Epigenetic mechanisms regulating fate specification of neural stem cells

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Neural stem cells (NSCs) possess the ability to self-renew and to differentiate along neuronal and glial lineages. These processes are defined by the dynamic interplay between extracellular cues including cytokine signalling and intracellular programmes such as epigenetic modification. There is increasing evidence that epigenetic mechanisms involving, for example, changes in DNA methylation, histone modification and non-coding RNA expression are closely associated with fate specification of NSCs. These epigenetic alterations could provide coordinated systems for regulating gene expression at each step of neural cell differentiation. Here we review the roles of epigenetics in neural fate specification in the mammalian central nervous system.

Keywords: neural stem cell; epigenetics; DNA methylation; chromatin remodelling; histone modification; non-coding RNA

1. INTRODUCTION

Most adult tissues retain a reservoir of self-renewing, multipotent stem cells that can generate differentiated tissue components. Until recently, the adult brain had been thought to represent an exception to this general concept. For decades, neurobiologists had subscribed to the idea that neural stem cells (NSCs) are depleted in the perinatal brain and that neurogenesis ceases during this period. Over the past 40 years, however, it has become clear that the adult brain also retains stem cells that produce neurons and glial cells throughout life (Altman 1962, 1963; Altman & Gopal 1965; Altman & Das 1966). This gradual realization has challenged former preconceptions about brain development, and has provided an opportunity to explore experimentally the identity of NSCs and the mechanisms by which they generate differentiated progeny.

NSCs are defined as cells that possess the ability to self-renew and to generate the three major cell types in the central nervous system (CNS): neurons; astrocytes; and oligodendrocytes (reviewed in Gage (2000), Temple (2001) and Okano (2002)). During brain development, telencephalic neuroepithelial cells including NSCs divide symmetrically in early gestation to increase their own numbers (Fujita 1963, 1986, 2003). These cells then undergo neurogenesis through mostly asymmetric division, giving rise to two distinct daughter cells: another NSC with the same potential as its mother cell and a neuron. Towards the end of the neurogenic phase, NSCs eventually acquire the multipotentiality to generate astrocytes and oligodendrocytes in addition to neurons (Qian *et al.* 2000). Adult NSCs

have been found in the two principal neurogenic regions, the subgranular zone of the hippocampus and the subventricular zone, both *in vivo* and *in vitro* (Kaplan & Bell 1984; Cameron *et al.* 1993; Gage 1998; García-Verdugo *et al.* 1998). Even in certain non-neurogenic regions, including the spinal cord, there exist cells harbouring the traits of NSCs when cultured *in vitro* (Gage 2000; Horner & Gage 2000). Although the mechanisms of NSC fate determination are not yet fully understood, it is gradually becoming apparent that both extracellular cues including cytokine signalling and intracellular programmes, such as epigenetic gene regulation, are deeply involved in the fate specification of NSCs. Epigenetic mechanism 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 non-coding RNA expression. These epigenetic modifications ensure appropriate gene activation at each step of NSC differentiation.

In this review, we focus on recent reports investigating the role of epigenetic mechanisms in the determination of neural cell fate in the mammalian CNS.

2. DNA METHYLATION SWITCHING THE FATE OF NSCs

In vertebrates, cytosine methylation of genomic DNA at CpG dinucleotides is one of the major epigenetic factors, regulating a diverse array of cellular events including developmental gene regulation, X chromosome inactivation, genome defence and genomic imprinting (Jaenisch & Bird 2003). DNA methylation-mediated gene regulation is thought to occur through two mechanisms. First, CpG methylation within a transcription factor-binding element interferes directly

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Figure 1. An astrocyte-specific gene promoter becomes demethylated at the stage when astrocytogenesis starts in the developing brain. (a) Developmental stage-dependent CpG methylation status in the STAT3-binding site within the GFAP gene promoter. Methylation status of the STAT3 recognition sequence in the GFAP promoter was investigated in freshly prepared or 4-day-cultured neuroepithelial cells from E11 (filled circles) and E14 (open circles) mouse telencephalon. (b) At midgestation, when neuroepithelial cells differentiate only into neurons, the STAT3-binding site in the astrocyte-specific GFAP promoter is highly methylated. Therefore, even if the cells are surrounded by STAT3-activating cytokine, STAT3 cannot activate target gene transcription. As gestation proceeds, the STAT3-binding site becomes demethylated, enabling STAT3 to bind its recognition sequence and to induce gene expression. Thus, DNA methylation in a cell type-specific gene promoter is a critical determinant for NSCs to acquire multipotentiality for differentiation during development. Me, methyl group.

