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Co-localization of *Nkx6.2* and *Nkx2.2* Homeodomain Proteins in Differentiated Myelinating Oligodendrocytes

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Abstract

Recent studies have suggested that *Nkx6.2/Gtx* and *Nkx2.2* homeodomain transcription factors are involved in the regulation of oligodendrocyte maturation and/or myelination which occur predominantly in postnatal stages. However, their cellular specificity in postnatal central nervous system has not been characterized and their dynamic expressional relationship during oligodendrocyte lineage progression has not been determined. Here we report that both *Nkx2.2* and *Nkx6.2* are selectively expressed in Olig2+ cells of oligodendrocyte lineage in postnatal spinal cords. While *Nkx6.2* is specifically expressed in the APC+ mature oligodendrocytes, *Nkx2.2* is initially expressed in differentiating oligodendrocyte precursor cells (OPCs) but quickly down-regulated as OPCs undergo terminal differentiation. Intriguingly, *Nkx2.2* expression is up-regulated in mature myelinating oligodendrocytes at later stages. The co-expression of *Nkx2.2* and *Nkx6.2* transcription factors in myelinating oligodendrocytes suggests their functional interactions in the regulation of myelin sheath formation and/or maintenance.

Keywords

gene expression; homeobox gene; transcription factor; spinal cord; Oligodendrocyte

INTRODUCTION

Recent studies have revealed that transcription factors play important roles in the fate specification and differentiation of oligodendrocyte lineage in the developing central nervous system (CNS). Oligodendrocyte progenitor cells (OPCs or OLPs), regardless of their developmental origins, initially express the bHLH transcription factor *Olig2* (Lu et al., 2000; Takeyabashi et al., 2000; Zhou et al., 2000). Genetic and molecular studies have implicated *Olig2* as the key determinant of oligodendrocyte lineage specification (Lu et al., 2002; Takeyabashi et al., 2002; Zhou and Anderson, 2002). Soon after OPCs are generated from the ventricular zone, they are rapidly dispersed into the surrounding regions where they undergo further proliferation and differentiation. Along with OPC migration and differentiation, they

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gradually acquire the expression of other oligodendrocyte lineage-specific transcription factors, notably *Sox10* and *Nkx2.2* (Stolt et al., 2002; Xu et al., 2000; Soula et al., 2001). In rodents, *Sox10* expression can be detected in early OPCs, whereas *Nkx2.2* expression is acquired later by OPCs after they migrate into the white matter (Fu et al., 2002; Liu et al., 2003). *Sox10* and *Nkx2.2* appear to have similar functions in the control of oligodendrocyte differentiation, and targeted mutations of these two genes caused similar phenotypes with retarded oligodendrocyte maturation and myelin gene expression (Qi et al., 2001; Stolt et al., 2002).

Despite the significant progress in our understanding of the transcriptional control of oligodendrocyte specification and differentiation, transcription factors that regulate the myelination process of oligodendrocytes remain to be defined. There is emerging evidence that *Nkx6.2/Gtx* homeodomain transcription factor is involved in this developmental process. It was previously reported that *Nkx6.2* was expressed in differentiating oligodendrocytes (Awatramani et al., 1997), and possibly in astrocytes as well (Komuro et al., 1993). The multiple *Nkx6.2* binding sites in the MBP and PLP promoters suggested that it may be involved in the regulation of myelin-specific gene expression (Awatramani et al., 1997). Contrary to this suggestion, oligodendrocyte differentiation and myelin gene expression are normal in *Nkx6.2* null mutants (Cai et al., 2001). Subsequent studies demonstrated that *Nkx6.2* mutation leads to small myelination defects in association with mild neurological and behavioral disorders (Southwood et al., 2004).

However, it has not been determined whether *Nkx6.2* expression is restricted to cells of oligodendrocyte lineage and it is not clear whether *Nkx6.2* collaborates with other related transcription factors such as *Nkx2.2* to regulate the complicated process of myelin sheath formation. In this study, we performed detailed studies on the dynamic expression of *Nkx6.2* in postnatal CNS, and demonstrated that *Nkx6.2* is specifically expressed in cells of oligodendrocyte lineage, specifically in APC+/cytoplasmic Olig1+ mature oligodendrocytes. Interestingly, after a transient and rapid down-regulation in differentiated oligodendrocytes in early postnatal spinal cord, *Nkx2.2* expression is restored in the *Nkx6.2*+ mature oligodendrocytes at later stages. While the expression of *Nkx2.2* in early differentiating OPCs controls oligodendrocyte differentiation, the late phase of *Nkx2.2* expression in mature oligodendrocytes suggests its possible role in the transcriptional regulation of myelin sheath formation and/or maintenance of myelin structures.

