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A Critical Cell-Intrinsic Role for Serum Response Factor in Glial Specification in the CNS

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Astrocytes and oligodendrocytes play crucial roles in nearly every facet of nervous system development and function, including neuronal migration, synaptogenesis, synaptic plasticity, and myelination. Previous studies have widely characterized the signaling pathways important for astrocyte differentiation and unveiled a number of transcription factors that guide oligodendrocyte differentiation in the CNS. However, the identities of the transcription factors critical for astrocyte specification in the brain remain unknown. Here we show that deletion of the stimulus-dependent transcription factor, serum response factor (SRF), in neural precursor cells (NPCs) (Srf–Nestin–cKO) results in nearly 60% loss in astrocytes and 50% loss in oligodendrocyte precursors at birth. Cultured SRF-deficient NPCs exhibited normal growth rate and capacity to self-renew. However, SRF-deficient NPCs generated fewer astrocytes and oligodendrocytes in response to several lineage-specific differentiation factors. These deficits in glial differentiation were rescued by ectopic expression of wild-type SRF in SRF-deficient NPCs. Interestingly, ectopic expression of a constitutively active SRF (SRF–VP16) in NPCs augmented astrocyte differentiation in the presence of pro-astrocytic factors. However, SRF–VP16 expression in NPCs had an inhibitory effect on oligodendrocyte differentiation. In contrast, mice carrying conditional deletion of SRF in developing forebrain neurons (Srf–NEX–cKO) did not exhibit any deficits in astrocytes in the brain. Together, our observations suggest that SRF plays a critical cell-autonomous role in NPCs to regulate astrocyte and oligodendrocyte specification in vivo and in vitro.

Introduction

Astrocytes play multiple crucial roles in brain development and functioning (Barres, 2008), and abnormalities in astrocyte development have been implicated in neurological disorders, including epilepsy, neurodegenerative disorders, and brain tumors (Lobsiger and Cleveland, 2007; Oberheim et al., 2008). Oligodendrocytes are essential for myelination (Hirano, 1968; Emery, 2010), and dysregulated myelination causes multiple sclerosis and leukodystrophy (Emery, 2010). Elucidating the molecular mechanisms regulating astrocyte and oligodendrocyte development is critical to our understanding of how these mechanisms might go awry in these disorders.

The onset of astrogliogenesis begins toward the end of neurogenesis and is regulated by several ligand–receptor complexes (Okano and Temple, 2009; Freeman, 2010). Widely studied cytokines among them include ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and cardiotrophin-1 (CT-1), which activate the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway to promote astrocyte differentiation (Johe et al., 1996; Bonni et al., 1997; Rajan and McKay, 1998; Barnabé-Heider et al., 2005). Activation of bone morphogenetic protein 2/4 (BMP-2/4) signaling results in phosphorylation of SMAD (Sma and Mad-related proteins) transcription factors and their association with STAT3 to regulate expression of astrocyte-specific genes (Nakashima et al., 1999). However, perturbation of JAK/STAT signaling caused only a 45–70% reduction in astrocytes in vivo, suggesting contributions from other pathways. Notch is another widely studied pathway and gain-of-function studies have shown that Notch receptors play a permissive role in neural precursor cells (NPCs) to promote astrocyte differentiation (Morrison et al., 2000; Tanigaki et al., 2001; Gaiano and Fishell, 2002).

Transcriptional control of astrocyte specification has been studied mainly in the spinal cord. Sox9 loss causes deficits in both astrocyte and oligodendrocyte generation (Stolt et al., 2003), whereas nuclear factor I-A/B and bHLH transcription factors, stem cell leukemia, and KLF15 (Krüppel-like factor 15) were found to be necessary and/or sufficient for astrocyte specification (Muroyama et al., 2005; Deneen et al., 2006; Fu et al., 2009). Oligodendrocyte specification occurs at late embryonic and early postnatal periods (Miller, 1996; Lee et al., 2000). Extracellular sonic hedgehog (Shh) signal in ventral telencephalon is necessary and sufficient for inducing commitment to oligodendrocyte precursors (Alberta et al., 2001; Tekki-Kessaris et al., 2001). Downstream to Shh signaling, two bHLH transcription factors, Olig1 and Olig2, are particularly important for oligodendrocyte development (Lu et al., 2001, 2002; Zhou et al., 2001; Zhou and Anderson, 2002). Other transcription factors, including Ying Yang 1 and members of Sox family of transcription factors, including Sox9, Sox10, and Sox17, play critical roles in oligodendrocyte
specification (Stolt et al., 2002, 2003; Sohn et al., 2006; He et al., 2007).

