49. **Wei Q, Miskimins WK, Miskimins R** (2004) Sox10 acts as a tissue-specific transcription factor enhancing activation of the myelin basic protein gene promoter by $p27^{Kip1}$ and Sp1. J Neurosci Res 78: 796–802.

50. **Nishiyama H, Takemura M, Takeda T, Itohara S** (2002) Normal development of serotonergic neurons in mice lacking S100B. Neurosci Lett 321: 49–52.

51. **Jessen KR, Mirsky R** (2010) Control of Schwann cell myelination. F1000 Biol Rep 2: 19.

52. **Kao SC, Wu H, Xie J, Chang CP, Ranish JA, et al.** (2009) Calcineurin/NFAT signaling is required for neuregulin-regulated Schwann cell differentiation. Science 323: 651–654.

53. **Marenholz I, Heizmann CW, Fritz G** (2004) S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). Biochem Biophys Res Commun 322: 1111–1122.

STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

HMGN Family Proteins Promote Astrocyte Differentiation of Neural Precursor Cells

Motoshi Nagao[1,*], Darin Lanjakornsiripan[2], Yasuhiro Itoh[2], Yusuke Kishi[2], Toru Ogata[1] & Yukiko Gotoh[2]

[1]Department of Rehabilitation for the Movement Functions, Research Institute, National Rehabilitation Center for Persons with Disabilities, 4-1 Namiki, Tokorozawa, Saitama 359-8555, Japan; [2] Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

*Correspondence: Motoshi Nagao Ph.D., Department of Rehabilitation for the Movement Functions, Research Institute, National Rehabilitation Center for Persons with Disabilities, 4-1 Namiki, Tokorozawa, Saitama 359-8555, Japan, Phone, +81-4-2995-3100; Fax, +81-4-2995-3132; E-mail, nagao-motoshi@rehab.go.jp

Received January 24, 2014; accepted for publication June 23, 2014;

1066-5099/2014/$30.00/0

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/stem.1787

Key words. Astrocytes • Neural stem cells • Chromatin • HMGN proteins • STAT3 transcription factor

ABSTRACT

Astrocytes are the most abundant cell type in the mammalian brain and are important for the functions of the central nervous system (CNS). Although previous studies have shown that the STAT signaling pathway or its regulators promote the generation of astrocytes from multipotent neural precursor cells (NPCs) in the developing mammalian brain, the molecular mechanisms that regulate the astrocytic fate decision have still remained largely unclear. Here, we show that the high mobility group nucleosome-binding (HMGN) family proteins, HMGN1, 2 and 3, promote astrocyte differentiation of NPCs during brain development. HMGN proteins were expressed in NPCs, Sox9$^+$ glial progenitors and GFAP$^+$ astrocytes in perinatal and adult brains. Forced expression of either HMGN1, 2 or 3 in NPCs in cultures or in the late embryonic neocortex increased the generation of astrocytes at the expense of neurons. Conversely, knockdown of either HMGN1, 2 or 3 in NPCs suppressed astrocyte differentiation and promoted neuronal differentiation. Importantly, overexpression of HMGN proteins did not induce the phosphorylation of STAT3 or activate STAT reporter genes. In addition, HMGN family proteins did not enhance DNA demethylation and acetylation of histone H3 around the STAT-binding site of the *gfap* promoter. Moreover, knockdown of HMGN family proteins significantly reduced astrocyte differentiation induced by gliogenic signal ciliary neurotrophic factor (CNTF), which activates the JAK-STAT pathway. Therefore, we propose that HMGN family proteins are novel chromatin regulatory factors that control astrocyte fate decision/differentiation in parallel with or downstream of the JAK-STAT pathway through modulation of the responsiveness to gliogenic signals. STEM CELLS *2014; 00:000–000*

INTRODUCTION

Astrocytes are associated with diverse functions such as recycling neurotransmitters, storing energy, forming the blood-brain barrier and regulating synapse formation in the CNS [1-3]. Astrocytes, as well as neurons and oligodendrocytes, arise from NPCs. Astrogenesis occurs towards the end of the neurogenesis period [4]. Although recent findings have shown that astrocyte development is regulated by extrinsic signals such as growth factors/cytokines and cell-intrinsic programs such as chromatin modifications [5-8], the molecular mechanisms of the astrocyte fate decision are still poorly understood.

It has been well established that activation of STAT signaling promotes astrocyte differentiation [9-12]. Indeed, the interleukin-6 (IL-6) family of cytokines including leukemia inhibitory factor (LIF), CNTF and cardiotrophin-1 (CT-1) that activates the JAK-STAT pathway has been shown to promote astrocyte differentiation. Moreover, astrocyte differentiation in the developing CNS is accompanied by the increased expression and activation of the STAT signaling components [13], and the deletion of gp130, the co-receptor of the IL-6 family receptors, results in impaired astro-

cyte differentiation [14]. Furthermore, the bone morphogenetic protein (BMP) and Notch pathways cross-talk with the JAK-STAT pathway and promote astrocyte differentiation [15-17]. DNA methylation at astrocytic gene loci maintained by Dnmt1 is essential for antagonizing STAT signaling during the neurogenic phase, and the Notch-NFIA pathway has been shown to overcome this effect of Dnmt1 in inducing astrocyte differentiation [18-21]. Other molecules unrelated to STAT signaling have also been implicated in the regulation of astrocyte differentiation, including Sox9, SCL, REST, SRF, MEK-Etv5 and Nfe2l1 [22-29]. However, it remains largely unclear how these factors regulate astrocyte differentiation.

HMGN family proteins (HMGN1-5) are architectural non-histone chromosomal proteins that bind to nucleosomes and modulate the structure and function of chromatin [30]. HMGN family proteins compete with the linker histone H1 for chromatin binding sites and reduce the H1-mediated compaction of the chromatin fiber [30]. Although HMGN1, 2 and 3 are highly expressed in the CNS [31-33], their roles in brain development are unknown.

In this study, we found that HMGN family proteins promote astrocyte differentiation in neocortical NPCs. Consistent with this observation, HMGN family proteins are expressed in NPCs, glial progenitors and astrocytes in the perinatal and adult brains. Given that knockdown of HMGN family proteins reduced astrocyte differentiation induced by CNTF, HMGN family proteins appear to regulate astrocyte differentiation through modulation of the responsiveness to gliogenic signals.

