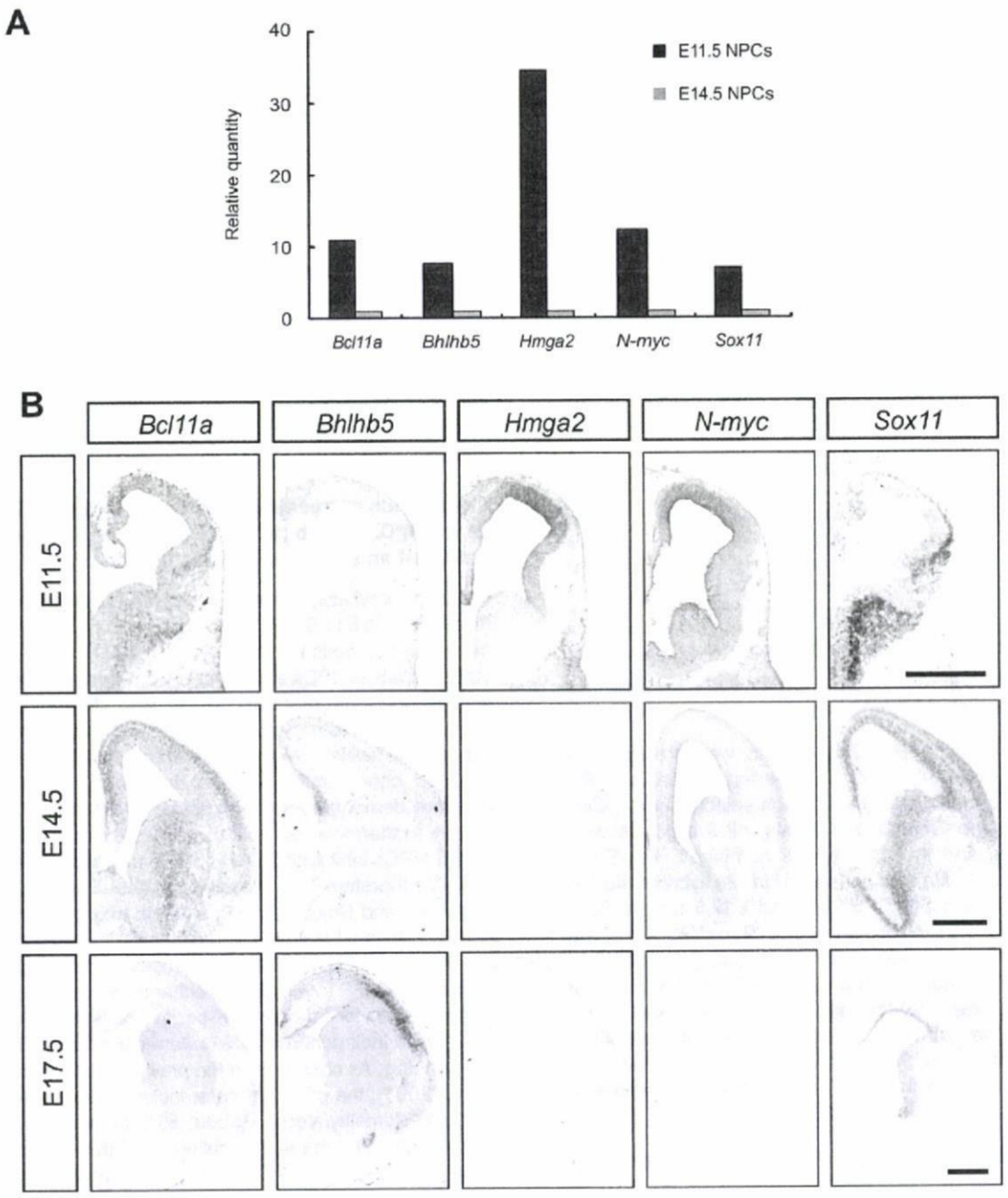

Fig. 2. *N-myc* and *Hmga2* are highly expressed in the VZ of E11.5 mouse brain. (A) Gene-specific real-time RT-PCR was performed to validate GeneChip analysis data. (B) *In situ* hybridization was performed for E11.5, E14.5 and E17.5 mouse brain sections. No signal was detected when sense-probes for each gene were used (data not shown). Scale bar=500 μm.

DISCUSSION

In this study, we compared NPC gene expression profiles at different developmental stages using Affymetrix GeneChips and the Percellome method, and then analyzed by *in situ* hybridization the spatio-temporal expression patterns of genes which were highly expressed in E11.5 NPCs. We found that *N-myc* and *Hmga2* were specifically expressed in E11.5 NPC both *in vivo* and *in vitro* and, furthermore, that the transduction of these genes into NPCs suppressed LIF-induced astrocytic differentiation without affecting DNA demethylation of the astrocyte-specific *gfap* gene promoter.

The basic HLH leucine zipper transcription factor N-myc,a member of the myc family of oncogenes, is a nuclear phosphoprotein exhibiting site-specific DNA-binding activity (Ramsay et al., 1986; Alex et al., 1992), and has

