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SOX10 Maintains Multipotency and Inhibits Neuronal Differentiation of Neural Crest Stem Cells

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Summary

The mechanisms that establish and maintain the multipotency of stem cells are poorly understood. In neural crest stem cells (NCSCs), the HMG-box factor SOX10 preserves not only glial, but surprisingly, also neuronal potential from extinction by lineage commitment signals. The latter function is reflected in the requirement of SOX10 in vivo for induction of MASH1 and PHOX2B, two neurogenic transcription factors. Simultaneously, SOX10 inhibits or delays overt neuronal differentiation, both in vitro and in vivo. However, this activity requires a higher *Sox10* gene dosage than does the maintenance of neurogenic potential. The opponent functions of SOX10 to maintain neural lineage potentials, while simultaneously serving to inhibit or delay neuronal differentiation, suggest that it functions in stem or progenitor cell maintenance, in addition to its established role in peripheral gliogenesis.

Introduction

Stem cells are self-renewing progenitor cells with the capacity to generate multiple differentiated derivatives (reviewed in Morrison et al., 1997). In developing embryos, stem cells serve to construct tissues and organs de novo, while in adults they maintain ongoing cellular turnover and provide regenerative capacity in certain tissues. Different categories of stem cells have been described, with different self-renewal and developmental capacities, in different tissues, and at different stages of development (reviewed in Weissman et al., 2001). While much has been learned about the cellular and molecular control of stem cell differentiation, especially in the nervous system (reviewed in Gage, 2000; Anderson, 2001; Temple, 2001; Tsai et al., 2002), rather less is known about factors that maintain the stem cell state.

Maintenance of the stem cell state involves at least three distinct functions: (1) inhibition of overt differentiation, (2) maintenance of proliferative capacity, and (3) maintenance of multipotency. Relatively few molecules have been identified for these functions. In the central nervous system (CNS), components of the Notch signaling pathway and its downstream transcriptional effectors, such as *Hes* genes, inhibit neuronal differentiation (Ohtsuka et al., 1999; Nakamura et al., 2000). However, such inhibition does not necessarily maintain multipotency. For example, in the peripheral nervous system (PNS), Notch restricts neural crest stem cells (NCSCs) to nonneuronal fates and promotes glial differentiation (Morrison et al., 2000). Studies of CNS stem cells have yielded conflicting data on this point (Tanigaki et al., 2001; Hitoshi et al., 2002). The HLH protein Id has been shown to inhibit differentiation and proliferative arrest (Benezra et al., 1990; Wang et al., 2001), but whether it maintains multipotency has not been determined.

Although multipotency is a key property of stem cells, it has been difficult to study. That is because multipotency is a latent property, which can be revealed only by exposing stem cells to conditions that elicit overt differentiation. While in vivo transplantation is the most general method for assessing developmental potential, in the nervous system it does not readily lend itself to clonogenic assays (reviewed in Anderson, 2001). Consequently, apparent changes in the multipotency of heterogenous populations of transplanted precursors may reflect either changes in stem cell potential or in the proportion of lineage-restricted progenitors (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996). In vitro clonogenic culture systems can help to distinguish between these alternatives. However, such an approach requires that instructive differentiation signals for the stem cells be identified in order to assess their developmental capacities. There are relatively few systems in which this extent of characterization has been achieved (e.g., Shah et al., 1994, 1996; Johe et al., 1996; reviewed in Temple, 2001).

One such system is that established for neural crest stem cells (NCSCs). NCSCs behave in vitro as multipotent, self-renewing PNS neural progenitors (Stemple and Anderson, 1992) that can differentiate to autonomic neurons, glia, and smooth muscle cells (reviewed in Anderson, 1997). (NCSCs do not generate sensory neurons [Greenwood et al., 1999; White and Anderson, 1999; White et al., 2001; Lo et al., 2002] and therefore contribute to a subset of neural crest derivatives in vivo [Le Douarin and Kalcheim, 1999]). The differentiation of NCSCs in vitro can be promoted by specific instructive extracellular signals. Glial growth factor II (GGFII), also known as Neuregulin-1 (NRG-1), promotes Schwann cell (glial) differentiation, BMP2/4 promotes autonomic neuronal and smooth muscle differentiation, while TGFβ promotes smooth muscle differentiation (Shah et al., 1994, 1996). The hierarchical influences and relative kinetics of lineage restriction promoted by these factors have also been characterized (Shah and Anderson, 1997).

Here we have employed these instructive signals together with retrovirus-mediated gene transfer to investigate the role of a transcriptional regulator, SOX10, in the control of multipotency. *Sox10* is a member of the high-mobility (HMG) group gene family, which includes the testis-determining factor *Sry* (Koopman et al., 1991) and *Sox2*, a marker of CNS stem cells (Kuhlbrodt et al., 1998; Wegner, 1999; Bowles et al., 2000; Zappone et

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al., 2000). *Sox10* is specifically expressed in neural crest cells, just as they migrate from the dorsal neural tube (Southard-Smith et al., 1998). Loss-of-function mutations in the *Sox10* (or *Dom*) locus cause dominant, cell-autonomous defects in Schwann cell and melanocyte differentiation, while homozygous mutants exhibit embryonic lethality with failure of migration and/or differentiation of multiple neural crest derivatives, including glia and autonomic neurons (Herbarth et al., 1998; Southard-Smith et al., 1998; Britsch et al., 2001; Potterf et al., 2001; Sonnenberg-Riethmacher et al., 2001). Studies of neural crest cells cultured from *Sox10*$^{-/-}$ mouse embryos have shown that the gene is required for glial differentiation (Paratore et al., 2001). However, more extensive analysis of *Sox10* loss-of-function phenotypes in such cultures is impeded by the extensive apoptosis caused by the mutation. Therefore, whether SOX10 plays a broader role in maintaining multipotency could not be determined.

Gain-of-function experiments would complement the understanding of SOX10 function gleaned from loss-of-function analysis. However, previous studies have reported that overexpression of *Sox10* yields no obvious phenotype in cultured rodent neural crest cells or in chick embryos (Paratore et al., 2001). Here we report that constitutive expression of SOX10 in NCSCs preserves both glial and, surprisingly, neuronal differentiation potentials. The maintenance of neurogenic potential is opposed by an independent function of SOX10 to inhibit or delay overt neuronal differentiation. SOX10 also prevents TGFβ-induced proliferative arrest. Together, these data suggest that SOX10 contributes to the maintenance of stem cell properties in the neural crest.

Results

SOX10 Is Expressed in Multipotent NCSCs and Downregulated in Nonglial Progeny

Using a monoclonal antibody to SOX10 (Lo et al., 2002; Zirlinger et al., 2002), we confirmed that the protein is expressed early in neural crest migration in rat embryos (Figure 1A) and maintained in satellite glia and Schwann cell precursors, while being downregulated in neuronal derivatives (Figures 1B and 1C). In vitro, virtually all p75^{+}, lin^{-} (lineage marker, e.g., neurofilament, GFAP, etc.) neural crest cells derived from rat E10.5 neural tube explants expressed SOX10 when plated at clonal density (Figures 1D and 1E). Since most or all of these cells can clonogenically generate neurons, glia, and smooth muscle cells as well as self-renew (Stemple and Anderson, 1992), these data support the idea that SOX10 is expressed by multipotent NCSCs. Similar conclusions have been drawn indirectly in mouse (Paratore et al., 2001), although NCSCs have not been clonogenically defined in that system.

When NCSCs were allowed to differentiate to all three lineages under standard culture conditions (Stemple and Anderson, 1992), SOX10 expression was extinguished in smooth muscle cells (Figures 1F and 1G) and neurons (Figures 1H and 1I) but maintained in Schwann cells (Figures 1J and 1K). These results are consistent with the pattern of SOX10 expression in vivo (see above), as well as with earlier studies in mouse neural crest cells (Paratore et al., 2001). As neuronal and smooth muscle differentiation can be promoted by BMP2/4 and TGFβ (Shah et al., 1996), we asked whether these factors promoted downregulation of SOX10 expression in advance of such overt differentiation. Application of BMP2 or TGFβ1 caused an extinction of SOX10 expression in 90% and 75% of NCSCs, respectively, within 24 hr (Figures 1L–1Q), while it was maintained in GGFII/NRG-1, as expected (Figures 1R and 1S). Thus, downregulation of SOX10 expression is an early event in the rapid lineage restriction promoted by BMP2 and TGFβ1 (Shah and Anderson, 1997).

SOX10 Maintains Gliogenic Potential in NCSCs

The observation that SOX10 expression is maintained in Schwann cell precursors, but rapidly downregulated by factors that promote differentiation to nonglial fates, raised the question of whether it is required for the maintenance of glial potential. To address this question, we first asked whether BMP2 promoted a loss of gliogenic differentiation potential in NCSCs, and if so, whether this could be prevented by constitutive expression of *Sox10*. To achieve such constitutive expression, we infected NCSCs with pseudotyped retroviruses (Kinsella and Nolan, 1996) expressing either a SOX10-IRES-GFP cassette or GFP alone. Under the conditions used (see Experimental Procedures), most or all (>90%) of the colonies were infected. After 24 hr to permit expression of the virally encoded transgene, the cells were preincubated with or without 1 nM BMP2 for a further 24 hr (Figure 2, flow diagram). Following washout of BMP2, glial differentiation potential was assayed by culturing the cells for several additional days in GGFII/NRG-1 and staining for GFAP. In some experiments, the cells were further cultured in a maturation medium (Stemple and Anderson, 1992; Shah et al., 1994) (see Experimental Procedures) and stained for O4, a surface marker of more mature Schwann cell precursors (Sommer and Schachner, 1981).