MATERIALS AND METHODS

Genotyping of *Nkx2.2* and *Nkx6.2* mutant mice

The *Nkx2.2* or *Nkx6.2* homozygous null mice and their wild-type littermates were obtained by the interbreeding of heterozygous animals. Genomic DNA extracted from mouse tails was used for genotyping by Southern analysis or by PCR. Genotyping of *Nkx2.2* and *Nkx6.2* loci was described in Sussel et al., (1998) and Cai et al., (2001), respectively.

Immunofluorescent staining

Postnatal mice from day 0 (P0) to the adult were fixed by cardiac perfusion with 4% paraformaldehyde (PFA). Brain and spinal cord tissues were dissected out and submerged in 4% PFA at 4°C overnight. Following fixation, tissues were transferred to 20% sucrose in PBS overnight, embedded in OCT media and then sectioned (16 µm thickness) on a cryostat. Double and triple immunofluorescent procedures were modified from the previous description in Xu et al., (2000). The dilution ratio of antibodies is as follows: anti-mouse A2B5 (1:50), anti-mouse PDGFRα (1:300, BD), anti-NG2 (1:1500, Chemicon), anti-mouse O4 (1:1000, Chemicon), anti-mouse *Nkx2.2* (1:50, DSHB; 1:5000, gift from Dr. Ericson), anti-mouse

Nkx6.2 (1:6000, gift from Dr. Ericson), anti- mouse Olig2 (1:3000, gift from Drs. Stiles and Alberta), anti- mouse Olig1 (1:100, gift from Drs. Stiles and Alberta), anti- mouse Sox10 (1:3000, gift from Dr. Wegner), anti-mouse APC/CC1 (1:50, Oncogene), anti-mouse MAG (1:300, Chemicon), anti-mouse MBP (1:5000, Chemicon), anti-mouse NeuN (1:1000, Chemicon), anti-rat GFAP (1:300, Chemicon), anti-mouse Glast (1:500, Abcam) and anti-mouse S100B (1:1000, BD).

OPC culture and differentiation assays

OPCs were obtained from newborn rat brain tissues by the simple shaking method as described previously (McCarthy and de Vellis, 1980). Briefly, the dissected forebrain tissues were minced into 1-mm³ pieces and incubated in Hanks' balanced salt solution containing 0.1% papain, 0.1% neutral protease, and 0.01% DNase for 30 minutes at 37°C. The digestion was stopped by the addition of an equal volume of Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS). The medium was changed after 24 hr of plating until the cells reached confluence after 8–12 days. At this stage, a loosely adherent superficial layer of cells that represents OPCs and microglia lying on a confluent astrocyte layer was removed by vigorous shaking of the cultures at 200rpm for 60min, followed by a change to fresh medium. Top-dwelling OPCs were dislodged by overnight (18–22 h) shaking of the cultures at 200 rpm and placed into uncoated tissue culture dishes for 60 min to allow adherence of any residual microglia. The suspension was filtered through 70-um nylon mesh and pelleted at 800 rpm for 5 min. The pellets were re-suspended in BDM/CYS+ medium (DMEM, 5 µg/ml insulin, 50 µg/ml apo-transferrin, 1mg/ml BSA, 30 ng/ml sodium selenite, 200 µM L-cysteine, 10 ng/ml D-biotin, and 100 µg/ml penicillin-streptomycin-glutamine, 10 nM hydrocortisone). In these cell preparations, about 90% of the bound cells expressed A2B5 and were therefore suitable for the subsequent differentiation experiments. OPC cells were subsequently cultured in fresh growth medium containing PDGF-AA (10 ng/ml) and FGF-2 (10 ng/ml) every other day. For differentiation assays, cells were switched to MBP differentiation medium containing T3 (15 ng/ml) and CNTF (100 ng/ml or 4 ng/ml) for another week.

Myelination assays in OPC-DRG COCULTURE

For co-culture studies, O4⁺ oligodendrocytes were immunopanned from adult spinal cord tissues using O4 antibody as described in the previous study (Cheng et al., 2007). DRG neurons were isolated from E15 Sprague-Dawley rat embryos (Charles River Laboratories). Briefly, the DRGs were treated with 0.25% trypsin (Invitrogen) for 8 mins followed by neutralization with 40% FBS. After washing twice with 10% FBS in L-15, DRG explants were resuspended in DRG-growth medium.

