

et al., 2004). Induction of the *Hes1* gene is particularly interesting because HES1 can act as a safeguard against irreversible cell cycle exit during quiescence, thereby possibly preventing premature senescence and inappropriate differentiation of AH-NSCs (Sang et al., 2008). As opposed to the BMP treatment, the addition of the antagonist Noggin to neurosphere cultures increased the number of spheres, their diameter, and the percentage of cells per sphere that were in the cell cycle, indicating that AH-NSCs secrete BMPs, which may act to regulate proliferation in an autocrine manner.

A relevant aspect of our *in vivo* results is that although blockade of BMP signaling in the hippocampus first stimulates proliferation, it leads subsequently to the loss of the regenerative capacity of the radial stem cell population. The early impact of Noggin infusion is an increase in proliferation, but the consequence after 3 weeks is a reduction in proliferation and a depletion of newborn neurons (which were also detected in *Smad4*- and *Bmpr1a*-deleted animals). Importantly, by consecutively labeling cells with CldU and IdU, we observed that CldU<sup>+</sup>IdU<sup>+</sup> double-positive cells in the SGZ were diminished 4 weeks after the onset of Noggin infusion, pointing to a restriction in the proliferative activity of the LRCs that were challenged to divide earlier. This finding differs from a recent report showing that continuous Noggin release from neuronal cells increases the number of slowly dividing LRCs (Bonaguidi et al., 2008). However, in that study, the interval between the administration of the two thymidine analogs was shorter, perhaps explaining their different results. Alternatively, it is possible that acute delivery of Noggin by pump infusion may have caused a response that differs from the effect triggered by a continuous Noggin release. Interestingly, we also observed that 4 weeks after blocking the BMP pathway, the number of nonradial SOX2<sup>+</sup> cells was reduced, whereas the number of SOX2<sup>+</sup> GFAP<sup>+</sup> radial cells remained constant. This observation is compatible with a model in which the production of nonradial cells by adult hippocampal radial NSCs *in vivo* becomes compromised after a certain number of mitotic rounds, resembling the behavior of NSCs in the aged hippocampus (Hattiangady and Shetty, 2008; Aizawa et al., 2009). In conclusion, our interpretation of the data presented herein is that a prolonged blockade of the BMP pathway can lead to a premature loss in the ability of activated hippocampal stem cells to proliferate and generate progeny *in vivo*. However, at later time points, feedback mechanisms elicited by the decline in stem cell activity could recruit a backup of dormant NSCs to replenish the niche.

Together, our findings reveal an aspect of BMP signaling in adult NSC niches, as a master regulator of quiescence, and may provide a key to understanding the maintenance of neurogenesis in the adult hippocampus. At the same time, our studies raise several questions regarding BMP function. First, it is tempting to speculate that the effects of diverse neurogenic external stimuli, such as learning, voluntary exercise, enriched

environment, or treatment with antidepressant drugs (reviewed by Ming and Song, 2005; Zhao et al., 2008), may converge mechanistically on an increase in inhibitory factors that block BMP signaling. In support of this view, it has been reported that running increases *Noggin* expression and decreases *Bmp4* expression in hippocampal tissue, and the resulting blockade of BMP signaling is crucial for the effects of exercise on neurogenesis and cognition in mice (Gobeske et al., 2009). In contrast, it has been shown that decreased neurogenesis in a transgenic mouse model of Alzheimer's disease is accompanied by increased expression of *Bmp4* and decreased expression of *Noggin* (Tang et al., 2009), pointing to a broad role for the niche's balance of BMP antagonists/ligands in controlling adult NSCs. However, the identification of the cellular source of BMP ligands and antagonists *in vivo* needs to be investigated further. Second, as shown in our *in vitro* studies, BMPs counteract mitogenic signals such as FGF2; thus, understanding the interplay between the BMP pathway and other signaling pathways may be crucial to unraveling how adult NSC division is balanced. Among the endogenous molecules that are expressed in the dentate gyrus of rodents and that have been shown to regulate hippocampal NSCs, Shh (Favaro et al., 2009) and Wnt3 (Lie et al., 2005) may be relevant (Figure S7). Given the antagonistic function of BMP and Shh in mediating the development of some central nervous system structures, and given the role of the fine-tuned activity of the BMP and Wnt pathways in the switch from stem cell quiescence to active proliferation in several adult organs (reviewed by Li and Clevers, 2010), future attempts should be made to understand the function of BMP signaling in a context-dependent manner.

## EXPERIMENTAL PROCEDURES

### Cell Culture

For proliferation and electroporation assays, AH-NSCs were grown as described in Supplemental Experimental Procedures. Recombinant BMP2/BMP4 (R&D Systems) and Noggin (Sigma) were added at the indicated concentrations.

### Immunostaining

Tissue and cultured cells were fixed with 4% paraformaldehyde and processed for immunostaining. Primary and secondary antibodies employed are listed in Supplemental Experimental Procedures. Stained specimens were analyzed with either a Bio-Rad Radiance confocal imaging system (Hercules, CA), a Leica TCS-SL spectral confocal (Heidelberg, Germany), or a Nikon Eclipse 800 microscope (Nikon, Melville, NY).

### Flow Cytometry

Analysis was performed on Hoechst 33342 (Sigma) and Pyronin Y (Sigma) stained cells with a FACSVantage cytometer (Becton Dickinson), as described in Supplemental Experimental Procedures. Oregon Green 488 carboxylic acid diacetate succinimidyl ester (DFFDA, Molecular Probes) was used for staining neurospheres. DFFDA<sup>high</sup> cells were sorted in FACSAria (Becton Dickinson) equipment.

(G) Diagram illustrating the experimental design employed for the selective deletion of *Smad4* in GLAST-expressing adult radial stem cells. BrdU was injected 10 days after the administration of tamoxifen. Animals were either sacrificed immediately after or 3 weeks later.

(H) Soon after tamoxifen treatment, proliferation in the SGZ of *Smad4* conditional knockouts was increased.

(I and J) Three weeks later, the number of nonradial precursors diminished but the number of radial NSCs remained unchanged. The confocal image shows the maximal projection of a 17-micron stack. GFAP staining is shown in red, SOX2 is shown in green, and DAPI is shown in blue. Unpaired Student's *t* test: \**p* < 0.05. The scale bar represents 40 μm.

**Intracerebroventricular Infusion**

Infusion was performed using Alzet osmotic mini-pumps (see Supplemental Experimental Procedures for additional details). In one experimental paradigm, animals were injected intraperitoneally with 50 mg BrdU/kg of body weight (seven times, once every 2 hours) on the last day of infusion and were sacrificed 1 hr after the last injection. In a second experimental paradigm, CldU (42.5 mg/kg) was injected on the last day of infusion (three times every 2 hr). Next, the catheter connecting the pump to the cannula was sealed. After 3 additional weeks, animals received IdU (57.5 mg/kg) over 3 days (six times every 12 hr) and were sacrificed 12 hr after the last injection. Handling of mice was carried out in accordance with 86/609/EEC guidelines. The animal procedures were performed in accordance with protocols approved by the Instituto de Salud Carlos III animal care and use committee.

**Lentiviral Vectors and In Vivo Injections**

Lentiviral vectors expressed either GFP or GFP/Cre fusion protein under the control of *Sox2* promoter. A total of 1.5  $\mu$ l of the viral concentrate was stereotactically injected into the right hippocampal dentate gyrus (see Supplemental Experimental Procedures for additional details). Ten days post-surgery, animals were injected intraperitoneally with BrdU four times at 2 hr intervals and were sacrificed two hours after the last injection. A separate group of animals was injected with BrdU four weeks post-surgery, once daily for three consecutive days, at a dose of 100 mg/kg body weight. Animals were perfused 24 hr after the final BrdU injection. All animal procedures were performed according to protocols approved by The Salk Institute for Biological Studies animal care and use committee.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.stem.2010.04.016.

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# Chromatin remodeling in neural stem cell differentiation

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Chromatin remodeling is a dynamic alteration of chromatin structure that regulates several important biological processes. It is brought about by enzymatic activities that catalyze covalent modifications of histone tail or movement of nucleosomes along the DNA, and these activities often require multisubunit protein complexes for its proper functions. In concert with DNA methylation and noncoding RNA-mediated processes, histone modification such as acetylation and methylation regulates gene expression epigenetically, without affecting DNA sequence. Recent advances have revealed that this intrinsic regulation, together with protein complexes such as RE1 silencer of transcription/neuron-restrictive silencer factor (REST/NRSF) and switch/sucrose nonfermentable (SWI/SNF), play critical roles in neural stem cell fate determination.

## Addresses

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## Introduction

Differentiated cells in the nervous system are generated from common neural stem cells (NSCs) which possess the ability to self-renew and to give rise to the three major cell types: neurons, astrocytes, and oligodendrocytes. During development, the fate of NSCs is orchestrated both by intrinsic programs operating within the cells and by cues arriving from outside the cells. Epigenetic mechanisms, such as DNA methylation, histone modification, and noncoding RNA-mediated processes, are examples of these intrinsic programs, which can generate variable patterns of gene expression from an invariant regulatory DNA sequence. Meanwhile, extrinsic molecules such as cytokines and growth factors can bind to their cognate receptors on the cell surface and transduce their signals into the cell. These signals are eventually integrated into

existing transcription factor (TF) regulatory networks that control gene expression. Recent advances have revealed that, in order to progress to a subsequent stage of development, cells along the neural lineage can use components of both intrinsic and extrinsic environments to alter their chromatin structure, thereby either enhancing or repressing gene expression. This regulated alteration of chromatin structure is called chromatin remodeling. Here we discuss new insights into the mechanisms that underlie chromatin remodeling, and its significance in determining NSC fate.

