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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Stem cell factor Sox2 and its close relative Sox3 have differentiation functions in oligodendrocytes

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ABSTRACT

Neural precursor cells of the ventricular zone give rise to all neurons and glia of the central nervous system and rely for maintenance of their precursor characteristics on the closely related SoxB1 transcription factors Sox1, Sox2 and Sox3. We show in mouse spinal cord that, whereas SoxB1 proteins are usually downregulated upon neuronal specification, they continue to be expressed in glial precursors. In the oligodendrocyte lineage, Sox2 and Sox3 remain present into the early phases of terminal differentiation. Surprisingly, their deletion does not alter precursor characteristics but interferes with proper differentiation. Although a direct influence on myelin gene expression may be part of their function, we provide evidence for another mode of action. SoxB1 proteins promote oligodendrocyte differentiation in part by negatively controlling miR145 and thereby preventing this microRNA from inhibiting several pro-differentiation factors. This study presents one of the few cases in which SoxB1 proteins, including the stem cell factor Sox2, are associated with differentiation rather than precursor functions.

KEY WORDS: Glia, Myelin, Transcriptional control, High mobility group, MicroRNA

INTRODUCTION

Sox2 has attracted special attention as a stemness and pluripotency factor in embryonic stem cells (ESCs) (Masui et al., 2007) and as part of the transcription factor cocktail that re-establishes pluripotency during the generation of induced pluripotent stem cells (Takahashi and Yamanaka, 2006). Besides maintaining ESCs in an undifferentiated, pluripotent state, Sox2 promotes neural over epidermal fate in ectoderm (Zhao et al., 2004; Takemoto et al., 2011). In neural progenitor cells (NPCs), Sox2 continues to be expressed and is required for proliferation (Bylund et al., 2003), maintenance of pan-neural progenitor identity and inhibition of precocious differentiation (Graham et al., 2003). Sox2 expression in the central nervous system (CNS) is thus largely associated with an undifferentiated neural progenitor state.

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In NPCs Sox2 shares many functions with Sox1 and Sox3, which together constitute the SoxB1 subgroup of the Sox transcription factor family (Wegner, 2010). These factors exhibit redundancy, such that one member of the SoxB1 subgroup can often compensate for the loss of another (Graham et al., 2003). With exit from the cell cycle upon neuronal specification and differentiation, SoxB1 expression declines in most neurons (Bylund et al., 2003). Interestingly, some subtypes of neurons continue or restart SoxB1 expression as they differentiate. In hypomorphic *Sox2* mutants, GABAergic neurons are not only reduced in numbers, but also altered in morphology (Cavallaro et al., 2008), and retinal ganglion cells fail to differentiate properly (Taranova et al., 2006). This argues that SoxB1 proteins may also have functions during differentiation and cell maturation as first postulated for Sox1 (Ekonomou et al., 2005). However, little is known about such roles.

SoxB1-positive NPCs not only generate neurons, but also oligodendrocytes and astrocytes as the two main CNS glia. Oligodendrocyte development is intimately linked to, and depends on, the expression of several Sox proteins for the generation, maturation and maintenance of the lineage. Sox9 is necessary for oligodendroglial specification (Stolt et al., 2003), whereas Sox10, although already expressed in oligodendrocyte precursors (OPCs) and therefore regarded as a general marker of the oligodendrocyte lineage, becomes important during terminal differentiation (Stolt et al., 2002). In both these events, Sox5 and Sox6 counteract Sox9 or Sox10 (Stolt et al., 2006). Sox17 again supports oligodendrocyte differentiation by promoting OPC cell cycle exit (Sohn et al., 2006).

This strong interconnection of Sox protein expression and oligodendrocyte development prompted us to analyze the influence of SoxB1 factors on oligodendrocyte development. Overexpression of Sox2 in cultured OPCs has previously been shown to cause fate reversal and conversion to neural stem cells (Kondo and Raff, 2004). Our study shows that Sox2 and Sox3 are expressed in OPCs and early differentiating oligodendrocytes. These SoxB1 proteins have no obvious function in OPCs, but are instead involved in oligodendrocyte differentiation. Their role can at least in part be ascribed to a cross-regulatory mechanism with a microRNA. Thus, we demonstrate for the first time that Sox2 and Sox3 play a role in CNS glia and provide mechanistic insights into their function during differentiation processes.

RESULTS

Sox2 and Sox3 are expressed in oligodendroglia

We analyzed the expression of the three SoxB1 proteins in mouse neural tube at different embryonic and postnatal stages (Fig. 1). NPCs in the spinal cord (SC) ventricular zone expressed the three SoxB1 proteins throughout embryonic development, most strongly at 10.5 days post-coitum (dpc) and 12.5 dpc (Fig. 1A,B,G,H,M,N). In the mantle zone, Sox1-, Sox2- and Sox3-positive cells had a mainly ventral localization at 15.5 dpc (Fig. 1C,I,O), followed by a

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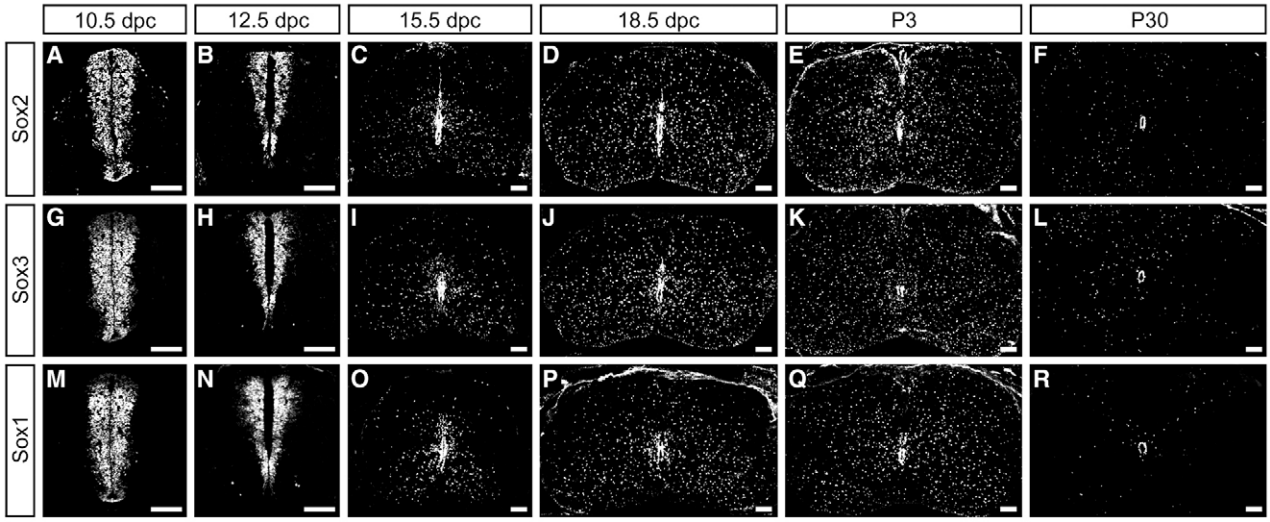

Fig. 1. SoxB1 transcription factors are expressed in ventricular and mantle zones of embryonic and postnatal mouse SC. Immunohistochemistry was performed on transverse sections of wild-type SC at 10.5 dpc (A,G,M), 12.5 dpc (B,H,N), 15.5 dpc (C,I,O), 18.5 dpc (D,J,P), P3 (E,K,Q) and P30 (F,L,R) with antibodies against Sox2 (A-F), Sox3 (G-L) or Sox1 (M-R). Scale bars: 100 µm.

more uniform distribution at 18.5 dpc and postnatal day (P) 3 (Fig. 1D,E,J,K,P,Q). By P30, there was only sparse SoxB1 expression remaining in the SC, most conspicuously in cells lining the central canal (Fig. 1F,L,R).

Co-immunohistochemical stainings were performed on SC sections to identify the SoxB1-expressing cell types (Fig. 2). Both major macroglia populations of the CNS, i.e. astroglia and oligodendroglia, showed Sox2 and Sox3 expression as evident from co-localization with the astroglial markers glutamine synthetase [GlnS; glutamate-ammonia ligase (Glul) – Mouse Genome Informatics] and glial fibrillary acidic protein (Gfap) (Fig. 2B,C,I) and the oligodendroglial markers NG2 (Cspg4 – Mouse Genome Informatics), Olig2 and Sox10 (Fig. 2D-F,J; data not shown). Quantification revealed that 78-81% of GlnS-positive astrocytes expressed Sox2 and Sox3 at 15.5 dpc and 69-74% of Gfap-positive astrocytes at 18.5 dpc (Fig. 2S,T). Among Sox10-positive oligodendroglial cells, 92-95% contained Sox2 and Sox3, while 89-91% of platelet derived growth factor receptor alpha (Pdgfra)-positive OPCs expressed Sox2 and Sox3 during embryonic development (Fig. 2O,P). There was also substantial co-expression of Sox2 and Sox3 with myelin proteins during perinatal and early postnatal times (Fig. 2G,K); 54-67% of myelin basic protein (Mbp)-positive cells expressed Sox2 and Sox3 at 18.5 dpc (Fig. 2Q). Overlap between Sox2 or Sox3 and the neuronal marker NeuN (Rbfox3 – Mouse Genome Informatics), by contrast, was restricted to very few cells in the ventral SC (Fig. 2A,H,R). Similar to Sox2 and Sox3, Sox1 was detectable in astrocytes (Fig. 2M,S,T) and in a minor fraction of neuronal cells (Fig. 2L,R), but not in oligodendroglia (Fig. 2N,O). Thus, Sox2 and Sox3, but not Sox1, are expressed in oligodendroglia throughout embryonic and early postnatal development.