Serum response factor (SRF) is a stimulus-dependent transcription factor required for differentiation and development of several different cell types, including keratinocytes, cardiac, and smooth muscle cells (Miano et al., 2004; Parlakian et al., 2004; Niu et al., 2005; Verdoni et al., 2010). Within the CNS, SRF regulates axon growth, tangential neuronal migration, activity-dependent gene expression, synaptic plasticity, and learning and memory (Ramanan et al., 2005; Etkin et al., 2006; Knöll et al., 2006; Stern et al., 2009; Johnson et al., 2011; Lu and Ramanan, 2011). Here, we report a previously unidentified role for SRF in astrocyte and oligodendrocyte differentiation in the brain.

Materials and Methods

Animals. Srf<sup>f/f</sup> mice (control) were maintained as a homozygous colony as described previously (Ramanan et al., 2005). The Srf<sup>Nestin–cKO</sup> and Srf<sup>NEX–cKO</sup> were generated as described previously (Lu and Ramanan, 2011) using a Nestin–Cre transgenic mouse line (Tronche et al., 1999) and a NEX–Cre line (Goebbels et al., 2006). The Srf<sup>+/+</sup>NesCre double-heterozygous mice did not exhibit any discernible phenotype. Pups of either sex (n = 3–5) from at least two different litters were used in all experiments. All experiments were approved by the Animals Studies Committee, Division of Comparative Medicine, Washington University School of Medicine (St. Louis, MO).

Immunostaining. cell counts, and statistical analyses. Immunohistochemistry was performed as described previously (Ramanan et al., 2005). Primary antibodies used included the following: Aldh1L1 (1:100; catalog #H9252; Sigma-Aldrich), Srf (1:1500; Sigma-Aldrich), Nestin (1:200; Developmental Studies Hybridoma Bank), Tuj1/β-tubulin III (1:1000; Sigma-Aldrich), adenosomatous polyposis coli (APC) (1:1000; Millipore), β-gal (1:1500; Aves Lab), β-gal (1:1500; Millipore), Olig2 (1:1000; Sigma-Aldrich), Oligl (1:50; NeuroMab), activated-Caspase 3 (1:1500; Millipore), Sox2 (1:100; Santa Cruz Biotechnology), and platelet-derived growth factor receptor α (PDGFRα) (1:750; Thermo Fisher Scientific). Secondary antibodies included the following: anti-goat Cy3 (1:300; Jackson Immunoresearch), anti-mouse and anti-rabbit Alexa Fluor 594 and Alexa Fluor 488 (1:500; Invitrogen). Biotinylated anti-rabbit and anti-mouse secondary antibodies (1:500; Vector Laboratories) were used along with ABC-Elite or VIP staining kits (Vector Laboratories). Cell count and statistical analyses were performed as described previously (Lu and Ramanan, 2011).

Western blotting. Total protein, 25 μg, prepared from neurospheres was immunoblotted by standard procedures. Primary antibodies were anti-SRF (1:5000; Santa Cruz Biotechnology) and anti-tubulin (1:10,000; Sigma-Aldrich). Secondary antibodies include anti-HRP (1:20,000; anti-mouse; Jackson Immunoresearch) and anti-rabbit (Invitrogen). Chemiluminescence detection was performed using Immobilon reagent (Millipore).

