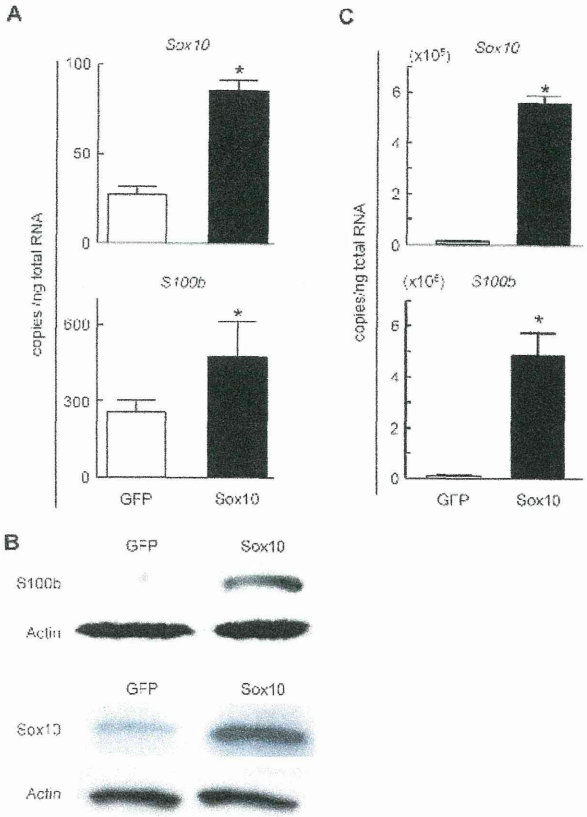

Fig. 2. Modulation of S100B expression by SOX10 in Schwann cells. (A) mRNA levels of *Sox10* (top) and *S100b* (bottom) in stable lines of primary rat Schwann cells retrovirally transfected with SOX10 or control GFP. (B) Protein level of S100B and Sox10 in stable lines of primary rat Schwann cells retrovirally transfected with SOX10 or control GFP. (C) Modulation of *S100b* expression by SOX10 in ROS cells. mRNA levels of *Sox10* (top) and *S100b* (bottom) in stable lines of rat non-neurogenic ROS cells retrovirally transfected with SOX10 or control GFP. Experiments were repeated independently three times with data shown as the mean ± SEM. $^{*}P<0.05$ versus control.

doi:10.1371/journal.pone.0115400.g002

Contribution of SOX10-S100B signaling to proliferation and myelination of Schwann cells

We next examined the function of S100B in the proliferation of Schwann cells. When Schwann cells were cultured under the proliferation or differentiation conditions, i.e., with or without NGF and forskolin treatment, respectively [28], the expression of both *Sox10* and *S100b* was markedly suppressed under the proliferation condition but increased under the differentiation condition (Fig. 6). When we knocked down either *S100b* or *Sox10* with shRNA, BrdU incorporation significantly increased (Fig. 7A–B, 7D–E). In the CCK-8 assay, knocking down either *S100b* or *Sox10* in the Schwann cells or non-glial cells (C3H10T1/2) also increased cell proliferation (Fig. 7C and Fig. 8). These results suggest that SOX10-S100B signaling negatively regulates Schwann cell proliferation.