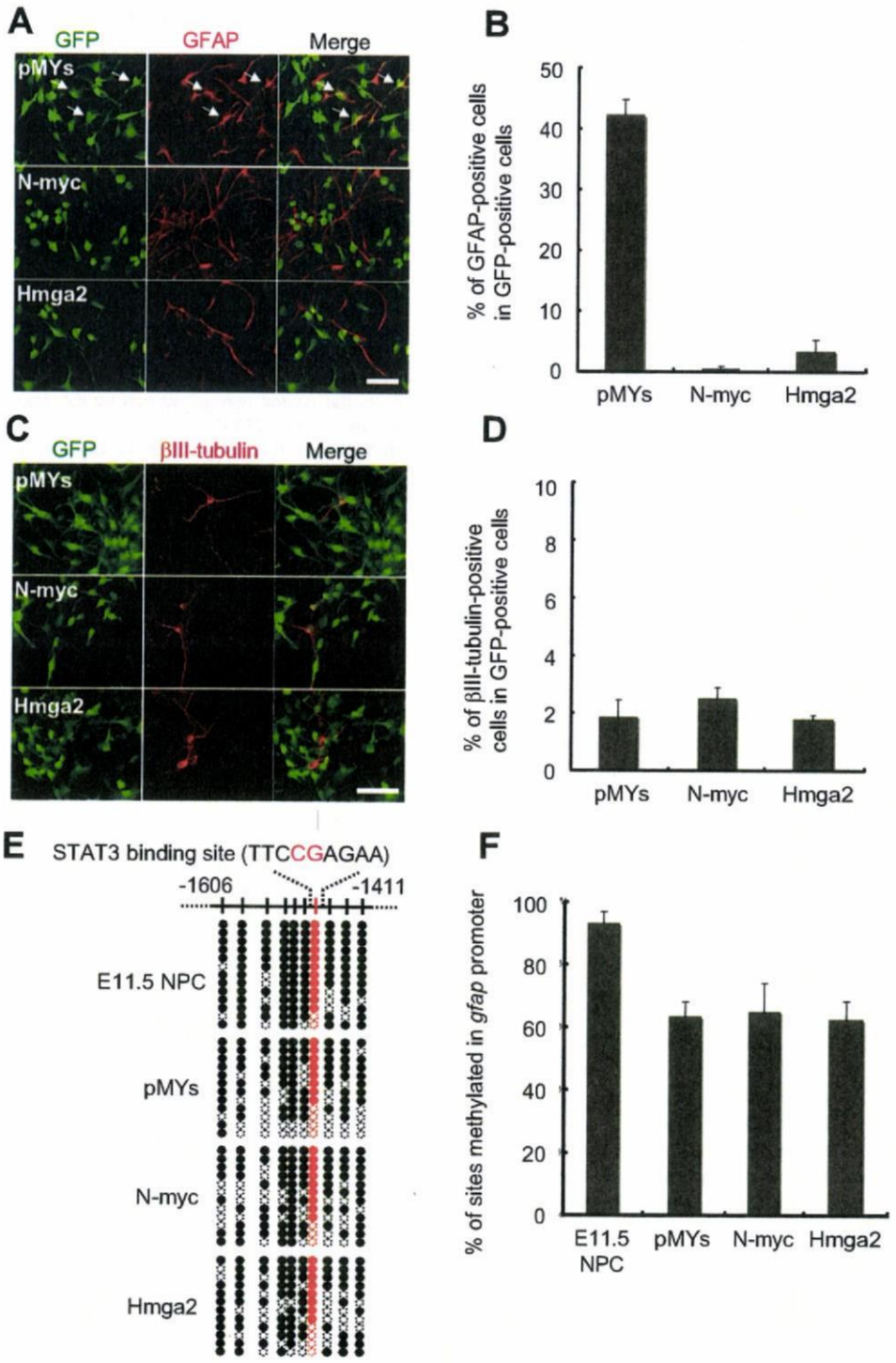

Fig. 3. N-myc and Hmga2 inhibit astrocyte differentiation of NPCs even in the presence of LIF. (A–D) E14.5 NPCs were infected with recombinant retroviruses engineered to express *EGFP* alone (pMYs), and *EGFP* together with either *N-myc* (N-myc) or *Hmga2* (Hmga2), and cultured with LIF (50 ng/ml) for 4 days to induce astrocyte differentiation. The cells were then stained with antibodies against GFP (green) and GFAP (red) (A). Arrows indicate GFP/GFAP double-positive cells. The percentage of GFAP-positive astrocytes in GFP-positive cells was quantified (B). Mean±S.D. The cells were also stained with antibodies against GFP (green) and βIII-tubulin (red) (C). The percentage of βIII-tubulin-positive neurons in GFP-positive cells was quantified (D). Mean±S.D. (E) E11.5 NPCs were infected with recombinant retroviruses engineered to express *EGFP* alone (pMYs), and *EGFP* together with either *N-myc* (N-myc) or *Hmga2* (Hmga2), and subsequently cultured for 4 days. GFP-positive cells were then sorted based on GFP fluorescence, and genomic DNA was extracted for bisulfite sequencing. White and black circles indicate unmethylated and methylated CpG sites, respectively. The E11.5 NPC sample was freshly prepared NPCs from telencephalon at E11.5. The CpG dinucleotide within the STAT3 binding site is indicated in red. (F) Methylation frequency in the *gfap* promoter. Mean±S.D. Scale bars=50 μm (A, C). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

been reported to be expressed in a wide range of vertebrate tissues, primarily during embryogenesis (Schreiber-Agus et al., 1993). The mice deficient for functional *N-myc* are embryonic lethal (Stanton et al., 1992). Since N-myc has been shown to be a transcriptional activator, it may inhibit astrocyte differentiation via induction of neurogenic bHLH factors such as *Ngn1* (Sun et al., 2001), which have already been suggested to inhibit astrocyte differentiation in midgestational NPCs. However, this scenario seems unlikely because *N-myc* expression in NPCs did not affect neuronal differentiation, as assessed by monitoring expression of the neuronal marker βIII-tubulin (Fig. 3C, D). On the other hand, Hmga2 possesses an acidic C-terminal tail and three individual DNA-binding domains which bind short stretches of AT-rich DNA with high affinity (Reeves, 2001). *Hmga2* is expressed in pluripotent embryonic stem (ES) cells and in most tissues and organs during embryogenesis, but at very low levels or not at all in adult tissues (Zhou et al., 1995). Its function appears to be critical for cell growth, because mice lacking functional *Hmga2* exhibit a pygmy phenotype (Zhou et al., 1995). Recently, it was reported that Hmga2 specifically accumulates on senescent cell chromatin and that it functions as a structural component of senescence-associated heterochromatin foci and as a repressor of proliferation-associated genes (Narita et al., 2006). We therefore expected that Hmga2 would maintain the hypermethylation status of the astrocyte-specific *gfap* promoter via transcription-repressive heterochromatin formation in E11.5 NPCs. However, our results indicate that this is not the case. The mechanism(s) whereby N-myc and Hmga2 inhibit astrocyte differentiation must await further investigation.

Although DNA methylation is a critical cell-intrinsic determinant for the neurogenic-to-astrogliogenic switch and/or astrocyte differentiation of NPCs, many other spatio-temporally expressed extracellular factors such as CT-1, Notch and Wnt1 (Barnabe-Heider et al., 2005; Hirabayashi and Gotoh, 2005; Nagao et al., 2007) and intracellular factors including Ngn (Sun et al., 2001), N-CoR (Hermanson et al., 2002), N-myc and Hmga2 (this study) complement DNA methylation to ensure the sequential differentiation of NPCs during development. Thus, to better understand the mechanism underlying these processes, this study emphasizes the need to take cell-extrinsic cues, cell-intrinsic programs and factors, and their interaction into consideration.

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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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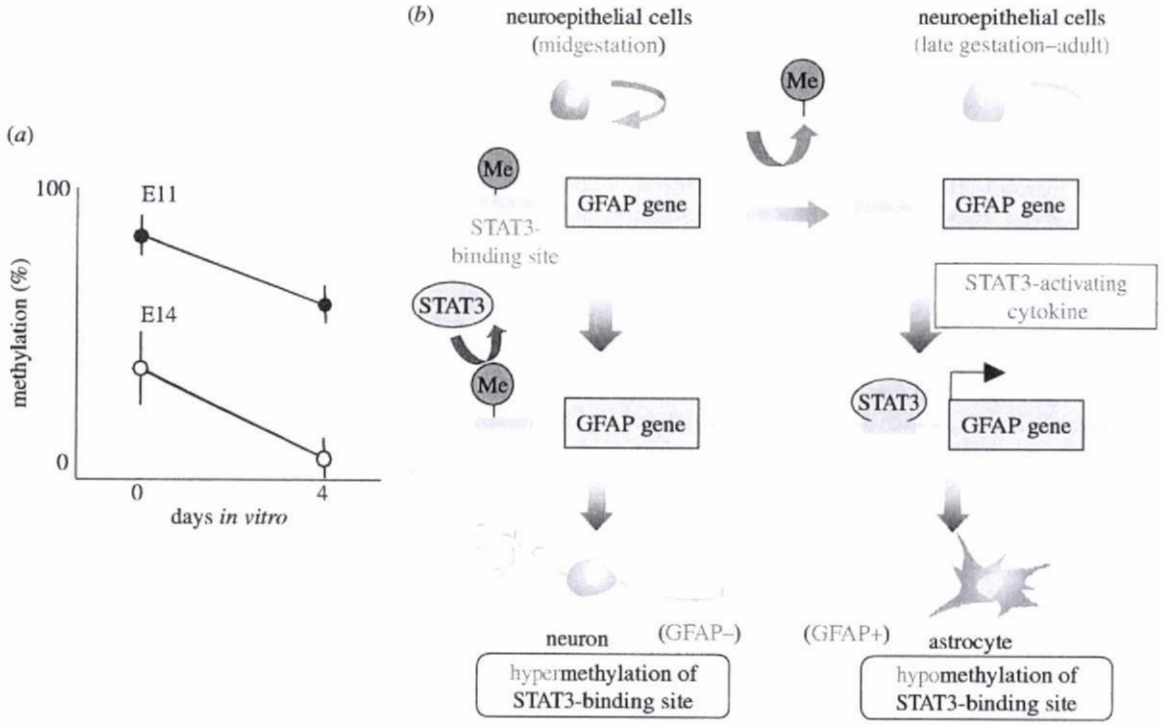