In myelination assays, O4⁺ OPCs were added to DRG neuron cultures in OPC-growth medium (DMEM/F12, 1×N2, 1×B27, 1×P/S, 20 ng/ml FGF-2, 10 ng/ml PDGF-AA, and 30 ng/ml Insulin). On the following day, the cultures were changed to the differentiation medium (DMEM/F12, 1×N2, 1×B27, 1×P/S, 30 ng/ml insulin) for one week to induce adult OPC differentiation. Immunostaining was performed in 2-week-old cultures with antibodies against MBP and Nkx2.2.

RESULTS

Selective expression of *Nkx2.2* and *Nkx6.2* in the postnatal spinal cord

To investigate the possible functional collaboration between *Nkx2.2* and *Nkx6.2* during oligodendrocyte myelination stages, we compared the *Nkx2.2* and *Nkx6.2* immunostaining in the mouse spinal cord at various developmental stages. *Nkx2.2* expression in the ventral white matter was first detected at E17.5 with a rapid increase afterwards (Fig. 1 A–E). At postnatal

day 8 (P8), *Nkx2.2*-expressing cells were found throughout the entire spinal cord, including both grey and white matter (Fig. 1 B). The robust expression was continuous to P30 before it started to be down-regulated to a lower level thereafter (Fig. 1 C–E, data not shown). In contrast, *Nkx6.2* expression was not observed in the white matter before birth (P0) (Fig S1). However, a small number of *Nkx6.2*+ small nuclei started to show up in the ventral white matter from P1 to P4 (arrowheads in Fig 1F, 1G; Fig S1). At these stages, a few *Nkx6.2*+ large nuclei were also observed in the ventral gray matter (Fig. 1 F and G, arrows; Fig S2), and these cells are likely to represent the V1 ventral interneurons (Vallstedt et al., 2001) because they were co-labeled with pan-neuronal marker NeuN (Fig S2). Interestingly, the number of *Nkx6.2*+ cells in the white matter were rapidly up-regulated from P4 to P15 in a ventral-to-dorsal gradient (Fig 1 F–H, arrowheads; Fig S1), peaked at around P34 and then gradually declined in P68 adult spinal cord (Fig 1 I, J, Fig S1). At the same time, the number of *Nkx6.2*+ small nuclei in the gray matter was also increased with time after P8 (Fig. 1 G, arrowheads). Similarly, up-regulation of *Nkx6.2* after *Nkx2.2* was also observed in the white matter of the cerebral cortex at postnatal stages. While *Nkx2.2* expression could be detected Olig2+ oligodendrocytes in P15 corpus callosum, *Nkx6.2* expression in Olig2+ cells was not seen until P30 (Fig 2).

***Nkx6.2* is selectively expressed in mature differentiated oligodendrocytes**

Previous *in vitro* studies suggested that *Nkx6.2* homeodomain factor was expressed in both astrocytes and oligodendrocytes (Komuro et al., 1993). To examine whether *Nkx6.2* is selectively expressed in oligodendrocyte lineage *in vivo*, we performed anti-*Nkx6.2* double immunofluorescent staining in conjunction with the anti-GFAP and anti-Olig2 antibodies in postnatal mouse spinal cords. *Nkx6.2* expression in the white matter was never observed in GFAP+, Glast+ or S100 β + astroglial cells after birth (Fig 3,). Olig2 is expressed in both OPC cells and mature oligodendrocytes, and can be used as a specific and reliable marker for oligodendrocyte lineage identification in the spinal cord (Lu et al., 2000; Takeyabashi et al., 2000; Zhou et al., 2000, Arnett et al., 2004). At early postnatal stages (P2), an increasing percentage of Olig2+ cells expressed *Nkx6.2* (Fig 4 A–C, 4M); and all *Nkx6.2*+ nuclei co-expressed Olig2 except for a few large *Nkx6.2*+ interneurons in the ventral gray matter (Fig 4 A–C; Fig S2). However, at later stages, an increasing percentage of *Nkx6.2*+ cells exhibited weak expression of Olig2 (Fig. 4 D–I, arrows in F and I), suggesting that Olig2 expression is down-regulated in myelinating oligodendrocytes. Meanwhile, many Olig2+ cells, especially those in the gray matter region, did not co-express *Nkx6.2* (Fig. 4 F and I, arrowheads) at all postnatal stages examined. These Olig2+/*Nkx6.2*- cells are likely to represent undifferentiated OPCs.