Materials and Methods

Plasmid constructs

The retrovirus vectors pMX-IRES-EGFP (pMXIG) [34] and pcUXIE were kindly provided by T. Kitamura and H. Song, respectively. pCAG-IRES-EGFP (pCAGIG) was a kind gift from C.L. Cepko and T. Matsuda. Mouse Hmgn1, Hmgn2 and Hmgn3b cDNAs were amplified by PCR from a cDNA library prepared from mouse NPCs. Hmgn3a cDNA was purchased from OriGene. STAT3-C construct was kindly provided by J.F. Bromberg. An activated mutant of Notch1 was described previously [17]. These cDNAs were subcloned into pMXIG, pcUXIE and pCAGIG. The pSIREN-shLuc (control short hairpin (sh) RNA), pSIREN-shHmgn1 (Hmgn1 shRNA), pSIREN-shHmgn2 (Hmgn2 shRNA) and pSIREN-shHmgn3 (Hmgn3 shRNA) retroviral constructs were generated as specified by the manufacturer (BD Biosciences and Clontech). The target sequences were as follows: Hmgn1 shRNA#1: 5′-GCGGGAAAGGATAAAGCATCA-3′, #2: 5′-GCATCCATCACGTCTGTCAGT-3′; Hmgn2 shRNA#1: 5′-GCGAAGAAGGGAGAGAAGGTA-3′, #2: 5′-GGTTTCCCAGTCCATTGAGTG-3′; Hmgn3 shRNA#1: 5′-GGAACAAAGATTAGCAGAGGT-3′, #2: 5′-GCAAATACCAATTTCACATCG-3′. All these viral vectors were transfected into the packaging cell line Plat-E or Plat-GP to produce retroviruses as described previously [34].

Animals

All female mice were maintained according to protocols approved by the Animal Care and Use Committee of the University of Tokyo.

Cell culture

Neurosphere culture was performed as described previously [17, 35]. The cells obtained from the forebrains of mouse embryos at E11.5 and E16.5 were cultured in DMEM/F-12 (1:1) (Life Technologies) supplemented with the B-27 supplement (Life Technologies), 20 ng/ml fibroblast growth factor 2 (FGF2) (Life Technologies), 20 ng/ml epidermal growth factor (EGF) (Millipore) and 2 □g/ml heparin (Sigma-Aldrich).

To infect cells with virus, primary neurospheres were dissociated at day 2 after seeding and replated to yield secondary spheres. The cells were harvested at day 4 and subjected to retrovirus infection by incubating with high-titer virus to yield 60-80% infection efficiency. In the differentiation assay, the virus-infected neurospheres were dissociated and seeded on poly-D-lysine (100 □g/ml, Sigma-Aldrich)-coated glass chambers, and subsequently the cells were cultured for 6 days without FGF2 and EGF and subjected to immunostaining. Clonal analysis was performed as described previously [36]. In the proliferation assay, dividing cells were labeled with 5-ethynyl-2′-deoxyuridine (EdU 4 □M, Life Technologies) for 2 h. In some experiments, cells were treated with 50 ng/ml CNTF (Sigma-Aldrich). The quantitative results are expressed as mean ± standard deviation (SD) of 3-5 independent culture experiments, and statistical analyses were performed with a two-tailed unpaired t-test.

Immunostaining and histological analysis

Sixteen □m-thick cryosections were prepared from mouse embryonic and postnatal forebrains, and adult mouse brains. Immunostaining of brain sections and cultured cells was performed as described previously [17, 35] using the following antibodies: TuJ1 (mouse, 1:5000; Covance); GFAP (mouse, 1:1000; Millipore, and rabbit, 1:1000; Dako); S100β (rabbit, 1:500; Dako); O4 (mouse IgM, 1:1000; Millipore); Sox9 (rabbit, 1:1000; Millipore, and goat, 1:100; Santa Cruz Biotechnology); Sox10 (goat, 1:500; Santa Cruz Biotechnology); Sox2 (goat, 1:200; Santa Cruz Biotechnology); GFP (rabbit, 1:2000; MBL, and chicken, 1:2000; Abcam); HMGN1 (rabbit, 1:10000; Abcam); HMGN2 (mouse, 1:10000; Millipore); HMGN3 (rabbit, 1:100; Santa Cruz Biotechnology); Cux1 (rabbit, 1:200; Santa Cruz Biotechnology); cleaved caspase3 (rabbit, 1:1000; Cell Signaling Technology); Tbr2 (rabbit, 1:2000; Abcam); NeuN (mouse, 1:200; Millipore, and rabbit, 1:1000; Millipore); CC1 (mouse, 1:500; Millipore); MBP (rabbit, 1:200; Millipore); MAG (mouse, 1:400; Millipore); Doublecortin

(Dcx) (goat, 1:200; Santa Cruz Biotechnology). EdU labeling and staining were performed using the Click-iTEdU Imaging Kit (Life Technologies). Immunoreactive cells were visualized by staining with secondary antibodies conjugated with Alexa Fluor 488 and 594 (1:400; Life Technologies). Cell nuclei in cultured samples and in tissue sections were stained with Hoechst 33342 (Sigma-Aldrich), TOTO-3 or TO-PRO-3 (Life Technologies). *In utero* electroporation experiments, NPC differentiation in the cortex of P7 mouse brains was examined by counting the number of marker$^+$/GFP$^+$ cells in defined areas (1.2 mm x 1.2 mm = 1.44 mm^2). From each animal, 2-5 coronal sections were chosen to cover comparable regions of the anterior and posterior parts of the forebrain.

In utero and postnatal electroporation

For *in utero* electroporation, introduction of plasmid DNA into neuroepithelial cells of the mouse embryonic brain was performed as described previously [36]. pCAGIG-HMGN vectors were injected at a concentration of 2.5 mg/ml into the lateral ventricle of mouse brain at E16.5. Electrodes were positioned at the flanking ventricular regions of each embryo, and four pulses of 40 V for 50 ms were applied at intervals of 950 ms with an electroporator (CUY21E; Tokiwa Science). The brains were isolated at postnatal day 7 (P7). For postnatal electroporation, pSIREN knockdown vectors for HMGN proteins were injected at a concentration of 2.5 mg/ml into the lateral ventricle of P0 mouse pups. Eight pulses of 80 V for 50 ms were applied at intervals of 950 ms. The brains were isolated at P3 and P7.