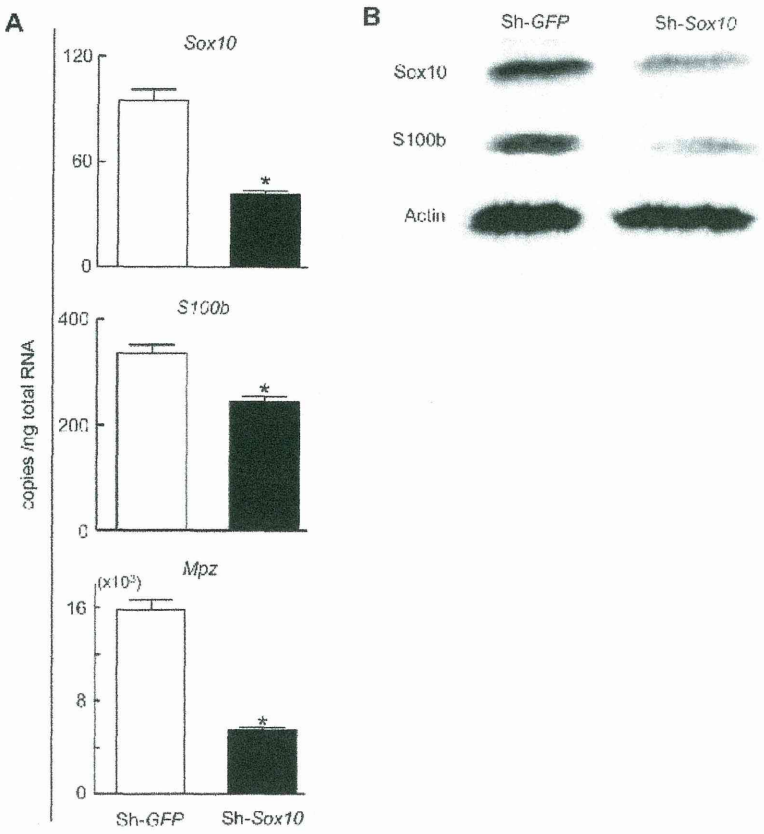

Fig. 3. Suppression of S100B and Mpz expression by SOX10 insufficiency in Schwann cells. (A) mRNA levels of *Sox10* (top), *S100b* (middle) and *Mpz* (bottom) in stable lines of primary rat Schwann cells retrovirally transfected with shRNA specific for *Sox10* or *GFP*. All experiments were repeated independently three times with data shown as the mean ± SEM. *$P<0.05$ versus *GFP* or sh-*GFP*. (B) Protein levels of SOX10 and S100B in stable lines of primary rat Schwann cells retrovirally transfected with shRNA specific for SOX10 or control GFP.

doi:10.1371/journal.pone.0115400.g003

Finally, we examined the involvement of S100B in myelination using dissociated DRGs. Compared to control cocultures, knocking down *S100b* in Schwann cells impaired the myelination of rat DRG neurons (Fig. 9A), and we quantified this by calculating the number of MBP-positive myelinating cells (Fig. 9B). This result suggests that S100B in Schwann cells plays a critical role in myelination.

Discussion

During vertebrate development, SOX10 is first highly expressed in the emerging neural crest and later in glial cells, where SOX10 is involved in the differentiation of both the peripheral nervous system (PNS) and CNS [7, 29]. SOX10 also plays an essential role in maturing and maintaining Schwann cells [30] by directly

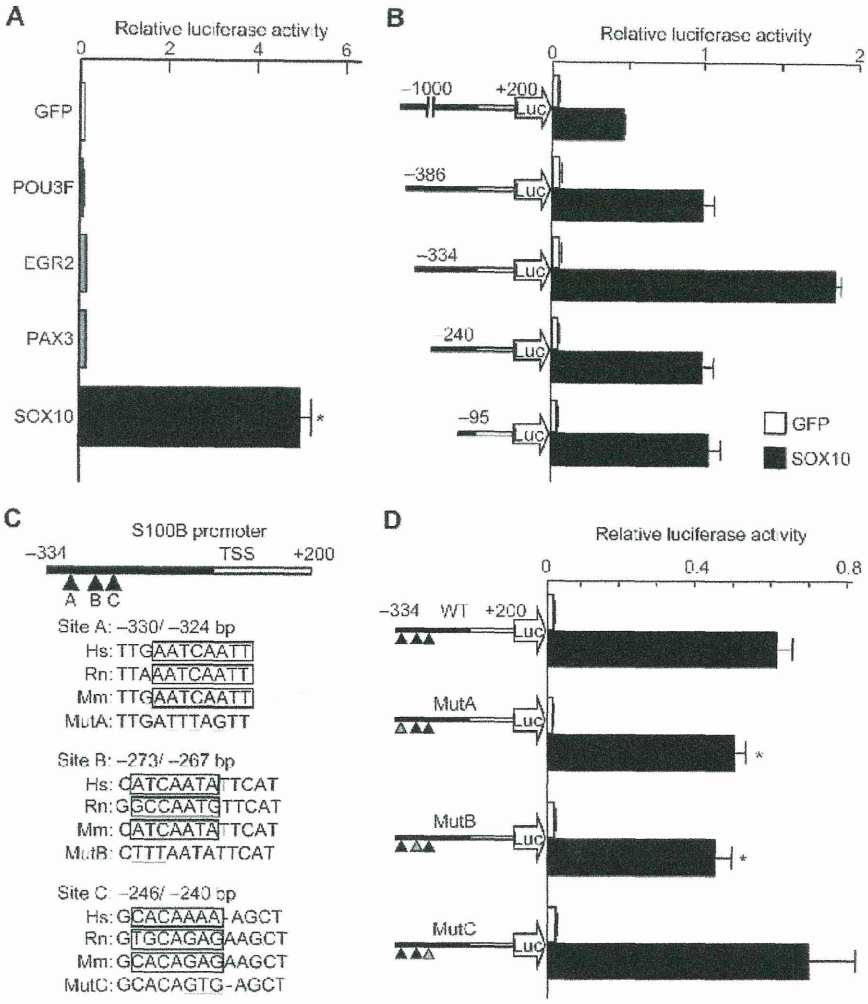

Fig. 4. Identification of putative SOX10-response elements in S100B. (A) Luciferase activities after transfection of putative Schwann cell-related transcription factors into HeLa cells with a reporter construct containing a fragment (−1,000 to +200 bp) of the *S100B* gene. **P*<0.05 versus GFP. (B) Deletion analysis using luciferase-reporter constructs containing a series of deletion fragments of the *S100B* gene in HeLa cells transfected with SOX10 or control GFP. (C) Comparison of human (Hs), rat (Rn), and mouse (Mm) sequences in three putative SOX motifs in the S100B promoter and mutated sequences (Mut A, Mut B, and Mut C), used in the following mutagenesis analysis. (D) Site-directed mutagenesis analysis using luciferase-reporter constructs containing −334 to +200 bp of the *S100B* gene with mutations as in Fig. 3C within the three SOX motifs in the cells above. **P*<0.05 versus wild-type (WT) with SOX10. All experiments were repeated independently three times with data shown as the mean ± SEM.