## Histone modification

Chromatin structure can be dynamically changed by covalent modifications that take place on histones. These modifications display a level of diversity and complexity whose combinatorial existence is read out as a ‘code’ for accessible/active chromatin (euchromatin) or condensed/inactive chromatin (heterochromatin) states [1]. In particular, two of the core histones, H3 and H4, have long amino-terminal tails that protrude from the nucleosome and are subject to an array of post-translational modifications: methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, glycosylation, biotinylation, carbonylation, and ADP-ribosylation [2]. Of these modifications, lysine (K) acetylation and methylation are the best understood. Histone acetyl transferases (HATs) and deacetylases (HDACs) are the two groups of enzymes that govern the acetylation status of lysine residues in histone tails (Table 1). In general, an increase of histone acetylation by HATs induces the remodeling of chromatin from a tightly to a loosely packed configuration, which facilitates transcriptional activation. Conversely, a decrease of histone acetylation by HDACs yields a condensed chromatin structure and thus transcriptional silencing (Figure 1). Patterns of histone methylation are less straightforward, because methylation can result in either activation or repression of gene transcription depending on the residue on which it occurs. For example, histone H3 lysine 4 (H3K4) methylation is a well-known marker for transcriptionally active chromatin, while methylated H3K9 and H3K27 mark transcriptionally inactive chromatin (Figure 1). Methylation and demethylation at lysine residues are carried out by histone methyl transferases (KMTs) and histone demethylases (KDMs), respectively.

More than a dozen HDACs have been characterized to date, and they can be classified into at least three major classes. In particular, HDAC1 and HDAC2, belonging to the class I group, are clearly implicated in NSC differentiation. NSCs and their lineage-committed progenitor cells express high levels of HDAC1, while only some of

Table 1

## Chromatin remodeling factors and complexes with their roles in NSC differentiation

Factor/complex	Subunit	Catalytic activity	Role in NSC	Reference
HATs (e.g. p300/CBP)		Add acetyl groups to histone tails	Promote neurogenesis	[50]
HDACs		Remove acetyl groups from histone tail		
HDAC1			Inhibits neurogenesis (?), reduce global acetylation during gliogenesis (?)	[3-5]
HDAC2			Inhibits gliogenesis	[6]
KMTs		Add methyl groups to lysine residue of histone H3		
G9a		H3K9	Represses neuronal gene expression when recruited by REST/NRSF	[30]
SUV39H1		H3K9	Same as above	[30]
KDMs		Remove methyl groups from lysine residue of histone H3		
Jmjd3		H3K27	Promotes activation of NSC marker genes in ESCs	[18]
LSD1		H3K4	Represses neuronal genes expression when recruited by REST/NRSF	[41]
TrxG		Adds methyl groups to lysine residue of histone for gene activation		
MLL	Mll1	H3K4	Promotes adult neurogenesis	[19**]
PcG		Modifies lysine residue of histone for gene repression		
PRC1	Ring1B	Adds ubiquitin to histone H2A	Promotes switch from neurogenesis to gliogenesis at late gestation	[21*]
PRC2	Ezh2 Eed	Adds methyl groups to H3K27 Required for the function of Ezh2	Same as above Same as above	[21*] [21*]
REST/NRSF		Represses neuronal genes expression in non-neuronal cell types	Inhibits neurogenesis	[31,32,36]
SWI/SNF		ATP-dependent reposition of nucleosomes		
BAF	Brm BAF60 Brg1		Promotes neurogenesis Same as above Inhibits neurogenesis	[46] [46] [48,49]

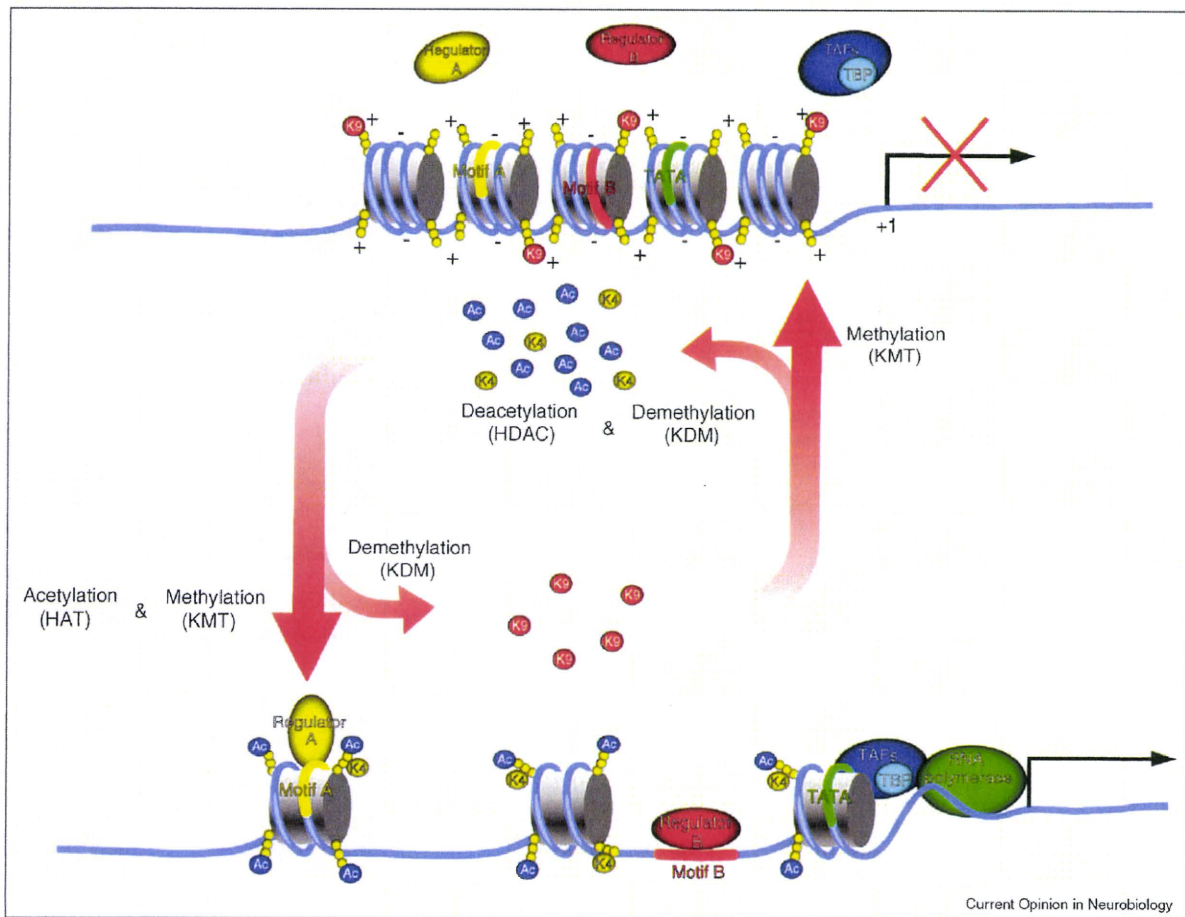
Note: '(?)' needs further confirmation.

them express low levels of HDAC2 [3] (Figure 2). Interestingly, as NSCs commit to the neuronal lineage, expression of HDAC2 is upregulated but that of HDAC1 is downregulated to the extent that in most postmitotic neurons it becomes undetectable, except only in some types of neurons [3,4]. On the other hand, HDAC1 expression is sustained in the majority of glial lineage cells (astrocytes and oligodendrocytes), in which HDAC2 is not detected [3,5] (Figure 2). Moreover, HDAC2, but not HDAC1, was found to inhibit astrocytic differentiation [6]. Intriguingly, despite the abundance of HDAC1 in NSCs, the level of histone acetylation in these cells is actually higher than that in their differentiated progeny [7].

The effect of HDAC1 on cell proliferation probably differs among brain regions where NSCs reside, as HDAC

inhibition using valproic acid has been reported both to inhibit [7,8] and to stimulate NSC proliferation [9]. HDAC1 may begin to deacetylate histones, and thus to compact chromatin structure, at the onset of gliogenesis, as mature glial cells eventually display a very low level of global histone acetylation [7]. It has been shown that progression in the oligodendrocyte lineage depends on HDAC activity [10]. Moreover, a recent study revealed that HDAC1/2 contributes to this progression by disrupting the activator complex formation of  $\beta$ -catenin and TCF at inhibitor of differentiation genes *Id2* and *Id4*, thereby preventing the synthesis of Id2/4 proteins, which inhibit myelin gene expression [11\*\*]. Treatment with HDAC inhibitors can stall oligodendrocytic differentiation and cause hypomyelination in the corpus callosum of postnatal rats [5]. In this relation, it has been shown that HDAC inhibitors induce neuronal differentiation at the

Figure 1



Dynamic histone tail modifications convert chromatin structure from a repressive to an active state to regulate gene expression. Histone acetylation and H3K4 methylation allow relaxation of chromatin and enable RNA polymerase and specific regulators to access DNA and initiate transcription. Histone deacetylation and H3K9 methylation contribute to the formation of repressive chromatin. Ac, acetyl group; K4 and K9, lysine methyl group; HAT, histone acetyl transferase; HDAC, histone deacetylase; KMT, lysine methyl transferase; KDM, lysine demethylase; TBP, TATA-binding protein; TAF, TBP-associated factor.