OPC expansion and migration occur independently of Sox2 and Sox3

By combining floxed *Sox2* (Smith et al., 2009) and *Sox3* (Rizzoti et al., 2004) alleles with a *Sox10::Cre* BAC transgene (Matsuoka et al., 2005), conditional deletion was achieved in the CNS of mutant mice, specifically in oligodendroglial cells. Sox10::Cre-mediated deletion of Sox2 or Sox3 had already occurred in the large majority of OPCs in the ventricular zone by 12.5 dpc (Fig. 3C,D,G,H). At 15.5 dpc, Sox10-positive oligodendrocytes were virtually devoid of Sox2 or Sox3 in the conditional mutants (Fig. 3K,L,O,P). Deletion rates were determined to be 97±1% for Sox2 and 94±2% for Sox3 at this stage. The mutant mice are hereafter referred to as $Sox2^{\Delta Sox10}$ or $Sox3^{\Delta Sox10}$.

At 15.5 dpc, oligodendroglial cells labeled by Sox10 or Olig2 were present in wild-type numbers in $Sox2^{\Delta Sox10}$ and $Sox3^{\Delta Sox10}$ mice (Fig. 4A-F,M,N). By 18.5 dpc, there was still no difference to the wild type in oligodendroglial cell numbers (Fig. 4G-N). In agreement, quantification of proliferative Ki67-positive oligodendroglia (i.e. cells positive for Ki67 and Sox10) and TUNEL-labeled apoptotic cells did not reveal any difference between wild-type and mutant animals (Fig. 4O,P; supplementary material Fig. S1A-F,I-N). This argues that Sox2 and Sox3 do not influence the proliferation, cell-cycle exit or survival of OPCs.

All oligodendrocytes arise from the ventricular zone and have to migrate to their final locations in the mantle and marginal zones. At 15.5 and 18.5 dpc, Sox2- or Sox3-deficient oligodendroglia showed a distribution pattern in the SC similar to that of wild-type oligodendroglia (Fig. 4A-L). Likewise, analysis of *Pdgfra* expression revealed no differences between the genotypes (data not shown). We conclude that OPC development, including expansion and emigration from the ventricular zone, occurs independently of Sox2 or Sox3.

Sox2 and Sox3 influence the terminal differentiation of oligodendrocytes

Transcripts of the major myelin proteins can already be detected in the SC by *in situ* hybridization (ISH) before birth, as shown for *Mbp* and proteolipid protein (*Plp*; *Plp1* – Mouse Genome Informatics) (Fig. 5). At 15.5 dpc, the first *Mbp*- and *Plp*-positive cells have appeared in wild-type animals (Fig. 5A,J). This onset of myelin gene expression was neither prematurely increased nor reduced in Sox2- or Sox3-deficient oligodendrocytes (Fig. 5D,G,M,P,S,U). At 18.5 dpc, however, differences became apparent. Major myelin genes were still sparsely expressed in the mutant SC, but were abundant throughout wild-type white matter. In Sox2- and Sox3-deficient animals, the number of *Mbp*-positive cells was reduced to 47±4%

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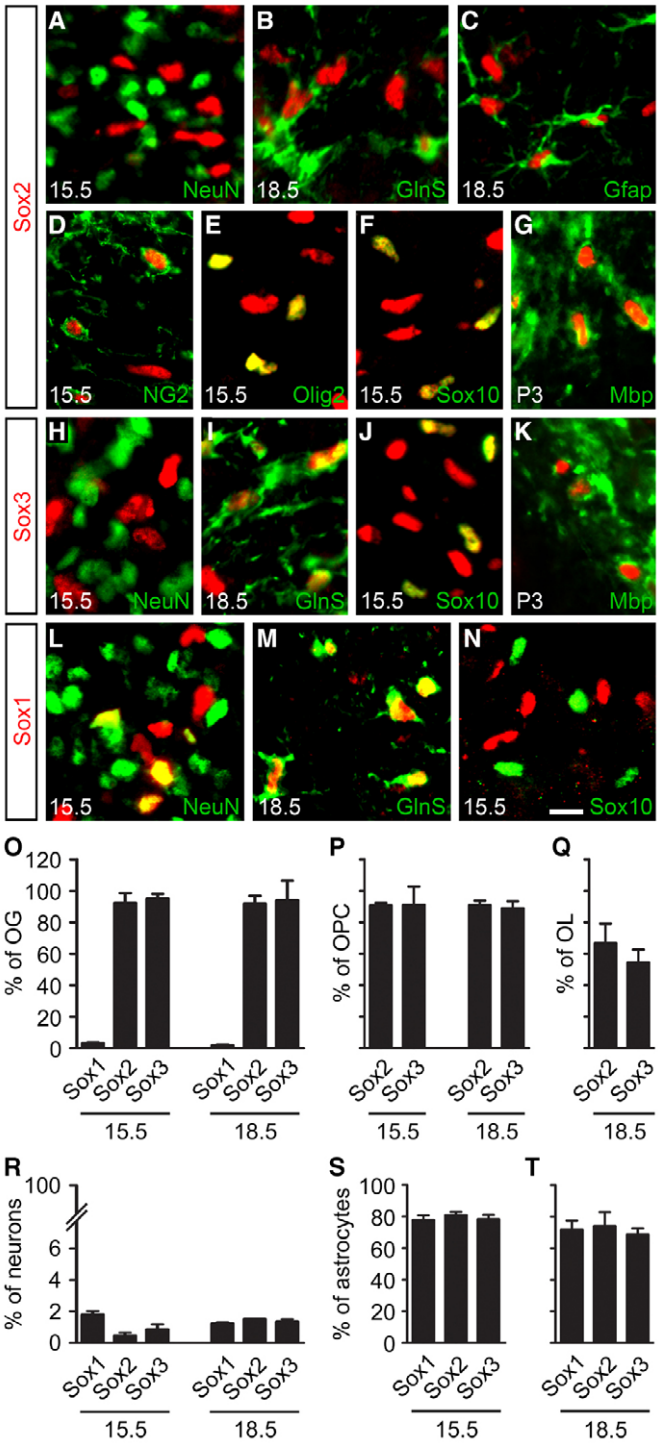

Fig. 2. SoxB1 transcription factors are differentially expressed in neurons and glia. (A-N) Co-immunohistochemistry on transverse sections of wild-type SC at 15.5 dpc, 18.5 dpc and P3 (as labeled in the lower left corner of panels) with antibodies against Sox2 (A-G), Sox3 (H-K) or Sox1 (L-N) (all in red) and the cell type-specific markers NeuN (A,H,L), GlnS (B,I,M), Gfap (C), NG2 (D), Olig2 (E), Sox10 (F,J,N) and Mbp (G,K) (all in green). Scale bar: 20 µm. (O-T) Quantification of Sox1, Sox2 and Sox3 expression in Sox10-positive oligodendroglia (OG) (O), Pdgfra-positive oligodendrocyte precursors (OPCs) (P), Mbp-positive oligodendrocytes (OL) (Q), NeuN-positive neurons (R), GlnS-positive astrocytes (S) or Gfap-positive astrocytes (T). Cell numbers are presented as mean percentage ± s.e.m. relative to the number of cells positive for the respective cell type marker (100%). Numbers on the *y*-axis in O are valid for O-Q, those on the *y*-axis in S for S,T.

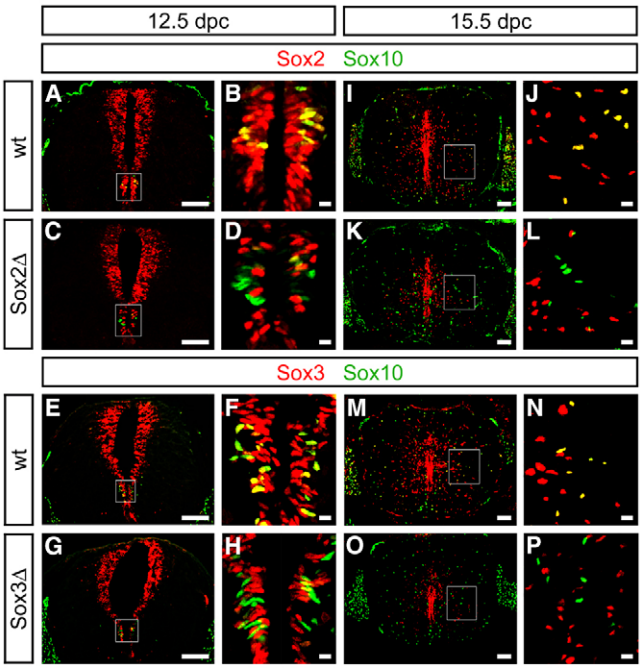

Fig. 3. Efficiency of Sox2 and Sox3 deletion in *Sox2*$^{\Delta Sox10}$ and *Sox3*$^{\Delta Sox10}$ oligodendroglia. Co-immunohistochemistry on transverse sections of wild-type (wt) (A,B,E,F,I,J,M,N), *Sox2*$^{\Delta Sox10}$ (Sox2Δ) (C,D,K,L) and *Sox3*$^{\Delta Sox10}$ (Sox3Δ) (G,H,O,P) SC at 12.5 dpc (A-H) and 15.5 dpc (I-P) with antibodies against Sox2 (A-D,I-L) or Sox3 (E-H,M-P) (red) and Sox10 (A-P) (green). Higher magnifications of framed areas from A,C,E,G,I,K,M,O (scale bars: 100 µm) are shown in B,D,F,H,J,L,N,P (scale bars: 10 µm).