doi:10.1371/journal.pone.0115400.g004

regulating MPZ, c-Ret, ciliary neurotrophic factor (CNTF), dopachrome tautomerase (DCT), microphthalmia-associated transcription factor (MITF), connexin32, connexin47, EGR2, SP1, and SP3 [31–39]. In the present study, we demonstrated that during Schwann cell differentiation, SOX10 is involved in the transcriptional induction of S100B. SOX10 belongs to the SOX transcription

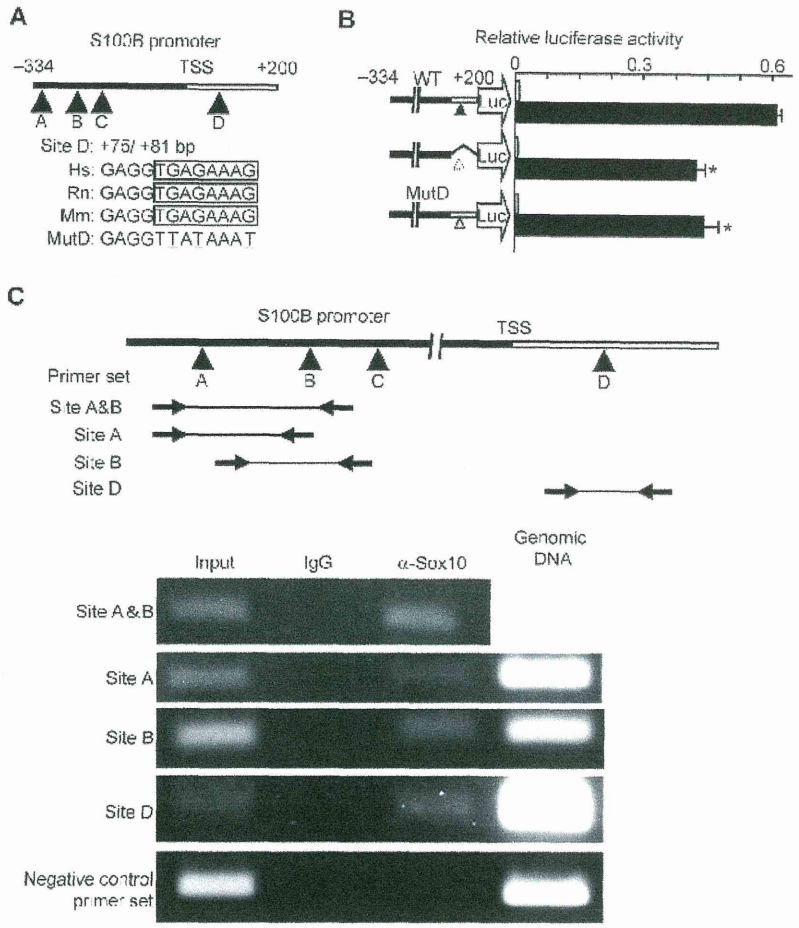

Fig. 5. Identification of putative response elements in S100B intron 1 by SOX10 and direct binding of SOX10 to the response elements. (A) Comparison of human (Hs), rat (Rn) and mouse (Mm) sequences in the putative SOX motif of the S100B intron 1 and mutated sequence (Mut D), used in the following mutagenesis analysis. (B) Deletion and site-directed mutagenesis analysis using luciferase-reporter constructs containing −334 to +200 bp of the *S100B* gene in HeLa cells transfected with SOX10 or control GFP. **P*<0.05 versus wild-type (WT) with SOX10. All experiments were repeated independently three times with data shown as the mean ± SEM. (C) ChIP assay performed using cell lysates of Schwann cells that were amplified by a primer set spanning the identified regions; sites A & B (top), site A (second row), site B (third row), and site D (fourth row), or not spanning the region (bottom) before (input) and after immunoprecipitation with antibodies to Sox10 (α-Sox10) or non-immune IgG (IgG). Genomic DNA was amplified as a positive control.

doi:10.1371/journal.pone.0115400.g005

factor family that contains high-mobility group (HMG) domain(s) [40, 41]. The SoxE family contains SOX10, SOX9, and SOX8. The family members are structurally similar and are known to have functional redundancies [42, 43]. We previously reported that SOX9 regulates S100B expression in chondrocytes through direct binding to the *S100b* promoter(s) [17]. Here, we found that *Sox10* is predominantly expressed in Schwann cells as compared to *Sox9* (Fig. 1A), and the expression levels of SOX8 and SOX9 are lower than that of SOX10 in

