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Review

Chromatin in embryonic stem cell neuronal differentiation

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Summary. Chromatin, the basic regulatory unit of the eukaryotic genetic material, is controlled by epigenetic mechanisms including histone modifications, histone variants, DNA methylation and chromatin remodeling. Cellular differentiation involves large changes in gene expression concomitant with alterations in genome organization and chromatin structure. Such changes are particularly evident in self-renewing pluripotent embryonic stem cells, which begin, in terms of cell fate, as a *tabula rasa*, and through the process of differentiation, acquire distinct identities. Here I describe the changes in chromatin that accompany neuronal differentiation, particularly of embryonic stem cells, and discuss how chromatin serves as the master regulator of cellular destiny.

Key words: Chromatin, Embrionic stem cells, DNA, Histones, Neuron, Development, Differentiation

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of the developing embryo at the blastocyst stage (Fig. 1). During development, the ICM gives rise to the three embryonic germ layers, endoderm, mesoderm and ectoderm, which comprise the embryo proper. Surrounding the ICM are the trophoblast cells, which make up the epithelial compartment of the placenta. When ES cells are successfully cultured, they have the potential to both self-renew indefinitely, and to differentiate into multiple cell types of all germ layers. Given the right conditions, the initial stages of ES cell *in vitro* differentiation are reminiscent of early embryogenesis, forming so-called embryoid bodies. When ES cells are allowed to differentiate spontaneously in the absence of any external signals it is believed that they enter the neuronal lineage by default (Munoz-Sanjuan and Brivanlou, 2002). Evidently, mouse ES cells can begin their transformation into primitive neural stem cells as early as 4 hours after the onset of differentiation (Smukler et al., 2006). Neuronal differentiation involves changes in gene expression and nuclear architecture as well as cellular-morphological changes such as neurite extension and synapse formation (Fig. 2). Therefore, ES cells must be plastic enough to accommodate such rapid and substantial changes. One of the likely keys to their success is their unique chromatin. It is becoming increasingly clear that chromatin holds some of the secrets for pluripotency, stem cell identity, regulation of differentiation and cellular fate. Here I review the role of chromatin structure and epigenetic regulation in ES cell neuronal differentiation and discuss the implications of chromatin organization for a variety of different processes ranging from cloning and nuclear transfer to neuronal activity and behavior.

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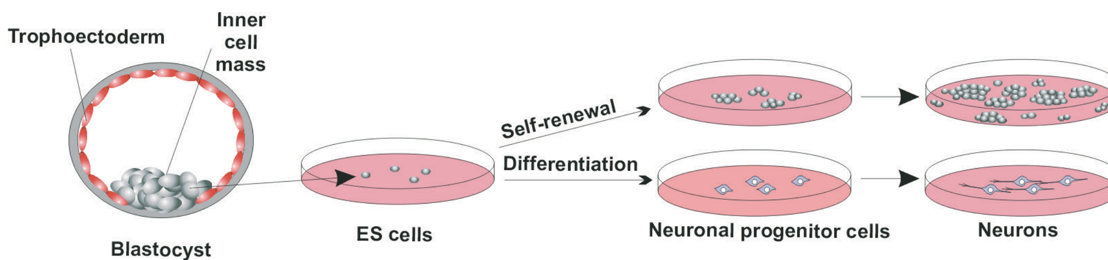
Chromatin structure

The fundamental unit of chromatin is the nucleosome, which comprises two copies each of the four core histones, H2A, H2B, H3 and H4. Together, they form a histone octamer, which is wrapped inside 147 bp of genomic DNA. The DNA bridging two adjacent nucleosomes is termed linker DNA, and is normally bound by the linker histone H1. This primary structural sequence is referred to as the ‘beads on string’ structure as perceived through the electron microscope (Robinson and Rhodes, 2006; Woodcock et al., 2006). In vivo, in the eukaryotic nucleus, chromatin is folded into higher order structures, of which our understanding is rudimentary at best (Robinson and Rhodes, 2006). What is clear though, is that chromatin is dynamic and its active nature directly influences genome activity and nuclear functions (Gasser, 2002; Misteli, 2001). Chromatin binding proteins associate with chromatin only transiently (Phair and Misteli, 2000). This is particularly evident in undifferentiated embryonic stem cells and other multipotent cells, where a loosely bound fraction of chromatin binding proteins is present in their nucleosplasms (Fig. 3A). Even the normally tightly associated core histones possess an unbound or loosely