and 47±2%, respectively, of wild-type levels (Fig. 5B,E,H,S). Similarly, *Plp* expression was reduced to 55±5% in *Sox2* mutants and to 46±2% in *Sox3* mutants (Fig. 5K,N,Q,U). Since *Sox2*$^{\Delta Sox10}$ animals survived for several weeks and *Sox3*$^{\Delta Sox10}$ animals until late adulthood, it was also possible to analyze the early postnatal SC at a time when myelin is rapidly increasing in the wild type. At P3, the numbers of Mbp- and Plp-positive cells were still reduced in the *Sox2*$^{\Delta Sox10}$ and *Sox3*$^{\Delta Sox10}$ mutants as judged both by ISH (Fig. 5C,F,I,L,O,R,S,U) and immunohistochemistry (Fig. 5T,V,W). This suggests that a large fraction of OPCs cannot properly enter the terminal differentiation program during the early postnatal period in the absence of either Sox2 or Sox3.

Sox2 and Sox3 act redundantly during oligodendrocyte differentiation

We did not observe major phenotypic differences between *Sox2*$^{\Delta Sox10}$ and *Sox3*$^{\Delta Sox10}$ animals. Together with the similar expression patterns of Sox2 and Sox3, this argues for functional redundancy between the proteins. To prevent the two Sox proteins from compensating for the loss of each other, we combined the targeted alleles of *Sox2* and *Sox3* with the *Sox10::Cre* transgene and obtained double deficiency in the oligodendroglial population. *Sox2*$^{\Delta Sox10}$ *Sox3*$^{\Delta Sox10}$ animals did not survive P1, but embryos could be analyzed up to 18.5 dpc. Similar to the single mutants, the early identity or numbers of OPCs were not affected by the combined loss of Sox2 and Sox3 as judged from the normal expression of Sox10, Olig2 and *Pdgfra* (Fig. 6A-H,Q-U). The number of proliferative Ki67-positive OPCs was also unaltered in the double mutant (Fig. 6V; supplementary material Fig. S1G,H). At all stages, the distribution of mutant OPCs throughout the SC was similar to

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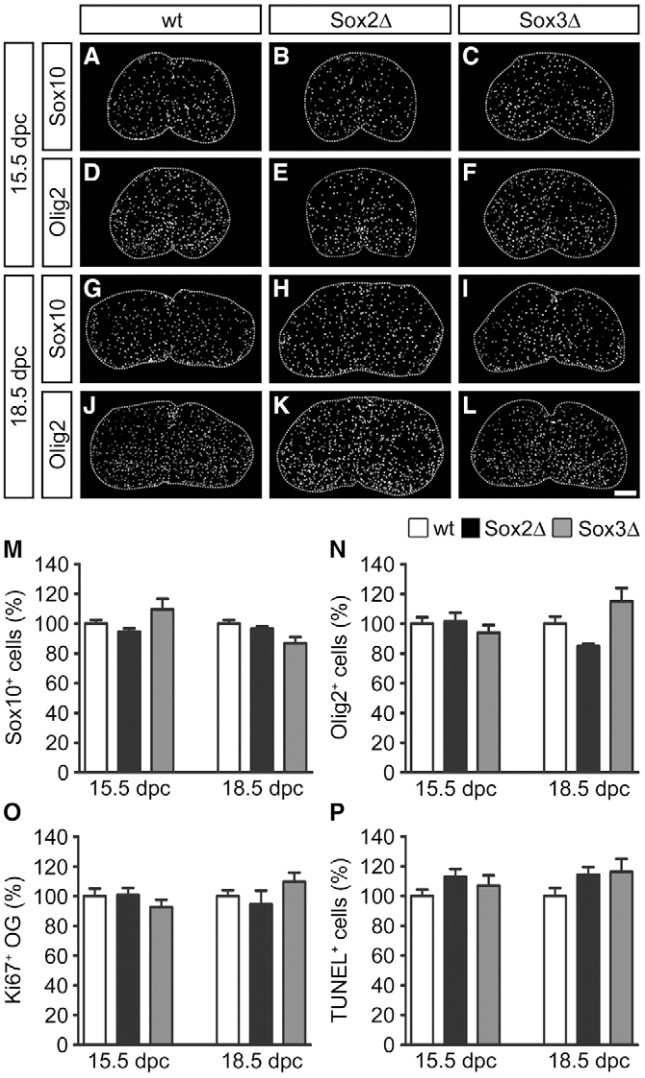

Fig. 4. OPCs are normal in the absence of Sox2 and Sox3.
(A-L) Immunohistochemistry on transverse sections of wild-type (A,D,G,J), *Sox2*$^{\Delta Sox10}$ (B,E,H,K) and *Sox3*$^{\Delta Sox10}$ (C,F,I,L) SC at 15.5 dpc (A-F) and 18.5 dpc (G-L) with antibodies against Sox10 (A-C,G-I) or Olig2 (D-F,J-L). Scale bar: 200 µm. (M-P) Quantification of Sox10-positive cells (M), Olig2-positive cells (N), Ki67-positive Sox10-expressing oligodendroglia (OG) (O) and TUNEL-positive cells (P). Cell numbers are presented as mean percentage ± s.e.m. relative to wild type (100%).

that in the wild type. The combined loss of Sox2 and Sox3 did not cause a significantly higher rate of apoptosis in the SC (supplementary material Fig. S1O,P), nor among oligodendroglial cells as assessed by combining TUNEL or cleaved caspase 3 staining with Sox10 immunohistochemistry (Fig. 6W,X).

The myelination defect observed in *Sox2*$^{\Delta Sox10}$ or *Sox3*$^{\Delta Sox10}$ single-mutant animals was dramatically aggravated upon concomitant loss of both Sox proteins. Although the first *Mbp*- and *Plp*-positive cells appeared on time and in normal numbers at 15.5 dpc in *Sox2*$^{\Delta Sox10}$ *Sox3*$^{\Delta Sox10}$ double-mutant embryos (Fig. 6I,J,M,N,Y,Z), myelin gene expression had not properly increased in the oligodendrocyte population of mutant animals by 18.5 dpc (Fig. 6K,L,O,P,Y,Z). There were only very few *Mbp*- or *Plp*-positive cells in mutant white matter at 18.5 dpc, corresponding to ~20% of wild-type levels and therefore being significantly lower than in the single mutants (compare Fig. 6Y,Z with Fig. 5S,U). Since the numbers of Sox10-positive cells and rates of OPC proliferation and apoptosis were unaltered, we assume that oligodendroglial cells are arrested at a stage after cell cycle exit and before the onset of terminal differentiation.

SoxB1 proteins may directly influence myelin gene expression

Sox2 and Sox3 may exert part of their effects during oligodendrocyte differentiation by acting directly on regulatory elements of myelin genes and thereby changing their transcription rates. One such known regulatory element is the *Mbp* promoter, which contains several Sox protein binding sites and is known to be activated by Sox10 (Stolt et al., 2002) (Fig. 7A). Using chromatin immunoprecipitation (ChIP), we found that the *Mbp* promoter was selectively enriched in chromatin precipitated with anti-Sox2 antibodies (Fig. 7A), indicating that Sox2 is bound to this region *in vivo*. A distal region 3 kb upstream of the *Mbp* transcription start site did not show enrichment (Fig. 7A).

By reporter gene assays, we analyzed whether binding of Sox2 to the *Mbp* promoter is an essential component of *Mbp* gene activation, as observed for Sox10 (Stolt et al., 2002). In 33B and Neuro2a cells, Sox2 activated luciferase expression from the *Mbp* promoter, but at substantially lower levels than Sox10 (Fig. 7B,C). Activation largely depended on the integrity of the major Sox binding sites in the *Mbp* promoter, with residual promoter responses likely to be due to remaining low-affinity sites (Fig. 7B,C). Sox2 and Sox10 did not synergize, as combined transfection did not further increase reporter activity. In 33B cells, activation rates remained at levels obtained with Sox10 alone (Fig. 7B), whereas they were between the rates obtained with either Sox10 or Sox2 in Neuro2a cells (Fig. 7C). Given that Sox10 is the stronger activator of *Mbp* expression and still present at normal levels in *Sox2*$^{\Delta Sox10}$, *Sox3*$^{\Delta Sox10}$ or double-mutant animals, it appears unlikely that the observed reduction in *Mbp* expression predominantly results from absence of direct transcriptional activation by Sox2 or Sox3.