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was obtained using TRIzol (Life Technologies). Reverse transcription (RT) was performed with 1 Eg of total RNA and ReverTra Ace qPCR RT kit (TOYOBO). The resulting cDNA was subjected to real-time PCR in a Roche LightCycler with SYBR Premix Ex Taq (Takara). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The following sense and antisense primers were used: mouse HMGN1: 5′-GCGGGAAAGGATAAAGCATCA-3′ and 5′-CTCTGTGGTTTGCTGGTC-3′; mouse HMGN2: 5′-AACCAAGGTGAAGGACGA-3′ and 5′-GTTTGGGCTCTGGCTTT-3′; mouse HMGN3: 5′-ACACAGAGGGCAAAGATG-3′ and 5′-GGAACAGGTTTCGCGGA-3′; mouse GAPDH: 5′-TGGGTGTGAACCACGAG-3′and 5′-AAGTTGTCATGGATGACCTT-3′.

Western blot analysis

The expression of pSTAT3-Y705, STAT3 and HMGN family proteins was examined by Western blot analyses as described previously [17] using the following antibodies: pSTAT3-Y705 (rabbit, 1:1000; Cell Signaling Technology); STAT3 (rabbit, 1:1000; Millipore); HMGN1 (rabbit, 1:10000; Abcam); HMGN2 (mouse, 1:10000; Millipore); and HMGN3 (rabbit, 1:100; Santa Cruz Biotechnology). GAPDH (mouse, 1:1000; Millipore) was used as an internal control.

Luciferase assay

Gfap promoter-Luc (GF1L-pGL3) [15] was kindly provided by K. Nakashima. NPCs were transfected with STAT-dependent (4 x APRE) Luc plasmid or *gfap* promoter-Luc plasmid, expression plasmids for HMGN family proteins or STAT3-C and the pRL-SV40 plasmid encoding Renilla luciferase (Promega) with the use of Lipofectamine 2000 (Life Technologies). The cells were cultured for 24 h with FGF2 and EGF, and then without FGF2 and EGF for 24 h. Cell extracts were subsequently prepared and assayed for luciferase activity (Promega). Firefly luciferase activity was normalized relative to the activity of Renilla luciferase.

Bisulfite sequencing

Genomic DNA was isolated from the virus-infected NPCs using the DNeasy Blood & Tissue kit (Qiagen) and was treated with sodium bisulfite with the EpiTect Bisulfite kit (Qiagen). The region in the *gfap* promoter containing the STAT-binding site of the bisulfite-treated genomic DNA was amplified by PCR using the following primers: 5′-GGGATTTATTAGGAGAATTTTAGTAAGTAG-3′ and 5′-TCTACCCATACTTAAACTTCTAATATCTAC-3′. The PCR products were cloned into pGEM-T vector (Promega), and at least 12 randomly selected clones were sequenced. Sequence data were analyzed using the QUMA quantification tool for methylation analysis.

Chromatin immunoprecipitation assay

NPCs were cultured without FGF2 and EGF for 2 days. The cells were fixed in a 0.5% paraformaldehyde solution, suspended in lysis solution (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1]) and sonicated to shear genomic chromatin into DNA fragments of ~0.5 to 1.0 kb. The lysate was incubated overnight at 4°C with anti-acetylated (K9/K14) histone H3 (Millipore) and normal rabbit IgG (Santa Cruz Biotechnology) antibodies. After the addition of Dynabeads Protein A (Life Technologies), the mixture was incubated with rotation for 1 h. The beads were then isolated and washed consecutively with a low-salt solution (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), a high-salt solution (0.1%SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), a LiCl solution (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]), and twice with a Tris-EDTA solution (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Immune complexes were then eluted from the beads with a solution containing 10 mM dithiothreitol, 1% SDS and 0.1 M NaHCO3, after which NaCl was added to a final concentration of 0.2 M and the eluate was incubated at 65°C overnight. The proteins were eliminated by digestion with proteinase K at

45°C for 1 h, and the DNA was purified with a QIAquick spin column (Qiagen). The eluted DNA was subjected to real-time PCR in a Roche LightCycler with SYBR Premix Ex Taq (Takara). The amount of target genome DNA was normalized relative to that of the input. The following sense and antisense primers were used: *gfap* promoter: 5′-ACCTTGGCATAGACATAATGG-3′ and 5′-TAGACTTCTCGGAAAGCATCG-3′; *gapdh* promoter: 5′-TGCAGTCCGTATTTATAGGAACC-3′ and 5′-CTTGAGCTAGGACTGGATAAGCA-3′; *Mage-a2*: 5′-GGACTCTGCGCCATTTTGTTCTGG-3′ and 5′-GCTAGGCAGGCTAAAGGTTGACC-3′.

Microarray analysis

NPCs derived from E11.5 mouse forebrains were infected with control (GFP alone) or HMGN1 virus. Three days after infection, the virus-infected neurospheres were dissociated and seeded on poly-D-lysine-coated dishes, and subsequently the cells were cultured for 1 day without FGF2 and EGF. The sample preparation and microarray analysis were carried out by Cell Innovator Inc. with Affymetrix Gene-Chip Mouse gene 1.0 ST Array. The microarray data are available in the Gene Expression Omnibus (GEO) database (accession number: GSE57534). The lists of up-regulated genes (Z-score ≥ 2.0 and ratio ≥ 1.50) and down-regulated genes (Z-score ≤ −2.0 and ratio ≤ 0.66) were uploaded into Database for Annotation, Visualization and Integrated Discovery (DAVID), and Gene ontology (GO) and pathway analyses were performed.

Results

HMGN family proteins are expressed in NPCs, glial progenitors and astrocytes in perinatal and adult brains

