

- Inoue, K., Terashima, T., Nishikawa, T., Takumi, T., 2004. Fez1 is layer-specifically expressed in the adult mouse neocortex. *European Journal of Neuroscience* 20, 2909–2916.
- Jenuwein, T., Forrester, W.C., Fernandez-Herrero, L.A., Laible, G., Dull, M., Grosschedl, R., 1997. Extension of chromatin accessibility by nuclear matrix attachment regions. *Nature* 385, 269–272.
- Jeong, J.Y., Einhorn, Z., Mathur, P., Chen, L., Lee, S., Kawakami, K., Guo, S., 2007. Patterning the zebrafish diencephalon by the conserved zinc-finger protein Fez1. *Development* 134, 127–136.
- Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., McKay, R.D., 1996. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes and Development* 10, 3129–3140.
- Joshi, P.S., Molyneaux, B.J., Feng, L., Xie, X., Macklis, J.D., Gan, L., 2008. Bhlhb5 regulates the postmitotic acquisition of area identities in layers II–V of the developing neocortex. *Neuron* 60, 258–272.
- Juliandi, B., Abematsu, M., Nakashima, K., 2010a. Epigenetic regulation in neural stem cell differentiation. *Development, Growth and Differentiation* 52, 493–504.
- Juliandi, B., Abematsu, M., Nakashima, K., 2010b. Chromatin remodeling in neural stem cell differentiation. *Current Opinion in Neurobiology* 20, 408–415.
- Juliandi, B., Abematsu, M., Sanosaka, T., Tsujimura, K., Smith, A., Nakashima, K., 2012. Induction of superficial cortical layer neurons from mouse embryonic stem cells by valproic acid. *Neuroscience Research* 72, 23–31.
- Kanold, P.O., Shatz, C.J., 2006. Subplate neurons regulate maturation of cortical inhibition and outcome of ocular dominance plasticity. *Neuron* 51, 627–638.
- Koester, S.E., O'Leary, D.D., 1993. Connectional distinction between callosal and subcortically projecting cortical neurons is determined prior to axon extension. *Developmental Biology* 160, 1–14.
- Kowalczyk, T., Pontious, A., Englund, C., Daza, R.A., Bedogni, F., Hodge, R., Attardo, A., Bell, C., Huttnner, W.B., Hevner, R.F., 2009. Intermediate neuronal progenitors (basal progenitors) produce pyramidal-projection neurons for all layers of cerebral cortex. *Cerebral Cortex* 19, 2439–2450.
- Kwan, K.Y., Lam, M.M., Krsnik, Z., Kawasawa, Y.I., Lefebvre, V., Sestan, N., 2008. SOX5 postmitotically regulates migration, postmigratory differentiation, and projections of subplate and deep-layer neocortical neurons. *Proceedings of the National Academy of Sciences of the United States of America* 105, 16021–16026.
- Lai, T., Jabaudon, D., Molyneaux, B.J., Azim, E., Arlotta, P., Menezes, J.R., Macklis, J.D., 2008. SOX5 controls the sequential generation of distinct corticofugal neuron subtypes. *Neuron* 57, 232–247.
- Lamb, A.N., Rosenfeld, J.A., Neill, N.J., Talkowski, M.E., Blumenthal, I., Girirajan, S., Keelean-Fuller, D., Fan, Z., Pouncey, J., Stevens, C., Mackay-Loder, L., Terespol-sky, D., Bader, P.I., Rosenbaum, K., Vallee, S.E., Moeschler, J.B., Ladda, R., Sell, S., Martin, J., Ryan, S., Jones, M.C., Moran, R., Shealy, A., Madan-Khetarpal, S., McConnell, J., Surti, U., Delahaye, A., Heron-Longe, B., Pipiras, E., Benzacken, B., Passemar, S., Verloes, A., Isidor, B., Le Caignec, C., Glew, G.M., Oheim, K.E., Descartes, M., Eichler, E.E., Morton, C.C., Gusella, J.F., Schultz, R.A., Ballif, B.C., Shaffer, L.G., 2012. Haploinsufficiency of SOX5 at 12p12.1 is associated with developmental delays with prominent language delay behavior problems, and mild dysmorphic features. *Human Mutation* 33, 728–740.
- Leone, D.P., Srinivasan, K., Chen, B., Alcamo, E., McConnell, S.K., 2008. The determination of projection neuron identity in the developing cerebral cortex. *Current Opinion in Neurobiology* 18, 28–35.
- Li, N., Hornbruch, A., Klafke, R., Katzenberger, B., Wizenmann, A., 2005. Specification of dorsoventral polarity in the embryonic chick mesencephalon and its presumptive role in midbrain morphogenesis. *Developmental Dynamics* 233, 907–920.
- Mairet-Coello, G., Tury, A., Van Buskirk, E., Robinson, K., Genestine, M., DiCicco-Bloom, E., 2012. p57(KIP2) regulates radial glia and intermediate precursor cell cycle dynamics and lower layer neurogenesis in developing cerebral cortex. *Development* 139, 475–487.
- Mallamaci, A., Muzio, L., Chan, C.H., Parnavelas, J., Boncinelli, E., 2000. Area identity shifts in the early cerebral cortex of Emx2-/- mutant mice. *Nature Neuroscience* 3, 679–686.
- Mallamaci, A., Stoykova, A., 2006. Gene networks controlling early cerebral cortex arealization. *European Journal of Neuroscience* 23, 847–856.
- Martin, O., Rubenstein, J.L., 2001. A long, remarkable journey: tangential migration in the telencephalon. *Nature Reviews Neuroscience* 2, 780–790.
- McKenna, W.L., Betancourt, J., Larkin, K.A., Abrams, B., Guo, C., Rubenstein, J.L., Chen, B., 2011. Tbr1 and Fezf2 regulate alternate corticofugal neuronal identities during neocortical development. *The Journal of Neuroscience* 31, 549–564.
- Molyneaux, B.J., Arlotta, P., Hirata, T., Hibi, M., Macklis, J.D., 2005. Fez1 is required for the birth and specification of corticospinal motor neurons. *Neuron* 47, 817–831.
- Molyneaux, B.J., Arlotta, P., Menezes, J.R., Macklis, J.D., 2007. Neuronal subtype specification in the cerebral cortex. *Nature Reviews Neuroscience* 8, 427–437.
- Mutch, C.A., Funatsu, N., Monuki, E.S., Chenn, A., 2009. Beta-catenin signaling levels in progenitors influence the laminar cell fates of projection neurons. *The Journal of Neuroscience* 29, 13710–13719.
- Nakashima, K., Wiese, S., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Yoshida, K., Kishimoto, T., Sendtner, M., Taga, T., 1999. Developmental requirement of gp130 signaling in neuronal survival and astrocyte differentiation. *The Journal of Neuroscience* 19, 5429–5434.
- Namihira, M., Kohyama, J., Abematsu, M., Nakashima, K., 2008. Epigenetic mechanisms regulating fate specification of neural stem cells. *Philosophical Transactions of the Royal Society of London B Biological Sciences* 363, 2099–2109.
- Namihira, M., Kohyama, J., Semi, K., Sanosaka, T., Deneen, B., Taga, T., Nakashima, K., 2009. Committed neuronal precursors confer astrocytic potential on residual neural precursor cells. *Developmental Cell* 16, 245–255.
- Nieto, M., Monuki, E.S., Tang, H., Imitola, J., Haubst, N., Khoury, S.J., Cunningham, J., Götz, M., Walsh, C.A., 2004. Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II–IV of the cerebral cortex. *The Journal of Comparative Neurology* 479, 168–180.
- Noctor, S.C., Martínez-Cerdeno, V., Ivic, L., Kriegstein, A.R., 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nature Neuroscience* 7, 136–144.
- Pereira, J.D., Sansom, S.N., Smith, J., Dobenecker, M.W., Tarakhovskiy, A., Livesey, F.J., 2010. Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex. *Proceedings of the National Academy of Sciences of the United States of America* 107, 15957–15962.
- Peters, A., Jones, E.G., 1984. Classification of cortical neurons. In: Peters, A., Jones, E.G. (Eds.), *Cerebral Cortex. Cellular Components of the Cerebral Cortex*, vol. 1. Plenum Press, New York, pp. 107–121.
- Pinto, L., Götz, M., 2007. Radial glial cell heterogeneity – the source of diverse progeny in the CNS. *Progress in Neurobiology* 83, 2–23.
- Polleux, F., Dehay, C., Kennedy, H., 1997. The timetable of laminar neurogenesis contributes to the specification of cortical areas in mouse isocortex. *The Journal of Comparative Neurology* 385, 95–116.
- Potkin, S.G., Turner, J.A., Guffanti, G., Lakatos, A., Fallon, J.H., Nguyen, D.D., Mathalon, D., Ford, J., Lauriello, J., Macciardi, F., 2009. FBIRN, A genome-wide association study of schizophrenia using brain activation as a quantitative phenotype. *Schizophrenia Bulletin* 35, 96–108.
- Rajan, P., McKay, R.D., 1998. Multiple routes to astrocytic differentiation in the CNS. *The Journal of Neuroscience* 18, 3620–3629.
- Rakic, P., 1971. Guidance of neurons migrating to the fetal monkey neocortex. *Brain Research* 33, 471–476.
- Rakic, P., 2003. Developmental and evolutionary adaptations of cortical radial glia. *Cerebral Cortex* 13, 541–549.
- Robertson, K.D., 2005. DNA methylation and human disease. *Nature Reviews Genetics* 6, 597–610.
- Rouaux, C., Arlotta, P., 2010. Fezf2 directs the differentiation of corticofugal neurons from striatal progenitors in vivo. *Nature Neuroscience* 13, 1345–1347.
- Rosenfeld, J.A., Ballif, B.C., Lucas, A., Spence, E.J., Powell, C., Aylsworth, A.S., Torchia, B.A., Shaffer, L.G., 2009. Small deletions of SATB2 cause some of the clinical features of the 2q33.1 microdeletion syndrome. *PLoS ONE* 4, e6568.
- Rosenfeld, J.A., Ballif, B.C., Torchia, B.S., Sahoo, T., Ravnun, J.B., Schultz, R., Lamb, A., Bejjani, B.A., Shaffer, L.G., 2010. Copy number variations associated with autism spectrum disorders contribute to a spectrum of neurodevelopmental disorders. *Genetics in Medicine* 12, 694–702.
- Rubenstein, J.L., Shimamura, K., Martinez, S., Puelles, L., 1998. Regionalization of the proencephalic neural plate. *Annual Review of Neuroscience* 21, 445–477.
- Rubenstein, J.L., Anderson, S., Shi, L., Miyashita-Lin, E., Bulfone, A., Hevner, R., 1999. Genetic control of cortical regionalization and connectivity. *Cerebral Cortex* 9, 524–532.
- Russo, V.E.A., Martienssen, R.A., Riggs, A.D., 1996. *Epigenetic Mechanisms of Gene Regulation*, vol. 32. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sahara, S., O'Leary, D.D., 2009. Fgf10 regulates transition period of cortical stem cell differentiation to radial glia controlling generation of neurons and basal progenitors. *Neuron* 63, 48–62.
- Sawamoto, K., Yamamoto, A., Kawaguchi, A., Yamaguchi, M., Mori, K., Goldman, S.A., Okano, H., 2001. Direct isolation of committed neuronal progenitor cells from transgenic mice coexpressing spectrally distinct fluorescent proteins regulated by stage-specific neural promoters. *Journal of Neuroscience Research* 65, 220–227.
- Schwartz, Y.B., Pirrotta, V., 2007. Polycomb silencing mechanisms and the management of genomic programmes. *Nature Reviews Genetics* 8, 9–22.
- Seuntjens, E., Nityanandam, A., Miquelajauregui, A., Debruyne, J., Stryjewska, A., Goebbels, S., Nave, K.A., Huylebroeck, D., Tarabykin, V., 2009. Sip1 regulates sequential fate decisions by feedback signaling from postmitotic neurons to progenitors. *Nature Neuroscience* 12, 1373–1380.
- Shen, Q., Wang, Y., Dimos, J.T., Fasano, C.A., Phoenix, T.N., Lemischka, I.R., Ivanova, N.B., Stifani, S., Morrissy, E.E., Temple, S., 2006. The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells. *Nature Neuroscience* 9, 743–751.
- Shen, X., Liu, Y., Hsu, Y.J., Fujiwara, Y., Kim, J., Mao, X., Yuan, G.C., Orkin, S.H., 2008. EZH1 mediates methylation on histone H3 lysine 27 and complements EZH2 in maintaining stem cell identity and executing pluripotency. *Molecular Cell* 32, 491–502.
- Shim, S., Kwan, K.Y., Li, M., Lefebvre, V., Sestan, N., 2012. Cis-regulatory control of corticospinal system development and evolution. *Nature* 486, 74–79.
- Shimogori, T., Banuchi, V., Ng, H.Y., Strauss, J.B., Grove, E.A., 2004. Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. *Development* 131, 5639–5647.
- Stancik, E.K., Navarro-Quiroga, I., Sellke, R., Haydar, T.F., 2010. Heterogeneity in ventricular zone neural precursors contributes to neuronal fate diversity in the postnatal neocortex. *The Journal of Neuroscience* 30, 7028–7036.
- Takahashi, T., Nowakowski, R.S., Caviness Jr., V.S., 1997. The mathematics of neocortical neurogenesis. *Developmental Neuroscience* 19, 17–22.

