Introduction

Recent molecular and genetic studies have established that oligodendrocytes originate from specific loci of the ventral neuroepithelium along the entire neuroaxis (Noll and Miller, 1993; Pringle and Richardson, 1993; Timsit et al., 1995; Spassky et al., 1998). In the developing rodent spinal cord, expression of early oligodendrocyte marker genes, such as Olig2, Pdgfra and Sox10, is initially detected in the motoneuron precursor domain (pMN) of the ventral neuroepithelium (Sun et al., 1998; Lu et al., 2000; Zhou et al., 2000; Fu et al., 2002). Thus, it is thought that motoneurons and oligodendrocytes are sequentially generated from the same precursor cells at different time windows, with motoneurons being generated first followed by oligodendrocyte production (Richardson et al., 1997; Richardson et al., 2000). After oligodendrocyte progenitor cells (OPCs) are born from the ventricular neuroepithelial cells, they migrate rapidly into the surrounding gray and white matter regions where they undergo further proliferation and differentiation. Interestingly, the Olig2+/Pdgfra+ OPCs start to acquire Nkx2.2 expression either before (in chicken) or after (in rodents) their migration into the white matter (Fu et al., 2002). The co-expression of Olig2 and Nkx2.2 appears to be a prerequisite for oligodendrocyte differentiation and maturation (Zhou et al., 2001; Sun et al., 2001; Fu et al., 2002).

During early neural development, the Nkx6.1 homeodomain neural progenitor gene is specifically expressed in the ventral neural tube, and its activity is required for motoneuron generation in the spinal cord. We report that Nkx6.1 also controls oligodendrocyte development in the developing spinal cord, possibly by regulating Olig gene expression in the ventral neuroepithelium. In Nkx6.1 mutant spinal cords, expression of Olig2 in the motoneuron progenitor domain is diminished, and the generation and differentiation of oligodendrocytes are significantly delayed and reduced. The regulation of Olig gene expression by Nkx6.1 is stage dependent, as ectopic expression of Nkx6.1 in embryonic chicken spinal cord results in an induction of Olig2 expression at early stages, but an inhibition at later stages. Moreover, the regulation of Olig gene expression and oligodendrogenesis by Nkx6.1 also appears to be region specific. In the hindbrain, unlike in the spinal cord, Olig1 and Olig2 can be expressed both inside and outside the Nkx6.1-expressing domains and oligodendrogenesis in this region is not dependent on Nkx6.1 activity.

Summary

Region-specific and stage-dependent regulation of Olig gene expression and oligodendrogenesis by Nkx6.1 homeodomain transcription factor

Rugao Liu1,*, Jun Cai1,*, Xuemei Hu1,*, Min Tan1, Yingchuan Qi1, Michael German2, John Rubenstein3, Maike Sander4 and Mengsheng Qiu1,†

1Department of Anatomical Sciences and Neurobiology, School of Medicine, University of Louisville, Louisville, KY 40292, USA
2Department of Medicine, University of California, San Francisco, CA 94143, USA
3Department of Psychiatry, University of California, San Francisco, CA 94143, USA
4Department of Developmental and Cell Biology, University of California at Irvine, 4228 McGaugh Hall, Irvine CA 92697-2300, USA

*These authors contributed equally to this work
†Author for correspondence (e-mail: m0qiu001@louisville.edu)

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Summary

During early neural development, the Nkx6.1 homeodomain neural progenitor gene is specifically expressed in the ventral neural tube, and its activity is required for motoneuron generation in the spinal cord. We report that Nkx6.1 also controls oligodendrocyte development in the developing spinal cord, possibly by regulating Olig gene expression in the ventral neuroepithelium. In Nkx6.1 mutant spinal cords, expression of Olig2 in the motoneuron progenitor domain is diminished, and the generation and differentiation of oligodendrocytes are significantly delayed and reduced. The regulation of Olig gene expression by Nkx6.1 is stage dependent, as ectopic expression of Nkx6.1 in embryonic chicken spinal cord results in an induction of Olig2 expression at early stages, but an inhibition at later stages. Moreover, the regulation of Olig gene expression and oligodendrogenesis by Nkx6.1 also appears to be region specific. In the hindbrain, unlike in the spinal cord, Olig1 and Olig2 can be expressed both inside and outside the Nkx6.1-expressing domains and oligodendrogenesis in this region is not dependent on Nkx6.1 activity.