Sox2 and Sox3 influence Sox9 expression post-transcriptionally

In wild-type SC at 15.5 and 18.5 dpc, Sox9 is present in all astroglia and oligodendroglia (Fig. 8A-D). Sox9-positive oligodendroglia (i.e. cells positive for both Sox9 and Sox10) were significantly decreased in *Sox2*$^{\Delta Sox10}$ embryos at 15.5 and 18.5 dpc and mildly affected in *Sox3*$^{\Delta Sox10}$ embryos (Fig. 8E-L,Q). In double-deficient animals, Sox9-positive oligodendroglia were dramatically reduced to 5±1% of wild-type numbers at 18.5 dpc (Fig. 8M-Q). Accordingly, protein extracts from whole SC of double-deficient animals exhibited reduced Sox9 levels despite the continued presence of the protein in astrocytes (Fig. 8R). This suggests that Sox2 and Sox3 are jointly involved in maintaining Sox9 expression in oligodendrocytes. However, Sox9 reduction cannot explain the phenotype in *Sox2*$^{\Delta Sox10}$ *Sox3*$^{\Delta Sox10}$ animals because oligodendroglial deletion of Sox9 is phenotypically unapparent (Finzsch et al., 2008).

In contrast to Sox9 protein levels, mRNA levels were unchanged in the SC of *Sox2*$^{\Delta Sox10}$ *Sox3*$^{\Delta Sox10}$ animals at 18.5 dpc according to qRT-PCR analysis (Fig. 8S) and ISH (Fig. 8T). These results were reproducible in cellular systems. In OLN93 cells, short hairpin RNA (shRNA)-dependent knockdown of Sox2 reduced Sox9 protein (Fig. 8U) but failed to lower *Sox9* mRNA levels (Fig. 8V). If anything, there was even a slight increase in *Sox9* mRNA. Reduced levels of Sox9 protein were also observed in a large proportion of primary oligodendroglial cells after shRNA-dependent knockdown of Sox2 (Fig. 8W). Compared with the control, only 40-45% of cells had

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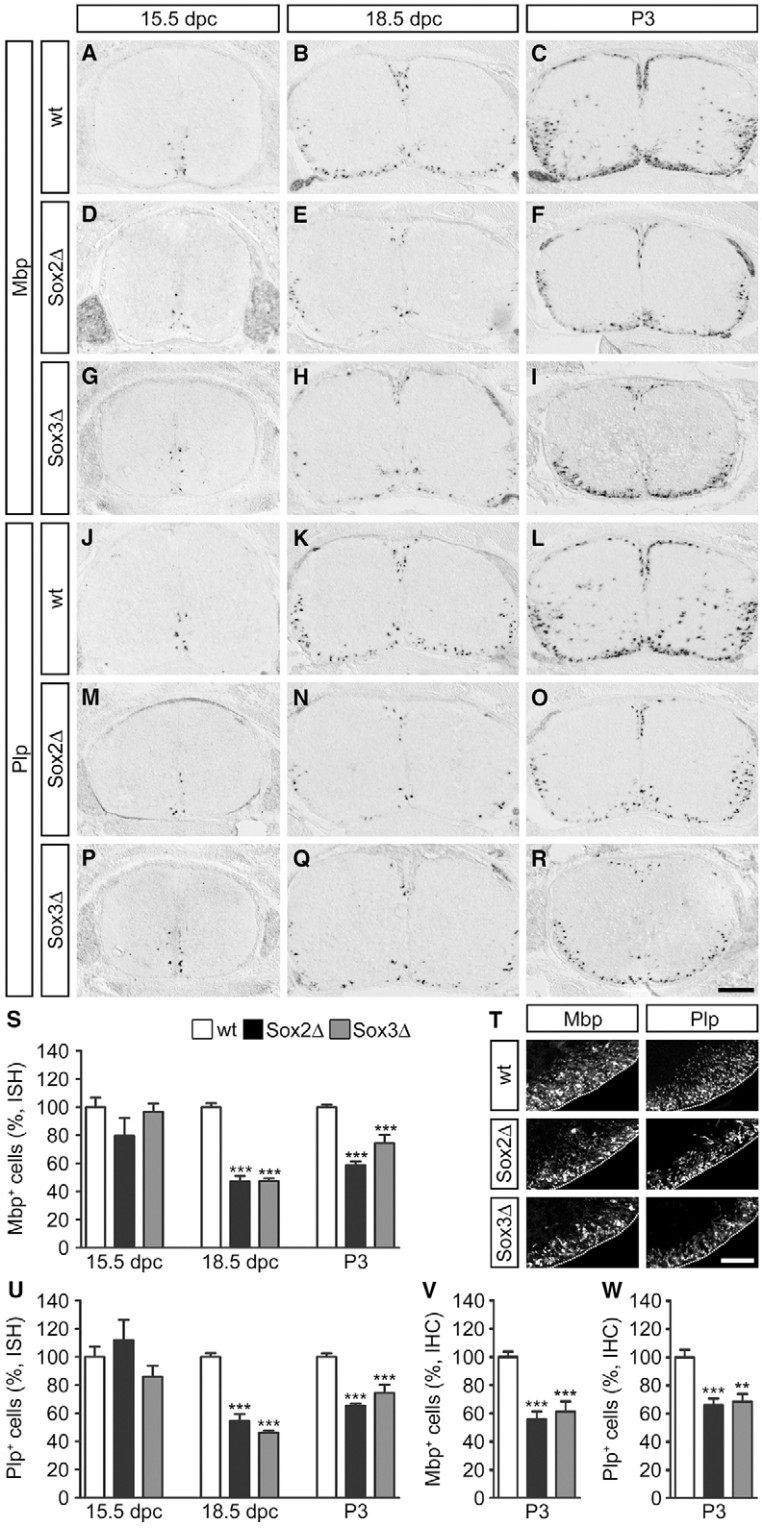

Fig. 5. Myelination is impaired in the absence of Sox2 or Sox3. (A-R) ISH on transverse sections of wild-type (A-C,J-L), *Sox2*$^{\Delta Sox10}$ (D-F,M-O) and *Sox3*$^{\Delta Sox10}$ (G-I,P-R) SC at 15.5 dpc (A,D,G,J,M,P), 18.5 dpc (B,E,H,K,N,Q) and P3 (C,F,I,L,O,R) with probes against *Mbp* (A-I) or *Plp* (J-R). (S,U) Quantification of *Mbp*-positive (S) and *Plp*-positive (U) cells from ISH. (T) Immunohistochemistry on transverse sections of wild-type, *Sox2*$^{\Delta Sox10}$ and *Sox3*$^{\Delta Sox10}$ SC at P3 with antibodies against Mbp or Plp. High magnifications of the white matter region are shown with the right ventral SC boundary marked by a dotted line. (V,W) Quantification of Mbp and Sox10 double-positive (V) and Plp and Sox10 double-positive (W) cells from immunohistochemistry (IHC). Cell numbers are presented as mean percentage ± s.e.m. relative to wild type (100%). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ (Student's *t*-test). Scale bars: 200 µm in A-R; 50 µm in T.

detectable levels of Sox9 protein. This differential impact of Sox2 on Sox9 mRNA and protein points to regulation at the level of mRNA translation rather than transcription. Such post-transcriptional regulation classically involves microRNA.

A database search (TargetScan) revealed that *Sox9* mRNA is potentially targeted by miR145. This microRNA is expressed in cultured primary OPCs and its levels decrease upon differentiation (Fig. 9A). To investigate whether dysregulated miR145 expression may contribute to Sox9 reduction, we overexpressed miR145 in primary oligodendroglial cells. Similar to shRNA-dependent Sox2 knockdown, miR145 led to a substantial reduction of Sox9 protein (Fig. 9B). We also cloned the *Sox9* 3′UTR region with the putative miR145 recognition site behind the luciferase open reading frame in a CMV-driven expression plasmid. In the presence of miR145 expression plasmid, luciferase activity decreased to 57±10% of control levels in HEK293 cells (Fig. 9C). This repressive effect of miR145 was confirmed in OLN93 cells (Fig. 9D). When the miR145 recognition site within the *Sox9* 3′UTR was mutated,