The observations that *Nkx6.2*+ cells emerge in the white matter region later than *Nkx2.2* have raised the possibility that *Nkx6.2* is selectively expressed in myelinating mature oligodendrocytes after birth. To examine this possibility, we performed the anti-*Nkx6.2* and anti-APC double immunostaining in postnatal spinal cord tissues. The antibody to APC protein (CC-1) specifically labels differentiated oligodendrocytes (Bhat et al., 1996; Lu et al., 2000). At all postnatal stages examined, nearly all *Nkx6.2*+ cells were immunoreactive to APC in the cytoplasm in both the gray and white matter regions (Fig. 4 J–L, data not shown). Interestingly, between P4 and P8, some APC+ cells in the white matter were not co-labeled with anti-*Nkx6.2* antibody (Fig. 4 J, K, N; represented by asterisks in the insets), suggesting that APC expression in differentiated oligodendrocytes occurs slightly earlier than *Nkx6.2*. Based on these observations, we concluded that *Nkx6.2* is selectively expressed in mature differentiated oligodendrocytes. In support of this notion, *Nkx6.2*+ oligodendrocytes co-expressed other mature markers MBP and MAG (Fig S3 C and D, arrows), but not OPC markers PDGFR α and NG2 (Fig S3 A and B, arrowheads).

Nkx2.2 is transiently down-regulated in the differentiating oligodendrocytes but restored in Nkx6.2+ differentiated oligodendrocytes

In the developing mouse spinal cord, *Nkx2.2* was previously shown to be up-regulated in Olig2+ differentiating immature oligodendrocytes at neonatal stages (Qi et al., 2001; Fu et al., 2002). To determine whether *Nkx2.2* expression persisted in myelinating mature oligodendrocytes, we carried out *Nkx2.2* double immunostaining with other oligodendrocyte lineage-specific markers in the spinal cord at various postnatal stages. At P2, all *Nkx2.2*+ oligodendrocytes co-expressed Olig2 transcription factor (Fig. 5 A). Surprisingly, at later stages, an increasing number of *Nkx2.2*+ cells started to lose Olig2 expression (Fig. 5 B–D, arrows; 5M). This result suggested the gradual down-regulation of Olig2 expression in mature oligodendrocytes at late postnatal stages.

Double labeling experiments also revealed that there was no overlapping expression of *Nkx2.2* with APC or *Nkx6.2* at P2. At this stage, *Nkx2.2* and *Nkx6.2*/APC were expressed in distinct populations of cells in the ventral white matter (Fig. 5 E, I). Two days later, *Nkx2.2* expression started to be detected in a few APC+ and *Nkx6.2*+ oligodendrocytes (Fig. 5 F and J, arrows; Fig 6. D–E, arrowheads). At P15, a larger number of APC+ and *Nkx6.2*+ oligodendrocytes co-expressed *Nkx2.2* (Fig. 5 G and K, arrows), despite the presence of many *Nkx2.2*+ /APC- and *Nkx2.2*+ /*Nkx6.2*- cells (Fig. 5 G and K, arrowheads). By P30, *Nkx2.2* expression was observed in the majority of APC+ /*Nkx6.2*+ cells (Fig. 5 H, L, N). In adult spinal cord (P68), virtually all *Nkx6.2*+ oligodendrocytes co-expressed the *Nkx2.2* transcription factor (data not shown).

Recent studies also showed that Olig1 protein is localized in the nucleus in immature oligodendrocytes but translocated to the cytoplasm in mature oligodendrocytes (Arnett et al., 2004; Kitada and Rowitch, 2006). Consistently, at P4, most *Nkx2.2*+ cells had nuclear Olig1 staining and a few had weak cytoplasmic Olig1 staining (Fig. 6 A, F, arrows). In mature oligodendrocytes with strong cytoplasmic Olig1 staining, *Nkx2.2* expression was not detected, suggesting that *Nkx2.2* is rapidly down-regulated in differentiating oligodendrocytes when Olig1 underwent cytoplasmic translocation (Fig. 6 A, F, arrowheads). In support, *Nkx2.2* expression was only detected in newly differentiated oligodendrocytes with weak MAG expression, but not in mature oligodendrocytes with strong MAG expression in both mouse and chicken spinal cord (Fig S4). At later stages (P15 and P40), while some *Nkx2.2*+ cells with nuclear or weak cytoplasmic Olig1 staining remained to be observed, weak *Nkx2.2* expression could be detected in oligodendrocytes with strong cytoplasmic Olig1 expression (Fig. 6 B, C), suggesting that mature oligodendrocytes regained *Nkx2.2* expression at later postnatal stages.