In control GFP retrovirus-infected NCSCs, BMP2 preincubation caused a complete loss of gliogenic potential, as reflected in a lack of both GFAP (Figures 2B versus 2D; Figure 2Q, lavender bars) and O4 expression (Figures 2J versus 2L; Figure 2R, lavender bars). Rather, most NCSCs differentiated to neurons (Figures 2C and 2K) and smooth muscle cells (data not shown). The rapid extinction of glial differentiation capacity was not due to a selective killing of glial progenitors by BMP2, since this factor causes little or no cell death in a 24 hr incubation (Shah et al., 1996; Shah and Anderson, 1997) (data not shown).

Constitutive expression of SOX10 prevented the extinction of gliogenic potential by BMP2 in >80% of infected clones, as evidenced by the recovery of GFAP expression (Figures 2D versus 2H; Figure 2Q, "+BMP2," purple bar). Recovery of O4 expression in SOX10-infected cells was also observed after sequential incubation in GGFII/NRG-1 and maturation medium (Figure 2L versus 2P), although the extent was not as great as for GFAP (Figure 2R, "+BMP2," magenta bar). These data indicate that constitutive expression of SOX10 can prevent BMP2 from extinguishing gliogenic potential in NCSCs. SOX10 also preserved gliogenic potential from extinction by TGFβ (see below).

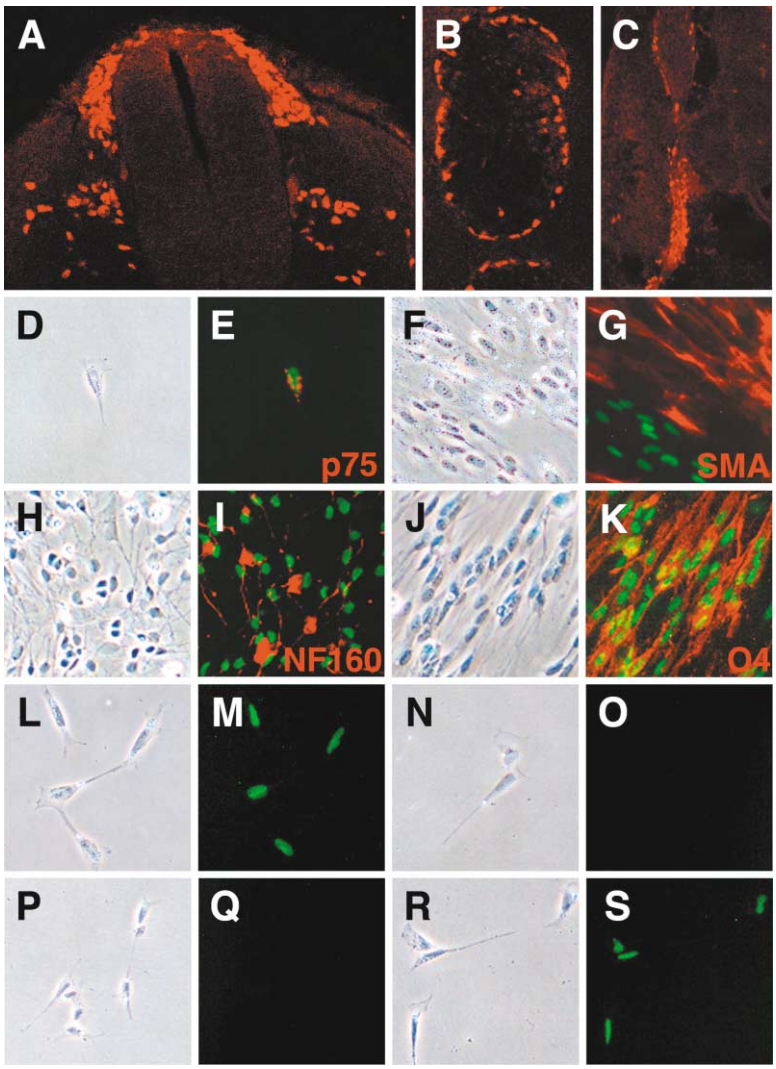

Figure 1. Expression of SOX10 Protein by Neural Crest Cells In Vivo and In Vitro

(A–C) Cross-sections through developing rat spinal cord at E10.5 (A) and E13.5 (B and C) showing expression of SOX10 in migrating neural crest cells (A) and in satellite glia at the perimeter of the DRG (B) as well as in Schwann cell precursors in peripheral nerve (C).

(D–S) Expression of SOX10 (green nuclei in all panels) by NCSCs and their derivatives in vitro. (D), (F), (H), (J), (L), (N), (P), and (R) represent phase-contrast images of the epifluorescence images shown in (E), (G), (I), (K), (M), (O), (Q), and (S), respectively. (D and E) Coexpression of SOX10 by a single $p75^+$ NCSC shortly after plating. (F–I) Downregulation of SOX10 in differentiating smooth muscle cells (G) and neurons (I). (J and K) Maintenance of SOX10 in differentiating glia (K). (L–S) Expression of SOX10 in NCSCs after 24 hr of exposure to: control medium (L and M); TGFβ1 (N and O); BMP2 (P and Q); and GGFII/NRG-1 (R and S).

As a preliminary step toward characterizing the molecular mechanism by which SOX10 maintains gliogenic potential, we examined the expression of the transmembrane receptor tyrosine kinase erbB3, a receptor for GGFII/NRG-1 (Garratt et al., 2000). In vivo, *Sox10* is required for maintenance of *erbB3* expression in neural crest cells (Britsch et al., 2001). RT-PCR analysis of NCSCs in vitro revealed that preincubation for 24 hr in BMP2 caused a striking (~10-fold) downregulation of *erbB3* mRNA (Figure 2S, "GFP, ±BMP2"). By contrast, in NCSCs infected with the SOX10 retrovirus, this downregulation was greatly attenuated (Figure 2S, "Sox10, +BMP2"), and *erbB3* mRNA levels were restored to ~50% of those in cultures lacking BMP2 (Figure 2S, "Sox10, −BMP2"). Thus, loss of gliogenic potential caused by preincubation in BMP2 is correlated with downregulation of a coreceptor for GGFII/NRG-1, and forced expression of SOX10 partially rescues this downregulation. These data suggest (although they do not causally establish) that SOX10 may protect glial differentiation potential from extinction by BMP2, at least in part, by maintaining GGFII/NRG-1 responsiveness in NCSCs. Consistent with this interpretation, responsiveness to NRG-1 is lost in neural crest cultures from *Sox10*$^{-/-}$ embryos (Paratore et al., 2001).

SOX10 Maintains Neurogenic Potential in NCSCs

The ability of SOX10 to maintain glial potential left open the question of whether it plays a broader role in maintaining other differentiation potentials of NCSCs. To address this question, we exploited the fact that pretreatment of NCSCs with TGFβ, a smooth muscle-inducing signal, causes a rapid and irreversible loss of neuronal differentiation capacity, as revealed by subsequent challenge with BMP2 (Shah and Anderson, 1997). Since TGFβ also rapidly downregulates SOX10 (Figure 1), we asked whether constitutive expression of SOX10 could prevent such a TGFβ-induced extinction of neurogenic potential. To do this, NCSCs plated at clonal density were infected with either the SOX10 or GFP retroviruses, and incubated for 24 hr to allow expression of the retroviral transgene. Subsequently, the cells were preincubated for 24 hr in different concentrations of TGFβ, washed, and then cultured in 1 nM BMP2 for a further 5–6 days to promote neurogenesis, and then stained for neurofilament expression (Figure 3, schematic).