bound fraction in pluripotent cells (Meshorer et al., 2006). This free pool becomes tightly associated with chromatin following ES cell neuronal differentiation (Meshorer et al., 2006) (Fig. 3B). Since undifferentiated but lineage-restricted cells do not possess this characteristic loosely bound pool, it seems to be a unique feature of multipotent or pluripotent cells (Meshorer and Misteli, 2006; Meshorer et al., 2006). Consistent with tighter binding of histones to chromatin in differentiated cells, terminally differentiated neurons have a lower core histone turnover, demonstrated by a decrease in their rate of synthesis (Cestelli et al., 1992b). The hyperdynamic association of chromatin-binding proteins with chromatin in ES cells raises questions regarding the nature of the mobility of chromatin itself in ES vs. somatic cells. Movement of chromatin per se was postulated to be more dynamic in ES cells than in differentiated cells (Gasser, 2002), but as yet this has not been confirmed experimentally. Chromatin motion is believed to be restrained by its physical association with the nuclear lamina, providing anchorage sites for chromatin fibers at the nuclear periphery (Goldberg et al., 1999; Gotzmann and Foisner, 1999). Some of the major constituents of the nuclear lamina are the lamin proteins, which both interact with chromatin and serve as key determinants of structural nuclear integrity (Gruenbaum et al., 2005). Interestingly, undifferentiated ES cells lack lamin A/C expression (Constantinescu et al., 2005). This brings forward an attractive proposal that the absence of lamin A/C in undifferentiated ES cells contributes to their unique dynamic chromatin.

One of the determining factors of chromatin structure is the spacing between two adjacent nucleosomes, or the nucleosome repeat length (NRL). This fundamental property of chromatin structure changes during cellular differentiation and was linked to the associated changes in gene expression (Sperling and Weiss, 1980). In cortical neurons, NRL changes during early post-natal development (Ermini and Kuenzle, 1978) and during the course of neuronal differentiation in the rat brain (Jaeger and Kuenzle, 1982), demonstrating global regulation of chromatin structure during the course of differentiation. ES cells in particular have a relatively low NRL (Fan et al., 2005) compared to that of cells from differentiated tissues such as liver or thymus (Fan et al., 2003). Interestingly, mouse ES cells lacking three of the six H1 genes display decondensed chromatin and shorter NRL (Fan et al., 2005),



Fig, 1. Derivation of embryonic stem cells. ES cells are extracted from the inner cell mass (ICM) of the developing embryo at the blastocyst stage (day 3.5 in mouse and day 4.5 in human). When cultured in vitro, depending on the conditions, ES cells can either self-renew indefinitely (top) or differentiate into multiple cell types, including neurons (bottom).