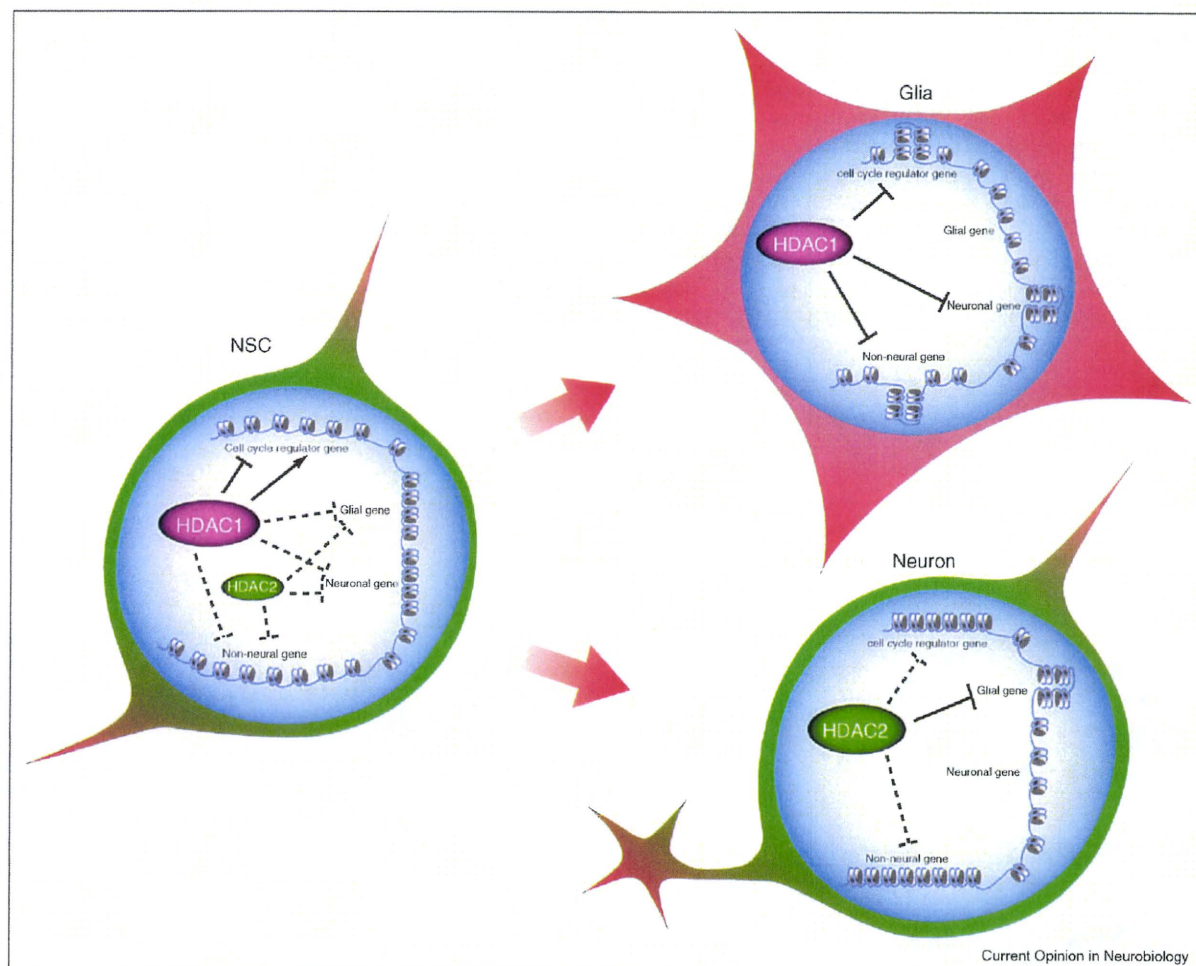
expense of glial differentiation in *in vitro* conditions that favor glia-specific differentiation [7]. Further, this HDAC inhibition upregulated the neuron-specific gene *NeuroD*, a neurogenic basic helix-loop-helix TF [7], and to activate the Ras-ERK pathway [12], resulting in the induction and suppression of neuronal and glial differentiation, respectively.

Under optimal culture conditions, pluripotent embryonic stem cells (ESCs) can be promoted to differentiate into NSCs, and subsequently into neurons. Histone acetylation is, at least partially, involved in the progression of ESCs to NSCs and neurons. ESCs appear to have a higher global level of histone acetylation than lineage-restricted stem cells and differentiated cells, which is consistent

with their higher levels of transcription and more open chromatin configuration [13]. For example, heterochromatin markers such as HP1 are highly dynamic and dispersed in the nuclei of ESCs, and then become more concentrated at specific loci as differentiation proceeds [14,15].

Differentiation-associated genes in ESCs are not completely silenced but are kept in a poised state by both activating and repressive histone methylation, ready for rapid activation during differentiation. Members of the Trithorax group (TrxG) and polycomb group (PcG) proteins have been shown to possess specific KMT activity which methylates H3K4 and H3K27, respectively (Table 1). In ESCs, while pluripotency-associated genes

Figure 2



A model of HDAC expression and function during NSC differentiation. In NSCs, HDAC1 (purple oval) is more abundant than HDAC2 (green oval) and probably functions mainly as a regulator of the cell cycle. During glial differentiation, global histone acetylation, and the amount of HDAC2 are reduced dramatically. In contrast, only a slight decrease of global histone acetylation occurs during neuronal differentiation with a persistent abundance of HDAC2. HDAC1 may contribute to global histone deacetylation in glial cells, while HDAC2 may have a specific function to repress glial genes in neurons. Solid and broken lines represent strong and moderate regulation of histone acetylation, respectively.

are marked by activating H3K4 trimethylation (H3K4me3), genes required for NSC differentiation are marked by both activating H3K4me3 and repressive H3K27me3 [16,17]. During the progression of ESCs to NSCs, this bivalent histone methylation in the promoters of NSC marker genes, such as *Nestin*, is then resolved by Jmjd3, a specific H3K27 KDM [18] (Table 1). Meanwhile, neuronal and glial genes retain their bivalent state and ESC pluripotency-associated genes are marked only by repressive H3K9 methylation. This histone methylation dynamics is also essential as NSCs differentiate into terminal progeny: the TrxG member mixed-lineage leukemia 1 (Mll1), for example, is required to remodel

chromatin during neurogenesis and its deficiency leads to a glial lineage preference [19\*\*] (Table 1). *Dlx2*, a key downstream regulator of neurogenesis, is not expressed in Mll1-deficient NSCs due to a change in histone methylation in the promoter from a single high level of H3K4me3 to a bivalent poised state marked by both H3K4me3 and H3K27me3 [19\*\*].

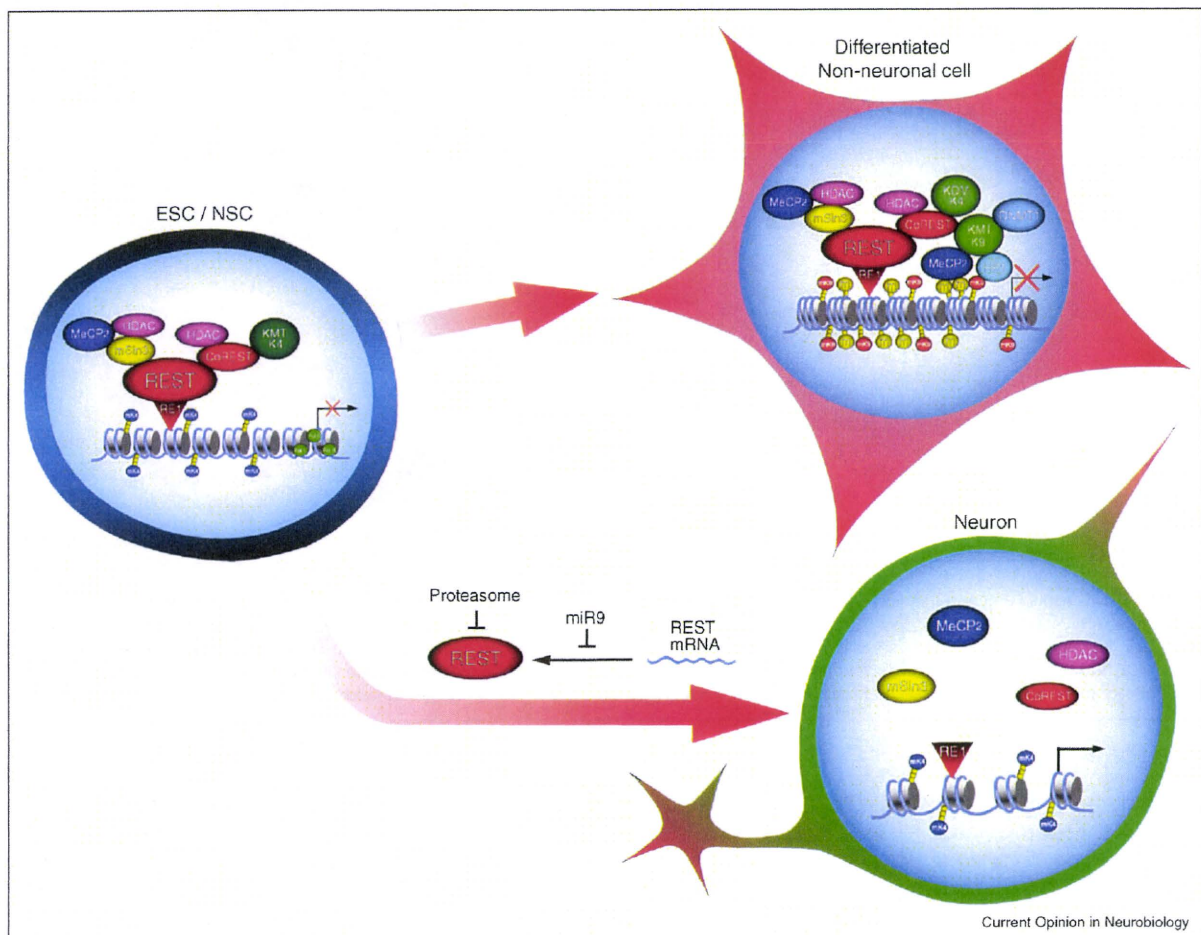
The switch of NSC fate from neurogenic to astroglial, which occurs in late gestation, is also dependent on histone methylation. Proneuronal basic helix-loop-helix (bHLH) genes such as *Neurog1*, are expressed during the neurogenic but not in the astroglial period of

