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MicroRNA-153 Regulates the Acquisition of Gliogenic Competence by Neural Stem Cells

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SUMMARY

Mammalian neural stem/progenitor cells (NSPCs) sequentially generate neurons and glia during CNS development. Here we identified miRNA-153 (miR-153) as a modulator of the temporal regulation of NSPC differentiation. Overexpression (OE) of miR-153 delayed the onset of astrogliogenesis and maintained NSPCs in an undifferentiated state in vitro and in the developing cortex. The transcription factors nuclear factor I (NFI) A and B, essential regulators of the initiation of gliogenesis, were found to be targets of miR-153. Inhibition of miR-153 in early neurogenic NSPCs induced precocious gliogenesis, whereas NFIA/B overexpression rescued the anti-gliogenic phenotypes induced by miR-153 OE. Our results indicate that miR-mediated fine control of NFIA/B expression is important in the molecular networks that regulate the acquisition of gliogenic competence by NSPCs in the developing CNS.

INTRODUCTION

Development of the vertebrate CNS is directed by the generation of various types of neurons and glia from multipotent neural stem/progenitor cells (NSPCs) in spatially and temporally regulated patterns (Okano and Temple, 2009). The temporal regulation of NSPC fate largely depends on the intrinsic regulation of cellular differentiation potential (Miller and Gauthier, 2007; Okano and Temple, 2009). For instance, NSPCs in early gestation cannot differentiate into astrocytes because they lack the competency to respond to gliogenic differentiation signals, including signals from the interleukin-6 (IL-6)/Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (Koblar et al., 1998; Nakashima et al., 1999), the bone morphogenic protein 2/4 (BMP2/4)/SMAD pathway (Gross et al., 1996; Nakashima et al., 2001), and the Notch signaling pathway (Ge et al., 2002; Grandbarbe et al., 2003). Therefore, gliogenesis starts during late embryonic stages after the acquisition of gliogenic competence by NSPCs to respond to these assorted signals at midgestation. Therefore, they generate neurons first in the developing brain, followed by glia.

The neurogenic-to-gliogenic transition of NSPCs is probably governed by a multi-layered system. The epigenetic status of astrocyte-specific (Takizawa et al., 2001) and neurogenic genes changes during development (Hirabayashi et al., 2009; Kishi et al., 2012; Pereira et al., 2010), critically determining NSPC responsiveness to extrinsic differentiation signals. Several transcription factors regulate the acquisition of gliogenic competence. For example, nuclear factor IA (NFIA) acts as a key regulator for the initiation of gliogenesis (das Neves et al., 1999; Deneen et al., 2006; Kang et al., 2012). *Nfia* is induced by the high-mobility group (HMG) box family member SOX9 and forms a SOX9/NFIA complex to control the induction of a subset of glial-specific genes (Kang et al., 2012). Moreover, NFIA is required for Notch signaling-induced demethylation of the glial fibrillary acidic protein (*Gfap*) gene promoter in NSPCs (Namihira et al., 2009). Our group has reported previously that chicken ovalbumin upstream promoter-transcription factor (COUP-TF) I and COUP-TFII are triggers of the neurogenic-to-gliogenic competence switch in NSPCs (Naka et al., 2008). However, in vitro knockdown (KD) of *Coup-tfI/II* in NSPCs did not substantially affect the expression levels of *Nfia* (Naka et al., 2008). Therefore, multiple transcriptional regulatory cascades act together to control NSPC acquisition of gliogenic competence.

MicroRNAs (miRNAs) are small endogenous non-coding RNAs found in many different organisms, including animals that regulate gene expression mainly at the post-transcriptional level (Bartel, 2004). In vertebrates, miRNAs base-pair with target sequences typically located within the 3′ UTR of target mRNAs by using 5′ "seed" regions. Furthermore, miRNAs stimulate RNA-silencing complexes to induce degradation, destabilization, and/or translational inhibition of target mRNAs (Bartel, 2009; Guo et al., 2010; Huntzinger and Izaurralde, 2011) and are seemingly involved in almost all cellular events, including the determination of cell fate (Ebert and Sharp, 2012; Friedman et al., 2009). In the developing mammalian CNS, various miRNAs participate in the control of neural

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stem cell self-renewal, proliferation, and differentiation (Balzer et al., 2010; Cimadamore et al., 2013; Li and Jin, 2010; Naka-Kaneda et al., 2014; Neo et al., 2014; Qureshi and Mehler, 2012; Shibata et al., 2011; Visvanathan et al., 2007; Yoo et al., 2009; Zhao et al., 2009).

This study identifies miR-153 as a regulator of the initiation of gliogenesis in the developing CNS. Although miR-153 is implicated in synaptic function, neurodegenerative disorders, and fetal ethanol exposure (Chi et al., 2009; Doxakis, 2010; Liang et al., 2012; Tsai et al., 2014; Wei et al., 2013), no reports to date have described a function for miR-153 in gliogenesis by NSPCs. Here we demonstrate that miR-153 inhibits the acquisition of gliogenic competence in NSPCs by targeting *Nfia/b* mRNAs.

RESULTS

Identification of Anti-Gliogenic miRNAs in NSPCs

Because the NFI transcription factors have been shown to be key regulators of gliogenesis (Shu et al., 2003; Deneen et al., 2006; Barry et al., 2008), we utilized this pathway to identify miRNAs involved in the regulation of the neurogenesis-to-gliogenesis switch by NSPCs. We focused on miRNAs with expression levels that were differentially regulated downstream of NFIA and established a mouse embryonic stem cell (ESC) line that expresses NFIA in a doxycycline (Dox)-dependent manner (the so-called "tet-off" system; Figures S1A and S1B). We also took advantage of a mouse pluripotent stem cell culture system that recapitulates the temporal specification of NSPCs (Miura et al., 2009; Naka-Kaneda et al., 2014; Naka et al., 2008; Okada et al., 2008).

To confirm the gliogenic role and cell-autonomous actions of NFIA in the promotion of gliogenesis, we cocultured multipotent NSPCs derived from tet-off ESCs and wild-type (WT) ESCs to form aggregated and mixed (WT/NFIA-mix) neurospheres (Figure S1C). The differentiation phenotype of the neurospheres was then assessed after the induction of exogenous NFIA expression by withdrawing Dox. NFIA overexpression (OE) promoted astrogliogenesis only after at least one passage of ESC-generated primary neurospheres (PNs) derived via embryoid body (EB) formation (data not shown). Therefore, the following in vitro functional studies of ESC-derived NSPCs were carried out using secondary or tertiary neurospheres (SNs and TNs). As expected, NFIA OE significantly facilitated astrocytic differentiation and suppressed neuronal differentiation of NSPCs in WT/NFIA-mix neurospheres (Figures S1D and S1E). On the other hand, intriguingly, we did not observe any significant increase in astrocytic differentiation but, rather, inhibition of neuronal differentiation of NSPCs derived from neurospheres composed of only NFIA OE cells (Figures S1D and S1E). This phenotypic difference may be due to a difference in the amount of endogenous gliogenic factors such as cardiotrophin-1, which is secreted from neurons (Barnabé-Heider et al., 2005). No significant differences were observed between WT cells within WT/NFIA-mix neurospheres and NFIA knockin cells in the presence of Dox in terms of their neuronal and glial differentiation phenotypes (Figures S1D and S1E).