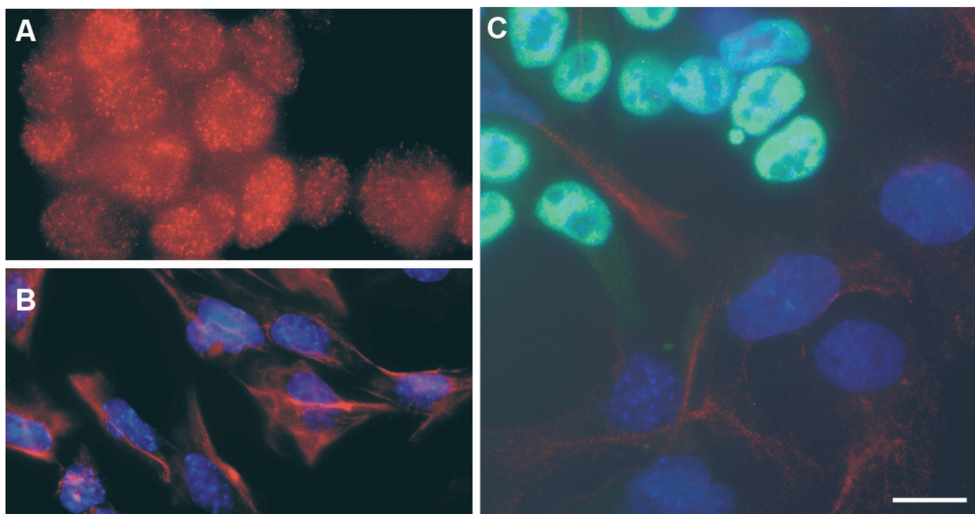


Fig. 2. Differentiation of mouse embryonic stem cells. **A.** Undifferentiated ES cells are stained with the stem cell marker Oct-4 (red). **B.** ES-derived neuronal progenitor cells (NPCs), differentiated for 7 days are stained with the neural stem cell marker nestin (red). DNA (DAPI) is shown in blue. **C.** During early differentiation, nestin-positive NPCs (red) emanate from the original colony, which contains Oct-4 positive undifferentiated ES cells (green). Bar: 10μm

demonstrating inverse correlation between chromatin condensation and NRL. It also appears that the ratio between the NRL and the number of H1 molecules per nucleosome is conserved in different cell types (Woodcock et al., 2006), implying that H1 is a determining factor of NRL. Supporting this view, in ES cells, the fraction of H1 that is bound to chromatin is smaller (Robinson and Rhodes, 2006; Woodcock et al., 2006), resulting in a correspondingly smaller NRL. But at the same time, a relatively high proportion of H1 molecules in ES cells are present in an unbound or loosely bound state (Meshorer et al., 2006) likely contributing to the dynamic chromatin structure (Catez et al., 2006; Meshorer et al., 2006), suggesting that the relationship between H1, NRL and chromatin structure might be more complex than previously perceived.

Global morphological changes of chromatin structure are also evident during neuronal differentiation of ES cells (Meshorer and Misteli, 2006). Light microscopy revealed marked changes in the number, size and distribution of heterochromatin foci (Meshorer et al., 2006), while electron microscopy demonstrated an increase in compact heterochromatin following retinoic acid stimulation of human ES cells (Park et al., 2004b) as well as reduced homogeneity and a decrease in the abundance of RNPs following neuronal differentiation of mouse ES cells (EM, unpublished observations). Chromatin reorganization during differentiation is by all means not unique to ES cells (Arney and Fisher, 2004), but since ES cells can transition to any cell type, the amplitude of changes in their chromatin structure is vast. Moreover, the unique properties of ES cell chromatin lead to recent suggestions that chromatin holds some of the secrets of pluripotency (Szutorisz and Dillon, 2005; Buszczak and Spradling, 2006; Meshorer and Misteli, 2006) and we should stay tuned for future developments in this dynamic field.

Histone modifications

Chromatin structure is greatly influenced by histone modifications. Through the action of specialized proteins, core histones can be methylated, acetylated, phosphorylated, ubiquitynated and sumoylated on multiple residues. This combinatorial complexity brought forward the idea of 'histone code' (Turner, 1993; Jenuwein and Allis, 2001). In support of this view, several modifications, such as H3 acetylation on lysine 9 or H3 tri-methylation on lysine 4, were shown to be highly associated with transcriptional activity; while other modifications, such as H3 tri-methylated on lysine 9 (H3-triMeK9) or H4 tri-methylated on lysine 20 are correlated with inactive chromatin (Nightingale et al., 2006). However, exceptions to the histone code rule are beginning to accumulate (Vakoc et al., 2005; Shi et al., 2006) and in several cases of histone modifications, the histone code was shown to be highly redundant (Schubeler et al., 2004; Dion et al., 2005). Regardless, what is clear from these studies is that histone modifications play important roles in the epigenetic status of chromatin and the regulation of chromatin structure during cellular events such as development, differentiation and reprogramming.