Although it has been reported that HMGN1, 2 and 3 are expressed in the mouse brain and spinal cord [31-33], the detailed expression patterns of HMGN proteins in the mouse brain have not been described. We thus performed immunohistochemistry of mouse forebrains at embryonic day (E) 18.5 and found that HMGN1, 2 and 3 were highly expressed in the ventricular zone/subventricular zone (VZ/SVZ) (Fig. 1A). HMGN1 and 3 were more expressed in the VZ than in the SVZ, whereas HMGN2 was expressed to a similar extent in both the VZ and SVZ (Fig. 1A). A large proportion of the cells positive for HMGN1, 2 and 3 in the VZ/SVZ co-expressed Sox9, a member of the Sox family of high mobility group (HMG)-box transcription factors (The percentage of HMGN1+ cells among Sox9+ cells (HMGN1/Sox9), 95.5%; HMGN2/Sox9, 69.6%; HMGN3/Sox9, 95.0%; Sox9/HMGN1, 72.6%; Sox9/HMGN2, 46.2%; Sox9/HMGN3, 90.1%) (Fig. 1A). Since previous reports have shown that Sox9 is expressed in multipotent neural stem cells and glial progenitors in the embryonic and adult CNS [22, 37-39], it is plausible that HMGN1, 2 and 3 are expressed in NPCs and glial progenitors. HMGN1, 2 and 3 were also expressed in some cells outside of the VZ/SVZ (Fig. 1A-1D). In the neocortex, these cells included both Sox9+ and Sox9− cells. Importantly, at postnatal day (P) 3 and P7, the majority of Sox9+ cells in the cortical plate co-expressed HMGN1 and 3, but not HMGN2 (Fig. 1B-1D). Interestingly, the majority of Sox9+ cells co-expressed HMGN2 as well as HMGN1 and 3 in the adult cortex (Fig. 1B-1D). Most GFAP+ astrocytes co-expressed HMGN1, 2 and 3 in the P7 and adult cortices (Fig. 1E). These results indicate that the expression of HMGN family proteins was maintained in glial progenitors and astrocytes in the perinatal and adult cortices. Together, HMGN family proteins were expressed in NPCs and glial lineage cells at later stages of brain development and in the adult brain. However, HMGN proteins were also expressed in the VZ/SVZ, preplate and cortical plate at earlier stages of brain development, for instance at E12.5 (Fig. 1F) and E16.5 (Supporting Information Fig. S1), indicating that HMGN family proteins were expressed in neuronal lineage cells. We further examined the expression of HMGN family proteins in neurons and astrocytes in vitro. HMGN1 and 2 were highly expressed in both Dcx+ immature neurons and Dcx− cells (Supporting Information Fig. S2A). In contrast, the expression level of HMGN3 was very low in Dcx+ immature neurons compared with that in Dcx− cells (Supporting Information Fig. S2A). The vast majority of GFAP+ astrocytes expressed HMGN1, 2 and 3, whereas NeuN+ mature neurons did them at very low levels (Supporting Information Fig. S2B, S2C). Consistent with this, NeuN+ mature neurons expressed much lower levels of HMGN1, 2 and 3 than NeuN− cells in the P7 cortex (Supporting Information Fig. S3). These results suggest that total expression levels of HMGN1, 2 and 3 are higher in astrocytic lineage cells than in neuronal lineage cells although HMGN family proteins are expressed in both lineage cells.

HMGN family proteins promote astrocyte differentiation of NPCs *in vitro*

Since HMGN family proteins were expressed in the glial lineage cells at perinatal stages, we next examined whether HMGN family proteins regulate glial differentiation. We labeled undifferentiated NPCs by infecting cells with the green fluorescent protein (GFP)-expressing recombinant retroviruses after expanding NPCs derived from the E16.5 mouse forebrain by neurosphere formation [17, 34, 35]. To induce their differentiation, these cells were cultured in the absence of FGF2 and EGF for 6 days. GFP^- and GFP^- cells in the control virus-infected culture did not show significant differences in their differentiation capacity (Fig. 2B). Overexpression of HMGN1 resulted in a higher fraction of $GFAP^+$ astrocytes among GFP^+ cells compared to the control (Fig. 2A, 2B). Overexpression of HMGN1 also increased the fraction of cells positive for Sox9, a marker of glial lineage cells, but did not affect the fraction of $O4^+$ oligodendrocytes (Fig. 2A, 2B). Conversely, overex-

pression of HMGN1 decreased the fraction of TuJ1$^+$ neurons among GFP$^-$ cells (Fig. 2A, 2B). Overexpression of HMGN2 or HMGN3 had similar effects on NPC differentiation (Fig. 2A, 2B). It is reported that the HMGN3 protein has two splice variants, HMGN3a and HMGN3b, the latter lacking most of the C-terminal chromatin regulatory domain [40]. Both HMGN3 isoforms increased the fraction of astrocytes and suppressed that of neurons (Fig. 2A, 2B), suggesting that the C-terminal chromatin regulatory domain of HMGN3a might be dispensable for this function. When we used NPCs isolated from the E11.5 mouse forebrains, which have higher neurogenic potential than E16.5 NPCs, we also observed that overexpression of HMGN members increased the fraction of astrocytes and decreased that of neurons compared to control (Supporting Information Fig. S4). We further asked whether HMGN family proteins affect the proliferation and survival of NPCs. Cell proliferation was evaluated by labeling with EdU for 2 h. No significant difference in the percentage of EdU$^+$ cells among GFP$^-$ cells was observed between control and HMGN-overexpressing cultures (Fig. 2C, 2D). The percentage of cells positive for cleaved Caspase3, a measure of apoptosis, among GFP$^+$ cells was less than 2% in both control and HMGN-overexpressing cultures (Fig. 2C, 2E). Therefore, selective proliferation or elimination of particular cell lineages does not appear to account for the observed effects of HMGN proteins on NPC differentiation propensity. Together, these results suggest that HMGN family proteins promote astrocyte differentiation of NPCs. To determine whether the increase in GFAP$^+$ astrocytes is due either to the promotion of astrocyte fate commitment by HMGN family proteins or to the selective expansion of progenitors already committed to astrocyte fate, we further performed a clonal assay by using NPCs infected with control or HMGN1 viruses at a low titer. Overexpression of HMGN1 increased the percentage of astrocyte-only and decreased that of neuron-only clones (Supporting Information Fig. S5A). HMGN1 overexpression did not significantly affect the size of the clones (Supporting Information Fig. S5B). These results suggest that HMGN family proteins control astrocyte fate decision of multipotent NPCs.

We next investigated the effects of knockdown of HMGN family proteins on NPC differentiation. Knockdown of HMGN1, 2 or 3 by shRNAs for each HMGN was confirmed by quantitative reverse-transcription PCR analysis (Fig. 3A-3C) and immunocytochemistry (Supporting Information Fig. S6). Each shRNA did not reduce the expression of other family members of HMGN (Supporting Information Fig. S7), indicating that each shRNA specifically knocked down the expression of its target. GFP+ and GFP– cells in the control virus (shRNA for luciferase)-infected culture did not show significant differences in NPC differentiation capacity (Fig. 3E). Knockdown of either HMGN1, 2 or 3 resulted in an increase in the percentage of TuJ1+ neurons and a decrease in that of GFAP+ astrocytes or Sox9+ cells compared to control (Fig. 3D, 3E). Knockdown of HMGN family proteins had little effect on the population of O4+ oligodendrocytes (Fig. 3E). We further knocked down all three members of HMGN proteins simultaneously in NPCs (Supporting Information Fig. S8A). Triple knockdown of HMGN1, 2 and 3 slightly enhanced single knockdown phenotypes (Supporting Information Fig. S8B). These results suggest that HMGN proteins are required for astrocyte differentiation of NPCs. To confirm the specificity of HMGN proteins knockdown effects on NPC differentiation, we performed rescue experiments using HMGN1, 2 and 3 constructs lacking their 3'-UTR regions because HMGN proteins shRNAs #2 were designed to target the 3'-UTR regions of HMGN1, 2 and 3 mRNAs. The expression of HMGN1, 2 or 3 was restored by their overexpression in HMGN1, 2 or 3 knockdown cells, respectively (Fig. 3F-3H). Overexpression of HMGN1, 2 or 3 reversed the suppression of astrocyte differentiation and the promotion of neuronal differentiation by knockdown of HMGN1, 2 or 3 (Fig. 3I, 3J). These results suggest that the observed phenotypes by expressing HMGN1, 2 or 3 shRNA are attributable to specific knockdown of HMGN proteins, rather than off-target effects.

