Ascl1 is required for oligodendrocyte development in the spinal cord

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Development of oligodendrocytes, myelin-forming glia in the central nervous system (CNS), proceeds on a protracted schedule. Specification of oligodendrocyte progenitors (OLPs) begins early in development, whereas their terminal differentiation occurs at later embryonic and postnatal periods. How these distinct steps are controlled remains unclear. Our previous study demonstrated an important role of the helix-loop-helix (HLH) transcription factor Ascl1 in early generation of OLPs in the developing spinal cord. Here, we show that Ascl1 is also involved in terminal differentiation of oligodendrocytes late in development. Ascl1” mutants showed a deficiency in differentiation of myelin-expressing oligodendrocytes at birth. In vitro culture studies demonstrate that the induction and maintenance of co-expression of Olig2 and Nkx2-2 in OLPs, and thyroid hormone-responsive induction of myelin proteins are impaired in Ascl1” mutants. Gain-of-function studies further showed that Ascl1 collaborates with Olig2 and Nkx2-2 in promoting differentiation of OLPs into oligodendrocytes in vitro. Overexpression of Ascl1, Olig2 and Nkx2-2 alone stimulated the specification of OLPs, but the combinatorial action of Ascl1 and Olig2 or Nkx2-2 was required for further promoting their differentiation into oligodendrocytes. Thus, Ascl1 regulates multiple aspects of oligodendrocyte development in the spinal cord.

KEY WORDS: Cell fate, Oligodendrocyte, Myelin, Glia, Stem cell, Transcription factor, HLH factor, Spinal cord, Mouse

INTRODUCTION

Oligodendrocytes are the major constituent of myelin in the vertebrate CNS, and play important roles in its formation and function (Raff, 1989). Although myelin is formed mostly in the postnatal period in mammals, specification of oligodendrocytes occurs relatively early during development (Noble et al., 2004; Richardson et al., 2006). Early cells in the oligodendrocyte lineage retain the capacity of cell divisions, and thus are called oligodendrocyte progenitors (OLPs) (Barres and Raff, 1994). OLPs are first specified from multipotent progenitors in the ventricular zone (VZ) located on the inner side of the developing neural tube. These specified OLPs subsequently migrate to the forming outer layer called the mantle zone (MZ) and spread throughout the CNS parenchyma (Richardson et al., 2006). These migratory OLPs remain undifferentiated until the perinatal period, and begin to undergo terminal differentiation and express myelin genes mostly in the postnatal period. Thus, oligodendrocyte development occurs on a protracted time course.

Recent studies have revealed that each of these steps occurs at distinct stages in different regions. For example, OLPs in the developing spinal cord had long been thought to arise from a restricted ventral progenitor domain (Rowitch, 2004; Richardson et al., 2006). It has recently been shown, however, that OLPs arise in multiple progenitor domains along the dorsoventral axis at distinct developmental stages (Spassky et al., 1998; Liu et al., 2003; Cai et al., 2005; Vallstedt et al., 2005; Fogarty et al., 2005; Sugimori et al., 2007). Recent studies have also demonstrated multiple origins of oligodendrocytes in the developing forebrain (Kessaris et al., 2006; Yue et al., 2006). In the spinal cord, these specified OLPs express either Nkx2-2 or Olig2, thereby comprising two molecularly distinct populations at early stages (Lu et al., 2002; Zhou and Anderson, 2002; Fu et al., 2002; Liu and Rao, 2004; Danesin et al., 2006; Sugimori et al., 2007). The terminal differentiation of these OLPs also occurs at specific stages in distinct regions. In the rodent spinal cord, myelin gene expression is initiated in the dorsal and ventral regions near the midline around birth (Fu et al., 2002; Wang et al., 2006). Subsequently, it spreads laterally along the forming white matter, and then gradually proceeds in the inner gray matter postnatally. Thus, both specification and terminal differentiation of oligodendrocytes are under precise spatiotemporal control.

Previous studies have demonstrated that multiple classes of transcription factors are involved in this process. They include the HLH factors Olig1 and Olig2 (Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002), Ascl1 (Kondo and Raff, 2000a; Wang et al., 2001; Parras et al., 2004; Parras et al., 2007; Gokhan et al., 2005; Sugimori et al., 2007), and Id2, Id4 and Hex5 (Kondo and Raff, 2000b; Wang et al., 2001; Samanta and Kessler, 2004; Liu et al., 2006), the homeodomain factors Nkx2-2 and Dlx1/2 (Qi et al., 2001; Fu et al., 2002; Liu et al., 2007; Petryniak et al., 2007), zinc-finger factor Zfp488 (Wang et al., 2006), and multiple members of the Sox family (Stolt et al., 2002; Stolt et al., 2003; Stolt et al., 2004; Stolt et al., 2006; Sohn et al., 2006). How these molecules control the timing of oligodendrocyte development, however, is not yet fully understood. Our recent studies have shown that Ascl1 controls specification of OLPs at an early embryonic stage in the spinal cord and forebrain (Sugimori et al., 2007; Parras et al., 2007). Interestingly, recent studies have shown that Ascl1 is also expressed in postnatal and adult OLPs (Parras et al., 2007; Aguirre et al., 2007; Kim et al., 2007). Its in vivo role in oligodendrocyte development,
however, remains unclear. Here, we show that Ascl1 plays an important role in differentiation of OLPs into myelin-expressing oligodendrocytes at late embryonic stages in the spinal cord.