Undifferentiated ES cells possess a unique epigenetic landscape. Recent efforts to characterize the histone modifications associated with pluripotency revealed a bivalent character: an abundance of active chromatin marks (Fig. 3), which are also associated with the repressive mark H3 tri-methylated on lysine 27 (Azuara et al., 2006; Bernstein et al., 2006; Meshorer et al., 2006). These opposing chromatin marks presumably prime ES cells for lineage-specific expression upon stimulation, but maintain them transcriptionally restricted at the undifferentiated state. Polycomb group (PcG) proteins, which are known to be associated with H3-triMeK27, bind promoter regions of numerous lineage specific genes in ES cells (Boyer et al., 2006; Lee et al., 2006). These genes otherwise promote differentiation and the PcG proteins keep them repressed but poised, in the undifferentiated state (Boyer et al., 2006; Lee et al., 2006). Supporting this view, loss of the PcG protein Bmi1, whose expression is limited to neural stem cells, leads to a decrease in the neural stem cell population and proliferation (Zencak et al., 2005) and to post-natal depletion of neural stem cells (Molofsky et al., 2003), causing neurological and growth defects.

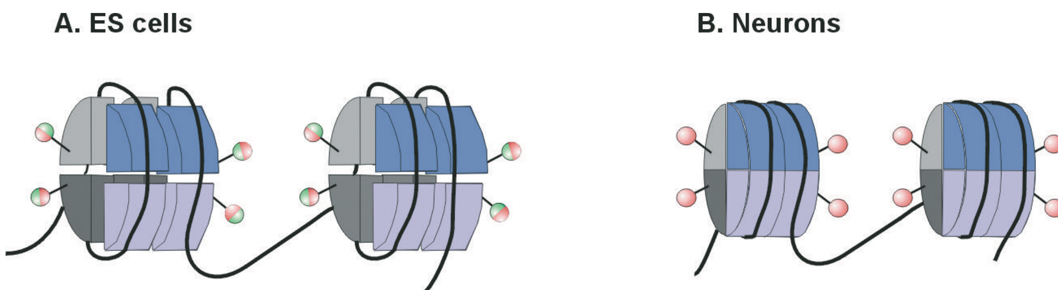


Fig. 3. Chromatin structure in ES cells and neurons. Shown are two transcriptionally inactive nucleosomes from either undifferentiated ES cells (**A**, left) or neuronally differentiated ES cells (**B**, right). In undifferentiated ES cells, a fraction of the histones is loosely bound to DNA and the histone modifications are bivalent, including both active (green) and inactive (red) chromatin marks. Following neuronal differentiation, the histones are bound tightly and inactive regions are marked with silenced histone marks (red).

Intriguingly, Bmi1 is not required for survival or differentiation of neural stem cells, but for their self-renewal capacity (Molofsky et al., 2003), highlighting the important role of Bmi1 and PcG proteins in maintaining multipotency and self-renewal (Park et al., 2004a; Valk-Lingbeek et al., 2004).

Differentiation of mouse ES cells is accompanied by global changes in histone modifications, including, for example, an increase in H3-triMeK9 (Meshorer et al., 2006), a decrease in H3-triMeK4 (Azuara et al., 2006; Bernstein et al., 2006; Lee et al., 2004) as well as a decrease in pan-acetylation of histones H3 and H4 (Lee et al., 2004; Meshorer et al., 2006). Therefore, stem cell neuronal differentiation, or any type of differentiation for that matter, requires the concerted action of chromatin modifying enzymes. Such enzymes include, but are not limited to, histone acetyl transferases (HATs), histone deacetylases (HDACs), histone methyl transferases (HMTs) and the recently discovered histone demethylases. Class II HDACs, for example, are up-regulated during neuronal differentiation of cultured hippocampal neural progenitor cells (Ajamian et al., 2003), but somewhat contrastingly, HDAC inhibition promoted neuronal differentiation of similar cultured progenitor cells (Hsieh et al., 2004). In ES cells, HDAC inhibition blocked differentiation (Lee et al., 2004), suggesting that these two cell types differ significantly in their global acetylation level and differentiation potential.