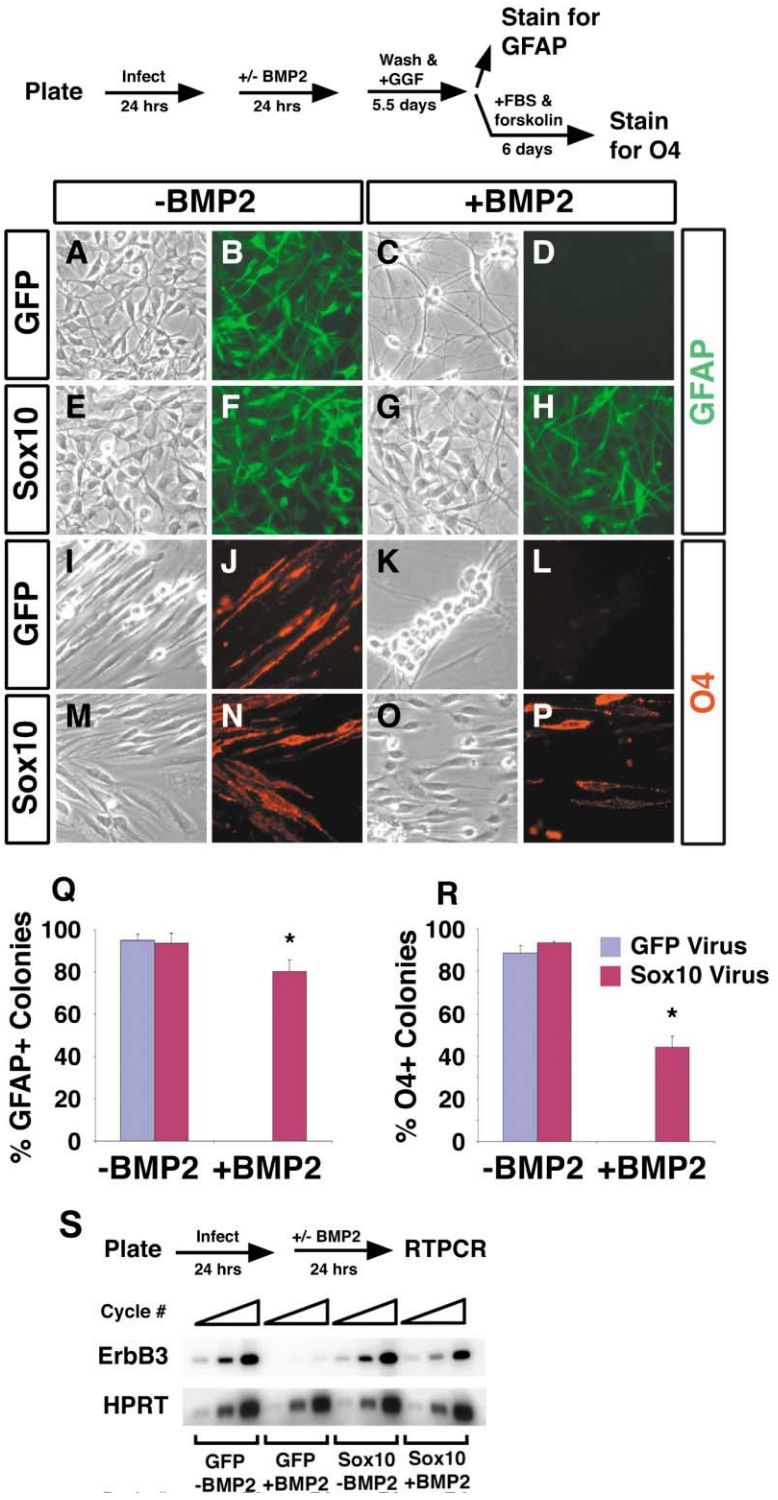

Figure 2. SOX10 Preserves Glial Potential from Extinction by BMP2

In this and other figures, the experimental design is illustrated in the flow diagram. NCSCs were infected immediately after plating with either GFP or SOX10-IRES-GFP retroviruses ("Infect"). (A–P) "−BMP2" or "+BMP2" indicates that the infected cultures were preincubated for 24 hr with or without 1 nM BMP2, followed by washout and further incubation under glial differentiation conditions (see Experimental Procedures). Note that the 24 hr preincubation in BMP2 extinguishes gliogenic potential as assessed by loss of both GFAP (B versus D) and O4 expression (J versus L), and forced expression of SOX10 rescues this potential (D versus H; L versus P). Quantification (Q and R) of the percentage of colonies containing any $GFAP^+$ (Q) or $O4^+$ (R) cells. *$p < 0.05$ by Student's t test. (S) SOX10 protects *erbB3* from downregulation by BMP2. Shown are RT-PCR measurements of *erbB3* and control *HPRT* mRNAs in NCSCs infected with either the control GFP or the SOX10-IRES-GFP retroviruses and then further incubated for 24 hr with or without 1 nM BMP2. PCR cycle number ranges from 26 to 30, in 2-fold steps.

In control GFP virus-infected cultures, preincubation with increasing doses of TGFβ caused a progressive loss of neuronal potential (Figure 3I, GFP virus), consistent with earlier studies (Shah and Anderson, 1997). At 20 pM, TGFβ preincubation completely extinguished the ability of BMP2 to subsequently elicit neuronal differentiation (Figure 3B versus 3D). By contrast, in SOX10 virus-infected cultures preincubated in 20 pM TGFβ, neuronal differentiation was recovered following BMP2 treatment (Figure 3D versus 3H; Figure 3I, SOX10 virus). Although the extent of BMP2-induced neuronal differentiation in such cultures was lower than in controls preincubated without TGFβ (Figure 3I), it was greater at lower concentrations of TGFβ and was always higher than in GFP-infected, TGFβ-preincubated controls (Figure 3I, magenta versus lavender bars). The ability of SOX10 to rescue

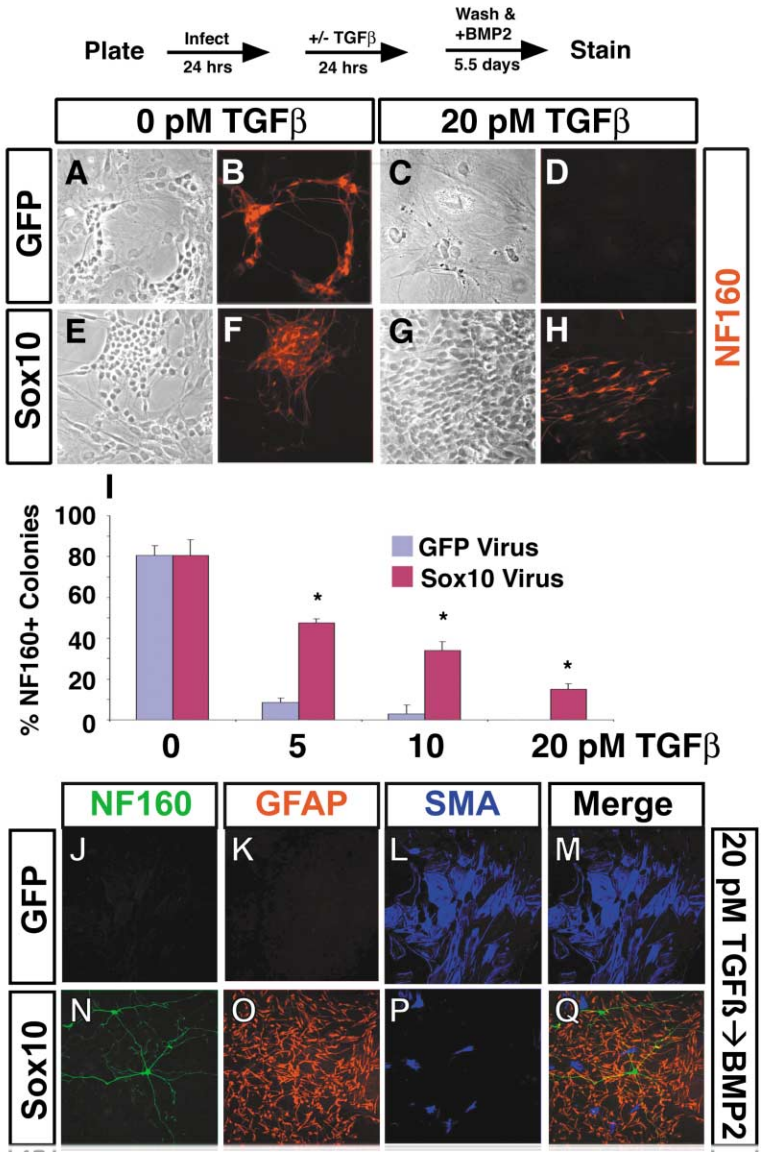

Figure 3. SOX10 Preserves Neurogenic and Gliogenic Potential from Extinction by TGFβ

Preincubation of GFP-infected NCSCs for 24 hr in TGFβ causes a dose-dependent loss of neuronal differentiation potential, tested by exposure to 1 nM BMP2 ([B versus D]; [I], GFP virus; *$p < 0.05$). Neuronal potential is maintained in SOX10-infected cells ([D versus H]; [I], SOX10 virus). All colonies in (A)–(H) were virally infected, as determined by counter staining with antibodies to GFP (data not shown). (J–Q) SOX10 preserves glial as well as neuronal differentiation potential from extinction by 20 pM TGFβ. (J)–(L) and (N)–(P) are individual fluorescence channels from the triple-labeled fields shown in (M) and (Q), respectively. Note that SOX10 preserves not only neuronal (J and N) but also glial (K and O) differentiation capacity, while reducing smooth muscle differentiation (L and P). For additional data and quantification see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/38/1/17/DC1.

neuronal differentiation capacity does not reflect an effect to prevent killing of neurogenic precursors by TGFβ, as determined by serial observation of identified founder cells (see below). Rather, SOX10 prevents TGFβ from extinguishing neuronal differentiation potential in NCSCs.

Colonies in SOX10-infected cultures exposed sequentially to TGFβ and BMP2 contained not only neurons, but also many nonneuronal cells (Figures 3G and 3H). To identify these cells, similar cultures were triple labeled with antibodies to NF160, α-smooth muscle actin (SMA), and GFAP. These experiments revealed that 57% of the colonies in such cultures contained GFAP$^+$ glia (Figure 3O), in addition to some smooth muscle cells (Figure 3P; see Supplementary Figure S1 at http://www.neuron.org/cgi/content/full/38/1/17/DC1). In striking contrast, in control GFP virus-infected cultures, no glia were observed (Figure 3K) and essentially all (98% ± 4%) of the colonies consisted exclusively of smooth muscle cells (Figures 3L and 3M; see Supplementary Figure S1 at http://www.neuron.org/cgi/content/full/38/1/17/DC1). Thus, constitutive expression of SOX10 preserves glial, as well as neuronal, potential from extinction by TGFβ (Figures 3J and 3K versus 3N and 3O).

