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Sox9 and Sox10 influence survival and migration of oligodendrocyte precursors in the spinal cord by regulating PDGF receptor α expression

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Specification of the myelin-forming oligodendrocytes of the central nervous system requires the Sox9 transcription factor, whereas terminal differentiation depends on the closely related Sox10. Between specification and terminal differentiation, Sox9 and Sox10 are co-expressed in oligodendrocyte precursors and are believed to exert additional functions. To identify such functions, we have deleted Sox9 specifically in already specified oligodendrocyte precursors of the spinal cord. In the absence of Sox9, oligodendrocyte precursors developed normally and started terminal differentiation on schedule. However, when Sox10 was additionally deleted, oligodendrocyte precursors exhibited an altered migration pattern and were present in reduced numbers because of increased apoptosis rates. Remaining precursors continued to express many characteristic oligodendroglial markers. Aberrant expression of astrocytic and neuronal markers was not observed. Strikingly, we failed to detect PDGF receptor α expression in the mutant oligodendrocyte precursors, arguing that PDGF receptor α is under transcriptional control of Sox9 and Sox10. Altered PDGF receptor α expression is furthermore sufficient to explain the observed phenotype, as PDGF is both an important survival factor and migratory cue for oligodendrocyte precursors. We thus conclude that Sox9 and Sox10 are required in a functionally redundant manner in oligodendrocyte precursors for PDGF-dependent survival and migration.

KEY WORDS: Sry, High-mobility group, Redundancy, SoxE, PDGF, Glia, Transgenic mice

INTRODUCTION

Rapid saltatory conductance in the vertebrate nervous system relies on the presence of myelinating glia and the formation of myelin sheaths. In the central nervous system (CNS), myelination is carried out by oligodendrocytes. These oligodendrocytes are generated at defined times and positions in the developing CNS (Richardson et al., 2006). In the developing mouse spinal cord, the vast majority of oligodendrocytes arises as proliferative precursor cells from multipotent neuroepithelial cells of the pMN domain in the ventral part of the ventricular zone around 12 days post coitum (dpc). Oligodendrocyte precursors (OLPs) then migrate to colonize the spinal cord and reach a particularly high density in the white matter region where they start to undergo terminal differentiation at the end of embryogenesis (Rowitch, 2004).

Several transcription factors have been identified that regulate different steps of oligodendrocyte development including bHLH proteins of the Olig family, homeodomain proteins of the Nkx family, the zinc-finger protein Zfp488 and HMG-domain proteins of the Sox family (Rowitch et al., 2002; Sohn et al., 2006; Wang et al., 2006; Wegner, 2001; Wegner and Stolt, 2005).

Among Sox proteins, two highly related group E (SoxE) proteins with pleiotropic roles in development are particularly important. Sox9, which also influences chondrogenesis, male sex determination and neural crest development (Akiyama et al., 2002; Chaboissier et al., 2004; Cheung and Briscoe, 2003), exerts a strong effect on the specification of OLPs (Stolt et al., 2003). Sox10, by contrast, is a major determinant in terminal oligodendrocyte differentiation (Stolt et al., 2002) besides having many functions in the neural crest (Britsch et al., 2001; Herbarth et al., 1998; Southard-Smith et al., 1998). Sox8 as the third SoxE protein is only of minor importance in oligodendrocyte development and appears to support Sox9 and Sox10 in their function (Stolt et al., 2004; Stolt et al., 2005).

We have previously shown that Sox9 continues to be expressed after specification in OLPs, while Sox10 is present in OLPs long before they undergo terminal differentiation. Sox9 and Sox10 thus jointly occur in cells of the oligodendrocyte lineage between these events. Oligodendrocyte development during this period progressed fairly normally in the absence of Sox10 (Stolt et al., 2002). Likewise, the few OLPs that were specified in a Sox9-deficient spinal cord thrived and replenished the oligodendrocyte pool during late embryogenesis (Stolt et al., 2003). We therefore concluded that if Sox9 and Sox10 are important for oligodendrocyte development during the period after specification and before terminal differentiation, they must have largely redundant functions and thus be able to reciprocally compensate each other's loss.

To uncover novel functions for Sox9 and Sox10 in OLPs, we generated mice that were not only deficient for Sox10, but in addition selectively lost Sox9 in OLPs after the specification event. As a result, normal numbers of OLPs were born. However, these cells soon developed aberrantly. This involved altered patterns of precursor cell migration and a decrease in precursor cell numbers that went along with an increased rate of apoptosis and a severe reduction of PDGF receptor α (Pdgfra). We conclude that Sox9 and Sox10 are crucial for migration and survival of OLPs, at least partly through regulating PDGF responsiveness.

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MATERIALS AND METHODS

Animal husbandry, genotyping and BrdU labelling

Mice with two *Sox9*^{loxP} alleles (Akiyama et al., 2002) were maintained as a colony or alternatively bred with mice carrying a *Sox10*^{lacZ} allele (Britsch et al., 2001) or a *Sox10::Cre* transgene (Stolt et al., 2006) on a mixed

C57Bl6J/C3H background. Sox9 deficiency in OLPs was achieved by crossing *Sox9*$^{loxP/loxP}$ mice with *Sox9*$^{loxP/+}$ mice that additionally carried a *Sox10::Cre* transgene. For the generation of embryos with combined deficiency of Sox9 and Sox10 in OLPs, *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/+}$ mice were crossed with *Sox9*$^{loxP/+}$, *Sox10*$^{lacZ/+}$, *Sox10::Cre* mice. Embryos from 12.5 dpc to 18.5 dpc were from staged pregnancies. To obtain *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* embryos beyond 15.5 dpc, drinking water of pregnant female mice was supplemented with 100 μg/ml L-phenylephrine, 100 μg/ml isoproterenol and 2 mg/ml ascorbic acid from 8.5 dpc onwards (Pattyn et al., 2000). This treatment prevented lethality due to developmental defects in the autonomic nervous system of the double mutant unrelated to the analyzed oligodendrocyte phenotype. All embryos older than 15.5 dpc in this study underwent the same treatment. Comparison of treated control, *Sox10*$^{lacZ/lacZ}$ or *Sox9*$^{loxP/loxP}$, *Sox10::Cre* embryos with untreated embryos revealed that the treatment did not influence oligodendrocyte development in these genotypes. Because oligodendrocyte development was identical in *Sox9*$^{+/+}$ and *Sox9*$^{loxP/loxP}$ genotypes, both wild-type and floxed alleles were used in control and *Sox10*$^{lacZ/lacZ}$ embryos. Genotyping was performed by PCR using primers a (5′-GGGGCTTGTCTCCTTCAGAG-3′), b (5′-TGGTAATGAGTCATACACAGTAC-3′), c (5′-ACACAGCATAG-GCTACCTG-3′), d (5′-GTCAAGCGACCCATGAACGC-3′) for *Sox9*; primers e (5′-GAGGTGGGCGTTGGGCTCTT-3′), f (5′-CAGAGCT-TGCCTAGTGTCTT-3′), g (5′-TAAAAATGCGCTCAGGTCAA-3′) for *Sox10*; and primers h (5′-ATGCTGTTTCACTGGTTATG-3′), i (5′-ATTGCCCCTGTTTCACTATC-3′) for the *Sox10::Cre* transgene (Fig. 1A), as described previously (Britsch et al., 2001; Stolt et al., 2003; Stolt et al., 2006). For BrdU labelling, pregnant mice were injected intraperitoneally with 100 μg BrdU (Sigma) per gram body weight 1 hour or 24 hours before embryo preparation depending on the experiment (Stolt et al., 2003).

Preparation of spinal cord cultures and tissue sections, immunohistochemistry, TUNEL and in situ hybridization

For primary cell cultures, spinal cords were dissected from embryos at 18.5 dpc and immediately triturated into single cell suspensions. Equal numbers of cells were seeded for each embryo into 35 mm dishes containing polylysine-coated cover slips in DMEM containing 10% foetal calf serum. After incubation for 3 hours, cells were adherent. For immediate analysis, cultures from wild-type, *Sox10*$^{lacZ/lacZ}$ and *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* embryos underwent fixation in 3% paraformaldehyde. For 48 hour cultures, medium was switched after 3 hours to serum-free DMEM containing N2 supplement, 20 ng/ml bFGF and in some cases 20 ng/ml PDGF-AA (Strathmann Biotec). After fixation of cells and extensive washing, cover slips were processed for immunocytochemistry.