An intriguing example of differentiation-associated neuronal gene regulation by chromatin is provided by the RE1 silencing transcription factor REST (also known as neuron-restrictive silencer factor, NRSF), which suppresses key neuronal genes by binding to a conserved 23 bp DNA motif called repressor element 1 (RE1) (Chong et al., 1995; Schoenherr and Anderson, 1995). REST maintains suppression through recruitment of the co-repressor mSin3A and HDAC1 (Huang et al., 1999) as well as an additional protein termed CoREST (Andres et al., 1999). Remarkably, while in neurons and primary neural progenitor cells REST acts concomitantly with H3-K9 methylation as well as DNA methylation at the REST binding site, in ES cells, REST operates through a different mechanism, independent of both histone and DNA methylation, thus, supposedly, leaving the ES cell chromatin repressed but poised for activity (Ballas et al., 2005). These results might also help explaining the opposing effects of HDAC inhibition on ES cells and neural progenitor cells: while the ES cell chromatin is globally acetylated and deacetylation is required for differentiation, in neural progenitor cells, deacetylase activity (e.g. HDAC1) is required to suppress neuronal genes, and inhibition of HDAC is therefore likely to drive the progenitors towards neuronal commitment.

Histone variants

A powerful means by which cells can regulate their chromatin is by the use of histone replacement proteins for either H2A or H3. These minor histone variants are unlike the major histone proteins. They are transcribed from a poly-adenylated mRNA and can be deposited unto chromatin in a replication-independent manner. They include H2A.Z, H2A.X, macroH2A and H2A.Bdb as well as H3.1, H3.2, H3.3 and CenpA. Analysis of the histone composition during neuronal differentiation revealed changes in core histone variants, as well as in the subtypes of the linker histone H1 (Pina and Suau, 1985, 1987; Pina et al., 1987; Ponte et al., 1994). Specifically, four subtypes of H1, H1a-d gradually decrease during postnatal development, while H1e becomes the dominant form in the adult brain (Dominguez et al., 1992). Interestingly, $H1^0$ accumulates in a period restricted to neuronal terminal differentiation (Ponte et al., 1994), suggesting a role for this specific variant in the chromatin-related changes underlying neuronal commitment (Cestelli et al., 1992a). The H3 variants, H3.1 and H3.2 exponentially decrease during neuronal differentiation, while the levels of the transcription-associated variant H3.3 increases (Bosch and Suau, 1995). H3.3 protein accumulation also occurs in differentiating cortical neurons, despite an unexpected decrease in its mRNA levels (Scaturro et al., 1995), suggesting post-transcriptional regulation of H3.3 in neurons. Since H3.3 marks actively transcribing chromatin regions (Ahmad and Henikoff, 2002), its deposition in neuronal chromatin might reflect the need for a dynamic transcriptional machinery, which must respond quickly to neuronal activation (West et al., 2002). An additional variant that accumulates during neuronal differentiation is the DNA damage-associated variant H2A.X (Bosch and Suau, 1995). H2A.X is rapidly phosphorylated following NMDA receptor activation in rat cortical neurons (Crowe et al., 2006). The abundance of H2A.X in neurons might be related to the accumulation of chromosomal aneuploidy in differentiating and post-mitotic neurons in the mammalian brain (Rehen et al., 2001). Taken together, these data demonstrate the active participation of histone variants in regulating chromatin structure and function during neuronal differentiation.

A recent comprehensive study of the three mammalian H3 variants, H3.1, H3.2 and H3.3 revealed that each of these histones is enriched for a distinct set of post-translational modifications (Hake et al., 2006). In agreement with what was initially observed during neuronal differentiation (Bosch and Suau, 1995), although to a lesser extent, in retinoic acid induced differentiation of mouse ES cells, the relative fraction of H3.3 slightly increases, while that of H3.2 and H3.1 slightly decreases (Hake et al., 2006). Proteomic analysis of the modification associated with each of the three variants revealed that H3.1 is enriched in a combination of both active and repressive marks, H3.2 is mostly enriched in inactive marks, while H3.3 contains histone modifications that are associated with transcriptional activity (Hake et al., 2006). Based on these observations, the authors speculate that these variants identify the

nature of their surrounding chromatin, with H3.1 defining reversibly silent chromatin, H3.2 irreversibly silent chromatin and H3.3 active chromatin regions.