with the binding of certain transcription activators to the target sequence (Watt & Molloy 1988; Takizawa *et al.* 2001). Second, and more generally, methylated genes are regulated through the action of methyl-CpG-binding domain (MBD)-containing protein family members such as MeCP2 and MBD1, which preferentially bind to methylated CpG(s) to suppress the gene expression (Lewis *et al.* 1992; Cross *et al.* 1997; Nan *et al.* 1997). These MBD proteins are themselves transcriptional repressors, and are further coupled to other corepressor proteins and histone modification enzymes, leading to repressive chromatin remodelling and gene silencing (Jones *et al.* 1998; Nan *et al.* 1998; Fuks *et al.* 2003). The DNA methylation pattern in the genome is established during embryogenesis by a family of DNA methyltransferases. Either single disruption of the maintenance methyltransferase Dnmt1 gene or compound disruption of the two de novo methyltransferase Dnmt3a and Dnmt3b genes in mice lead to drastic demethylation in the genome and the mice died at midgestation, indicating that DNA methylation is essential for embryogenesis (Goto *et al.* 1994; Okano *et al.* 1999; Robertson & Wolffe 2000). Mutations in genes encoding components of DNA methylation-associated machinery have been linked to human diseases such as cancer and several neurological disorders, including Rett, ICF (immunodeficiency-centromeric instability-facial anomalies), Fragile-X and ATRX (α -thalassaemia mental retardation)

syndromes, suggesting an important role for DNA methylation in brain development and function (Robertson & Wolffe 2000). Furthermore, it has become increasingly evident that DNA methylation also participates in the acquisition of multipotentiality by NSCs during development.

As described above, NSCs lack multipotentiality in early gestation and differentiate only into neurons during midgestation. NSCs then gradually acquire multipotentiality and differentiate into astrocytes and oligodendrocytes in late gestation (Fujita 1986, 2003; Miller 1996; Temple 2001). It has been suggested that DNA methylation in the astrocyte-specific gene promoters is a critical cell-intrinsic determinant for enabling NSCs to differentiate into astrocytes in the foetal brain (figure 1; Takizawa *et al.* 2001). It is generally known that members of the interleukin-6 (IL-6) family of cytokines, including leukaemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF), efficiently induce astrocyte differentiation of NSCs through the activation of Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3) pathway (Bonni *et al.* 1997; Rajan & McKay 1998; Nakashima *et al.* 1999a,b). However, in contrast to the cultures of neuroepithelial cells prepared from mouse telencephalon at embryonic day (E)14 (relatively late gestation), expression of a typical astrocytic marker, glial fibrillary acidic protein (GFAP), was not induced in a culture of telencephalic

neuroepithelial cells at E11 (midgestation) even when stimulated with the JAK–STAT3 pathway-activating cytokine LIF (Takizawa *et al.* 2001). Furthermore, the methylation target sequence CpG occurs in the STAT3-binding element (TTCGGAGAA) itself within the GFAP promoter, and this element is conserved in mouse, rat and human (Bonni *et al.* 1997; Nakashima *et al.* 1999b). Given these findings, the methylation status of this particular STAT3-binding site in the GFAP promoter was compared between E11 and E14 neuroepithelial cells. These experiments revealed that the STAT3-binding site was hypermethylated in E11 neuroepithelial cells, which do not respond to LIF to induce GFAP expression, but was barely methylated in E14 neuroepithelial cells, which have the potential to express GFAP in response to LIF stimulation (figure 1a; Takizawa *et al.* 2001). Moreover, it was found that STAT3 does not bind to the methylated form of its target sequence, explaining the lack of response of the GFAP promoter to cytokine stimulation (Takizawa *et al.* 2001). S100 β is also an astrocytic marker and is expressed during the earlier stages of astrocyte differentiation than GFAP. A particular cytosine residue in the S100 β promoter becomes demethylated, correlating with the onset of S100 β expression in the brain at E14 (Namiyama *et al.* 2004). In addition, another astrocyte-inducing cytokine, bone morphogenetic protein 2 (BMP2), increases histone acetylation around the CpG site in neuroepithelial cells at E14, but not at E11 when S100 β expression is not yet observed *in vivo*. Thus, it is conceivable that DNA methylation plays an important role in defining the timing of NSC fate specification (figure 1b).