Next, global miRNA expression patterns were compared between control and NFIA OE cells isolated from WT/NFIA-mix neurospheres. We identified ten candidate miRNAs (with 11 probes) that were expressed in a temporally regulated manner during the in vitro development of ESC-derived NSPCs and showed altered expression by NFIA OE in the manner of accelerated temporal changes (Figures 1A and 1B; Table S1; Supplemental Experimental Procedures). To assess the function of these miRNAs, lentiviral vectors expressing each miRNA were generated and infected into SNs, followed by induction of differentiation (Figure 1A). Of these miRNAs, only OE of miR-124, miR-153, and miR-219 significantly decreased the proportion of GFAP-positive (+) astrocytes (Figures 1C and 1D). However, only miR-124 OE significantly increased the appearance of βIII-TUBULIN+ neurons. All three miRNAs were highly expressed in PNs and downregulated during neurosphere passage and by NFIA OE (Table S1). Of note, our screening did not identify any miRNAs promoting astrogliogenesis. The miR-124 OE phenotype is consistent with previous results showing that miR-124 is crucial for neurogenesis (Kawahara et al., 2012). However, the roles of miR-153 and miR-219 in mammalian NSPCs have not yet been elucidated. Therefore, we focused on miR-153 and miR-219 for further investigation.

To confirm the anti-gliogenic function of miR-153 and miR-219, we examined the differentiation phenotype of the more gliogenic TNs after lentiviral transduction at the time of plating for SN formation. Under these conditions, miR-153 OE, but not miR-219 OE, still significantly inhibited astrocytic differentiation (Figures 1E and 1F). Therefore, miR-124, miR-153, and miR-219 differentially influence the timing of gliogenesis in developing NSPCs.

Spatiotemporal Expression of miR-153 in Developing NSPCs

To determine whether miR-153 and miR-219 could inhibit gliogenesis during the neurogenic phase of cortical development, we analyzed their expression patterns in the developing mouse CNS by detecting mature miRNAs by in situ hybridization (ISH) with locked nucleic acid (LNA) probes. On embryonic days (E) 9.5 and E10.5, expression of miR-153 was broadly detected throughout the CNS, including in the ventricular zone (VZ) (Figures 2A and 2B). In the E14.5 forebrain, miR-153 was observed

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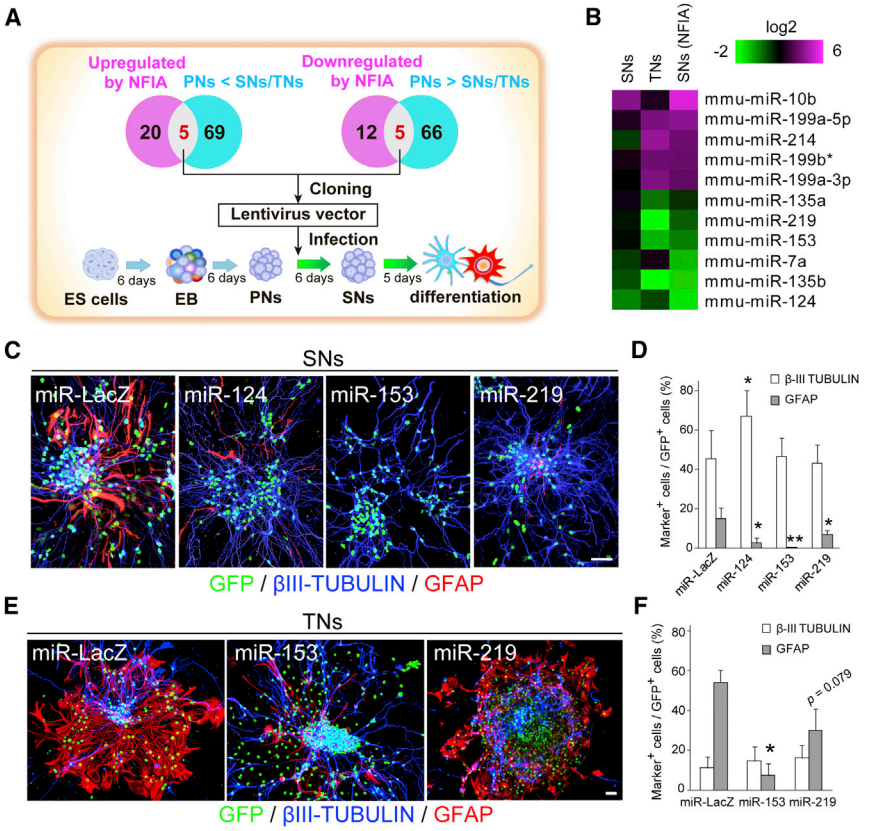

Figure 1. Identification of Anti-Gliogenic miRNAs

(A) Schematic of the screening process for miRNAs involved in the temporal specification of NSPCs. PNs generate only neurons. SNs and TNs can also generate astrocytes. Left: Venn diagram depicting the overlap between miRNAs upregulated (>1.5-fold) by NFIA OE in SNs and miRNAs with a >1.5-fold higher expression level in late versus early NSPCs. Right: Venn diagram depicting the overlap between miRNAs downregulated (<0.75-fold) by NFIA OE in SNs and miRNAs with a >1.5-fold higher expression level in early versus late NSPCs (see Supplemental Experimental Procedures). Ten miRNAs with 11 probes extracted by the comparative analysis were functionally analyzed by OE via lentiviral vectors in SNs.

(B) Heatmap of the ten candidate miRNAs differentially expressed during the passage of ESC-derived neurospheres and responsive to NFIA OE. Expression levels are shown relative to that in PNs.

(C and D) OE of miR-124, miR-153, or miR-219 inhibits astrocytic differentiation from SNs. Percentages of β-III TUBULIN+ and GFAP+ cells among the lentivirus-infected GFP+ cells are shown (n = 4).

(E and F) OE of miR-153 (but not miR-219) in TNs inhibits astrocytic differentiation (n = 3). Lentiviruses were transduced at the time of plating for SN formation.

Data represent the means ± SD (*p < 0.05, **p < 0.01 versus miR-LacZ control; n = number of independent experiments). Scale bars, 50 μm.

throughout the ventral region, including the VZ and the cortical plate (CP), but was only faintly detected in the cortical VZ (Figure 2C). In contrast, miR-219 was found in the E9.5 mesencephalon, rhombencephalon, and spinal cord but not in the prosencephalon (Figure S2). Because miR-153 displayed stronger anti-gliogenic potential (Figures 1E and 1F) and miR-219 is not expressed in the developing forebrain/telencephalon/cortex, we focused only on the role of miR-153 in the remainder of this study.

Next we verified the expression levels of miR-153 in ESC-derived NSPCs at different developmental stages in vitro by RT-qPCR analysis. Consistent with the miRNA array data (Figure 1B), miR-153 expression levels decreased as the number of neurosphere passages increased (Figure S3). We also analyzed miR-153 expression in NSPCs in the developing mouse forebrain by RT-qPCR. NSPCs were purified from *Nestin*- EGFP transgenic mouse embryos (Kawaguchi et al., 2001) via fluorescence-activated cell sorting (FACS). Compared with the E10.5 telencephalon, miR-153 expression levels in NSPCs derived from the E14.5 and E18.5 cortex were significantly attenuated. Nevertheless, similar expression levels were maintained in the E10.5 telencephalon and the E14.5 and 18.5 lateral ganglionic eminence (LGE) (Figure 2D), consistent with the ISH results (Figures 2A–2C). Moreover, because the LGE and, subsequently, the adult subventricular zone (SVZ) constantly generate neurons throughout life (Alvarez-Buylla et al., 2008), these findings indicate that miR-153 expression in NSPCs is related to prolonging the neurogenic potential of NSPCs.