HMGN family proteins promote astrocyte differentiation of NPCs *in vivo*

We examined the roles of HMGN family proteins in NPC differentiation *in vivo*. We introduced plasmids encoding either GFP alone or GFP with HMGN1, 2, 3a or 3b into mouse neocortical NPCs by *in utero* electroporation at E16.5 and analyzed their brains at P7. Under the control at P7, the majority of GFP$^+$ cells differentiated into neurons positive for Cux1, a marker of upper layer neurons, and a very small number of GFP$^+$ cells became astrocytes positive for GFAP or another astrocyte marker S100β (Fig. 4A-4C). Forced expression of HMGN1, 2, 3a or 3b markedly increased the number of GFAP$^+$ or S100β$^+$ astrocytes in the cortex (Fig. 4A, 4B). In addition, overexpression of HMGN family members also increased the number of Sox9$^+$ cells residing in the cortex (Fig. 4A, 4C). Conversely, overexpression of HMGN family members decreased the number of Cux1$^+$ neurons (Fig. 4A, 4C). These results suggest that HMGN family proteins promote astrocyte differentiation at perinatal stages *in vivo*. Importantly, we found that the introduction of HMGN-expressing plasmids into neocortical NPCs by *in utero* electroporation before E16.5 (cf. E15.5) failed to increase the number of astrocytes *in vivo* (data not shown).

We next examined the effects of knockdown of HMGN family proteins on NPC differentiation *in vivo*. We introduced knockdown plasmids encoding either shRNA for luciferase with GFP or shRNA for HMGN1, 2 or 3 with GFP into mouse neocortical NPCs by postnatal electroporation at P0 and analyzed their brains at P3 and P7. Knockdown of either HMGN1, 2 or 3 markedly decreased the fraction of GFAP$^+$ astrocytes in the cortex at P7 compared to control (Fig. 5A, 5B). Conversely,

knockdown of HMGN family proteins significantly increased that of immature neurons or neuronal progenitors positive for the marker Tbr2 in the SVZ at P3 (Fig. 5A, 5B). To rule out the off-target effects of these shRNAs, we carried out rescue experiments *in vivo*. Suppression of astrocyte differentiation by knockdown of either HMGN1, 2 or 3 was rescued by overexpression of HMGN1, 2 or 3 (Fig. 5C, 5D). These results suggest that HMGN family proteins are necessary for astrocyte differentiation at postnatal stages *in vivo*.

HMGN family proteins do not activate the JAK-STAT pathway in NPCs

The JAK-STAT pathway regulates *gfap* gene expression. STAT binds to the promoter region of *gfap* and directly induces its expression [10, 15]. Therefore, we examined whether HMGN family proteins activate the JAK-STAT pathway. Although CNTF stimulation markedly enhanced tyrosine phosphorylation of STAT3 at position 705 (STAT3 pY705), which is crucial for activation of STAT3, overexpression of HMGN1, 2, 3a or 3b failed to do so (Fig. 6A, 6B). We further examined whether HMGN family proteins directly activate STAT-mediated transcription and induce the activation of the *gfap* gene promoter by using a reporter gene assay. Overexpression of either HMGN1, 2, 3a or 3b did not increased the activity of a STAT-dependent promoter (4 x APRE) [41] or that of the *gfap* promoter (GF1L) [15] in NPCs in contrast to overexpression of an activated mutant of STAT3 (STAT3-C) [42] (Fig. 6C). These results suggest that HMGN family proteins are unlikely to promote the activation of JAK-STAT pathway.

HMGN family proteins do not directly regulate the *gfap* gene locus in NPCs

The binding of STAT1/3 proteins to the *gfap* promoter is regulated by DNA methylation of a STAT-binding site within the promoter region [18-20]. At early stages of neural development, the *gfap* gene locus is methylated, thereby suppressing its activation. At later stages, Notch-NFIA signaling reduces the DNA methylation level, which allows STAT1/3 proteins activated by LIF/CNTF/CT-1 to access the *gfap* gene locus [18-20]. Therefore, we examined whether HMGN1 affects the methylation status of the STAT-binding site at the *gfap* promoter. The amount of demethylation of this site was increased in NPCs expressing an active form of Notch1, as reported previously [20], whereas overexpression of HMGN1 did not change the methylation status of the site compared with control (Fig. 6E). Therefore, HMGN1 does not appear to regulate the *gfap* gene locus through the methylation of this site.

FGF2 signaling induces lysine 4 methylation of histone H3 (H3K4me2) and suppresses lysine 9 methylation of histone H3 (H3K9me2), and retinoic acid increases acetylation of histone H3 around the STAT-binding site of the *gfap* promoter, resulting in the induction of GFAP expression [43, 44]. It has been reported that HMGN family proteins stimulate lysine 14 acetylation of histone H3 (H3K14ac) by p300/CBP-associated factor (PCAF) and induce the expression of target genes [45-47]. We then sought to investigate whether HMGN family proteins enhance histone H3 acetylation at the *gfap* promoter. Overexpression of HMGN1, 2, 3a or 3b did not increase H3K9/K14ac around the STAT-binding site at the *gfap* promoter 2 days after the induction of differentiation (Fig. 6F). These data indicate that HMGN family proteins do not directly regulate the *gfap* gene locus.

HMGN family proteins modulate the response to gliogenic signal CNTF

Since HMGN family proteins did not activate the JAK-STAT pathway (Fig. 6), we next investigated whether HMGN family proteins act downstream of the JAK-STAT pathway in NPCs. We stimulated NPCs with CNTF to examine whether the JAK-STAT pathway regulates the expression of HMGN family proteins. We found that CNTF stimulation did not change the expression level of HMGN1, 2 and 3 (Fig. 6D), suggesting that the STAT signaling does not regulate the expression of HMGN family proteins. We further examined whether HMGN family proteins modulate the responsiveness of NPCs to CNTF. CNTF stimulation resulted in an increase in the percentage of GFAP+ astrocytes and a decrease in that of $TuJ1^+$ neurons compared to control (Fig. 7A, 7B). Knockdown of either HMGN1, 2 or 3 reduced the increase in the fraction of $GFAP^+$ astrocytes induced by CNTF and had an opposite effect on that of $TuJ1^+$ neurons (Fig. 7A, 7B). These results suggest that HMGN family proteins promote astrocyte differentiation through modulation of the responsiveness to CNTF.