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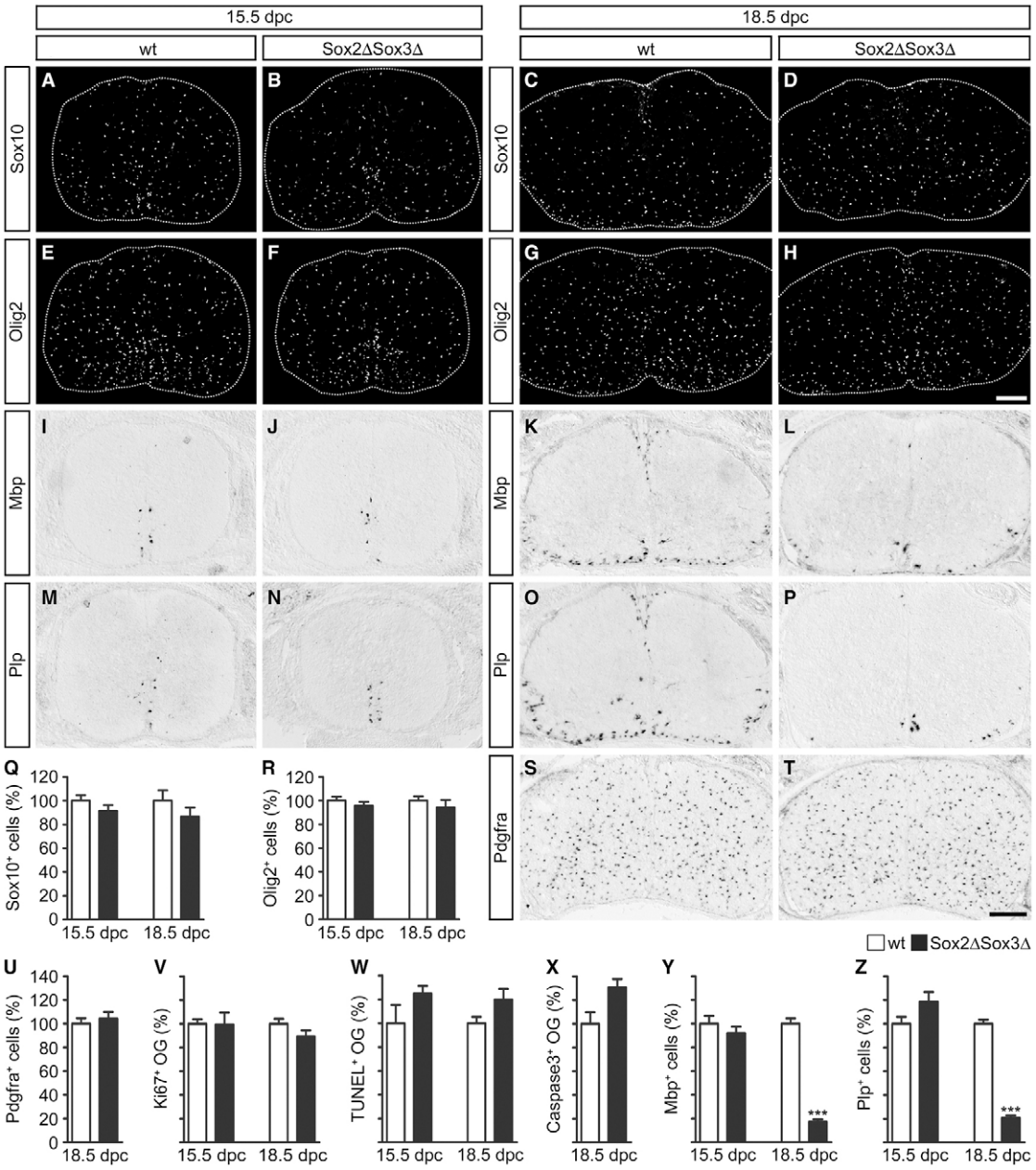

Fig. 6. Sox2 and Sox3 act redundantly during oligodendrocyte terminal differentiation. (A-P,S,T) Immunohistochemistry (A-H) and ISH (I-P,S,T) on transverse sections of wild-type (A,C,E,G,I,K,M,O,S) and *Sox2*$^{\Delta Sox10}$ *Sox3*$^{\Delta Sox10}$ (Sox2ΔSox3Δ) (B,D,F,H,J,L,N,P,T) SC at 15.5 dpc (A,B,E,F,I,J,M,N) and 18.5 dpc (C,D,G,H,K,L,O,P,S,T) with antibodies against Sox10 (A-D) and Olig2 (E-H), or probes against *Mbp* (I-L), *Plp* (M-P) and *Pdgfra* (S,T). Scale bars: 200 µm (in H for A-H, in T for I-P,S,T). (Q,R,U-Z) Quantification of Sox10-positive cells (Q), Olig2-positive cells (R), *Pdgfra*-positive cells (U), Ki67-positive Sox10-expressing oligodendroglia (OG) (V), TUNEL-positive Sox10-expressing oligodendroglia (W), cleaved caspase 3-positive Sox10-expressing oligodendroglia (X), *Mbp*-positive cells (Y) and *Plp*-positive cells (Z) at 15.5 dpc (Q,R,V,W,Y,Z) and 18.5 dpc (Q,R,U-Z). Cell numbers are presented as mean percentage ± s.e.m. relative to wild type (100%). The *y*-axis scale of U is valid for U-Z. ***$P \leq 0.001$ (Student's *t*-test).

miR145 no longer repressed luciferase expression in either cell line (Fig. 9C,D). This argues that recognition of the *Sox9* 3′UTR by miR145 can reduce *Sox9* translation.

Interestingly, Sox2 influenced miR145 levels. Upon Sox2 transfection into OLN93 cells, endogenous miR145 levels decreased (Fig. 9E), whereas transfection of a *Sox2*-specific shRNA led to an induction of miR145 expression (Fig. 9F). miR145 is located in intronic sequences of the *Gm16908* gene (also known as *Mir143hg*), and we analyzed whether Sox2 influences the activity of the *Gm16908* promoter and thereby miR145 levels. In reporter gene assays, the *Gm16908* promoter was 6-fold more active in the absence of Sox2, pointing to an inhibitory function of Sox2 (Fig. 9G). The sixfold activation of the Gm16908 promoter in the absence of Sox2 was substantially higher than the determined 1.8-fold induction of miR145 levels after Sox2 knockdown. Currently, we do not know whether this quantitative difference is biologically relevant or simply caused by the different types of assay employed. Nevertheless, these results link Sox2 to miR145 expression in oligodendroglia and raise the possibility that Sox2 may exert part of its effects on the terminal differentiation of oligodendrocytes through microRNA inhibition.

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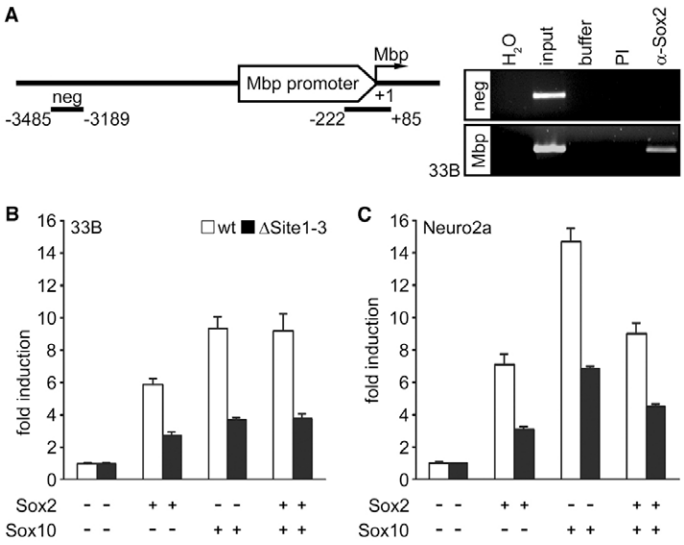

Fig. 7. Sox2 binds and activates the *Mbp* promoter. (A) ChIP was performed on chromatin from 33B cells with antibodies against Sox2 and pre-immune serum (PI) to assess Sox2-dependent enrichment of *Mbp* promoter and control (neg) regions (left) in precipitate over input by PCR (right). Water and buffer served as further controls. (B,C) Luciferase reporter gene assays were performed in 33B (B) or Neuro2a (C) cells. Cells were co-transfected with the *Mbp*-luciferase plasmid in wild-type (wt) or mutant (ΔSite1-3) version and with expression plasmids for Sox2 and Sox10 as indicated beneath. Fold induction ± s.e.m. relative to empty vector is shown.

miR145 negatively regulates the expression of factors required for oligodendrocyte differentiation

MicroRNAs usually have multiple, functionally relevant targets. Considering that miR145 expression is higher in OPCs than in differentiated oligodendrocytes, these targets could be mRNAs of pro-differentiation factors, the translation of which needs to be inhibited in OPCs to prevent precocious maturation. Loss of Sox2 and the ensuing upregulation of miR145 could then interfere with regular oligodendrocyte differentiation by preventing timely induction of these pro-differentiation factors.

In line with such an assumption, myelin regulatory factor (*Myrf*) was predicted as a target for miR145 in a TargetScan search. Gene deletion studies in mouse have shown that Myrf is of vital importance for the differentiation of oligodendroglia (Emery et al., 2009). Myrf expression was also strongly reduced in $Sox2^{\Delta Sox10}$ $Sox3^{\Delta Sox10}$ SC, with numbers of Myrf and Sox10 double-positive oligodendrocytes in the marginal zone reduced to 50±4% of wild-type levels (Fig. 10A,B). A similar reduction was observed in primary oligodendroglial cells treated with a *Sox2*-specific shRNA and then cultured under differentiating conditions for 3 days (Fig. 10C). Compared with the control, only 15-20% of the cells had detectable levels of Myrf protein. Overexpression of miR145 under identical conditions also led to a substantial reduction of Myrf protein and comparable changes in cell morphology as signs of impaired differentiation (Fig. 10C). In reporter gene assays with fusions between luciferase and *Myrf* 3′UTR sequences, miR145 decreased luciferase activity to 54±5% of control levels, confirming a direct inhibitory effect of miR145 (Fig. 10D).

Another factor recently implicated in myelination is mediator complex subunit 12 (Med12) (Vogl et al., 2013). This factor was also identified as a potential target of miR145. In this case, the amount of luciferase produced from a fusion between the luciferase open reading frame and the *Med12* 3′UTR was reduced to 66±4% in the presence of miR145 (Fig. 10E). miR145 was also able to repress luciferase production via the 3′UTR of *Sox2* (Fig. 10F), in accord with a recent report of miR145 function in glioblastoma cells (Fang et al., 2011), but did not affect luciferase production via the 3′UTR of *Sox10* (Fig. 10G). These results suggest that miR145 interferes with at least three other factors that occur in oligodendrocytes, of which two are furthermore required for oligodendrocyte differentiation.