Neurosphere culture. Neurosphere cultures and culture media were prepared as described previously (Dasgupta and Gutmann, 2005). Neonatal cortices were dissected and trypsinized at 37°C for 15 min. Cells were mechanically dissociated by pipetting and incubated in 10% fetal calf serum medium for 10 min and pelleted by centrifugation. Dissociation medium was used to wash the cells before resuspending in growth medium containing 5 ng/ml FGF and EGF. Samples were cultured at 250,000–500,000 cells per 60 mm dish at 37°C with 5% CO₂ for 4 d.

NPC proliferation analysis. Srf<sup>f/f</sup>, Srf<sup>+/+</sup>NesCre, and Srf<sup>+/+</sup>NesCre mice-derived neurospheres were dissociated by trypsin digestion and seeded at similar cell densities in 24-well plates with fresh growth medium. Every 12 h, neurospheres from triplicate wells were collected and dissociated, and cell counts were determined. Cell growth was monitored over 48 h, and results were plotted with sample mean and SEM. The experiment was repeated at least three times using NPCs generated from animals from different litters.

Secondary neurosphere analysis. NPCs from control and Srf<sup>Nestin–cKO</sup> brains were grown as neurospheres. After 4 DIV, neurospheres were dissociated and seeded as single cells. Approximately 50–70 single NPCs from each background were plated in 96-well plates and grown in fresh growth medium. The number of new neurospheres was monitored every 24 h and over 5 d to measure the percentage of cells that gave rise to a secondary neurosphere. The experiment was repeated twice using animals from different litters.

NPC differentiation. Neurospheres were trypsinized and washed with dissociation medium and were plated at 150,000 cells per well in 24-well plates in differentiation medium (growth medium without FGF and EGF). To enrich for astrocytes, the following pro-astrocytic growth factors and cytokines were supplemented either individually or collectively: CTNT, 100 ng/ml (Peprotech); LIF, 40 ng/ml (Millipore); CT-1, 50 ng/ml (Peprotech); BMP-2, 30 ng/ml (Peprotech); interleukin-6 (IL6), 20 ng/ml (Peprotech); sIL6R, 25 ng/ml (Peprotech); and Jagged-1 (Jag-1), 500 ng/ml (R & D Systems). Cells were fixed using 4% sucrose in 4% paraformaldehyde in PBS and immunostained 4 d after induction. To enrich for oligodendrocytes, PDGFRα at 10 ng/ml (Peprotech) and 3,5,3-triiodo-L-thyronine (T3) at 30 ng/ml (MP Biomedicals) were added to NPCs. The percentage of oligodendrocyte precursor cells (OPCs) generated was analyzed at 2 d after induction, and the percentage of differentiated oligodendrocytes was analyzed at 4 d after induction.

Generation of murine stem cell virus. The retroviral murine stem cell virus vector (MSCV) was obtained as a gift from Dr. David Gutmann (Washington University School of Medicine) (Dasgupta and Gutmann, 2005). SRF and a constitutively active variant of SRF (SRF–VP16) were cloned into the MSCV plasmid. For generating virulent particles, HEK293T cells were transfected with each of the MSCV constructs along with complementary T-helper plasmid using Fugene HD (Roche). Culture supernatant containing the virus particles was collected at 48 and 72 h and filtered through a 0.4 μm syringe filter. For viral transduction, NPCs were trypsinized and grown in a mixture of 20% fresh NPC growth medium and 80% viral supernatant for 2 d before switching to 100% NPC growth medium for an additional 3–4 d. The empty MSCV was used as a control for analyzing the effects of MSCV–SRF and MSCV–SRF–VP16 in rescue experiments.