The observation that BMP2 can promote the development of GFAP$^+$ glia, as well as neurons, in SOX10-infected colonies may seem paradoxical, given that transient BMP2 exposure extinguishes glial potential in control NCSCs (Figure 2). However, BMP2 promotes not only neuronal, but also smooth muscle differentiation (see Supplementary Figure S1 at http://www.neuron.org/cgi/content/full/38/1/17/DC1) (Shah et al., 1996). If BMP2-induced downregulation of endogenous SOX10 (Figure 2) is overcome by constitutive retroviral expression of the HMG-box factor, then the nonneuronal fate promoted by BMP2 is shifted from smooth muscle toward glial (0% glial-containing colonies in controls versus ~60% glial-containing colonies in SOX10-infected cultures; see Supplementary Figures S1A, S1C, and S1E at http://www.neuron.org/cgi/content/full/38/1/17/

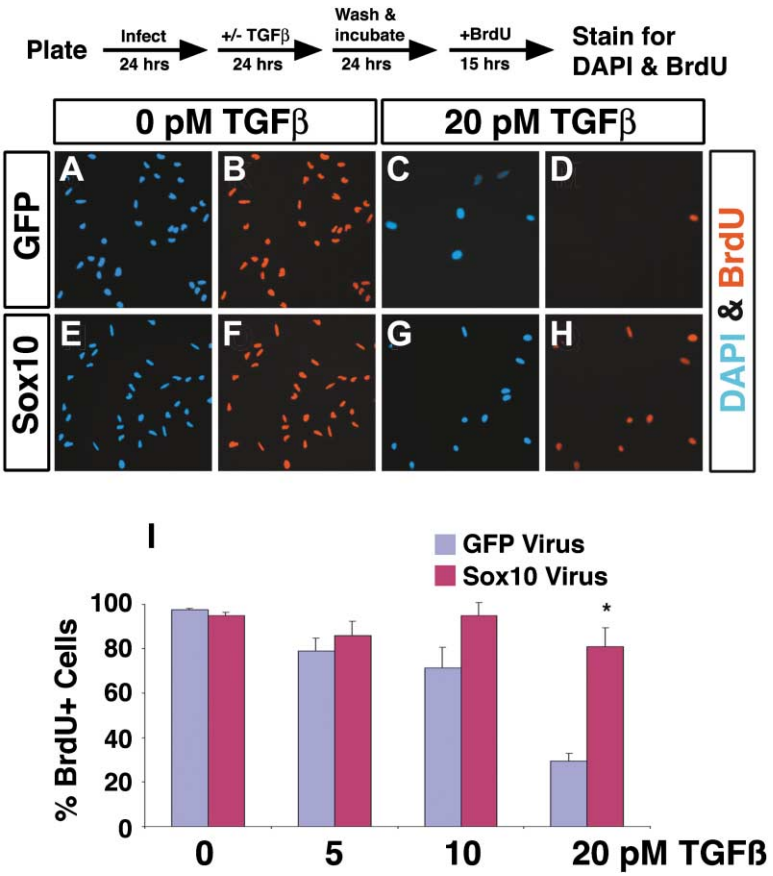

Figure 4. SOX10 Inhibits TGFβ1-Induced Cell Cycle Arrest

(A) and (B), (C) and (D), (E) and (F), and (G) and (H) represent pairs of images from the same fields, double labeled for DAPI to reveal all cell nuclei (blue) and BrdU to reveal dividing cells (red). Note the dose-dependent decrease in BrdU incorporation caused by TGFβ ([B versus D]; [I], GFP virus). SOX10 largely restores BrdU incorporation even at 20 pM TGFβ ([D versus H]; [I], SOX10 virus; *$p < 0.05$).

DC1). The ability of BMP2 to promote GFAP$^+$ glial differentiation in SOX10-expressing NCSCs is consistent with the ability of this growth factor to promote GFAP expression in CNS glial precursors (Nakashima et al., 1999). Thus, if SOX10 is constitutively expressed, BMP2 can reveal glial as well as neuronal differentiation potentials. In control NCSCs, both potentials are abolished by TGFβ preincubation (Figures 3J and 3K; Supplementary Figure S1E at http://www.neuron.org/cgi/content/full/38/1/17/DC1), and all NCSCs differentiate to a smooth muscle fate (Figures 3J–3M, Supplementary Figures S1B and S1E at http://www.neuron.org/cgi/content/full/38/1/17/DC1). Constitutive expression of SOX10 overrides this effect of TGFβ (Figures 3N–3Q) and maintains both neuronal and glial lineage potentials, as revealed by their response to BMP2.

SOX10 Prevents TGFβ-Induced Proliferative Arrest in NCSCs

In the course of these experiments, we noted that colony sizes appeared significantly larger in SOX10 virus-infected cultures preincubated in TGFβ than in GFP virus-infected cultures (Figures 3C and 3G and data not shown). As TGFβ strongly inhibits proliferation in NCSCs (Shah et al., 1996), this observation suggested that SOX10 might inhibit such proliferative arrest. To address this question directly, GFP or SOX10 virus-infected cultures were incubated in TGFβ for 24 hr, washed, and then further incubated for 24 hr before being pulsed for 15 hr with BrdU (Figure 4, schematic). Preincubation of GFP-infected NCSCs with TGFβ for 24 hr caused a dose-dependent reduction in BrdU labeling (Figures 4A–4D; Figure 4I, GFP virus). This effect was to a large extent overcome by forced expression of SOX10 (Figure 4D versus 4H; Figure 4I, SOX10 virus). Serial observation of identified founder cells indicated that there was little or no cell death caused by the 24 hr preincubation in TGFβ (see below). Despite the rescue of BrdU incorporation, there were somewhat fewer cells in SOX10-infected cultures incubated with TGFβ than without (Figure 4E versus 4G); nevertheless, the number of cells was still ~50% greater than in control TGFβ-treated cultures (Figure 4C versus 4G). These data suggest that SOX10 not only preserves neuronal and glial differentiation potentials, but also proliferative activity, from extinction by TGFβ. Consistent with these activities, SOX10 inhibited smooth muscle differentiation induced by TGFβ (Supplemental Figure S2 at http://www.neuron.org/cgi/content/full/38/1/17/DC1).

SOX10 Is Essential for the Induction of Transcriptional Determinants of Autonomic Neurogenesis

The observation that SOX10 preserves neuronal differentiation potential from extinction by TGFβ (Figure 3) was unexpected, because genetic analysis has suggested that *Sox10* plays a primary role in supporting peripheral glial differentiation and survival (Britsch et al., 2001; Paratore et al., 2001). Our observations therefore raised the questions of the molecular mechanism under-

lying this activity, as well as whether it occurs in vivo. As a first step, we examined the relationship of SOX10 to the induction of MASH1, a proneural gene for the autonomic neuronal lineage (Guillemot et al., 1993; Lo et al., 1998). Preincubation of control NCSCs in 20 pM TGFβ, which extinguishes neurogenic potential (Figure 3), also strongly attenuated the induction of MASH1 by BMP2 (Figures 5A and 5C, GFP), consistent with earlier studies (Shah and Anderson, 1997). The induction of MASH1 was to a large extent restored by constitutive expression of SOX10 (Figure 5B, arrowheads; Figure 5C, SOX10). Observation of individual, identified founder cells for 48 hr, followed by fixation and DAPI staining to detect apoptotic nuclei, indicated that <10% of the cells died during the course of this experiment. Therefore, the parallel effects of SOX10 to preserve both MASH1 inducibility and neurogenic potential do not reflect a protection from TGFβ-mediated killing of neuronal precursors.

These results indicated that SOX10 attenuates the effect of TGFβ preincubation to inhibit induction of MASH1 by BMP2. This raised the question of whether SOX10 is normally required for MASH1 expression. In vitro, SOX10 by itself had no effect to increase *Mash1* expression in the absence of BMP2 (Figure 5C), suggesting that it may play a permissive role in the expression of this proneural gene. To determine whether SOX10 is required for MASH1 induction in vivo, we examined the expression of MASH1 in SOX10-expressing neural crest cells at the time they first migrate to the dorsal aorta, a site of autonomic (sympathetic) neurogenesis. Double labeling for MASH1 and SOX10 at E9.5 revealed that a high proportion of $SOX10^+$ cells were $MASH1^+$ near the dorsal aorta in wild-type embryos (Figures 5D and 5H, arrowheads; 50.3% ± 15.2% of $SOX10^+$ cells $MASH1^+$; n = 254 $SOX10^+$ cells analyzed in two embryos).

To determine whether MASH1 induction in neural crest cells is dependent on SOX10 in vivo, we examined its expression in *Dom/Dom* embryos, which are genotypically null for *Sox10* (Britsch et al., 2001). To do this, we exploited the fact that our monoclonal antibody to SOX10 recognizes an N-terminal epitope on the protein, which is spared by the C-terminal truncation of the *Sox10* coding sequence in *Dom* mutants (Herbarth et al., 1998; Southard-Smith et al., 1998) (L.L. and D.J.A., unpublished data). This antibody therefore allowed us to directly examine the expression of MASH1 in SOX10-trunc (truncated)-expressing neural crest cells in *Dom/Dom* embryos. Strikingly, although in *Dom/Dom* embryos at E9.5 there were many SOX10-$trunc^+$ cells adjacent to the dorsal aorta (Figure 5F), the vast majority of these cells did not express MASH1 (1 $MASH1^+$ cell/516 SOX10-$trunc^+$ cells, n = 2 embryos). Nevertheless, MASH1 expression was maintained in the ventral neural tube (Figures 5D and 5F, arrows), providing an internal positive control for antibody staining. These data suggest that SOX10 is required for the initial induction of MASH1 in neural crest-derived autonomic precursors in vivo. By E10.5, however, many $MASH1^+$ cells near the dorsal aorta no longer expressed SOX10 (Figure 5J, arrows), suggesting that the requirement of SOX10 for expression of MASH1 is transient.

We also examined the relationship of SOX10 to PHOX2B, a paired homeodomain transcription factor that, like MASH1, is essential for autonomic neurogenesis in vivo (Pattyn et al., 1999). Double labeling of mouse embryo sections with antibodies to SOX10 and PHOX2B revealed that, as in the case of MASH1, a high proportion (64% ± 19.2%) of $SOX10^+$ neural crest-derived cells near the dorsal aorta initially coexpress PHOX2B, at E9.5 (Figures 5E and 5I, arrowheads). In *Dom/Dom* embryos, however, PHOX2B expression was essentially lost in the SOX10-$trunc^+$ population at E9.5 (Figure 5G; 1 $PHOX2B^+$ cell/817 SOX10-$trunc^+$ cells analyzed). These data suggest that the initial expression of PHOX2B in neural crest cells, like that of MASH1, requires SOX10 function in vivo. At later stages, however, SOX10 and PHOX2B are expressed in largely nonoverlapping cell populations near the dorsal aorta (Figure 5K, arrows), as is the case for $MASH1^+$ cells (Figure 5J). Thus, SOX10 is required for the induction, but not the maintenance, of both MASH1 and PHOX2B in neural crest cells in vivo.