DNA methylation

DNA cytosine methylation is a major epigenetic factor influencing gene regulation and chromatin structure (Hsieh, 2000; Lorincz et al., 2004; Lande-Diner and Cedar, 2005). Mammalian cells possess two types of DNA methyltransferases. Dnmt1 is a maintenance DNA methyltransferase, whereas both Dnmt3a and Dnmt3b are de novo DNA methyltransferases (Kaneda et al., 2004). As ES cells are derived from the developing embryo at the blastocyst stage, following the almost complete erasure of genomic DNA methylation marks (Monk et al., 1987; Li, 2002), they are nearly devoid of DNA methylation when initially cultured, and still express high levels of Dnmt3a and Dnmt3b, which are required for and are reduced during ES cell differentiation (Jackson et al., 2004; Richards et al., 2004). Methylation levels in ES cells typically rise during prolonged culturing (Maitra et al., 2005), and in the same manner that methylation is re-established in the embryo proper after implantation, cultured ES cells re-acquire specific methylation patterns during differentiation. For example, neuronal differentiation of human embryonal carcinoma (EC) cells, which similarly to ES cells express the stem cell markers Nanog and Oct-4, involve sequential DNA methylation of these gene promoters concomitant to their differentiation-induced silencing (Deb-Rinker et al., 2005). DNA methylation seems to play an important role in neuronal differentiation and neurogenesis in particular. Cre/loxP conditional knock-out of DNMT1 has no effect on post-mitotic neurons, but severely impairs neurogenesis (Fan et al., 2001). Conversely, the *de novo* methyltransferase DNMT3b has a central role in terminal neuronal differentiation, demonstrated by the failure of NGF-stimulated rat pheochromocytoma PC12 cells to produce post-mitotic neurons when Dnmt3b expression was knocked-down or depleted (Bai et al., 2005). Mice lacking the methyl-CpG binding protein 1 (MBD1), a transcriptional repressor, display neural stem cell differentiation as well as adult neurogenesis defects (Zhao et al., 2003), and the methyl-CpG binding protein MeCP2 is the causative agent of the neurological defect Rett syndrome (Guy et al., 2001), serving as a key player in neuronal differentiation (Jung et al., 2003; Matarazzo et al., 2004) and maturation (Kishi and Macklis, 2004). These examples demonstrate the role of DNA methylation in neuronal commitment and differentiation. Interestingly, MeCP2 also binds the catalytic subunit of the SWI/SNF complex Brahma (Brm) (Harikrishnan et al., 2005), thus linking DNA methylation with chromatin remodeling and gene suppression during neurogenesis (Zlatanova, 2005). MeCP2 also serves as an intriguing link between neuronal activity and chromatin structure. In resting neurons, MeCP2 binds the methyl-CpG islands of the BDNF promoter. Neuronal activation leads to MeCP2 phosphorylation and relocalization of MeCP2 to heterochromatin foci in the nucleus. This releases MeCP2 from the BDNF promoter, allowing activation of BDNF transcription (Chen et al., 2003; Martinowich et al., 2003). These observations demonstrate the dynamic interplay between methylated DNA and methyl-CpG binding proteins in neurons, and emphasize the close relationship between neuronal activity and chromatin structure.

Chromatin remodeling

One possibility for maintaining a decondensed chromatin structure in undifferentiated ES cells is through the combined action of chromatin remodeling factors. These ATP-dependent proteins form chromatin-associated complexes that remodel the chromatin to allow access of the transcription machinery to the DNA (Cairns, 2005; Kimmins and Sassone-Corsi, 2005; Mellor, 2005; Smith and Peterson, 2005). Genomic disruption of chromatin remodeling proteins, including Brg1 (Bultman et al., 2000), Snf5 (Klochendler-Yeivin et al., 2000), SSRP1 (Cao et al., 2003) and Snf2h (Stopka and Skoultchi, 2003), all result in premature embryonic death at the blastocyst stage prior to implantation. This alone already suggests an imperative role for chromatin remodeling during early differentiation and development. Moreover, ATP-dependent chromatin-remodeling factors are abundant in undifferentiated ES cells (Kurisaki et al., 2005) and are implicated in early ES cell differentiation (Puente et al., 2006). The chromatin remodeling NuRD complex, for example, is specifically important for ES cell differentiation (Kaji et al., 2006).