- Takizawa, T., Nakashima, K., Namihira, M., Ochiai, W., Uemura, A., Yanagisawa, M., Fujita, N., Nakao, M., Taga, T., 2001. DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. *Developmental Cell* 1, 749–758.
- Tarabykin, V., Stoykova, A., Usman, N., Gruss, P., 2001. Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression. *Development* 128, 1983–1993.
- ten Donkelaar, H.J., Lammens, M., Wesseling, P., Hori, A., Keyser, A., Rotteveel, J., 2004. Development and malformations of the human pyramidal tract. *Journal of Neurology* 251, 1429–1442.
- Thomas, T., Voss, A.K., Chowdhury, K., Gruss, P., 2000. Querkopf, a MYST family histone acetyltransferase, is required for normal cerebral cortex development. *Development* 127, 2537–2548.
- Topark-Ngarm, A., Golonzhka, O., Peterson, V.J., Barrett Jr., B., Martinez, B., Crofoot, K., Filtz, T.M., Leid, M., 2006. CTIP2 associates with the NuRD complex on the promoter of p57KIP2, a newly identified CTIP2 target gene. *The Journal of Biological Chemistry* 281, 32272–32283.
- Vaccarino, F.M., Schwartz, M.L., Raballo, R., Nilsen, J., Rhee, J., Zhou, M., Doetschman, T., Coffin, J.D., Wyland, J.J., Hung, Y.T., 1999. Changes in cerebral cortex size are governed by fibroblast growth factor during embryogenesis. *Nature Neuroscience* 2, 246–253.
- Wu, H., Sun, Y.E., 2006. Epigenetic regulation of stem cell differentiation. *Pediatric Research* 59, 21R–25R.
- Zembrzycki, A., Griesel, G., Stoykova, A., Mansouri, A., 2007. Genetic interplay between the transcription factors Sp8 and Emx2 in the patterning of the forebrain. *Neural Development* 2, 8.
- Zhao, C., Guan, W., Pleasure, S.J., 2006. A transgenic marker mouse line labels Cajal-Retzius cells from the cortical hem and thalamocortical axons. *Brain Research* 1077, 48–53.
- Zhou, C., Tsai, S.Y., Tsai, M.J., 2001. COUP-TFI: an intrinsic factor for early regionalization of the neocortex. *Genes and Development* 15, 2054–2059.
- Zimmer, C., Tiveron, M.C., Bodmer, R., Cremer, H., 2004. Dynamics of Cux2 expression suggests that an early pool of SVZ precursors is fated to become upper cortical layer neurons. *Cerebral Cortex* 14, 1408–14020.