For the preparation of cryotome sections, genotyped, age-matched embryos were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded in OCT compound at –80°C. As previously described, 10 μm sections were used for immunohistochemistry, 14 μm sections for in situ hybridization according to standard protocols (Stolt et al., 2003; Stolt et al., 2002). For better comparison, all spinal cord sections were from the forelimb level. For immunochemistry, the following primary antibodies were used in various combinations: anti-NeuN mouse monoclonal (1:500 dilution, Chemicon), anti-PCNA mouse monoclonal (1:100 dilution, Roche Biochemicals), anti-Nkx2.2 mouse monoclonal (1:400 dilution, Developmental Studies Hybridoma Bank), anti-DCC mouse monoclonal (1:1000 dilution, BD Pharmingen), anti-O4 mouse monoclonal (1:500 dilution, R&D Systems), anti-Sox10 guinea pig antiserum [1:1000 dilution (Maka et al., 2005)], anti-Sox9 guinea pig antiserum [1:500 dilution (Stolt et al., 2003)], anti-Ki67 rabbit monoclonal (1:500 dilution, Neomarkers), affinity-purified anti-Sox9 rabbit antiserum [1:2000 dilution (Stolt et al., 2004)], anti-NG2 rabbit antiserum (1:1000 dilution, gift of J. Trotter, Universität Mainz), anti-Pdgfra rabbit antiserum (1:80 dilution, Neomarkers), anti-Olig2 rabbit antiserum (1:50,000 dilution, gift of D. Rowitch, UCSF, San Francisco), anti-B-FABP rabbit antiserum, (1:10,000 dilution, gift of C. Birchmeier and T. Müller, MDC, Berlin), anti-Glast rabbit antiserum (1:1000 dilution, BD Transduction Laboratories), anti-β-galactosidase rabbit antiserum (1:500 dilution, ICN) or anti-β-galactosidase goat antiserum (1:500 dilution, Biotrend). Detection of immunoreactivity was with secondary antibodies conjugated to Cy2, Cy3 or Alexa Fluor immunofluorescent dyes (Dianova and Molecular Probes). Incorporated BrdU was visualized by an Alexa-488-coupled mouse monoclonal antibody directed against BrdU (Molecular Probes) at a 1:20 dilution. TUNEL assays were performed according to the manufacturer's protocol (Chemicon). In situ hybridization was performed with DIG-labelled antisense riboprobes for *Mbp* (myelin basic protein gene), *Plp* (proteolipid protein gene; *Plp1* – Mouse Genome Informatics) and *Pdgfra* (Stolt et al., 2002). Samples were analyzed and documented using either a Leica TCS SL confocal microscope or a Leica inverted microscope (DMIRB) equipped with a cooled SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI).

Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed as described (Schlierf et al., 2006). Briefly, cellular protein and genomic DNA from freshly prepared spinal cords of 4-day-old mice were crosslinked in 1% formaldehyde before chromatin extraction and sonication to an average fragment length of 300 to 600 bp. Immunoprecipitations were performed overnight at 4°C using polyclonal control IgG or anti-Sox9 IgG from rabbit (Stolt et al., 2003). DNA was purified from precipitates after crosslink reversal and subjected to PCR. For detection of sequence elements within the 5′ flanking region of the *Pdgfra* gene, the following primer pairs were used in 34 cycles of standard PCR using an annealing temperature of 58°C: 5′-TCTGGTTGCCCATGGTGGCT-3′ and 5′-AGCTCAGCCTTCTG-AGTGGC-3′ for positions –7999 to –7721 relative to the start of exon 1 (N1); 5′-ACAGGCGTTGTCTGCCCAAC-3′ and 5′-CTGGTCGTC-CGATTCCCTCT-3′ for positions –4060 to –3781 (N2); 5′-ATT-TGCTTGCCTGCTCCACC-3′ and 5′-CCACAAATGGTAACTTCA-CAGT-3′ for positions –2100 to –1959 (C1); 5′-CCCTCGCTC-CGTGTGTGTG-3′ and 5′-ACCGTGGGGATATCAGGCTC-3′ for positions –1609 to –1443 (C2); 5′-GAAGAGGTCTTGAGCCTGAG-3′ and 5′-CTCCTTCTATGTCAATTTGCAAA-3′ for positions –133 to +67 (C3).

RESULTS

Selective deletion of Sox9 in OLPs of the spinal cord

To analyze potential roles of Sox9 in the oligodendrocyte lineage after the initial specification event, we used a floxed *Sox9* allele (Akiyama et al., 2002) and a *Sox10::Cre* BAC transgene (Kessaris et al., 2006; Stolt et al., 2006) (Fig. 1A). As Sox10 is restricted in the CNS to cells of the oligodendrocyte lineage and starts to be expressed immediately upon oligodendrocyte specification (Stolt et al., 2002), Cre recombinase activity and resulting *Sox9* deletion is predicted to occur selectively in cells of the oligodendrocyte lineage and should commence after specification from ventricular zone cells.

To verify the expected deletion pattern, we analyzed the occurrence of Sox9 in the developing spinal cord by immunohistochemistry (Fig. 1B-M). At 12.5 dpc, Sox9-expressing cells are preferentially localized to the ventricular zone (Stolt et al., 2003). Their overall number was comparable in the spinal cord of wild-type and *Sox9*$^{loxP/loxP}$, *Sox10::Cre* embryos (Fig. 1B,E).

As spinal cord OLPs are predominantly derived from the pMN domain, we focused on this region of the ventral ventricular zone. Again, Sox9 expression was unaltered in pMN domain cells of *Sox9*$^{loxP/loxP}$, *Sox10::Cre* embryos, which are marked by Olig2 expression (Fig. 1C,F). Of the Olig2-expressing cells, few are also marked by Sox10 (Stolt et al., 2003). These newly specified OLPs occurred in normal numbers in *Sox9*$^{loxP/loxP}$, *Sox10::Cre* embryos and still contained Sox9 protein (Fig. 1D,G).

At 15.5 dpc, Sox9-positive cells were not only present in the ventricular zone, but also throughout the mantle zone in a dispersed pattern. When overall number and distribution of Sox9-positive cells in *Sox9*$^{loxP/loxP}$, *Sox10::Cre* embryos were now compared with the wild type, there still was no obvious difference in the ventricular