Results

SRF ablation results in reduced astrocyte numbers in vivo

SRF has been shown to regulate cell-fate specification in a cell-autonomous manner in several organ systems in mice (Miano et al., 2004; Parlakian et al., 2004; Sandbo et al., 2009; Verdoni et al., 2010), but whether SRF has similar roles in the nervous system has remained unexplored. To address this, we conditionally deleted SRF within NPCs using a Nestin–Cre transgenic mouse line (Srf<sup>Nestin–cKO</sup>) (Lu and Ramanan, 2011). The Srf<sup>Nestin–cKO</sup> mice died neonatally and did not exhibit any defects in cell survival, neurogenesis, or neuronal subtype specification (Lu and Ramanan, 2011). Because neurogenesis was unaffected in Srf<sup>Nestin–cKO</sup> mice, we examined astrocytes at P0.5 by immunostaining for the astrocyte marker GFAP. We found that Srf<sup>Nestin–cKO</sup> mice exhibited nearly 60% reduction in astrocytes in multiple brain regions, including neocortex, hippocampus, corpus callosum, and thalamus (Fig. 1A and data not shown). We next assessed SRF deletion in astrocytes. Coimmunostaining for GFAP and SRF revealed that SRF is robustly expressed in astrocytes of control mice but not in Srf<sup>Nestin–cKO</sup> mice (Fig. 1B). We also confirmed that reduced astrocytes in Srf<sup>Nestin–cKO</sup> mice were not attributable to diminished GFAP expression. Immunostaining brain sections from Srf<sup>Nestin–cKO</sup> and control mice using two other astrocyte markers, Aldh1L1 (Cahoy et al., 2008) and S100β, showed that control mice had significantly more astrocytes in several brain regions, including neocortex and hippocampus, compared with Srf<sup>Nestin–cKO</sup> mice (Fig. 1C–F, n = 5 mice).

Our recent findings revealed that Srf<sup>Nestin–cKO</sup> mice did not exhibit any deficits in neurogenesis or increased apoptosis during development (Lu and Ramanan, 2011). However, there
was an increase in proliferative precursor cells in the subventricular zone in Srf–Nestin–cKO mice (Lu and Ramanan, 2011), which is possibly a result of impairment in glial lineage commitment. Together, these findings suggest that the decrease in astrocytes in neonatal Srf–Nestin–cKO mice reflects a critical requirement of SRF for astrocyte differentiation in vivo.

**SRF deficiency in NPCs impairs astrocyte specification in vitro**

To study the role of SRF in astrocyte specification further, we used the neurosphere culture system (Dasgupta and Gutmann, 2005). NPCs cultured from P0.5 cortices of control and Srf–Nestin–cKO brains formed neurospheres within 2–3 d in the presence of EGF and FGF as mitogens and were physically indistinguishable from one another (Figs. 2A, 3A and data not shown). Coimmunostaining for the NPC marker Nestin and SRF showed that neurospheres from control mice exhibited strong immunoreactivity for both SRF and Nestin. In contrast, neurospheres from Srf–Nestin–cKO mice were positive for Nestin but lacked SRF (Fig. 2A). Immunoblotting of total protein isolated from control and SRF-deficient neurospheres further confirmed SRF deletion in NPCs from Srf–Nestin–cKO mice (Fig. 2B).
Likewise, SRF was completely ablated in NPCs isolated from cortices of E12.5 Srf–Nestin–cKO mice (data not shown). We next analyzed the effect of SRF deletion on NPC proliferation and found that SRF-deficient and SRF-heterozygous NPCs had rates of proliferation comparable with NPCs from control mice (Fig. 2C). We analyzed the capacity of SRF-deficient NPCs to self-renew by assessing their ability to form clonal secondary neurospheres from single NPCs. SRF loss did not affect the capacity of NPCs to form secondary neurospheres (Fig. 2D). These findings demonstrate that SRF deletion in NPCs does not affect NPC growth rate and capacity for self-renewal in vitro.

NPCs are multipotent and are capable of differentiating into neurons, astrocytes, and oligodendrocytes following mitogen withdrawal (Rao, 1999; Liu and Rao, 2004; Ahmed, 2009). We found that SRF-deficient NPCs were capable of generating neurons, astrocytes, and oligodendrocytes as identified by the expression β-tubulin III (Tuj1+) in neurons, GFAP+ in astrocytes, and coexpression of APC and O4 in differentiated oligodendrocytes (Hegedus et al., 2007; Fancy et al., 2011) (Fig. 2E). Similar to that observed in vivo, SRF-deficient NPCs generated significantly fewer astrocytes and oligodendrocytes compared with wild-type NPCs, whereas similar number of neurons was generated (Fig. 2F). These results confirm that SRF is dispensable for neurogenesis but is critical for glial specification.

