direct demonstrations of reprogramming activities within ES cells come from experiments involving intentional cell fusions between ES and somatic cells. Fusions between male ES cells and adult female thymocytes led to demonstrated alterations in the chromatin of inactivated X chromosomes originating from the thymocyte nucleus. These alterations included the abolition of late replication timing of the thymocyte Xi, and destabilization of the Xist RNA transcript. In addition, a silenced *Oct4-GFP* transgene was reactivated in these fusion cells, a transcriptional state reminiscent of primitive cells [44]. Significantly, ES-thymocyte fusions gained pleuripotency since they contributed to all three germ layers in chimeric embryos [44]. However fusions between ES cells and thymocytes failed to alter methylation of the imprinted *Igf2-H19* region, which occurs readily when embryonic germ cells and thymocytes are fused [45].

Hints from adult stem cells and developmental biology

Studies of adult stem cells provide ample evidence that chromatin functions in specialized differentiation events. A classic view of development posits that the commitment of primitive multipotent cells to specific lineages is mediated by key transcription factors that activate downstream tissue-specific genes. For instance, retroviral-mediated transfer of transcription factor MyoD into a variety of non-muscle cell types can convert them into cells that resemble striated myoblasts as judged by cellular morphology and expression of muscle-specific markers [46]. This is but one of many demonstrations that transcription factors can effectively initiate developmental programs that culminate in directed differentiation (even trans-differentiation) of cells. In contrast, recent evidence shows that heterochromatin formation can be a key mechanism for lineage restriction during development.

The differentiation of hematopoietic stem cells (HSCs) seems to occur by means of selective silencing (i.e. restriction) of lineage-specific genes. Hematopoietic stem cells and their immediate progenitors exhibit a promiscuous pattern of gene expression. Uncommitted HSCs simultaneously express genes previously thought to be transcribed exclusively in either the myeloid or erythroid lineages [47]. Developmentally-regulated restriction of gene expression also occurs during formation of glial cells of the brain. The formation of oligodendrocytes from glial cell precursors requires exit from mitosis and histone deacetylase (HDAC) activity shortly thereafter. Oligodendrocyte formation is abolished by use of the HDAC inhibitor trichostatin A, suggesting that hypoacetylation of histones in glial stem cells is a normal occurrence during neurogenesis [48].

The regulation of globin gene expression is also mediated by chromatin remodeling. The locus control region (LCR) is a non-coding regulatory domain that lies adjacent to the globin gene cluster. Lineage-specific expression of globin genes is modulated by a complex set of chromatin events involving the LCR and individual globin gene promoters. Interestingly, DNase I sensitive sites within the LCR that are observed in erythrocyte lineages can be found much earlier in multipotent hematopoietic stem cells, suggesting that chromatin rearrangements precede transcriptional activation of lineage-specific genes during hematopoiesis [49]. This "primed" configuration of the LCR is also evident in embryonic yolk sac, where acetylated histones are present at the LCR and globin gene promoters (whether active or inactive) while in brain, the LCR is hypoacetylated and globin genes are transcriptionally silent [50]. Interestingly, the LCR is present in an acetylated state in ES cells [51]. Together, these data suggest that aspects of globin gene expression may be regulated by a restriction mechanism involving the LCR.

Finally, the intranuclear position of chromatin seems to have a bearing on its regulation. In many mammalian cell types, it has been observed that transcriptionally silent genes reside in a position near the nuclear periphery [52], or interphase centromeres (chromocenters) [53], while active genes are maintained near the center of nuclei. Nuclear location of genes may therefore lead to changes in their transcription state and there is some evidence that

this is a dynamic process. For instance, the zinc-finger protein Ikaros is expressed in lymphoid cells and seems to be a regulator of gene silencing by recruiting genes to repressive heterochromatin [54]. Deletion of Ikaros leads to a loss of differentiative potential in hematopoietic stem cells in mice [55].

Conclusions

The dynamic assembly and disassembly of specialized chromatin is a likely mechanism for the epigenetic regulation of gene expression during ES cell differentiation and development *in vivo*. Put another way, the transcriptional state of a gene can be viewed as a reflection of its underlying chromatin state. Indeed, it seems possible that all stem cells share common epigenetic mechanisms given the recent demonstration that hematopoietic, neural, and embryonic stem cells express a significant number of non-housekeeping genes in common [56]. Because stem cell differentiation is essentially an attempt to achieve tissue-specific patterns of gene expression in vitro, it seems reasonable that studies of chromatin in differentiating ES cells will greatly aid the subsequent clinical development of stem cell therapies. The analysis of chromatin factors and modifications that exhibit tissue-specific genomic distribution patterns will likely yield substantial insights into the use of stem cells of all types.

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