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Developmental stage dependent regulation of DNA methylation and chromatin modification in a immature astrocyte specific gene promoter

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Abstract Astrocytes are generated from neuroepithelial cells after neurons during brain development. However, the mechanism of this sequential generation is not fully understood. Here, we show that a particular cytosine residue in the promoter of the gene encoding the immature astrocyte marker, S100β, becomes demethylated, correlating with the time when the S100β expression commences at embryonic day (E) 14. In addition, astrocyte-inducing cytokine, BMP2, increased histone acetylation around the CpG site in neuroepithelial cells at E14 but not E11 when S100β expressing astrocytes are absent. Furthermore, binding of a methyl DNA binding protein, MeCP2, to the S100β gene promoter in neuroepithelial cells was reduced at E14 compared to E11. Thus, demethylation of specific CpG site is suggested to be a critical determinant in regulating astrocyte differentiation in the developing brain.

Keywords: Neural stem cell; Astrocyte; Differentiation; Development; DNA-methylation; Chromatin modification

1. Introduction

During the development of the central nervous system (CNS), neurons and astrocytes are generated from common precursor cells present in the neuroepithelium [1–3]. In the mammalian CNS, neurons are generated primarily during the embryonic period [4]. For example, in the mouse cerebral cortex, neurogenesis commences around embryonic day 11 (E11), peaks at about E15, and finishes around birth [5,6]. While transient radial glia are present during the early stages of development, macroglial production in the cortex is delayed until mid-gestation and only occurs at low levels. While cortical astrocytes are first apparent at E16 and oligodendrocytes around birth, the vast majority of both cell types are produced during early neonatal development [7]. Although the separate timing of neurogenesis and gliogenesis in the CNS has been known for many years, the molecular mechanisms underlying these processes remain largely unknown.

The specification of cell lineages in the developing brain is thought to be regulated by cell-external cues and cell-intrinsic programs. Cell-intrinsic programs include epigenetic modification such as chromatin remodeling and DNA methylation. The cytosine in CpG dinucleotides of vertebrate genomes is prone to modification into 5-methylcytosine. This methylation has been proposed as a means of transcriptional silencing [8,9]. The establishment of normally regulated DNA methylation is essential for development [10], and abnormalities in regulating DNA methylation are frequently associated with tumorigenesis [11] and cell aging [12].

Glial fibrillary acidic protein (GFAP) and S100β are two proteins often used to monitor astrocyte differentiation and maturation. GFAP is considered the major component of glial fibrils and provides a viable index of astrocyte maturation, while S100β is a soluble calcium-binding protein synthesized in astrocytes and is known as a marker for immature astrocytes [13]. During brain development, GFAP expression in neural precursor cells is dependent on the activation of the transcription factor, signal transducer and activator of transcription 3 (STAT3) [14–16]. We have previously shown that a CpG dinucleotide within a STAT3 binding element in the GFAP promoter is highly methylated in E11.5 neuroepithelial cells, post-mitotic neurons, and cells outside the nervous system, but is demethylated in cells that exhibit STAT3-induced expression of GFAP [17]. Based on this finding, we proposed that DNA methylation is a pivotal event in regulating astrocyte differentiation during brain development. However, it remains unclear whether this developmental stage-dependent demethylation is confined to the GFAP gene promoter or is more widespread in astrocyte-specific gene promoters in the fetal brain. S100β is also known as an astrocytic marker and expressed during earlier stages of astrocytic development than GFAP. We thus determined the methylation status of the promoter for the S100β gene and profiled S100β gene expression during brain development.