Effects of miR-153 on the Acquisition of NSPC Gliogenic Competence

Although miR-153 OE inhibited astrogliogenesis even in TNs (Figures 1E and 1F), no significant changes in neurogenesis were observed. Therefore, we further analyzed the fate of miR-153 OE NSPCs after the induction of differentiation in vitro. Differentiated TNs were first immunostained with antibodies against the SOXB family of transcription factors (SOX1/2/3), which are specifically expressed in

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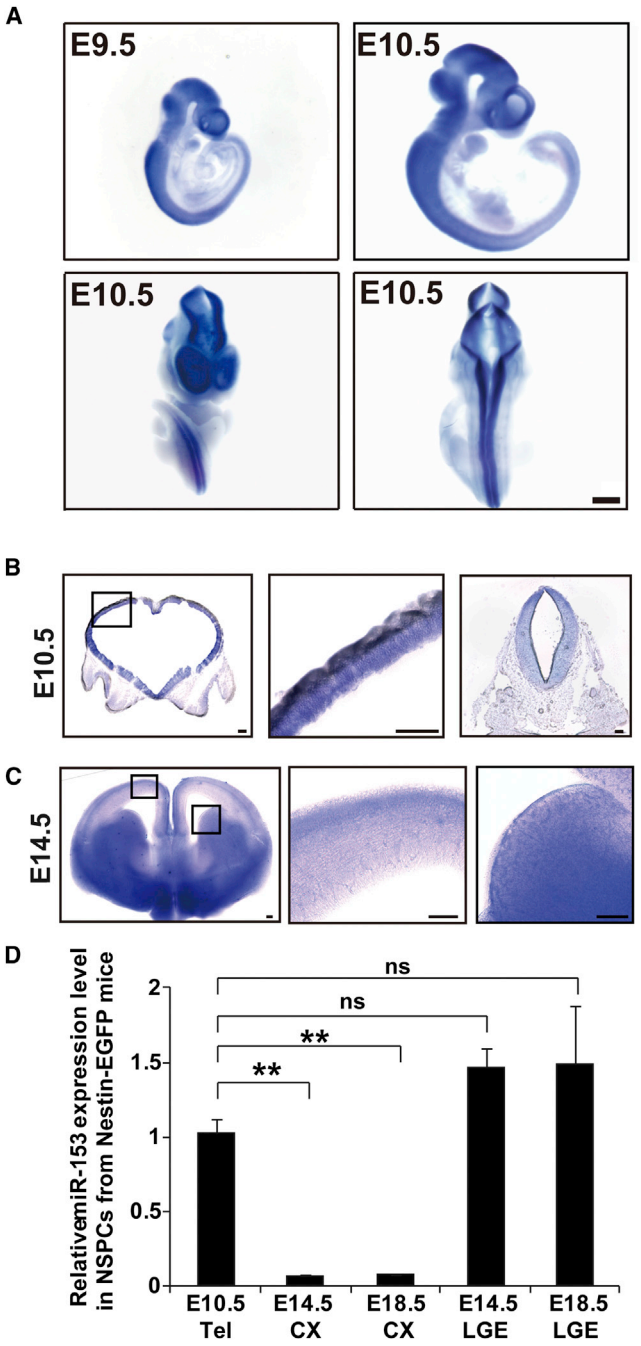

Figure 2. Spatiotemporal Regulation of miR-153 Expression in the Developing CNS

(A) Whole-mount ISH showing the expression patterns of mature miR-153 at E9.5 and E10.5. Scale bars, 500 μm.

(B and C) Coronal sections of an E10.5 embryo and E14.5 forebrain after ISH to mature miR-153. Scale bar, 100 μm.

(D) qPCR analysis of mature miR-153 expression in NSPCs from *Nestin*-EGFP mouse embryos at E10.5, E14.5, and E18.5. Expression levels relative to that in E10.5 telencephalon are presented as fold differences. Tel, telencephalon; CX, cortex; LGE, lateral ganglionic eminence. Data represent the means ± the SD (n = 3 embryos; *p < 0.05, **p < 0.01; ns, not significant).

undifferentiated neural progenitors (Wood and Episkopou, 1999). The antibody used recognized mainly SOX1/3 but was faintly reactive against SOX2 (Okada et al., 2004; Tanaka et al., 2004). Notably, a SOXB+ undifferentiated population was increased significantly in TNs by miR-153 OE (Figures 3A and 3B).

Next, we asked whether miR-153 OE affects the responsiveness of NSPCs to leukemia inhibitory factor (LIF) and BMP2, two well-known astrogliogenic cytokines. miR-153 OE inhibited astrocytic differentiation of SNs and TNs, even in the presence of LIF and BMP2 (Figures 3C–3E), but to a lesser extent than in the absence of the cytokines (Figures 1C–1F). Unexpectedly, neuronal differentiation of TNs was increased significantly by miR-153 OE in the presence of LIF and BMP2. Collectively, these results indicate that miR-153 suppresses the acquisition of gliogenic competence by NSPCs and maintains their neurogenic potential, although to a limited extent. However, miR-153 may also inhibit the differentiation of committed glial precursors, even in the face of glial differentiation signals. The increase in neuronal numbers only in the presence of LIF and BMP2 may be due to the actions of BMP2 as a neurogenic signal. In this regard, BMP can enhance astrocytic as well as neuronal differentiation, depending on the developmental stage (Hegarty et al., 2013; Li et al., 1998). If that is the case, some of miR-153 OE NSPCs may have maintained an early neurogenic status. Alternatively, miR-153 may reduce the sensitivity of NSPCs to various neurogenic signals, including BMPs.

Suppression of Astrogliogenesis by miR-153 in the Developing Cortex

To explore the in vivo function of miR-153, we overexpressed miR-153 in the developing mouse cortex via lentiviral infection and analyzed the fate of the infected NSPCs by immunohistochemistry with anti-NeuN antibody, a marker for neurons; anti-Acyl-CoA synthetase, bubblegum family, member 1 (ACSBG1) antibody, a marker for astrocytes (Cahoy et al., 2008); and anti-SOXB antibody, a marker for undifferentiated progenitors. Lentivirus vectors were injected into the lateral ventricle (LV) on E12.5, and brains were fixed on postnatal day 14 (P14). miR-153 OE significantly increased the number of NeuN+ neurons and SOXB+ undifferentiated cells in the CP, and significantly decreased the number of ACSBG1+ astrocytes (Figures 4A–4G), similar to the in vitro results of Figure 3. The SOXB+ miR-153 OE cells in the CP potentially included undifferentiated glial precursors. Importantly, miR-153 OE did not influence NSPC migration (Figure S4).