Discussion

This study provides evidence for the involvement of HMGN family proteins in regulating astrocyte differentiation. We found that HMGN1, 2 and 3 are expressed in NPCs, glial progenitors and astrocytes in the perinatal and adult brains and that they promote astrocyte differentiation during brain development. Although various signaling pathways that promote astrocyte differentiation converge on the activation of the STAT transcription factors, HMGN family proteins do not appear to do so. Our findings may thus reveal that HMGN family proteins are novel components that promote astrocyte differentiation in parallel to or downstream of the STAT signaling pathway during development.

How do HMGN family proteins control astrocyte differentiation, then? Although HMGN proteins display little or no DNA sequence specificity when binding to nucleosomes [48], several reports have indicated that HMGN proteins target specific genomic loci and can regulate the expression of particular target genes. For instance, a microarray analysis of the mouse hepatoma cell line

Hepa-1 overexpressing HMGN3a and HMGN3b showed that HMGN3a and HMGN3b regulate the expression of individual genes, rather than having a broader, more general effect on transcription [49]. Interestingly, previous studies have shown that HMGN proteins regulate astrocyte-related genes (cf. Sox9 in limb buds, MeCP2 in $CD4^+$ T cells and in the brain, Glyt1 in Hepa-1 cells) [32, 47, 49, 50]. We therefore performed a microarray analysis of HMGN1-overexpressing NPCs to investigate whether HMGN proteins can regulate the expression of astrocyte-related genes in NPCs. However, there was little difference in the expression of genes involved in astrocyte differentiation between control and HMGN1-overexpressing NPCs (Fold change in HMGN1/control: Sox9, 0.97; MeCP2, 1.04; Glyt1, 1.08; STAT1, 1.09; STAT3, 1.06; JAK1, 1.15; JAK2, 1.06; gp130, 1.10; LIFR, 0.97; CNTFR, 1.07; NFIA, 1.07; NFIB, 1.04; NFIX, 0.96; REST, 1.01; SRF, 1.02; Etv5, 1.12; Nfe2l1, 1.13; S100β, 1.01; GLT1, 1.06; Aldh1L1, 1.03) under a differentiation-inducing condition in which NPCs were cultured without FGF2 and EGF for 1 day although a significant increase in the expression of GFAP (1.51-fold) was observed. We further performed GO and pathway analyses using DAVID software tools. GO terms enriched in HMGN1-overexpressing cells were, for instance, neurological system process, cell surface receptor linked signal transduction and acetylation (Supporting Information Fig. S9), and were not associated with nervous system development. These results suggest that overexpression of HMGN1 alone is not sufficient to directly induce astrocytic genes.

Because HMGN proteins are chromatin-associated factors that can modulate high order chromatin structure in part through competing with the linker histone H1 and through regulating modifications of core histones [30, 51-54], it is possible that HMGN proteins alone do not induce transcription of their target genes, but rather change the chromatin structure so that other transcription factors can induce target gene expression. This may be consistent with our results that overexpression of HMGN proteins alone did not induce a marked change of expression profiles in NPCs, although it promoted astrocyte differentiation, and that HMGN proteins promoted astrocyte differentiation in late stage NPCs but not in early stage NPCs *in vivo* (data not shown). It will be worth investigating the possibility that HMGN proteins mediate chromatin permissiveness for transcriptional regulation at astrocyte-related gene loci and thus provide NPCs with the potential to differentiate into the astrocytic lineage. Actually, a similar idea has been proposed for another group of chromatin-associated factors, high mobility group protein A (HMGA) family proteins, in promoting the potential of NPCs to differentiate into the neuronal lineage [55]. Namely, in the developing neocortex, HMGA proteins modulate the chromatin structure of neocortical NPCs, promote neuronal differentiation, and suppress astrocyte differentiation, although the causal relationship between the HMGA-regulated chromatin structure and the differentiation potential has not been directly demonstrated. Locus-specific analyses of the chromatin accessibility to the transcription factors should shed light on the roles of HMGN proteins in regulating the astrogenic potential of NPCs. Interestingly, it is recently reported that the absence of DNA methylation and/or repressive histone modifications such as H3K9me2 or H3K27me3 around the STAT-binding site of the *gfap* promoter is insufficient to induce the expression of GFAP and that alteration of chromatin accessibility around STAT-binding site plays a critical role in the STAT binding to its target site and the regulation of GFAP expression [56]. Since knockdown of HMGN family proteins significantly reduced astrocyte differentiation induced by CNTF (Fig. 7), it is likely that HMGN family proteins modulate the responsiveness to gliogenic signals by altering chromatin accessibility around the STAT-binding sites of the astrocyte-related gene promoters.

We revealed that HMGN1, 2 and 3 were expressed in NPCs, $Sox9^+$ glial progenitors and $GFAP^+$ astrocytes during the astrocytic phase of brain development (Fig. 1), which is consistent with the notion that HMGN proteins contribute to astrocyte differentiation. However, HMGN proteins were also expressed in NPCs and neuronal lineage cells at earlier stages of brain development even though NPCs in these stages mainly produce neurons, not astrocytes (Fig. 1F and Supporting Information Fig. S1). We further found that overexpression of HMGN family proteins in neocortical NPCs before E16.5 (cf. E15.5) failed to increase the number of astrocytes *in vivo* (data not shown). These results suggest that an additional factor(s) present at late developmental stages is required for, or an inhibitory factor(s) present at early developmental stages suppresses, HMGN-mediated astrocyte differentiation *in vivo*. Because Sox9 expression has been shown to be associated with bipotent progenitors for astrocytes and oligodendrocytes in the spinal cord [22], we examined whether HMGN family proteins are expressed in oligodendrocyte progenitors that express another Sox family protein Sox10 and oligodendrocytes. The majority of Sox10+ cells in the P7 cortex co-expressed HMGN1 and 3, but not HMGN2 (Supporting Information Fig. S10A) and mature oligodendrocytes positive for CC1 or MBP did not expressed HMGN1, 2 and 3 at P7 (Supporting Information Fig. S10B). Additionally, HMGN1, 2 and 3 were expressed in Sox10+ cells at high, low and moderate levels, respectively (Supporting Information Fig. S11A) and mature oligodendrocytes positive for MAG or MBP expressed HMGN1, 2 and 3 at very low levels in vitro (Supporting Information Fig. S11B). From these observation, we can not rule out the possibility that HMGN family proteins are involved in oligodendrocyte differentiation although overexpression or knockdown of HMGN family proteins had no or a little effect on the population of oligodendrocytes, respectively, at least under our experimental condition (Fig. 2B, 3E, 3J).