Supplemental data available online

Key words: Oligodendrocyte development, Nkx6.1 mutation, In ovo electroporation, Spinal cord, Olig2, Nkx2.2
domain expressing a unique combination of transcription factors and generating a particular class of neurons (V0-V3 interneurons and motoneurons) (McMahon, 2000; Briscoe and Ericson, 2001). Motoneurons are produced from the pMN progenitor domain which expresses Nkx6.1/Pax6/Olig2. Recent genetic studies have revealed that these transcription factors play important roles in controlling the development of both motoneurons and oligodendrocytes. For example, misexpression of both Olig2 and Pax6 genes can cause aberrant development of motoneurons and oligodendrocytes (Ericson et al., 1997; Sun et al., 1998; Sun et al., 2001; Zhou et al., 2001; Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Nkx6.1 also has an instructive role in controlling motoneuron specification, as overexpression of the Nkx6.1 homeodomain protein induces ectopic motoneuron formation in embryonic chicken spinal cord (Briscoe et al., 2000) and mutation of Nkx6.1 leads to a drastic reduction of motoneuron genesis (Sander et al., 2000).

To determine the role of Nkx6.1 expression in oligodendrocyte development, we examined the specification and differentiation of oligodendrocytes in Nkx6.1 loss- and gain-of-function studies. Our studies have provided genetic and molecular evidence that Nkx6.1 regulates Olig2 expression in the ventral neuroepithelial cells and the development of oligodendrocyte progenitors. In Nkx6.1 mutant embryos, there is a delay in OPC specification and differentiation in the spinal cord, but not in the hindbrain region. Overexpression studies in chicken embryos demonstrated that Nkx6.1 can either activate or inhibit Olig2 gene expression in the developing spinal cords, depending on developmental stages.

Materials and methods

Genotyping of Nkx6.1 mutant mice

The Nkx6.1 homozygous mouse embryos were obtained by the interbreeding of heterozygous animals. For genotyping, genomic DNA extracted from embryonic tissues or mouse tails was used for genotyping by Southern analysis as previously described (Sander et al., 2000).

In situ RNA hybridization and immunofluorescent staining

Spinal cord tissues from E10.5 to E18.5 mouse embryos were fixed directly in 4% paraformaldehyde (PFA) at 4°C overnight. For P0 pups, animals were fixed by cardiac perfusion with 4% PFA. Following fixation, tissues were transferred to 20% sucrose in PBS overnight, embedded in OCT media and then sectioned (20 μm) using a cryostat. Adjacent sections from the wild-type and Nkx6.1 mutant embryos were subsequently subjected to in situ hybridization or immunofluorescent staining. In situ hybridization was performed as described in Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers and Gerfin-Moser, 1993) with minor modifications, and the detailed protocol is available upon request. Immunofluorescent procedures were previously described in Xu et al. (Xu et al., 2000). Anti-Nx2.2 (1:50, DBSH), anti-Mnr2/Hb9 (1:100; DBSH), anti-Ng2 (1:1500, Chemicon) and anti-P-PECAM-1 (1:50 from Pharmingen) were obtained from commercial sources. Anti-Olig2 (1:10000) was generously provided by Drs Chuck Stiles and David Rowlitch.

Spinal cord explant culture

Segments of spinal cord tissue were isolated from E13.5 embryos at the thoracic region and grown on 8.0 μm nucleopore polycarbonate membranes (Costar) floating on culture medium (DMEM + N2 supplement + T3 30 ng/ml + T4 40 ng/ml + BSA 1 mg/ml + FBS 0.5% + Pen-Strep). After 7 days culture in vitro, explants were then fixed in 4% PFA and processed for whole-mount in situ RNA hybridization with MBP riboprobe, as described in Cai et al. (Cai et al., 1999).

In ovo electroporation

A full-length hamster Nkx6.1 cDNA (Rudnick et al., 1994) was subcloned in replication-competent retroviral vector RCASBP(B) (Morgan and Fekete, 1996). For in ovo electroporation, about 1.5 μl (1 μg/μl) of expression vectors was injected into stage 11-13 (embryonic day 2 or E2) white horn chicken embryos with the aid of Picospritzer III instrument. The injected embryos were then subjected to three short pulses of electrical shock (25V, 50 mseconds for each pulse) and allowed to develop for two (E4) or four (E6) more days before they are fixed in 4% PFA for gene expression studies.