Oxygen Levels Epigenetically Regulate Fate Switching of Neural Precursor Cells via Hypoxia-Inducible Factor 1 α -Notch Signal Interaction in the Developing Brain

TETSUJI MUTOH, TSUKASA SANOSAKA, KEI ITO, KINICHI NAKASHIMA

Laboratory of Molecular Neuroscience, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, Japan

Key Words. Neural precursor cell • Astrocyte differentiation • DNA methylation • Epigenetic gene regulation • Notch signaling • Oxygen concentration

ABSTRACT

Oxygen levels in tissues including the embryonic brain are lower than those in the atmosphere. We reported previously that Notch signal activation induces demethylation of astrocytic genes, conferring astrocyte differentiation ability on midgestational neural precursor cells (mgNPCs). Here, we show that the oxygen sensor hypoxia-inducible factor 1 α (HIF1 α) plays a critical role in astrocytic gene

demethylation in mgNPCs by cooperating with the Notch signaling pathway. Expression of constitutively active HIF1 α and a hyperoxic environment, respectively, promoted and impeded astrocyte differentiation in the developing brain. Our findings suggest that hypoxia contributes to the appropriate scheduling of mgNPC fate determination. *STEM CELLS* 2012;30:561–569

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The mammalian embryonic brain contains multipotent neural precursor cells (NPCs) that can self-renew and give rise to all three major cell types in the nervous system, that is, neurons, astrocytes, and oligodendrocytes. NPC fate determination is regulated by the collaboration between cell-external cues, from molecules such as cytokines and growth factors, and cell-internal epigenetic programs [1].

We and others have shown that members of the interleukin-6 (IL-6) family of cytokines, including leukemia inhibitory factor (LIF), efficiently induce astrocyte differentiation of late-gestational (lg) NPCs by activating the janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway [2, 3]. In contrast to lgNPCs, midgestational (mg) NPCs differentiate only into neurons because the promoters of astrocytic genes, such as that encoding the typical astrocytic marker glial fibrillary acidic protein (GFAP), are hypermethylated at this stage [4, 5]. These findings indicate that DNA methylation is a critical determinant for the acquisition of astrocyte differentiation ability by NPCs [6]. Notch receptors and their ligands, molecules best known for influencing cell fate decisions through direct cell-cell contact, participate in a wide variety of biological processes, including fate determination of NPCs. As gestation proceeds, mgNPCs generate com-

mitted neuronal precursors (or young neurons) expressing Notch ligands, and these cells activate Notch signaling in neighboring NPCs. Notch signal activation induces the demethylation of astrocytic gene promoters in NPCs, which explains, at least in part, how the neuronal-to-glial cell fate switch occurs in NPCs during brain development [1, 6, 7].

Oxygen is essential for cell survival as the final electron acceptor in the electron transport chain of aerobic respiration. Sensing and controlling oxygen localization are of extreme importance because normal metabolic processes generate radical intermediates. When oxygen levels exceed the capacity of endogenous antioxidant systems, radicals including reactive oxygen species attack cellular components such as nucleic acids, the sulfhydryl groups of proteins, or the unsaturated fatty acid moieties of phospholipids [8].

Hypoxia-inducible factors (HIFs) are known to play critical roles as molecular sensors of oxygen tension. HIF1 α activity in cells is controlled by oxygen levels in multiple ways, such as transcriptional regulation and hydroxylation-dependent binding by Von Hippel-Lindau tumor suppressor protein followed by proteasomal degradation [9–11]. A HIF1 complex composed of HIF1 α and HIF1 β (also known as ARNT) binds to the hypoxia response element and activates the transcription of target genes such as *inhibitor of differentiation 2* [12], *insulin-like growth factor binding protein, p21*, *phosphoglycerate kinase 1*, and *vascular endothelial growth factor* [13].

Author contribution: T.M.: conception and design, financial support, administrative support, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; T.S.: administrative support, collection and/or assembly of data, and data analysis and interpretation; K.I.: provision of study material or patients; and K.N.: conception and design, financial support, administrative support, data analysis and interpretation, and final approval of manuscript. T.M. and T.S. contributed equally to this study.

Correspondence: Kinichi Nakashima, Ph.D., Laboratory of Molecular Neuroscience, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan. Telephone: 81-743-72-5471; Fax: 81-743-72-5479; e-mail: kin@bs.naist.jp Received July 19, 2011; accepted for publication December 2, 2011; first published online in *STEM CELLS EXPRESS* December 29, 2011. © AlphaMed Press 1066-5099/2011/\$30.00/0 doi: 10.1002/stem.1019

HIF1 α has also been reported to be crucial for normal brain development [14]. Moreover, HIFs may act cooperatively with other signaling molecules, such as Notch [15] and mammalian target of rapamycin [16, 17], thereby influencing a wide range of processes including tumor malignancy and NPC growth, maintenance and differentiation [11, 18-20].

In this study, we focused on how oxygen tension affects the DNA methylation status of astrocytic genes in mgNPCs. Oxygen levels in the microenvironment around NPCs in the embryonic brain at midgestation were comparable to those seen under hypoxic culture conditions (2-5% O₂). Bisulfite sequencing revealed that these conditions promoted demethylation of the *gfap* promoter. This hypoxia-induced demethylation was mediated by cooperation between HIF1 α and the Notch signaling pathway. Furthermore, ectopic expression of a constitutively active form of HIF1 α in the embryonic brain induced precocious astrocyte differentiation of NPCs. By contrast, when embryos developed in hyperoxic conditions, astrocyte differentiation of NPCs was suppressed. These findings suggest that oxygen levels in the embryonic brain play a critical role in fine-tuning the timing of NPC fate switching during development.

MATERIALS AND METHODS

Cell Culture

mgNPCs were prepared from telencephalons of E11.5 embryos and cultured as described previously [5]. E15.5 ventricular zone (VZ) containing NPCs was manually separated from other parts of the brain using a hand-made microknife (Supporting Information Fig. S1). As described previously [5], NPCs were cultured with basic fibroblast growth factor (bFGF; 1×10^6 cells per dish) in poly-L-ornithine/fibronectin-coated 6-cm culture dishes. Hand-made chambers were used to obtain atmospheres of different oxygen levels [21]. Notch signal activation was inhibited with the γ -secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT; Calbiochem-MERCK, Darmstadt, Germany, www.merckgroup.com, 10 μ M).

Bisulfite Sequencing

Genomic DNA was extracted from NPCs and subjected to bisulfite sequencing as previously described [5]. Specific DNA fragments were amplified by polymerase chain reaction (PCR) using primers described previously [5, 7]. The PCR products were cloned into pT7Blue vector (Novagen, Darmstadt, Germany, www.merckgroup.com/en/index.html), and 10-20 randomly picked clones were sequenced. Each experiment was performed at least three times.

Lentivirus Production

A mouse *HIF1 α* -targeting short hairpin RNA (shRNA) sequence [22] was cloned into pLLX vector [23]. Lentiviruses were pseudotyped with the vesicular stomatitis virus-G envelope and concentrated by centrifugation as previously described [24]. *HIF1 α* mRNA degradation was confirmed by quantitative PCR (qPCR). All the recombinant DNA experiments in this manuscript followed the guidelines by Ministry of Education, Culture, Sports, Science and Technology of Japan, which conform to the National Institutes of Health Guidelines.