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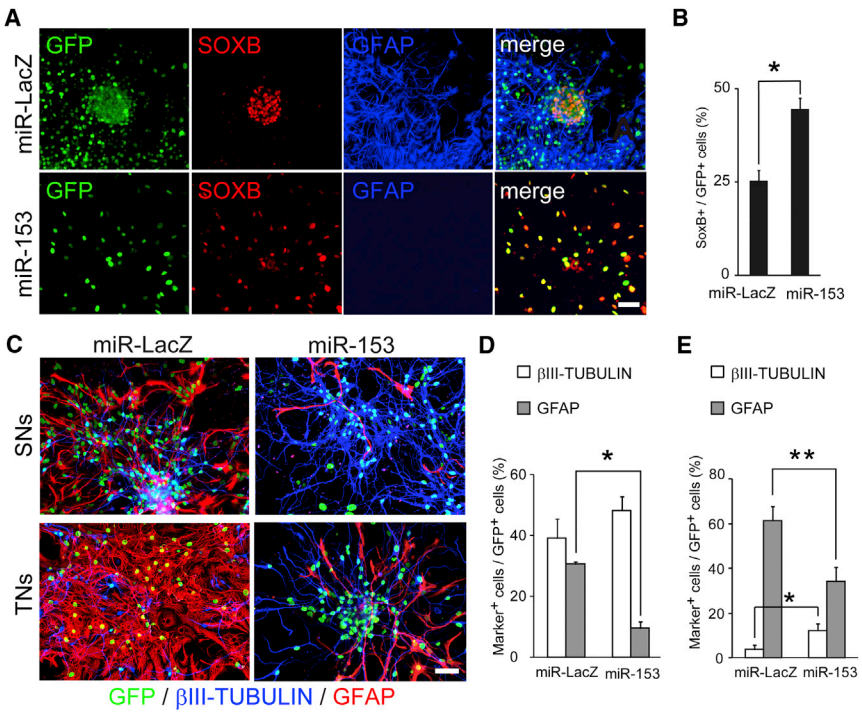

Figure 3. Suppression of Gliogenic Competence by miR-153 OE

TNs infected with lentiviruses expressing miR-LacZ or miR-153 were obtained and allowed to differentiate as described in Figure 1.

(A) Immunocytochemical analysis of SOXB and GFAP expression after induction of differentiation.

(B) Percentages of SoxB+ cells among GFP+ cells.

(C) Immunocytochemical analysis of differentiated TNs exposed to LIF (10 ng/ml) and BMP2 (100 ng/ml) during differentiation.

(D and E) Relative percentages of GFAP+ and βIII-TUBULIN+ cells among GFP+ cells in the SNs (D) and TNs (E).

Data in (B), (D), and (E) represent the mean ± SD (n = 3 independent experiments; *p < 0.05, **p < 0.01). Scale bars, 50 μm.

Therefore, miR-153 apparently suppresses the differentiation of NSPCs into astrocytes, even after radial migration in the developing cortex.

The short-term effects of miR-153 OE were also assessed on NSPC proliferation and neurogenesis in the late embryonic cortex by in utero electroporation (IUE) of a miR-153-expression vector into the E15.5 cortical VZ. Two days after electroporation, pregnant mice were administered a single intraperitoneal injection of 5-bromodeoxyuridine (BrdU, 50 mg/kg) and sacrificed 0.5 hr later. miR-153 OE did not alter the numbers of TBR2 (T-box, brain, 2)+ neuronal precursors (Englund et al., 2005) or BrdU-labeled S phase cells (Figures 4H–4J), indicating that miR-153 enhances neither progenitor proliferation nor neurogenesis in the late embryonic cortex. Collectively, the increase in NeuN+ neurons (Figures 4A–4G) may simply result from the prolonged maintenance of the neurogenic state of NSPCs.

Nfia and *Nfib* Are Targets of miR-153

We next set out to identify the direct molecular targets of miR-153 underlying the miR-153-mediated regulation of gliogenesis in NSPCs. Because miRNAs generally bind to and induce the degradation of their target mRNAs (Guo et al., 2010), global gene expression was compared in miR-153 OE and NFIA OE NSPCs at different developmental stages (i.e., ESC-derived PNs and TNs) via gene expression microarray analysis. Candidate genes were restricted by using web-based miRNA target prediction programs, and based on their developmental expression patterns and their loss-of-function (LOF) phenotypes reported previously (Table S3). Interestingly, *Nfia* and *Nfib* were among the seven genes fitting these stringent criteria (see Supplemental Experimental Procedures), where both are well-established gliogenic factors (Deneen et al., 2006; Piper et al., 2014; Steele-Perkins et al., 2005).

Nfi genes were recently identified as potential miR-153-regulated genes in another system (Tsai et al., 2014). We confirmed that *Nfi* genes contain putative miR-153 binding sites in their 3′ UTRs and that they are upregulated during passage of ESC-derived neurospheres and downregulated by miR-153 OE (Figures 5A and 5B; Table S3).

To test whether miR-153 can indeed decrease NFIA/B protein levels in NSPCs, we overexpressed miR-153 in SNs and TNs as described in Figure 3 and subjected the neurospheres to western blotting analysis with anti-NFIA/B antibodies. Consistent with the microarray data, expression levels of both *Nfia* and *Nfib* were decreased by miR-153 OE (Figure 5C). To examine whether *Nfia* and *Nfib* are directly regulated by miR-153 via their 3′ UTRs, we followed up with a luciferase reporter gene assay. The full-length 3′ UTR for each gene with or without mutations in all of the putative miR-153 binding sites was cloned into a luciferase reporter vector and cotransfected with a miR-153 expression vector into HEK293A cells. Reporter gene activities were significantly reduced in miR-153-cotransfected cells compared with cells cotransfected with a control miR-LacZ expression vector targeting the LacZ gene, whereas mutations in the putative *Nfia/b* miR-153 binding

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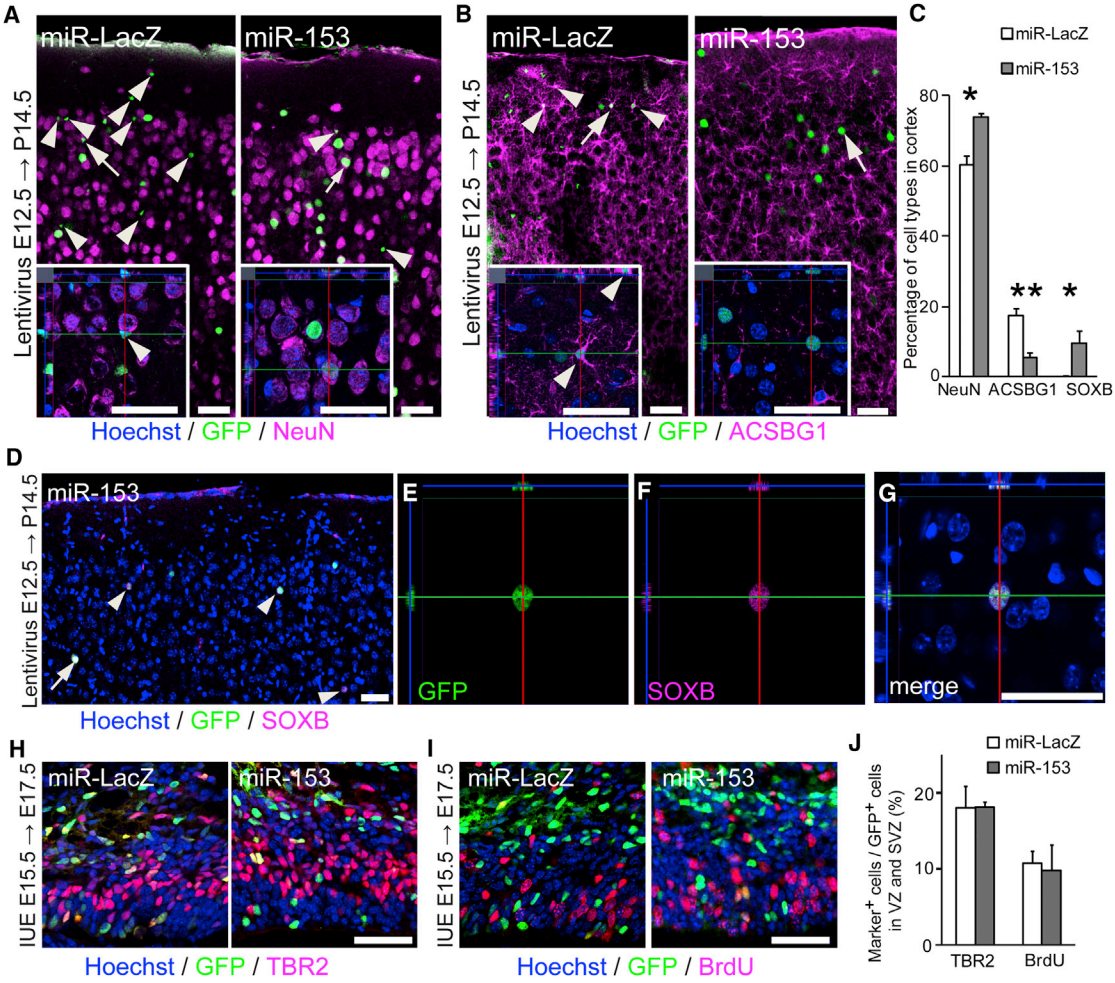