Results

Nkx6.1 mutation leads to a reduced Olig2 expression in the ventral neuroepithelium and a delayed production of oligodendrocyte progenitors

As motoneurons and oligodendrocytes may share the same lineage and Nkx6.1 homeodomain protein controls motoneuron development, we expect that the Nkx6.1 gene may also regulate oligodendrocyte genesis in the spinal cord. To test this idea and further investigate the role of Nkx6.1 in oligodendrocyte development, we first examined the expression of the motoneuron/oligodendrocyte precursor gene, Olig2, in Nkx6.1 mutant spinal cords at the thoracic level. At embryonic day 12 (E12.0), immediately preceding the onset of oligodendrogenesis, Olig2 expression was normally expressed in the pMN domain of ventral ventricular zone (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000) (Fig. 1A). In the mutants, however, Olig2 expression in the ventral neuroepithelium was dramatically reduced and often asymmetrical with a much broader expression domain at one side of the lumen (Fig. 1B). At E13.5, while numerous Olig2+ OPCs had migrated out into the surrounding gray matter in control embryos, few Olig2+ cells were detected outside the ventricular zone in the mutants (Fig. 1C; Fig. 3G,H). However, at E15.5, a small number of Olig2+ migratory OPCs were observed in the mutant spinal cord. Interestingly, at this stage, while the Olig2 expression in wild-type embryos remained strong, the ventricular expression of Olig2 in the mutants was not observed (Fig. 1E,F), indicating that the temporal window for oligodendrogenesis from the ventricular zone in Nkx6.1 mutant spinal cords is much shortened as well. Together, these observations demonstrated that the initial production of OPCs from the Olig2+ ventricular cells is both delayed and reduced Nkx6.1 mutants. As embryogenesis proceeded, the number of Olig2+ OPCs in the spinal cord parenchyma increased over time in both genotypes (Fig. 1G,H). By perinatal stages, there was no obvious difference in the number or density of Olig2+ cells between normal and mutant spinal cords (Fig. 1I), possibly owing to an increased proliferation of progenitor cells in the mutants.

To confirm the initial delay of OPC production, we examined the expression of Pdgfra, a well-characterized OPC marker (Hall et al., 1996; Calver et al., 1998), in the embryonic spinal cord. At E13.5, while Pdgfra expression was observed in the ventral ventricular zone and in many migratory OPCs in
Defective oligodendrogenesis in Nkx6.1 mutants

Wild-type embryos, no Pdgfra expression was found in Nkx6.1 mutants (Fig. 2A,B). However, the Pdgfra+ OPCs did emerge in the mutant spinal cord by E15.5, although their number was much smaller as compared to the wild-type embryos (Fig. 2C,D). At late gestation, the number of Pdgfra+ OPCs in Nkx6.1 mutants increased rapidly (Fig. 2E,F), so that by perinatal stages (P0), no apparent difference was found in the wild-type and mutant animals (Fig. 2G,H). The initial reduction of OPCs was further verified with the expression of another OPC marker NG2 (Nishyama et al., 1996). There were many fewer NG2+ cells in Nkx6.1 mutant spinal cords at E15.5 (Fig. 2I,J); but a comparable number of NG2+ cells was observed in both genotypes at P0 (Fig. 2K,L).

Nkx2.2 expression in the white matter is delayed in Nkx6.1 mutant spinal cords

Previous studies have demonstrated that the Nkx2.2 homeobox gene is expressed in both OPC cells and differentiating oligodendrocytes (Xu et al., 2000; Soula et al., 2001; Zhou et al., 2001; Fu et al., 2002), and that expression of Nkx2.2...
plays a determinative role for the terminal differentiation of oligodendrocytes (Qi et al., 2001; Sun et al., 2001; Zhou et al., 2001). To investigate whether Nkx2.2 expression is affected by the Nkx6.1 mutation during oligodendrogenesis, we examined Nkx2.2 expression in the thoracic region of the spinal cord at various embryonic stages. At E13.5 and E15.5, Nkx2.2 was similarly expressed in the p3 domain and adjacent ventral gray matter in the wild-type and Nkx6.1 mutant embryos (Fig. 3A-D,G,H). Double immunofluorescent staining revealed that the p3-derived Nkx2.2+ cells in the ventral gray matter did not co-express Olig2 at E13.5 (Fig. 3G,H). Surprisingly, at E18.5, these Nkx2.2+ cells remained as Olig2 negative, and did not disperse into the white matter regions (Fig. 3I,J). This observation is contrary to the previous hypothesis that the Nkx2.2+ cells originating from the p3 domain would later migrate into the white matter, gain Olig2 expression and differentiate into oligodendrocytes (Fu et al., 2002). The confinement of these Nkx2.2+/Olig2– cells to the ventral gray matter would rather suggest a neuronal identity for these cells. To examine this possibility, we performed the Nkx2.2 and NeuN (Neuna60 – Mouse Genome Informatics) double immunolabeling and found that the Nkx2.2+ cells in the ventral gray matter started to co-express the pan-neuronal marker NeuN (Mullen et al., 1992) at later stages of embryogenesis (Fig. 3K-N). Based on these observations, we now propose that the Nkx2.2+ cells originating from the p3 domain give rise to neurons instead of OPCs. The co-expression of Nkx2.2 and NeuN in the ventral gray matter was also observed in other species such as rat and chicken (see Fig. S1 at http://dev.biologists.org/supplemental/).