Immunostaining

Cells were fixed with 4% paraformaldehyde and processed for immunostaining as described [5]. The following primary antibodies were used: chick anti-green fluorescent protein (anti-

GFP; 1:500, Aves Labs, Tigard, OR, www.aveslab.com), rat anti-hemagglutinating (anti-HA) (1:500, Roche Applied Science, Indianapolis, IN, www.roche-applied-science.com), rat anti-bromodeoxyuridine (1:250, AbD Serotec, Raleigh, NC, www.abdserotec.com) chicken anti-Nestin (1:1000, Aves Labs), mouse anti-Map2ab (1:500, Sigma, St. Louis, MO, www.sigmaldrich.com), and mouse anti-GFAP (1:500, Sigma). Secondary antibodies were Alexa488-conjugated goat anti-rat IgG (1:500), Alexa488-conjugated goat anti-chick IgY (1:500), Alexa555-conjugated goat anti-rabbit IgG (1:500), Alexa555-conjugated goat anti-rat IgG (1:500), Alexa555-conjugated goat anti-mouse IgG (1:500), or Alexa647-conjugated goat anti-mouse IgG (1:500, Invitrogen, Carlsbad, CA, www.invitrogen.com). Nuclei were stained using bisbenzimidide H33258 fluorochrome trihydrochloride (Hoechst; Nacalai Tesque, Kyoto, Japan, www.nacalai.co.jp). All experiments were independently replicated at least three times. A Hypoxyprobe-1 Plus kit (Hypoxyprobe-Millipore, Billerica, MA, www.millipore.com) was used, following the manufacturer's protocol, to determine the hypoxicity of cultured cells and the developing brain. After culturing NPCs for 4 days in 2%, 5%, or 21% O₂, pimonidazole-HCl was added to the culture medium for 1.5 hours and the cells were then fixed. For brain sections, pimonidazole-HCl was injected intraperitoneally into pregnant mice (E15.5). Embryos were fixed 2 hours later with 4% paraformaldehyde and the brains were cryosectioned at 20- μ m intervals. Pimonidazole adducts were detected with a fluorescein isothiocyanate-conjugated specific antibody supplied with the kit (1:500). Stained sections were visualized with a confocal microscope (Fluoview FV10i, Olympus, Tokyo, Japan, www.olympus.co.jp) or a fluorescence microscope (Zeiss Axiovert 200M, Zeiss, Jena, Germany, www.zeiss.com).

Animal Procedures and Electroporation

All aspects of animal care and treatment were conducted according to the guidelines of the Experimental Animal Care Committee of Nara Institute of Science and Technology. The surgical procedures performed on pregnant ICR mice and embryo manipulations in utero were conducted as previously described [25]. E11.5 pregnant mice were deeply anesthetized by intraperitoneal injection with sodium pentobarbitone (50 μ g/g b.wt.). HA-constitutively active HIF1 α (HA-caHIF1 α) cDNA was cloned into the EcoRI site of pCAGGS vector. After the uterus was exposed, approximately 1-2 μ l of plasmid solution (1 μ g/ μ l in phosphate-buffered saline) was injected into the lateral ventricle of the telencephalon with a glass micropipette. The embryos were held with the tips of a tweezers-type electrode with a diameter of 5 mm (CUY650-P3; Tokiwa Science, Fukuoka, Japan), and five electronic pulses (27V, 50 ms, at intervals of 950 ms) were given to each embryo with an electroporator (CUY21SC; Tokiwa Science). The embryos were reinserted into the abdominal cavity and the abdominal wall was closed with surgical sutures.

Exposure to High-Oxygen Atmosphere and Tissue Oxygen Measurement

The animal cage was placed in a hyperoxic oxygen chamber (Terucom, Kanagawa, Japan, www.terucom.co.jp). Oxygen tension was measured using a portable oxygen meter (Terucom) in the same chamber. E11.5 pregnant mice were exposed to 60% O₂ for 2 days, and the oxygen tension was then raised to 80% (the upper nonlethal concentration limit) and maintained at that level until fixation at E17.5.

Local tissue oxygen tension corresponding to each atmospheric oxygen level was measured with an OxyLab pO₂ monitor (Oxford Optronix, Oxford, UK, www.oxford-optronix.com) [26] (Supporting Information Fig. S4Q). The probe was

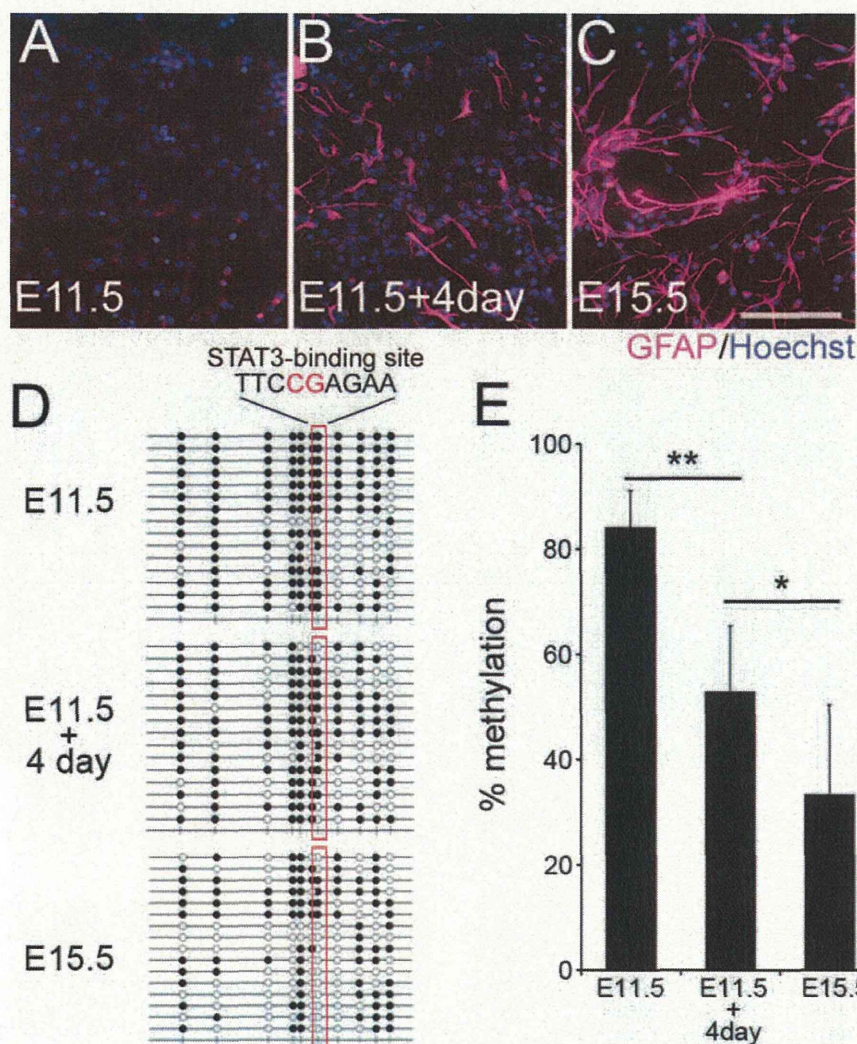


Figure 1. Astrocyte differentiation of midgestational neural precursor cells is retarded in vitro. (A–C): Leukemia inhibitory factor (LIF)-induced GFAP-positive astrocyte differentiation in E11.5 (A), in vitro 4-day-cultured E11.5 (B) and E15.5 (C) NPCs. After a 4-day culture in the presence of LIF, the cells were stained with anti-GFAP antibody (magenta) and with Hoechst to identify nuclei (blue). Scale bar = 100 μ m. (D, E): Genomic DNA was extracted from the cells, and the methylation status of the *gfap* promoter around the STAT3-binding site was examined by bisulfite sequencing. Closed and open circles indicate methylated and unmethylated CpG sites, respectively (D). E11.5 and E15.5 indicate the results obtained for freshly prepared neural precursor cells (NPCs) from E11.5 cortex and E15.5 VZ. The STAT3-binding site is marked by red rectangles. Methylation at the STAT3-binding site in the *gfap* promoter is higher in 4-day in vitro-cultured E11.5 NPCs than in freshly prepared E15.5 NPCs (E). Data are shown as means \pm SD ($n = 3$). Statistical significance was evaluated by the Student's *t* test. *, $p < .05$; **, $p < .01$. Abbreviations: GFAP, glial fibrillary acidic protein; STAT3, signal transducer and activator of transcription.

guided to the E15.5 brain using a 20-gauge needle with a plastic canula (Terumo, Tokyo, Japan, www.terumo.co.jp).