neocortical development [20]. Recently, *Neurog1* has been shown to be trimethylated at H3K27 by PcG proteins in late-gestational NSCs and this suppression is attributable to both polycomb repressive complexes, PRC1 and PRC2 [21\*] (Table 1). This repressive methylation is important for NSCs to acquire astrocytic competency, because *Neurog1* expression can suppress astrocytic gene expression, in part by sequestering the p300/CBP-Smads activator complex to prevent it from binding to STAT3, a prominent TF for astrocytic differentiation [20,22–24]. It is thus conceivable that the dynamics of histone methylation and the bivalent state generated by TrxG and PcG proteins are common

mechanisms for the maintenance of differentiation potential in many stem/progenitor cells [25].

Several extrinsic factors affect chromatin structure by modifying histone tails in NSCs. For example, it has been suggested that fibroblast growth factor 2 (FGF2) signaling is directly responsible for the acquisition of glial competency during NSC culture by increasing H3K4 and reducing H3K9 methylation around the STAT3-binding sites of the astrocyte-specific *gfap* promoter [26]. Since FGF2 is a common factor used for maintaining NSCs *in vitro*, protocols that include FGF2 should be used with caution as the cells may acquire astrocytic potential over

Figure 3



Chromatin remodeling of neuronal genes by the REST/NRSF complex. In stem cells and differentiated non-neuronal cells, chromatin is relatively compact and neuronal genes are actively repressed by REST/NRSF (REST, red oval) and its corepressors. However, the presence of KMT K4, which generates methylated histone H3 lysine 4 (mK4, blue circles), and stalled RNA polymerase II (Pol II, light green circles), renders stem cell chromatin permissive for very low basal transcription of neuronal genes (thin red cross). In contrast, neuronal genes are not expressed (thick red cross) in differentiated non-neuronal cells because the DNA around the RE1 sequence is methylated (m, yellow circles), which facilitates the binding of MeCP2 and other corepressors including KDM K4, which demethylates mK4, and KMT K9, which methylates histone H3 lysine 9 (mK9, red circles). During neuronal differentiation, REST/NRSF is removed by proteasomal degradation and miR-9-mediated transcriptional repression. REST/NRSF corepressors also dissociate from RE1, resulting in a more loosely packaged chromatin and the expression of neuronal genes in mature neurons.



time in culture. Bone morphogenetic protein 2 signaling also changes chromatin structure in midgestational NSCs by increasing histone acetylation around the *S100 $\beta$*  promoter [27]. Another recent study has shown that activation of the retinoic acid (RA) signaling pathway can induce histone H3 acetylation around the STAT3-binding site in the *gfap* promoter, thus allowing STAT3 to gain efficient access to the promoter and to induce astrocyte differentiation, probably through the relaxation of chromatin caused by this acetylation [28\*].

### Role of REST/NRSF and SWI/SNF

The recognition that multisubunit protein complexes are recruited to numerous genes during regulation of their expression has sparked intense efforts to characterize the complexes and their specific functions. Interestingly, although some of these proteins do not bind directly to DNA, they possess chromatin structure-modifying capacities that can lead to either permission or restriction of its target gene's transcription.

In ESCs and non-neuronal cells, neuronal genes are repressed by the binding of RE1 silencing transcription factor/neuron restricted silencing factor (REST/NRSF) to its DNA response element (RE1) and the subsequent formation of a repressor complex by the recruitment of HDAC1/2 and Sin3A at the amino-terminal repressor domain of REST/NRSF [29–32] (Table 1). Another protein, CoREST, interacts directly with the carboxy-terminal repressor domain of REST/NRSF [33,34] and recruits HDACs for more stable repression [34,35]. Nevertheless, neuronal gene chromatin in ESCs and NSCs is poised, ready for expression, by the presence of H3K4 KMT and RNA polymerase II (Pol II), that permits a very low level of basal transcription [32] (Figure 3). During neuronal differentiation, the key switch for releasing neuronal genes from this poised state into an active one is the disappearance of REST/NRSF, most likely by proteasomal degradation, leading to the removal of REST/NRSF-associating transcriptional repressors from the genes [32,36] (Figure 3).

A neuron-specific microRNA, miR-9, has been shown to target *REST/NRSF* mRNA [37\*\*] (Figure 3). Indeed, overexpression of miR-9 promotes neuronal differentiation, while its downregulation has the opposite effect [38]. Perturbation of HDAC activity may also contribute to the alleviation of the poised state, as HDAC inhibition results in an enhancement of neuronal differentiation of NSCs by upregulation of the proneuronal *NeuroD* gene, whose promoter contains an RE1 site [7,39]. In contrast, the REST–CoREST complex in differentiated non-neuronal cells can recruit several chromatin modifiers, such as G9a and SUV39H1 (H3K9 KMTs), MeCP2 (a methyl DNA-binding protein), HP1 (a heterochromatin protein), and LSD1 (an H3K4 KDM), for further compaction of chromatin

during differentiation [30,40,41] (Figure 3). The complex composition is different among cell types, probably due to the methylation of RE1 and its surrounding region in the promoters of neuronal genes in differentiated non-neuronal cells [32]. This methylated DNA provides a binding site for MeCP2, which further recruits other transcriptional repressors (Figure 3).

Several proteins belonging to the SWI/SNF complex possess the ability to remodel chromatin by an ATP-dependent mechanism that utilizes energy to reposition nucleosomes. Recent studies have revealed that BAF complexes containing Brg1 or Brm ATPases play crucial roles in neural development by means of combinatorial assembly and subunit switching (reviewed in [42\*\*]). Brg1 is consistently expressed throughout differentiation, whereas Brm expression is very low in NSCs and only rises during differentiation into neurons and astrocytes [43,44] (Table 1). Brg1 associates with two proneuronal bHLH proteins, Neurogenin-related1 and NeuroD, and is essential for their function in neurogenesis [45]. Meanwhile, Brm and BAF60 can bind to *Neurog1* gene during neuronal differentiation of RA-treated P19 cells (mouse embryonic carcinoma cell line) [46]. Moreover, expression of *empty spiracles homeobox 2* and *forkhead box g1*, two critical genes in neurogenesis, is also regulated by Brg1 [47]. Brg1 and other BAF complex subunits were also shown to form a larger complex with REST/NRSF and its corepressors, and inhibition of Brg1 activity can increase REST/NRSF target gene expression [48]. Furthermore, Brg1 can enhance deacetylation of histone H4 around RE1, thereby providing another mechanism to repress REST/NRSF target genes [49].

### Conclusions

It is now well established that chromatin remodeling plays a substantial role in the differentiation of NSCs. Combinatorial and cell type specific regulation of subunit composition also emerged as a crucial factors to control targeting and function of chromatin remodeling complexes during neural differentiation. However, our understanding about this relationship is still in its infancy and many interesting avenues remain to be explored. For example, it is unclear why a global increase of histone acetylation, which occurs after treatment with HDAC inhibitors, upregulates only specific genes such as neuronal genes in NSCs. Could this be due to a unique subunit composition of HDAC-containing chromatin remodeling complexes at the promoters of neuronal genes? If so, what are these subunits and what are their specific features? How are different molecules and signaling pathways that affect chromatin structure integrated to promote NSCs differentiation? Given the potential of NSCs to treat neurological diseases and dysfunctions, answers to these and other outstanding questions are eagerly awaited.

## Acknowledgements

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## Review Article

## Epigenetic regulation in neural stem cell differentiation

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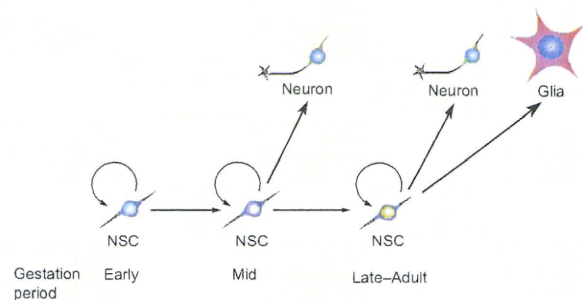
The central nervous system (CNS) is composed of three major cell types – neurons, astrocytes, and oligodendrocytes – which differentiate from common multipotent neural stem cells (NSCs). This differentiation process is regulated spatiotemporally during the course of mammalian development. It is becoming apparent that epigenetic regulation is an important cell-intrinsic program, which can interact with transcription factors and environmental cues to modulate the differentiation of NSCs. This knowledge is important given the potential of NSCs to produce specific CNS cell types that will be beneficial for clinical applications. Here we review recent findings that address molecular mechanisms of epigenetic and transcription factor-mediated regulation that specify NSC fate during CNS development, with a particular focus on the developing mammalian forebrain.