Mouse pluripotent embryonic stem (ES) cells remain undifferentiated in LIF-containing culture medium. However, ES cells do not express GFAP in the presence of LIF, unlike neuroepithelial cells at late gestation. To explain this phenomenon, it has been suggested that the STAT3-binding site in the GFAP gene promoter is highly methylated in ES cells (Shimozaki *et al.* 2005). Demethylation of this site occurs only when pluripotent cells are committed to a neural lineage that is capable of producing astrocytes (Shimozaki *et al.* 2005). By contrast, the hypermethylated status of the STAT3-binding site is sustained in ES cell-derived endodermal and mesodermal cells. Hypermethylation of the STAT3 site is also observed in adult tissues outside the nervous system, such as liver, heart and femoral muscle (Takizawa *et al.* 2001). Furthermore, the incidence of CpG methylation of a specific site in the S100 β promoter was very high in ES cells, but low in ES cell-derived neural progenitors (Shimozaki *et al.* 2005). These data reinforce the suggestion that astrocyte gene-specific demethylation is not confined to the GFAP gene promoter, but is rather common among astrocyte-specific genes (Shimozaki *et al.* 2005).

On the other hand, it was demonstrated that hypomethylation-induced precocious astrocyte differentiation is not simply due to the demethylation of the STAT3-binding site in the GFAP promoter, but is also attributable to the elevation of overall JAK–STAT signalling activity. Precocious astrocyte differentiation was observed in Dnmt1-deficient CNS (Fan *et al.* 2005). This was explained by the fact that the

activation of JAK–STAT signalling was enhanced by the accelerated demethylation of gene promoters involved in the JAK–STAT pathway resulting in the upregulation of the genes' expression. These data suggest that DNA methylation regulates the timing and magnitude of astrocyte differentiation through both modulation of JAK–STAT activity and direct inhibition of glial marker genes via inactive chromatin remodelling (Fan *et al.* 2005).

Members of the MBD family have been also shown to play important roles in CNS development and function. Sequence homology searches using the conserved MBD of MeCP2 and MBD1 identified three additional members, MBD2, MBD3 and MBD4 (Hendrich & Bird 1998). MBD4 has turned out to be a DNA T : G mismatch repair enzyme and may function to minimize mutation at methylated CpG sites (Hendrich *et al.* 1999). Biochemical assays have shown that MBD3 cannot directly bind to methylated DNA, formally disqualifying MBD3 as a methyl-CpG-binding protein (Hendrich & Bird 1998). MeCP2 is particularly abundant in the mature CNS (Nan *et al.* 1997; Shahbazian *et al.* 2002b; Cassel *et al.* 2004; Mullaney *et al.* 2004), and transcripts of MBD1–MBD3 are easily detected by northern blot analysis of the adult brain (Hendrich & Bird 1998). More specifically, MBD proteins were found to be expressed predominantly in neurons, but not in astrocytes or oligodendrocytes, in the CNS (Coy *et al.* 1999; Jung *et al.* 2002; Shahbazian *et al.* 2002b; Kishi & Macklis 2004). Mutations in MeCP2 have been linked to the neurological disorder Rett syndrome (Shahbazian *et al.* 2002a; Kriaucionis & Bird 2003). Rett syndrome patients are characterized by normal development until 1 year of age, followed by a rapid deterioration involving loss of acquired speech and motor skills, microcephaly, seizures, autism, ataxia, intermittent hyperventilation and characteristic stereotypic movements (Nomura & Segawa 1990; Guy *et al.* 2001; Jung *et al.* 2003; Segawa & Nomura 2005). Mice deficient for MBD1 showed decreased neurogenesis, defects in spatial learning and a reduction in long-term potentiation in the dentate gyrus of the hippocampus (Zhao *et al.* 2003). MBD1-deficient NSCs generated fewer neurons than wild-type cells, suggesting a role for MBD1 in neuronal fate specification. The absence of MBD1 in NSCs also resulted in increased aneuploidy and upregulated expression of intracisternal A particle, a type of endogenous virus whose expression levels are frequently elevated in cancer cells with genomic instability (Walsh *et al.* 1998).