To further verify the transient down-regulation of *Nkx2.2* expression in newly differentiated oligodendrocytes, we first purified OPC cells from rat brain or spinal cord tissues and cultured them in differentiation medium for various time lengths prior to gene expression assays. Double immunostaining revealed that about 90% A2B5+ OPC cells expressed *Nkx2.2* (Fig 7A, A', E), about 35% O4+ pro-oligodendrocytes expressed *Nkx2.2* (Fig 7 B, B', E), whereas fewer than 2% MBP+ oligodendrocytes co-expressed *Nkx2.2* (Fig 7 C, C', E). However, when oligodendrocytes formed myelin sheaths around DRG axons, a higher percentage of oligodendrocytes in DRG-OPC co-culture co-expressed *Nkx2.2* protein (Fig 7D, D', E'). Together, our *in vitro* studies demonstrated that *Nkx2.2* expression was progressively down-regulated as OPCs underwent terminal differentiation, but up-regulated again in oligodendrocytes during myelination stage. These findings are consistent with the notion of biphasic *Nkx2.2* expression during oligodendrocyte differentiation and myelination stages.

***Nkx6.2* expression in the white matter is reduced in *Nkx2.2* mutant spinal cords**

The non-overlapping expression of *Nkx2.2* and *Nkx6.2* in Olig2+ cells in P2 spinal cord could be explained by the transient down-regulation of *Nkx2.2* in differentiated *Nkx6.2*+/APC+ oligodendrocytes. However, it remains possible that they are initially expressed in two distinct oligodendrocyte subpopulations (Kitada and Rowitch 2006) which differentiate independently at early postnatal stages, but gain the expression of each other at later stages. To distinguish these two possibilities, we examined *Nkx6.2* and APC expression in the early postnatal spinal cords of *Nkx2.2* null mutants, in which oligodendrocyte differentiation is severely inhibited (Qi et al., 2001). At P4, APC expression was detected in wild-type but not mutant tissues (Fig. 8 A–B). Likewise, *Nkx6.2* expression was also absent in the white matter region of the mutants (Fig. 8 C, D and G), although a small number of *Nkx6.2*+ oligodendrocytes started to emerge in P6 mutant spinal cord (Fig. 8 E–G). Contrarily, *Nkx2.2* and APC expression are normal in *Nkx6.2* mutant spinal cord (data not shown). The absence of *Nkx6.2* and APC may be the consequence of inhibited oligodendrocyte differentiation due to *Nkx2.2* disruption, providing evidence that *Nkx2.2*+ and *Nkx6.2*+ oligodendrocyte cells are from the same lineage and that *Nkx6.2* expression is dependent on *Nkx2.2*. This observation is consistent with the concept that *Nkx2.2* expression is transiently down-regulated in newly differentiated oligodendrocytes before they progress into *Nkx6.2*+/APC+ myelinating oligodendrocytes.

DISCUSSION

In this study, we characterized the spatiotemporal pattern of *Nkx6.2* and *Nkx2.2* expression in postnatal spinal cords in relation to other stage-specific oligodendrocyte lineage markers. Detailed analyses revealed that both *Nkx* genes are selectively expressed in oligodendrocyte lineage. Specifically, *Nkx6.2* is expressed in mature oligodendrocytes, whereas *Nkx2.2* is initially expressed in differentiating OPCs and then transiently down-regulated in newly differentiated oligodendrocytes before it is re-expressed in myelinating oligodendrocytes.

Specific expression of *Nkx6.2* in mature oligodendrocytes downstream of *Nkx2.2* in postnatal spinal cord