2. Materials and methods

2.1. Animals and cell preparation

Time-pregnant ICR mice were used to prepare neuroepithelial cells. Mice were treated in accordance with the guidelines of Kumamoto University Center for Animal Resources and Development. Neuroepithelial cells were prepared from telencephalons of E11.5 or E14.5 mice and cultured as described previously [18]. Briefly, the telencephalons were triturated in Hank's balanced salt solution (HBSS) by mild pipetting with a 1 ml pipette tip (Gilson). Dissociated cells were cultured for four days in N2-supplemented Dulbecco's Modified Eagle's Medium with F12 (Gibco) containing 10 ng/ml basic FGF (R&D

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Systems) (N2/DMEM/F12/bFGF) on culture dishes pre-coated with poly-L-ornithine (Sigma) and fibronectin (Life Technologies).

2.2. Immunohistochemistry

Mouse embryos were fixed in a 4% paraformaldehyde PBS solution for 24 h, and serially submerged in PBS containing 10% and 20% sucrose at 4 °C for 24 h. The tissue was cut into 15 μm thick sections using a cryostat. The sections were then stained with one of the following primary antibodies: mouse monoclonal antibodies specific for S100β (Sigma) and polyclonal antibodies for MAP2 (Chemicon). The following secondary antibodies were used: Cy3-conjugated anti-mouse IgG (Jackson Laboratory), FITC-conjugated anti-rabbit (Chemicon). Nuclei were stained with bisbenzimide H33258 fluorochrome trihydrochloride (Nakaraitesque).

2.3. Immunocytochemistry

Cells were cultured on chamber slides (Nunc) either with or without BMP2 (80 ng/ml, Yamanouchi Pharmaceutical) for 4 days, washed with PBS, fixed in 4% paraformaldehyde in PBS, and stained with one of the following primary antibodies: anti-S100β (Sigma), anti-MAP2 (Chemicon). The following secondary antibodies were used: Cy3-conjugated anti-mouse IgG (Jackson Laboratory), FITC-conjugated anti-rabbit (Chemicon). Nuclei were stained with bisbenzimide H33258 fluorochrome trihydrochloride (Nakaraitesque).

2.4. RT-PCR

Total RNA was isolated from E11.5 or E14.5 neuroepithelial cells cultured either with or without BMP2 (80 ng/ml) for 4 days. Reverse transcriptions were performed using the RNA as a template and Superscript II reverse transcriptase (GibcoBRL). PCRs were performed using AmpliTaq Gold (Perkin–Elmer) with the following settings: 95 °C for 9 min; 28 cycles at 94 °C for 20 s, at 60 °C for 20 s, at 72 °C for 30 s; one cycle at 72 °C for 5 min. The following primers were used: S100β S, 5′-AGAGGACTCCAGCAGCAAAGG-3′; S100β AS, 5′-AGAGAGCTCAGCTCCTTCGAG.

2.5. Bisulfite sequencing

Sodium bisulfite treatment of genomic DNA was performed essentially as described previously [19]. Briefly, 5 μg of genomic DNA was digested with *Sac*I, denatured with 0.3 M NaOH at 37 °C for 15 min and incubated with 3.1 M sodium bisulfite and 0.5 mM hydroquinone at 55 °C for 16 h. The samples were purified using a desalting column (Promega) in accordance with the manufacturer's instructions and eluted in 50 μl of H_2O. 3 M NaOH (5.5 μl) was added and the samples were incubated at 37 °C for 15 min. The samples were first neutralized by the addition of 3 M ammonium acetate, then ethanol precipitated, and dissolved in H_2O. There are four CpG sites at positions −818, −318, −207, and −64 relative to the transcription start site. Two DNA fragments containing the former one and the latter three were amplified, respectively, by PCR using the following sets of primers: S100818MS, 5′-GTTGTTGGGAATTGAATTTAGGATTTTTGG-3′; and S100818MAS, 5′-ATCTTAAAACTCTCTCTCCCTACCCTAATC-3′; and S100MS, 5′-AAGTTGGTAGATAAGTAAGATGTTTAAGAG-3′; and S100MAS, 5′-ATCACCTTTACTACTAAAATCCTCTCTAAC-3′. The PCR products were cloned into pT7Blue and 11–13 clones were randomly chosen from each of the three independent PCRs and sequenced.