Figure 1. An astrocyte-specific gene promoter becomes demethylated at the stage when astrocytogenesis starts in the developing brain. (*a*) Developmental stage-dependent CpG methylation status in the STAT3-binding site within the GFAP gene promoter. Methylation status of the STAT3 recognition sequence 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.* 1999*a*,*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 (TT*CG*GAGAA) itself within the GFAP promoter, and this element is conserved in mouse, rat and human (Bonni *et al.* 1997; Nakashima *et al.* 1999*b*). 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 1*a*; 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). S100β is also an astrocytic marker and is expressed during the earlier stages of astrocyte differentiation than GFAP. A particular cytosine residue in the S100β promoter becomes demethylated, correlating with the onset of S100β expression in the brain at E14 (Namihira *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 S100β 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 1*b*).

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 S100β 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.* 2002*b*; 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.* 2002*b*; Kishi & Macklis 2004). Mutations in MeCP2 have been linked to the neurological disorder Rett syndrome (Shahbazian *et al.* 2002*a*; 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 2*a*; Setoguchi *et al.* 2006). The exon 1 region of the GFAP gene remains highly

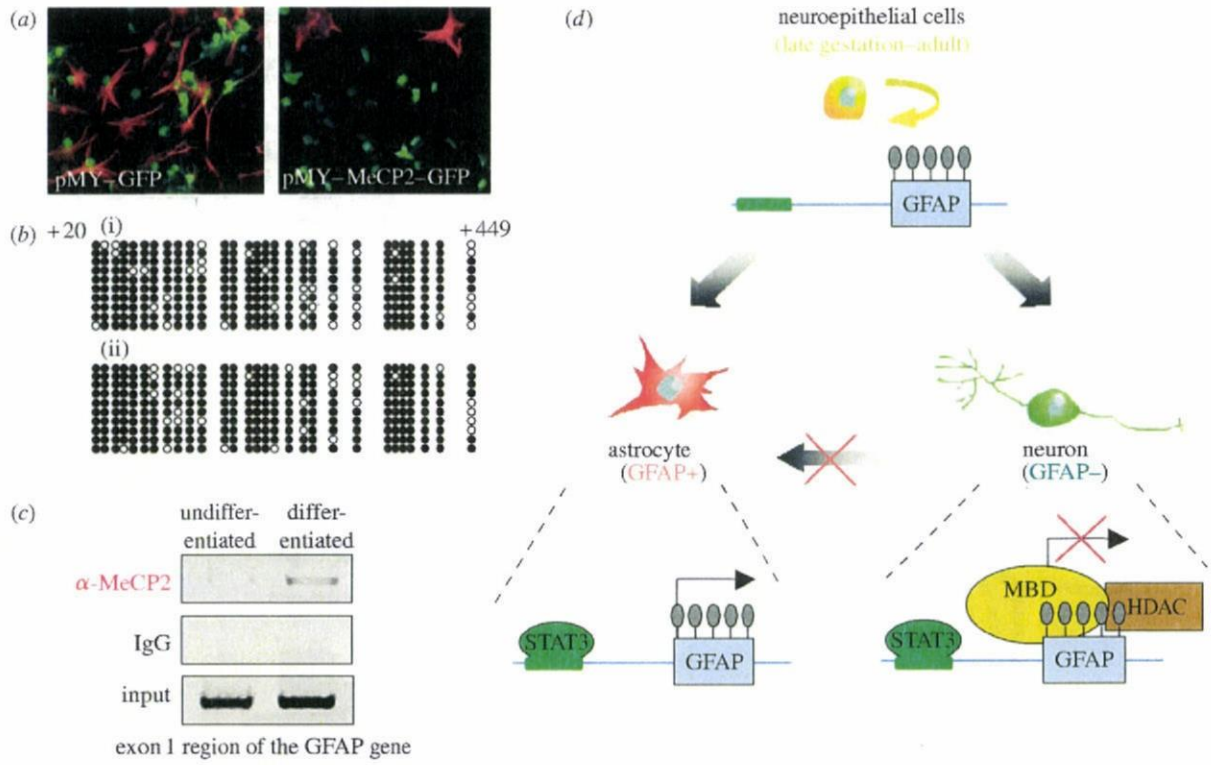

Figure 2. Region-specific DNA methylation and MBD proteins restrict differentiation plasticity of neurons. (*a*) Four-day *in vitro*-cultured E14 neuroepithelial cells were infected with recombinant retrovirus engineered to express only GFP (pMY–GFP), or MeCP2 together with GFP (pMY–MeCP2–GFP), and stimulated 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.

methylated, 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 2*b*). Furthermore, MeCP2 indeed binds to the highly methylated exon 1 of the GFAP gene in neurons (figure 2*c*), and to hypermethylated CpG sites around the transcription start site of the astrocyte-specific S100β 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 2*d*).