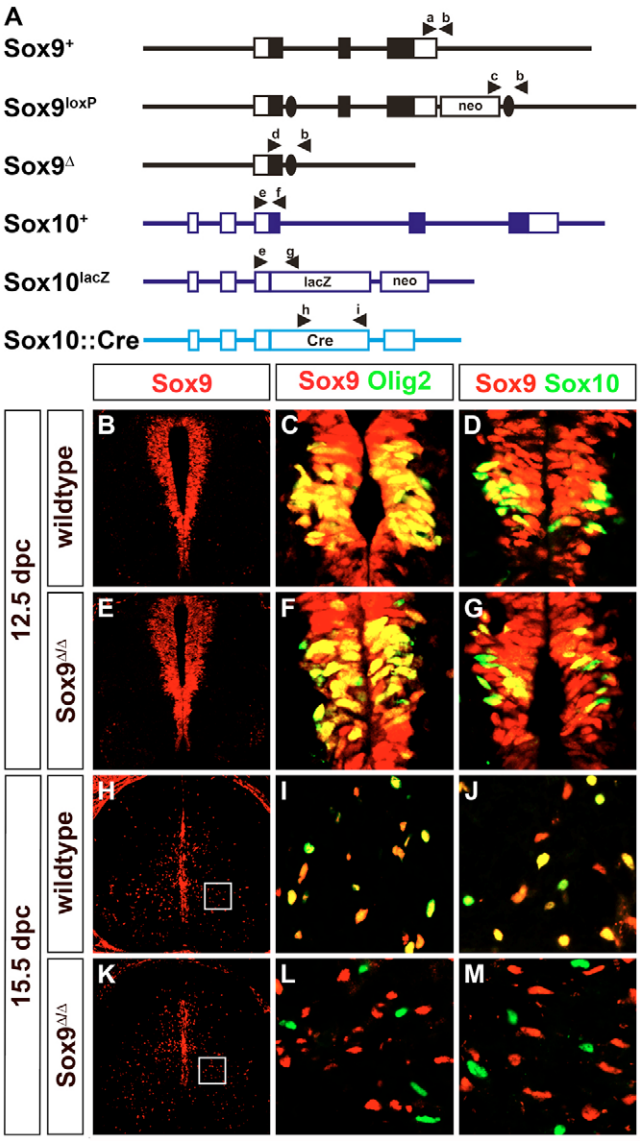

Fig. 1. Sox9 deletion in the oligodendrocyte lineage. (**A**) Schematic representation of *Sox9* and *Sox10* alleles and the *Sox10::Cre* transgene used in this study. Boxes represent exons with translated parts being filled or expression cassettes for neomycin resistance (neo), β-galactosidase (lacZ) and Cre recombinase (Cre). Ellipses indicate positions of loxP sites, arrowheads those of genotyping primers a-i. (**B-M**) Sox9 immunoreactivity (red) was detected at 12.5 dpc (B-G) and 15.5 dpc (H-M) alone (B,E,H,K) or together with Olig2 (C,F,I,L) or Sox10 (D,G,J,M) immunoreactivity (green) in transverse spinal cord sections from the forelimb region of wild-type (B,C,D,H,I,J) or *Sox9*$^{loxP/loxP}$, *Sox10::Cre* (*Sox9*$^{Δ/Δ}$) (E,F,G,K,L,M) embryos. The region from which I,J,L,M were taken is boxed in H and K.

zone. However, Sox9-positive cells in the mantle zone appeared to be slightly reduced in number (Fig. 1H,K). Closer inspection revealed that Sox9 was selectively lost from cells in the mantle zone that express Olig2 and Sox10 and, thus, represent OLPs (compare Fig. 1I,J with Fig. 1L,M). The very few cells that still co-expressed Sox9 and Sox10 were predominantly found in close vicinity to the ventricular zone and thus likely corresponded to newly specified, late-born OLPs (data not shown). We thus conclude that Sox9 is both effectively and selectively lost from OLPs in spinal cords of

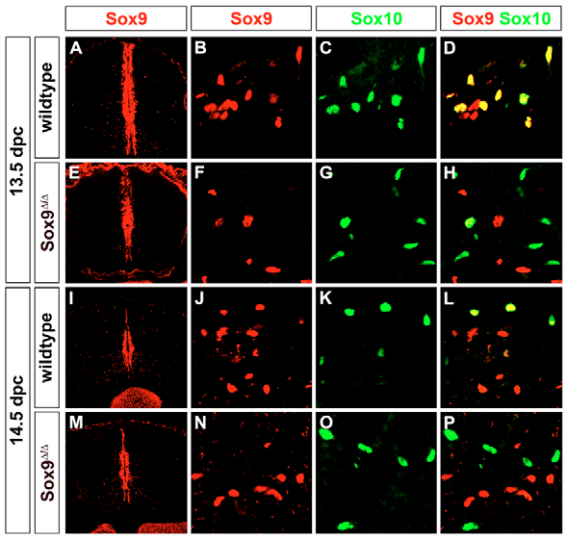

Fig. 2. Progression of Sox9 deletion in *Sox9*$^{loxP/loxP}$, *Sox10::Cre* spinal cords. Sox9 immunoreactivity (red) was detected and compared with Sox10 immunoreactivity (green) at 13.5 dpc (**A-H**) and 14.5 dpc (**I-P**) in transverse spinal cord sections from the forelimb region of wild-type (A-D,I-L) or *Sox9*$^{loxP/loxP}$, *Sox10::Cre* (*Sox9*$^{Δ/Δ}$) (E-H, M-P) embryos. Both low-magnification overviews (A,E,I,M) and high-magnifications (B,C,D,F,G,H,J,K,L,N,O,P) are provided. A,B,E,F,I,J,M,N show the Sox9 staining; C,G,K,O show the Sox10 staining; D,H,L,P represent merged pictures.

Sox9$^{loxP/loxP}$, *Sox10::Cre* embryos by 15.5 dpc. A more detailed analysis revealed that OLPs in the mantle zone were already devoid of Sox9 at 14.5 dpc (Fig. 2I-P). Even at 13.5 dpc, the Sox9-expressing OLPs amounted to a mere 10-15% of the whole OLP population in the mantle zone (Fig. 2A-H).

Normal development of Sox9-deficient OLPs

Using several stage-specific markers, we analyzed oligodendrocyte development in spinal cords of *Sox9*$^{loxP/loxP}$, *Sox10::Cre* embryos after the specification event. Immunohistochemistry with antibodies against Sox10 and Olig2 at 15.5 dpc revealed that in the absence of Sox9 OLPs effectively colonized the mantle zone (compare Fig. 3A,E with Fig. 3B,F). At 15.5 dpc, expression of Pdgfra as an additional OLP marker revealed no significant differences to the wild type (Fig. 3I,J), although Sox9 was no longer co-expressed with Pdgfra in *Sox9*$^{loxP/loxP}$, *Sox10::Cre* spinal cords (Fig. 4A,B,E,F). Proliferation rates and apoptosis were likewise comparable between wild-type and *Sox9*$^{loxP/loxP}$, *Sox10::Cre* spinal cords at 15.5 dpc.

Even at 18.5 dpc, Sox9-deficient OLPs still exhibited normal distribution throughout the spinal cord and were present in normal numbers as evident from Sox10, Olig2 and Pdgfra expression patterns (compare Fig. 3C,G,K with Fig. 3D,H,L). At 18.5 dpc, several OLPs in the marginal zone of the spinal cord furthermore downregulate Pdgfra expression and start to activate myelin gene expression as part of their terminal differentiation program (Stolt et al., 2002). Again, no difference was detected between the *Sox9*$^{flox/flox}$, *Sox10::Cre* and the wild-type spinal cord in the onset of myelin gene expression as evident from in situ hybridization with *Plp*- or *Mbp*-specific probes (Fig. 3M,N and data not shown). We therefore conclude, that in stark contrast to the specification event, Sox9 is dispensable in OLPs for their further timely development during embryogenesis.