Neuroepithelial cells at late gestation, which have already lost the methylation in the STAT3-binding site within the GFAP promoter, can still generate neurons that do not respond to a STAT3-activating cytokine to express GFAP (Setoguchi *et al.* 2006). Recently, we reported that DNA methylation and MBD proteins are involved in the silencing of astrocytic genes in neurons to restrict the differentiation plasticity of the cell. In support of this, ectopic expression of MBDs actually inhibits astrocytic genes' expression and differentiation of embryonic neuroepithelial cells, which normally differentiate into astrocytes under the control of STAT3-activating cytokines (figure 2a; Setoguchi *et al.* 2006). The exon 1 region of the GFAP gene remains highly

Figure 2. Region-specific DNA methylation and MBD proteins restrict differentiation plasticity of neurons. (a) Four-day *in vitro*-cultured E14 neuroepithelial cells were infected with recombinant retrovirus engineered to express only GFP (pMY-GFP), or MeCP2 together with GFP (pMY-MeCP2-GFP), and stimulated with LIF for 4 days to induce astrocyte differentiation. The cells were then stained with antibodies against GFP (green) and GFAP (red). MeCP2 expression inhibited astrocyte differentiation of neuroepithelial cells. (b) Four-day *in vitro*-cultured E14 neuroepithelial cells (undifferentiated neuroepithelial cells) were differentiated in medium containing 0.5% FBS for 4 days (differentiated cells). Under these conditions, over 30% of the cells become positive for the neuronal marker Tuj-1. The methylation status of CpG sites within the GFAP gene exon 1 region (+20 to +449 base pair (bp)) in (i) undifferentiated neuroepithelial cells and (ii) differentiated cells was analysed by bisulphite sequencing. Open and filled circles indicate unmethylated and methylated CpG sites, respectively. (c) ChIP assays were performed using anti-MeCP2 antibody and PCR primers to detect a DNA fragment spanning –18 to +510 bp of the GFAP gene in undifferentiated neuroepithelial cells (left lanes) and differentiated cells (right lanes). (d) Neuroepithelial cells, which have already lost STAT3 site methylation in the GFAP promoter, can still differentiate into neurons (right). MBD proteins including MeCP2 are predominantly expressed in neurons, suppressing astrocyte-specific GFAP gene expression through their binding to highly methylated regions of the gene.

methyated, even in neuroepithelial cells that have already lost methylation in the STAT3-binding site within the GFAP promoter and in neurons generated from these cells (figure 2b). Furthermore, MeCP2 indeed binds to the highly methylated exon 1 of the GFAP gene in neurons (figure 2c), and to hypermethylated CpG sites around the transcription start site of the astrocyte-specific S100 β gene to suppress their expression. Ectopic MBD1 expression inhibits astrocyte differentiation of neuroepithelial cells as well, demonstrating some functional redundancy among MBD proteins. From these studies, it is plausible that region-specific DNA methylation and MBD proteins play an important role in the regulation of differentiation plasticity to maintain the identity of neurons, in which MBD proteins are predominantly expressed (figure 2d).

3. HISTONE MODIFICATION AND NEURAL DIFFERENTIATION

It has become apparent that chromatin modification plays a critical role in the regulation of cell-type-specific gene expression. One of the best-characterized histone modifications to date is lysine acetylation, which is

mediated by two groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs; figure 3a). HATs induce acetylation of N-terminal histone tails, which decreases the interaction of the positively charged histone tails with the negatively charged phosphate backbone of DNA and, hence, results in the relaxation of the nucleosomes. HDACs catalyse the reverse reaction; in the deacetylated state, histones package DNA into condensed chromatin, which in turn prevents access of transcriptional activators to their target sequences, thus resulting in transcriptional repression (figure 3a; Hsieh & Gage 2004). The well-known anti-epileptic valproic acid (VPA) functions as an inhibitor of HDACs. VPA has been shown to induce neuronal differentiation of adult NSCs (Hsieh *et al.* 2004). In addition, VPA inhibits glial cell differentiation of NSCs, even under conditions that favour lineage-specific differentiation. Among the VPA-upregulated neuron-specific genes, a neurogenic basic helix-loop-helix transcription factor, NeuroD, was identified. Overexpression of NeuroD in NSCs resulted in the induction and suppression of neuronal and glial differentiation, respectively. Taken together, these