**MATERIALS AND METHODS**

**Animals**

All animal procedures were performed according to the guidelines and regulations of the Institutional Animal Care and Use Committee and the National Institute of Health. The maintenance and genotyping of Ascl1<sup>−/−</sup> (Parras et al., 2002) and Ascl1::GFP mice (Gong et al., 2003) were described previously (Parras et al., 2007). Embryos and pups of the wild-type and mutant mice and Sprague-Dawley rats were collected from timed-pregnant females.

**Immunostaining**

Rabbit anti-Nkx2-2 and guinea pig anti-Olig2 antibodies were kind gifts from Dr T. Jessell at Columbia University. Mouse monoclonal antibody for Nkx2-2 was obtained from the Developmental Studies Hybridoma Bank at the Iowa University. Rabbit antibodies for Ascl1, Olig1, Olig2 and Pax6 have been described previously (Mizuguchi et al., 2001). Antibodies for following antigens were purchased from commercial sources: Ascl1, platelet-derived growth factor receptor α (PDGFRα) and activated caspase 3 (BD Bioscience); O4, galactocerebrosidase (GalC), NG2 (chondroitin sulfate proteoglycan 4, Cspg4), myelin basic protein (MBP) and myelin-associated glycoprotein (MAG) (Millipore); β-3′-cyclic-nucleotide 3′-phosphodiesterase (CNP; CNP1) and glial fibrillary acidic protein (GFAP) (Sigma-Aldrich); β-tubulin type III (TuJ1) (Babco); Sox10 (Santa Cruz); and green fluorescent protein (GFP) (Invitrogen). Labeling of dividing cells with 5-bromo-2′-deoxyuridine (BrDU) was performed by administering BrdU (50 mg/kg) to pregnant animals 2 hours before sampling of embryos. Staining was visualized with secondary antibodies conjugated with Alexa Fluor 488, 555, 568, 594 and 633 (Invitrogen), and images were obtained using Zeiss LSM-510 confocal microscope or Apotome as described previously (Sugimori et al., 2007; Parras et al., 2007).

**Reverse transcriptase polymerase chain reaction (RT-PCR) and in situ hybridization**

The expression of Ascl3 and Ascl5 in the developing spinal cord was examined by RT-PCR and in situ hybridization. The following primers were used to obtain cDNAs encoding the predicted full-length open reading frames of Ascl3 and Ascl5 (sequences corresponding to the predicted initiation and termination codons are underlined): Ascl3, 5′-GAAAGGATGATCACCCAGAAACGCT-3′ and 5′-CTGTTCCTAATGACTTCACGAG-3′; and Ascl5, 5′-CATTGACACAGTAC-3′ and 5′-GCCAGATCGAAGGCTGGGT-3′. cDNAs reverse transcribed from total RNAs isolated from embryonic (E)10.5 and E16.5 mouse spinal cords were used as templates. The identity of PCR products was verified by sequencing. These cDNAs were used for in situ hybridization as described previously (Mizuguchi et al., 2001).

**Cell culture**

Neurosphere culture and infection of recombinant retroviruses were performed as described previously (Sugimori et al., 2007). The titers of retroviruses used for neurosphere formation were adjusted to infect ~70% of total cells in culture. In double infections, retroviruses were co-expressed to ensure that more than 85% of GFP+ cells co-expressed both viruses to ensure that more than 85% of GFP+ cells co-expressed both viruses. In double infections, retroviruses were co-expressed to ensure that more than 85% of GFP+ cells co-expressed both viruses.

**RESULTS**

**Expression of Ascl1 in OLPs at late embryonic stages**

Our previous study has shown that Ascl1 expression in OLPs occurs transiently at the time when they are specified in the VZ (Sugimori et al., 2007). Its expression pattern at later stages, however, has not yet been examined in details. Thus, we first followed the developmental time course of Ascl1 expression in the rat spinal cord.