Figure 4. miR-153-Induced Suppression of Astrogliogenesis in the Developing Cortex

(A, B, and D–G) Lentiviruses expressing miR-LacZ or miR-153 were injected into the E12.5 lateral ventricle.

(A) Infected cells within the P14.5 CP of the frontal cortex were analyzed immunohistochemically for NeuN in differentiated neurons.

(B) ACSBG1 in differentiated astrocytes.

(D–G) SOXB in undifferentiated neural progenitors. Arrowheads indicate non-neuronal cells, including ACSBG1+ astrocytes. Arrows show cells in enlarged images. miR-153 OE impaired astrocytogenesis, inhibited NSPC differentiation, and increased neurogenesis.

(H–J) IUE of miR-LacZ- or miR-153-expressing vector into cortical NSPCs at E15.5. miR-153 OE did not significantly alter NSPC proliferation (as assessed by BrdU incorporation) or neurogenesis (as defined by TBR2 expression).

Data in (C) and (J) represent the mean ± SD (n = 3 embryos; *$p < 0.05$, **$p < 0.01$). Scale bars, 50 μm.

sites decreased the actions of miR-153 (Figure 5D). Therefore, miR-153 directly downregulates NFIA/B expression.

Nfia and *Nfib* knockout mice are phenotypically similar, with significantly reduced GFAP expression in the brain and an increased undifferentiated progenitor fraction at the perinatal stage (Barry et al., 2008; Piper et al., 2014; Steele-Perkins et al., 2005), suggesting a delay in gliogenesis. Therefore, we confirmed the contribution of NFIA and NFIB to astrogliogenesis by performing *Nfia* or *Nfib* KD in mouse cortical NSPCs derived from E14.5 embryos in vitro. *Nfia* or *Nfib* KD was accomplished by lentiviral vector-mediated expression of the corresponding short hairpin RNAs (shRNAs), leading to a significant reduction in astrocytic differentiation of the infected NSPCs (Figure S5).

Next, we verified that *Nfia/b* are the downstream effectors of miR-153 by testing whether NFIA/B OE without the 3′ UTR could rescue the anti-gliogenic phenotype of miR-153 OE in vitro. Co-overexpression of miR-153 and either NFIA or NFIB in ESC-derived TNs via lentiviral vector introduction partially rescued the astrocytic differentiation that was inhibited by miR-153 OE versus control Kusabira

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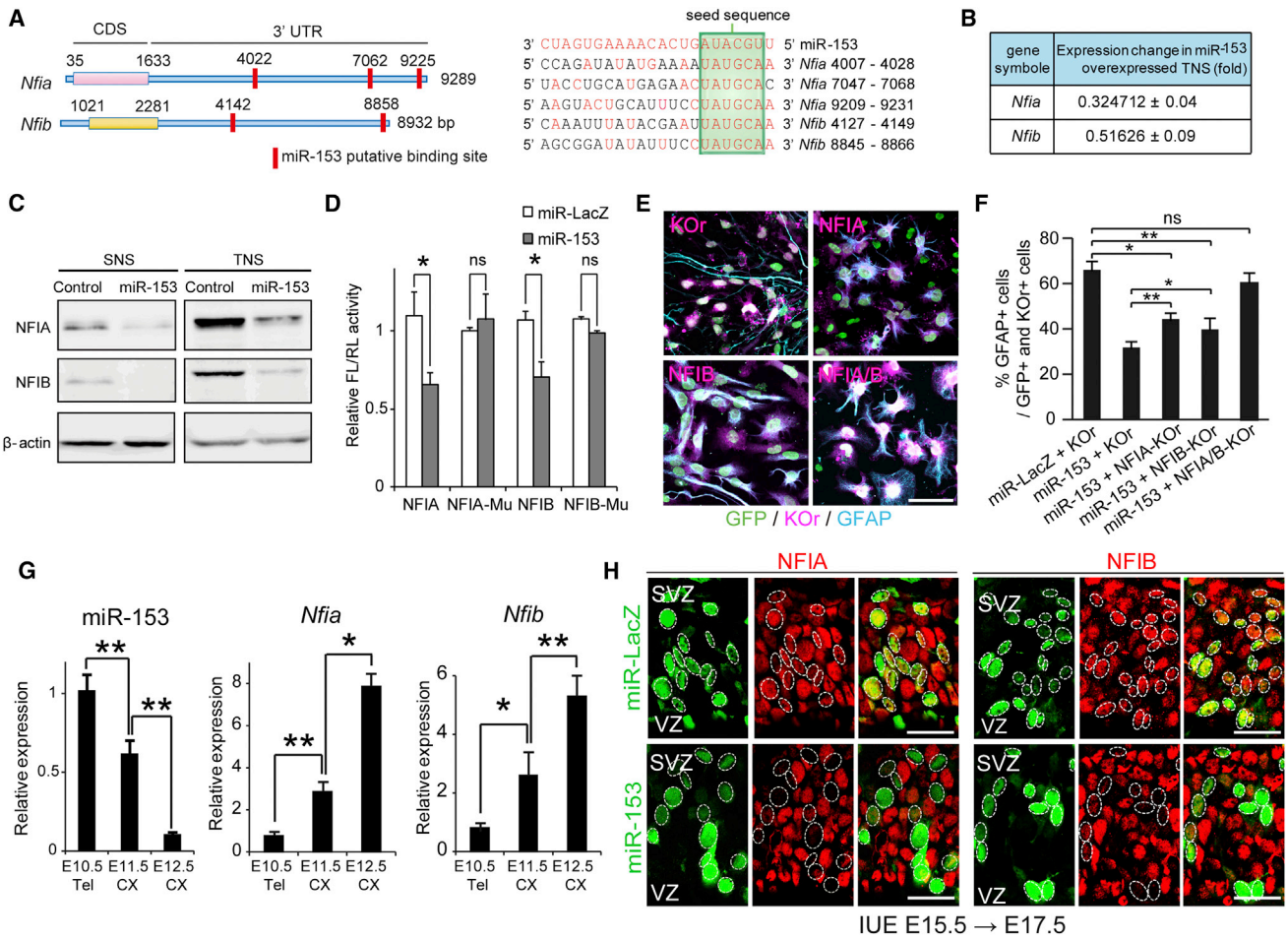

Figure 5. Identification of *Nfia/b* as miR-153 Targets in the Regulation of Gliogenesis

(A) Left: schematic view of putative miR-153 binding sites in *Nfia/b* mRNAs. Right: miR-153 sequences and complementary binding sites in *Nfia/b* 3′ UTRs. Seed sequences and complementary sequences are boxed in green. miR-153-complementary *Nfia* (GenBank: NM_001122952) and *Nfib* (GenBank: NM_008687) 3′ UTR sequences are highlighted in red.