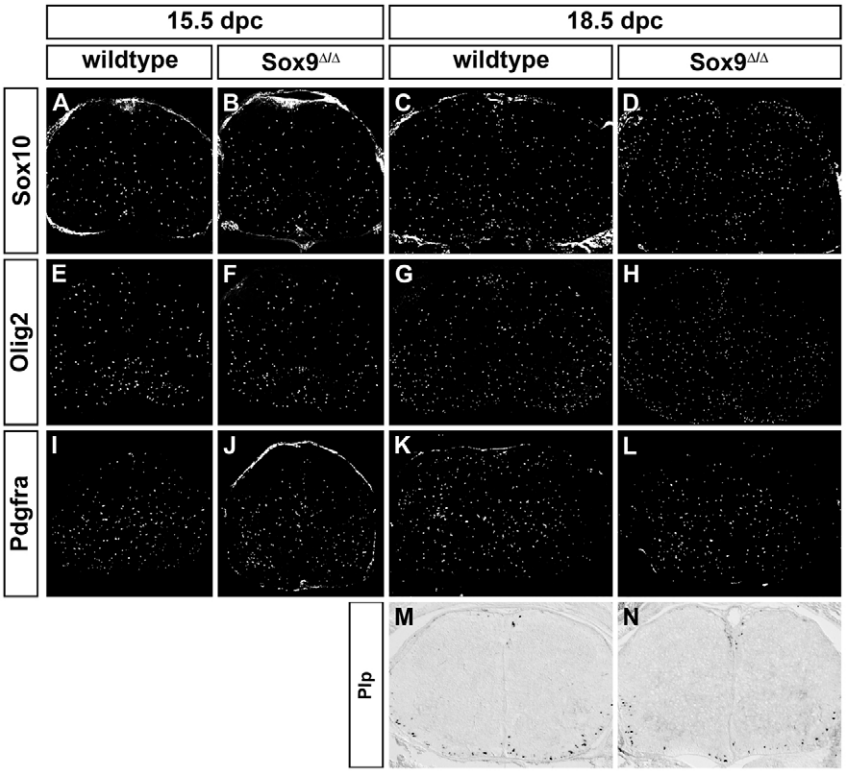

Fig. 3. Oligodendrocyte development in *Sox9$^{loxP/loxP}$, Sox10::Cre* spinal cords. Immunohistochemistry with antibodies specific for Sox10 (**A-D**), Olig2 (**E-H**), Pdgfra (**I-L**) and in situ hybridizations with a *Plp*-specific probe (**M**,**N**) were performed on transverse sections from the forelimb region of wild-type (A,C,E,G,I,K,M) or *Sox9$^{loxP/loxP}$, Sox10::Cre (Sox9$^{Δ/Δ}$)* (B,D,F,H,J,L,N) embryos at 15.5 dpc (A,B,E,F,I,J) and 18.5 dpc (C,D,G,H,K,L,M,N).

Abnormal OLP distribution and reduced number in the combined absence of Sox9 and Sox10

The apparently normal development of Sox9-deficient OLPs could be explained by the ability of Sox10 to compensate for the loss of Sox9. A reciprocal compensatory mechanism has previously been invoked to explain the unaltered development of Sox10-deficient OLPs until they undergo terminal differentiation (Stolt et al., 2002). To clarify whether Sox9 and Sox10 are indeed functionally redundant in OLPs, we generated *Sox9$^{loxP/loxP}$*, *Sox10$^{lacZ/lacZ}$*, *Sox10::Cre* embryos.

At 12.5 dpc, there was no significant difference between *Sox9$^{loxP/loxP}$*, *Sox10$^{lacZ/lacZ}$*, *Sox10::Cre* embryos and wild-type littermates, in accordance with the fact that Sox9 had not yet been deleted from OLPs (Fig. 5A-D). Approximately equal numbers of OLPs were specified from the Olig2-positive pMN domain in *Sox9$^{loxP/loxP}$*, *Sox10$^{lacZ/lacZ}$*, *Sox10::Cre* spinal cords, the single mutants and the wild type, as evident from the comparison of Sox10-positive and β-galactosidase-positive cells in the respective genotypes (Fig. 5U).

At 13.5 dpc, alterations in OLP distribution (Fig. 5E-H), but not in OLP number (Fig. 5V) became evident in the double mutant using either Olig2 or β-galactosidase as a marker. This altered distribution was even more pronounced at 14.5 dpc and 15.5 dpc (Fig. 5I-P). Whereas OLPs spread throughout the mantle zone and became more or less equally distributed throughout the wild-type spinal cord with a slightly lower density in the dorsal than in the ventral half (Fig. 5I,K,M,O), Olig2- and β-galactosidase-positive cells in the *Sox9$^{loxP/loxP}$*, *Sox10$^{lacZ/lacZ}$*, *Sox10::Cre* spinal cord were preferentially localized around the pMN domain and in the ventral region of the spinal cord, as if they had a decreased ability to migrate away from their region of origin (Fig. 5J,L,N,P). In contrast to the situation at 13.5 dpc, OLP numbers were now also reduced in *Sox9$^{loxP/loxP}$*, *Sox10$^{lacZ/lacZ}$*, *Sox10::Cre* embryos. Quantification revealed a 46-62% reduction in β-galactosidase-positive cells and a similar decrease in Olig2-positive cells (Fig. 5V,W and data not shown). By contrast, no statistically significant alteration of OLP numbers was observed in *Sox9$^{loxP/loxP}$*, *Sox10::Cre* embryos or *Sox10$^{lacZ/lacZ}$* littermates, indicating that their reduction is specific to the double mutant (Fig. 5W and data not shown). OLP numbers in *Sox10$^{lacZ/lacZ}$* embryos had previously been reported to be normal in one study (Stolt et al., 2002), but reduced in another (Liu et al., 2007). Although the reason for this discrepancy is not clear at the moment, it could be caused by differences in genetic backgrounds.

Neither OLP numbers nor their distribution recovered to the wild-type situation at 18.5 dpc with Olig2- and β-galactosidase-positive cells still being preferentially localized in the ventral part of the

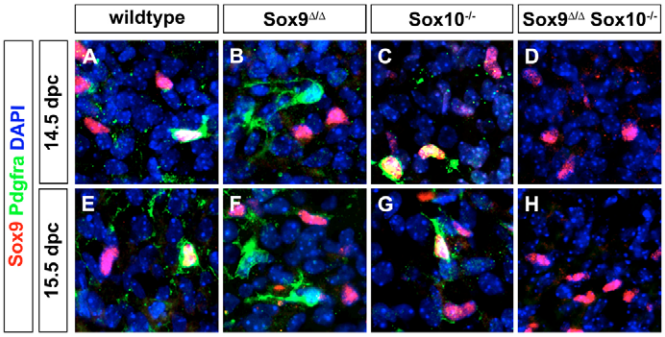

Fig. 4. Correlation of Sox9 and Pdgfra expression. Co-immunohistochemistry was performed on transverse spinal cord sections from the forelimb region of wild-type (A,E), *Sox9$^{loxP/loxP}$, Sox10::Cre (Sox9$^{Δ/Δ}$)* (B,F), *Sox10$^{lacZ/lacZ}$ (Sox10$^{-/-}$)* (C,G) and *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre (Sox9$^{Δ/Δ}$ Sox10$^{-/-}$)* (D,H) embryos at 14.5 dpc (**A-D**) and 15.5 dpc (**E-H**) with antibodies directed against Sox9 (red) and Pdgfra (green). Nuclei were counterstained with DAPI.