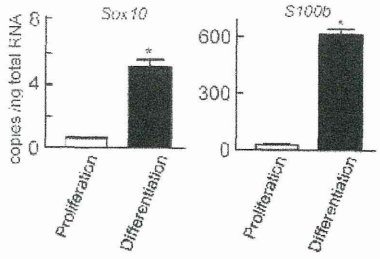

Fig. 6. Suppressed Schwann cell proliferation by SOX10-S100B signaling. Comparison of *Sox10* and *S100b* mRNA levels between conditions of proliferation and differentiation in rat sciatic nerve Schwann cells. All experiments were repeated independently three times with data shown as the mean ± SEM. *$P<0.05$ versus proliferation condition.

doi:10.1371/journal.pone.0115400.g006

differentiated glial cells [36]. In addition, previous reports showed that the expression of S100B as well as MZP and MBP is suppressed in SOX10-deficient sciatic nerves [30]. Therefore, in the present study, we focused on the role of SOX10 in Schwann cell differentiation.

It has been reported that SOX transcription factors determine cell fate by enhancing transcriptional activity through interaction with their co-factors; several factors such as PAX3 [33], SP1, SP3, heterogeneous nuclear ribonucleoprotein K, pur-alpha [35], MITF [34], EGR2 [31, 36], and POU3F [44] have already been shown to function as co-factors of SOX10. In addition, because Sox10 has several known targets, such as Krox20/Egr2 and ErbB3, we could not exclude the possibility that these molecules also play a role in the regulation of proliferation and differentiation together with S100B. The cooperation of SOX10 with other factors should be analyzed to elucidate the mechanism of S100B induction in Schwann cells in further detail.

Although S100B is involved in energy metabolism, cell cycling, apoptosis, extracellular signaling, and regulating the cytoskeleton [11, 45, 46], its function in Schwann cells has not been fully clarified. We found that the expression of SOX10 and S100B was decreased under the proliferation condition, while it was increased under the differentiation condition (Fig. 6A). Moreover, knockdown of SOX10 or S100B increased the proliferation of Schwann cells (Figs. 6B, 7A–7B). A previous report showed a significant increase in the number of proliferating cells in the sciatic nerve from Schwann cell-specific *Sox10*-ablated mice [30]. By analyzing cyclin-dependent kinase (Cdk)-deficient mice, it was discovered that Cdk4 controls postnatal Schwann cell proliferation [47], although the exact role of SOX10 or S100B as a regulator of cell cycle-related molecules in Schwann cells has not been established. Interestingly, S100B promotes cell cycling in the CNS [9] and S100B levels are high in neuronal tumor cells as compared to normal parental cells. Nevertheless, other studies demonstrated that the Cdk inhibitor $p21^{WAF1}$ was induced by S100B via AKT activation in PC12 neuronal cells [48] and that another Cdk inhibitor, $p27^{Kip1}$, activated the MBP promoter in cooperation with SOX10 in oligodendrocytes [49]. Therefore, SOX10-S100B signaling may