(B) Microarray analysis indicating the decrease of *Nfia/b* expression by miR-153 OE in TNs. The microarray contained two probes for *Nfia* and five probes for *Nfib*.

(C) Reduction of NFIA/B protein expression by miR-153 OE in SNs and TNs was confirmed by western blot analysis.

(D) Dual-luciferase reporter assays were performed to investigate the interaction between miR-153 and *Nfia/b* 3′ UTRs by using reporter constructs containing putative miR-153 binding sites (NIFA/B) and mutated miR-153 binding sites (NFIA/B-Mu). Data are from three independent experiments with triplicate measurements. Firefly luciferase (FL) activity was normalized to *Renilla* luciferase (RL) activity as an internal control.

(E) Immunocytochemistry of differentiated TNs co-transduced with miR-153 and NFIA- and/or NFIB-KOr-expressing lentivirus vectors. Lentivirus infection and neurosphere differentiation were performed as described in Figure 1. Single and combinatorial infections of NFIA- and NFIB-KOr vectors were conducted at the same multiplicity of infection.

(F) The miR-153 OE-induced anti-gliogenic phenotype was rescued partially and fully by single and combinatorial NFIA and NFIB OE, respectively. Scale bar, 50 μm.

(G) qPCR analysis of mature miR-153 and *Nfia/b* expression levels in developing NSPCs from *Nestin*-EGFP mouse forebrains at E10.5, E11.5, and E12.5. Expression levels are presented as fold differences relative to that in E10.5 telencephalon.

(H) Reduction of NFIA/B by miR-153 OE in the VZ/SVZ of mouse embryonic cortex. miR-LacZ- and miR-153-expressing plasmid vectors were transfected into NSPCs in the E15.5 mouse cortex by IUE and processed for NFIA/B immunohistochemistry at E17.5. Transfected cells are circled in white. Scale bar, 20 μm.

Data in (D), (F), and (G) represent the mean ± SD (n = 3 independent experiments; *p < 0.05, **p < 0.01).

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Orange (KOr) expression. Combined expression of NFIA and NFIB with the same dosage as NFIA or NFIB OE alone resulted in an almost complete rescue of the astrocytic phenotype (Figures 5E and 5F). Therefore, the function of NFIA and NFIB in the regulation of astrogliogenesis appears to be additive, despite their similar protein sequences and functions (Barry et al., 2008; das Neves et al., 1999; Namihira et al., 2009; Piper et al., 2010, 2014). Simultaneous NFIA/B expression, therefore, seems to be essential for normal astrogliogenesis. Taking all of the in vitro data together, these experiments indicate that suppression of astrocytic differentiation of NSPCs is caused by miR-153-mediated downregulation of both *Nfia* and *Nfib* as direct miR-153 targets, at least in part.

We next investigated the correlation between miR-153 and *Nfia/b* in developing NSPCs in vivo by performing RT-qPCR analysis of these genes in FACS-purified NSPCs derived from the embryonic telencephalon and cortex of E10.5–E12.5 *Nestin*-EGFP mice. As expected from our in vitro results (Figures 1B and 5C; Figure S3), temporal changes in miR-153 and *Nfia/b* expression were inversely correlated in developing NSPCs (Figure 5G). Furthermore, expression levels of NFIA and NFIB were increased dramatically in E12.5 VZ cells compared with E11.5 VZ cells (Figures S6D and S6E). Next, to assess whether miR-153 targets *Nfia/b* in vivo, the miR-153 expression vector was electroporated into the E15.5 mouse cortex, and NFIA/B expression was immunohistochemically examined at E17.5. miR-153 OE clearly reduced the expression levels of both factors in a cell-autonomous fashion (Figure 5H), suggesting that miR-153 downregulation is required for increased NFIA and/or NFIB content in the developing cortex.

Stimulation of Astrogliogenesis and NFIA/B Expression via miR-153 Inhibition

Finally, we investigated the role of miR-153 in gliogenesis by inhibiting miR-153 function with the aid of a synthetic tough decoy (S-TuD) system that can suppress specific miRNA activity (Haraguchi et al., 2012). S-TuD inhibition of miR-153 was first tested by performing a luciferase reporter assay that employed a reporter construct containing three tandem repeats of the complementary target sequence of miR-153 as a miRNA sensor. Cotransfection of the miR-153 expression vector and the S-TuD complementary to miR-153 (S-TuD-153) rescued the miR-153-mediated reduction of reporter activity in HEK293A cells (Figure 6A). Moreover, transfection of S-TuD-153 into ESC-derived PNs significantly increased the number of GFAP+ astrocytes (Figures 6B and 6C), indicating that miR-153 is required to prevent precocious gliogenesis.

The involvement of miR-153 in the regulation of NFIA and/or NFIB expression in developing NSPCs was further explored by electroporating S-TuD-153 into the mouse cortex at E10.5, and we assessed NFIA/B protein expression levels at E11.5, when their expression is mostly undetectable with our antibodies in the VZ (Figure S6D). S-TuD-153 induced a significant increase in NFIA/B content in the cortical VZ (Figures 6D and 6E). Next, to assess whether miR-153 inhibition can induce competence change of NSPCs to respond to gliogenic signals, we cultured cortical NSPCs from mouse E11.5 brains electroporated with S-TuD-153 at E10.5 in the presence of the gliogenic cytokines LIF and BMP2 for 4 days. The introduction of S-TuD-153 significantly increased the number of GFAP+ astrocytes (Figures 6F and 6G) and decreased the number of SOX1+ progenitor cells (Figure S7). Moreover, co-electroporation of S-TuD-153 and the LIF expression vector into the E10.5 mouse cortex induced ectopic expression of GFAP at E12.5, whereas no expression of GFAP was detected at all in any other electroporations, including S-TuD-153 only and a combination of the LIF expression vector and control S-TuD (Figure 6H). These results indicate that miR-153 is involved in the modulation of the acquisition of gliogenic competence by developing NSPCs via the regulation of NFIA and NFIB.

DISCUSSION

Here we demonstrated that miR-153 plays a crucial role in the regulation of acquisition of gliogenic competence by NSPCs as an upstream regulator of NFIA/B. The inverse correlation of miR-153 and NFIA/B expression revealed here is indicative of the requirement of miR-153 for the prevention of gliogenesis by NSPCs in the early neurogenic period and strongly suggests that the regulation of NFIA/B expression levels by miR-153 is one of the critical factors for the timing of astrogliogenesis (Figure 7). Importantly, miR-153 has been shown to be able to regulate the expression of NFIA/B in the immature brain during the course of this study (Tsai et al., 2014). However, the spatiotemporal expression of miR-153 in the developing CNS, the LOF analysis of miR-153 to clarify its physiological function in the developing CNS, and the association of miR-153 with astrogliogenesis have not been reported.