RESULTS

It is well known that IgNPCs but not mgNPCs differentiate into astrocytes in response to stimulation with IL-6 family cytokines [2–5, 7]. When we cultured IgNPCs prepared from the VZ of mouse telencephalon at E15.5 (Supporting Information Fig. S1), we observed their LIF-induced astrocyte differentiation, as judged by the expression of the astrocytic marker GFAP (Fig. 1C, $26.8 \pm 6.5\%$). By contrast, hardly any LIF-treated E11.5 mgNPCs differentiated into astrocytes (Fig. 1A). Interestingly, E11.5 mgNPCs cultured in vitro for 4

days (nominally corresponding to E15.5) did undergo astrocyte differentiation (Fig. 1B, $12.2 \pm 1.9\%$), albeit to a lesser extent than E15.5 IgNPCs.

Since an inverse correlation exists between the potential of NPCs to express *gfap* and the methylation status of the *gfap* promoter, which includes a STAT3-binding site [5, 27], we examined whether the in vitro culture conditions of mgNPCs delayed the demethylation of the promoter compared to its demethylation in vivo. Bisulfite sequencing for the *gfap* promoter of E11.5, E11.5 + 4-day culture in vitro, and E15.5 NPCs revealed that this was indeed the case (Fig. 1D, 1E). These data indicate that the in vitro culture conditions retard the demethylation of the astrocytic gene promoter in NPCs.

In terms of the physical conditions surrounding cells, one of the biggest differences between in vitro and in vivo

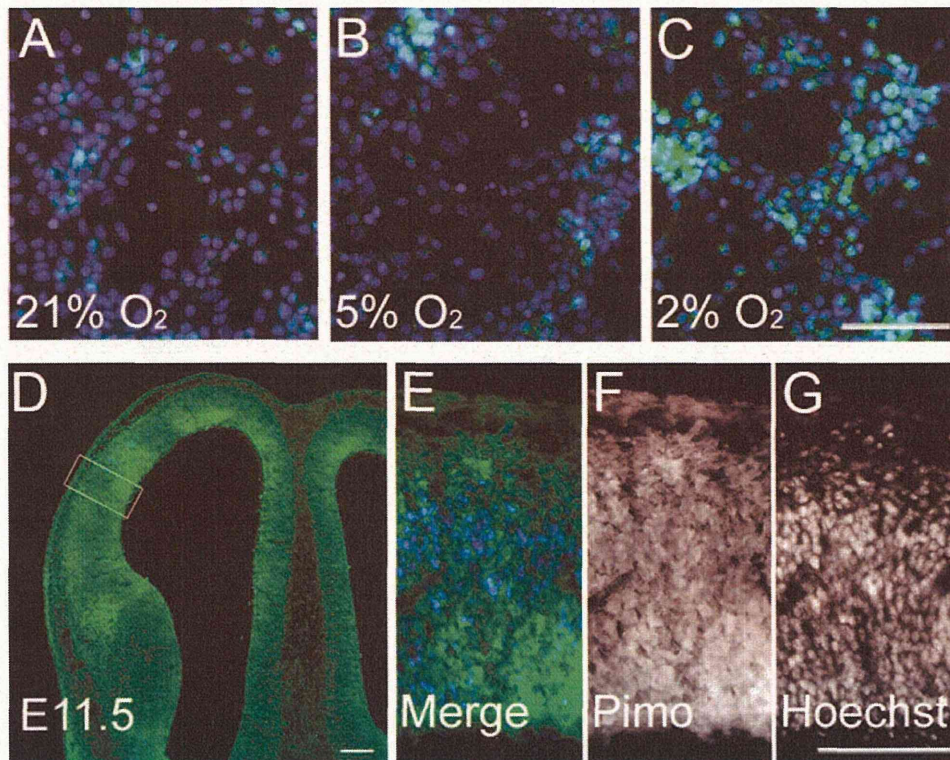


Figure 2. Oxygen levels are low in the embryonic brain. (A–C): Neural precursor cells cultured in 21% (A), 5% (B), or 2% O₂ (C) were stained with Hypoxyprobe. Pimonidazole adduct formation under hypoxic conditions (green) was observed clearly in 2% O₂ (C), less markedly in 5% O₂ (B), but not in 21% O₂ (A) (bar = 100 μ m). (D–G): E11.5 embryonic brain was stained with Hypoxyprobe. Hypoxyprobe (green); Hoechst (blue). The image in (E) is an enlargement of the area in (D) marked by the rectangle. Bar = 100 μ m.

environments is oxygen tension. The atmosphere contains 21% O₂ (160 mmHg), whereas interstitial oxygen concentration ranges from 1 to 5% (7–40 mmHg) in mammalian tissues including the embryonic brain [11, 20]. Although hypoxia is generally considered a pathological phenomenon, mammalian embryos develop naturally in a mildly hypoxic environment [28]. To determine the tissue oxygen tension in the embryonic brain, where NPCs reside, we used the chemical reagent pimonidazole. Pimonidazole is reductively activated in hypoxic cells and forms stable adducts with sulfhydryl groups in amino acids, at around or below 10 mmHg, which can be detected with specific antibodies [29, 30]. Pimonidazole adducts were weakly but clearly detected in NPCs cultured in 2% and 5% O₂ conditions, respectively, but not in NPCs cultured in 21% O₂ (Fig. 2A–2C). Pimonidazole adducts were also abundant in midgestational embryonic brain, indicating that oxygen levels there are low, particularly in the NPC-containing VZ (Fig. 2D–2G).

From the above findings, we hypothesized that culturing mgNPCs at a low oxygen level might accelerate their differentiation into astrocytes. To test this idea, mgNPCs were cultured in 2% or 21% O₂ atmospheres for 4 days in the presence of bFGF, and subsequently stimulated with LIF for an additional 4 days. As shown in Figure 3A–3C, a higher proportion of mgNPCs cultured in the presence of LIF under 2% O₂ than under 21% O₂ became GFAP-positive astrocytes. Reflecting the promotion of astrocyte differentiation, NPC (Nestin) and neuron (Map2ab) marker-positive cell numbers were lower under 2% O₂ than under 21% O₂ (Supporting Information Fig. S2A). The proliferation of cells in our normoxic and hypoxic culture was similar as judged by BrdU staining (Supporting Information Fig. S2B). Furthermore, in the 2% O₂ condition, significantly fewer CpG sites

in the *gfap* promoter were methylated than in the 21% O₂ condition (Fig. 3D); this was also the case for CpG sites in the promoter of another astrocytic marker, *S100 β* (Supporting Information Fig. S3A). Consistent with the lower methylation, *S100 β* expression in hypoxic culture in the presence of LIF was higher than that in normoxic condition (Supporting Information Fig. S3B). These results suggest that low oxygen levels facilitate the DNA demethylation of astrocytic genes in mgNPCs.

We have shown previously that Notch signal activation induces the demethylation of astrocytic genes in mgNPCs [7]. Intriguingly, *hairy* and *enhancer of split 1* (*Hes1*) and *Hes5*, two known targets of Notch signaling, were markedly upregulated in the 2% O₂ culture condition (Fig. 4A), indicating that Notch signaling is more active at lower O₂ levels. Therefore, we examined whether low oxygen levels enhance the astrocyte differentiation of mgNPCs via Notch signal activation. An inhibitor of Notch signal activation, DAPT, was added to the culture medium during the first 4-day expansion phase of mgNPCs with bFGF in the hypoxic condition. The expression of *Hes1* and *Hes5* was dramatically reduced, confirming that hypoxia-induced elevation of Notch signaling was inhibited by DAPT (Supporting Information Fig. S3C). Furthermore, as shown in Figure 4B and 4C, the astrogenic potential of NPCs was almost completely suppressed by DAPT, and the enhanced demethylation of the *gfap* and *S100 β* promoters observed in the hypoxic condition was no longer seen after DAPT treatment (Fig. 4D and Supporting Information Fig. S3D).