Figure 3. Chromatin modification with histone acetylation and methylation. (a) HATs induce acetylation of N-terminal tails of histones, decreasing the interaction between the positively charged histone tails and the negatively charged phosphate backbone of DNA and hence resulting in relaxation of the nucleosomes. HDACs catalyse the reverse reaction; in the deacetylated state, histones package DNA into condensed chromatin, which, in turn, prevents the access of transcriptional activators to their target sites, thus resulting in transcriptional repression. Ac, acetyl group. (b) In E18 rat cortical cultures, co-treatment with FGF2 and CNTF leads to a marked increase in the fraction of cells that express GFAP. FGF2 facilitates access of the STAT/CBP complex to the GFAP promoter by inducing H3K4 methylation and suppressing H3K9 methylation around the STAT3-binding site. Me, methyl group.

results suggest that VPA promotes neuronal fate and inhibits glial fate simultaneously through the induction of neurogenic transcription factors including NeuroD (Hsieh *et al.* 2004).

In contrast to histone acetylation, which appears to be reversible and dynamic and is most often associated with the expression of individual genes, epigenetic regulation by histone methylation had been thought to be stable and involved in the long-term maintenance of certain regions of the genome. However, the recent discovery of histone demethylases has revealed that the histone methylation is in fact much more flexible than previously thought. Lysine methylation has been directly linked to epigenetic inheritance: histone H3 methylation at lysine 4 (K4), K36 and K79 leads to transcriptional activation, whereas histone H3 methylation at K9 and K27 as well as histone H4 methylation at K59 is associated with transcriptional silencing.

Recently, FGF2, which by itself is not capable of inducing astrocyte-specific gene expression, has been reported to positively affect the ability of CNTF, an IL-6 cytokine family member, to induce the expression of GFAP (figure 3b; Song & Ghosh 2004). FGF2 promotes the access of the complex involving STAT3 and a HAT, CREB-binding protein (CBP), to the

GFAP promoter by inducing H3K4 methylation while suppressing H3K9 methylation around the STAT-binding site. These findings suggest that extracellular signals can modulate the accessibility of transcription factors to gene promoters by local histone modification (figure 3b).

Epigenetic strategies control the orderly acquisition and maintenance of neuronal traits. A growing number of reports indicate that a complex network of transcriptional repressors and corepressors mediates specific gene expression for these strategies.

It has been demonstrated that epigenetic modifications via a zinc-finger domain-containing transcriptional repressor protein, RE1 silencing transcription factor (REST)/neuron restrictive silencing factor (NRSF), plays a fundamental role in the progression of pluripotent cells to lineage-restricted neural progenitors (Ballas & Mandel 2005; Ballas *et al.* 2005). REST/NRSF binds to a conserved 21- to 23-base pair (bp) cognate sequence, referred to as RE-1/NRSE (Chong *et al.* 1995; Schoenherr & Anderson 1995; Schoenherr *et al.* 1996), to mediate negative regulation of many different neuronal genes, including those encoding ion channels, neurotransmitter receptors, synaptic vesicle proteins and adhesion molecules in

Figure 4. REST/NRSF-mediated regulation of neuronal gene expression. (a) Class I neuronal genes are expressed by default relying solely on the dissociation of the REST/NRSF repressor complex from the RE-1/NRSE site for maximal expression. (b) In addition to the REST/NRSF complex at the RE-1/NRSE site, class II genes are bound by CoREST and MeCP2 complexes on adjacent methylated CpGs in their promoters throughout neuronal differentiation. Only NRSF-containing complex is dissociated from the gene during neuronal differentiation. Upon membrane depolarization, MeCP2 becomes phosphorylated and dissociates from the chromatin of the induced genes, resulting in a maximal level of expression. Me, methyl group; P, phosphorylated.