2.6. ChIP assay

Chromatin immunoprecipitation was performed as previously described [20] with some minor modifications. Cells were exposed to formaldehyde at a final concentration of 1% added directly to the tissue culture medium. Cells were centrifuged into a pellet after 10 min of formaldehyde exposure, lysed in lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris–HCl, pH 8.1) containing protease and phosphatase inhibitors [3 mM pAPMSF, 5 mg/ml aprotinine, 1 mg/ml pepstatin A (Wako Chemicals), and 2 mM sodium orthovanadate] and then incubated for 5 min on ice. Cell lysate was sonicated using a microtip until the DNA fragments were 600–1000 base pairs in length. Chromatin samples were diluted 1:10 with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, and 167 mM NaCl). 1% of the total volume was stored as input at −20 °C for later use. After the samples were pre-cleared with protein A Sepharose beads (Amersham) containing 5 μg of sonicated phage lambda DNA (Toyobo), immunoprecipitation was performed overnight at 4 °C with 1 μg of the antibody for either acetylated H3, H4 or MeCP2 (Upstate Biotechnology). Immune complexes were collected by protein A–Sepharose beads and washed with the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, and 150 mM NaCl), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, and 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris–HCl, pH 8.1) and TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0). Immune complexes were disrupted with elution buffer (1% SDS and 50 mM $NaHCO_3$) and the covalent links between the immunoprecipitates and input chromatin were reversed by incubation with 300 mM NaCl at 65 °C for 4 h. DNA was further incubated with proteinase K, purified by phenol extraction, and then ethanol-precipitated. DNA pellets were dissolved in 50 μl of H_2O and used as a template for PCR with the following set of primers. Primers for the proximal promoter region of S100β gene were as follows: S100β promS, 5′-TCCAGCACTCAGCATGAGAAG-3′; S100β promAS, 5′-GTCAGTGGCTTTCTCACCTCC-3′. Primers for the −318 CpG site of the 100β promoter region were as follows: S100β318S, 5′-TTACTGCATGCTGGTCCCTG-3′; S100β318AS, 5′-TAGAGTCAGCTTCTCTGCACC-3′.

3. Results

E11.5 or E14.5 mouse brains were fixed and sections stained for S100β and MAP2 proteins using immunohistochemistry. S100β positive cells were detected in the subventricular layer of the telencephalic cortex of E14.5 brains (Fig. 1F), but not in the E11.5 brain, except for non-neural tissues such as the brain meninges (Fig. 1B). MAP2 positive cells were observed in the E11.5 preplate and E14.5 cortical plate (Fig. 1A and E). No GFAP positive cells were observed in these sections (data not shown). These results demonstrate developmental stage dependent expression of S100β in the fetal mouse brain.

We have previously shown that cells expressing S100β develop from E14.5 mouse neuroepithelial cells after being cultured with BMP2 for 2 days [21]. This suggests that BMP2 plays an important role in the development of S100β positive cells from neural progenitors. To determine whether the responsiveness of neural progenitors to BMP2 is dependent on the developmental stage, neuroepithelial cells were acutely dissociated from E11.5 and E14.5 telencephalons and cultured either with or without BMP2 for 4 days, and S100β and MAP2 proteins were labeled using immunohistochemistry and S100β mRNA levels analyzed by RT-PCR.

As shown in Fig. 1J, S100β positive cells were induced by BMP2 in a culture of acutely prepared E14.5 neuroepithelial cells. S100β mRNA was also induced in the same culture (Fig. 1M, lane 2). These results suggest that E14.5 neuroepithelial cells express S100β following exposure to BMP2. In contrast, no S100β immunoreactivity (Fig. 1L) and mRNA were detected in E11.5 neuroepithelial cells cultured under the same conditions (Fig. 1M, lane 4). These results indicate that the responsiveness of neural progenitors to BMP2 is dependent on the developmental stage. BMP receptor expression in E11.5 neuroepithelial cells was confirmed by RT-PCR analysis (data not shown). The lack of S100β expression in BMP2-stimulated E11.5 neuroepithelial cells may be due to inactivation of the promoter region of the S100β gene. Using bisulfite genomic sequencing analysis of acutely prepared or 4-day cultured neuroepithelial cells from either E11.5 or E14.5 telencephalons, we determined the methylation status of the