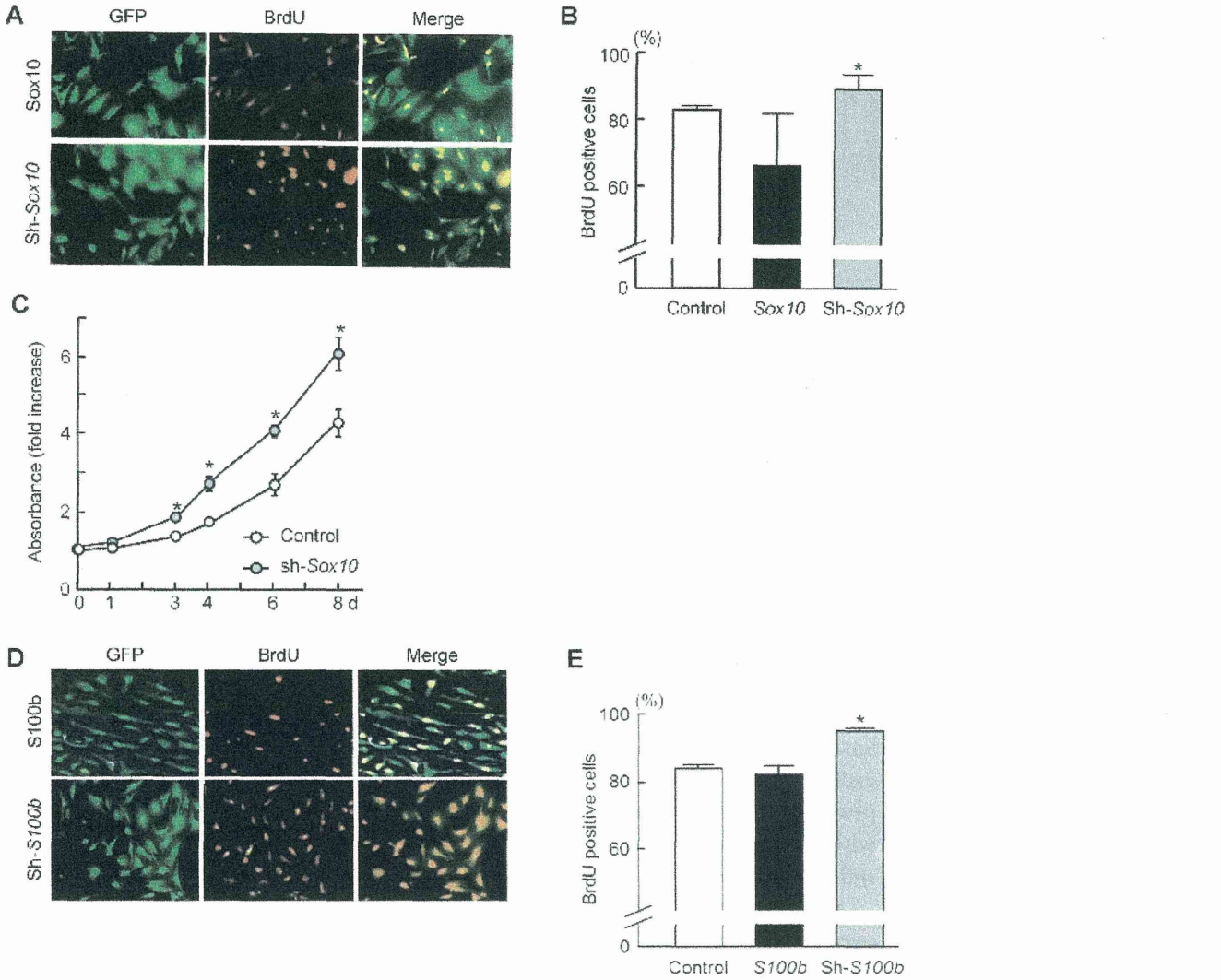

Fig. 7. Enhanced proliferation by knockdown of *Sox10* or *S100b* in Schwann cells. (A, B) BrdU labeling of stable lines of Schwann cells retrovirally transfected with *SOX10* or shRNA specific for *SOX10* and *GFP* (A). Ratio of BrdU-positive cells to total cells was quantified after 3 d culture of stable lines of Schwann cells transfected with *Sox10* expressing vector, shRNA vector specific for *Sox10*, and control *GFP* vector (B). (C) Growth curves using the CCK-8 assay of stable lines of Schwann cells retrovirally transfected with sh-*Sox10* or control *GFP*. Experiments were repeated independently three times with data shown as the mean ± SEM. *$P<0.05$ versus control. (D, E) BrdU labeling of stable lines of Schwann cells retrovirally transfected with *S100b* or shRNA specific for *S100b* and *GFP* (D). Ratio of BrdU-positive cells to total cells were quantified after 3-day-old cultures of stable lines of Schwann cells were transfected with *S100b* expressing vector, shRNA vector specific for *S100b*, and control *GFP* vector (E). Experiments were repeated independently three times with data shown as the mean ± SEM. *$P<0.05$ versus control.

doi:10.1371/journal.pone.0115400.g007

negatively regulate cell-cycle progression in Schwann cells by activating inhibitors of Cdks.

We also show that S100B is involved in Schwann cell myelination (Fig. 9). Our findings confirm a previous study that found that myelination is delayed in *S100b*-deficient mice [50]. How S100B functions to stimulate Schwann cell myelination