Our previous studies have suggested that in rodents, a majority of Olig2+ OPCs derived from the pMN acquire Nkx2.2 expression after they migrate into the white matter (Fu et al., 2002). Interestingly, upregulation of Nkx2.2 expression in the white matter OPCs occurred only in the wild-type but not mutant spinal cords (Fig. 3E,F). Double immunolabeling confirmed that all Nkx2.2+ cells in the white matter co-expressed Olig2 (Fig. 3I,J). Together, these results indicated a delayed or defective Nkx2.2 upregulation in Olig2+ OPCs in Nkx6.1 mutant spinal cords.

Myelin gene expression is delayed and reduced in Nkx6.1 mutants

Recent studies have demonstrated that the Nkx2.2 expression in white matter precedes and regulates oligodendrocyte differentiation (Qi et al., 2001; Sun et al., 2001; Zhou et al., 2001; Fu et al., 2002). Thus, the absent Nkx2.2 expression in the white matter of mutant spinal cords would predict retarded oligodendrocyte differentiation and maturation. To examine this possibility, we investigated the expression of the mature oligodendrocyte markers myelin basic protein (MBP) and proteolipid protein (PLP) in mutant spinal cords at the thoracic level. At E17.5, a small number of MBP+ oligodendrocytes were found in the ventral spinal cord of wild-type embryos, but not in the mutants (Fig. 4A,B). By P0, although the number of MBP+ oligodendrocytes in wild-type mice increased, MBP+ and PLP+ oligodendrocytes were still not

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Fig. 3. Altered expression of Nkx2.2 in Nkx6.1 mutant spinal cord at the thoracic level. (A-F) Spinal cord sections from E13.5 (A,B), E15.5 (C,D) and E18.5 (E,F), wild-type (A,C,E) and mutant (B,D,F) animals were hybridized with Nkx2.2 riboprobe. (G-L) Double immunofluorescent staining of E13.5 (G,H) and E18.5 (I-L) spinal cord sections with anti-Nkx2.2 and anti-Olig2 (G-J), or with anti-Nkx2.2 and NeuN (K,L). In the ventral gray matter, most Nkx2.2+ cells co-express NeuN but not Olig2. In the white matter, Nkx2.2+ cells co-express Olig2, but not NeuN. Representative Nkx2.2+ cells are indicated by arrowheads, whereas the double-positive cells are indicated by arrows. (M,N) Higher power view of the NeuN/Nkx2.2 double-positive cells (yellow) in the wild-type (M) and Nkx6.1 mutant (N) spinal cords.
Defective oligodendrogenesis in Nkx6.1 mutants

Detected in the mutant spinal cords (Fig. 4C-F). Unfortunately, further development of oligodendrocytes in postnatal mutant cords can not be assessed owing to the neonatal death of mutant animals.

There are two plausible explanations for the absence of expression of Nkx2.2 and myelin genes in the white matter of the Nkx6.1 mutant spinal cords. One possibility is that oligodendrocyte differentiation is simply delayed in the mutants, in parallel with the delayed generation of OPCs. Alternatively, in the absence of Nkx6.1 activity, OPCs generated in the mutants have restrictive or defective developmental potential and can not differentiate under any circumstances. To distinguish between these two possibilities, spinal cord explants from E13.5 wild-type (G) and mutant (H) embryos and cultured in vitro for 7 days before whole-mount in situ hybridization with Mbp riboprobe.