SRF-deficient NPCs fail to respond to pro-astrocytic stimuli

Astrocyte cell fate can be induced by several different extracellular ligands. We tested the ability of SRF-deficient NPCs to generate
astrocytes in the presence of these known pro-astrocytic ligands. After mitogen withdrawal and in the presence of CNTF and LIF, wild-type NPCs differentiated predominantly into astrocytes at 4 DIV with characteristic stellar morphology along with neurons and oligodendrocytes (Fig. 3A, A', A" and data not shown). In contrast, SRF-deficient NPCs generated significantly fewer astrocytes compared with wild-type NPCs, and a large proportion of cells remained spherical and precursor cell like (Fig. 3B).

**Figure 3.** SRF-deficient NPCs fail to differentiate in response to pro-astrocyte stimuli. **A,** NPCs from control and Srf–Nestin–cKO mice were cultured as neurospheres for 4 DIV. The neurospheres were trypsinized and induced with CNTF (100 ng/ml) and LIF (40 ng/ml) [A', A'']. SRF-deficient NPCs generated fewer astrocytes compared with wild-type cells as visualized by GFAP immunostaining [A'']. The SRF-deficient astrocytes also appeared smaller and less stellar [A', A'']. **B,** NPCs from control and Srf–Nestin–cKO mice were induced into astrocytes by several pro-astrocytic ligands as indicated. At 4 d after induction, SRF-deficient NPCs generated significantly fewer astrocytes compared with NPCs from control littermates as visualized by GFAP immunolabeling. Scale bar, 25 µm. **C,** Quantification of GFAP + astrocytes in B shows that SRF is critically required under all these induction conditions for astrocyte specification (n = 3 experiments). **D,** Control and SRF-deficient NPCs were cultured in the presence of all ligands shown in B, and cells were immunostained at 4 DIV for astrocytes (GFAP and S100β) and NPCs (Sox2). Arrows point to S100β + cells. Scale bar, 25 µm. **E,** Cell count analyses for D show that loss of SRF impairs commitment to astrocytes even in the presence of several pro-astrocyte signals and that more SRF-deficient NPCs remain as Sox2− precursors in culture.
Furthermore, the small population of SRF-deficient cells that were GFAP positive (GFAP

We also tested the ability of other cytokines and pro-astrocytic ligands, including CT-1, IL6, BMP-2, and Jag-1 to promote astrocyte fate choice (Kahn and De Vellis, 1994; Ma¨rz et al., 1999; Nakashima et al., 2001; Ochiai et al., 2001; Takizawa et al., 2001; Yanagisawa et al., 2001; Grandbarbe et al., 2003; Barnabe´-Heider et al., 2005; Namihira et al., 2009). When cultured in the presence of each of the above ligands, SRF-deficient NPCs generated significantly fewer astrocytes compared with control NPCs (Fig. 3

Because SRF deletion in NPCs does not lead to an increase or impairment in neurogenesis but rather a decrease in astrocytes and oligodendrocytes, we sought to determine the fate of NPCs remaining in culture. Immunosnoating for Sox2, a transcription factor expressed specifically by NPCs, showed that, compared with 63% of control cells, 76% of SRF-deficient cells were Sox2

Figure 4. SRF deletion in neurons does not affect astrocyte differentiation. A, Brain sections from P0.5 control and Srf–NEX– cKO mice were immunostained using anti-Aldh1L1 antibody to label all astrocytes (arrows). Neocortex and hippocampus are shown. Scale bar, 20 µm. B, Quantification of astrocyte cell count from A shows no significant difference in astrocytes in Srf–NEX– cKO mice compared with control littermates (n = 3 mice). C, Immunofluorescence staining for GFAP and Aldh1L1 to label astrocytes in 3-month-old control and Srf–NEX– cKO mice. No GFAP