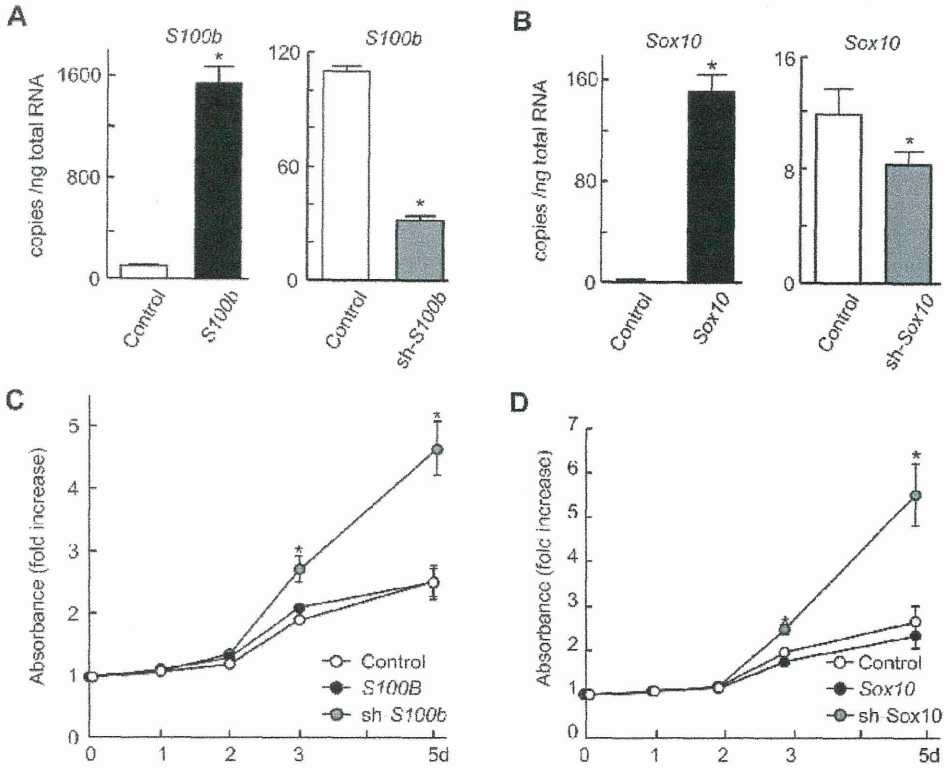

Fig. 8. Enhanced proliferation by knockdown of *S100b* or *Sox10* in C3H10T1/2 cells. (A) mRNA levels of *S100b* determined by real-time RT-PCR in stable lines of mouse mesenchymal C3H10T1/2 cells retrovirally transfected with *S100b*, shRNA for *S100b*, or control *GFP*. (B) mRNA levels of *Sox10* determined by real-time RT-PCR in stable lines of mouse mesenchymal C3H10T1/2 cells retrovirally transfected with *Sox10*, shRNA for *Sox10*, or control *GFP*. (C and D) Growth curves using the CCK-8 assay of stable lines of C3H10T1/2 cells as mentioned above. All experiments were repeated independently three times with data shown as the mean ± SEM. *$P<0.05$ versus control.

doi:10.1371/journal.pone.0115400.g008

is still unclear. Recent work suggests that because S100B is a Ca^{2+} binding protein, S100B controls intracellular Ca^{2+} concentration crucial for myelination induced by neuregulin-dependent phosphorylation of calcineurin. Neuregulin signaling controls myelination by increasing Ca^{2+} levels in Schwann cells in order to activate the phosphatase calcineurin [51, 52]. Thus, because Ca^{2+} levels regulate myelination, as a Ca^{2+} binding protein, S100B may also influence myelination in Schwann cells.

We conclude that SOX10 directly transactivates S100B to inhibit proliferation and to promote myelination during Schwann cell differentiation. It has been reported that the function of S100B changes depending on its expression levels. While at nanomolar levels S100B promotes axon extension via RAGE receptors, at millimolar levels S100B triggers apoptosis in neurons. Furthermore, S100B expression levels can indicate the malignant grade of malignant tumors [11, 53]. Together, these lines of evidence suggest that modulating the SOX10-S100B axis may be a viable therapeutic target for various neuronal disorders including demyelinating disease, neuropathy, and nerve injury.

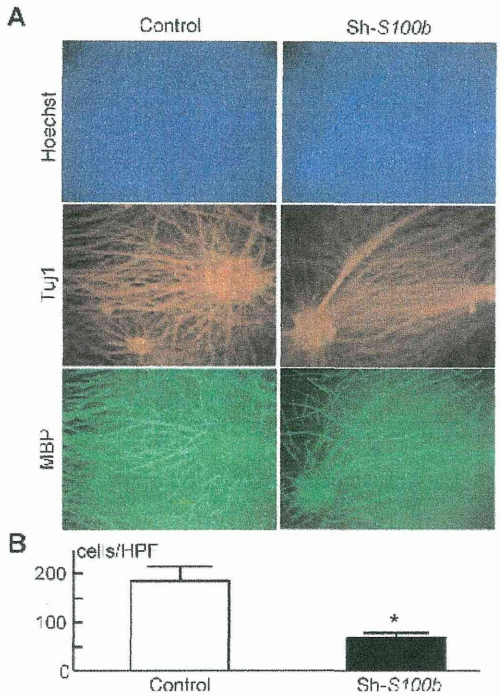

Fig. 9. Impaired myelination by knockdown of *S100b*. (A) Immunocytochemistry of neurons and stable lines of Schwann cells retrovirally transfected with shRNA specific for *S100b* or control *GFP* in DRG dissociated cultures. Staining of Tuj1 (red), MBP (green) and Hoechst (blue) in neurons, Schwann cells, and nuclei, respectively. (B) The number of MBP-positive Schwann cells in a high-power field of the immunocytochemistry as in Fig. 9A. Experiments were repeated independently three times with data shown as the mean ± SEM. *$P<0.05$ versus control.

doi:10.1371/journal.pone.0115400.g009

Acknowledgments

We thank R. Yamaguchi and H. Kawahara for excellent technical assistance.

Author Contributions

Conceived and designed the experiments: SF SH HK KN ST TO. Performed the experiments: SF SH TU MH. Analyzed the data: SF SH TU MH. Contributed reagents/materials/analysis tools: SF SH TU MH TS TI. Wrote the paper: SF MH ST TO.