The differences in functions between HMGN1, 2 and 3 are still unclear. HMGN1 and HMGN2 show similar ex-

pression patterns in some tissues [57]. Loss of HMGN1 has been reported to increase the amount of HMGN2 bound to the *sox9* gene locus in the limb bud of HMGN1 knockout mice, suggesting a functional redundancy among these proteins [32]. On the other hand, HMGN1 knockout mice exhibit defects in the development of the corneal epithelia [58] and HMGN1-deficient MEFs are hypersensitive to both UV and ionizing radiation [59, 60], suggesting that HMGN2 fails to fully compensate for the loss of HMGN1 in these contexts. In the developing forebrain, we found that HMGN1, 2, 3a and 3b appear to have similar functions in NPCs, since overexpression of each member showed similar effects on the differentiation propensity of NPCs (Fig. 2A, 2B). HMGN3b promoted astrocyte differentiation like other HMGN proteins despite not having the C-terminal chromatin regulatory domain (Fig. 2A, 2B). Since HMGN3b as well as HMGN1, 2 and 3a has nucleosome-binding domain, it can compete with the linker histone H1 for chromatin binding sites. Therefore, it may be important to alter chromatin accessibility by competing with the linker histone H1 for the promotion of astrocyte differentiation by HMGN family proteins. We also found that knockdown of each member suppressed astrocyte differentiation of NPCs (Fig. 3D, 3E, 3I, 3J). This may suggest that HMGN family members cannot compensate for each other's functions in promoting astrocyte differentiation or that there might be a threshold for the total level of HMGN family members required for astrocyte differentiation. Total expression levels of HMGN1, 2 and 3 appeared to be higher in astrocytic lineage cells than in neuronal lineage cells although HMGN family proteins were expressed in both lineage cells (Fig. 1 and Supporting Information Fig. S1-S3). In addition, the expression of total HMGN family proteins appeared to be maintained at a certain level in astrocytic lineage cells although it diminished in neuronal lineage cells during differentiation process (Fig. 1 and Supporting Information Fig. S1-S3). These observations may suggest that total expression levels of HMGN family proteins should be maintained above a certain threshold in astrocytic lineage cells and that a higher amount of HMGN proteins are required for altering chromatin accessibility at astrocyte-related gene loci. Therefore, NPCs may fail to differentiate into astrocytes when the total levels of HMGN family proteins are less than the threshold by their knockdown.

Since we found that HMGN proteins facilitate astrocyte differentiation, it is possible that upstream signaling molecules that regulate the expression or activity of HMGN proteins, if any, are involved in regulating the differentiation of astrocytes. So far, we could not find any evidence that astrocyte-inducing cytokines such as CNTF and BMP increase the levels of HMGN family members (Fig. 6D and data not shown). However, future studies on the pathways upstream as well as downstream of HMGN proteins may provide new insights into the control of astrocyte differentiation in the developing forebrain.

CONCLUSION

In this study, we have demonstrated that HMGN family proteins were expressed in NPCs, glial progenitors, and astrocytes in the perinatal and adult brains and that they promote astrocyte differentiation of NPCs. Although various signaling pathways that promote astrocyte differentiation are reported to converge on the activation of STAT, we have shown that HMGN family proteins did not activate STAT signaling in NPCs. Since knockdown of HMGN family proteins blocked the action of CNTF to promote astrocyte differentiation, HMGN proteins are likely to function as modulators of the response to CNTF in NPCs. Thus, our findings suggest that HMGN family proteins are novel components that promote astrocyte differentiation in the CNS through modulation of the responsiveness to gliogenic signals and provide a new insight into the regulation of NPC differentiation during brain development.

ACKNOWLEDGMENTS

We thank C.L. Cepko (Harvard University), T. Matsuda (Kyoto University), T. Kitamura (The University of Tokyo), H. Song (Johns Hopkins University), K. Nakashima (Nara Institute of Science and Technology) and J.F. Bromberg (Memorial Sloan-Kettering Cancer Center) for plasmids, K. Tyssowski for editing the manuscript, Y. Hirabayashi and H. Sakai for reagents and technical assistance, J. Yamauchi (National Research Institute for Child Health and Development) and members of the Gotoh laboratory for helpful discussion. This work was supported by Grant-in-Aid for Scientific Research on Innovative Areas "Neural Diversity and Neocortical Organization" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and Grants-in-Aid for Scientific Research (C) of MEXT.

DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

The authors indicate no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

M.N.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript; D.L.: collection and/or assembly of data and data analysis and interpretation; Y.I.: collection and/or assembly of data and data analysis and interpretation; Y.K.: collection and/or assembly of data and data analysis and interpretation; T.O.: financial support and administrative support; Y.G.: conception and design and manuscript writing.

References

1 Volterra A, Meldolesi J. Astrocytes, from brain glue to communication elements: the revolution continues. Nat Rev Neurosci 2005;6:626-640.

2 Eroglu C, Barres BA. Regulation of synaptic connectivity by glia. Nature 2010;468:223-231.

3 Molofsky AV, Krencik R, Ullian EM, et al. Astrocytes and disease: a neurodevelopmental perspective. Genes Dev 2012;26:891-907.

4 Temple S. The development of neural stem cells. Nature 2001;414:112-117.

5 Miller FD, Gauthier AS. Timing is everything: making neurons versus glia in the developing cortex. Neuron 2007;54:357-369.

6 Freeman MR. Specification and morphogenesis of astrocytes. Science 2010;330:774-778.

7 Rowitch DH, Kriegstein AR. Developmental genetics of vertebrate glial-cell specification. Nature 2010;468:214-222.

8 Namihira M, Nakashima K. Mechanisms of astrocytogenesis in the mammalian brain. Curr Opin Neurobiol 2013;23:921-927.

9 Johe KK, Hazel TG, Muller T, et al. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev 1996;10:3129-3140.

10 Bonni A, Sun Y, Nadal-Vicens M, et al. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 1997;278:477-483.

11 Rajan P, McKay RD. Multiple routes to astrocytic differentiation in the CNS. J Neurosci 1998;18:3620-3629.

12 Barnabe-Heider F, Wasylnka JA, Fernandes KJ, et al. Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. Neuron 2005;48:253-265.