3A’,A”). Furthermore, the small population of SRF-deficient cells that were GFAP positive (GFAP

Because SRF deletion in NPCs does not lead to an increase or impairment in neurogenesis but rather a decrease in astrocytes and oligodendrocytes, we sought to determine the fate of NPCs remaining in culture. Immunosnoating for Sox2, a transcription factor expressed specifically by NPCs, showed that, compared with 63% of control cells, 76% of SRF-deficient cells were Sox2

SRF is required cell autonomously for astrocyte differentiation in vivo
Recent observations have shown that newborn neurons and committed NPCs promote astrocyte specification by secreting Notch ligands, such as Jag-1, and thus activating Notch signaling in neighboring uncommitted NPCs (Namihira et al., 2009). Because SRF is also deleted in neurons in Srf–Nestin–cKO mice, the deficits in astrocytes could be attributable to non-cell-autonomous effect from SRF-deficient neurons. To address whether SRF-deficient neurons contribute to the deficits in astrocytes observed in Srf–Nestin–cKO mice, we analyzed astrocytes in Srf–NEX–cKO mice, in which SRF deletion was restricted to glutamatergic neurons of the neocortex and hippocampus starting at approximately E11.5 (Lu and Ramanan, 2011). Immunostaining of P0.5 brains from Srf–NEX–cKO mice and control littermates using anti-GFAP and anti-Aldh1L1 antibodies showed no differences in the number of astrocytes localized in neocortex and hippocampus of Srf–NEX–cKO mice compared with control littermates (Fig. 4A,B and data not shown). Unlike the Srf–Nestin–cKO mutant mice, the Srf–NEX–cKO mice do not exhibit neonatal lethality. Analyzing the brains of 3-month-old Srf–NEX–cKO mice, we observed no deficits in astrocytic numbers compared with control littermates (Fig. 4C,D). Together, these observations indicate that SRF plays a cell-autonomous role within NPCs to promote astrocyte specification both in vitro and in vivo and that SRF deletion in neurons does not have any discernible effect on astrocyte specification and maintenance.

Srf–Nestin–cKO mutant mice also exhibit a reduction in oligodendrocyte precursors
SRF-deficient NPCs generated fewer astrocytes and oligodendrocytes during mitogen withdrawal in culture (Fig. 2E,F). We asked whether this also reflected a role for SRF in oligodendrocyte specification in vivo. Oligodendrocytes are mostly generated in the first few weeks after birth (Sauvageot and Stiles, 2002). However, OPCs can be seen in the brain as early as E10.5–E14.5 (Woodruff et al., 2001). Immunostaining for OPCs using Olig1 and Olig2 antibodies (Lu et al., 2000; Zhou et al., 2000) revealed significantly (40–50%) fewer OPCs in multiple regions of Srf–Nestin–cKO brains compared with wild-type control littermates (Fig. 5A–D). These findings suggest that SRF is also critical for oligodendrocyte specification in vivo.
SRF-deficient NPCs show impairment in oligodendrocyte specification

Astrocytes have been shown to promote OPC survival in vitro by the secretion of PDGF (Gard et al., 1995). Therefore, the reduced OPCs observed in Srf–Nestin–cKO mutant brains could be attributable to reduced astrocytes or this could reflect a cell-autonomous role for SRF in oligodendrocyte specification. PDGF, which promotes OPC proliferation (McKinnon et al., 1990; Robinson and Miller, 1996), and T3, which drives oligodendrocyte differentiation (Almazan et al., 1985), were supplemented to induce and enrich for differentiated oligodendrocytes. We found that SRF-deficient NPCs generated twofold to threefold less PDGFRα+ and Olig2+ OPCs at 2 d after induction and similar deficits in APC+O4+ and MBP+ differentiated oligodendrocytes at 4 d after induction compared with wild-type NPCs (Fig. 6A–D). We observed <0.5% of neurons and astrocytes under these pro-oligodendrocyte induction conditions (data not shown). These findings recapitulate the in vivo observations made in Srf–Nestin–cKO brains and suggest that SRF plays a cell-autonomous role in oligodendrocyte specification and maturation.