During the early neural development, *Nkx6.2* is transiently transcribed in p1 progenitor domain of the ventral spinal cord and later in the V1 interneurons as well (Cai et al., 1999; Vallstedt et al., 2001; Fig 1). At later stages, *Nkx6.2* expression is down-regulated in V1 neurons and neural progenitor cells but up-regulated in glial cells; however, its expression specificity in glial lineage has not been fully characterized. It was reported that *Nkx6.2*/Gtx was expressed in astrocytes (Kumoro et al., 1993), differentiating or differentiated oligodendrocytes (Awatramani et al., 1997; Southwood et al., 2004). In this study, we provided evidence that *Nkx6.2* is exclusively expressed in mature oligodendrocytes in postnatal spinal cord (Fig 1 and 4) and brain (Fig 2), as all *Nkx6.2*+ cells co-expressed the mature marker APC (Fig 4) and had strong cytoplasmic Olig1 staining (Fig 6). *Nkx6.2* starts to be expressed in the white matter region of spinal cord at postnatal day 0 (Fig. S1), about two days later than *Nkx2.2* whose expression can be detected in the white matter as early as E17.5 (Qi et al., 2001; Fu et al., 2002). Previous studies showed that *Nkx2.2* is selectively expressed in differentiating OPC cells, and its activity is required for the timely differentiation of oligodendrocytes (Qi et al., 2001). Surprisingly, there was no overlapping expression of *Nkx2.2* and *Nkx6.2* during the early stages of oligodendrocyte differentiation, and *Nkx2.2* expression was not detected in *Nkx6.2*+ oligodendrocytes prior to P4 when oligodendrocytes undergo rapid differentiation (Fig 5). One possibility for the complete lack of overlapping expression of these two genes is that they are initially expressed by two distinct oligodendrocyte populations or lineages which develop independently at early postnatal stages, as suggested by Kitada et al., (2006). However, the dramatic inhibition of *Nkx6.2* expression in *Nkx2.2* mutant spinal cord (Fig 8) strongly argues against this possibility and supports the alternative idea that *Nkx2.2* is quickly down-

regulated once OPCs become fully differentiated into *Nkx6.2*+/*APC*+ mature oligodendrocytes. Consistent with this notion, there was no co-expression of *Nkx2.2* with *APC* in newly differentiated oligodendrocytes at P4 (Fig 6), although expression of these two mature markers is also dependent on *Nkx2.2* activity. Additional evidence for the down-regulation of *Nkx2.2* in mature oligodendrocytes came from the observation that at P4, *Nkx2.2* expression is co-localized with nuclear *Olig1* staining, but not with strong cytoplasmic *Olig1* staining (Fig 6A–C). Furthermore, *Nkx2.2* expression was detected in newly differentiated oligodendrocytes with a weak *MAG* expression, but not in mature oligodendrocytes with strong *MAG* expression in both chicken and mouse spinal cord tissues (Fig S4).

Re-expression of *Nkx2.2* in myelinating oligodendrocytes

Starting at P4, *Nkx2.2* expression can be detected in an increasing number of differentiated *APC*+/*Nkx6.2*+ oligodendrocytes with cytoplasmic *Olig1* localization (Fig 5, 6). The expression of *Nkx2.2* in mature oligodendrocytes is relatively weak compared to its initial expression in immature oligodendrocytes, and persists in adult spinal cord (Fig 1, 5, 6; Kitada et al., 2006). Since *Nkx6.2*+ mature oligodendrocytes do not co-express *Nkx2.2* prior to P4, it is most likely that the *Nkx6.2*-expressing mature oligodendrocytes regain *Nkx2.2* expression.

The up-regulation of *Nkx2.2* in differentiated oligodendrocytes suggests that there is a biphasic *Nkx2.2* expression in oligodendrocytes lineage in the postnatal spinal cord. At perinatal stages (prior to P4), *Nkx2.2* expression is restricted to differentiating OPCs. Between P4 and P30, *Nkx2.2* is expressed by both differentiating OPCs and mature oligodendrocytes. In adult spinal cord, *Nkx2.2* is co-expressed with *Nkx6.2* and *APC* in mature myelinating oligodendrocytes. The temporal sequence of *Nkx2.2* and *Nkx6.2* expression in relation to other oligodendrocyte stage-specific markers is summarized in Figure 9. In the postnatal spinal cord, the *Nkx2.2*+/*Nkx6.2*- cells represent the differentiating immature population, the *Nkx2.2*-/*Nkx6.2*+ cells represent the newly differentiated but non-myelinating oligodendrocytes, whereas the *Nkx2.2*+/*Nkx6.2*+ cells represent mature myelinating oligodendrocytes. It should be noted that there is heterogeneity among OPC cells in the timing of their differentiation and thus the expression of *Nkx2.2*, *Nkx6.2* and other maturation-related genes. Once OPC cells enter the maturation and myelination program, they undergo biphasic pattern of *Nkx2.2* expression. The heterogeneity of oligodendrocyte differentiation may offer an alternative explanation for the existence of different oligodendrocyte populations that express distinct combinations of transcription factors at postnatal stages (Kitada et al., 2006).