DISCUSSION

SoxB1 proteins, especially Sox2, are best known for their function as pluripotency and stemness factors and for maintenance of the undifferentiated precursor state (Graham et al., 2003; Takahashi and Yamanaka, 2006; Masui et al., 2007). Their presence usually inhibits precocious differentiation (Bylund et al., 2003) and exerts a positive influence on proliferation by preventing cells from premature cell cycle exit (Graham et al., 2003; Ferri et al., 2004). In the context of neural development, SoxB1 proteins have primarily been studied in NPCs as they develop into neurons. However, NPCs also give rise to glia. The present study shows that, in contrast to their downregulation in most neurons (Bylund et al., 2003; Graham et al., 2003), SoxB1 proteins continue to be expressed after specification in both astrocyte and oligodendrocyte lineages throughout embryonic development. Whereas astrocytes express Sox1, Sox2 and Sox3, oligodendroglial cells do not contain Sox1 and exclusively rely on Sox2 and Sox3. There expression patterns have been determined in SC and confirmed on forebrain tissue. All other studies have exclusively been performed on SC tissue.

Despite strong expression in OPCs, neither Sox2 nor Sox3 seems to have any obvious function in these cells. However, fewer cells initiated myelin gene expression in mice that lacked either Sox2 or Sox3. The effect was especially strong in the combined absence of Sox2 and Sox3. Considering that induction of myelin gene expression is a reliable indicator of terminal differentiation in oligodendrocytes, we conclude that the two SoxB1 proteins function as differentiation factors in these cells. This is compatible with their expression well into the early phases of oligodendrocyte differentiation. Such a function is not completely unexpected. In some subsets of neurons, SoxB1 proteins are known to be continuously expressed or to reappear after their initial downregulation. Several observations on SoxB1 mouse mutants have indicated that these proteins might participate in the differentiation of select neuron subtypes (Ekonomou et al., 2005; Taranova et al., 2006; Cavallaro et al., 2008). However, evidence for a role in differentiation remained circumstantial. Our study is thus one of the first to directly show such a function.

SoxB1 proteins act in a redundant manner during oligodendrocyte differentiation. This is obvious from the fact that the full myelination defect becomes apparent only after deletion of both Sox2 and Sox3. Such functional redundancy had already been observed for SoxB1 proteins in NPCs, as overexpression of Sox1, Sox2 or Sox3 in the chicken neural tube was equally efficient in preventing neuronal specification (Bylund et al., 2003; Graham et al., 2003).

Among Sox proteins, functional redundancy during oligodendrocyte development is not restricted to SoxB1 factors and has previously been observed for SoxE and SoxD proteins (Stolt et al., 2003; Stolt et al., 2004; Stolt et al., 2006; Finzsch et al., 2008). Usually, functional redundancy of Sox proteins is restricted to members of a specific subgroup. In particular, it has not been described between the SoxB1 factors Sox2 and Sox3 and the SoxE protein Sox9. Furthermore, Sox9 is largely redundant with Sox10, which is unaltered in the absence of Sox2 and Sox3. Therefore, we

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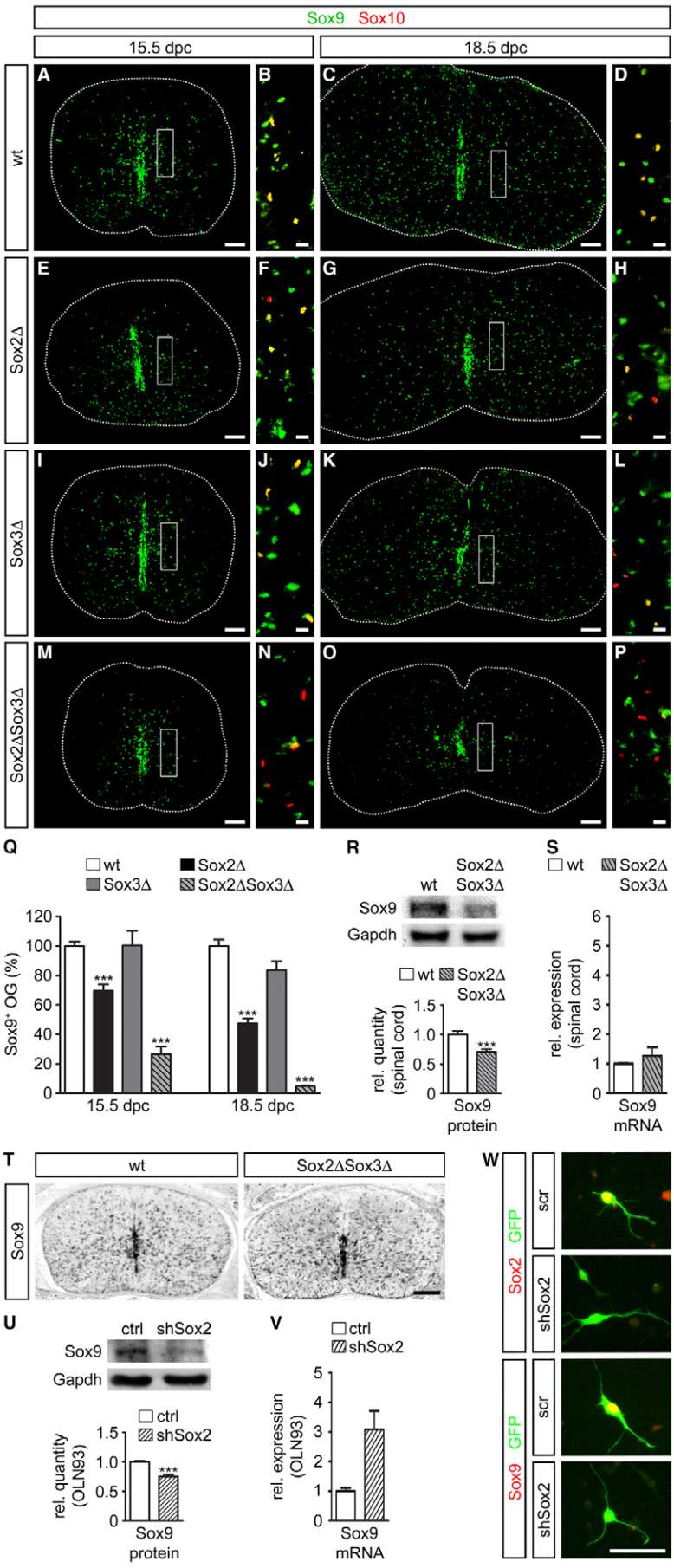

Fig. 8. Sox9 is post-transcriptionally downregulated in oligodendrocytes. (A-P) Co-immunohistochemistry on transverse sections of wild-type (A-D), *Sox2*$^{\Delta Sox10}$ (E-H), *Sox3*$^{\Delta Sox10}$ (I-L) and *Sox2*$^{\Delta Sox10}$ *Sox3*$^{\Delta Sox10}$ (M-P) SC at 15.5 dpc (A,B,E,F,I,J,M,N) and 18.5 dpc (C,D,G,H,K,L,O,P) with antibodies against Sox9 (A-P) (green) and Sox10 (B,D,F,H,J,L,N,P) (red). Higher magnifications of framed areas from A,C,E,G,I,K,M,O are shown in B,D,F,H,J,L,N,P. (Q) Quantification of Sox9 and Sox10 double-positive oligodendroglia (OG). Cell numbers are presented as mean percentage ± s.e.m. relative to wild type (100%). (R) Sox9 protein levels were determined by western blot on extracts of SC at 18.5 dpc. Quantity ± s.e.m. is shown relative to Gapdh with wild-type values set to 1. (S) *Sox9* mRNA levels were determined by qPCR on cDNA of SC at 18.5 dpc. Expression ± s.e.m. is shown relative to *Rpl8* with wild-type values set to 1. (T) ISH on transverse SC sections at 18.5 dpc with probes against *Sox9*. (U) Sox9 protein levels determined by western blot on extracts from control (ctrl) or *Sox2* shRNA-treated (shSox2) OLN93 cells. Quantity ± s.e.m. is shown relative to Gapdh with control values set to 1. (V) *Sox9* mRNA levels were determined by qPCR on cDNA from control or *Sox2* shRNA-treated OLN93 cells. Expression ± s.e.m. is shown relative to *Rpl8* with control values set to 1. (W) Immunocytochemistry with antibodies against Sox2 or Sox9 (red) on primary OPCs nucleofected with scrambled (scr) or *Sox2*-specific shRNA (shSox2) expression plasmid. Electroporated cells were identified by GFP expression (green). ***$P \leq 0.001$ (Student's *t*-test). Scale bars: 200 µm in T; 100 µm in A,C,E,G,I,K,M,O; 50 µm in W; 10 µm in B,D,F,H,J,L,N,P.