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 3*a*). 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 3*a*; 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

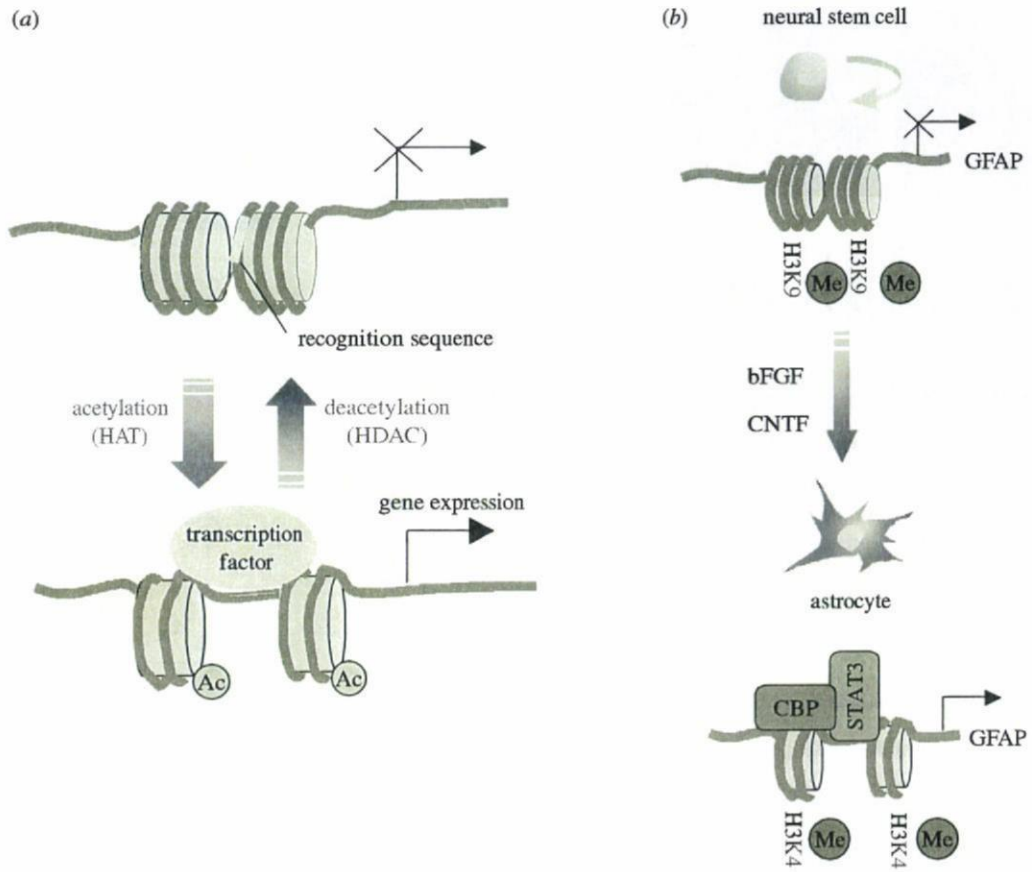

Figure 3. Chromatin modification with histone acetylation and methylation. (*a*) HATs induce acetylation of N-terminal tails of histones, decreasing the interaction between the positively charged histone tails and the negatively charged phosphate backbone of DNA and hence resulting in relaxation of the 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 3*b*; 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 3*b*).

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

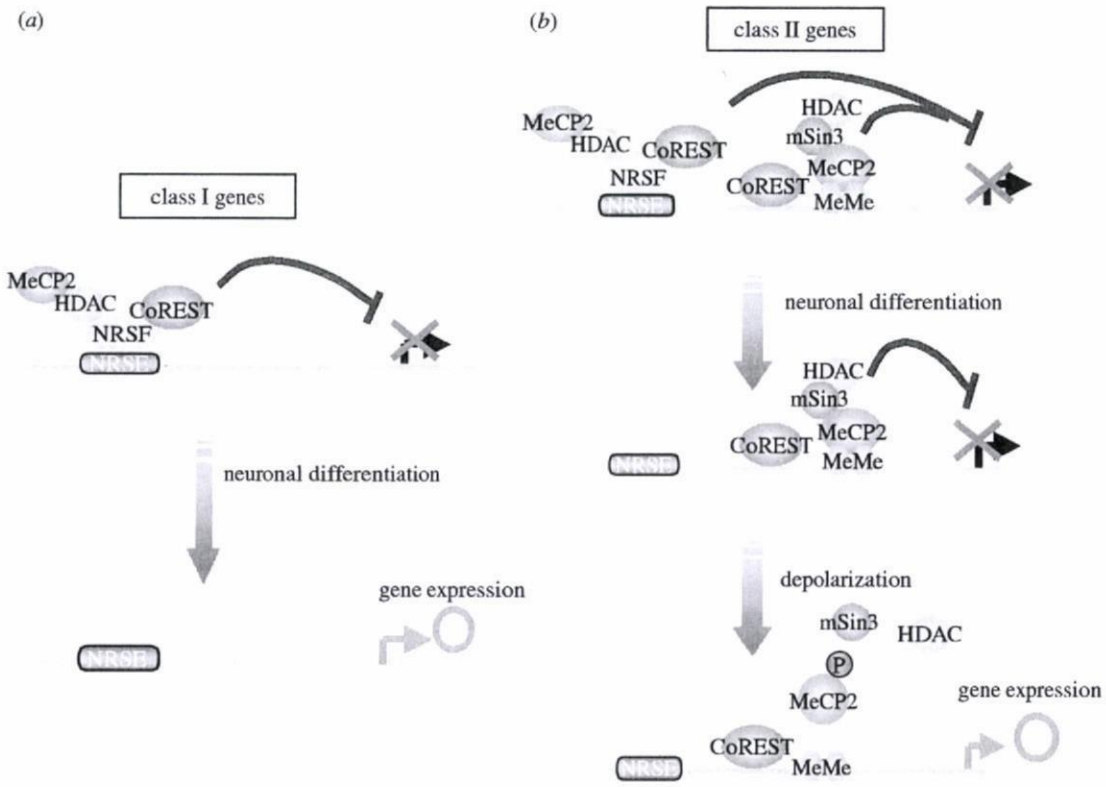

Figure 4. REST/NRSF-mediated regulation of neuronal gene expression. (*a*) Class I neuronal genes are expressed by default relying solely on the dissociation of the REST/NRSF repressor complex from the RE-1/NRSE site for maximal expression. (*b*) In addition to the REST/NRSF complex at the RE-1/NRSE 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 βIII-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 4*a*). 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 4*b*). 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 4*b*). 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

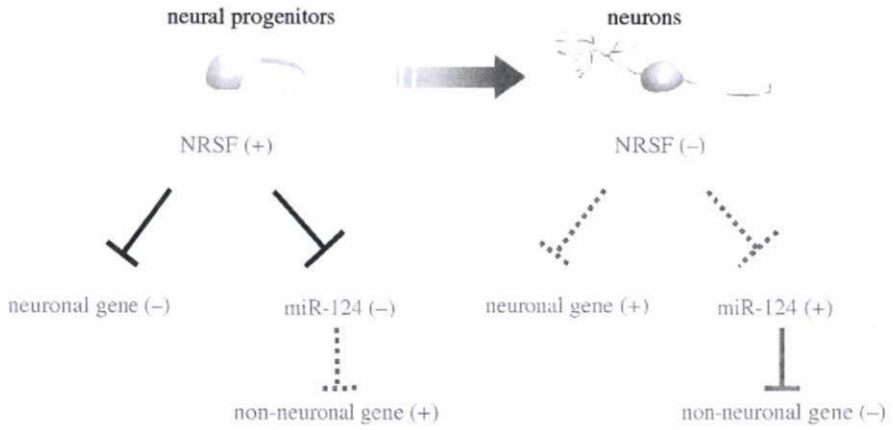

Figure 5. Reciprocal function of REST/NRSF and miRNA in neuronal identity. In neural progenitors, REST/NRSF is expressed and inhibits miR-124a expression, allowing the persistence of non-neuronal transcripts. When the progenitors differentiate into neurons, REST/NRSF dissociation from the miR-124a gene 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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Astrocyte-Specific Genes Are Generally Demethylated in Neural Precursor Cells Prior to Astrocytic Differentiation