Activation of the Notch signaling pathway in mgNPCs induces expression of the transcription factor nuclear factor IA (NFIA), which leads to demethylation of astrocyte-specific genes including *gfap* [7]: NFIA binds to astrocytic gene

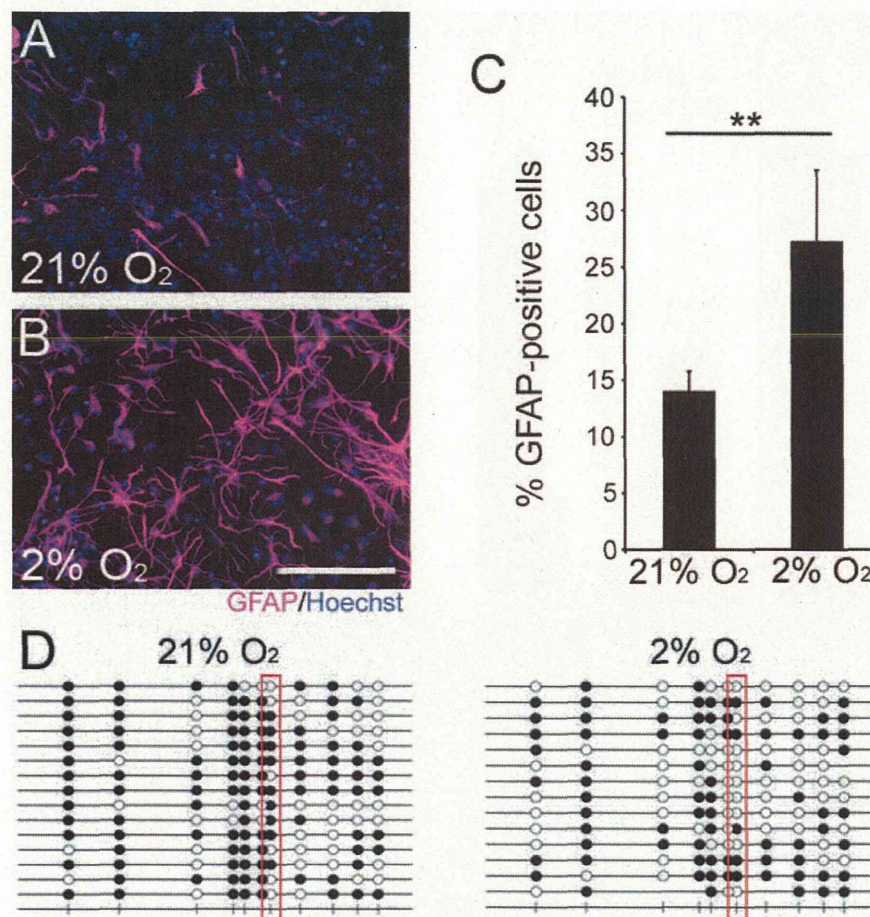


Figure 3. Culturing under 2% O₂ accelerates leukemia inhibitory factor (LIF)-induced astrocyte differentiation of neural precursor cells (NPCs). (A–C); E11.5 NPCs were cultured for 4 days under 2% O₂ (A) or 21% O₂ (B), followed by a 4-day LIF stimulation to induce astrocyte differentiation. GFAP (magenta), Hoechst (blue); bar = 100 μ m. Proportions of GFAP-positive cells in the total cell populations were quantified (C). Data are shown as means \pm SD ($n = 3$). Statistical significance was evaluated by the Student's t test. **, $p < .01$. (D); CpG methylation at the signal transducer and activator of transcription 3 (STAT3)-binding site in the *gfap* promoter is lower under 2% O₂ than under 21% O₂ after a 4-day culture of E11.5 NPCs. The STAT3-binding site is indicated by red rectangles. Abbreviation: GFAP, glial fibrillary acidic protein.

promoters and induces dissociation of DNA methyltransferase 1 (DNMT1) from the promoters, resulting in their demethylation. Therefore, we examined the expression of *Nfia* and found that it increased dramatically under the hypoxic condition (Supporting Information Fig. S4A). Moreover, we did not observe astrocyte differentiation of NFIA-deficient NPCs even when cultured with LIF under the hypoxic condition (Supporting Information Fig. S4B). Taken together, these findings suggest that activation of the Notch-NFIA cascade is the mechanism whereby hypoxia induces demethylation of the *gfap* promoter.

HIF1s have been shown to play important roles in cellular adaptation to hypoxia [11, 13, 31]. *HIF1 α* and *HIF1 β* are expressed in the developing mouse brain [32], and HIF1 α protein accumulates to a higher level in hypoxic than in normoxic conditions as a result of increased transcription and protein stabilization [33–35]. Consistent with those observations, we found that both *HIF1 α* transcript and nuclear HIF1 α protein levels in E11.5 mgNPCs were upregulated under the 2% O₂ culture condition (Supporting Information Fig. S5A, S5B). Expression of the HIF1 α target gene *Id1* was also upregulated (Supporting Information Fig. S5C), as was observed in neuroblastoma cells [12].

To determine whether HIF1 α contributes to the hypoxia-promoted astrogenic potential of mgNPCs, we suppressed *HIF1 α* mRNA using a specific shRNA. mgNPCs were prepared from E11.5 telencephalon and were infected the following day with lentiviruses expressing *HIF1 α -shRNA*, cultured under 2% O₂ for 3 days, and then stimulated with LIF for a further 4 days. The level of *HIF1 α* mRNA was reduced greatly in cells infected with *HIF1 α* shRNA-expressing viruses (Supporting Information Fig. S6A). Moreover, *HIF1 α* shRNA expression markedly diminished both the astrogenic potential of mgNPCs and the degree of demethylation in the *gfap* promoter (Fig. 5A–5G, and Supporting Information Fig. S6B).

caHIF1 α is a hydroxylation-resistant, constitutively active mutant form of HIF1 α in which two amino acids (prolines 402 and 564) are substituted with alanines [36]. One-day in vitro-cultured E11.5 mgNPCs were infected with lentiviruses expressing HA-tagged caHIF1 α (HA-caHIF1 α) and cultured for a further day, and then subjected to LIF stimulation for 3 days under the normoxic condition. HA-caHIF1 α protein was clearly detected in the nucleus (Fig. 5M). In contrast to control virus-infected mgNPCs (Fig. 5H–5K), cells transduced to express HA-caHIF1 α became GFAP-positive astrocytes in response to LIF stimulation, even under the 21% O₂ condition