At E14.5, Olig2<sup>+</sup> and Nkx2-2<sup>+</sup> cells scattered in the spinal cord did not express Ascl1 at a detectable level, except for those in and adjacent to the VZ (see Sugimori et al., 2007). The majority of them were positive for O4 and PDGFRα, and thus considered to be OLPs (Fu et al., 2002; Liu and Rao, 2004; Danesin et al., 2006; Sugimori et al., 2007). From E16.5 onwards, however, Olig2<sup>+</sup> and Nkx2-2<sup>+</sup> cells beneath the pial surface began to express Ascl1. In particular, the majority of Olig2<sup>+</sup> (95%, 112/118 cells examined) and Nkx2-2<sup>+</sup> (58%, 88/153) cells were Ascl1<sup>+</sup> near the dorsal and ventral midline areas and the lateral margin of the MZ (Fig. 1A, boxed areas and inset, Fig. 1B,C). In the same areas, 68% (131/194) of Olig2<sup>+</sup> cells expressed Nkx2-2, and, conversely, 92% (131/142) of Nkx2-2<sup>+</sup> cells were Olig2<sup>+</sup> (Fig. 1D).

At E18.5, the frequency of co-expression of Ascl1, Olig2 and Nkx2-2 increased in both inner and outer parts of the MZ (Fig. 1E-H). Yet, OLPs that just began to leave the VZ at this late stage did not co-express Olig2 and Nkx2-2, and remained negative for Ascl1 (Fig. 1I, and data not shown). At E20.5 and P0, however, the majority of OLPs appeared to co-express these transcription factors throughout the MZ (Fig. 2A-D,LI). Following their co-expression, the first population of GalC<sup>+</sup> and MBP<sup>+</sup> oligodendrocytes emerged at E20.5 (Fig. 2E-H) and increased in number at P0 (Fig. 2M-O) beneath the pial surface. These myelin-expressing cells expressed Ascl1 and Olig2 (Fig. 2E-H,K-O).

To further examine the relationship between Ascl1 expression and OLP development, we used Ascl1::GFP mice in which GFP expression is driven by the Ascl1 locus on a transgene (Gong et al., 2003). In this reporter line, GFP protein sustains longer than endogenous Ascl1 so that progeny of Ascl1<sup>+</sup> progenitors can be transiently marked as GFP<sup>+</sup> cells (Parras et al., 2007). In fact, more GFP<sup>+</sup> cells than Ascl1<sup>+</sup> cells were detected in Ascl1::GFP mice at E16.5, the stage when both neurons and OLPs are generated (Fig. 3A-A′). (Helms et al., 2005; Mizuguchi et al., 2006; Sugimori et al., 2007). Many of these GFP<sup>+</sup> cells, especially those in the dorsal MZ, were negative for PDGFRα and expressed Ascl1, Nkx2-2, Olig2, and a significant fraction of PDGFRα<sup>+</sup> cells were negative for GFP in the dorsal MZ (Fig. 3C-C′, arrows). These dorsal GFP/PDGFRα<sup>+</sup> cells might have derived from Ascl1<sup>+</sup> progenitors or downregulated GFP during migration. Nevertheless, most of the PDGFRα<sup>+</sup> cells detected near the pial surface were GFP<sup>+</sup> in both the ventral and dorsal aspects of the spinal cord, and some of them expressed...
endogenous Ascl1 (Fig. 3G-H''). These results demonstrate that Ascl1 expression occurs in a significant fraction of OLPs late in development.

**Temporal correlation between Ascl1 and OLP differentiation**

We next used dissociated culture to quantify the frequency of co-expression of Ascl1 with other oligodendrocyte markers. As observed in sections, the total numbers of Ascl1+ Olig2+ and Nkx2-2+ cells markedly increased between E18.5 and E20.5, which occurred in parallel to the emergence of MBP+ and GalC+ oligodendrocytes (Fig. 4A,B). At E18.5, 89% of Nkx2-2+ cells and 63% of Olig2+ cells were NG2+ OLPs (Fig. 4D). Yet, a significant fraction of Nkx2-2+ cells remained Olig2–, and many Olig2+ cells were Nkx2-2– at this stage. At E20.5, however, a higher fraction of cells co-expressed Olig2 and Nkx2-2. In parallel to their co-expression, the percentage of Ascl1+ cells in Olig2+ and Nkx2-2+ cells also increased. We could not directly determine the number of triple-positive cells for technical reasons, and therefore performed a series of double staining to estimate the number of triple-positive cells (Fig. 4C, parts a-c, D). Their estimated number remained small until E18.5 (Fig. 4A). At E20.5, however, more than half of total Olig2+ cells were estimated to be Ascl1+/Nkx2-2+, and about two-thirds of the Nkx2-2+ cells were considered to be Ascl1+/Olig2+.

Moreover, the majority of NG2+, GalC+ and MBP+ cells expressed these markers.