Although NFIB participates critically in the regulation of NSPC differentiation and astrogliogenesis during CNS development (Steele-Perkins et al., 2005), no information was available previously regarding the regulation of NFIB expression in developing NSPCs, although a recent report demonstrated miR-153-facilitated control of NFIA/B levels in the fetal brain (Tsai et al., 2014). Here we demonstrated independently the direct regulation of NFIA/B expression by miR-153 in developing NSPCs. The synergistic actions of the two factors to rescue the anti-gliogenic phenotype provoked by miR-153 in NSPCs (Figures 5E and 5F) and

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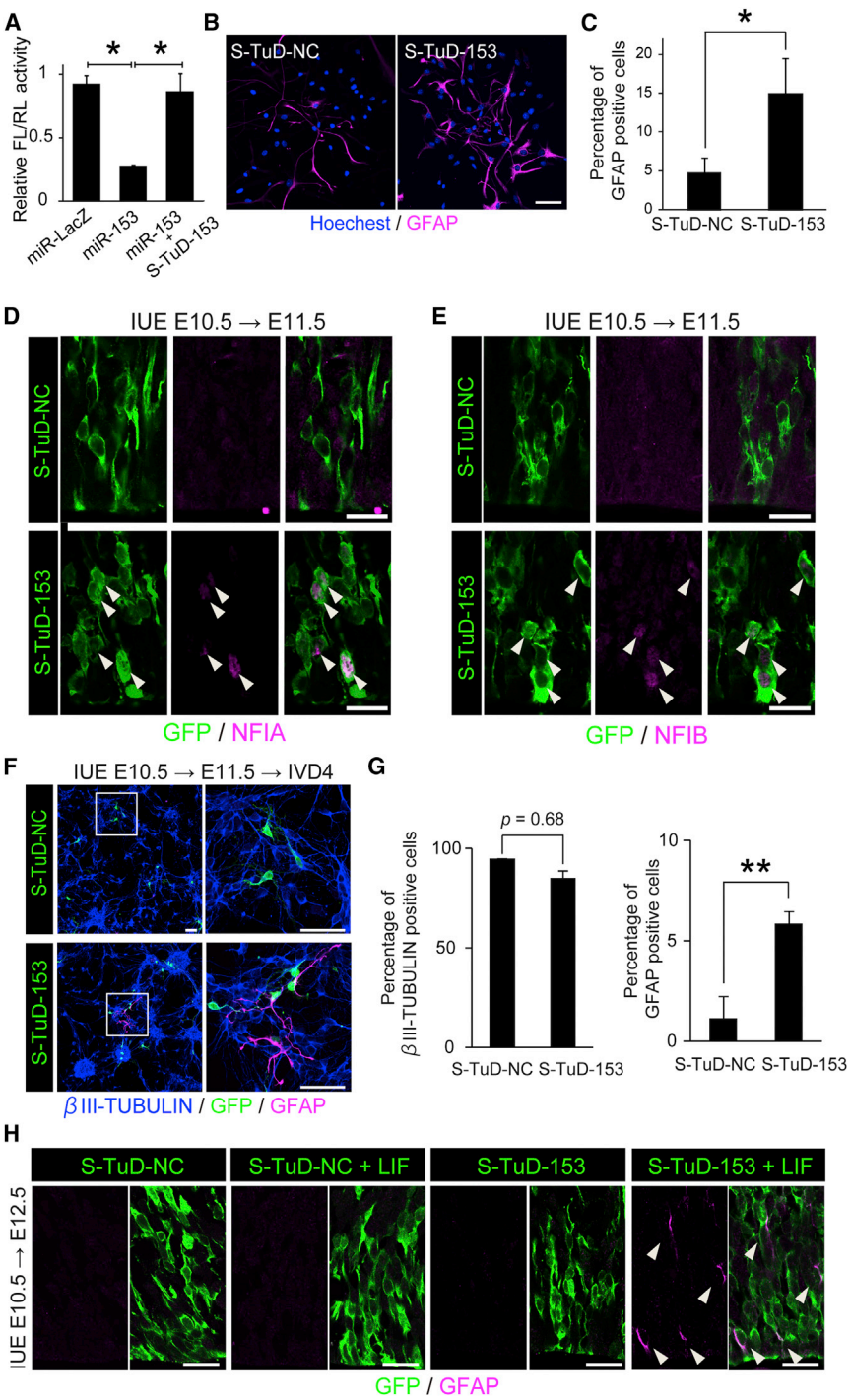

Figure 6. Inhibition of miR-153-Accelerated Progression of NSPC Development

(A) HEK293A cells were cotransfected with a miR-153 sensor vector containing three tandem miR-153 binding sequences downstream of a luciferase reporter gene with the miR-153 inhibitor S-TuD-153 RNA or with a miR-153- or miR-LacZ-expressing plasmid vector without S-TuD-153 RNA. Luciferase activities were then measured. S-TuD-153 successfully abolished the suppressive effect of miR-153 on miR-153 sensor vector reporter activity. FL activity was normalized to RL activity as an internal control.

(B and C) Dissociated EB cells were transfected with an S-TuD-negative control (NC) or miR-153 and cultured for 2 days in the presence of fibroblast growth factor 2 (FGF2). The cells were then allowed to differentiate in the presence of LIF for 5 days. S-TuD-153 accelerated the onset of astrogliogenesis in the early NSPCs. Scale bar, 50 μm.

(D and E) IUE of S-TuD-153 into the E10.5 cortex increased the expression of NFIA/B. Brains were processed for immunocytochemistry of NFIA (D) and NFIB (E) 24 hr post-electroporation. Arrowheads indicate NFIA+ or NFIB+ cells. Scale bars, 20 μm.

(F and G) In vitro differentiation of cortical NSPCs after IUE of S-TuDs at E10.5 in vivo. Cortices from E11.5 mouse embryos were dissociated and cultured in the presence of LIF (10 ng/ml) and BMP2 (100 ng/ml) for 4 days. A significant increase of GFAP+ astrocytes by introduction of S-TuD-153 compared with that of S-TuD-NC was observed. Scale bar, 50 μm.

(H) Precocious expression of GFAP was induced by a combination of miR-153 inhibition and LIF overexpression. E10.5 mouse brains were electroporated with S-TuDs and/or a LIF expression vector (pCAGGS-LIF) at the cortical VZ and processed for immunocytochemistry of GFAP at E12.5. Arrowheads indicate GFAP+ cells. Scale bars, 20 μm.