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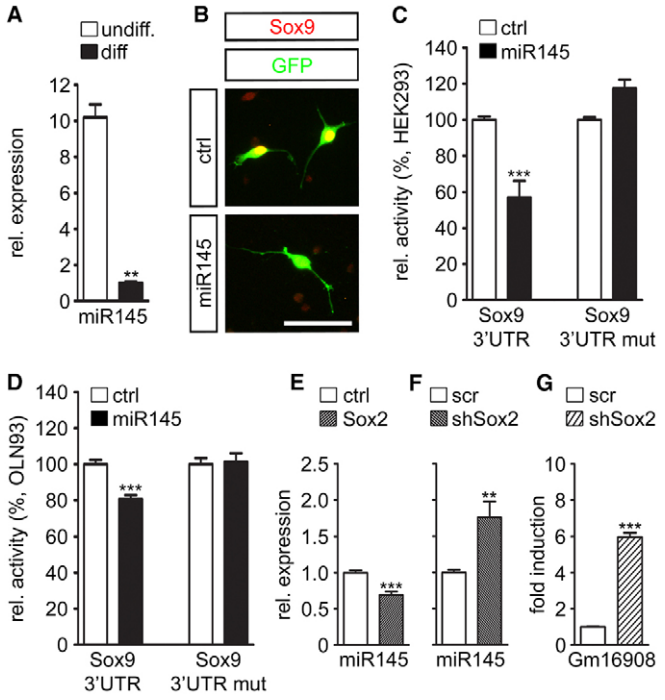

Fig. 9. Cross-regulation of Sox2, miR145 and Sox9. (A) Endogenous expression of miR145 was assessed by qPCR on cDNA from undifferentiated and differentiated primary rat oligodendroglia. Expression ± s.e.m. is shown relative to U6 RNA as control, with values of differentiated cells set to 1. (B) Immunocytochemistry with antibodies against Sox9 (red) on primary OPCs electroporated with control (ctrl) or miR145 expression plasmid. Electroporated cells were identified by GFP expression (green). Scale bar: 50 µm. (C,D) For reporter gene assays, HEK293 (C) or OLN93 (D) cells were co-transfected with a fusion between luciferase and the *Sox9* 3′UTR in wild-type or mutant version and control (ctrl) or miR145 expression plasmid. Luciferase activity ± s.e.m. is shown relative to control (100%). (E,F) Endogenous expression of miR145 was assessed by qPCR on cDNA from transfected OLN93 cells overexpressing Sox2 (E) or depleted of endogenous Sox2 by shRNA (F). Expression ± s.e.m. is shown relative to U6 RNA with values of the respective controls set to 1. Numbers on the *y*-axis of E are also valid for F. (G) For reporter gene assays, OLN93 cells were co-transfected with a *Gm16908*-luciferase reporter and scrambled (scr) or *Sox2*-specific shRNA (shSox2) expression plasmids. Fold induction ± s.e.m. is shown relative to the scrambled control which was set to 1. ***P*≤0.01, ****P*≤0.001 (Student's *t*-test).

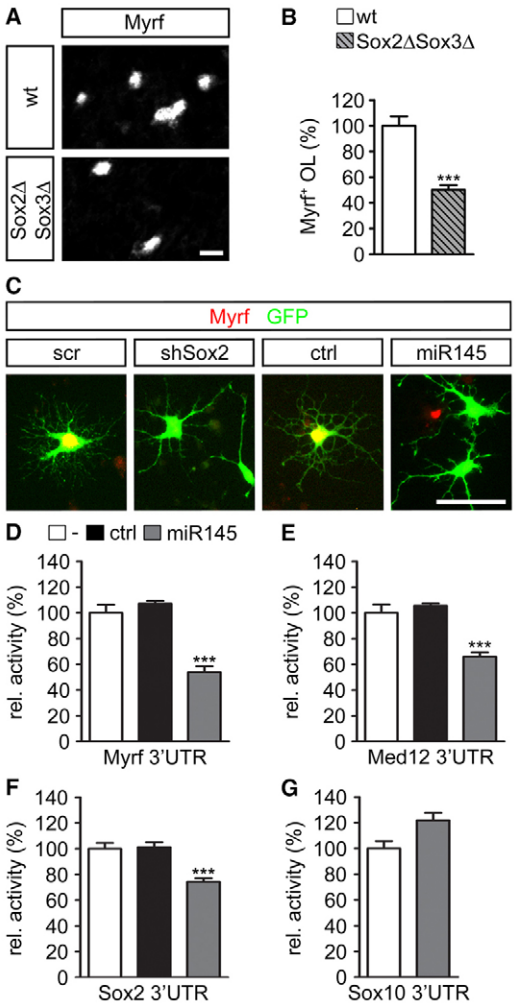

Fig. 10. Target genes of miR145. (A) Immunohistochemistry on transverse SC sections at 18.5 dpc with antibodies against Myrf. High magnifications of the white matter region are shown. (B) Quantification of Myrf-positive Sox10-expressing oligodendrocytes at 18.5 dpc. Cell numbers are presented as mean percentage ± s.e.m. relative to wild type (100%). (C) Immunocytochemistry with antibodies against Myrf (red) on primary OPCs nucleofected with expression plasmids for scrambled (scr) or *Sox2*-specific shRNA (shSox2), control (ctrl) or miR145. Electroporated cells were identified by GFP expression (green). (D-G) For reporter gene assays, HEK293 cells were co-transfected with fusions between luciferase and 3′UTR sequences of *Myrf* (D), *Med12* (E), *Sox2* (F) or *Sox10* (G) and empty (–), control (ctrl) or miR145 expression plasmids. Luciferase activity ± s.e.m. is shown relative to that of the empty expression vector (100%). ****P*≤0.001 (Student's *t*-test). Scale bars: 10 µm in A; 50 µm in C.

consider it unlikely that functional redundancy between Sox2 and Sox3 on the one hand and Sox9 on the other is a major cause of the phenotype, but cannot completely rule out such a possibility.

With so many different Sox proteins expressed during oligodendroglial development it is important to determine how SoxB1 proteins fit in and how they perform their function. Considering that Sox2 and Sox3 have a positive influence on oligodendrocyte differentiation, one option is that these proteins directly activate genes that are associated with the differentiated state, such as myelin genes. Indeed, we were able to show that Sox2 is bound to the promoter region of the *Mbp* gene *in vivo* and can activate this promoter in cell culture. However, it has to be taken into account that the same *Mbp* promoter is bound and activated by other Sox proteins, such as Sox10 or Sox8 (Stolt et al., 2002; Stolt et al., 2004). Transfection data furthermore mark Sox2 as a much weaker activator of the *Mbp* promoter than Sox10. Considering that Sox10 is still expressed normally in differentiating oligodendrocytes after deletion of both Sox2 and Sox3, it appears unlikely that SoxB1 proteins primarily function as direct activators of myelin gene expression.

The expression of myelin genes and of SoxB1 factors overlap for only a short time in the early phases of oligodendrocyte differentiation, such that SoxB1 transcription factors can, at most, only be responsible for the induction and early phase of expression of *Mbp* and other myelin genes. SoxB1 proteins have previously been shown to function as pioneer factors during neuronal development and to keep the chromatin of neuronal differentiation genes in a poised state in NPCs so that they can be activated once cells commit to a neuronal fate (Bergsland et al., 2011; Wegner, 2011). Therefore, it is attractive to speculate that SoxB1 proteins function similarly in the oligodendroglial lineage by keeping the

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chromatin of myelin and other terminal differentiation genes in a poised state as a prerequisite for subsequent Sox10-dependent activation.

Our current study does not address this issue. Instead, it provides evidence for a link between Sox2 and a microRNA as part of its action. There is evidence that microRNA function is essential for oligodendrocyte development. Cell type-specific deletion of the microRNA-processing enzyme Dicer, for instance, leads to severe myelination defects in the corresponding mouse mutant (Dugas et al., 2010; Zhao et al., 2010). Multiple microRNAs have furthermore been reported to be differentially expressed during oligodendrocyte development (Emery, 2010; Letzen et al., 2010).

Here we show that Sox2 influences miR145 levels in oligodendrocytes at least in part by reducing expression of the miR145-harboring *Gm16908* gene. This complements previous findings in glioblastoma cells showing that Sox2 depletion results in miR145 upregulation (Fang et al., 2011). In our mice, loss of Sox2 leads to an increase in miR145 levels, which in turn reduces Sox9 protein levels. Oligodendroglia are thus the third cell type after chondrocytes and melanocytes in which miR145 has been shown to influence Sox9 levels (Yang et al., 2011; Dynoodt et al., 2013). However, absence of Sox9 cannot explain the differentiation defect in SoxB1-deficient oligodendrocytes (Finzsch et al., 2008). This prompted us to search for other potential miR145 targets of relevance for oligodendrocyte differentiation. The transcription factor Myrf and the mediator subunit Med12 constitute two such targets (Emery et al., 2009; Vogl et al., 2013).

It has previously been shown that miR145 is present in oligodendroglial cells and that its abundance decreases during the differentiation of ESC-derived oligodendrocytes (Letzen et al., 2010). In agreement with these data, we find higher miR145 levels in undifferentiated than in differentiated oligodendroglia. This argues that miR145 normally functions in OPCs by preventing precocious translation of pro-differentiation factors such as Myrf, Med12 and possibly others. miR145 thus works in the opposite manner to miR219 and miR338, which are induced concurrent with oligodendrocyte differentiation and target factors that maintain OPCs in the undifferentiated state (Dugas et al., 2010; Zhao et al., 2010). Our results thus provide evidence that microRNAs have pro-differentiating functions as well as anti-differentiating functions during oligodendrocyte development.

We propose that the loss of SoxB1 factors leads to increased miR145 levels in OPCs, which in turn prevent the timely translation of factors that are required for the terminal differentiation of oligodendrocytes. We do not know at present whether SoxB1 proteins also influence the expression of other microRNAs, but consider this an attractive possibility to be studied in future experiments.