References

1. **Ogata T, Iijima S, Hoshikawa S, Miura T, Yamamoto S, et al.** (2004) Opposing extracellular signal-regulated kinase and Akt pathways control Schwann cell myelination. J Neurosci 24: 6724–6732.
2. **Bhatheja K, Field J** (2006) Schwann cells: origins and role in axonal maintenance and regeneration. Int J Biochem Cell Biol 38: 1995–1999.
3. **Kioussi C, Gruss P** (1996) Making of a Schwann. Trends Genet 12: 84–86.

4. **Haldin CE, LaBonne C** (2010) SoxE factors as multifunctional neural crest regulatory factors. Int J Biochem Cell Biol 42: 441–444.

5. **Stolt CC, Wegner M** (2010) SoxE function in vertebrate nervous system development. Int J Biochem Cell Biol 42: 437–440.

6. **Wegner M, Stolt CC** (2005) From stem cells to neurons and glia: a Soxist's view of neural development. Trends Neurosci 28: 583–588.

7. **Kuhlbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M** (1998) Sox10, a novel transcriptional modulator in glial cells. J Neurosci 18: 237–250.

8. **Donato R** (1999) Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. Biochim Biophys Acta 1450: 191–231.

9. **Selinfreund RH, Barger SW, Pledger WJ, Van Eldik LJ** (1991) Neurotrophic protein S100 beta stimulates glial cell proliferation. Proc Natl Acad Sci U S A 88: 3554–3558.

10. **Azmitia EC, Dolan K, Whitaker-Azmitia PM** (1990) S-100B but not NGF, EGF, insulin or calmodulin is a CNS serotonergic growth factor. Brain Res 516: 354–356.

11. **Donato R, Sorci G, Riuzzi F, Arcuri C, Bianchi R, et al.** (2009) S100B's double life: intracellular regulator and extracellular signal. Biochim Biophys Acta 1793: 1008–1022.

12. **Mrak RE, Griffin WS** (2004) Trisomy 21 and the brain. J Neuropathol Exp Neurol 63: 679–685.

13. **Tateishi N, Shimoda T, Yada N, Shinagawa R, Kagamiishi Y** (2006) [S100B: astrocyte specific protein]. Nihon Shinkei Seishin Yakurigaku Zasshi 26: 11–16.

14. **Allore R, O'Hanlon D, Price R, Neilson K, Willard HF, et al.** (1988) Gene encoding the beta subunit of S100 protein is on chromosome 21: implications for Down syndrome. Science 239: 1311–1313.

15. **Gonzalez-Martinez T, Perez-Pinera P, Diaz-Esnal B, Vega JA** (2003) S-100 proteins in the human peripheral nervous system. Microsc Res Tech 60: 633–638.

16. **Jessen KR, Mirsky R** (2002) Signals that determine Schwann cell identity. J Anat 200: 367–376.

17. **Saito T, Ikeda T, Nakamura K, Chung UI, Kawaguchi H** (2007) S100A1 and S100B, transcriptional targets of SOX trio, inhibit terminal differentiation of chondrocytes. EMBO Rep 8: 504–509.

18. **Roh J, Cho EA, Seong I, Limb JK, Lee S, et al.** (2006) Down-regulation of Sox10 with specific small interfering RNA promotes transdifferentiation of Schwannoma cells into myofibroblasts. Differentiation 74: 542–551.

19. **Inoue K, Shilo K, Boerkoel CF, Crowe C, Sawady J, et al.** (2002) Congenital hypomyelinating neuropathy, central dysmyelination, and Waardenburg-Hirschsprung disease: phenotypes linked by SOX10 mutation. Ann Neurol 52: 836–842.

20. **Verheij JB, Sival DA, van der Hoeven JH, Vos YJ, Meiners LC, et al.** (2006) Shah-Waardenburg syndrome and PCWH associated with SOX10 mutations: a case report and review of the literature. Eur J Paediatr Neurol 10: 11–17.

21. **Shimotake T, Tanaka S, Fukui R, Makino S, Maruyama R** (2007) Neuroglial disorders of central and peripheral nervous systems in a patient with Hirschsprung's disease carrying allelic SOX10 truncating mutation. J Pediatr Surg 42: 725–731.

22. **Mathon NF, Malcolm DS, Harrisingh MC, Cheng L, Lloyd AC** (2001) Lack of replicative senescence in normal rodent glia. Science 291: 872–875.

23. **Yano F, Kugimiya F, Ohba S, Ikeda T, Chikuda H, et al.** (2005) The canonical Wnt signaling pathway promotes chondrocyte differentiation in a Sox9-dependent manner. Biochem Biophys Res Commun 333: 1300–1308.

24. **Kitamura T** (1998) New experimental approaches in retrovirus-mediated expression screening. Int J Hematol 67: 351–359.