Forced Expression of SOX10 Inhibits Overt Neuronal Differentiation in NCSCs

The observation that SOX10 expression is ultimately extinguished in $MASH1^+$ and $PHOX2B^+$ cells (Figures 5H–5K) suggested that its continued expression might interfere with neuronal differentiation. To address this possibility directly, we examined neuronal differentiation in clonal cultures of NCSCs constitutively expressing SOX10. Under standard culture conditions, the first neurons (identified as process-bearing, $NF160^+$ cells) are detected in NCSC clones after ~7–7.5 days of incubation, and the percentage of neuron-containing colonies increases thereafter, reaching >90% after ~12 days (Stemple and Anderson, 1992). Similar kinetics were observed in cultures infected with the control GFP retrovirus (Figure 6A, GFP). By contrast, there was a clear inhibition of neuronal differentiation in SOX10 virus-infected cultures at all time points (Figure 6A, SOX10). The extent of neuronal differentiation increased slightly in SOX10-infected cultures at longer incubation times, but this increase was correlated with a spontaneous extinction of proviral gene expression in many cells by this time point (Figure 6B, cf. DAPI versus GFP). The expression of neuronal markers in such SOX10-infected clones occurred preferentially in those cells that had extinguished transgene expression (Figure 6B, arrow versus arrowhead; 38 GFP^+/589 $NF160^+$ cells). By contrast, the majority of neurofilament-positive cells in control GFP virus-infected colonies coexpressed the transgene (Figure 6B, large arrow; 848 GFP^+/1011 $NF160^+$ cells). Thus, SOX10 inhibits spontaneous neuronal differentiation in NCSCs. SOX10 also inhibited BMP2-induced neuronal differentiation, albeit to a lesser extent (data not shown).

Inhibition of Neurogenesis by SOX10 Is Correlated with Its Repression of PHOX2A, Both In Vitro and In Vivo

We wished to determine whether SOX10 functions to inhibit overt neurogenesis in vivo as well as in vitro. If so, then loss of SOX10 function might be expected to cause derepression and/or precocious induction of some neuronal differentiation markers in autonomic pre-

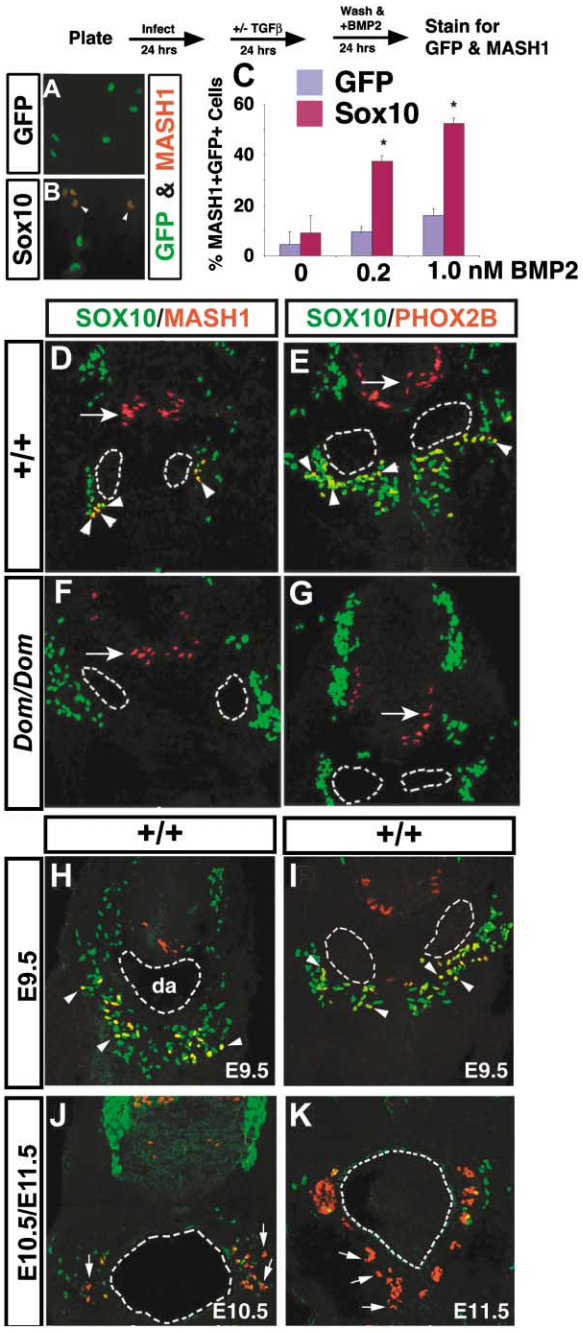

Figure 5. SOX10 Is Required for the Induction of Neurogenic Transcription Factors

(A and C) Preincubation in 20 pM TGFβ strongly attenuates subsequent induction of MASH1 by BMP2, in control GFP-infected NCSCs ([C], lavender bars). Typically ~90% of cells are MASH1$^+$ in these conditions (Shah and Anderson 1997) (data not shown). (B and C) Forced expression of SOX10 preserves MASH1-inducibility in many infected cells ([B], arrowheads; [C], magenta bars; *$p < 0.05$). (D–G) Transverse sections of wild-type (D and E) and *Sox10*$^{-/-}$ (*Dom/Dom*) embryos at E9.5, double labeled for SOX10 (green) and either MASH1 ([D and F]; red) or PHOX2B ([E and G]; red). The dorsal aortae are outlined by dashed lines. MASH1 and PHOX2B expression initially occurs in cells that coexpress SOX10 ([D and E]; arrowheads), and is lost in *Dom/Dom* mutants (F and G). Arrows indicate expression of MASH1 (D and F) and PHOX2B (E and G) in ventral spinal cord. (H–K) Transient coexpression of MASH1 (H and J) and PHOX2B (I and K) with SOX10 in wild-type embryos. Note that at stages older than E9.5, many MASH1$^+$ and PHOX2B$^+$ cells no longer coexpress SOX10 ([J and K]; arrows).

cursors. To this end, we analyzed the expression of PHOX2A, a close relative of PHOX2B that is also expressed by developing autonomic neuroblasts (Pattyn et al., 1997). PHOX2A is expressed 1 day later than PHOX2B in sympathetic ganglia primordia and is never coexpressed with SOX10 at E9.5 (Figures 7A and 7D; 0 PHOX2A$^+$ cells/982 SOX10$^+$ cells analyzed in n = 2 embryos). Rather, from its onset at E10.5, PHOX2A expression is mutually exclusive with that of SOX10 (Figure 7B; inset, arrows). These data suggested that SOX10 might negatively regulate PHOX2A expression (or vice-versa). To test this, we first asked whether forced expression of SOX10 might interfere with the induction of PHOX2A by BMP2 (Lo et al., 1998) in cultured NCSCs. Indeed, constitutive expression of SOX10 in NCSCs caused a >2-fold inhibition of PHOX2A induction by BMP2, in comparison to controls (Figure 7C, SOX10 versus GFP virus). By contrast, no such inhibition of MASH1 or PHOX2B induction by SOX10 was observed (Figure 5C and data not shown).

To determine whether SOX10 is a negative regulator of PHOX2A expression in vivo as well as in vitro, we examined *Dom/Dom* embryos by antibody double labeling for SOX10-trunc and PHOX2A. In contrast to wild-type embryos (Figure 7D), many SOX10-trunc$^+$ cells coexpressed PHOX2A at E9.5 in *Dom/Dom* embryos, at axial levels anterior to the bifurcation of the dorsal aorta (Figures 7E and 7F). (At more posterior axial levels, derepression of PHOX2A was not detected; however, depletion of neural crest cells has been reported to be more severe around the caudal dorsal aorta in *Dom/Dom* embryos [Britsch et al., 2001].) Near the dorsal aorta, approximately 20% (19% ± 15.5%; mean ± SD) of SOX10-trunc$^+$ cells were PHOX2A$^+$ (Figures 7E and 7F, arrowheads; 970 SOX10$^+$ cells analyzed in 16 sections from 3 mutant embryos). Furthermore, many SOX10-trunc$^+$, PHOX2A$^+$ cells could be observed at ectopic sites in the neural crest migration pathway. In wild-type embryos, PHOX2A is normally not detected until neural crest cells have aggregated near the dorsal aorta to form the primordia of the sympathetic ganglia (Figure 7B). By contrast, in E9.5 *Dom/Dom* embryos, we observed numerous instances of SOX10-trunc$^+$, PHOX2A$^+$ cells at more dorsal positions in the crest migration pathway, adjacent to the neural tube (Figures 7E, 7F, and 7F′, arrows; 16.4% ± 10.2% of SOX10-trunc$^+$ cells PHOX2A$^+$, 1117 SOX10$^+$ cells analyzed). Thus, PHOX2A is precociously and ectopically derepressed in a subset of SOX10$^+$ neural crest cells in *Dom/Dom* embryos.