Data in (A), (C), and (G) represent the mean ± SD (n = 3 independent experiments; *p < 0.05, **p < 0.01).

the astrogliogenesis defects generated in various NFIA/B LOF analyses (Figure S5; das Neves et al., 1999; Shu et al., 2003; Steele-Perkins et al., 2005) suggest that NFIA and NFIB function cooperatively yet in parallel to promote astrogliogenesis. For instance, NFIA/B may modify different transcriptional targets to achieve this goal. NFIB is a demonstrated transcriptional repressor of the Polycomb group (PcG) protein *Ezh2*, which is itself required to prevent the premature onset of gliogenesis in developing NSPCs (Pereira et al., 2010; Piper et al., 2014). For its part, NFIA functions as a transcriptional activator for several gliogenic genes, including *Gfap*. Notably, EZH2 binds to

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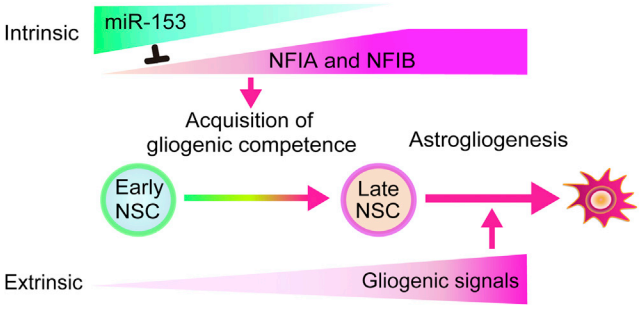

Figure 7. Schematic Showing the Role of miR-153 in the Timing of NSPC Gliogenesis during Development

In the early neurogenic stage, miR-153 is highly expressed and prevents competence change for the initiation of gliogenesis by repressing NFIA/B expression. Downregulation of miR-153 causes increased levels of NFIA/B and the acquisition of gliogenic competence so that NSPCs can respond to gliogenic signals at midgestation stage. The downregulation of miR153 may stem from a progression of NSPCs into the gliogenic phase, facilitated by increased NFI levels, which may, in turn, contribute to the "one-way street" nature of this developmental process.

the *Gfap* promoter together with chromodomain helicase DNA binding protein 4 to repress *Gfap* transcription (Sparmann et al., 2013). Therefore, NFIB-mediated repression of EZH2 and removal from the *Gfap* promoter, as well as direct transcriptional activation of *Gfap* by NFIA, probably contribute to the procurement of glial gene expression.

Based on the above evidence, miR-153, which downregulates both NFIA and NFIB, likely acts to fine-tune gliogenic timing. The increase in undifferentiated NSPCs with a limited enhancement of neurogenesis by miR-153 OE in vitro and in vivo (Figures 3 and 4A–4G) as well as the decrease in progenitor cells by S-TuD-153 (Figure S7) may also be explained by the inhibition of NFIA/B. To this end, NFIA is required for the differentiation of astrocyte precursors (Deneen et al., 2006), and knockout mice for any of these genes exhibit an increase in undifferentiated progenitors in the perinatal brain (Pereira et al., 2010; Piper et al., 2010, 2014). Nonetheless, there is a clear phenotypic difference in NSPC proliferation between these *Nfi* knockout mice and miR-153 OE in that proliferation is increased in the former and unaltered in the latter (Figures 4H–4J). This may occur because NFIs function to both inhibit proliferation and induce differentiation. In addition, other unidentified miR-153 targets may account for the difference. In fact, miR-153 reportedly plays both positive and negative roles in cell proliferation depending on the cellular context (Wu et al., 2013; Zhao et al., 2013). Alternatively, the difference may be caused by difference of NFIA/B protein levels and/or duration of their LOF. Regardless, because miR-153 expression decreases dramatically before the onset of gliogenesis in the developing cortex (Figures 2B–2D and 5G), the primary role for miR-153 in the modulation of the neurogenesis-to-gliogenesis switch in the developing CNS is probably to block precocious gliogenesis via repression of NFIA/B expression in NSPCs.

Elucidation of the regulatory mechanisms for miR-153 expression is required to further understand the neurogenesis-to-gliogenesis switch. Although NFIA OE decreased miR-153 expression in ESC-derived NSPCs (Figure 1B), no significant change was observed in LOF studies of NFIA and NFIB in vitro and in *Nfia* knockout mice (Figure S8). Most likely, the reduction in miR-153 levels by NFIA OE stems from an enhanced transition to the gliogenic phase. In agreement, miR-153 expression is maintained in the VZ of the LGE (Figure 2C), which becomes the highly neurogenic adult SVZ, where only subpopulations of SVZ astrocytes and neuroblasts express NFIA/B (Plachez et al., 2012).

This study indicates that miR-124 and miR-219 may also be involved in the neurogenesis-to-gliogenesis switch by NSPCs. miR-124 promotes neurogenesis via downregulation of several genes (*Sox9*, *Scp1*, and *Ezh2*) that are positive regulators of gliogenesis or negative regulators of neurogenesis in the developing and adult brain (Neo et al., 2014; Papagiannakopoulos and Kosik, 2009; Visvanathan et al., 2007). Depression of the neurogenic cascade, including miR-124-mediated regulation, may participate in the neurogenesis-to-gliogenesis switch. miR-219 stimulates both neurogenesis and gliogenesis during CNS development in the zebrafish (Hudish et al., 2013) and terminal differentiation of oligodendrocytes in the mouse (Dugas et al., 2010; Zhao et al., 2010). In our work, however, miR-219 OE only suppressed astrocytic differentiation of NSPCs in a limited developmental time window (Figures 1C–1F). The region-specific expression pattern of miR-219 in the early embryonic CNS (Figure S2) suggests that miR-219 functions differently in different CNS regions.

We showed recently that miR-17/106 inhibits gliogenesis by NSPCs via downregulation of p38 MAP kinase downstream of COUP-TFs (Naka-Kaneda et al., 2014). miR-17 OE in highly gliogenic quaternary neurospheres from ESCs even induced a marked restoration of neuropotency, which is unlike the miR-153 OE phenotype. Because the expressions of both *Nfia* and *Nfib* are not changed significantly by *Coup-tfI/II*-KD in developing NSPCs (Naka et al., 2008), the miR-17/106-p38 axis and the miR-153-NFIA/B axis may function in parallel to control the neurogenesis-to-gliogenesis transition.

In conclusion, our results, together with those of others, indicate that the neurogenesis-to-gliogenesis switch in developing NSPCs is governed by precise control of the expression of many different proteins by a number of miRNAs in a multi-layered regulatory cascade.

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EXPERIMENTAL PROCEDURES

Cell Culture

Mouse ESC (EB3 and EBRTch3) culture, EB formation with Noggin treatment, neurosphere formation, and neurosphere differentiation were performed as described previously (Naka-Kaneda et al., 2014).

Lentivirus Preparation

Lentivirus vectors were prepared as described previously (Naka et al., 2008). Details, including the sequences of the shRNAs and the artificial microRNAs, are described in the Supplemental Experimental Procedures and Tables S1–S5.

Animals

Animal experiments were performed with Institute of Cancer Research (ICR) strain mice (CD-1) and *Nfia*-deficient mice (Shu et al., 2003). All experimental procedures were approved by the Ethics Committee of Keio University (approval no. 09091) and were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH).

Statistical Analyses

At least three independent experiments were included in each statistical analysis. Statistical significance was determined via two-tailed t test. In all cases, $p < 0.05$ was considered statistically significant.

ACCESSION NUMBERS

The GEO accession number for the microarray data reported in this paper is GSE70131.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.06.006.

AUTHOR CONTRIBUTIONS

All experiments were designed by J.T. and T.S. J.T. performed all experiments and acquired the data. J.B. provided a heatmap of the microRNA array. J.B. and L.J.R. provided embryonic brains of NFIA knockout mice and contributed to the writing of the manuscript. H.I., Y.M., and T.H. generated antibodies against NFIA and NFIB. J.T., T.S., and H.O. wrote the paper. T.S. and H.O supervised the project.

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