At P0, a significant fraction of Olig2+ cells became negative for Ascl1 and Nkx2-2 (Fig. 2I, asterisks; Fig. 4D). These cells could become astrocytes at postnatal stages (Masahira et al., 2006; Cai et al., 2007) or remain as undifferentiated OLPs up to adulthood (Yamamoto et al., 2001; Kitada and Rowitch, 2006; Ohori et al., 2006). Moreover, many MBP+ cells became negative for Ascl1 at
this stage (Fig. 4D). This could be because Ascl1 expression in myelin+ cells is transient or, alternatively, because a separate population of oligodendrocytes emerges from Ascl1-negative OLPs postnatally. In summary, these results demonstrate a temporal correlation between the co-expression of Ascl1 with Olig2 and Nkx2-2, and terminal differentiation of oligodendrocytes.

**Recovery of OLPs in late development in Ascl1−/− mutant spinal cords**

We next examined late-stage OLP development in Ascl1−/− mutant mice. Despite a significant reduction of OLPs at early stages in the mutant (Sugimori et al., 2007), we found no obvious difference in the density or distribution pattern of Olig2+ and Nkx2-2+ cells isolated from E18.5 mutants were similar to those in the wild type (1.85±0.22×10^5 Olig2+ cells per spinal cord, corresponding to 103±12% of the wild-type level; 1.54±0.19×10^5 Nkx2-2+ cells and 101±12%, n=3). The majority of these cells were NG2+, PDGFRα+, and Sox10+, indicating that they retained the characteristics of OLPs.

To follow the recovery of OLPs in Ascl1−/− mutants, we examined earlier stages. In our previous study (Sugimori et al., 2007), we detected a significant reduction (60-90%) in the number of OLPs in both the VZ and MZ at E12.5 and E14.5, the stages when early OLPs are generated in the ventral spinal cord. The number of OLPs recovered at later stages in Ascl1 mutants, probably because of continuous production of OLPs in multiple progenitor domains over a prolonged period late in development.

The above results suggest that molecules other than Ascl1 promote OLP specification Ascl1−/− mice. We hypothesized that HLH factors related to Ascl1 exert such a function. In fact, we found that two Ascl1-related genes, Ascl3 and Ascl5 (Mouse Genome Informatics: http://www.informatics.jax.org/) are expressed in the developing spinal cord at both E10.5 and E16.5 (Fig. 5A). At E10.5, the expression of Ascl3 and Ascl5 mRNAs was detected in both the dorsal and ventral aspects of the VZ, but excluded from the Olig2+ motoneuron progenitor domain (pMN), similarly to Ascl1 (Fig. 5B-D). We also examined the activity of Ascl3 and Ascl5 in vitro. Our previous study has shown that Ascl1 promotes both neurogenesis and oligodendrogenesis in E13.5 spinal cord-derived multipotent progenitors (Sugimori et al., 2007). Under the same conditions, retrovirus-mediated overexpression of Ascl3 and Ascl5 also promoted differentiation of TuJ1+ neurons and O4+ oligodendrocytes at the expense of GFAP+ astrocytes (Fig. 5E-G).
These results suggest that Ascl3 and Ascl5 are involved in oligodendrogenesis in Ascl1–/– spinal cords. Their in vivo function, however, awaits further studies.

Defect in oligodendrocyte differentiation in Ascl1–/– mice

We next asked whether Ascl1 plays any role in differentiation of oligodendrocytes late in development. As described above, the numbers of Olig2+ and Nkx2-2+ cells were similar between the wild type and mutant at E18.5. Staining for activated caspase 3 and labeling of dividing cells with BrdU did not detect any significant difference in the rate of cell proliferation or death between the wild-type and mutant spinal cords. Nevertheless, Ascl1–/– spinal cords showed a severe defect in myelin protein expression at birth. In the wild type, oligodendrocytes expressing CNP, MAG and MBP were detected at P0 in the ventrolateral and dorsomedial outer layer, which corresponded to the forming white matter (Fig. 6A-D, arrowheads). In Ascl1–/– mutants, however, only a few myelin+ oligodendrocytes were found in the corresponding regions (Fig. 6E-H). Quantitative analyses demonstrated a more than 80% reduction of myelin+ oligodendrocytes in Ascl1–/– mice (Fig. 5I).