Regulation of Olig2 and Sox10 gene expression by Nkx6.1 in embryonic chicken spinal cord

Recent studies have indicated that Olig1 and Olig2 genes are required for the initial specification of the oligodendrocyte lineage in the pMN domain (Lu et al., 2002; Zhou et al., 2002), raising the possibility that the effects of the Nkx6.1 mutation on oligodendrocyte development in the spinal cord is mediated by its direct regulation of Olig gene expression in the ventral neuroepithelium. To investigate whether expression of Nkx6.1 can activate Olig gene expression and oligodendrogenesis in the developing spinal cord, we overexpressed Nkx6.1 gene in stage 11-13 (E2) chicken spinal cord by in ovo electroporation and then examined its effects on the expression of Olig2 and oligodendrocyte marker gene

Fig. 4. (A-F) Disrupted expression of Mbp and Plp in Nkx6.1 mutant spinal cord. Spinal cord sections at the thoracic level were prepared from E17.5 (A,B) and P0 (C-F) wild-type (A,C,E) and mutant (B,D,F) animals and hybridized with Mbp (A-D) and Plp (E,F) riboprobes. (G-H) Similar Mbp expression in spinal cord explants in normal and Nkx6.1 mutants. Spinal cord explants were isolated from the thoracic regions of the E13.5 wild-type (G) and mutant (H) embryos and cultured in vitro for 7 days before whole-mount in situ hybridization with Mbp riboprobe.

Fig. 5. Reduced expression of Nkx2.2 and Mbp in P0 Nkx6.1 mutant spinal cord at the cervical region. Cross-sections of cervical spinal cord from P0 wild-type (A,C,E) and mutant (B,D,F) pups were hybridized with Nkx2.2 (A,B), Mbp (C,D) and Plp (E,F) riboprobes. Arrows indicate the MBP+/PLP+ oligodendrocytes in the mutants.
Sox10. As a positive control, the motoneuron marker Mnr2/Hb9 (Hlxb9 – Mouse Genome Informatics) was also included in the assay (Tanabe et al., 1998). Two days after Nkx6.1 electroporation (at E4), ectopic expression of Olig2, Mnr2/Hb9 and Sox10 was all induced in the electroporated side of the spinal cords (Fig. 6A-D; see Fig. S2 at http://dev.biologists.org/supplemental/). Normally, expression of Sox10 in the ventral neuroepithelium is not detectable until E6 (Fig. 6H). Thus, overexpression of Nkx6.1 can induce precocious expression of oligodendrocyte marker Sox10. However, induction of other markers such as O4 and MBP was not observed at this stage (data not shown), possibly owing to the lack of expression of the co-factor Nkx2.2 (Zhou et al., 2001). Surprisingly, 4 days after electroporation, no ectopic expression of Olig2 and Sox10 was observed in the electroporated side of the spinal cords, although ectopic expression of Mnr2/Hb9 remained in the adjacent section (Fig. 6E-H). Moreover, the endogenous expression of Olig2 in the ventral neuroepithelium was significantly reduced by Nkx6.1 overexpression (Fig. 6F; see Fig. S2 at http://dev.biologists.org/supplemental/). These results suggest that Nkx6.1 can function as either an activator or an inhibitor of Olig2 gene expression, depending on the developmental stage of the spinal cord tissue.

Oligodendrogenesis is not affected in the hindbrain

Previous studies have demonstrated that Nkx6.1 is also expressed in the motoneuron progenitor cells in the ventral hindbrain (Qiu et al., 1998; Puelles et al., 2001; Takanashi and Osumi et al., 2002). As in the spinal cord, mutation of Nkx6.1 also results in the loss of somatic motoneurons in the hindbrain, although the development of visceral motoneurons derived from the Nkx2.2+ ventral neuroepithelium is spared (Sander et al., 2000). As oligodendrocyte development is coupled to the development of somatic motoneurons (Richardson et al., 2000; Lu et al., 2002; Zhou and Anderson, 2002), it is expected that oligodendrocyte development might also be affected in the hindbrain. To examine this possibility, we first studied whether the differentiation of oligodendrocytes is similarly delayed in the hindbrain of Nkx6.1 mutants. At E15.5, expression of MBP was detected in the anterior hindbrain (the pons) flanking the midline in both the wild-type and mutant embryos (Fig. 7A,D). A similar pattern of MBP expression in the pontine area was also observed in the E17.5 embryos in both genotypes, despite the drastic decrease of MBP expression in the cervical spinal cord (Fig. 7B,E). At P0, MBP+ oligodendrocytes
were widely and similarly distributed in the rostral hindbrain region in wild-type and mutant pupps (Fig. 7C,F). Therefore, unlike in the spinal cord, oligodendrocyte differentiation in the hindbrain region is not apparently affected by the Nkx6.1 mutation.