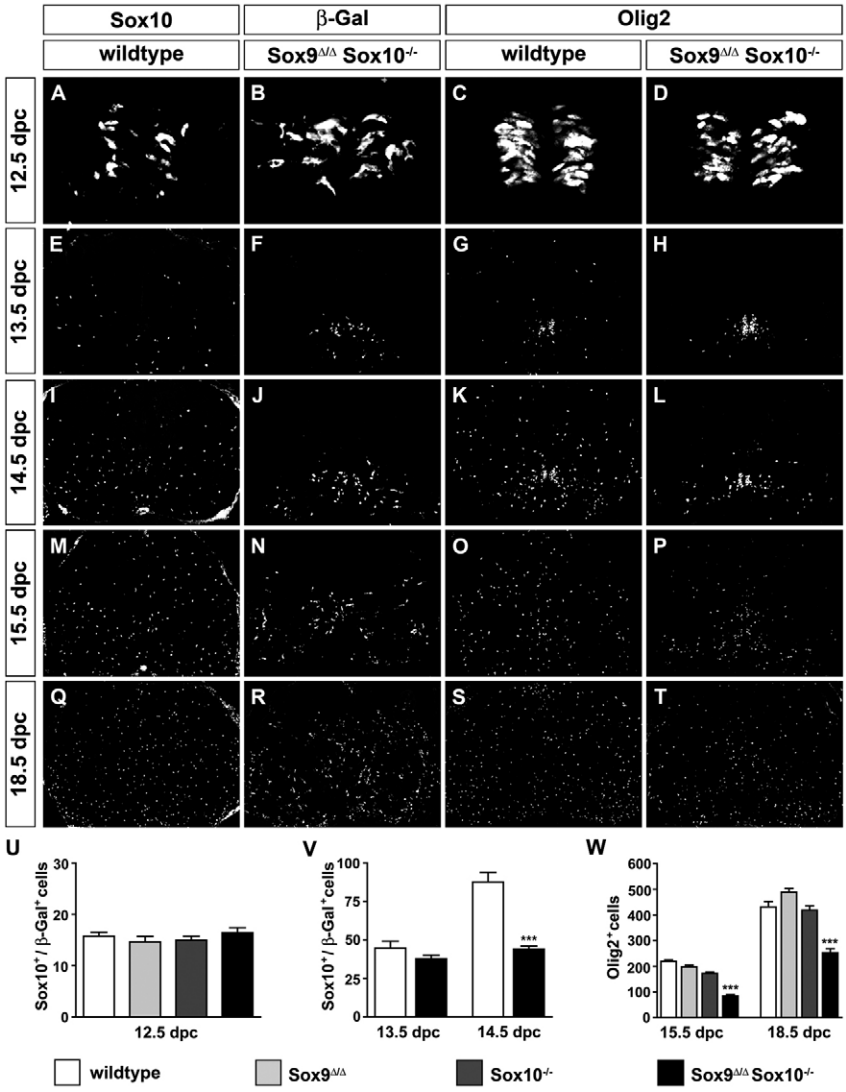

Fig. 5. Oligodendrocyte development in *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre* spinal cords. Immunohistochemistry with antibodies specific for Sox10 (A,E,I,M,Q), β-galactosidase (B,F,J,N,R) and Olig2 (C,D,G,H,K,L,O,P,S,T) were performed at 12.5 dpc (**A-D**), 13.5 dpc (**E-H**), 14.5 dpc (**I-L**), 15.5 dpc (**M-P**) and 18.5 dpc (**Q-T**) on transverse sections from the forelimb region of wild-type (A,C,E,G,I,K,M,O,Q,S) or *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre (Sox9$^{Δ/Δ}$ Sox10$^{-/-}$)* (B,D,F,H,J,L,N,P,R,T) embryos. Subcellular localization of Sox10 (nuclear) and β-galactosidase (cytoplasmic) causes a slightly altered appearance of cells in A,E,I,M,Q when compared with B,F,J,N,R. (**U-W**) OLP numbers were determined at 12.5 dpc (U), 13.5 dpc and 14.5 dpc (V) as Sox10- or β-galactosidase-positive cells, and at 15.5 dpc and 18.5 dpc (W) as Olig2-positive cells in wild-type (white bars), *Sox9$^{loxP/loxP}$, Sox10::Cre* (light-grey bars), *Sox10$^{lacZ/lacZ}$* (dark-grey bars) and *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre* (black bars) spinal cords as indicated. For these quantifications, at least 30 separate 10 μm sections from the forelimb region of two independent embryos were counted for each genotype. Data are presented as mean±s.e.m. Differences to the wild type were statistically significant for oligodendrocyte numbers in *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre* spinal cords from 14.5 dpc onwards, as determined by Student's *t*-test (***$P \leqslant 0.001$).

spinal cord (Fig. 5Q-T) and numbers of Olig2-positive cells being reduced by 42% (Fig. 5W). Very few OLPs ever reached the dorsal-most region of the spinal cord.

The lower number of OLPs was not due to decreased proliferation rates. Both at 15.5 and 18.5 dpc, the number of proliferating OLPs (determined as the number of Sox10- or β-galactosidase-positive cells also labelled by Ki67 or PCNA) was comparable with the wild type in *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre* spinal cords (Fig. 6A and data not shown). Similarly, there was no significant difference in the number of BrdU-labelled OLPs after a single injection of the nucleoside 1 hour before embryo preparation, indicating that the number of OLPs in S phase was unaltered (data not shown). As the fraction of proliferating cells that are in S phase also provides an estimation of the cell cycle length in mammalian cells in which S phase length is relatively constant and G1 phase length variable (Chenn and Walsh, 2002), we determined the fraction of Ki67-immunoreactive cells that were also labelled with BrdU. Again, we detected no difference between wild type and mutant at 15.5 dpc and 18.5 dpc (Fig. 6B), indicating that OLPs in *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre* spinal cords have an unaltered cell cycle length.

We also examined Ki67-immunoreactivity and BrdU incorporation at 15.5 dpc after a single BrdU pulse 24 hours earlier. By determining the fraction of BrdU-labelled cells that were no longer Ki67 positive, a measure was obtained for the rate of cell cycle exit. As this fraction was similar between wild type and double mutant, cell cycle exit was not significantly altered in *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre* spinal cords (Fig. 6C).

Differences were, however, detectable in the rate of apoptosis at 15.5 dpc and 18.5 dpc (Fig. 6D-G). In the *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre* spinal cord, there were ~60% more TUNEL-positive cells than in the wild type (Fig. 6H). TUNEL-positive cells were preferentially localized to those areas where OLPs were preferentially found in the *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre* spinal cord (Fig. 6D-G). Direct analysis furthermore revealed that OLPs had a significantly increased rate of apoptosis in the double mutant (Fig. 6I). Taking the unaltered rates of oligodendrocyte specification and proliferation into account, the lower number of Sox9/Sox10 double deficient OLPs is thus probably caused by reduced survival rates.

No transdifferentiation of OLPs in the combined absence of Sox9 and Sox10

OLPs in the *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre* spinal cord have so far been operationally defined as those cells that are positive for Olig2 and express β-galactosidase from the mutant *Sox10* locus. To characterize these cells in further detail, we first

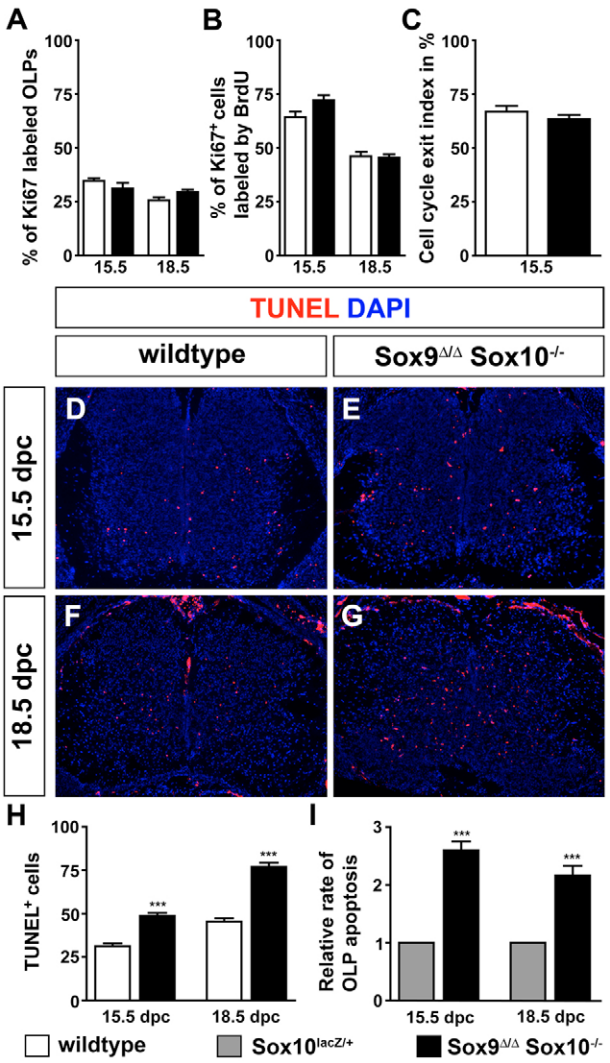

Fig. 6. Oligodendroglial proliferation and survival in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords. (**A**) Oligodendroglial proliferation was measured at 15.5 dpc and 18.5 dpc as the percentage of Sox10-positive cells (in the wild type, white bars) or β-galactosidase-positive cells (in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* embryos, black bars) that also expressed Ki67. (**B**) The fraction of Ki67-positive cells also labelled by a 1 hour pulse of BrdU was determined in the wild-type and in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* embryos as a measure of mitotic rates and cell cycle length. (**C**) The Ki67-negative fraction of cells labelled by a single BrdU pulse 24 hours before embryo preparation was determined in the wild-type and in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* embryos as a measure of cell cycle exit rates. (**D-G**) TUNEL was performed on transverse sections from the forelimb region of wild-type (D,F) and *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* (E,G) embryos at 15.5 dpc (D,E) and 18.5 dpc (F,G). (**H**) Apoptosis was quantified as the number of TUNEL-positive cells in wild-type (white bars) and *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* (black bars) spinal cords at 15.5 dpc and 18.5 dpc. (**I**) Apoptosis in OLPs was determined at 15.5 dpc and 18.5 dpc as the number of TUNEL-positive, β-galactosidase-labelled cells in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords (black bars) relative to the number in the *Sox10*$^{lacZ/+}$ spinal cords (grey bars) which was arbitrarily set to 1. For all quantifications, at least 30 separate 10 μm sections from the forelimb region of two independent embryos were counted for each genotype. Data are presented as mean±s.e.m. Differences were statistically significant for apoptosis rates in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords, as determined by Student's *t*-test (***$P \leqslant 0.001$).