Ascl1–/– mice die at birth, and therefore differentiation of Ascl1–/– OLPs was examined in vitro. Cells isolated from E18.5 spinal cords were cultured in the presence and absence of thyroid hormone (TH) to stimulate oligodendrocyte differentiation (Kondo and Raff, 2000a). When NG2+ OLPs undergo terminal differentiation, they first become NG2+/GalC+ intermediate cells, and subsequently differentiate into NG2–/GalC+ and MBP+ oligodendrocytes. In the wild-type culture, a significant fraction of cells was GalC+ at day 1 after plating (DAP1) (5.9±1.5% of total cells, n=3), and about a half of them (49±13%) had already proceeded to the NG2+/GalC– state. In the mutant culture, GalC+ cells were smaller in percentage, and the majority of them (89±11%) remained as NG2+/GalC– cells (Fig. 6K). Likewise, 3.6±0.8% (n=3) of wild-type cells already expressed MBP at DAP1, and their percentage significantly increased at DAP7 (Fig. 6L,N). MBP+ cells were further increased about twofold (2.4±0.5-fold) by treatment with TH (Fig. 6N). By contrast, few cells were MBP+ in the mutant culture at DAP1, and a much lower percentage of cells were MBP+ cells at DAP7 (Fig. 6M,N). Their percentage remained lower in the mutant culture (4.3±0.8%) than in the wild type (14.5±2.9%) at DAP14. Moreover, TH-dependent increase of MBP+ cells in the mutant culture (1.4±0.3-fold compared with untreated culture) was significantly smaller than that in the wild type (4.3±0.8%) at DAP14. However, the number of oligodendrocytes among total cells did not significantly differ between the wild-type and mutant cells (16.7±4.5% for the wild type and 15.8±2.9% for the mutant at DAP7). Thus, a loss of OLPs during culture is...
unlikely to account for the differentiation defect of mutant cells. These results suggest that the severe loss of myelin protein-expressing cells in the mutant is attributable to attenuated or delayed differentiation of OLPs.

Defect in the co-expression of Olig2 and Nkx2-2 in Ascl1−/− OLPs

We next asked whether the observed defect in Ascl1−/− mutants is related to the co-expression of Olig2 and Nkx2-2. At E16.5, many Olig2+ cells were Nkx2-2+ in the ventral white matter of the wild-type spinal cord (Fig. 7A, arrowheads). By contrast, only a few double-positive cells were detected in the corresponding region of the mutant. We further examined the co-expression of Olig2 and Nkx2-2 in vitro. Proliferative cells were enriched in culture of E16.5 spinal cords by treatment with growth factors for 7 days. At the time of growth factor withdrawal (DAP7), 57.6±15.7% (n=3) of the total cells were Olig2+. Most of these Olig2+ cells (>95%) were NG2+ and Sox10+, but few of them were Sox2+ (<0.5%), indicating that Olig2+ OLPs were enriched in this culture. At this stage of culture, only a small fraction of Olig2+ cells were Nkx2-2+ in both wild-type and mutant cultures (Fig. 7B). However, when the culture was continued for additional 4 days (DAP11), many Olig2+ cells became Nkx2-2+ in the wild-type culture, and the percentage of Olig2+/Nkx2-2+ cells among total cells increased from 1.7±0.8% to 18.1±1.8% (P<0.01). By contrast, a much smaller fraction of Olig2+ cells became Nkx2-2+ in culture of Ascl1−/− cells. The percentage of Olig2+ cells among total cells remained similar between the wild-type and mutant cultures at DAP11 (26.2±2.3% and 28.5±5.1%, respectively), and thus the loss of Olig2+ cells did not account for the lack of co-expression of Nkx2-2. These results suggest that the induction of Nkx2-2 expression is impaired in Ascl1−/− Olig2+ cells.

Nevertheless, 87.7±6.9% (n=3) of Olig2+ cells acutely isolated from E18.5 mutants co-expressed Nkx2-2 (Fig. 7C). Thus, the defect observed in vitro is likely to reflect a delay of the expression of Nkx2-2.

We also asked whether Ascl1 is involved in the maintenance of co-expression of Olig2 and Nkx2-2. The vast majority of Olig2+ cells isolated from E18.5 embryos co-expressed Nkx2-2 (Fig. 7C). Moreover, 84.6±8.6% of wild-type Olig2+ cells were Ascl1+ at this stage. The high percentage of co-expression Nkx2-2 in Olig2+ cells was maintained for 7 days in the wild-type culture, whereas only about a half of Ascl1−/− Olig2+ cells remained Nkx2-2+. However, most Nkx2-2+ cells remained Olig2+ (>90%) at both DAP1 and DAP7, and the number of Olig2+ cells did not significantly differ between the wild-type and mutant cultures (data not shown). Thus, mutant Olig2+ OLPs were defective in maintaining the expression of Nkx2-2. These results demonstrate that one of the actions of Ascl1 in late-stage OLPs is to properly induce and maintain the co-expression of Olig2 and Nkx2-2.