The normal differentiation of oligodendrocytes would suggest the normal specification and generation of OPC cells in Nkx6.1 mutant hindbrain. To investigate this possibility, we compared the expression of three OPC marker genes, Olig1, Olig2 and Pdgfra, in the rostral hindbrain of wild-type and mutant embryos during early stages of oligodendrogenesis. At E13.5, the expression of Olig1 and Olig2 in the rostral hindbrain was surprisingly detected in multiple domains of neuroepithelium in wild-type embryos (Fig. 8A,C), including the Nkx6.2 domain (Fig. 8J) and the ventricular cells dorsal to the Nkx6.1- and Nkx6.2-expressing domain (Fig. 8I). In the mutants, Olig1 and Olig2 are also expressed in multiple domains of ventricular zone in both dorsal and ventral territories (Fig. 8B,D). Thus, in contrast to their singular expression in the pMN domain in the spinal cord, Olig1 and Olig2 appeared to have multiple sites of ventricular expression in the rostral hindbrain in both normal and mutant embryos. Moreover, the number and distribution of Olig1/2+ and Pdgfra+ progenitors in this region were not appreciably altered by the mutation (Fig. 8A-F). At E15.5, numerous Olig1+, Olig2+ and Pdgfra+ OPC cells were distributed in the entire sections of the rostral hindbrain in both normal and mutant embryos (Fig. 9). From E15.5 to P0, the number of Pdgfra+ OPCs in the hindbrain increased steadily, and no apparent differences were detectable between wild-type and mutant animals (data not shown). Taken together, our expression studies indicate that in the rostral hindbrain, oligodendrocytes originate from multiple domains of the neuroepithelium and oligodendrogenesis is not affected by the loss of Nkx6.1 activity.

Discussion

Stage-dependent regulation of Olig2 expression and oligodendrocyte development by Nkx6.1 in the developing spinal cord

The present study has demonstrated that the Nkx6.1 neural progenitor gene also regulates oligodendrocyte specification and differentiation, in addition to motoneuron development, in the developing spinal cord. In the absence of Nkx6.1 gene activity, expression of Olig2 in the pMN domain is markedly reduced and can not be maintained during gliogenesis (Fig. 1). Because expression of Olig2 in the ventral spinal neuroepithelium is both necessary and sufficient for oligodendrocyte specification in the spinal cord (Sun et al., 2001; Zhou et al., 2001; Lu et al., 2002; Zhou and Anderson, 2002; Takebayashi et al., 2002), we hypothesize that the diminished Olig2 expression in the pMN domain is directly responsible for the delayed and reduced generation of OPCs in Nkx6.1 mutant spinal cords. A similar delay of oligodendrogenesis was also observed in Pax6 mutant spinal cords (Sun et al., 1998), in which Olig2 expression is greatly reduced (Novitch et al., 2001; Mizuguchi et al., 2001).

Consistent with our hypothesis that Nkx6.1 regulates spinal oligodendrogenesis by controlling Olig expression, overexpression of Nkx6.1 in embryonic chicken spinal cords lead to ectopic expression of Olig2, Mnr2 and the OPC marker Sox10 at E4, 2 days after in ovo electroporation (Fig. 6, Fig. S1 at http://dev.biologists.org/supplemental/). Ectopic Sox10 expression occurred 2 days earlier than its endogenous expression, which is not detectable until E6. This precocious induction of an OPC lineage marker probably resulted from the high level of Nkx6.1 protein expression or activity in our electroporation studies. However, ectopic expression of later oligodendrocyte markers was not induced at this stage, possibly