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Abstract

Epigenetic changes are thought to lead to alterations in the property of cells, such as differentiation potential. Neural precursor cells (NPCs) differentiate only into neurons in the midgestational brain, yet they become able to generate astrocytes in the late stage of development. This differentiation-potential switch could be explained by epigenetic changes, since the promoters of astrocyte-specific marker genes, glial fibrillary acidic protein (*Gfap*) and *S100β*, have been shown to become demethylated in late-stage NPCs prior to the onset of astrocyte differentiation; however, whether demethylation occurs generally in other astrocyctic genes remains unknown. Here we analyzed DNA methylation changes in mouse NPCs between the mid-(E11.5) and late (E14.5) stage of development by a genome-wide DNA methylation profiling method using microarrays and found that many astrocytic genes are demethylated in late-stage NPCs, enabling the cell to become competent to express these genes. Although these genes are already demethylated in late-stage NPCs, they are not expressed until cells differentiate into astrocytes. Thus, late-stage NPCs have epigenetic potential which can be realized in their expression after astrocyte differentiation.

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Introduction

DNA methylation usually occurs in mammalian cells at CpG dinucleotides and approximately 60–90% of cytosines at these sites are methylated [1]. Most CpG-rich DNA fragments, or CpG islands in genes, have been thought to remain unmethylated even in cell types that do not express the genes [2]. However, changes in DNA methylation have been sporadically observed in CpG islands during development and are thought to play important roles in the regulation of cell type-specific gene expression [3,4]. DNA methylation also participates in the regulation of differentiation and embryonic development [5]. For example, inactivation of *Oct3/4* (*Pou5f1*) and *Nanog* genes by DNA methylation is important for early development [6,7]. These genes are essential for maintaining pluripotency of embryonic stem (ES) cells and early embryos [8,9,10] and are also known as two of four genes which have been shown to reprogram somatic cells to pluripotent stem (iPS) cells with the essential characteristics of embryonic stem (ES) cells [11].

Epigenetic modification is also thought to play an important role in altering the differentiation potential of neural precursor cells (NPCs). NPCs differentiate only into neurons in the midgestational brain, while they become able to generate astrocytes as gestation proceeds [12]. The Janus kinase (JAK) signal transducer and activator of the transcription (STAT) pathway, which is activated by cytokines, including leukemia inhibitory factor (LIF), can effectively induce astrocyte differentiation [13,14]. A particular cytosine residue within a STAT3-binding site in the astrocyte-specific marker glial fibrillary acidic protein (*Gfap*) gene promoter is highly methylated in NPCs midgestation (E11.5) when astrogenesis does not normally occur, while it becomes demethylated in late-stage (E14.5) NPCs that are prone to differentiating into astrocytes [15]. Although these late-stage (E14.5) NPCs with demethylated promoter do not express *Gfap*, the expression can be upregulated upon differentiation by leukemia inhibitory factor (LIF), which induces STAT3-activating cytokines [13,14].

Previously, we developed a genome-wide DNA methylation analysis called Microarray-based Integrated Analysis of Methylation by Isoschizomers (MIAMI) using a microarray with genome-wide probes and used for several applications [16,17]. With this method, we detected DNA methylation using the methylation-sensitive restriction enzyme *Hpa* II and its methylation-insensitive isoschizomer *Msp* I.

Although the demethylation of *Gfap* is known in late-stage NPCs prior to astrocyte differentiation [15], it is not known whether other genes confer this astrocytic property to cells. To study the role of DNA methylation in altering the differentiation potential of NPCs, here we analyzed DNA methylation changes in mouse NPCs between the mid-(E11.5) and late (E14.5) stages of development by a genome-wide DNA methylation profiling method using microarrays. We also compared the methylation status with that of postnatal day 1 (P1) astrocytes.

Results and Discussion

Genome-wide profiling of DNA methylation

Methylation changes in the differentiation potential switch of NPCs were analyzed by comparing mid-(E11.5) and late (E14.5)-stage NPCs using the MIAMI method [16]. We also compared the

methylation status with that of postnatal day 1 (P1) astrocytes. Before analysis, the key genes involved in astrocyte differentiation, such as *Gfap* and *Stat3*, were analyzed for DNA methylation in the sample we used (Fig. 1). As described previously [15], the STAT3-binding site of *Gfap* gene was demethylated in E14.5 NPCs prior to astrocyte differentiation. On the other hand, the promoter of *Stat3* gene was unmethylated throughout differentiation. The microarray used consisted of probes chosen from the Agilent promoter array using an eArray system (http://earray.chem.agilent.com/earray/). The probes are located on *Hpa* II fragments of less than 1 kilobasepair (kb) and cover 14,543 genes. Probes which showed methylation changes at least in E14.5 NPCs or astrocytes compared to E11.5 NPCs are presented in Fig. 2A and Table S1 (Name of the probes and genes were indicated in Table S1). As shown in Fig. 2B, E14.5 NPCs are hypomethylated in 85 probes (80 genes) and hypermethylated in 15 probes (15 genes). On the other hand, astrocytes are hypomethylated in 275 probes (256 genes) and hypermethylated in 170 probes (152 genes). The reliability of the analysis was confirmed by bisulfite sequencing analysis of eight genes (Fig. 3). The methylation ratio analyzed by MIAMI (Fig. 3A) had good correlation with the methylation of two *Hpa* II sites adjacent to the probes (Fig. 3B). Interestingly 80% of the probes hypomethylated in E14.5 NPCs are also hypomethylated in astrocytes. (Fig. 2C). If we extend the hypomethylation change criteria to the threshold level (D10<0.5, usually we use D10<0.2 for hypomethylation), 89% of the probes hypomethylated in E14.5 NPCs are also hypomethylated in astrocytes. In other words, 48% of the hypomethylated probes in astrocytes are also hypomethylated in E14.5 NPCs. These include a probe for an astrocyte-specific marker gene, *Gfap*, which was previously shown to be hypomethylated both in E14.5 NPCs and astrocytes [15]. However, another hypomethylated astrocyte marker, S100β [18], was not detected by the MIAMI method because the DNA sequence with methylation change does not contain any *Hpa* II sites, leading to underestimation of the total number of genes actually demethylated in E14.5 NPCs. Nevertheless, including the genes undetectable by MIAMI, many hypomethylated sequences in astrocytes are already demethylated in E14.5 NPCs, which are competent to differentiate into astrocytes. These include important genes for astrocyte-specific function or phenotype in addition to an astrocyte-specific marker gene, *Gfap*. For example, Aldolase C encodes a member of the class I fructose-biphosphate aldolase gene family specific to astrocytes (*Aldoc*, 19). The demethylated region of *Aldoc* is located on exon 1. We further confirmed this result by bisulfite sequencing and found that *Aldoc* was methylated in E11.5 NPCs and demethylated in E14.5 NPCs and astrocytes (Fig. 3B). Another example, *Kcnj10* (*Kir4.1*), is widely expressed in astrocytes throughout the brain [20]. The product of this gene is absent in immature proliferating cells, and progressive expression of the genes correlates with astrocyte differentiation, which is characterized by the establishment of a negative membrane potential and exit from the cell cycle. *Kcnj10* (*Kir4.1*) encodes a member of the inward rectifier-type potassium channel family, characterized by having a greater tendency to allow potassium to flow into, rather than out of, a cell, resulting in negative membrane potential. The encoded protein is responsible for potassium buffering action, which is a major function of astrocytes and is also responsible for promoting differentiation and inhibiting cell growth [20,21]. Mutations in this gene have been associated with seizure susceptibility of common idiopathic generalized epilepsy syndromes [22,23]. *Sparcl1* is known as an astrocyte marker colocalized with *Gfap* [24] and is also known as a candidate gene for multiple sclerosis [25]. *Cbs* and *BC055107* are also known as astrocyte markers [26,27]. Thus, we found that demethylation