13 He F, Ge W, Martinowich K, et al. A positive autoregulatory loop of Jak-STAT signaling controls the onset of astrogliogenesis. Nat Neurosci 2005;8:616-625.

14 Nakashima K, Wiese S, Yanagisawa M, et al. Developmental requirement of gp130 signaling in neuronal survival and astrocyte differentiation. J Neurosci 1999;19:5429-5434.

15 Nakashima K, Yanagisawa M, Arakawa H, et al. Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science 1999;284:479-482.

16 Kamakura S, Oishi K, Yoshimatsu T, et al. Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling. Nat Cell Biol 2004;6:547-554.

17 Nagao M, Sugimori M, Nakafuku M. Cross talk between notch and growth factor/cytokine signaling pathways in neural stem cells. Mol Cell Biol 2007;27:3982-3994.

18 Takizawa T, Nakashima K, Namihira M, et al. DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. Dev Cell 2001;1:749-758.

19 Fan G, Martinowich K, Chin MH, et al. DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. Development 2005;132:3345-3356.

20 Namihira M, Kohyama J, Semi K, et al. Committed neuronal precursors confer astrocytic potential on residual neural precursor cells. Dev Cell 2009;16:245-255.

21 Deneen B, Ho R, Lukaszewicz A, et al. The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. Neuron 2006;52:953-968.

22 Stolt CC, Lommes P, Sock E, et al. The Sox9 transcription factor determines glial fate choice in the developing spinal cord. Genes Dev 2003;17:1677-1689.

23 Kang P, Lee HK, Glasgow SM, et al. Sox9 and NFIA coordinate a transcriptional regulatory cascade during the initiation of gliogenesis. Neuron 2012;74:79-94.

24 Martini S, Bernoth K, Main H, et al. A critical role for Sox9 in notch-induced astrogliogenesis and stem cell maintenance. Stem Cells 2013;31:741-751.

25 Muroyama Y, Fujiwara Y, Orkin SH, et al. Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. Nature 2005;438:360-363.

26 Kohyama J, Sanosaka T, Tokunaga A, et al. BMP-induced REST regulates the establishment and maintenance of astrocytic identity. J Cell Biol 2010;189:159-170.

27 Lu PP, Ramanan N. A critical cell-intrinsic role for serum response factor in glial specification in the CNS. J Neurosci 2012;32:8012-8023.

28 Li X, Newbern JM, Wu Y, et al. MEK Is a Key Regulator of Gliogenesis in the Developing Brain. Neuron 2012;75:1035-1050.

29 Molofsky AV, Glasgow SM, Chaboub LS, et al. Expression profiling of Aldh1l1-precursors in the developing spinal cord reveals glial lineage-specific genes and direct Sox9-Nfe2l1 interactions. Glia 2013;61:1518-1532.

30 Postnikov Y, Bustin M. Regulation of chromatin structure and function by HMGN proteins. Biochim Biophys Acta 2010;1799:62-68.

31 Ito Y, Bustin M. Immunohistochemical localization of the nucleosome-binding protein HMGN3 in mouse brain. J Histochem Cytochem 2002;50:1273-1275.

32 Furusawa T, Lim JH, Catez F, et al. Down-regulation of nucleosomal binding protein HMGN1 expression during embryogenesis modulates Sox9 expression in chondrocytes. Mol Cell Biol 2006;26:592-604.

33 Deng T, Zhu ZI, Zhang S, et al. HMGN1 modulates nucleosome occupancy and DNase I hypersensitivity at the CpG island promoters of embryonic stem cells. Mol Cell Biol 2013;33:3377-3389.

34 Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 2000;7:1063-1066.

35 Nagao M, Campbell K, Burns K, et al. Coordinated control of self-renewal and differentiation of neural stem cells by Myc and the p19ARF-p53 pathway. J Cell Biol 2008;183:1243-1257.

36 Hirabayashi Y, Itoh Y, Tabata H, et al. The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. Development 2004;131:2791-2801.

37 Pompolo S, Harley VR. Localisation of the SRY-related HMG box protein, SOX9, in rodent brain. Brain Res 2001;906:143-148.

38 Kordes U, Cheng YC, Scotting PJ. Sox group E gene expression distinguishes different types and maturational stages of glial cells in developing chick and mouse. Brain Res Dev Brain Res 2005;157:209-213.

39 Scott CE, Wynn SL, Sesay A, et al. SOX9 induces and maintains neural stem cells. Nat Neurosci 2010;13:1181-1189.

40 West KL, Ito Y, Birger Y, et al. HMGN3a and HMGN3b, two protein isoforms with a tissue-specific expression pattern, expand the cellular repertoire of nucleosome-binding proteins. J Biol Chem 2001;276:25959-25969.

41 Hocke GM, Barry D, Fey GH. Synergistic action of interleukin-6 and glucocorticoids is mediated by the interleukin-6 response element of the rat alpha 2 macroglobulin gene. Mol Cell Biol 1992;12:2282-2294.

42 Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. Cell 1999;98:295-303.

43 Song MR, Ghosh A. FGF2-induced chromatin remodeling regulates CNTF-mediated gene expression and astrocyte differentiation. Nat Neurosci 2004;7:229-235.

44 Asano H, Aonuma M, Sanosaka T, et al. Astrocyte differentiation of neural precursor cells is enhanced by retinoic acid through a change in epigenetic modification. Stem Cells 2009;27:2744-2752.

45 Lim JH, West KL, Rubinstein Y, et al. Chromosomal protein HMGN1 enhances the acetylation of lysine 14 in histone H3. EMBO J 2005;24:3038-3048.

46 Ueda T, Postnikov YV, Bustin M. Distinct domains in high mobility group N variants modulate specific chromatin modifications. J Biol Chem 2006;281:10182-10187.

47 Barkess G, Postnikov Y, Campos CD, et al. The chromatin-binding protein HMGN3 stimulates histone acetylation and transcription across the Glyt1 gene. Biochem J 2012;442:495-505.

48 Shirakawa H, Herrera JE, Bustin M, et al. Targeting of high mobility group-14/-17 proteins in chromatin is independent of DNA sequence. J Biol Chem 2000;275:37937-37944.

49 West KL, Castellini MA, Duncan MK, et al. Chromosomal proteins HMGN3a and HMGN3b regulate the expression of glycine transporter 1. Mol Cell Biol 2004;24:3747-3756.

50 Abuhatzira L, Shamir A, Schones DE, et al. The chromatin-binding protein HMGN1