Fig. 8. Multiple sites of origin of Olig1 and Olig2 expression in the rostral hindbrain during early stages of oligodendrogenesis. (A-H) Adjacent slide sections from E13.5 wild-type (A,C,E,G) and mutant (B,D,F,H) hindbrain at the pontine area were hybridized with Olig1 (A,B), Olig2 (C,D), Pdgfra (E,F) and Nkx2.2 (G,H) probes. Only the tissue staining surrounding the fourth ventricle is shown. In A-D, arrows indicate Olig1 and Olig2 expression in the Nkx6.1/Nkx2.2 domain of the ventral ventricular zone; arrowheads indicate Olig1/2 expression outside the Nkx6.1 domain. (I,J) Slide sections from E13.5 wild-type hindbrain at the same level were double-labeled with Nkx6.1 (blue) and Olig1 (orange), or Nkx2.2 (blue) and Olig1 (orange). In I, the blue arrow indicates the dorsal boundary of Nkx6.1 expression.
because it requires the co-expression of Nkx2.2 (Zhou et al., 2001) or a longer induction of Olig2 by Nkx6.1. Unexpectedly, 4 days after electroporation, the ectopic expression of Olig2 and Sox10 was not detected and the endogenous expression of Olig2 in the pMN domain was markedly inhibited (Fig. 6, see Fig. S2 at http://dev.biologists.org/supplemental/). The rapid downregulation of Olig2 in Nkx6.1-overexpressing cells after E4 might explain why we did not observe a further increase of oligodendrogenesis at E6 and later stages in the Nkx6.1-electroporated embryos. Thus, Nkx6.1 appears to switch its role from an activator to an inhibitor of Olig2 expression at later stages, possibly owing to the dynamic expression of its co-factors. This role switch might also explain why Nkx6.1 and Olig2 are not expressed in the same cells after oligodendrogenesis. Nkx6.1 expression is immediately downregulated in the Olig2+ OPC cells after they migrate out of the ventricular zone (Xu et al., 2000). By contrast, Nkx6.1 expression is retained in the ventricular cells throughout embryonic and postnatal animal development (Fu et al., 2003), while Olig2 expression is rapidly lost in the ventricular neuroepithelial cells after oligodendrogenesis stages (E9 in chicken or E16.0 in mouse). Interestingly, the ventricular Olig2 expression in Nkx6.1 mutant spinal cords is not increased or prolonged after oligodendrogenesis. One possible explanation is that the Olig2+ ventricular cells are prematurely depleted during oligodendrogenesis in the mutants because of its small pool size (Fig. 1B,D,F), before Nkx6.1 becomes inhibitory. In addition, the rapid and premature downregulation of Olig2 expression at E6 in our Nkx6.1 overexpression studies is likely to be due to an unregulated high-level Nkx6.1 protein expression or activity.

The stage-dependent regulation of gene expression was previously suggested for other homeodomain transcription factors such as Nkx2.2. During early neurogenesis stages, Nkx2.2 inhibits Olig2 expression in the pMN domain; however, during oligodendrogenesis stages, Nkx2.2 and Olig2 are co-expressed in the same OPC cells and Nkx2.2 becomes a co-activator, instead of an inhibitor, of Olig2 (Qi et al., 2001; Zhou et al., 2001).

Identity of Nkx2.2-expressing cells in embryonic mouse spinal cord

Recent molecular and genetic studies have established that the Olig2+/Pdgfra+ OPC cells originate from the pMN domain (Sun et al., 1998; Richardson et al., 2000; Fu et al., 2002; Lu et al., 2002; Zhou and Anderson, 2002). In rodents, the majority of Olig2+ OPCs appear to acquire Nkx2.2 expression after they migrate out into the white matter prior to their terminal differentiation (Fu et al., 2002). This study has provided additional support for this concept, as the delayed generation of Olig2+/Pdgfra+ OPCs in Nkx6.1 mutant spinal cords is associated with a delay of Nkx2.2 expression in the white matter (Fig. 3).

It has also been proposed that a second source of OPCs might be generated from the Nkx2.2+ p3 domain of ventral neuroepithelium (Soula et al., 2001; Zhou et al., 2001; Fu et al., 2002; Lee et al., 2003). Based on the migration pattern of Nkx2.2+ cells, it was hypothesized that OPC cells derived from the p3 domain are initially Nkx2.2+/Pdgfra- /Olig2-, but become Olig2+ after they migrate into the white matter (Fu et al., 2002). However, in contrast to the previous hypothesis, this group of Nkx2.2 cells did not appear to disperse into the white matter, and nor did they gain Olig2 expression (Fig. 3L). Instead, they remained in the ventral gray matter and started to co-express the panneuronal marker NeuN at later stages of embryogenesis (Fig. 3K,L; see Fig. S1 at http://dev.biologists.org/supplemental/). Collectively, these observations would strongly argue that Nkx2.2+ cells derived
from the p3 domain develop into neurons (possibly V3 ventral interneurons), rather than OPC cells. This new interpretation is consistent with the recent genetic evidence that oligodendrocyte development in embryonic mouse spinal cord is coupled to motoneuron development (Lu et al., 2002; Zhou and Anderson, 2002).