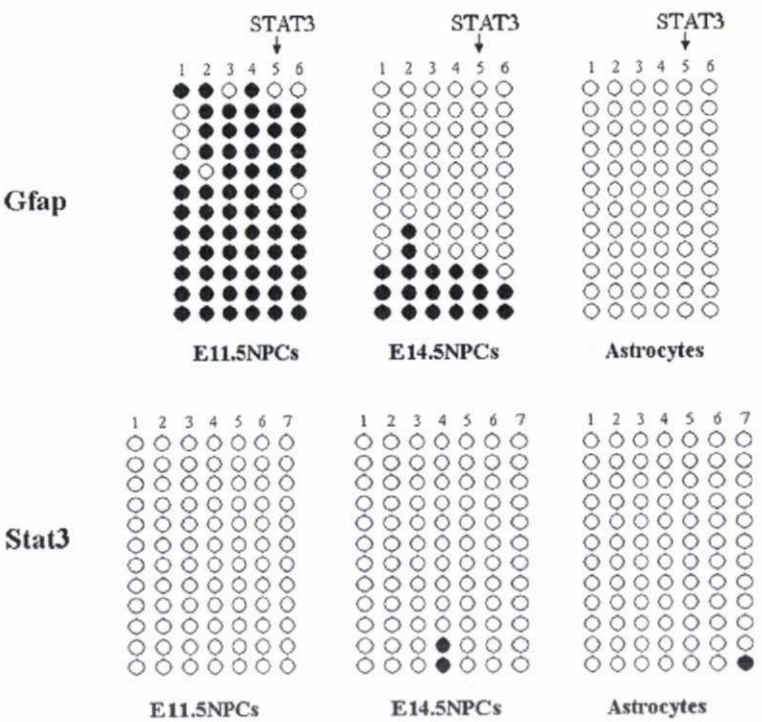

Figure 1. DNA methylation of *Gfap* and *Stat3* gene. DNA methylation was analyzed by bisulfite sequencing. Closed circles indicate methylated CpG sites and open circles indicate unmethylated CpG sites. STAT3 binding sites are indicated by arrows.
doi:10.1371/journal.pone.0003189.g001

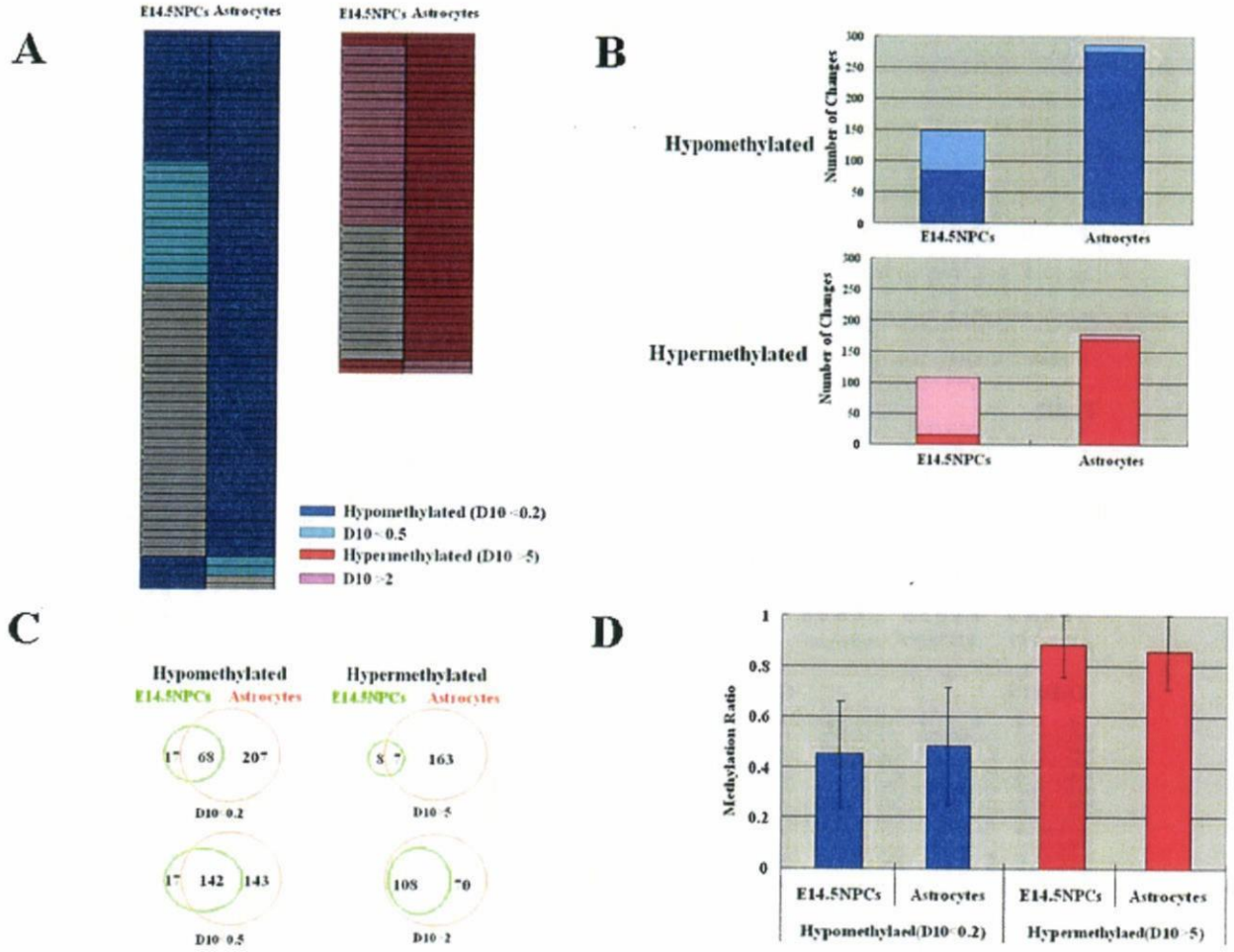

Figure 2. Genome-wide profiling of DNA methylation. (A) Hypomethylated and hypermethylated genes in E14.5 NPCs and astrocytes compared to E11.5 NPCs. Each row indicates each probe and each column indicates E14.5 NPCs or astrocytes. Probes with methylation changes are indicated in color. D10 is a value indicating the difference between methylation-sensitive *Hpa* II cleavage and methylation-insensitive *Msp* I cleavage. (B) Number of methylation changes in E14.5 NPCs and astrocytes compared to E11.5 NPCs. Numbers of methylation changes indicated in Fig. 1A were counted and indicated by bars. (C) Venn diagram of the number of probes with methylation changes in E14.5 NPCs and astrocytes compared to E11.5 NPCs. (D) Absolute levels of DNA methylation analyzed by MIAMI presented as methylation ratio. Methylation ratios of hypermethylated and hypomethylated probes are presented as average±standard deviation.
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occurred not only in *Gfap* but also in the other genes involved in astrogenesis prior to astrocyte differentiation.