extra-neuronal tissue. Accordingly, REST/NRSF prevents premature expression of terminal differentiation genes in neural precursors (for a review, see Schoenherr *et al.* 1996). ES cells can form embryoid bodies in culture that, upon exposure to retinoic acid, differentiate into neurons. REST/NRSF protein was present in the nuclei of dividing undifferentiated ES cells, none of which expresses the neuron-specific β III-tubulin gene (Ballas *et al.* 2005). REST/NRSF bound to the RE1 site of neuronal genes in ES cells and neuronal genes' chromatin in ES cells were maintained in a poised state via a specialized REST/NRSF repressor complex, including CoREST, HDAC1 and MeCP2, which permitted basal expression of neuronal genes at a very low level. However, in contrast to fibroblasts, in which the neuronal genes were completely silenced, di- and trimethylated H3K4, which are usually associated with actively transcribed genes, were present and dimethylated H3K9, usually associated with silenced genes, was absent at the RE-1/NRSE site of neuronal genes in ES cells (Roopra *et al.* 2004).

As ES cells differentiate into neural progenitors, REST/NRSF mRNA levels stay relatively constant, whereas the amount of REST/NRSF protein decreases to a quite low level. When REST/NRSF protein was downregulated by proteasomal degradation, the REST/NRSF repressor complex disappeared from the RE-1/NRSE site in the promoter regions of neuronal genes, with the result that the genes became

derepressed and activated in neurons (figure 4; Ballas *et al.* 2005). It was suggested that the loss of REST/NRSF in neurons led to the expression of two classes of RE-1/NRSE-containing neuronal genes (Ballas *et al.* 2005). Class I genes were expressed by default, relying solely on the dissociation of the REST/NRSF repressor complex from the RE-1/NRSE site for maximal expression (figure 4a). On the contrary, class II genes, typified by brain-derived neurotrophic factor (BDNF) and calbindin genes, contain, besides the REST/NRSF complex at the RE-1/NRSE site, CoREST and MeCP2 complexes on adjacent methylated CpGs in their promoters that remained bound throughout differentiation (figure 4b). Upon depolarization of neural cells, while CoREST remained bound to the chromatin, MeCP2 became phosphorylated and dissociated, together with HDAC and mSin3, from the chromatin of class II genes leading to a maximal level of expression. The calbindin and BDNF genes are representative of a large class of genes whose expression is upregulated in neurons by interfering with HDAC activity and, in some cases, also by depolarization (figure 4b). Many genes in this class are considered to be implicated in the functional plasticity of neurons *in vivo* (figure 4; Ballas & Mandel 2005).

A high-mobility group box-containing protein, BRAF35, is involved in a corepressor complex that has been previously reported to be required for the repression of REST/NRSF-regulated genes. Recently,

the BRAF35 homologue, an inhibitor of BRAF35 (iBRAF), has been shown to activate REST/NRSF-regulated genes through the modulation of histone methylation (Wynder *et al.* 2005). Analysis of mouse embryonic carcinoma P19 cells undergoing neuronal differentiation revealed iBRAF accumulation at the promoter of neuron-specific genes coincident with the augmented expression of neuron-specific synapsin, recruitment of the H3K4 methyltransferase mixed-lineage leukaemia (MLL) and enhanced trimethylation of H3K4. The ectopic expression of iBRAF was sufficient to induce neuronal differentiation of the cells through the recruitment of MLL, resulting in increased H3K4 trimethylation and activation of neuron-specific genes.

4. NON-CODING RNA AND NEURAL DIFFERENTIATION

Non-coding RNAs were recently identified as a new class of molecule exhibiting epigenetic effects on gene regulation (Grewal & Moazed 2003; He & Hannon 2004). Among several types of non-coding RNAs, microRNAs (miRNAs) have been reported to play roles in regulating target protein levels minimally, through either degradation of target mRNAs or inhibition of mRNA translation.