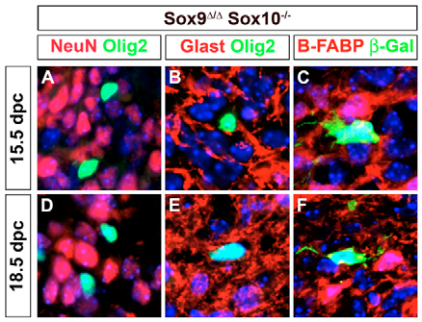

Fig. 7. Absence of ectopic marker gene expression in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords. In co-immunohistochemistry on transverse spinal cord sections from the forelimb region of *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* (*Sox9*$^{Δ/Δ}$ *Sox10*$^{-/-}$) embryos at 15.5 dpc (**A-C**) and 18.5 dpc (**D-F**). OLPs were identified by Olig2 (A,B,D,E) or β-galactosidase (C,F) expression (in green), and analyzed for the simultaneous occurrence of the cell-type specific markers (in red) NeuN (A,D), Glast (B,E) and B-FABP (C,F). Nuclei were counterstained with DAPI.

asked whether there is aberrant expression of neuronal, astroglial or radial glial markers in these Sox9/Sox10 double-deficient OLPs. In co-immunohistochemistry (30 sections from two independent embryos), we did not detect NeuN or Tuj1 expression in Olig2-positive cells of the *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cord at 15.5 dpc and 18.5 dpc, and very few Glast or glutamine synthetase-expressing Olig2-positive cells. As the latter were also present in the wild type, mutant OLPs did not aberrantly express neuronal and astroglial markers (Fig. 7A,B,D,E and data not shown). There was also no significant overlap between β-galactosidase and B-FABP expression (Fig. 7C,F), arguing that Sox9/Sox10 double-deficient OLPs are similarly negative for radial glia markers as wild-type precursors (Stolt et al., 2004).

Normal expression of many markers in OLPs after combined loss of Sox9 and Sox10

As we failed to detect ectopic marker expression in OLPs from *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords, we analyzed whether OLP markers other than Olig2 and β-galactosidase were still normally expressed in the mutant spinal cord at 18.5 dpc. Depending on the marker, its subcellular localization and the performance of the corresponding antibody, analysis was carried out on spinal cord sections by immunohistochemistry or on acutely dissociated spinal cord cultures by immunocytochemistry. When compared with the wild type, all markers were expressed in fewer cells of the *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cord in accordance with the lower number of OLPs (Fig. 8E and data not shown). However, when we focused on the Olig2-positive cells as the total OLP population and analyzed how many of them additionally expressed other oligodendroglial markers, we found that the relative number of NG2-, DCC- and O4-expressing OLPs was comparable between *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* and wild-type or *Sox10*$^{lacZ/lacZ}$ spinal cords (Fig. 8A-C).

By contrast, the relative number of Olig2-expressing cells that were also positive for Nkx2.2 was reduced to 50% (Fig. 8D). In the mouse spinal cord, Nkx2.2 expression commences fairly late in OLPs, which are predominantly localized in the marginal zone (Fig. 8E), and may rely on marginal zone signals. As proportionately fewer OLPs have entered the marginal zone in *Sox9*$^{loxP/loxP}$,

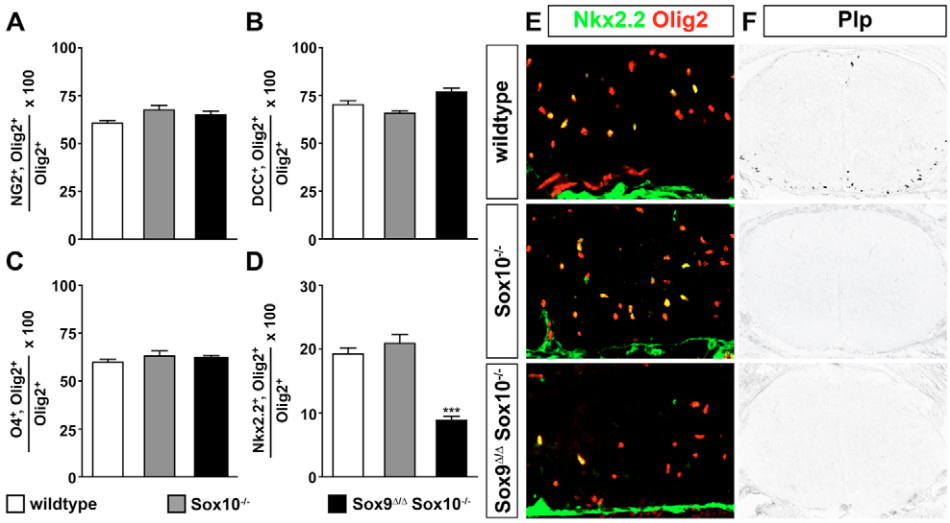

Fig. 8. Oligodendrocyte marker gene expression in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords. (**A-D**) Quantification of oligodendroglial marker gene expression at 18.5 dpc as the percentage of Olig2-positive cells that also expressed NG2 (A), DCC (B), O4 (C) and Nkx2.2 (D) in wild-type (white bars), *Sox10*$^{lacZ/lacZ}$ (*Sox10*$^{-/-}$; dark grey bars) and *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* (*Sox9*$^{\Delta/\Delta}$, *Sox10*$^{-/-}$; black bars) spinal cords at 18.5 dpc following immunocytochemistry on acutely dissociated spinal cord cultures (A-C) or immunohistochemistry on transverse spinal cord sections (D). For all quantifications, at least 10 separate cover slips or 30 separate 10 μm sections from the forelimb region were counted from two independent embryos for each genotype. Data are presented as mean±s.e.m. Differences from the wild-type were statistically significant for Nkx2.2 in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords, as determined by Student's *t*-test (***$P \leqslant 0.001$). (**E**) Co-immunohistochemistry with antibodies specific for Olig2 (in red) and Nkx2.2 (in green) were performed on transverse sections from the forelimb region of wild-type, *Sox10*$^{lacZ/lacZ}$ and *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords at 18.5 dpc. Comparable regions of the ventral marginal zone are shown. (**F**) In situ hybridization with a *Plp*-specific probe was performed on transverse sections from the forelimb region of wild-type, *Sox10*$^{lacZ/lacZ}$ and *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords at 18.5 dpc.

Sox10$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords at 18.5 dpc, the lower relative number of Nkx2.2-expressing OLPs may be caused by the altered migration pattern observed in the mutant genotype. Despite this reduction, Nkx2.2 was still detectable in OLPs.

In contrast to markers for OLPs, those for terminally differentiating oligodendrocytes such as *Plp* and *Mbp* were absent in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords (Fig. 8F). This is not surprising as the same lack of terminal differentiation markers was already observed in *Sox10*$^{lacZ/lacZ}$ spinal cords (Fig. 8F) (Stolt et al., 2002) and is thus not specific to the double deficient OLPs analyzed in this study.

Severely reduced Pdgfra expression in OLPs in the combined absence of Sox9 and Sox10

Taking the fairly normal expression of most OLP markers into account, we did not expect dramatic changes in Pdgfra occurrence. We analyzed wild-type, single mutant and double mutant spinal cords from 13.5 dpc to 18.5 dpc by in situ hybridization, and from 14.5 dpc to 18.5 dpc by immunohistochemistry for Pdgfra expression. Compared with the wild type, there was no significant difference in the single mutants regarding appearance, number or distribution of Pdgfra-positive cells at any of the analyzed time points by in situ hybridization or immunohistochemistry (Fig. 3I-L, Fig. 4A-C,E-G, Fig. 9A-C,E-G, Fig. 10A-C and data not shown). However, to obtain in situ hybridization signals for the single mutants that were comparable in intensity with the wild type, staining reactions had to be extended, especially at the earlier stages. This indicated that *Pdgfra* transcript levels were reduced in OLPs from the single mutants, as already reported for the Sox10-deficient OLPs (Stolt et al., 2002).