When comparing methylation status between E14.5 NPCs and astrocytes, it is important to know what percentage of the E14.5 NPCs actually gives rise to astrocytes. After 4 day-culture in the presence of LIF, 30–40% of E14.5 NPCs become GFAP-positive, yet this does not necessary mean that *Gfap* promoter is methylated in all GFP-negative cells. We have previously shown that STAT site within the *Gfap* promoter was virtually unmethylated in E14.5 NPCs during 4-day culture in the proliferating condition. They, however, could not give rise to 100% of GFAP-positive cells [15]. These findings suggest the existence of other factors that inhibit astrocyte differentiation. Thus, it is conceivable that demethylation is necessary but not sufficient for NPCs to differentiate into GFAP-positive astrocytes.

Absolute levels of DNA methylation were analyzed by MIAMI and were presented as average methylation ratio for hypomethylated and hypermethylated genes compared to E11.5 NPCs (Fig. 2D). On average, hypermethylated genes were nearly fully methylated as expected. However, hypomethylated genes had some methylation on average. In addition, some hypomethylated genes, specially, *Aldoc* had highly mosaic pattern, suggesting a possibility that equilibrium state may have been reached rather than DNA demethylation (Fig. 3B).

A hallmark for Polycomb-mediated repression is methylation of lysine 27 histone H3 (H3K27), which is set up by the Polycomb repressive complex2 [28,29]. Many polycomb targets in embryonic stem cells (ESCs) have been shown to reside in a chromatin state characterized not only by the dual presence of repressive H3K27 methylation but also by active H3K4 methylation [30]. In neural differentiation, the promoters marked by H3K27 methylation in ESCs are more likely to become de novo DNA methylated [31]. Therefore, we analyzed the histone methylation status in ESCs for our hypermethylated genes in astrocytes compared to E11.5 NPCs using the published ESCs data [32]. We found that promoter that were marked by H3K27 in ESCs were significantly enriched in hypermethylated genes in astrocytes ($P<1\times10^{-14}$).

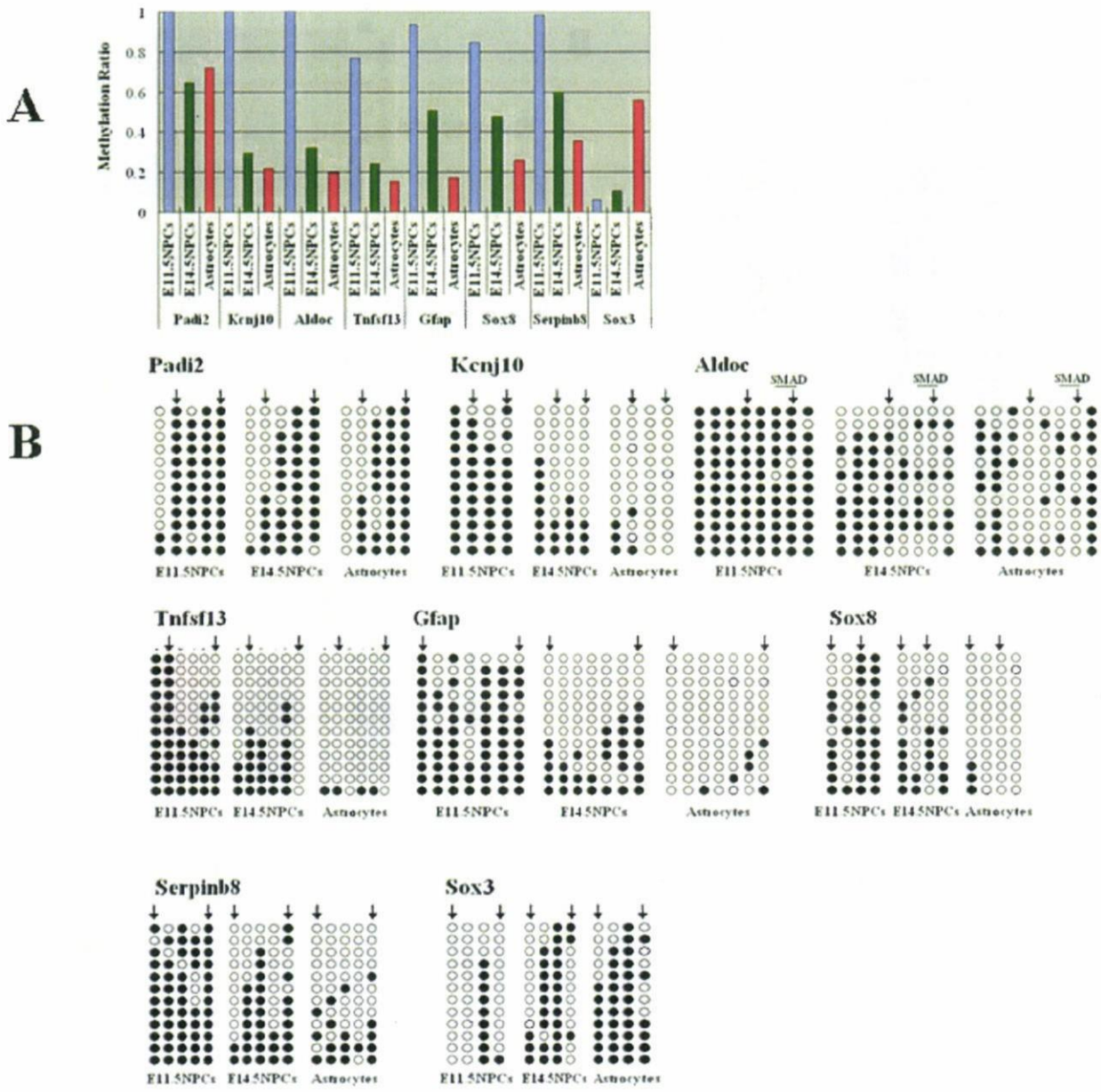

Figure 3. Confirmation of genome-wide methylation profiling by MIAMI. (A) Methylation ratios of seven hypomethylated and one hypermethylated gene compared to E11.5 NPCs. Methylation ratios are calculated by MIAMI data. (B) Confirmation of seven hypomethylated genes and one hypermethylated gene by bisulfite sequencing analysis. Closed circles indicate methylated CpG sites and open circles indicate unmethylated CpG sites. Positions of *Hpa* II sites are indicated as arrows.
doi:10.1371/journal.pone.0003189.g003