Collaborative actions of Ascl1, Olig2 and Nkx2-2

We next performed gain-of-function studies using neurosphere culture. Multipotent progenitors from E13.5 spinal cords were infected with GFP retroviruses that expressed Olig2, Nkx2-2 and Ascl1 either alone or in combination. Although the expression level of endogenous and exogenous factors varied in individual cells, virus-mediated expression conferred about the same or a two-fold higher level of respective transcription factors compared with endogenous proteins (Fig. 8A-D). In neurosphere culture, specified OLPs were first detected as NG2+ cells, and they subsequently differentiated into GalC+ oligodendrocytes (Fig. 8E,F). We first compared the percentages of NG2+ and GalC+ cells among virus-infected cells (Fig.

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**Fig. 5. Expression and activity of Ascl3 and Ascl5.** (A) Expression of Ascl3 and Ascl5 in the spinal cord at E10.5 and E16.5. mRNA expression was detected by RT-PCR using reverse-transcribed (RT+) and non-transcribed (RT−) RNA samples as templates. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as internal control. (B–D) Expression of Ascl1 protein, and of Ascl3 and Ascl5 mRNA in the VZ of the mouse spinal cord at E10.5. The neural tube is outlined in C,D. Brackets indicate the position of the Olig2+ motoneuron progenitor domain (pMN). The horizontal lines indicate the boundary of the dorsal and ventral aspects of the VZ. (E–G) Neurogenic and oligodendrogenic activity of Ascl3 and Ascl5. Neurospheres derived from E13.5 spinal cords were infected with GFP retroviruses expressing Ascl1, Ascl3 and Ascl5, and subsequently induced to differentiate for 4 (E) or 10 (F,G) days. The percentages of GFP+ cells that differentiated into TuJ1+ neurons, O4+ oligodendrocytes and GFAP+ astrocytes were quantified (mean±s.d., three independent experiments). *P<0.01 compared with control virus-infected culture. Scale bar: 100 μm.
Consistent with previous reports (Zhou et al., 2001; Sun et al., 2001; Liu et al., 2007), both Olig2 and Nkx2-2 increased the fraction of oligodendrocyte lineage cells among total cells. Olig2 increased both NG2+ OLPs and GalC+ oligodendrocytes, whereas Nkx2-2 increased NG2+ but not GalC+ cells. Like Olig2, Ascl1 increased both NG2+ and GalC+ cells. Thus, each of these transcription factors retained the ability to promote OLP specification. These effects were not observed in GFP- cells within the same culture (data not shown), indicating their cell-autonomous actions. The rate of cell death did not significantly differ between cultures infected with different viruses, indicating that elimination of cells with specific phenotypes is unlikely to account for the observed effects.

Fig. 6. Defect in oligodendrocyte differentiation in Ascl1−/− spinal cords. (A-H) Co-immunostaining of CNP, MAG and MBP with Olig2 in wild type (A-D) and Ascl1−/− mutants (E-H). Boxed areas in A and E indicate the regions shown in other panels. Arrowheads indicate double-positive cells. Asterisks indicate non-specific staining outside the spinal cord. (I) Reduction of myelin+ oligodendrocytes in Ascl1−/− mutants at P0. Data are mean±s.d. obtained from staining of five or six sections derived from three embryos for each genotype. The percentage of the mutant level compared with the wild type is shown for each marker. *P<0.01. (J-M) Expression of GalC and NG2 (J,K) and MBP (L,M) in culture of wild-type and Ascl1−/− embryos. Cells from E18.5 spinal cords were cultured for 7 days. In J and K, arrows indicate NG2+/GalC− oligodendrocytes, whereas arrowheads indicate NG2+/GalC+ intermediate cells. In L and M, arrows indicate MBP+ oligodendrocytes. Cell nuclei were stained with DAPI (blue). (N) Differentiation of MBP+ oligodendrocytes in vitro. Culture of E18.5 spinal cords was performed either the presence (+) or absence (−) of TH, and the percentage of MBP+ cells among total cells was quantified at DAP1 and DAP7 (mean±s.d., three independent experiments). Parentheses show the percentages of the mutant level compared with the wild type. *P<0.05, **P<0.01 compared with the wild type. Scale bars: in A,E, 200 μm; in D,H, 50 μm for B-D,F-H; in M, 50 μm for J-M.