MATERIALS AND METHODS

Plasmids

Expression plasmids for rat Sox10 and Sox2 were based on pCMV5 (Kuhlbrodt et al., 1998), and luciferase plasmids with the wild-type or mutant *Mbp* promoter fragment were derived from pGL2 (Promega) as described (Stolt et al., 2002). Similarly constructed from pGL2 was the reporter plasmid in which luciferase was placed under control of the mouse *Gm16908* promoter fragment (−464 to +12, relative to the transcription start site). Further reporter plasmids were based on pBI-EGFP (Clontech) and contained the 3′UTR of *Sox2*, *Sox10*, *Sox9*, *Myrf* or *Med12* generated by PCR and inserted immediately behind the luciferase open reading frame. A mutated version of the *Sox9* 3′UTR was also inserted, with the potential miR145 recognition site changed from 5′-AACUGGAA-3′ to 5′-AAGGTACC-3′ using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). pSuper.neo/gfp-based plasmids (OligoEngine) were used for knockdown experiments. The *Sox2*-specific shRNA was generated using 5′-GCTCGCAGACCTACATGAA-3′ as target sequence. For miR145 overexpression, mouse *Mir145* was amplified by PCR from genomic DNA with 200-300 bp flanking sequences and cloned into the pCAGGS-IRES-EGFP expression plasmid (Cossais et al., 2010). The corresponding control plasmid contained an unrelated sequence. For primer sequences see supplementary material Table S1.

Generation of mice, genotyping and tissue preparation

Mice carrying the floxed *Sox2* (Smith et al., 2009) or floxed *Sox3* (Rizzoti et al., 2004) alleles were crossbred with mice expressing Cre recombinase from a *Sox10::Cre* BAC transgene (Matsuoka et al., 2005). Genotyping was performed as described (Rizzoti et al., 2004; Matsuoka et al., 2005; Smith et al., 2009). For histology, tissue was fixed in 4% paraformaldehyde, transferred to 30% sucrose and frozen in Tissue Freezing Medium (Leica). SC from 18.5 dpc mice was homogenized in TRIzol reagent (Life Technologies) for RNA extraction or fresh frozen for protein extract preparation.

Immunochemistry, TUNEL, ISH and quantification of cell number

For immunochemistry, 10 µm cryotome sections from the forelimb level or plated cells were stained (Stolt et al., 2003) with primary antibodies against Sox2 [rabbit antiserum; 1:1000 (Thein et al., 2010)], Sox3 (rabbit antiserum; 1:500; gift of T. Edlund, Umea University, Sweden), Sox1 (rabbit antiserum; 1:1000; gift of S. Wilson, Columbia University, New York), Sox9 [rabbit antiserum; 1:2000 (Stolt et al., 2003)], Olig2 (rabbit antiserum; 1:1000; Chemicon, AB9610), Pdgfra (rabbit antiserum; 1:300; Santa Cruz, sc-338), Myrf [rabbit antiserum; 1:1000 (Hornig et al., 2013)], Mbp (mouse monoclonal; 1:100; Boehringer Mannheim, 1118099), Plp (rabbit antiserum; 1:400; gift of K.-A. Nave, Max Planck Institute, Göttingen), cleaved caspase 3 (rabbit antiserum; 1:200; Cell Signaling, 9661), Ki67 (rabbit monoclonal; 1:500; Neomarkers, RM-9106), NeuN (mouse monoclonal; 1:500; Chemicon, MAB377), Gfap (mouse monoclonal; 1:100; Chemicon, MAB3402), GlnS (mouse monoclonal; 1:1000; BD Transduction Laboratories, 610518), NG2 (rat monoclonal; 1:100; gift of J. Trotter, University of Mainz, Germany), Sox10 [guinea pig antiserum; 1:1000 (Maka et al., 2005)] and Sox9 [guinea pig antiserum; 1:500 (Stolt et al., 2003)]. For co-immunohistochemistry of Sox2 or Sox3 with Pdgfra or Olig2, Sox2 or Sox3 antibody treatment was followed by incubation with goat anti-rabbit IgG Fab fragment (1:100; Dianova, 111-007-003) before addition of the Pdgfra or Olig2 antibody. Secondary antibodies conjugated to Alexa Fluor 488 (Molecular Probes) or Cy3 immunofluorescent dyes (Dianova) were used for detection.

TUNEL assay was performed according to the manufacturer's protocol (Chemicon, S7165). Samples were documented on a Leica DMI 6000B inverted microscope equipped with a Leica DFC 360FX camera using Leica LAS-AF acquisition software. ISH was performed on 14 µm transverse cryotome sections from the forelimb level with DIG-labeled antisense riboprobes for *Mbp*, *Plp*, *Pdgfra* and *Sox9* (Stolt et al., 2002). Samples were documented on a Leica MZFLIII stereomicroscope equipped with a Plan-APO objective and a Zeiss AxioCam MRc camera using Zeiss AxioVision 4.7 acquisition software. Image processing was performed using Adobe Photoshop 7.0 software. For most images from immunohistochemical stainings, SCs were placed on a black background.

Cell culture, nucleofection, transient transfection, luciferase assays, FACS and RNA preparation

Rat 33B oligodendroglioma cells, rat OLN93 oligodendroglial cells, mouse Neuro2a neuroblastoma cells and HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum. Rat primary oligodendroglia were grown in Sato proliferation or differentiation medium (Bottenstein and Sato, 1979) and nucleofected with 2.5 µg plasmid DNA using Amaxa program O-017 (Lonza). Nucleofected cells were identified by co-electroporated GFP (one-tenth the amount of total DNA). For immunocytochemistry, cells were fixed in 4% paraformaldehyde.

For reporter assays with *Mbp*-luciferase plasmids, 33B and Neuro2a cells were transfected in triplicate with polyethylenimine (PEI) (Sigma) or Superfect reagent (Qiagen) using 1.0 µg luciferase plasmid per 35-mm plate and a total of 0.5 µg pCMV5-based expression plasmids (total maintained by adding empty vector). For reporter assays on luciferase fusions with 3′UTRs, HEK293 or OLN93 cells were transfected in triplicate with PEI. Amounts per 35-mm plate were 0.2 µg luciferase plasmid and 1.5-3.5 µg pCAGGS-IRES-EGFP-based expression plasmid. For reporter assays with *Gm16908*-luciferase plasmids, OLN93 cells were transfected in duplicate with PEI using 0.25 µg luciferase plasmid and 1.0 µg pSuper.neo/gfp-based plasmid per 35-mm plate. For all reporter assays, luciferase activities were measured 48 hours post-transfection and each transfection was performed at least three times.

For protein extract preparation, OLN93 cells were transfected with X-fect Reagent (Clontech) and harvested 72 hours post-transfection. For RNA extraction, OLN93 cells were transfected with Superfect reagent (Qiagen) and separated 48 hours post-transfection in a fluorescence activated cell sorter (FACS Aria II SORP, BD Biosciences) using GFP fluorescence. From the sorted fraction, RNA was extracted using TRIzol reagent or the miRNeasy Mini Kit (Qiagen).

Quantification and statistical analysis

For quantification of cell numbers, at least four SC sections from three to five independent embryos of each genotype were counted. In the case of cytoplasmic stainings (such as GlnS and Gfap), only signals immediately surrounding a DAPI-positive nucleus were included in the quantification. All statistical analysis was performed with Prism5 software (GraphPad). Statistically significant differences were determined by Student's *t*-test.

Western blotting

SC and OLN93 protein extracts were size fractionated on SDS-PAGE gels. Endogenous expression of Sox9 and Gapdh was verified by western blotting using anti-Sox9 rabbit antiserum [1:15,000 (Stolt et al., 2003)] or anti-Gapdh rabbit antiserum (1:3000; Santa Cruz, sc-25778) and horseradish peroxidase-coupled protein A. For detection, SuperSignal West Femto Chemiluminescent Substrate was applied (Thermo Scientific). Densitometric measurements were performed with ImageJ software (NIH).

Quantitative RT-PCR

Total RNA from mouse SC or OLN93 cells was reverse transcribed and used to analyze *Sox9* expression levels by quantitative PCR (qPCR) on a Bio-Rad CFX96 Real-Time PCR System (primer sequences are listed in supplementary material Table S1). Transcript levels were normalized to *Rpl8*. For miR145 quantification, mature miR145 and U6 RNA were reverse transcribed with specific primers for cDNA synthesis (Life Technologies, Ambion) and quantified by qPCR with TaqMan probes (Life Technologies, Ambion). miR145 levels were normalized to U6 spliceosomal RNA levels.

ChIP

ChIP assays were performed as described (Küspert et al., 2011) on 33B cells transfected with pCMV5-Sox2 using a polyclonal anti-Sox2 rabbit antiserum and control pre-immune serum. DNA from input and precipitated chromatin was probed by PCR for the presence of *Mbp* regions (for primer sequences see supplementary material Table S1). Two independent biological samples were analyzed in triplicate for each probe.

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.A.H., M.W. and S.R. conceived and designed the experiments. S.A.H., D.H., M.K. and S.R. performed the experiments. S.A.H., D.H., M.K., M.W. and S.R. analyzed the data. R.A.L. and R.L.-B. contributed reagents/materials/analysis tools. M.W. and S.R. wrote the paper.

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Supplementary material

Supplementary material available online at
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