**Key words:** cell differentiation, epigenetics, neural stem cells.

## Introduction

The mammalian central nervous system (CNS) consists of the brain and spinal cord; organs that have a highly complex structure. These organs develop from common multipotent neural stem cells (NSCs) that line the neural tube. NSCs have the capacity to self-renew and to differentiate into distinct cell types such as neurons, astrocytes and oligodendrocytes. During the differentiation process, some NSCs can also produce immediate progeny that are known as neural progenitor cells (NPCs). NPCs have a more limited self-renewal capacity, and behave as transit amplifying cells that expand the number of newly differentiated cells owing to their higher rate proliferation than the more quiescent stem cells (Kempermann 2006). Progression along the lineage from NSCs to its progenies is characterized by striking morphological and functional changes at each stage of lineage commitment. During brain development, NSCs in the ventricular zone (VZ) divide symmetrically in early gestation to increase their own numbers (Fujita 1963, 1986, 2003; Fig. 1). These cells then undergo neurogenesis through a mostly asym-

metric division, giving rise to two distinct daughter cells: another NSC and a neuron (Noctor *et al.* 2001, 2004). As gestation proceeds, some NSCs can acquire the ability to divide asymmetrically to produce another NSC and an NPC, which then resides in the



**Fig. 1.** Temporal development of neural stem cells (NSCs) exemplified by the ventricular zone of the cerebral cortex. Early-gestational NSCs divide symmetrically to increase their own number. These NSCs then undergo neurogenesis at midgestation, through mostly asymmetric divisions, each cell giving rise to two distinct daughter cells, another NSC and a neuron. As gestation proceeds, some NSCs can acquire the ability to divide asymmetrically to produce another NSC and a neural progenitor cell (NPC) (not depicted). Almost all of these NPCs will later divide symmetrically to produce two neurons. Towards the end of the gestational period, and perinatally, residual NSCs eventually acquire the multipotentiality to generate glia (astrocytes and oligodendrocytes) in addition to neurons.

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subventricular zone (SVZ) and almost all of these NPCs will subsequently divide symmetrically to produce two neurons (Noctor *et al.* 2004). Towards the end of the neurogenic phase, residual NSCs eventually acquire the multipotentiality to generate astrocytes and oligodendrocytes in addition to neurons (Qian *et al.* 2000; Fig. 1). Although the mechanisms of NSC fate determination are not yet fully understood, it is gradually becoming apparent that cell-intrinsic programs such as epigenetic regulation, together with transcription factors (TFs) and extracellular cues, are deeply involved in this fate specification of NSCs.

The term "epigenetic" refers to any heritable influence (in the progeny of cells or individuals) on chromosome or gene function that is not accompanied by a change in DNA sequence (Yoder *et al.* 1997). It includes processes such as DNA methylation, histone modification and noncoding RNA expression. Appropriate gene activation and/or silencing at each step of NSC progression is achieved by such epigenetic regulation. In this review, we survey recent reports investigating the role of epigenetic mechanisms in the determination of NSC fate in the mammalian CNS, with a special emphasis on the developing forebrain.

### DNA methylation

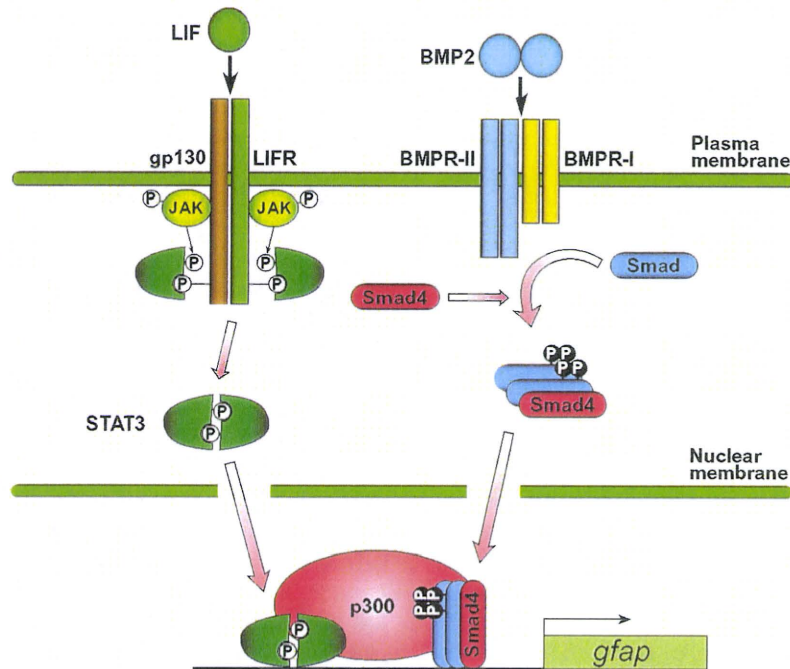
One of the major epigenetic mechanisms in vertebrates, which regulates a diverse array of cellular events including developmental gene regulation, X chromosome inactivation, genome defense and genomic imprinting, is cytosine methylation of genomic DNA at CpG dinucleotides (Jaenisch & Bird 2003). The DNA methylation pattern in the genome is established by a family of DNA methyltransferases (DNMTs). Maintenance of methylation patterns is achieved by a function of DNMT1 during DNA replication, while *de novo* methylation is primarily catalyzed by DNMT3a and DNMT3b. The DNMT family is essential for embryogenesis, as either single disruption of the *DNMT1* gene or compound disruption of *DNMT3a* and *DNMT3b* genes in mice led to drastic demethylation in the genome and the mice died at midgestation (Goto *et al.* 1994; Okano *et al.* 1999; Robertson & Wolffe 2000).

Regulation of gene expression by DNA methylation can be achieved by two mechanisms. First, methylation of CpG dinucleotides affects DNA structure and can directly interfere with the binding of TFs to their target sequences (Takizawa *et al.* 2001); second, a more pervasive effect, methyl-CpG-binding domain (MBD)-containing protein family members can bind to genes with methylated CpG dinucleotides, thereby suppressing the genes' expression (Lewis *et al.* 1992; Cross *et al.* 1997; Nan *et al.* 1997).

The acquisition of multipotentiality in NSCs during development is tightly regulated by DNA methylation. As described above, NSCs at early gestation can only self-renew and then differentiate exclusively into neurons during midgestation (Fig. 1). Gradually, NSCs begin to differentiate into glia (astrocyte and oligodendrocyte) at late gestation (Fujita 1986, 2003; Miller 1996; Qian *et al.* 2000; Temple 2001; Fig. 1). Two well studied pathways that act synergistically to promote astrocytic differentiation are those activated by the interleukin-6 (IL-6) family of cytokines (such as leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and cardiotrophin-1 (CT-1)) and bone morphogenetic protein (BMP) signaling (Bonni *et al.* 1997; Rajan & McKay 1998; Nakashima *et al.* 1999a,b; Sun *et al.* 2001; Barnabe-Heider *et al.* 2005; Fig. 2). LIF, CNTF and CT-1 can activate the Janus kinase (JAK)-signal transducer and activator of the transcription (STAT) pathway upon binding to their cognate receptors (Stahl & Yancopoulos 1994), while BMPs activate the downstream TF SMAD through their serine/threonine kinase type cognate receptors (Massague 2000). Synergistic activation of astrocytic genes is achieved by the formation of a complex involving activated STAT3 and SMADs bridged by the transcriptional coactivator p300/CBP (Nakashima *et al.* 1999b; Fig. 2). It has been suggested that, in early- and midgestational NSCs, astrocytic gene promoters such as glial fibrillary acidic protein (*gfap*) are hypermethylated, a status that impedes binding of the STAT3-p300/CBP-SMADs complex to its target sequence and thus prevents these NSCs from differentiating into astrocytes even when the cells are stimulated by astrocyte-inducing cytokines (Takizawa *et al.* 2001; Fig. 3a).

Mouse embryonic stem cells (mESCs) also do not express GFAP and remain in the undifferentiated state, even in the presence of LIF in its culture medium. Like NSCs at early- and midgestation, the *gfap* promoter is hypermethylated in mESCs (Shimozaki *et al.* 2005). Hypermethylation is maintained even after mESCs differentiate into endodermal and mesodermal lineages, and demethylation in this region only occurs when the cells are committed to a neural lineage that is capable of producing astrocytes (Shimozaki *et al.* 2005). *Gfap* promoter hypermethylation is also observed in adult tissues outside the nervous system, such as liver, heart and femoral muscle (Takizawa *et al.* 2001). In contrast, the STAT3 binding site-containing *gfap* promoter in NSCs at late gestation is barely methylated, so that upon LIF stimulation these NSCs can differentiate into astrocytes (Takizawa *et al.* 2001; Fig. 3a).