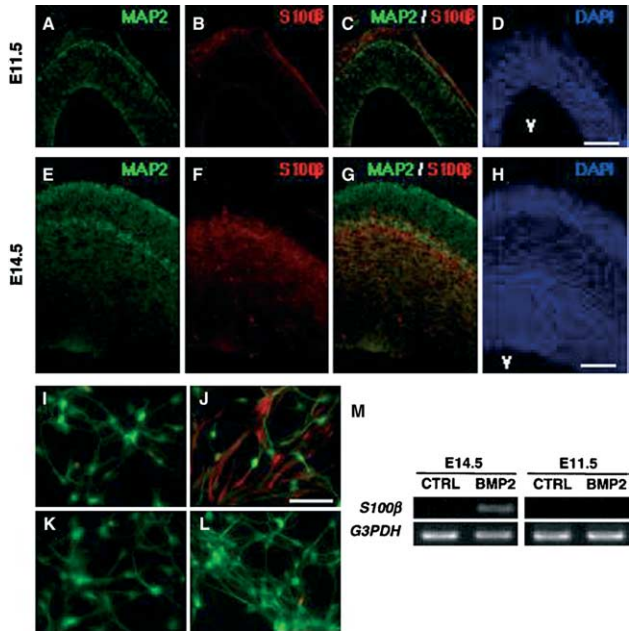

Fig. 1. S100β expression and its responsiveness to BMP signal are controlled by the developmental stage of the embryonic mouse brain. (A)–(H) Double immunofluorescence labeling of the proteins S100β (B and F, red) and MAP2 (A and E, green) in the cortex of E11.5 or E14.5 mouse brains. MAP2 immunoreactivity was detected in the cortex of both E11.5 and E14.5 mice. S100β immunoreactivity was only detected in the intermediate zone of the cortex in E14.5 mice. Superimposed views of A and B are shown in C, and superimposed views of E and F are shown in G. DAPI staining indicates nuclei (D, H). V indicates the ventricle. Bar = 50 μm. (I–L) Neuroepithelial cells prepared from E14.5 or E11.5 mouse telencephalons were cultured in the presence (J, L) or absence (I, K) of BMP2 (80 ng/ml) for 4 days. Cells were stained with antibodies for the astrocytic marker S100β (red) or the neuronal marker MAP2 (green). No S100β-positive astrocytes were induced by BMP2 in cultured E11.5 neuroepithelial cells (L), but were induced in E14.5 neuroepithelial cells under the same conditions (J). Bar = 50 μm. (M) Total RNA was extracted from E11.5 or E14.5 neuroepithelial cells cultured in either the presence or absence of BMP2 (80 ng/ml) for 4 days, and was analyzed by RT-PCR using specific primer for S100β and G3PDH.