Fig. 7. Defect in the co-expression of Olig2 and Nkx2-2 in Ascl1−/− spinal cords. (A) Co-expression of Olig2 and Nkx2-2 in vivo at E16.5. Arrowheads indicate Nkx2-2+/Olig2− cells in the ventral white matter. Scale bar: 50 μm. (B,C) Expression of Nkx2-2 in Olig2+ OLPs in vitro. Spinal cord cells were isolated from either E16.5 (B) or E18.5 (C) embryos, and the expression of Nkx2-2 in Olig2+ cells was compared between the wild type and Ascl1−/− mutants at indicated time points. Data are mean±s.d. (n=3). *P<0.01 compared with DAP7; %P<0.01 compared with the wild type.
To distinguish their actions on the early specification and late maturation steps, we next measured the shift from NG2+/GalC− OLPs to NG2−/GalC+ oligodendrocytes among total oligodendrocyte lineage cells (defined as NG2+ and/or GalC+ cells) (Fig. 9A-C). In control virus-infected culture, only a small fraction of GFP+ cells (3.9±2.3%, n=4) differentiated into cells in the oligodendrocyte lineage. Yet, 57.4% of them proceeded to the mature NG2−/GalC+ stage (Fig. 9D), and most of them expressed endogenous Olig2, Nkx2-2 and Ascl1 (data not shown). By contrast, this NG2−/GalC+ fraction was much smaller in Olig2- and Nkx2-2-virus infected culture (4.4% and 18.7%, respectively, P<0.01), despite a significant increase of oligodendrocyte lineage cells among total cells (Fig. 9D). Moreover, their co-overexpression did not stimulate the shift to NG2−/GalC+ cells: the vast majority of cells remained as NG2+/GalC− cells even after prolonged culture (data not shown).

Given that both Olig2 and Nkx2-2 are expressed in mature oligodendrocytes in vivo, it is unlikely that their virus-mediated sustained expression accounted for attenuated differentiation. Thus, although the co-expression of Olig2 and Nkx2-2 coincides with the emergence of myelin+ oligodendrocytes in vivo, increased expression of each of these factors or their combination was not sufficient to promote differentiation of OLPs into oligodendrocytes.
In Ascl1 virus-infected culture, the majority remained as NG2*/GalC* intermediate cells (69.5%), and only a small fraction (11.2%) proceeded to NG2*/GalC* cells (Fig. 9B,D). Importantly, the combinatorial expression of Ascl1 and Olig2 markedly stimulated this transition: 51.2% of total oligodendrocyte lineage cells become NG2*/GalC* cells. Similarly, a significantly higher percentage (34.8%) became NG2*/GalC* cells in culture co-infected with Ascl1 and Nkx2-2 viruses. Given that overexpression of Ascl1 alone promoted the generation of NG2* OLPs that expressed endogenous Olig2 or Nkx2-2, these results suggest that simultaneous elevated expression of Ascl1 and Olig2 or Ascl1 and Nkx2-2 is necessary for promoting the transition from OLPS to oligodendrocytes.

**DISCUSSION**

**Role of Ascl1 in late-stage oligodendrocyte development**

We have demonstrated that the HLH transcription factor Ascl1 plays an important role in late-stage development of oligodendrocytes in the spinal cord. Although Olig2* and Nkx2-2* OLPS migrating in the MZ did not express Ascl1 at early stages, they became Ascl1* around the perinatal stage in the forming white matter. This late Ascl1 expression coincided with the co-expression of Olig2 and Nkx2-2. Moreover, Ascl1* mutant Olig2* OLPS showed a defect in the induction and maintenance of co-expression of Nkx2-2. The subsequent expression of myelin proteins was also attenuated in the mutant at birth. In vitro, Ascl1 promoted differentiation of OLPS into oligodendrocytes in collaboration with Olig2 and Nkx2-2.

It is noteworthy that Ascl1 expression is biphasic during the course of oligodendrocyte development. This is in sharp contrast with the sustained expression of other transcription factors involved in oligodendrogenesis. For example, the expression of Olig2, Nkx2-2, Sox5 and Sox9 begins early in multipotent progenitors and continues in OLPS during late embryogenesis (Qi et al., 2001; Lu et al., 2002; Takebayashi et al., 2002; Stolt et al., 2003; Stolt et al., 2006). Olig1, Sox8 and Sox10 are induced in specified OLPS and maintained in mature oligodendrocytes (Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002; Stolt et al., 2002; Stolt et al., 2004). By contrast, Ascl1 expression is transient at the time of OLPS specification, and once specified, OLPS remain negative for Ascl1 until the onset of their terminal differentiation.

How does Ascl1 control late-stage oligodendrocyte differentiation? Given that the co-expression of Olig2 and Nkx2-2 is impaired in Ascl1* mutants, the severe reduction of myelin-expressing oligodendrocytes at birth could be attributable, at least in part, to this defect. In fact, the reported phenotypes of Nkx2-2* mice are reminiscent of those observed in Ascl1* mutants (Qi et al., 2001). However, our preliminary study suggests that the expression of Olig2 or Nkx2-2 is not under direct transcriptional control by Ascl1 (S.M. and M.N., unpublished). Our data also suggest that Ascl1 has a role other than their co-expression. Combinatorial overexpression of Olig2 and Nkx2-2 was not sufficient to promote the transition from OLPS to oligodendrocytes, whereas the combination of Ascl1 with Olig2 or Nkx2-2 strongly stimulated this maturation step. Moreover, differentiation of MBP* cells was poorly stimulated by TH in culture of Ascl1* cells, suggesting that Ascl1 is involved in regulating the responsiveness to TH. Interestingly, a previous study has shown that Ascl1 upregulates expression of the TH receptor TRβ1 in cultured OLPS (Kondo and Raff, 2000a). These results support the idea that Ascl1 regulates late-stage differentiation of oligodendrocytes at two steps: the co-expression of Olig2 and Nkx2-2, and the subsequent TH-responsive myelin gene expression (Fig. 10).