DNA demethylation as gestation proceeds is not exclusive to the *gfap* promoter, but is common for astrocyte-specific genes. For example, the earlier

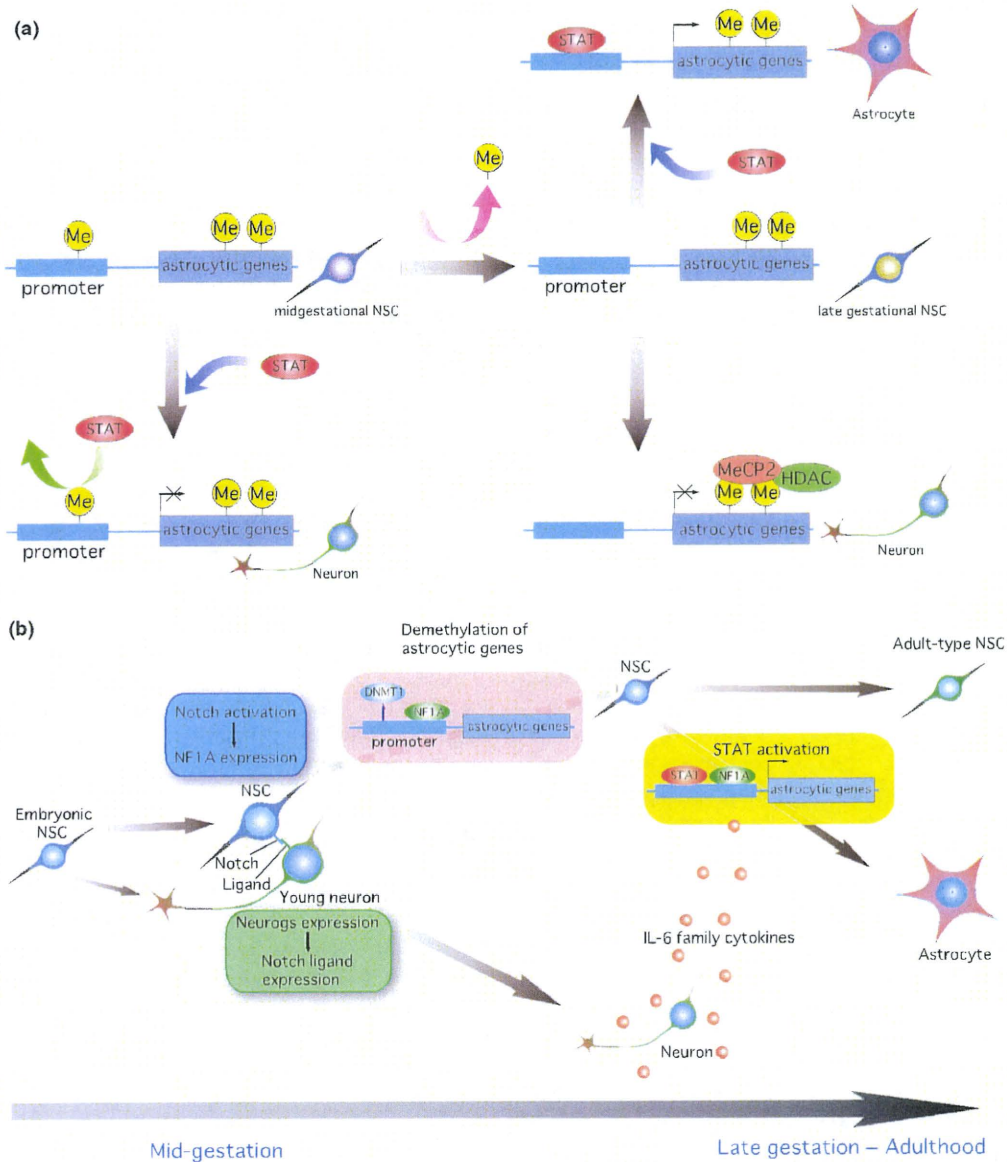


**Fig. 2.** Signaling crosstalk between two distinct cytokines to activate astrocytic genes. Leukaemia inhibitory factor (LIF), a member of the interleukin-6 (IL-6) cytokine family, binds to its specific receptor, LIFR, which dimerizes with a common signal transducer, gp130; this dimerization leads to activation of the JAK-STAT pathway. Activated STAT3 forms a homodimer and subsequently translocates into the nucleus. BMP2, a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, signals through a heterotetrameric serine/threonine kinase receptor complex composed of two type I (BMPR-I) and two type II (BMPR-II) receptor molecules. Activated BMPRs phosphorylate the downstream transcription factors Smad1, -5, and -8, which then bind to the common mediator Smad4 before accumulating in the nucleus. There, STAT3 and Smads form a complex bridged by the transcriptional coactivator p300/CBP to activate the transcription of astrocytic markers such as *gfap*.

astrocytic marker *S100 $\beta$*  also possesses a cytosine residue in its promoter that is highly methylated in mESCs but becomes demethylated as the cells differentiate into NSCs (Shimozaki *et al.* 2005). This demethylation also occurs at midgestation, coincident with the onset of *S100 $\beta$*  expression in the brain (Namihira *et al.* 2004). Furthermore, the genome-wide DNA methylation status of mid- and late gestational NSCs has recently been compared by a microarray profiling method (Hatada *et al.* 2008), confirming that many astrocytic genes become demethylated in late stage NSCs (Fig. 3a). Thus, it is apparent that DNA methylation plays an important role in defining the timing of NSC fate specification switch from neurogenesis to astrocytogenesis.

Although it is now clear that DNA demethylation determines the competency of astrocytic gene expression, the mechanisms underlying this process are not yet fully elucidated. The *gfap* promoter is hypermethylated at all developmental stages and in all lineages except late-gestational NSCs and its progenies. Therefore, demethylation of the *gfap* promoter is temporally

regulated and cell type-specific. The most likely candidate mechanism for DNA demethylation is the passive loss of CpG methylation due to successive rounds of DNA replication in the absence of DNMT1-catalyzed maintenance methylase activity (Bestor 2000). After conditional deletion of *DNMT1* from the neural lineage in mouse, which led to hypomethylation of the genome, neural development was shown to be precociously shifted toward astroglial differentiation (Fan *et al.* 2005). Further, this precocious astrocytic differentiation was attributable not only to demethylation of the STAT3-binding site in the *gfap* promoter, but also to the elevation of overall JAK-STAT signaling activity, due to the rapid demethylation of gene promoters that are involved in the JAK-STAT pathway and consequent upregulation of the genes' expression. These data suggest that DNA methylation can regulate the timing and magnitude of astrocytic differentiation, through both direct inhibition of TFs binding to astrocytic genes promoter and modulation of JAK-STAT activity (Fan *et al.* 2005). However, recent studies suggest that active DNA demethylation occurs in



**Fig. 3.** (a) Astrocytic gene methylation status during neural stem cell (NSC) development. Although STAT3 can be activated in midgestational NSCs, it cannot bind to astrocytic gene promoters such as *gfap* due to promoter hypermethylation, so these NSCs can only differentiate into neurons (left). As gestation proceeds, these promoters become demethylated, allowing STAT3 to bind and activate astrocytic genes, resulting in the differentiation of NSCs into astrocytes (upper right). MeCP2 blocks this activation in neurons (lower right). (b) Notch-induced demethylation of astrocytic genes. Activation of Notch signaling in residual NSCs by young neurons induces demethylation of astrocytic gene promoters by upregulation of NF1A and release of DNMT1 from astrocytic gene promoters. In turn, at late gestation, interleukin-6 (IL-6) family cytokines activate the STAT3 pathway and induce NSCs to differentiate into the astrocytic lineage.

vertebrates through the DNA excision repair process, which is mediated by several enzymatic machineries and non-enzymatic Gadd45 proteins (Ma *et al.* 2009).

Dynamic expression of the murine homologues of chicken ovalbumin upstream promoter transcription factors I and II (COUP-TFI/II) may also contribute to

the demethylation of the astrocytic gene promoter. COUP-TFI/II expression is transiently upregulated in the early neurogenic period but decreases markedly before the onset of astrocytogenesis (Naka *et al.* 2008). Using a mESC-derived NSC culture that recapitulates *in vivo* mouse CNS development (Okada

*et al.* 2008), Naka *et al.* (2008) showed that CpG methylation of the *gfap* promoter remained high after COUP-TFI/II composite knockdown and that the switch from neurogenesis to gliogenesis was thereby inhibited. COUP-TFI/II composite knockdown in the developing mouse forebrain also had the same effect. Taken together, these results indicate that COUP-TFI/II are important factors for *gfap* promoter demethylation, although the mechanism is not yet known.

Another contribution to the *gfap* promoter demethylation might come from the progeny of early- and mid-gestational NSCs (Fig. 3b). Several reports have indicated that neuronally committed NPCs and young neurons express Notch ligands (Campos *et al.* 2001; Kawaguchi *et al.* 2008; Yoon *et al.* 2008). Notch signaling is a conserved pathway from insects to mammals, which contributes to cell-to-cell communication (Nye & Kopan 1995; Simpson 1995; Bray 1998; Louvi & Artavanis-Tsakonas 2006) and controls cell fate determination in the CNS (Lundkvist & Lendahl 2001). Upon Notch activation by its ligands, the Notch intracellular domain (NICD) is released from the plasma membrane and translocates into the nucleus, where it converts a particular repressor complex into an activator complex (Wallberg *et al.* 2002; Nakayama *et al.* 2008). Namihira *et al.* (2009) confirmed that Notch ligands are indeed expressed in neuronally committed NPCs and young neurons, and that these ligands activate Notch signaling in the residual NSCs (Fig. 3b). Further, forced expression of NICD in midgestational NSCs induced the upregulation of nuclear factor 1A (NF1A), which in turn accelerated demethylation of astrocytic gene promoters by preventing DNMT1 from binding to them (Fig. 3b) and thus allowed precocious astrocytic differentiation in response to LIF stimulation (Namihira *et al.* 2009).