Chromatin remodeling proteins are also involved in a growing number of cases of neuronal differentiation (Hsieh and Gage, 2005). The ATRX protein is a critical remodeling factor during corticogenesis, and cortical progenitor cells isolated from Atrx-null mice undergo enhanced apoptosis when induced to differentiate (Berube et al., 2005). Notably, the catalytic subunit of the SWI/SNF chromatin remodeling complex brahma-related gene 1 (Brg1) is critical for neuronal differentiation in *Xenopus* (Seo et al., 2005), but in the mouse, it has an opposite effect; a targeted deletion of Brg1 results in precocious terminal neuronal differentiation of the neural stem cells in the subventricular zone (Matsumoto et al., 2006). Along the same lines, transcriptional profiling comparing neural stem cells from the mouse subventricular zone with fully differentiated brain regions revealed, using Gene Ontology analysis, chromatin remodeling as one of two prominent processes implicated during adult neurogenesis (Lim et al., 2006). These data together provide strong evidence for a vital role for chromatin remodeling during neuronal differentiation of both ES cells and neuronal progenitor cells, but additional investigation is required in order to identify the key

players involved.

Conclusions

Differentiation provides an excellent model system for studying epigenetics, since the same cell with its own fixed genetic material transforms from one state to another. This transition appears to involve the combined regulation of chromatin structure, chromatin modifications, histone variants and chromatin remodeling. ES cells in particular arguably provide the best model system as they are devoid of any cellular identity and are able to take the form of essentially any cell type, with the exception of some extra-embryonic tissues. Their lack of commitment makes them also the most suitable candidates for cloning experiments. When somatic cells are used for somatic cell nuclear transfer (SCNT), their chromatin must first be reprogrammed to a state that is reminiscent of the ES cell chromatin state for successful nuclear transfer (Jaenisch et al., 2004). Remarkably, even terminally differentiated, post-mitotic olfactory neurons are able to undergo the necessary reprogramming events and to re-enter the cell cycle following nuclear transfer and to subsequently develop into mature animals (Eggan et al., 2004). Reprogramming also occurs in human fibroblasts when fused with hES cells. Although the fused cells remain tetraploid, their transcriptional activity, allele-specific gene expression and DNA methylation patterns are undistinguishable from those of the parental ES cells (Cowan et al., 2005). Reprogramming, or de-differentiation as it is often referred to, clearly demonstrates the imperative role that chromatin plays during cell fate decisions and normal differentiation events. The recent discoveries in the field of stem cell chromatin biology clearly advances our understanding of the role that chromatin plays in stem cell fate and commitment, but at the same time emphasizes how much remains to be explored and discovered in this active field.

In neurons particularly, chromatin is not only central during neuronal differentiation, but, as we have seen, also plays a dynamic regulatory role in neuronal excitation. Neuronal epigenetic programming is involved in a number of intriguing physiological events. Increased grooming and nursing by female rats modifies chromatin structure at the glucocorticoid receptor gene of their offspring (Weaver et al., 2004). Chronic cocaine administration causes H3 hyperacetylation at the promoters of BDNF and Clk5, both of which are induced during and implicated in chronic drug abuse (Kumar et al., 2005). Epigenetic chromatin regulation is also the underlying mechanism affecting the changes in the different BDNF transcripts that occur following chronic social stress and depression (Tsankova et al., 2006); and finally, dietary choline deficiency alters global pattern of DNA methylation in the developing mouse hippocampus (Niculescu et al., 2006). This growing number of cases demonstrates how chromatin regulation is now becoming the focus of attention for a myriad of different phenomena, ranging from stem cell differentiation and reprogramming to mammalian stress responses and dietary habits. The next few years will no doubt vastly expand our knowledge on this exciting and stimulating field.

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