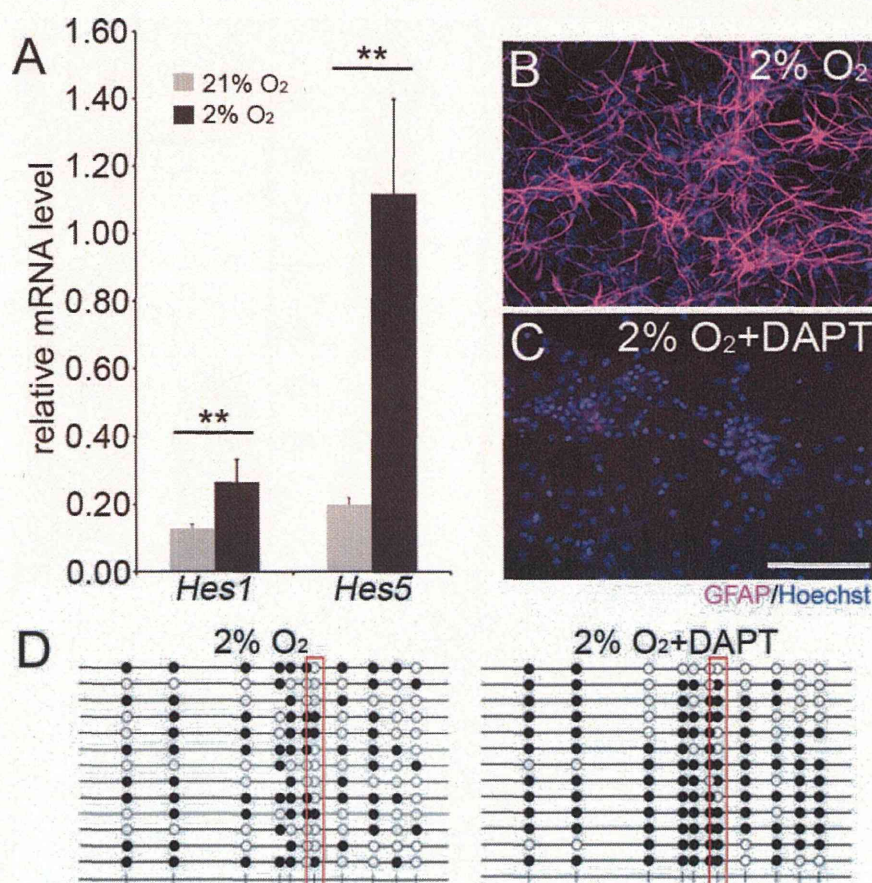


Figure 4. Notch signal activation is required for hypoxia-promoted acquisition of astrocytic potential by neural precursor cells (NPCs). (A): Quantitative polymerase chain reaction analysis of the expression of Notch signal target genes. Expression of the representative Notch signal targets *Hes1* and *Hes5* was upregulated in midgestational neural precursor cells cultured for 4 days under 2% O_2 . Statistical significance was evaluated by the Student's *t* test. **, $p < .01$. (B, C): Hypoxia-induced astrocytic potential of NPCs was blocked by Notch signal inhibition. E11.5 NPCs were cultured for 4 days in the 2% O_2 condition with or without DAPT (10 μ M), followed by a 4-day leukemia inhibitory factor stimulation. Bar = 100 μ m. (D): The methylation status of the *gfap* promoter in E11.5 NPCs cultured with or without DAPT (10 μ M) in the 2% O_2 condition was examined by bisulfite sequencing. The signal transducer and activator of transcription 3-binding site is indicated by red rectangles. Abbreviations: DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester; GFAP, glial fibrillary acidic protein.

(Fig. 5L–5O). HA-caHIF1 α expression also augmented Notch signal activation in these cells (Fig. 5P). Furthermore, HA-caHIF1 α -induced astrocyte differentiation and Notch signal activation were both abolished by DAPT treatment (Fig. 5P and Supporting Information Fig. S6C–S6J). Consistent with the results of these *in vitro* experiments, ectopic expression of HA-caHIF1 α in E11.5 brains led to precocious GFAP expression in NPCs (Supporting Information Fig. S6K–S6P). These experiments suggest that HIF1 α promotes the astrocytic potential of mgNPCs by enhancing Notch signal activation, probably because hypoxia-stabilized HIF1 α can form a complex with the Notch intracellular domain (NICD) to effectively induce the expression of Notch-target genes [15].

Given that the 21% O_2 condition delayed the acquisition of astrocyte differentiation ability by NPCs *in vitro*, we next asked whether a hyperoxic environment has a similar effect on NPCs *in vivo*. To address this, pregnant mice were housed in a normoxic or hyperoxic chamber for 6 days (E11.5 to E17.5). To confirm that the embryonic brain was indeed under hyperoxia, we measured the local brain oxygen tension at E15.5 using an oxygen electrode. When the pregnant mice were in the normoxic (21% O_2) and hyperoxic (80% O_2) con-

ditions, the oxygen tensions were 5.24 and 112.9 mmHg, respectively (Supporting Information Fig. S6Q), indicating that local brain oxygen tension was increased as a result of the atmospheric oxygen levels surrounding the pregnant mother mouse. After 6 days of hyperoxic housing, embryonic brains were then subjected to immunostaining and Western blotting. Interestingly, as shown in Figure 5Q–5S, exposure to high oxygen markedly decreased GFAP expression in the brains of embryos of mice housed in the hyperoxic condition.

These results suggest that a hypoxic environment is important for the proper timing of astrocyte differentiation during embryonic development.

DISCUSSION

HIF1 α is known to be an important factor in the response of various types of cells to hypoxic stress such as ischemia [11, 13] and to play a critical role in normal brain development [14]. In the present study, we have shown that astrocytic genes are progressively demethylated in the mildly hypoxic environment of the developing brain (Figs. 1–3). We further