Inhibition of Neuronal Differentiation, but Not Maintainance of Neurogenic Potential, Exhibits Haploinsufficiency for *Sox10*

The inhibition of neuronal differentiation caused by constitutive SOX10 expression in vitro exhibited incomplete penetrance (Figure 6). One possible explanation for this phenomenon is that the extent of such inhibition is a sensitive function of the level of SOX10 expression (the

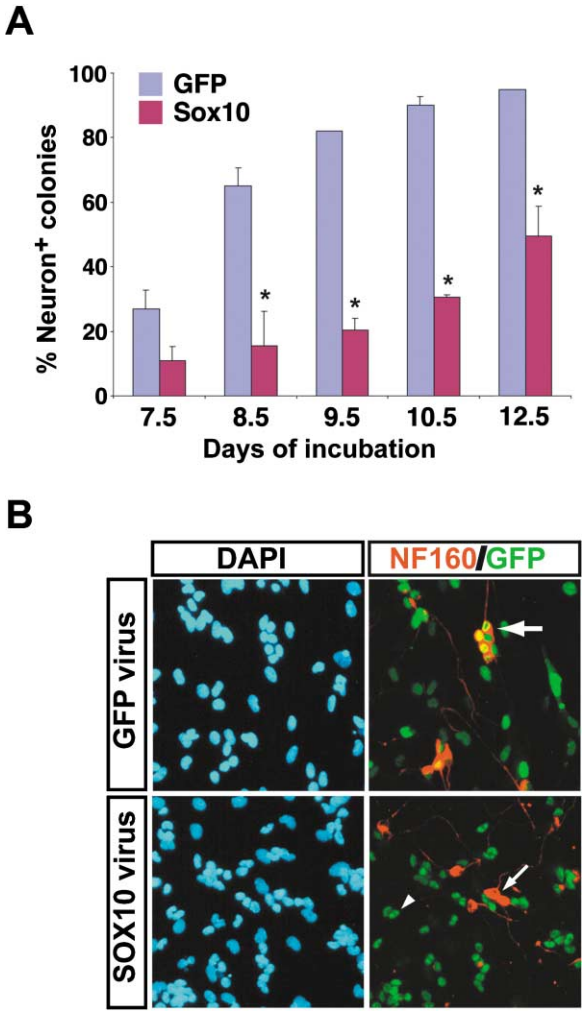

Figure 6. SOX10 Inhibits Neuronal Differentiation in NCSCs

(A) The percentage of infected (GFP^+) colonies containing neurons ($NF160^+$ cells) was quantified at the indicated time points. The results represent the mean ± SEM of two independent experiments. $^*p < 0.05$.

(B) Neuronal differentiation in 12.5 day SOX10-infected colonies occurs preferentially in cells that have extinguished proviral gene expression (lower right panel, arrow); see text for quantification. By contrast, the majority of neuronal cells in control GFP virus-infected colonies still express the transgene (large arrow).

level of SOX10 expression achieved using our retroviral expression system is only ~40% higher than endogenous levels; see Experimental Procedures). If so, then removal of a single copy of endogenous *Sox10* might be expected to cause a derepression of PHOX2A in vivo. There is precedent that some other functions of SOX10 in neural crest development (e.g., in melanogenesis and enteric gangliogenesis) exhibit such haploinsufficiency (Herbarth et al., 1998; Southard-Smith et al., 1998).

We therefore asked whether any derepression of PHOX2A in $SOX10^+$ cells could be detected in *Dom*/+ embryos. Indeed, at E9.5 such precocious expression of PHOX2A in $SOX10^+$ cells was clearly detectable near the dorsal aorta (Figure 7G versus 7H; inset, arrowheads). Precocious expression of the RNA binding protein HuD, a pan-neuronal precursor marker, was also detected in $SOX10^+$ cells in such $Sox10^{+/-}$ heterozygotes (Figure 7K″, arrows). Triple labeling indicated that derepression of HuD occurred in $SOX10^+$ cells in which $PHOX2A^+$ was also derepressed (Figures 7K–7K‴, arrows). Although we occasionally detected rare HuD^+ $PHOX2A^+$ cells near the dorsal aorta in wild-type embryos at this stage (E9.5+; Figures 7J″ and 7J‴, arrowhead), such cells were $SOX10^-$ (Figure 7J′, arrowhead). Thus, both PHOX2A and HuD are derepressed in $SOX10^+$ crest cells in *Dom*/+ mutants. By contrast, PHOX2B and MASH1 appeared normally expressed in such heterozygotes (Figure 7I and data not shown). Thus, removal of one copy of *Sox10* is sufficient to cause precocious expression of PHOX2A and HuD in $SOX10^+$ precursors, while removal of both copies causes a failure of MASH1/PHOX2B expression and consequently of autonomic neuronal differentiation (Figure 6). This latter phenotype is reflected in the lack of expression of HuD in *Dom*/*Dom* embryos (Figure 7L″).

MASH1 and PHOX2B Repress Expression of Endogenous SOX10 in NCSCs

The opposite effects of the *Dom*/*Dom* genotype on MASH1/PHOX2B and PHOX2A expression in neural crest-derived autonomic precursors suggested that SOX10 is required not only to maintain neurogenic differentiation potential but also to inhibit or delay overt neuronal differentiation. This raised the question of how this inhibition is ultimately relieved to permit neuronal differentiation to occur. One possibility is that MASH1, PHOX2B, or PHOX2A, once having been induced, in turn repress SOX10. To test this possibility, we examined the expression of endogenous SOX10 protein in NCSCs infected with retroviruses encoding these three neurogenic transcription factors. Both MASH1 and PHOX2B caused a slight reduction in SOX10 expression 48 hr after infection, in comparison to GFP virus-infected controls (Figure 8M, lavender bars). By 72 hr postinfection, the expression of SOX10 was severely reduced, especially in MASH1-infected cells (Figure 8C versus 8F and 8L; Figure 8M, magenta bars). In contrast, PHOX2A had no effect to suppress SOX10 expression (Figures 8I and 8M). The observation that MASH1 and PHOX2B can repress (directly or indirectly) the expression of SOX10, taken together with the fact that their initial expression is SOX10 dependent (Figure 6), suggests that a negative-feedback loop may extinguish SOX10 expression in autonomic neuronal precursors (Figure 9C).

Discussion

The evidence presented here suggests that SOX10 is a multifunctional protein that contributes to several important properties of NCSCs. First, SOX10 preserves both neurogenic and gliogenic differentiation capacity from extinction by lineage restriction factors. Second, SOX10 inhibits overt neuronal and smooth muscle differentiation. Third, SOX10 prevents TGFβ-induced proliferative arrest. The extent to which these functions are independent is discussed below. The notion that SOX10 exerts multiple functions in the neural crest and its derivatives is consistent with emerging evidence that SOX proteins