Members of the MBD family have been also shown to influence NSC differentiation. MBD proteins are expressed predominantly in neurons, and not in astrocytes or oligodendrocytes, in the CNS (Coy *et al.* 1999; Jung *et al.* 2002; Shahbazian *et al.* 2002; Kishi & Macklis 2004). As discussed above, late gestational NSCs have lost *gfap* promoter methylation and become competent to differentiate into astrocytes, in addition to neurons (Figs 1 and 3a). However, it was later found that exon1 of *gfap* are hypermethylated in all neural cell types and that only in neurons, methyl-CpG-binding protein 2 (MeCP2), a member of the MBD family, is highly expressed and binds to this methylated exon1 region (Setoguchi *et al.* 2006; Fig. 3a). Therefore, even if STAT3 binds to the hypomethylated *gfap* promoter in neurons, GFAP expression and thus astrocyte differentiation is blocked by the presence of MeCP2 at exon1 (Setoguchi *et al.*

2006). In neurons, MeCP2 also binds to hypermethylated CpG sites around the transcription start site of *S100 $\beta$* , thereby suppressing its expression and consequently astrocyte differentiation. Although recent studies have shown that MeCP2 expression also occurs in astrocytes, it is at a very low level (Kohyama *et al.* 2008; Ballas *et al.* 2009). Indeed, ectopic expression of MeCP2 directs NSCs to become neurons and inhibits astrocytic differentiation, even in the presence of astrocyte-inducing cytokines such as LIF and BMP2 (Tsujiura *et al.* 2009). Interestingly, such cytokines, if applied simultaneously with ectopic expression of MeCP2, actually induce NSCs to produce more neurons by as yet unknown mechanisms. Ectopic expression of other MBD family members, such as MBD1, also inhibits astrocyte differentiation of NSCs, demonstrating some functional redundancy among MBD proteins (Setoguchi *et al.* 2006). Moreover, MBD1-deficient NSCs generate fewer neurons than do wild-type NSCs, suggesting an important role for MBD1 in neuronal fate specification (Zhao *et al.* 2003). Hypomethylated *gfap* promoter can be also found in oligodendrocytes but, unlike neurons, these cells do not express MeCP2 at high levels, and upon astrocyte-inducing cytokine stimulation can change their fate and become astrocytes (Kohyama *et al.* 2008).

### Histone modification

Histone modification displays high levels of diversity and complexity compared with DNA methylation. Two of the core histones, H3 and H4, have long amino-terminal tails that protrude out of the nucleosome and can be subjected to a variety of posttranslational modifications: methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, glycosylation, biotinylation, carbonylation and adenosine diphosphate (ADP)-ribosylation (Strahl & Allis 2000). Among these, lysine (K) acetylation and methylation are the most studied and best understood histone modifications.

#### Acetylation

Acetylation and deacetylation of lysine residue in histone tails is mediated by histone acetyl transferases (HATs) and histone deacetylases (HDACs), respectively (Hsieh & Gage 2005). In general, an increase of histone acetylation by HATs causes remodeling of chromatin from a tightly to a loosely packed configuration (euchromatin), which subsequently leads to transcriptional activation. Conversely, a decrease of histone acetylation by HDACs results in a condensed chromatin structure (heterochromatin) and finally transcriptional silencing.



Histone acetylation is at least partially involved in the differentiation of mESCs into NSCs and neurons. Mouse ESCs appear to have a higher global level of histone acetylation than lineage-restricted stem cells and differentiated cells, which is consistent with their higher levels of transcription and more open chromatin configuration (Efroni *et al.* 2008). In fact, heterochromatin markers such as HP1 are highly dynamic and dispersed in the nuclei of mESCs, and become more concentrated at specific loci as differentiation proceeds (Meshorer & Misteli 2006; Meshorer *et al.* 2006).

In mESCs and non-neuronal cells, neuron-specific genes are repressed by the binding of neuron restricted silencing factor/RE-1 silencing transcription factor (NRSF/REST) to its DNA response element (RE-1); this binding permits the formation of a repressor complex upon recruitment of HDAC1/2 and Sin3A (Rice & Allis 2001; Lunyak *et al.* 2002; Ballas *et al.* 2005; Lunyak & Rosenfeld 2005). As the cells differentiate into neuronal progenitors and neurons, this HDAC-containing repressor complex dissociates from neuron-specific genes due to the degradation of NRSF/REST (Ballas *et al.* 2005).

Adult hippocampal-derived NSCs differentiate predominantly into neurons, at the expense of astrocytes and oligodendrocytes, when treated by the antiepileptic and HDAC inhibitor valproic acid (VPA) *in vitro*, even in conditions that favor glia-specific differentiation (Hsieh *et al.* 2004). VPA-mediated HDAC inhibition upregulates the neuron-specific gene *NeuroD*, a neurogenic basic helix-loop-helix TF, resulting in the induction and suppression, respectively, of neuronal and glial differentiation. In the developing rat brain and in cultured E14 NSCs, VPA treatment has also been shown to promote neurogenesis by activating the Ras-ERK pathway (Jung *et al.* 2008).

Progression of the oligodendrocyte lineage is also dependent on HDAC activity (Marin-Husstege *et al.* 2002). Postnatal administration of VPA was shown to delay the timing of NSC differentiation into myelin-forming oligodendrocytes in the developing forebrain (Shen *et al.* 2005); significant hypomyelination in the developing corpus callosum, together with sustained expression of progenitor markers and delayed expression of late differentiation markers, were observed in this study. However, HDAC inhibition by VPA after the onset of myelination resulted in comparable myelin gene expression to that seen in VPA-untreated rats, which was attributed to further transitions in nucleosomal histones from a state of reversible deacetylation to a more stably repressed state by histone methylation. It has also recently been shown that HDAC1/2 contributes to the progression of murine oligodendrocyte

differentiation by disrupting the  $\beta$ -catenin-T cell factor (TCF) activator complex at *id2/4*, inhibitor of differentiation genes, thereby preventing the synthesis of *Id2/4* proteins, which can inhibit myelin gene expression (Ye *et al.* 2009).

### Methylation

Histones are methylated and demethylated at lysine residues by histone methyl transferases (KMTs) and histone demethylases (KDMs), respectively. Methylation can result in either activation or repression of gene transcription, depending on which residue is methylated (Yoo & Jones 2006). For example, histone H3 lysine 4 (H3K4) methylation is a well-known marker for transcriptionally active chromatin, whereas methylated H3K9 and H3K27 mark transcriptionally inactive chromatin.

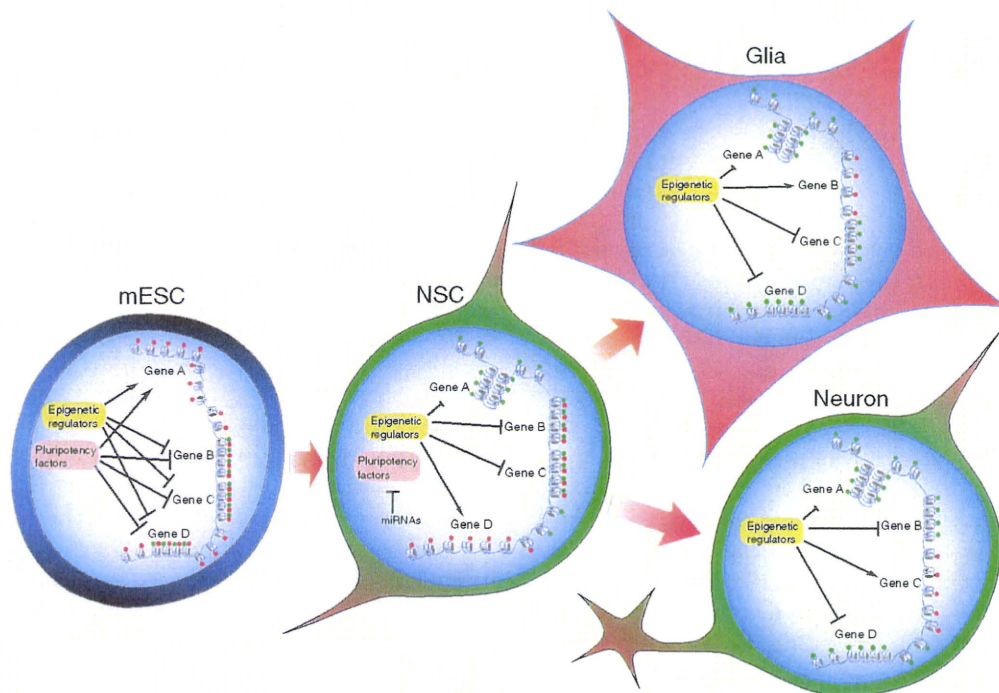
Several extrinsic factors affect the histone methylation status of NSCs. For example, it has been suggested that fibroblast growth factor 2 (FGF2) signaling is directly responsible for the acquisition of glial competency during NSC culture by increasing H3K4 and reducing H3K9 methylation around the STAT3-binding sites of the *gfap* and *S100 $\beta$*  promoters so that, upon CNTF stimulation, NSCs can differentiate into astrocytes (Song & Ghosh 2004). However, FGF2 is a common factor, when used either alone or together with epidermal growth factor (EGF), for maintaining NSCs in culture. Early gestational NSCs are initially responsive only to FGF2, and this signal then primes NSCs to become responsive to EGF later during development or culture *in vitro* (Trophepe *et al.* 1999; Lillien & Raphael 2000; Ciccolini 2001). How FGF2 and/or EGF signaling might influence KMTs and KDMs, resulting in the aforementioned changes in histone methylation, therefore remains an open question.