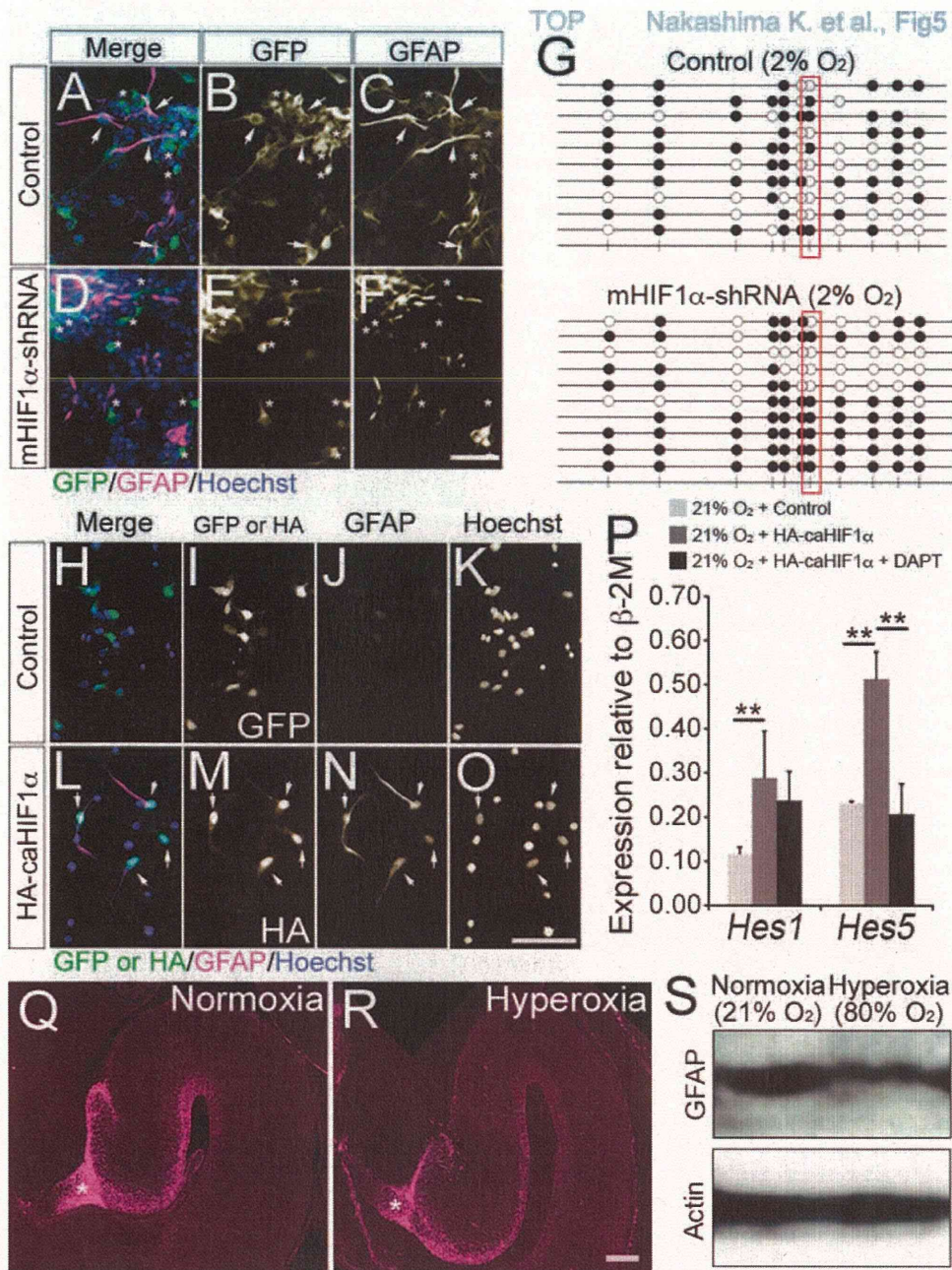


Figure 5. Astrogenic potential of neural precursor cells (NPCs) is regulated by HIF1 α and oxygen levels. (A–F): E11.5 NPCs were infected with lentiviruses encoding GFP alone or GFP together with mHIF1 α -shRNA, and cultured under 2% O₂ for 3 days followed by a 4-day leukemia inhibitory factor (LIF) stimulation. Virus-infected cells were identified by their GFP expression (green) and analyzed for GFAP expression (magenta) by immunocytochemistry. Hoechst, blue. GFP and GFAP double-positive cells (arrows) were observed in control virus-infected cells (A–C), whereas most of the mHIF1 α -shRNA-expressing virus-infected cells were positive for GFP only (asterisks) (D–F). Bar = 50 μ m. (G): The methylation status of the *gfap* promoter in 3-day-cultured virus-infected cells was examined by bisulfite sequencing. Demethylation in mHIF1 α -shRNA-expressing virus-infected cells was inhibited. The signal transducer and activator of transcription 3-binding site is indicated by red rectangles. (H–O): Hydroxylation-mediated degradation-resistant constitutively active HIF1 α (HA-caHIF1 α) was introduced into E11.5 NPC by lentivirus infection, and the cells were cultured for 1 day followed by a 3-day LIF stimulation under 21% O₂. Precocious GFAP expression was observed in HA-caHIF1 α -expressing virus-infected cells (arrows), even under 21% O₂ (L–O). Bar = 50 μ m. (P): E11.5 NPCs were infected with control or HA-caHIF1 α -expressing lentiviruses on the day after preparation and cultured in the presence of LIF for 3 days with or without DAPT under 21% O₂, and their RNAs were then subjected to quantitative polymerase chain reaction. *Hes1* and *Hes5* expression was normalized to that of β -2M. The expression of both of these genes was upregulated by HA-caHIF1 α expression, and this effect was abolished by DAPT treatment. Statistical significance was evaluated by the Student's *t* test. **, *p* < .01. (Q, R): GFAP expression in E17.5 brain was reduced when pregnant mice were housed in a hyperoxic atmosphere (80% O₂) from E11.5 to E17.5. Brain sections are stained with anti-GFAP antibody (magenta). Hippocampal regions are shown as representatives. Asterisks, fimbria. Bar = 100 μ m. (S): Whole brains of embryos from pregnant mice housed in normoxic (21% O₂) and hyperoxic (80% O₂) atmospheres were lysed and subjected to immunoblot to detect GFAP and β -actin (as loading control) expression. Abbreviations: β -2M, β -2 microglobulin; caHIF1 α , constitutively active HIF1 α ; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; HA, hemagglutinating; mHIF1 α , mouse HIF1 α ; shRNA, short hairpin RNA.

suggest that this demethylation process is executed by a collaboration between HIF1 α and Notch signal activation (Figs. 4, 5), probably through the formation of a complex between HIF1 α and NICD, to effectively induce the expression of Notch-target genes [15]. The augmentation of Notch signal activation under hypoxia led to higher expression of *Nfia* (Supporting Information Fig. S4A), which has been shown to induce dissociation of DNMT1 from astrocytic gene promoters, resulting in demethylation of the promoters [7]. It has been suggested that HIFs act cooperatively with other signaling molecules, thereby influencing a wide range of biological processes including NPC maintenance and differentiation [11, 18–20]. Nevertheless, we report for the first time, to the best of our knowledge, that oxygen levels not only modify the behavior of transcription factors but also affect the epigenetic status of genes. The promotion of astrocytic gene demethylation by the hypoxic condition contributes to specifying the appropriate timing of the neural-to-glial cell fate switch of NPCs, by ensuring a proper balance between neurons and astrocytes that are generated during brain development.

We also found in this study that oxygen tension affects the DNA methylation status of astrocytic genes in mgNPCs via HIF-Notch signaling. Strikingly, hypoxia, HIF1 α , Notch signaling and DNA methylation are all known to participate in the onset and/or progression of glioblastoma [12, 13, 15, 37], the most common and malignant type of brain tumor. Thus, a deeper understanding of glial cell-generating mechanisms, including astrocyte differentiation, may be of therapeutic interest.

As has been shown in a previous study [5] and the present work, DNA demethylation in the astrocytic gene promoters of NPCs is crucial for astrocyte differentiation, and the efficacy of astrocyte differentiation of NPCs is influenced by oxygen levels in the brain throughout development. Premature infants in neonatal intensive care units (NICUs) are often incubated under hyperoxic conditions to support their immature respiration. Oxygen concentration is strictly controlled in NICUs, since it has been shown that excess oxygen administration causes retinopathy of prematurity [38]. Furthermore, a few studies report other risks of hyperoxia to the central nervous system of extremely premature infants, including defects in mental and psychomotor development [39, 40]. The present study suggests that such effects may be attributable to an

imbalance in NPC differentiation caused by a high level of oxygen in the incubator, and identifying their underlying mechanism in future work could provide new approaches for clinical applications that address developmental abnormalities in the nervous system, particularly in the context of neonatal intensive care.

CONCLUSION

We showed that local oxygen concentration can affect the fate of NPCs through an epigenetic mechanism. Unraveling how actual microenvironmental oxygen levels in the embryo influence epigenetic gene regulation in NPCs will provide new aspects for the study of NPC regulation.

ACKNOWLEDGMENTS

We thank T. Kobayashi for the hyperoxic oxygen chamber, H. Harada, M. Hiraoka, T. Takizawa, M. Namihiro, Y. Bessho, T. Matsui, Y. Nakahata, and K. Semi for valuable discussions and technical advice, I. Smith for editing the manuscript, and M. Tano for secretarial assistance. This work was supported by a Grant-in-Aid for Young Scientists (Start-up), Grant-in-Aid for Scientific Research on Innovative Area: Neural Diversity and Neocortical Organization, Grant-in-Aid for Research Program of Innovative Cell Biology by Innovative Technology and by the NAIST Global COE Program (Frontier Biosciences: Strategies for survival and adaptation in a changing global environment) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Health Sciences Research Grants from the Ministry of Health, Labor and Welfare, Japan. T.M. is currently affiliated with Biological Sciences, Gunma Kokusai Academy Secondary School, Ota, Gunma, Japan.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

- Hirabayashi Y, Gotoh Y. Epigenetic control of neural precursor cell fate during development. *Nat Rev Neurosci* 2010;11:377–388.
- 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.
- 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.
- 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.
- Takizawa T, Nakashima K, Namihiro M et al. DNA methylation is a critical cell-intrinsic determinant of astrocyte differentiation in the fetal brain. *Dev Cell* 2001;1:749–758.
- Pierfelice T, Alberi L, Gaiano N. Notch in the vertebrate nervous system: An old dog with new tricks. *Neuron* 2011;69:840–855.
- Namihiro M, Kohyama J, Semi K et al. Committed neuronal precursors confer astrocytic potential on residual neural precursor cells. *Dev Cell* 2009;16:245–255.
- Slater TF. Free-radical mechanisms in tissue injury. *Biochem J* 1984; 222:1–15.
- Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 2001;294:1337–1340.
- Sang N, Fang J, Srinivas V et al. Carboxyl-terminal transactivation activity of hypoxia-inducible factor 1 alpha is governed by a von Hippel-Lindau protein-independent, hydroxylation-regulated association with p300/CBP. *Mol Cell Biol* 2002;22:2984–2992.
- Simon MC, Keith B. The role of oxygen availability in embryonic development and stem cell function. *Nat Rev Mol Cell Biol* 2008;9: 285–296.
- Lofstedt T, Jogi A, Sigvardsson M et al. Induction of ID2 expression by hypoxia-inducible factor-1: A role in dedifferentiation of hypoxic neuroblastoma cells. *J Biol Chem* 2004;279:39223–39231.
- Semenza G. Signal transduction to hypoxia-inducible factor 1. *Biochem Pharmacol* 2002;64:993–998.
- Tomita S, Ueno M, Sakamoto M et al. Defective brain development in mice lacking the Hif-1alpha gene in neural cells. *Mol Cell Biol* 2003;23:6739–6749.
- Gustafsson MV, Zheng X, Pereira T et al. Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell* 2005;9: 617–628.
- Dunlop EA, Tee AR. Mammalian target of rapamycin complex 1: Signaling inputs, substrates and feedback mechanisms. *Cell Signal* 2009; 21:827–835.
- Land SC, Tee AR. Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. *J Biol Chem* 2007;282:20534–20543.
- Jogi A, Ora I, Nilsson H et al. Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. *Proc Natl Acad Sci USA* 2002;99:7021–7026.