Region-specific regulation of Olig2 expression and oligodendrogenesis by Nkx6.1 expression

Previous expression studies have demonstrated that Nkx6.1 is also expressed in the ventral hindbrain, including motoneuron progenitor cells (Qiu et al., 1998; Puelles et al., 2001; Takahashi and Osumi, 2002). Loss of Nkx6.1 gene activity disrupts the development of somatic motoneurons derived from the Olig1/2+ neuroepithelium (Sander et al., 2000). Surprisingly, unlike in the spinal cord, the ventral expression of Olig genes in the mutant hindbrain is not altered during oligodendrogenesis stages. The generation of Pdgfra+ and Olig1/2+ OPCs in this region appears to be normal and their differentiation and maturation are also on schedule in Nkx6.1 mutants. One possible explanation for the differential effects of Nkx6.1 mutation on oligodendrogenesis in the ventral spinal cord and hindbrain is that the loss of Nkx6.1 is compensated by Nkx6.2, given that Nkx6.1 and Nkx6.2 have a similar expression pattern in the hindbrain but not in the spinal cord (Cai et al., 1999; Vallstedt et al., 2001). However, our preliminary studies have indicated that oligodendrocyte development in the hindbrain is not significantly compromised in the Nkx6.1/Nkx6.2 double mutants (data not shown). Together, our studies indicate a regional difference in the regulation of Olig2 expression and oligodendrocyte development by the Nkx6.1 homeodomain transcription factor.

Consistent with the previous suggestions on multiple origins of OPCs in the developing hindbrain (Spassky et al., 1998; Spassky et al., 2000; Davies and Miller, 2001), Olig1 and Olig2 are expressed in multiple ventricular and subventricular zones in the rostral hindbrain (metencephalon) at E13.5 (Fig. 8A-D), and OPC cells can be produced from the neuroepithelial cells both inside and outside the Nkx6.1-expressing domain (Figs 8, 9). The lack of Nkx6.1 expression in the dorsal metencephalon, together with the normal production of OPC cells from Nkx6.1 mutant hindbrain tissue, indicates the existence of an Nkx6.1-independent mechanism for regulation of Olig gene expression and oligodendrogenesis in the rostral CNS. This is particularly true for the developing telencephalon, where Nkx6.1 is not expressed and oligodendrogenesis occurs normally in Nkx6.1 mutant embryos (data not shown). In addition, the generation of OPC cells, but not motoneurons, from the dorsal Nkx6.1-negative neuroepithelial cells in the rostral hindbrain would also indicate the lack of an obligatory lineage relationship between oligodendrocytes and motoneurons in this region.

Regulation of oligodendrocyte differentiation and maturation

Previous studies have demonstrated that the Olig2+/Pdgfra+ OPC cells originate from the pMN domain (Sun et al., 1998; Richardson et al., 2000; Fu et al., 2002), and acquire Nkx2.2 expression as they migrate into the white matter prior to their terminal differentiation (Fu et al., 2002). The up-regulation of Nkx2.2 expression in OPC cells is required for normal myelin gene expression and oligodendrocyte differentiation (Qi et al., 2001; Sun et al., 2001; Zhou et al., 2001). In Nkx6.1 mutant spinal cords, the delayed production of OPC cells is accompanied by a delayed expression of Nkx2.2 in the white matter (Fig. 3) and myelin genes MBP/PLP (Figs 4, 5).

The mechanisms underlying the parallel defect of oligodendrocyte specification and differentiation remain to be determined at this stage. As Nkx6.1 per se is not expressed in migratory OPC cells (Xu et al., 2000) and the OPCs from the Nkx6.1 mutant spinal cord are capable of differentiating into MBP+ mature oligodendrocytes both in vitro (Fig. 4G,H) and in vivo (Fig. 5), the effect of Nkx6.1 mutation on oligodendrocyte differentiation is likely to be indirect or cell non-autonomous. One possibility is that oligodendrocyte differentiation in the spinal cord is controlled by an intrinsic clocking mechanism, as suggested by previous studies on the in vitro differentiation of OPC cells (Temple and Raff, 1986; Gao et al., 1997). It is possible that during development, OPC cells also exit the cell cycle and undergo terminal differentiation after a fixed number of cell divisions or a fixed amount of time after birth, and a delay of progenitor production is coupled with a delay of differentiation. Another possibility is that the decreased expression of neuregulin 1 (Nrg1) is responsible for the retarded oligodendrocyte differentiation in the spinal cord (Vartanian et al., 1999; Park et al., 2001). In Nkx6.1 mutants, motoneuron development is inhibited (Sander et al., 2000) and the production of Nrg1 by motoneurons is markedly reduced (data not shown). Finally, the terminal differentiation of oligodendrocytes could be regulated by a density-dependent mechanism. It is conceivable that OPC cells may secrete some autocrine differentiation-inducing factor whose concentration could be reduced in the mutants as compared with wild-type embryos, because there are fewer cells in the prenatal period. Future studies are needed to unravel the molecular mechanisms underlying the delayed oligodendrocyte differentiation and maturation in Nkx6.1 mutant spinal cords.

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