Ectopic expression of SRF rescues both astrocyte and oligodendrocyte specification in SRF-deficient NPCs

We asked whether the defects in astrocyte and oligodendrocyte specification observed in SRF-deficient NPCs reflects a fundamental defect in differentiation in the absence of SRF or whether these deficits suggest a requirement for SRF-dependent transcription for glial cell-fate specification. NPCs from Srf–Nestin–cKO and control littermates were transduced with MSCV expressing either wild-type SRF (MSCV–SRF) or empty vector (MSCV) alone. In control experiments using the MSCV–SRF virus, we found nearly 98% transduction efficiency of SRF-deficient NPCs at 4 d after infection using immunostaining for SRF (data not shown). We also found that MSCV infection did not affect proliferation rates of control and SRF-deficient NPCs (data not shown). Ectopic expression of SRF alone in wild-type NPCs did not potentiate glial differentiation (Fig. 7A–D). However, reintroduction of SRF into SRF-deficient NPCs was sufficient to rescue the deficits in both astrocyte and oligodendrocyte differentiation in the presence of CNTF/LIF and PDGF/T3, respectively, to the levels seen in wild-type NPCs (Fig. 7A–D).
SRF is not sufficient to promote glial differentiation but augments the effects of astrocyte induction

Our findings thus far suggest that SRF is required cell intrinsically to regulate glial specification. We next asked whether activation of SRF-dependent transcription alone is sufficient to promote glial specification. For this, we expressed constitutively active SRF, SRF–VP16, a chimeric protein generated by fusing the DNA binding domain of SRF with the strong transactivation domain of VP16 protein from herpes simplex virus (Johansen and Prywe, 1994; Schratt et al., 2002). SRF–VP16 binds to target SRF binding elements (termed the CArG box sequence) in the regulatory regions of its target genes and activates gene expression even in the absence of any stimuli that stimulate SRF-dependent transcription. SRF-deficient and control NPCs were transduced with MSCV expressing either SRF–VP16 or empty vector and were allowed to differentiate after mitogen withdrawal. At 4 d after mitogen withdrawal, we observed that SRF–VP16 expression alone was not sufficient to generate more astrocytes compared with empty vector and even exhibited an inhibitory effect on astrocyte specification (Fig. 8A, B). However, when NPCs were induced for astrocyte differentiation by the addition of CNTF and LIF, we found that SRF–VP16 significantly potentiated astrocyte differentiation to a similar extent in both control and SRF-deficient NPCs (Fig. 8A, B).

Similarly, we also found that SRF–VP16 was not sufficient to induce oligodendrocyte differentiation in the absence of mitogens (Fig. 8C, D). Interestingly, in contrast to that observed for astrocytes, expression of SRF–VP16 in NPCs inhibited oligodendrocyte differentiation in the presence of pro-oligodendrocyte factors T3 and PDGF (Fig. 8C, D). A recent study showed that SRF–VP16 expression in neurons inhibited oligodendrocyte maturation through a paracrine mechanism involving connective tissue growth factor (CTGF) (Stritt et al., 2009). Our results suggest that SRF–VP16 expression might exert a similar but an autocrine inhibitory effect on NPCs to regulate oligodendrocyte differentiation. In summary, our results identified SRF as a novel transcriptional regulator critical but not sufficient for astrocyte and oligodendrocyte differentiation in the brain.

Discussion

Astrocytes are the most abundant cell type in the mammalian brain and perform critical roles in nearly every facet of brain function, including synaptogenesis, neurotransmitter homeostasis, and synaptic plasticity. Oligodendrocytes are responsible for the generation and maintenance of myelination that facilitates neuronal salutary transmission as well as for providing trophic support for the survival of neurons and growth of axons. In this study, we found that mice with conditional deletion of SRF in NPCs (Srf–Nestin–cKO) exhibit deficits in astrocytes and oligodendrocyte specification in vivo. In contrast, SRF deletion in neurons (Srf–NEX–cKO) does not affect astrocyte development, suggesting a cell-autonomous role for SRF in astrocyte specification. Cultured SRF-deficient NPCs do not display any defects in proliferation or exhibit increased apoptosis. As observed in vivo, SRF-deficient NPCs were unable to properly differentiate into astrocytes and oligodendrocytes in response to lineage-specific differentiation signals. Thus, our study identifies a previously unknown cell-intrinsic role for SRF in glial cell-fate specification in the brain.