25. **Ikeda T, Kamekura S, Mabuchi A, Kou I, Seki S, et al.** (2004) The combination of SOX5, SOX6, and SOX9 (SOX Trio) provides signals sufficient for induction of permanent cartilage. Arthritis Rheum 50. 3561–3573.

26. **Einheber S, Hannocks MJ, Metz CN, Rifkin DB, Salzer JL** (1995) Transforming growth factor-beta 1 regulates axon/Schwann cell interactions. J Cell Biol 129: 443–458.

27. **Srinivasan R, Sun G, Keles S, Jones EA, Jang SW, et al.** (2012) Genome-wide analysis of EGR2/SOX10 binding in myelinating peripheral nerve. Nucleic acids research 40: 6449–6460.

28. **Monuki ES, Weinmaster G, Kuhn R, Lemke G** (1989) SCIP: a glial POU domain gene regulated by cyclic AMP. Neuron 3: 783–793.

29. **Paratore C, Goerich DE, Suter U, Wegner M, Sommer L** (2001) Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. Development 128: 3949–3961.

30. **Finzsch M, Schreiner S, Kichko T, Reeh P, Tamm ER, et al.** (2010) Sox10 is required for Schwann cell identity and progression beyond the immature Schwann cell stage. J Cell Biol 189: 701–712.

31. **Bondurand N, Girard M, Pingault V, Lemort N, Dubourg O, et al.** (2001) Human Connexin 32, a gap junction protein altered in the X-linked form of Charcot-Marie-Tooth disease, is directly regulated by the transcription factor SOX10. Hum Mol Genet 10: 2783–2795.

32. **Ito Y, Wiese S, Funk N, Chittka A, Rossoll W, et al.** (2006) Sox10 regulates ciliary neurotrophic factor gene expression in Schwann cells. Proc Natl Acad Sci U S A 103: 7871–7876.

33. **Lang D, Epstein JA** (2003) Sox10 and Pax3 physically interact to mediate activation of a conserved c-RET enhancer. Hum Mol Genet 12: 937–945.

34. **Ludwig A, Rehberg S, Wegner M** (2004) Melanocyte-specific expression of dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf transcription factors. FEBS Lett 556: 236–244.

35. **Melnikova IN, Yang Y, Gardner PD** (2000) Interactions between regulatory proteins that bind to the nicotinic receptor beta4 subunit gene promoter. Eur J Pharmacol 393: 75–83.

36. **Peirano RI, Goerich DE, Riethmacher D, Wegner M** (2000) Protein zero gene expression is regulated by the glial transcription factor Sox10. Mol Cell Biol 20: 3198–3209.

37. **Reiprich S, Kriesch J, Schreiner S, Wegner M** (2010) Activation of Krox20 gene expression by Sox10 in myelinating Schwann cells. J Neurochem 112: 744–754.

38. **Schlierf B, Werner T, Glaser G, Wegner M** (2006) Expression of connexin47 in oligodendrocytes is regulated by the Sox10 transcription factor. J Mol Biol 361: 11–21.

39. **Verastegui C, Bille K, Ortonne JP, Ballotti R** (2000) Regulation of the microphthalmia-associated transcription factor gene by the Waardenburg syndrome type 4 gene, SOX10. J Biol Chem 275: 30757–30760.

40. **Bowles J, Schepers G, Koopman P** (2000) Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. Dev Biol 227: 239–255.

41. **Wegner M** (1999) From head to toes: the multiple facets of Sox proteins. Nucleic Acids Res 27: 1409–1420.

42. **Schepers GE, Bullejos M, Hosking BM, Koopman P** (2000) Cloning and characterisation of the Sry-related transcription factor gene Sox8. Nucleic Acids Res 28: 1473–1480.

43. **Sock E, Schmidt K, Hermanns-Borgmeyer I, Bosl MR, Wegner M** (2001) Idiopathic weight reduction in mice deficient in the high-mobility-group transcription factor Sox8. Mol Cell Biol 21: 6951–6959.

44. **Ghislain J, Charnay P** (2006) Control of myelination in Schwann cells: a Krox20 cis-regulatory element integrates Oct6, Brn2 and Sox10 activities. EMBO Rep 7: 52–58.

45. **Donato R** (2003) Intracellular and extracellular roles of S100 proteins. Microsc Res Tech 60: 540–551.

46. **Salama I, Malone PS, Mihaimeed F, Jones JL** (2008) A review of the S100 proteins in cancer. Eur J Surg Oncol 34: 357–364.

47. **Atanasoski S, Boentert M, De Ventura L, Pohl H, Baranek C, et al.** (2008) Postnatal Schwann cell proliferation but not myelination is strictly and uniquely dependent on cyclin-dependent kinase 4 (cdk4). Mol Cell Neurosci 37: 519–527.

48. **Arcuri C, Bianchi R, Brozzi F, Donato R** (2005) S100B increases proliferation in PC12 neuronal cells and reduces their responsiveness to nerve growth factor via Akt activation. J Biol Chem 280: 4402–4414.