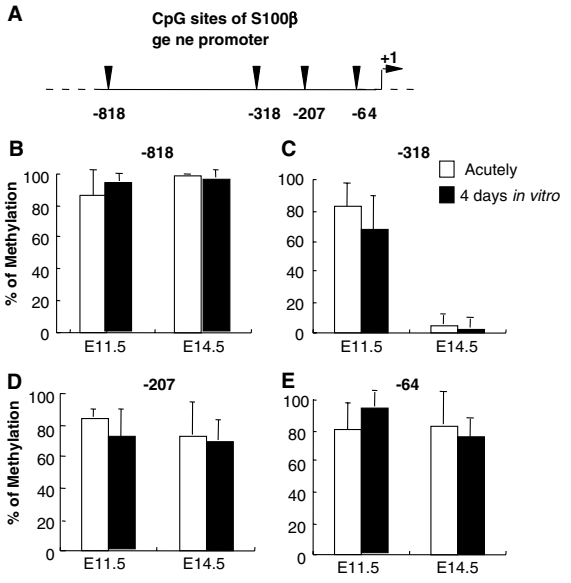

Fig. 2. Specific CpG site in a promoter region of S100β is demethylated during development. (A) CpG sites of the S100β gene promoter. Potential methylation sites are shown by arrowheads. Numbering begins with the transcription initiation site as +1. (B)–(D) The frequency of methylation at each potential methylation site in the S100β promoter gene was investigated using the bisulfite sequencing method. In this assay, either freshly prepared or 4-day cultured neuroepithelial cells from E11.5 or E14.5 mouse telencephalon were used.

four CpG dinucleotides present in an 860 base pair region of the S100β gene promoter just upstream of the transcriptional start site.

An 860 base pair-region within the mouse S100β promoter was analyzed. There are four CpG sites at −818, −318, −207, and −64 bases relative to the transcriptional start site (Fig. 2A). Methylation in each cell group was determined by sequencing 11 to 13 clones prepared from bisulfite-treated genomic DNA. The experiments were performed three times and the methylation frequency was calculated from the triplicate cell preparation. As shown in Fig. 2B-E, all four of the CpG sites were highly methylated in both acutely prepared and 4-day cultured E11.5 neuroepithelial cells, which express neither S100β in vivo nor S100β in vitro following exposure to BMP2. It is notable that the methylation frequency at the −318 CpG site was significantly reduced in E14.5 neuroepithelial cells (Fig. 2C), which normally express S100β in vivo and S100β in vitro after exposure to BMP2. In contrast, the methylation frequency remained high at the other three CpG sites. Hence, the methylation status of the CpG dinucleotide at −318 correlates with S100β expression levels in neuroepithelial cells in vivo and S100β expression in cultured neuroepithelial cells following stimulation by BMP2.

It is known that DNA methylation contributes to transcriptional silencing of gene expression both by interfering with the accessibility of transcription factors to their target DNA and by recruiting methyl DNA binding domain-containing proteins, which trigger the formation of inactive chromatin structures [8]. Since we did not detect any of the previously reported Smad-binding consensus sequences [22–24] around the −318 CpG site in the S100β gene promoter, methylation of this site in E11.5 neuroepithelial cells may contribute to the formation of inactive chromatin structures to inhibit transcriptional activation of the S100β gene. Among the many proteins that bind to methylated DNA, MeCP2 characteristically binds to single, symmetrical methylated CpG pairs in any sequence context and is linked to gene silencing by recruiting histone deacetylases (HDACs) and co-repressors, including mSin3A [25–27]. We thus investigated whether MeCP2 participates in the regulation of S100β gene repression.

ChIP was performed with E11.5- or E14.5-telencephalon-derived neuroepithelial cells using an anti-MeCP2 antibody and PCR primers to detect a DNA fragment spanning −550 and −250 in the S100β gene promoter. As shown in Fig. 3A and B, a significant level of MeCP2 binding to the −318 CpG-containing part of the S100β gene promoter was detected in E11.5-telencephalon-derived cells, but was less apparent in E14.5 cells. Downregulation of MeCP2 binding to this part of the promoter in E14.5 cells appears to be in accordance with the reduction in methylation frequency at the −318 CpG site. This result suggests that the binding of MeCP2 to the methylated −318 CpG site within the S100β gene promoter in E11.5 cells inactivates the gene.