**Cooperation of Ascl1 with Olig2 and Nkx2-2 in oligodendrocyte development**

Ascl1 cooperated with Olig2 and Nkx2-2 to promote differentiation of OLPS into oligodendrocytes. It remains unknown at present what mechanisms underlie their collaborative actions. These transcription factors could cooperatively regulate the same set of downstream genes or, alternatively, control independent sets of genes that, in turn, mediate their collaborative actions. Given that combinatorial overexpression of these transcription factors was required for differentiation of OLPS, their overall expression levels and/or ratios could be an important determinant for terminal differentiation of oligodendrocytes. Previous studies have shown that various inhibitory HLH factors negatively regulate myelin gene expression (Gokhan et al., 2005; Liu et al., 2006). Thus, it could be that the expression of Ascl1, Olig2 and Nkx2-2 needs to reach a certain level to counteract these inhibitors. Moreover, given that they act in both specification and differentiation of oligodendrocytes, additional molecules are likely to cooperate with them at each of these steps. A possible candidate is the Sox family of transcription factors. The reported defects in Sox9*-, Sox10* and their double mutants are reminiscent of those in Ascl1* mice (Stolt et al., 2003; Liu et al., 2007). Sox10 and Ascl1 have been shown to synergize to activate transcription of a MBP enhancer-driven reporter in vitro (Gokhan et al., 2005; Liu et al., 2006).

**Ascl1-dependent and -independent oligodendrocyte development**

Generation of OLPS at early stages is severely impaired in the Ascl1* mutant spinal cord (Sugimori et al., 2007). This could be attributable to a defect in either specification or proliferation of early OLPS. The number of Olig2* and Nkx2-2* OLPS, however, recovered to the wild-type level around the perinatal stage. These cells expressed PDGFRα, Sox10 and NG2, suggesting that they retained the characteristics of OLPS. Thus, the differentiation defect of Ascl1* OLPS at late stages appears not to be a mere consequence of their early specification defect. It remains possible, however, that mutant OLPS are defective in a manner that is not discernible using commonly used OLPS markers, at the time of their specification, and that such an early defect is responsible for impaired differentiation at later stages.

Several lines of evidence support the idea that Ascl1 is required cell-autonomously in oligodendrocytes. Recent studies have provided genetic evidence for the expression of Ascl1 in the
oligodendrocyte lineage (Battiste et al., 2007; Parras et al., 2007; Kim et al., 2007). Moreover, overexpression of Ascl1 stimulated differentiation of oligodendrocytes in a cell-autonomous manner in vitro. Our previous study has also shown that Ascl1–/– mutant cells poorly differentiate into oligodendrocytes when grafted into the wild-type mice (Parras et al., 2004). These results, however, do not exclude the possibility that Ascl1 also regulates oligodendrogenesis in a non-cell-autonomous manner, i.e. through the regulation of other cell lineages. It should also be noted that the defect in oligodendrocyte differentiation late in development was not complete in Ascl1–/– mutants. It remains unknown whether this is simply due to delayed differentiation of all OLPs or to the inability of a subpopulation of OLPS to differentiate. Our previous studies have shown a partial or transient defect in OLP specification in the mutant brain (Parras et al., 2007). Wang et al. (Wang et al., 2001) reported that OLPs isolated from postnatal optic nerves of Ascl1–/– mice show no noticeable defect in vitro. Thus, it is likely that there are Ascl1-dependent and -independent oligodendrocytes. The latter could originate from Ascl1 non-expressing cells or, alternatively, they could derive from Ascl1-expressing cells by Ascl1-independent mechanisms. We found that the Ascl1-related factors Ascl3 and Ascl5 are expressed in the developing spinal cord, and that like Ascl1, they are capable of promoting oligodendrogenesis in vitro. The redundant function of these Ascl factors may explain partial defects in both neurogenesis and oligodendrogenesis in many areas of the developing CNS in Ascl1–/– mutants (Parras et al., 2002; Casarosa et al., 1999; Tortii et al., 1999; Helms et al., 2005; Mizuguchi et al., 2006; Sugimori et al., 2007; Parras et al., 2007). Further understanding of the roles of Ascl1 and related HLH factors in oligodendrocytes should provide better insights into the mechanisms underlying development of this important glial cell type in the vertebrate CNS.

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References


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