Expression changes in NPCs and astrocytes

Expression changes during the differentiation potential switch of NPCs were analyzed by comparing mid-(E11.5) and late (E14.5)-stage NPCs, and astrocytes by expression microarray analysis (Fig. 4, Table S2). We found that 49% of the probes upregulated in E14.5 NPCs compared to E11.5 NPCs were also upregulated in astroctyes (Fig. 3B). This is smaller than the ratio of hypomethylation where 80% of the probes hypomethylated in E14.5 NPCs were also hypothylated in astrocytes (Fig. 2C). This implies that astrocyte-specific genes, including *Gfap, Aldoc,* and *Kcnj10* (*Kir4.1*), are demethylated at E14.5 NPCs, while they are not expressed until cells differentiate into astrocytes. To examine this hypothesis, we analyzed the expression of genes hypomethylated both in E14.5 NPCs and astrocytes. Among these genes, those that showed upregulation in E14.5 NPCs or astrocytes compared to E11.5 NPCs are shown in Fig. 5. Of these, 64% of the genes, including astrocyte-specific marker *Gfap*, showed more than twice the expression level in astrocytes compared to E14.5 NPCs. Thus, genes already demethylated, such as *Gfap, Aldoc,* and *Kcnj10* (*Kir4.1*), were competent but not highly expressed in E14.5 NPCs,

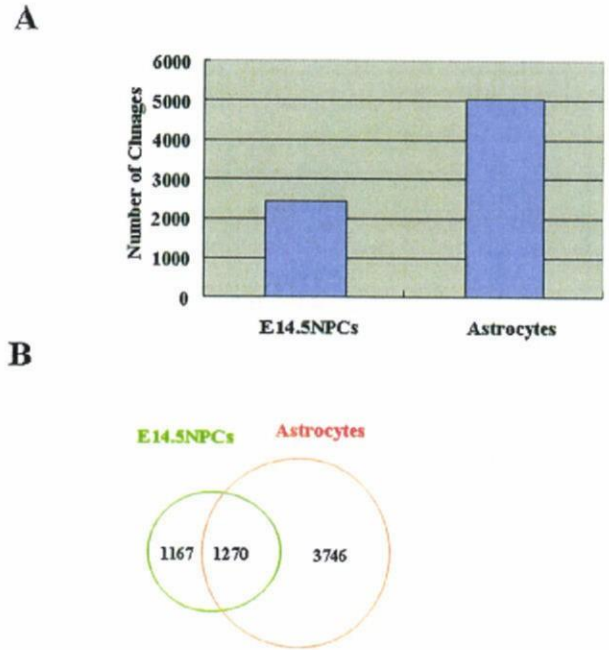

Figure 4. Expression analysis by microarray. (A) Number of upregulated genes in E14.5 NPCs and astrocytes compared to E11.5 NPCs. (B) Venn diagram of the number of upregulated genes in E14.5 NPCs and astrocytes compared to E11.5 NPCs.
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suggesting that their expression appears after differentiation into astrocytes (Fig. 6). Astrocyte-specific genes probably require the expression and/or activation of astrocyte-inducing transcription factors, such as STAT3, to be effectively expressed (Fig. 6).

To analyze the relation between the DNA methylation and the expression of genes, we plotted the methylation ratio calculated by MIAMI and the expression level calculated by expression microarray analysis for all genes examined (Fig. 7A). As expected, the methylation ratio is inversely correlated to the expression level.

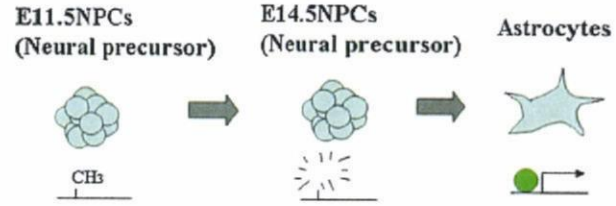

Figure 6. Model proposed for astrocyte-specific expression of the genes demethylated in E14.5 NPCs. Several astrocyte-specific genes are already demethylated in E14.5 NPCs; however, they are induced after diffentiation to astrocytes. This could be explained by astrocyte-specific transcription factors.
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Next we analyzed the average expression levels of hypermethylated and hypomethylated genes in astrocytes compared to E11.5 NPCs (Fig. 7B). Although there are some exceptions, we found the average expression levels of hypermethylated genes were low and those of hypomethylated genes were high in astrocytes. One of the reasons which could explain the existence of exceptions is the wide distribution of the probes (between −8000 to +2000 bases from the transcription start sites). Interestingly, the expression levels of hypomethylated genes were low in E14.5 NPCs compared to ones in astrocytes. This again indicates activation of expression appears after differentiation into astrocytes.

STAT and SMAD binding sites in demethylated and upregulated genes

A CpG sequence in a conserved STAT-binding site in the *Gfap* gene promoter is methylated in NPCs midgestation when astrogliogenesis does not normally commence; however, it becomes demethylated in late gestational NPCs that have gained the potential to differentiate into astrocytes [15]. Thus, the *Gfap* gene with the hypomethylated promoter can be expressed upon astrocyte differentiation induced by LIF, which activates the JAK/STAT signaling pathway [13]. Therefore, we searched for conserved STAT-binding sites in the genes hypomethylated both in E14.5 NPCs and astrocytes compared to E11.5 NPCs. To avoid omitting threshold level genes, the threshold used in E14.5 NPCs

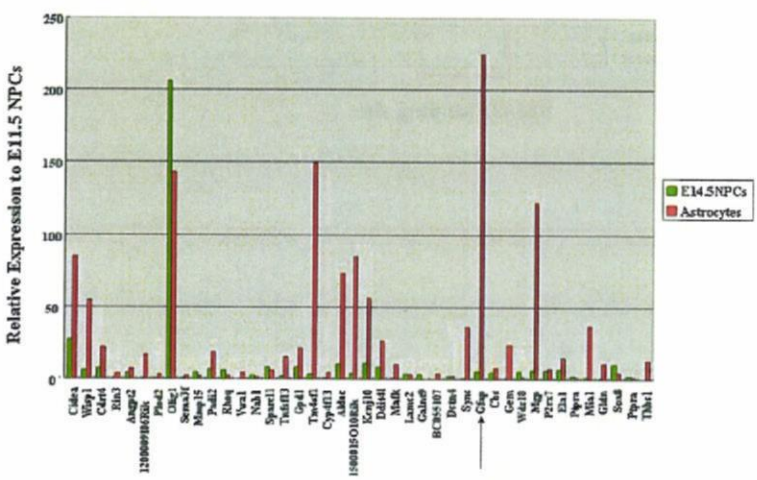

Figure 5. Expression of the genes hypomethylated both in E14.5 NPCs and astrocytes compared to E11.5 NPCs. Among hypomethylated genes, genes which showed upregulation in E14.5 NPCs or astrocytes compared to E11.5 NPCs are shown. Expression is calculated from data obtained by expression microarray analysis and presented as fold change compared to E11.5 NPCs. *Gfap* is indicated by an arrow.
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