Mixed-lineage leukemia (MLL), a member of the trithorax group (trxG) gene family, can either specifically methylate H3K4 for gene activation, by recruiting HATs such as MOF and CBP in various cell lines (Ernst *et al.* 2001; Milne *et al.* 2002; Dou *et al.* 2005), or repress target genes by recruiting polycomb group (PcG) proteins, HDACs and/or SUV39H1 (Xia *et al.* 2003). In the postnatal mouse brain, MLL1 is required for neurogenesis and its deficiency in NSCs in the subventricular zone (SVZ) leads to a glial lineage preference (Lim *et al.* 2009). One of the key downstream regulators of SVZ neurogenesis, *Dlx2*, is not expressed in MLL1-deficient NSCs. This is due to a change in histone methylation of *Dlx2*, from a single high level of H3K4 trimethylation (H3K4me3) to a bivalent poised state marked by both activating H3K4me3 and repressive H3K27me3 (Lim *et al.* 2009).

The existence of both activating and repressive histone methylation marks is necessary for the rapid activation of genes during differentiation. In mESCs, while pluripotency-associated genes are marked by active H3K4me3, those that are necessary for differentiation are marked by both active H3K4me3 and repressive H3K27me3 (Azuara *et al.* 2006; Bernstein *et al.* 2006; Fig. 4). It has been shown that a specific KDM, Jmjd3 (an H3K27me3 demethylase), which belongs to the jumonji protein family, can resolve the bivalent state of NSC marker genes promoter such as *nestin*, and thereby lead mESCs to NSC commitment (Burgold *et al.* 2008). Eventually, in mESC derived-NSCs, the ESC pluripotency-associated genes are repressed by H3K9 methylation and the bivalent state exists on neuronal and glial differentiation genes (Fig. 4). Thus, it is conceivable that the bivalent state generated by trxG and PcG proteins is a common mechanism for the

maintenance of differentiation potential in many stem/progenitor cell types (Mikkelsen *et al.* 2007).

PcG proteins have also been shown to participate in NSC differentiation by establishing the H3K27me3 repressive mark at proneuronal basic helix-loop-helix (bHLH) genes, such as Neurogenin1 (*Neurog1*) (Hirabayashi *et al.* 2009). *Neurog1* expression *in vitro* and *in vivo* can suppress astrocytic differentiation of NSCs in part because *Neurog1* sequesters the p300/CBP-Smads complex from STAT3, leading to suppression of STAT3 target genes (Cai *et al.* 2000; Sun *et al.* 2001; Guillemot 2007). Since *Neurog1* is expressed solely during the neurogenic and not the astrocytic period of neocortical development (Sun *et al.* 2001), *neurog1* repression, by means of an H3K27me3 mark applied by PcG proteins in late gestational NSCs, provides a mechanism for a switch in NSC differentiation from neurogenic to astrogenic (Fig. 4).

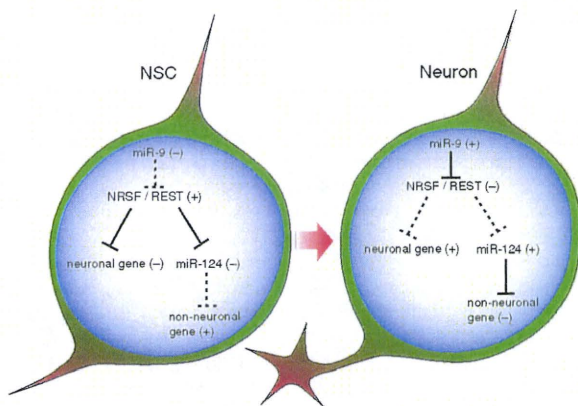


**Fig. 4.** Chromatin dynamics and histone modification status during stem cell development. Chromatin status changes from a highly to a less dynamic form during differentiation of mouse embryonic stem cells (mESCs) into neural stem cells (NSCs) and neural lineage cells. Genes associated with pluripotency (Gene A) are actively transcribed in pluripotent mESCs, while NSC multipotency- (Gene D) and differentiation-associated genes (Genes B, glial differentiation; Gene C, neuronal differentiation) are kept in a silent poised state. Several epigenetic regulators and pluripotency factors regulate this state, in part by a combination of the activating H3K4me3 (red circles) and the repressive H3K27me3 (green circles) methylation marks. In mESCs, only H3K4me3 is present at pluripotency-associated genes, while both H3K4me3 and H3K27me3 mark NSC multipotency- and differentiation-associated genes (bivalent marks). Upon progression to NSCs, miRNAs downregulate pluripotency factors. NSC multipotency genes, such as *nestin*, resolve bivalent marks into the single activating H3K4me3, while differentiation-associated genes retain their bivalent marks. During NSC differentiation to each neural lineage, NSC multipotency genes are kept silenced with H3K27me3, and differential methylation status in genes specific for each lineage determines NSC fate.

## Noncoding RNA

Noncoding RNAs are increasingly recognized as exerting epigenetic effects on gene regulation (Grewal & Moazed 2003; He & Hannon 2004). Among several types of such RNAs, microRNA (miRNA) has been studied intensively in the context of its role in NSC differentiation. miRNA is a 20–25-nucleotide single-stranded RNA that can bind to the 3' untranslated region (UTR) of target mRNAs, by an imperfect sequence match, to repress their translation and stability (Rana 2007) through the formation of a structure called the RNA-induced silencing complex (RISC). As well as the 3' UTR, miRNA may also target the coding region and the 5' UTR of target mRNAs (Lytle *et al.* 2007; Orom *et al.* 2008).

miR-124a is expressed predominantly in neural tissues and has been shown to participate in the *in vitro* differentiation of NSCs into neurons by mediating degradation of non-neuronal gene transcripts (Conaco *et al.* 2006). miR-124a expression is regulated by NRSF/REST, which is expressed only in NSCs and non-neuronal cells including mESCs (Fig. 5). In NSCs, therefore, since expression of the *miR-124a* gene is suppressed by NRSF/REST, the stability of non-neuronal gene transcripts can be increased, thus preventing NSCs from differentiating into neurons. When NRSF/REST is absent, the expression of *miR-124a* and neuronal genes is upregulated, leading to a preference for neuronal lineage differentiation (Fig. 5). miR-124a can also target small carboxy-terminal domain



**Fig. 5.** Regulation of neurogenesis by miRNA and neuron restricted silencing factor/RE-1 silencing transcription factor (NRSF/REST). In neural stem cells (NSCs), NRSF/REST is expressed and inhibits neuronal genes and miR-124 expression, allowing non-neuronal transcripts to persist. When NSCs differentiate into neurons, downregulation of NRSF/REST by miR-9 de-represses neuronal and miR-124 gene loci, resulting in the selective degradation of non-neuronal gene transcripts.

phosphatase 1 (SCP1), which, like NRSF/REST, is an anti-neuronal factor in non-neural tissues and is recruited to RE1-containing gene promoters by NRSF/REST (Yeo *et al.* 2005), thus providing another mechanism to induce neurogenesis (Visvanathan *et al.* 2007). Interestingly, NRSF/REST can also be down-regulated by two other neural tissue-specific miRNAs, miR-9 and miR-9\*, which target NRSF/REST and its cofactor CoREST, respectively (Packer *et al.* 2008; Fig. 5). Moreover, it has been shown that overexpression of both miR-124 and miR-9 promotes neuronal differentiation, while their downregulation has the opposite effect (Conaco *et al.* 2006).

Promotion of neurogenesis by miR-124 and miR-9 might also involve other mechanisms. Both of these miRNAs can stimulate neurogenesis *via* inhibition of STAT3 activation, which is responsible for astrocytogenesis (Krichevsky *et al.* 2006). miR-124 has also been shown to repress *Sox9*, a SRY-box TF that is important for glial cell specification, during the transition of NPCs to neuroblasts in the adult subventricular zone (Cheng *et al.* 2009). miR-124 can also reduce the levels of polypyrimidine tract binding protein 1 (PTBP1), a global repressor of alternative pre-mRNA splicing, leading to a switch from non-neuronal to neuronal alternative splicing patterns (Makeyev *et al.* 2007). In the case of miR-9, several studies have shown that it can form a regulatory loop with *Tlx*, a self-renewal gene in NSCs, that is critical for neurogenesis by each regulating the other's transcript (Shi *et al.* 2004; Zhao *et al.* 2009). miR-9 has also been shown to modulate Cajal-Retzius cell differentiation by targeting transcripts of *Foxg1*, which encodes a winged helix transcriptional repressor, in mouse medial pallium (Shibata *et al.* 2008). How these known mechanisms are coordinated and integrated during neurogenesis is an important topic for future studies.

Other miRNAs, such as miR-128, miR-129 and miR-298, are also expressed exclusively in the neuronal lineage (Lau *et al.* 2008), while miR-23 is restricted to the glial lineage, and miR-26 and miR-29 are more strongly expressed in glia than in neurons (Smirnova *et al.* 2005; Lau *et al.* 2008). Therefore, it is tempting to conclude that modulation of the expression of individual miRNAs may be cellular context-dependent and crucial for cell fate choice.

## Closing remarks

Neural stem cells hold great promise for clinical treatment of neurological diseases and dysfunctions, owing to their ability to self-renew and their potential to generate various neural cell types. We have now established that epigenetic regulation contributes

substantially, along with other mechanisms, to these properties of NSCs. However, our knowledge about the precise mechanisms that control NSC differentiation is still in its infancy and many avenues remain to be explored. We also have to evaluate carefully the various origins of NSCs, and the methods available for their induction, before we can generate specific CNS cell phenotypes that can be used reliably in clinical applications.

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