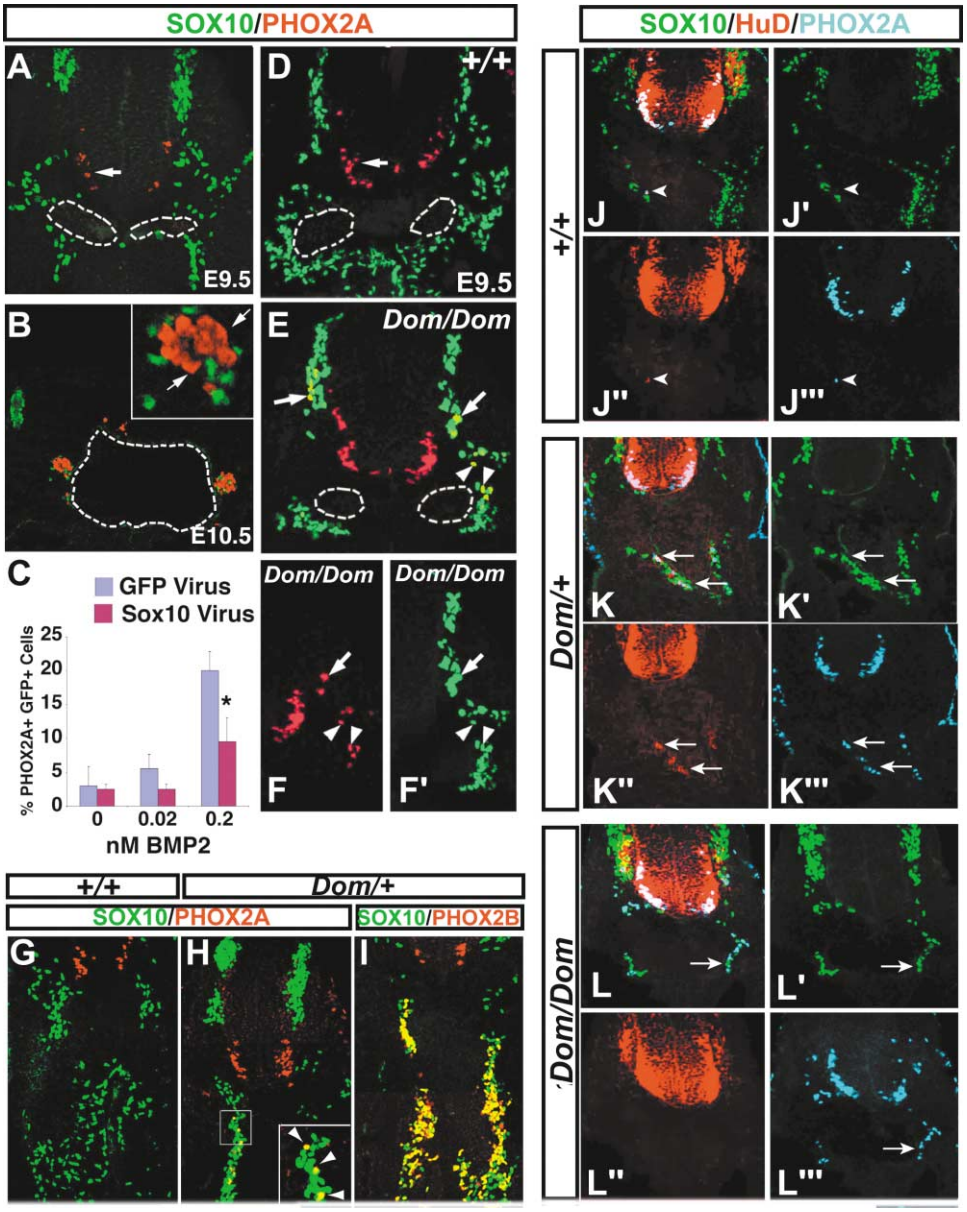

Figure 7. SOX10 Represses PHOX2A Expression Both In Vitro and In Vivo

(A and D) PHOX2A is not expressed in SOX10$^+$ cells at E9.5, and initial expression at E10.5 occurs in SOX10$^-$ cells ([B], inset, arrows). Dashed lines demarcate dorsal aortae; arrows in (A) and (D) indicate PHOX2A expression in ventral neural tube. (C) SOX10 inhibits induction of PHOX2A by BMP2 in vitro (magenta bars, *$p < 0.05$). Induction of MASH1 and PHOX2B is unaffected (data not shown). (D–F) PHOX2A is precociously derepressed in a subset of SOX10-trunc$^+$ cells in E9.5 *Dom/Dom* mutants (E–F′); (D) section from a wild-type littermate. Arrows (E and F) indicate ectopic expression in the neural crest migration stream (F′), arrowheads indicate premature expression near the dorsal aorta. (G–I) Derepression of PHOX2A in SOX10$^+$ cells occurs in *Sox10*$^{+/-}$ heterozygotes (*Dom/+*; [G and H] inset, arrowheads), while expression of PHOX2B (I) and MASH1 (data not shown) are unaffected. (J–K‴) Derepression of HuD is also observed in *Dom/+* embryos ([K″], arrows) and occurs in SOX10$^+$ cells ([K′], arrows) that also show derepression of PHOX2A ([K and K‴], arrows). At this stage (E9.5+) and axial level (anterior), rare HuD$^+$ PHOX2A$^+$ cells are seen near the dorsal aorta in wild-type littermates ([J–J‴], arrowheads), but do not coexpress SOX10 ([J and J′], arrowhead). (L–L‴) Expression of HuD is lost in *Dom/Dom* embryos (L″), while derepression of PHOX2A still occurs ([L′ and L‴], arrows).

are multifunctional (Akiyama et al., 2002; Wilson and Koopman, 2002). While other proteins undoubtedly contribute to stem cell properties, the combination of functions exhibited by SOX10 in NCSCs seems well suited to a role in the maintenance of the stem cell state.

SOX10 Is Required for Neurogenic as Well as Gliogenic Potential

We have employed an experimental paradigm in which NCSCs are preincubated with lineage commitment factors that cause a rapid loss of certain developmental

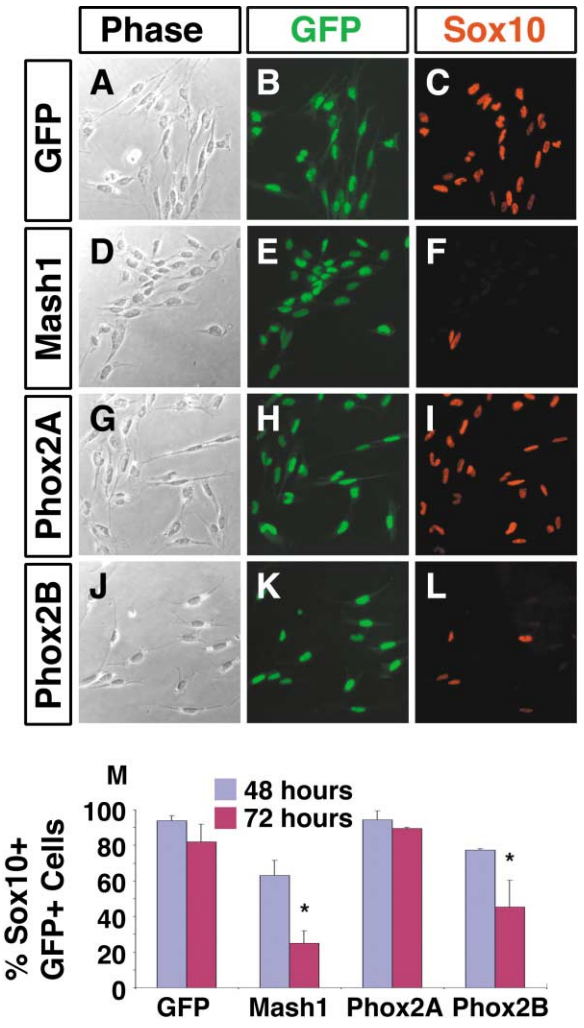

Figure 8. Sustained Expression of MASH1 and PHOX2B Causes Downregulation of Endogenous SOX10 in NCSCs

NCSCs were infected after plating with retroviruses encoding MASH1, PHOX2A, or PHOX2B, and the percentage of infected (GFP^+; [B, E, H, and K]) cells expressing SOX10 (C, F, I, and L) was measured 48 hr ([M], lavender bars) or 72 hr ([M], magenta bars) later. Note the progressive repression of SOX10 with time by MASH1 (C versus F; M), and to a lesser extent by PHOX2B (C versus L; M). PHOX2A has no effect (C, I, and M). *$p < 0.05$.

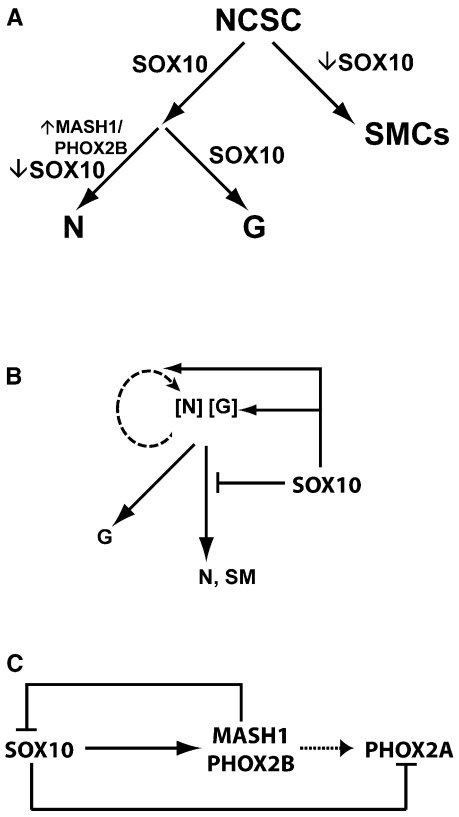

Figure 9. Schematic Illustrating Functions and Genetic Circuitry of SOX10 in Neural Crest Stem Cells

(A) Summary of results. Extinction of SOX10 in NCSCs (e.g., by TGFβ) causes them to exit the neural crest lineage and default to smooth muscle/myofibroblasts (SMCs) in vitro. Maintenance of SOX10 preserves neuronal and glial lineage potentials.

(B) SOX10 inhibits overt neuronal ("N") and smooth muscle ("SM") differentiation, while simultaneously maintaining multipotency ([N] and [G]) and inhibiting proliferative arrest (circular arrow).

(C) Interactions of SOX10 with neurogenic transcription factors. The arrows are genetic and not meant to imply direct interactions.

potentials (Shah and Anderson, 1997). This paradigm permitted us to ask whether constitutive SOX10 expression could prevent the loss of such potentials. For example, SOX10 prevented extinction of glial potential by BMP2. Such a permissive role in glial differentiation is consistent with earlier in vitro loss-of-function studies of SOX10 (Paratore et al., 2001) and fits with its requirement for the differentiation of all peripheral glia in vivo (Britsch et al., 2001).

Surprisingly, we also found that constitutive expression of SOX10 prevented the rapid loss of neurogenic (as well as gliogenic) potential caused by preincubation in TGFβ (Shah and Anderson, 1997). Although this effect was all-or-none with respect to GFP-infected controls, the extent of neurogenesis in SOX10-infected cultures exposed to TGFß was not fully restored to that in cultures incubated without TGFβ. Such incomplete penetrance may reflect the fact that SOX proteins require partners (Wilson and Koopman, 2002), which may be limiting in NCSCs. If so, then increasing SOX10 expression above a certain level would have no effect; however, removing SOX10 should have a fully penetrant phenotype.

The observation that loss of *Sox10* in vivo causes failure of expression of the neurogenic transcription factors MASH1 and PHOX2B, with essentially 100% penetrance, is consistent with this idea. This lack of MASH1/PHOX2B expression is unlikely to simply reflect cell death, rather than lack of gene expression, as double labeling with the TUNEL reagent indicates that the SOX10-$trunc^+$ cells in *Dom/Dom* mutant embryos at E9.5 are not apoptotic (data not shown). The conclusion that SOX10 is required for the initial expression of MASH1 in vivo is also supported by the fact that in *Drosophila*, expression of the MASH1 homolog ACHAETE in CNS neuroblasts is dependent on the SOX2-related gene SoxNeuro (Buescher et al., 2002; Overton et al., 2002). Since SOX10 maintains neurogenic

capacity in vitro, and is required in vivo for induction of *Mash1* and *Phox2b*, genes that are in turn required for neurogenic capacity (Guillemot et al., 1993; Pattyn et al., 1999), it follows that SOX10 is likely required for neurogenic capacity in vivo, as well as in vitro.

Our in vivo loss-of-function data are, therefore, consistent with the ability of SOX10 to preserve neurogenic potential from extinction by lineage commitment factors in vitro. Taken together with its ability to maintain gliogenic capacity, these data support the idea that SOX10 maintains multipotency in NCSCs. However, it is currently difficult to directly test whether SOX10 maintains NCSC multipotency in vivo. The idea that SOX10 maintains multipotency in stem cells is not inconsistent with a later function in committed glia (Peirano et al., 2000); this may reflect a developmental change in its partner proteins (Wilson and Koopman, 2002). In other systems, SOX proteins have been shown to play multiple, sequential roles within a developing lineage (Akiyama et al., 2002).