Pdgfra expression was much more dramatically altered in *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* spinal cords. Already at 13.5 dpc, very few *Pdgfra*-positive cells remained (compare Fig. 10A-C with Fig. 10D). These Pdgfra-positive cells were furthermore localized in close vicinity to the ventricular zone and thus probably corresponded to newly specified OLPs that had not yet lost Sox9. From 14.5 dpc onwards, Pdgfra was no longer detected in the double mutant either on the transcript (compare Fig. 10E,I,M with Fig. 10F,J,N) or on the protein level (compare Fig. 10G,K,O with Fig. 10H,L,P; see also Fig. 4D,H and Fig. 9D,H). Even at 18.5 dpc, Pdgfra expression had not recovered, indicating that Pdgfra expression was severely compromised in the double mutant and is probably under the control of Sox9 and Sox10 in spinal cord OLPs.

Chromatin immunoprecipitation experiments furthermore revealed that Sox9 is bound to elements C1, C2 and C3 within the proximal 5′ flanking region of the *Pdgfra* gene that are conserved among amniotes (http://ecrbrowser.dcode.org), but not to non-conserved regions N1 and N2 in the distal 5′ flanking part (Fig. 10Q). These results are compatible with a direct *Pdgfra* regulation by SoxE proteins. In primary cultures from spinal cords of 18.5 days old *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* embryos treated with saturating amounts of PDGF-AA, OLP numbers exhibited a PDGF-

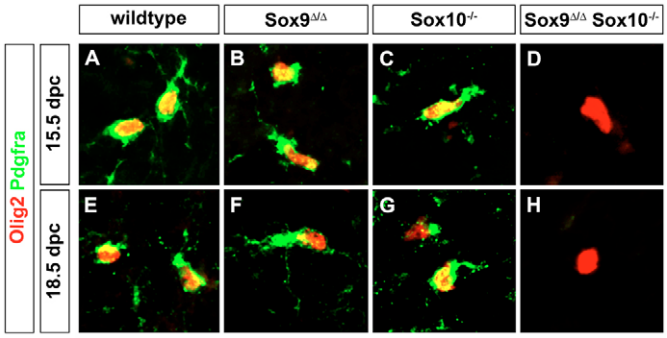

Fig. 9. Correlation of Olig2 and Pdgfra expression. Co-immunohistochemistry was performed on transverse spinal cord sections from the forelimb region of wild-type (A,E), *Sox9*$^{loxP/loxP}$, *Sox10::Cre* (*Sox9*$^{\Delta/\Delta}$) (B,F), *Sox10*$^{lacZ/lacZ}$ (*Sox10*$^{-/-}$) (C,G) and *Sox9*$^{loxP/loxP}$, *Sox10*$^{lacZ/lacZ}$, *Sox10::Cre* (*Sox9*$^{\Delta/\Delta}$ *Sox10*$^{-/-}$) (D,H) embryos at 15.5 dpc (**A-D**) and 18.5 dpc (**E-H**) with antibodies directed against Olig2 (red) and Pdgfra (green).

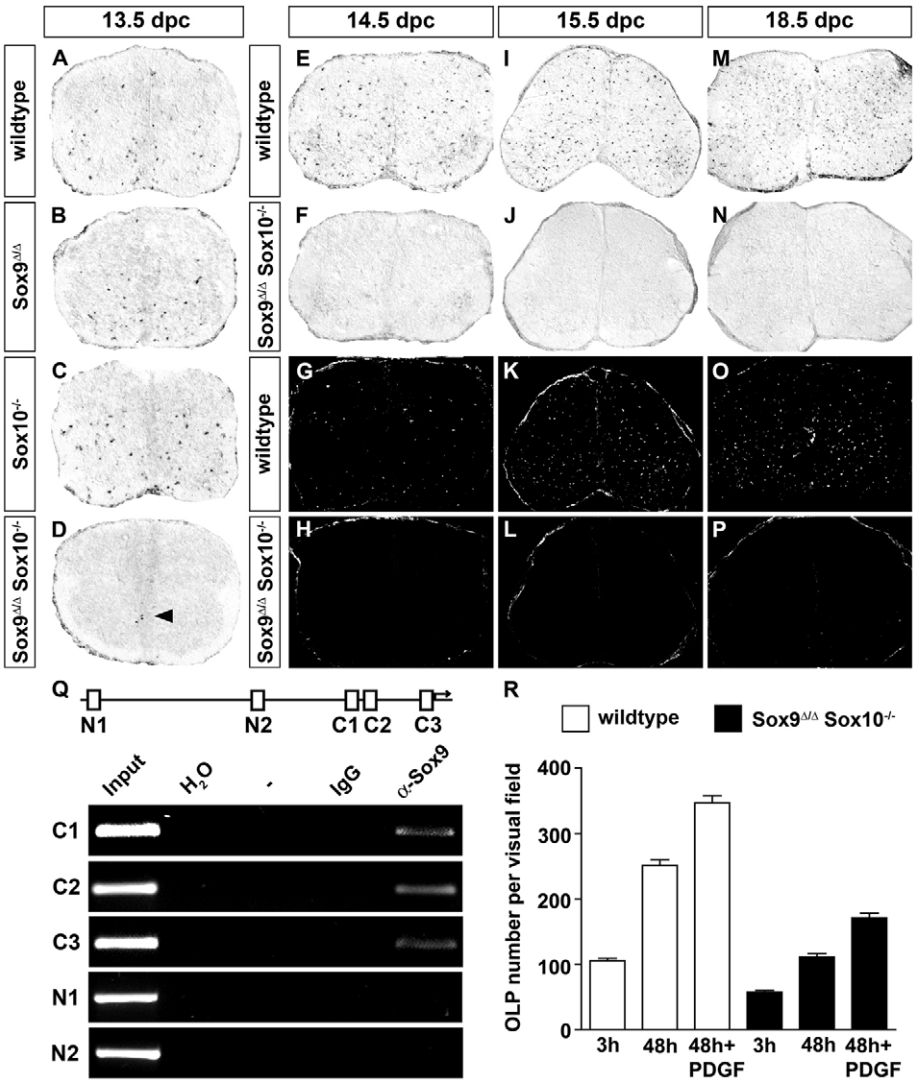

Fig. 10. Pdgfra expression in *Sox9$^{loxP/loxP}$*, *Sox10$^{lacZ/lacZ}$*, *Sox10::Cre* spinal cords. In situ hybridizations with a *Pdgfra*-specific probe (A,B,C,D,E,F,I,J,M,N) and immunohistochemistry with antibodies specific for Pdgfra (G,H,K,L,O,P) was performed at 13.5 dpc (**A-D**), 14.5 dpc (**E-H**), 15.5 dpc (**I-L**) and 18.5 dpc (**M-P**) on transverse sections from the forelimb region of wild-type (A,E,I,M,G,K,O), *Sox9$^{loxP/loxP}$, Sox10::Cre (Sox9$^{\Delta/\Delta}$)* (B), *Sox10$^{lacZ/lacZ}$ (Sox10$^{-/-}$)* (C) and *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre (Sox9$^{\Delta/\Delta}$ Sox10$^{-/-}$)* (D,F,H,J,L,N,P) embryos. The arrowhead in D indicates the Pdgfra-positive cells. (**Q**) Chromatin immunoprecipitation was performed on formaldehyde-fixed spinal cords from four day old wild-type mice in the absence (–) and presence of antibodies (IgG, control IgG; α-Sox9, anti-Sox9 antibodies). PCR was applied on the immunoprecipitate to detect evolutionary conserved sequence elements C1, C2 and C3 in the proximal 5′ flanking region of the *Pdgfra* gene, as well as non-conserved regions N1 and N2 in the distal 5′ flanking region. These fragments were also amplified from 1/20 of the material used for immunoprecipitation (Input). H_2O: water control. (**R**) OLP numbers were determined in cultures of dissociated spinal cords from wild-type (white bars) and *Sox9$^{loxP/loxP}$, Sox10$^{lacZ/lacZ}$, Sox10::Cre (Sox9$^{\Delta/\Delta}$ Sox10$^{-/-}$)* (black bars) 18.5-day-old embryos 3 hours after seeding or after 48 hours in the absence or presence of 20 ng/ml PDGF-AA. At least 30 visual fields were counted from two independent embryos for each genotype. Data are presented as mean OLP number per visual field±s.e.m.

dependent increase after 48 hours, arguing that at least under these conditions PDGF-AA retained mitogenic activity on Sox9/Sox10 double deficient OLPs (Fig. 10R).