SRF deletion in Srf–Nestin–cKO resulted in low of ~40–60% astrocytes and oligodendrocytes in vivo. The Srf–Nestin–cKO mice also exhibited neonatal hemorrhage beginning at ~12–16 h after birth and exhibited lethality soon after. The hemorrhagic phenotype could be attributable to reduced astrocytes, which are an essential component of the blood–brain barrier (Janzer and
Raff, 1987). Although not reported by several studies that have used the Nestin–Cre transgenic mouse line used in this study, it is also likely that there is leaky expression of Cre recombinase in endothelial cells. Because SRF has been shown to be important for vascular development (Franco et al., 2008), SRF deletion in endothelial cells could also compromise the integrity of the blood–brain barrier, resulting in hemorrhage. Future work will determine whether the hemorrhage is attributable to deficits in astrocyte numbers or vascular integrity or both.

Similar to the in vivo observations, SRF-deficient NPCs also exhibited a failure to generate astrocytes and oligodendrocytes when induced to differentiate in vitro. This could reflect a likely delay in glial specification, which is difficult to assess in Srf–Nestin–cKO mice because they exhibit neonatal lethality. However, we found that differentiation of SRF-deficient NPCs for longer periods (until 12 d) in culture did not yield more astrocytes or oligodendrocytes. Interestingly, SRF–VP16 appeared to have an inhibitory effect on astrocyte specification in the absence of SRF. SRF loss in NPCs alone caused a significant reduction in astrocyte differentiation even at 3 months of age. However, SRF loss in NPCs alone caused a significant reduction in astrocytes both in vivo and in vitro. Therefore, neuronal loss of SRF does not appear to have any effect on astrocyte specification and development.

Our findings suggest that SRF is required for specification of a particular glial-restricted precursor cell subpopulation or within a particular subpopulation for glial differentiation. A population of glial-restricted precursor cells that can generate both astrocytes and oligodendrocytes express the surface antigen A2B5 (Rao and Mayer-Proschel, 1997; Liu and Rao, 2004; Lin and Goldman, 2009). Recent studies have shown that astrocytes can be generated from more than one precursor cell type (Liu et al., 2004; Lin and Goldman, 2009; Cai et al., 2012). Future studies will aim to identify these precursor subpopulations in the brains of Srf–Nestin–cKO mice and study their capacity to generate astrocyte and oligodendrocytes in the absence of SRF.

Recent observations have shown that SRF-deficient neurons affect maturation of oligodendrocyte precursors in a non-cell-autonomous manner (Stritt et al., 2009). In the reported Srf mutant mice, widespread SRF loss in late-embryonic forebrain neurons caused deficits in tangential neuronal migration, leading to cell accumulation in the subventricular zone (Alberti et al., 2005). This led to massive apoptosis and consequently triggered an increase in astrocytes postnatally possibly indicative of reactive astrogliosis (Alberti et al., 2005; Stritt et al., 2009). We found that SRF ablation in developing glutamatergic neurons of the neocortex and hippocampus in Srf–Nex–cKO mice did not cause cell death or affect astrocyte differentiation even at 3 months of age.
signaling proteins that are critical for astrocyte specification and in the presence of appropriate extracellular stimuli this causes a potentiated response and subsequent increase in astrocyte differentiation. Future work will aim to identify the SRF target genes in NPCs that are important for astrocyte and oligodendrocyte specification. Together, our observations strongly suggest that SRF plays a cell-intrinsic role in NPCs to regulate astrocyte and oligodendrocyte specification in the brain.

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