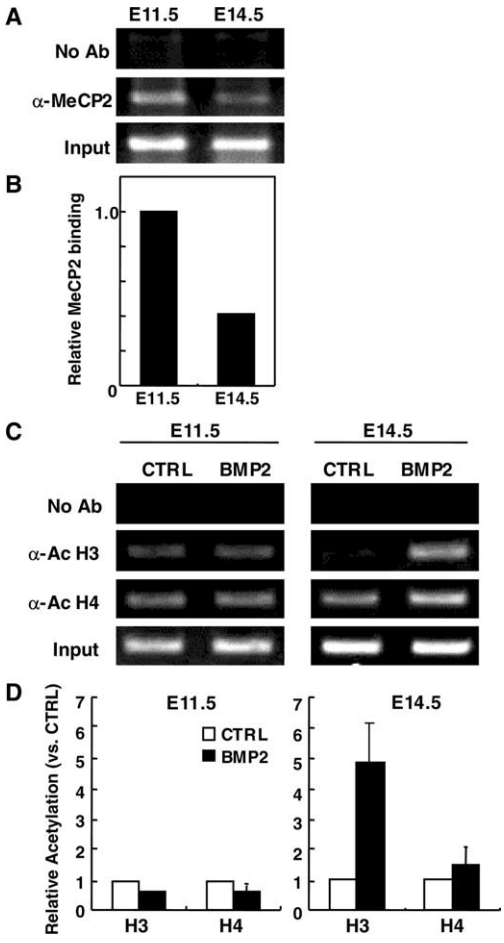

Fig. 3. The inactive status of the chromatin structure of the S100β promoter region in E11.5 neuroepithelial cells. (A and B) The abundant binding of MeCP2 to the methylated −318 CpG site within the S100β gene promoter region in E11.5 cells. (A) Results of an ChIP assay performed with an antibody for MeCP2 in E11.5 or E14.5 neuroepithelial cells cultured with bFGF for 4 days. (B) Quantification of MeCP2 ChIP PCR results. The results were scanned and analyzed by NIH Image. The signal intensity of each PCR band was measured and normalized by the Input. The signal intensity of the band detected in E11.5 cells was arbitrarily chosen as 1.0 and the MeCP2 binding levels of each ChIP product were referred to as a multiple of that value. (C and D) The chromatin structure of the S100β gene promoter in E11.5 neuroepithelial cells was in the inactivated state for interference with histone acetylation following stimulation with BMP2. (C) Chromatin immunoprecipitation assay performed using formaldehyde cross-linked moderately sonicated chromatin from non-stimulated or BMP2-stimulated (80 ng/ml) neuroepithelial cells. The DNA fragments of the S100β promoter were immunoprecipitated using an antibody for acetylated H3 and H4, and detected using PCR. (D) Quantification of the PCR products from chromatin preparation. The results of three experiments were scanned and analyzed by NIH Image. The intensity of each PCR band was measured and normalized by the Input. The signal of the band detected in CTRL (open bar) of E11.5 or E14.5 cells was arbitrarily chosen as 1.0 and the acetylation levels of the ChIP products of BMP2 stimuli (closed bar) were referred to as a multiple of that value.

To provide an indication of gene activation, we analyzed the S100β gene promoter in E11.5 and E14.5 neuroepithelial cells cultured for 4 days either with or without BMP2 for histone acetylation. ChIP assays were performed using an antibody specific to the acetylated histones H3 or H4, and a set of PCR primers to detect the promoter fragment spanning −362 and +11. BMP2 stimulation failed to induce acetylation of histones H3 or H4 at the S100β gene promoter in E11.5-derived cells (Fig. 3C and D, left-hand panels). This result suggests that in E11.5 neuroepithelial cells, the chromatin structure of the S100β gene promoter is in the inactivated state and cannot be modified by BMP2 stimulation. In marked contrast, exposure of E14.5-derived neuroepithelial cells to BMP2 for 4 days resulted in a significant increase in acetylation of histones H3, and to a lesser extent, H4 in the S100β gene promoter compared with non-treated cells (Fig. 3C and D, right-hand panels). TGF-β proteins activate transcription through both a physical and functional interaction of DNA binding Smads with other sequence-specific transcription factors as well as the coactivators CBP and p300. These coactivators possess (and can also recruit proteins with) histone acetyltransferase activity and acetylation of histones loosens the chromatin structure facilitating gene transcription. Thus, these coactivators may contribute to acetylation of histones in the S100β gene promoter following BMP2 stimulation and lead to sufficient transcriptional activation. The mechanism underlying BMP2-induced acetylation of histones in the S100β gene promoter in E14.5 neuroepithelial cells remains to be elucidated. Methylation of the −318 CpG site may keep a transcription factor complex away from the S100β gene promoter in E11.5 neuroepithelial cells due to the inactive status of the chromatin structure. Taken together, our results suggest that site-specific DNA demethylation of the S100β gene promoter during embryogenesis is required for S100β expression throughout brain development.