The biphasic *Nkx2.2* expression during oligodendrocyte lineage progression appears to be associated with its distinct functions in oligodendrocyte development. The early *Nkx2.2* expression in OPC cells is required for initiating oligodendrocyte differentiation program (Qi et al., 2001; Sun et al., 2001; Zhou et al., 2001; Sugimori et al., 2008), possibly by suppressing the expression of some unknown inhibitors (e.g. *Id* or *Hes* genes) in OPCs. As oligodendrocytes further differentiate along and produce myelin proteins, *Nkx2.2* expression is rapidly down-regulated. The functional significance for the rapid down-regulation of *Nkx2.2* in differentiated oligodendrocytes is not clear. Given that *Nkx2.2* functions as a transcription repressor (Muhr et al., 2001) and has an inhibitory effect on the expression of *MBP* gene in vitro (Wei et al., 2005), *Nkx2.2* down-regulation may be necessary to allow for the rapid transcription of myelin genes during active myelination stage.

The late phase of *Nkx2.2* expression in mature oligodendrocytes may be involved in regulation of myelin sheath formation and/or maintain myelin structures in collaboration with *Nkx6.2*. In support of this hypothesis, *Nkx2.2* heterozygosity in *Nkx6.2* mutant background (*Nkx2.2*+/-*Nkx6.2*-/-) resulted in severe neurological defects and abnormal ultra-structure of myelin sheaths (unpublished observations). Alternatively, it is also possible that *Nkx2.2* expression in mature myelinating oligodendrocytes may function as a negative regulator to prevent

excessive production of myelin proteins. In other words, *Nkx2.2* and *Sox10* may act concertly to fine tune the expression of myelin genes in myelinating OLs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Nkx2.2 and *Nkx6.2* expression in postnatal mouse spinal cords. Transverse sections from P1 (A, F), P8 (B, G), P15 (C, H), P34 (D, I) and P68 (E, J) wild-type spinal cords were subject to immunofluorescent staining with anti-*Nkx2.2* (A-E) or anti-*Nkx6.2* (F-J). Only the ventrolateral spinal cord positions were shown. The *Nkx6.2*+ cells with large nuclei in the grey matter (top-right inset in F) and those with small nuclei in the white matter (bottom-left inset in F) are represented by arrows and arrowheads, respectively. Scale bar: 100 μ m.

Figure 2. *Nkx2.2* and *Nkx6.2* expression in postnatal mouse brains. Transverse sections from P15 (A, C) and P30 (B, D) wild-type corpus callosum regions were subject to double immunofluorescent staining with anti-Nkx2.2 and anti-Olig2 (A, B) or anti-Nkx6.2 and anti-Olig2 (C, D). Scale bar: 100 μm.

Figure 3.

Non-overlapping of Nkx6.2 and astrocytic markers in postnatal mouse spinal cords. Cross sections from P2 (A and D), P6 (B) and P15 (C and E) wild-type pups were subject to double immunofluorescent staining with anti-Nkx6.2 and anti-GFAP (A-C), anti-Glial (D), or anti-S100B (E). Only the ventrolateral spinal cord positions were shown. Scale bar: 50 μ m.

Figure 4.

Co-expression of Nkx6.2 with Olig2 and APC in spinal cord oligodendrocytes. Spinal cord sections from P2 (A–C), P4 (J), P8 (K), P13 (D–F), P30 (G–I) and P34 (L) wild-type pups were subject to double immunofluorescent staining with anti-Nkx6.2 and anti-Olig2 (A–I), or anti-Nkx6.2 and anti-APC (J–L). Only the ventrolateral spinal cord positions were shown. The Nkx6.2⁺⁺⁺/Olig2⁺ and Olig2⁺⁺⁺/Nkx6.2⁻ single positive cells were represented by arrows and arrowheads, respectively. The percentage of Nkx6.2⁺ and Nkx6.2⁻ cells in APC⁺ or Olig2⁺ + population from P1 to P34 were shown in M and N. The Nkx6.2⁺ cells in C and J and represent the ventral interneurons. The APC⁺/Nkx6.2⁻ cells were indicated by asterisks. Inset in K showed the APC/Nkx6.2/Dapi triple labeling from the outlined region. Scale bar: 100 μ m.

Figure 5.