miRNA genes are transcribed by RNA polymerase II and subsequently 5'-capped and 3'-polyadenylated in primary miRNA transcripts (pri-miRNA; Cai *et al.* 2004). The RNase III enzyme Drosha initiates nuclear processing of the pri-miRNA into hairpin-like precursor miRNA (pre-miRNA; approx. 70 nucleotide (nt)) that contain a large (10 nt) terminal loop (Lee *et al.* 2004). The double-stranded RNA-binding protein DGCR8 interacts with Drosha to form the microprocessor complex (reviewed by Kim 2005). The 2 nt 3' overhang end structure of the miRNA precursor is recognized by Exportin-5, a Ran-GTP-dependent nuclear export factor (reviewed by Hutvagner 2005), and then the miRNA is transported into the cytoplasm. Maturation of the pre-miRNA (to approx. 22 nt) is catalysed by the cytoplasmic RNase Dicer (reviewed by Hammond 2005), and the mature miRNA then guides RNA-induced silencing complex to the 3'-untranslated region (UTR) of target mRNAs (reviewed by Hutvagner 2005).

Although the most straightforward strategy to uncover the importance of miRNAs in mammalian development would be to eliminate individual miRNAs by gene targeting, the depletion of a specific miRNA has not been reported so far. However, the production of all miRNA is completely abolished in mice deficient for Dicer (Bernstein *et al.* 2003). Mice deficient for the Dicer-1 die before E7.5, raising questions regarding the importance of miRNA in later organogenesis and homeostasis in the adult organism. Therefore, a conditional targeting of the gene would help us to unravel the functions of miRNAs in various aspects of development. Nevertheless, since at least some miRNAs are known to be expressed in a tissue-specific manner during development, it seems probable that miRNAs are implicated to some extent in the control of organ development (Kloosterman *et al.* 2006).

To explore the function of miRNAs, it is necessary to determine the spatio-temporal pattern of their expression. Recent miRNA array technology has paved a new way for general neurobiologists (Krichevsky *et al.* 2003; Miska *et al.* 2004). Screenings against miRNAs have identified at least 125 miRNAs expressed in mouse brain at different developmental phases. Of these, miRNAs termed miR-9, -29, -124a, -125b, -127, -128, -132, -137, -138 and -139 are highly expressed. For example, Kosik's group performed miRNA array analysis at various stages of cortical neurogenesis (Krichevsky *et al.* 2003). They examined the expression of miRNAs from the forebrain of prenatal (E12, E13 and E21), neonatal (P5) and adult rats. Among the miRNAs they examined, miR-128 was selectively expressed postnatally, whereas miR-19b was expressed only prenatally. The expression of miR-9, -124a, -131, -178 and -266 increased gradually during the embryonic period. Moreover, several miRNAs were found to be expressed in distinct types of neural cells. miR-124a and -128 were expressed specifically in neurons, while miR-23, -26 and -29 were in astrocytes (Smirnova *et al.* 2005).

Other strategies to examine the precise expression pattern of miRNAs are RNA *in situ* hybridization and the miRNA sensor system. Unfortunately, conventional RNA *in situ* hybridization using miRNAs have been unsuccessful for most miRNAs examined so far (Harfe 2005). However, the miRNA sensor system is a powerful and simple method to detect miRNA distribution *in vitro* and *in vivo* (Mansfield *et al.* 2004; Smirnova *et al.* 2005). The sensor construct is composed of a reporter gene such as LacZ or EGFP under a ubiquitously active promoter and with a non-specific 3'-UTR sequence. To identify the cellular expression pattern of a miRNA, a sequence that is perfectly complementary to the miRNA of interest is inserted in the 3'-UTR. The presence of miRNA in a specific cell thus triggers posttranscriptional suppression of the reporter gene product, resulting in the ablation of the reporter signal. This system may provide us with an efficient way to analyse the distribution and dynamism of miRNA expression relating to neural cell-type specification.

The importance of miRNA in stem cell regulation, including fate specification, is becoming evident from several lines of study in various somatic tissue stem cells. The first example of miRNA-mediated cell fate regulation was discovered in haematopoietic stem cells (Chen *et al.* 2004; Felli *et al.* 2005). One of the miRNAs (miR-181) was specifically expressed in B-lymphoid cells and its ectopic expression in haematopoietic stem cells induced an increase in the number of B cells, strongly implying that miRNAs can modulate lineage commitment of other tissue-specific stem cells.