4. Discussion

In the mammalian brain, the majority of neurons are born before glia cells develop [4]. A major question is therefore how neural progenitors in the neuroepithelium show such developmental stage dependent changes in the preference of cell-fate determination, i.e., from neurogenesis in the earlier stages to gliogenesis in later stages. In the present study, we show that CpG dinucleotides in the promoter region of the gene for immature astrocyte-specific protein S100β in E11.5 neuroepithelial cells, which express S100β neither in vivo nor in vitro after exposure to BMP2, are highly methylated and the chromatin structure in the S100β promoter region is in the inactive state. At the later developmental stage of E14.5, only one of the four CpG dinucleotides examined is substantially demethylated, and most likely facilitates BMP2-mediated induction of S100β expression. These results indicate that DNA demethylation of the specific CpG dinucleotide in the S100β gene promoter coincides with its expression in neural progenitors. Although we detected acetylation of histones H3 and H4 in the S100β gene promoter of 4-day cultured E11.5 cells not stimulated by BMP2, S100β expression was not detected in the E11.5 cells. This result suggests that negative regulators of astrocyte differentiation such as Olig2 [28] or neurogenin1 [29] may also contribute to the inhibition of astrocyte-specific S100β expression in E11.5 neuroepithelial cells through different mechanisms from the methylation of specific gene promoters.

In the present study, we show that S100β expression, which is known to be an early marker for astrocyte differentiation, is efficiently induced in E14.5 neuroepithelial cells by BMP2 and that S100β positive cells are present in the subventricular layer of the telencephalic cortex in the E14.5 brain, but no GFAP

positive cells are present. Previous studies have shown that GFAP expression in neural precursor cells is dependent on the activation of STAT3 [14–16]. Furthermore, we have shown that the CpG dinucleotide within a particular STAT3 binding element in the GFAP promoter is highly methylated in E11.5 neuroepithelial cells, post-mitotic neurons and cells outside the nervous system, but is demethylated in cells where STAT3 induces the expression of GFAP [17]. GFAP is known as an index of astrocyte maturation [13]. Our data on the S100β gene promoter support the idea that DNA demethylation of astrocyte specific gene promoters in neural progenitors is a key event during mid-gestation as part of a cell-intrinsic program for astrogenesis. Thereafter, cell-external cues such as BMP2 may help neural progenitors to switch from neurogenesis to astrogenesis and result in STAT3 activation signals promoting maturation of astrocytes in the last stage of CNS development.

In this paper, we show that MeCP2 binds to the specific methylated CpG site within the S100β gene promoter and suggest that the binding of MeCP2 inactivates the gene in E11.5 neuroepithelial cells. It has been reported that regulation of the interaction between MeCP2 and methylated DNA is critical for neurogenesis during Xenopus development and for neuronal activity-dependent recent BDNF expression [30–32]. Thus, it is becoming increasingly apparent that MeCP2-binding to the methylated CpG site in a specific promoter plays a key role in the development of CNS and in the establishment of functional neural network. More studies are needed to fully explain how methylation or demethylation of specific gene promoters is regulated.

In conclusion, taken together with our previous report on the GFAP gene promoter [17], methylation of cell type-specific gene promoters may be a pivotal event in regulating lineage specification during brain development.

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