Expression of *Nkx2.2* in the oligodendroglial cells of postnatal spinal cords. Spinal cord sections at the thoracic level were prepared from P2 (A, E, I), P4 (F, J), P15 (B, G, K), P34 (C, H, L), and adult (D) wild-type mice. A-D. Immunofluorescence with anti-*Nkx2.2* (red) and anti-Olig2 (green). Arrows indicated the *Nkx2.2*+/*Olig2*- or weak *Olig2*+ cells. E-H. Double immunostaining with anti-*Nkx2.2* (red) and anti-APC (green). I-L. Double immunolabeling with anti-*Nkx6.2* (red) and anti-*Nkx2.2* (Green). Arrows in F-H and J-L represented the *Nkx2.2*+/*APC*+ and *Nkx6.2*+/*Nkx2.2*+ (either strong or weak) cells, respectively. Arrowheads in G and K indicated the *Nkx2.2*+/*APC*- or *Nkx6.2*- cells. The

percentage of Nkx2.2⁺ and Nkx2.2⁻ cells in APC⁺ or Olig2⁺ population from P1 to P34 were shown in **M** and **N**. Scale bar: 100 μ m.

Figure 6.

Definition of *Nkx2.2* and *Nkx6.2* expression by triple immunostaining with stage-specific markers in oligodendrocytes. Spinal cord sections were collected from P4 (**A, D-F**), P15 (**B**), and P40 (**C**) wild-type pups. Only the ventrolateral spinal cord positions were shown. **A-C**. Immunofluorescent staining with anti-*Nkx2.2* (red), anti-Olig1 (green) and Dapi (blue). Arrows delineated the *Nkx2.2*+/*nuclear* Olig1+. The *Nkx2.2*-/*cytoplasmic* Olig1+ and *Nkx2.2*+/*cytoplasmic* Olig1+ cells were represented by arrowheads and asterisks, respectively. **D**. Triple labeling with anti-*Nkx2.2* (red), anti-*Sox10* (green), and anti-APC (blue). **E**. Immunofluorescence with anti-*Nkx6.2* (red), anti-*Nkx2.2* (green), and anti-APC (blue). **F**. Triple staining with anti-*Nkx6.2* (red), anti-Olig1 (green) and anti-*Nkx2.2* (blue). Arrows represented either *Nkx2.2*+/*Sox10*+ cells in **D** or *Nkx2.2*+/*nuclear* Olig1+ in **F**. Open arrows

represented Nkx2.2+/Sox10+/APC- cells in **D**, Nkx6.2-/Nkx2.2+/APC- in **E**, or Nkx6.2-/Nkx2.2+/nuclear Olig1+ in **F**. Leis in **F** delineated the Nkx6.2+/cytoplasmic Olig1+ cells. Scale bar: 50 μ m.

Figure 7.

Up-regulation of *Nkx2.2* in OPCs and pro-oligodendrocytes but down-regulation in mature oligodendrocytes *in vitro*. Immunofluorescence with anti-*Nkx2.2* (red) and A2B5 (green), O4 (green) or anti-MBP (green) were performed in dissociated oligodendrocytes at A2B5 (A, A'), O4 (B, B'), and MBP (C, C') stages, or co-culture of DRG neurons and oligodendrocytes (D, D'). The percentage of *Nkx2.2*+ cells oligodendrocyte population at different differentiation stages was summarized in E and E'. ***: $p < 0.001$. Arrows represented either *Nkx2.2*-/Dapi+ nuclei in A and A' or *Nkx2.2*+ /Dapi+ nuclei in D and D'. Arrowheads in B, B', D, and D' or stars in C and C' indicated *Nkx2.2*+ (weak)/Dapi+ cells.

Figure 8.

Delayed expression of *Nkx6.2* and *APC* in *Nkx2.2* mutant spinal cords. Cross sections of cervical spinal cord from wild-type (**A**, **C**, **E**) and *Nkx2.2* mutant (**B**, **D**, **F**) pups at P4 (**A-D**) and P6 (**E**, **F**) were labeled with anti-APC (**A**, **B**) and anti-*Nkx6.2* (**C-F**) antibodies. **G**. *Nkx6.2* + cells per section in P4 and P6 wild-type and *Nkx2.2* null mutant spinal cords (n=3). ***: $p < 0.001$.

Figure 9. Schematic illustration of oligodendrocyte development and stage-specific expression of transcription factors during oligodendroglial lineage progression. NPC, neural progenitor cells; OPC, oligodendrocyte progenitor cell; OL, oligodendrocyte; VZ, ventricular zone.