Among miRNAs expressed predominantly in neural tissues, miR-124a has been shown to participate in the differentiation of NSCs into neurons by mediating degradation of non-neuronal gene transcripts (figure 5; Conaco *et al.* 2006). This study indicated that miR-124a expression is regulated by REST/NRSF, which is expressed in neural progenitors and non-neuronal cells but not in neurons. Many neuronal genes are actively repressed in non-neuronal cells through a conserved 21–23 bp repressor element in their promoter that

Figure 5. Reciprocal function of REST/NRSF and miRNA in neuronal identity. In neural progenitors, REST/NRSF is expressed and inhibits miR-124a expression, allowing the persistence of non-neuronal transcripts. When the progenitors differentiate into neurons, REST/NRSF dissociation from the miR-124a gene loci induces derepression of the gene, resulting in the selective degradation of non-neuronal gene transcripts. Thus, REST/NRSF links transcriptional and posttranscriptional events to fine-tune the balance of phenotype between neuronal and non-neuronal cells.

is recognized by REST/NRSF (Chong *et al.* 1995; Schoenherr & Anderson 1995). As described above, REST/NRSF is known to recruit HDACs via corepressors associated with its repressor domains to suppress target gene expression (Andres *et al.* 1999; Roopra *et al.* 2000; Battaglioli *et al.* 2002). Therefore, in neural progenitors expressing REST/NRSF, suppression of miR-124a expression by REST/NRSF permits the existence of non-neuronal gene transcripts in the cells. On the other hand, derepression of the miR-124a gene locus induced by the absence of REST/NRSF leads to the degradation of non-neuronal gene transcripts in neurons. REST/NRSF thus links transcriptional and posttranscriptional events to fine-tune the balance of phenotype between neuronal and non-neuronal cells (Figure 5).

More recently, another example of miRNA function in cell fate determination of neural progenitors was reported (Krichevsky *et al.* 2006). STAT3 activation induces astrocyte differentiation but inhibits neuronal differentiation of neural progenitors (Gu *et al.* 2005). The authors focused on the function of miR-124a and -9, and found that these miRNAs promote neurogenesis via the inhibition of STAT3 tyrosine phosphorylation, which is critical for STAT3 activation, by an as yet unknown mechanism.

Although most non-coding RNAs have been reported to play roles in gene silencing, Kuwabara and colleagues have found that one type of non-coding RNA can function in gene activation (Kuwabara *et al.* 2004). It was revealed that this non-coding RNA, referred to as small modulatory double-stranded RNA (smRNA), appears to be transiently expressed during the course of neuronal differentiation of adult hippocampal NSCs. The sequence defined by this smRNA is RE-1/NRSE (described above), which is recognized by REST/NRSF. Ectopic expression of the NRSE dsRNA in adult NSCs promoted neuronal differentiation, whereas the ablation of NRSE dsRNA by a ribozyme targeting the dsRNA inhibited neuronal differentiation. As described above, REST/NRSF has so far been considered to be a repressor for neuronal genes in

non-neuronal cells. However, the NRSE dsRNA switches the function of REST/NRSF from repressor to activator through interaction with REST/NRSF, leading to the activation of neuron-specific genes in early neuronal progenitors.

5. CLOSING REMARKS

Owing to their ability to self-renew and a remarkable potential for differentiation into neural derivatives, NSCs hold great promise for therapeutic applications in neurological dysfunctions such as Parkinson's disease, amyotrophic lateral sclerosis and spinal cord injury (Ogawa *et al.* 2002; Okano 2002; Lindvall & Kokaia 2006). However, we still do not know the precise mechanism by which cell fate is controlled. By introducing the concept of epigenetics, we have become able to provide mechanistic explanations for many biological phenomena that formerly could only be described as cell context dependent. For example, although LIF activates the same JAK–STAT signalling pathway in both E11 and E14 neuroepithelial cells, whereas only the latter can respond to LIF to express the astrocyte-specific GFAP gene, the unresponsiveness of E11 neuroepithelial cells to LIF can be now explained by the fact that the GFAP gene promoter is highly methylated in these cells. As we have described, an emerging body of evidence indicates that epigenetic mechanisms play critical roles in various aspects of NSC regulation; this trend, moreover, is not confined to the nervous system. However, pressing questions remain, such as how gene-specific epigenetic modifications are introduced and how they interact with signals from outside the cell. A comprehensive understanding of the precise mechanisms of NSC regulation must await further investigation.

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