SOX10 Inhibits Autonomic Neuronal Differentiation

In addition to maintaining the multipotency of NCSCs in vitro, SOX10 inhibits overt neuronal differentiation. It is possible that this inhibitory function is simply a secondary consequence of the effect of SOX10 to preserve multipotency. Arguing against this, however, is the fact that these two functions can be genetically separated in vivo, because of their different gene-dosage sensitivities. PHOX2A, a marker of autonomic neuronal differentiation, is derepressed in both *Dom*/+ and *Dom*/*Dom* embryos, while expression of MASH1 and PHOX2B is lost only in *Dom*/*Dom* homozygotes. Haploinsufficiency of SOX10 for the repression of neuronal differentiation markers has previously been reported in vitro (Paratore et al., 2001) as well as in the enteric nervous system in vivo (Paratore et al., 2002). Evidently, a higher level of SOX10 is required to inhibit neuronal differentiation than to maintain neurogenic potential. This may explain the incomplete penetrance of the effect of SOX10 to inhibit neurogenesis in gain-of-function experiments. Although its mechanistic basis remains to be determined, this genetic dissociation strongly suggests that these two functions of SOX10 are independent.

The derepression of *Phox2a* in *Sox10* mutants may appear paradoxical given that expression of *Phox2a* is lost in the sympathetic primordia of *Mash1*$^{-/-}$ or *Phox2b*$^{-/-}$ mutants (Hirsch et al., 1998; Lo et al., 1998; Pattyn et al., 1999), and that *Dom*/*Dom* mutants lack MASH1 and PHOX2B expression. However, the ability of MASH1 to repress SOX10 in vitro (Figure 8) suggests that in *Mash1*$^{-/-}$-sympathetic ganglia there might be a failure of normal SOX10 downregulation, permitting constitutive repression of PHOX2A (Figure 9C). If induction of PHOX2A normally requires repression of SOX10, then in *Dom*/*Dom* mutants lacking *Sox10* function, PHOX2A could be derepressed despite the failure of MASH1 and PHOX2B expression (Figure 9C). Such a mechanism does not, however, preclude additional enhancement of *Phox2a* expression by MASH1 and/or PHOX2B (Hirsch et al., 1998; Lo et al., 1998; Flora et al., 2001). Indeed, the fact that *Phox2a* is derepressed only in anterior regions of *Dom* embryos may reflect a greater dependence of its expression on PHOX2B and/or MASH1 at more posterior axial levels (Morin et al., 1997; Pattyn et al., 1997, 1999; Hirsch et al., 1998).

In contrast to autonomic neurons, the initial differentiation of sensory neurons appears largely unperturbed in *Sox10* mutant embryos, although glial precursor survival and differentiation in the dorsal root ganglia (DRG) is severely affected (Britsch et al., 2001; Sonnenberg-Riethmacher et al., 2001). This lack of an early sensory neurogenesis defect is not inconsistent with our results, since a variety of data suggest that NCSCs are restricted to generating autonomic derivatives (Greenwood et al., 1999; White et al., 2001; Lo et al., 2002). Thus, the opponent functions of SOX10 to maintain neurogenic potential and inhibit neuronal differentiation may be limited to progenitor cells of the autonomic sublineage.

The Role of SOX10 in Multipotency and Proliferation

Forced expression of SOX10 prevents TGFβ-induced mitotic arrest, at the same time as it preserves neuronal and glial potential. Is it possible that the preservation of such multipotency is a secondary consequence of the ability of SOX10 to maintain proliferation under such conditions? We think this is unlikely for several reasons. First, SOX10 preserves glial potential in BMP2 as well as in TGFβ, but BMP2 (unlike TGFβ) does not induce proliferative arrest (Shah et al., 1996). Second, such a model implies that neural crest-derived cells should cease proliferating at the time that they lose multipotency, but this is not the case. Autonomic neuronal precursors continue to proliferate for days after the stage when SOX10 is extinguished (DiCicco-Bloom et al., 1990; Verdi and Anderson, 1994). This implies that loss of SOX10 function in *Dom*/*Dom* embryos is unlikely to cause proliferative arrest (although we have not directly tested this). The requirement of SOX10 for MASH1 expression in vivo, independent of any requirement for proliferation, would suggest that the ability of SOX10 to maintain MASH1 inducibility in TGFβ is likely to be independent of its effect to maintain proliferation in vitro. To the extent that MASH1 inducibility is a marker of neurogenic potential, this suggests that the maintenance of multipotency by SOX10 is not secondary to its ability to prevent mitotic arrest. We cannot exclude, however, that the ability of SOX10 to inhibit TGFβ-induced proliferative arrest is secondary to its ability to inhibit smooth muscle differentiation.

SOX10 May Function in Multiple Progenitor Cell Compartments

In which cell type(s) does SOX10 function? In vivo, SOX10 is initially expressed in p75^{+}, MASH1^{-}, and PHOX2B^{-} neural crest cells, and its expression persists transiently in MASH1^{+}, PHOX2B^{+} cells. Therefore, SOX10 could exert its functions in either or both progenitor cell compartments. The fact that SOX10 is required for the initial induction of MASH1 and PHOX2B in vivo, and maintains MASH1 inducibility in vitro, suggests that it functions in a pre-MASH1-expressing stem cell compartment to maintain multipotency. On the other hand, the fact that SOX10 inhibits PHOX2A expression (which normally occurs after expression of MASH1 and

PHOX2B) suggests that it may also operate in MASH1$^+$, PHOX2B$^+$ cells to inhibit or delay their differentiation into neurons. The persistence of SOX10 expression in such cells is also likely to maintain their multipotency, as evidenced by the fact that constitutive expression of SOX10 preserves gliogenic capacity in BMP2-treated cells, which express MASH1 and PHOX2B. Whether the induction of MASH1 and PHOX2B is associated with a loss of self-renewal capacity, or whether MASH1, PHOX2B$^-$ and MASH1, PHOX2B$^+$ cells represent different types of self-renewing stem cells, remains to be determined.

The Glial Fate as a "Default" Fate for NCSCs

SOX10 inhibits neuronal and smooth muscle, but is permissive for glial differentiation. In keeping with this, SOX10 continues to be expressed in Schwann cells, while it is extinguished in the other derivatives. Interestingly, there are many other regulatory genes that, like *Sox10,* are expressed in NCSCs and Schwann cells but not in other crest derivatives (Stemple and Anderson, 1992; Britsch et al., 1998; Labosky and Kaestner, 1998; Shin et al., 1999). The genome of NCSCs may therefore be intrinsically biased toward a glial program of gene expression. Consistent with this idea, SOX10 is known to be a coactivator of glial-specific genes (Peirano et al., 2000; Bondurand et al., 2001; Stolt et al., 2002). These data suggest that the Schwann cell fate may be the "default" fate that NCSCs adopt once they have lost alternative developmental potentials. Similarly, the observation that some embryonic and adult CNS stem cells express genes such as *Gfap* and *Bfabp* (Doetsch et al., 1999; Malatesta et al., 2000) may indicate that the astrocyte fate is the "default" in the CNS (Zhou and Anderson, 2002).

The notion of the glial fate as a "default" state for NCSCs is not incompatible with the existence of instructive inducing signals for this fate, such as GGFII/NRG-1 (Shah et al., 1994) or Notch ligands (Morrison et al., 2000). Such signals may function primarily to promote the loss of neuronal (or other nonglial) differentiation potentials, rather than to activate a glial differentiation program. This would be analagous to the case of neural "inducers" in *Xenopus,* which promote a "default" neural fate by inhibiting epidermal differentiation (Hemmati-Brivanlou and Melton, 1997). The expression of SOX10 in NCSCs may provide a permissive genomic environment for eventual restriction to a glial fate, while simultaneously providing an extended window of opportunity for neuronal differentiation. It will be interesting to determine whether SOX2 plays a similar role in CNS stem cells (Uwanogho et al., 1995; Rex et al., 1997; Zappone et al., 2000) and , more generally, whether other SOX proteins contribute to the maintenance of tissue-specific stem cell properties in other lineages.

Experimental Procedures

NCSC Culture

NCSCs were obtained form E10.5 rat embryos as previously described (Stemple and Anderson, 1992). Cells were typically allowed to attach for 2 hr prior to the first manipulation (retroviral infection and/or growth factor addition). Recombinant human TGFβ1 was purchased from R&D Systems, recombinant human BMP2 was a gift from Genetics Institute, and recombinant human GGFII was a gift from CeNeS Pharmaceuticals. In some experiments, NCSCs grown in GGFII for $\sim$5 days were allowed to undergo further differentiation to O4$^+$ Schwann cells, by incubation for 6 days in maturation medium containing 10% fetal bovine serum (FBS) and 5 μM forskolin (Stemple and Anderson, 1992). Procedures for generating pseudotyped retroviruses and additional methods can be found in Supplemental Data at http://www.neuron.org/cgi/content/full/38/1/17/DC1.

Antibody Staining

Dom/Dom embryos were generated from heterozygous intercrosses and genotyped as described previously (Britsch et al., 2001). Staining procedures and sources of commercial antibodies are provided as Supplemental Data at http://www.neuron.org/cgi/content/full/38/1/17/DC1. Results were quantified by scoring the percentage of colonies containing $\geq$1 cell of the indicated phenotype (Shah et al., 1996; Shah and Anderson, 1997; Lo et al., 2002); this represents a conservative parameter because we are measuring the effect of SOX10 to prevent extinction of differentiation. All experiments were repeated at least twice, and 100–300 clones were scored per condition per experiment.

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