DISCUSSION

We have previously shown that Sox9 has an important role in OLP specification (Stolt et al., 2003), whereas the related Sox10 is required for their differentiation into mature oligodendrocytes (Stolt et al., 2002). The presence of Sox9 and Sox10 in OLPs has furthermore led us to postulate additional roles for both Sox proteins during lineage progression. To address this issue, we deleted Sox9 in already specified OLPs, thereby leaving Sox9 function during specification intact. The resulting loss of Sox9 did not lead to significant alterations in the development of spinal cord OLPs in the corresponding mouse mutant. Defects became detectable only after the combined loss of Sox9 and Sox10. Taken together with the already reported normal development of OLPs in Sox10-deficient mice until terminal differentiation (Stolt et al., 2002), we conclude that Sox9 and Sox10 have additional functions in developing OLPs, but exert them in a functionally redundant manner. Our analysis focused on pMN-derived OLPs. However, our findings can very probably be extrapolated to the dorsally derived OLPs of the spinal cord (Richardson et al., 2006), because SoxE expression is very similar in all spinal cord oligodendrocytes.

Interestingly, many OLP markers continued to be expressed in the absence of Sox9 and Sox10. These included Olig2, DCC, NG2, O4 and Nkx2.2, although the latter was expressed in proportionately fewer OLPs. Even expression of β-galactosidase from the mutant *Sox10* locus continued in OLPs in the absence of Sox9 and Sox10. Although our results do not exclude an earlier essential role for Sox9 in the induction of Sox10 expression similar to what has been observed in the early neural crest (Cheung et al., 2005), neither Sox9 nor Sox10 are essential for maintained expression from the *Sox10* locus in OLPs. Whether Sox9 and Sox10 are substituted in this function by Sox8, which is closely related and also expressed in OLPs (Stolt et al., 2004; Stolt et al., 2005), is unclear at present. Alternatively, SoxE proteins are completely dispensable for maintaining Sox10 expression in OLPs.

OLPs not only continued to express many of their characteristic markers, they also failed to ectopically express other markers that are normally found in neurons, astroglia or radial glia. Thus, there is no reason to assume that OLPs in the absence of Sox9 and Sox10

have been partially transformed into different CNS cell types. On the contrary, OLPs seem to preserve their identity in the absence of Sox9 and Sox10.

On this background, our failure to detect Pdgfra in most OLPs at 13.5 and all OLPs at later stages is particularly intriguing and argues that Pdgfra signalling is one of the main pathways by which Sox9 and Sox10 regulate OLP lineage progression. Although our study proves that Sox9 and Sox10 are genetically upstream of Pdgfra in spinal cord OLPs, it does not allow a firm conclusion of whether *Pdgfra* represents a direct or an indirect target gene. In vivo binding of Sox9 to the proximal 5′ flanking region of the *Pdgfra* gene, especially to the core promoter supports the assumption that Sox9 directly activates *Pdgfra* expression. It has to be stressed, however, that the 5′ flanking region is not sufficient for expression in OLPs (Reinertsen et al., 1997). Sox9 binding to this region may therefore be relevant to aspects of Pdgfra expression other than oligodendroglial expression. Full clarification of this issue has to await a better understanding of *Pdgfra* gene regulation, in particular the identification of the regulatory regions that are responsible for oligodendroglial expression. So far, they have only been mapped to a 380 kb region (Sun et al., 2000).

Pdgfra remained detectable in OLPs after Sox9 deletion, indicating that Sox10 occurrence in OLPs is sufficient to permit *Pdgfra* expression, although at reduced levels. The same Sox10, however, fails to maintain *Pdgfra* expression during terminal differentiation and thereafter in mature oligodendrocytes. In a reciprocal manner, several myelin genes have been shown to be upregulated by Sox10 with the onset of terminal differentiation, but are not expressed earlier in OLPs, despite the fact that Sox10 is already present (Bondurand et al., 2001; Schlierf et al., 2006; Stolt et al., 2002).

This argues that the activity of Sox10, and probably also of Sox9, has to be modulated in a stage-specific manner during oligodendrocyte development. Most likely, Sox10 requires additional transcription factors that are differentially present in OLPs and in mature oligodendrocytes. The reliance on partner transcription factors is a general property of Sox proteins (Kamachi et al., 2000; Wegner, 2005). We have recently shown that the presence of Sox5 and Sox6 in OLPs prevents a premature Sox10-dependent activation of myelin gene expression (Stolt et al., 2006). Sox5 and Sox6 may also be involved in allowing Sox10-dependent *Pdgfra* activation.

The role of PDGF signalling in oligodendrocyte development has been intensely studied both in animal models and cell culture systems. On cultured OLPs, PDGF-AA acts as a mitogen (Noble et al., 1988; Richardson et al., 1988), a survival factor (Barres et al., 1992) and a chemoattractant (Armstrong et al., 1990). Thus, it is very likely that the altered migration pattern that we observed for OLPs in the combined absence of Sox9 and Sox10, and the increased apoptosis are direct consequences of the changed *Pdgfra* expression in these mice. Supporting evidence comes from the close resemblance of OLP defects in Sox9/Sox10 double-deficient mice and in mice harbouring Pdgfra mutants with eliminated PI3 kinase-dependent or Src-dependent downstream signalling (Klinghoffer et al., 2002). Klinghoffer and colleagues also found altered migration and reduction of OLP numbers, although the cause for the latter is not clear, as both proliferation and apoptosis were found to be normal. Interestingly, both the Sox9/Sox10 double-deficient and *Pdgfra* mutant phenotypes are, however, less severe than the one caused by PDGF-AA loss, in which OLP numbers are reduced to less than 10% of the wild type and OLP proliferation is severely disturbed (Fruttiger et al., 1999). This phenotypic divergence is surprising, as PDGF-AA is thought to act preferentially on OLPs through Pdgfra (Hoch and Soriano, 2003).

The mitogenic function of PDGF-AA on OLPs has been impressively confirmed in both loss-of-function and gain-of-function studies in mice (Calver et al., 1998; Fruttiger et al., 1999). Interestingly, Sox9/Sox10 double-deficient OLPs proliferated normally in vivo (where PDGF-AA levels are limiting) and exhibited a mitogenic response to PDGF-AA in mixed spinal cord cultures (where PDGF-AA is present in saturating amounts). PDGF-AA thus retained its mitogenic activity on Sox9/Sox10 double-deficient OLPs. The mitogenic effect of PDGF-AA may therefore not be exclusively mediated by Pdgfra, but additionally by yet unknown pathways. Alternatively, *Pdgfra* expression may not be completely turned off in OLPs in the combined absence of Sox9 and Sox10, but simply below our detection limits. Low residual levels may then be sufficient for mediating the mitogenic effect of PDGF-AA, but not for the survival effect in vivo.

Whatever the exact mechanism, this study clearly shows that in addition to their respective functions during OLP specification and terminal differentiation, Sox9 and Sox10 additionally influence survival and migration of OLPs through their effect on PDGF signalling. Comparable influences on survival and migration have already been described for Sox10 in the neural crest and its derivatives (Kim et al., 2003; Maka et al., 2005; Paratore et al., 2001), but never before in the oligodendrocyte lineage.

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