

Olig bHLH proteins interact with homeodomain proteins to regulate cell fate acquisition in progenitors of the ventral neural tube

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Background: Organizing signals such as Sonic hedgehog are thought to specify neuronal subtype identity by regulating the expression of homeodomain proteins in progenitors of the embryonic neural tube. One of these, *Nkx2.2*, is necessary and sufficient for the development of V3 interneurons.

Results: We report that *Olig* genes, encoding basic helix-loop-helix (bHLH) proteins, are expressed in a subset of *Nkx2.2* progenitors before the establishment of interneurons and oligodendroglial precursors. Gain-of-function analysis in transgenic mouse embryos indicates that *Olig* genes specifically inhibit the establishment of *Sim1*-expressing V3 interneurons. Moreover, coexpression of *Olig2* with *Nkx2.2* in the chick neural tube generated cells expressing *Sox10*, a marker of oligodendroglial precursors. Colocalization of Olig and *Nkx2.2* proteins at the dorsal extent of the *Nkx2.2* expression domain is consistent with regulatory interactions that define the potential of progenitor cells in the border region.

Conclusions: Interactions between homeodomain and Olig bHLH proteins evidently regulate neural cell fate acquisition and diversification in the ventral neural tube. In particular, interactions between Olig and *Nkx2.2* proteins inhibit V3 interneuron development and promote the formation of alternate cell types, including those expressing *Sox10*.

Background

The generation of cellular diversity in the central nervous system (CNS) is thought to depend on graded inductive and repressive signals derived from local organizing centers. The secreted glycoprotein Sonic hedgehog (Shh), produced in ventral midline structures such as the notochord and floor plate, is essential for the induction and maintenance of many ventral neural cell types (reviewed in [1, 2]). Along the dorsal-ventral axis of the spinal cord, neural progenitors have been distinguished by regionally restricted expression of distinct sets of homeodomain (HD) proteins. It has been proposed that expression of a given set of HD proteins constitutes a neural code that eventually leads to the production of specific neuronal subtypes, such as interneurons and somatic motor neurons [2–6]. Indeed, functional studies indicate that genes encoding HD-containing proteins of the *Nkx* family can specify particular neuronal subtypes (reviewed in [7]). For example, *Nkx2.2* is both necessary and sufficient for the generation of V3 interneurons, recognized by the expression of the Pas domain transcription factor *Sim1* [4, 8, 9].

In addition to HD proteins, basic helix-loop-helix (bHLH) transcription factors have many established and conserved

roles during neural development, including the determination of neuronal versus glial cell fate [10–15]. bHLH proteins are expressed contemporaneously with HD proteins in discrete domains along the dorsal-ventral axis of the CNS [16–18]. However, functional interactions between homeodomain- and bHLH-containing proteins in cell fate specification during ventral neural tube development have not been described. The expression of *Olig* genes, encoding bHLH proteins, is regulated by Shh in the embryonic spinal cord [19]. *Olig* expression is tightly associated with cells of the oligodendrocyte lineage after 12.5 days postcoitum (dpc) in the mouse spinal cord, and ectopic *Olig* expression is sufficient to promote the formation of oligodendrocyte precursors (OLPs) [19, 20]. However, *Olig* genes are expressed from 9–12.5 dpc, raising the possibility that Olig proteins function in the development of other neural lineages [19–21]. Indeed, this period of time is typically associated with the production of motor neurons and interneurons in the ventral neural tube (reviewed in [2, 6]). In this study, we have focused on the possible interactions between Olig proteins and homeodomain transcription factors within neural progenitors during two distinct phases of *Olig* expression associated with the formation of neurons and oligodendroglial precursors in the vertebrate spinal cord.

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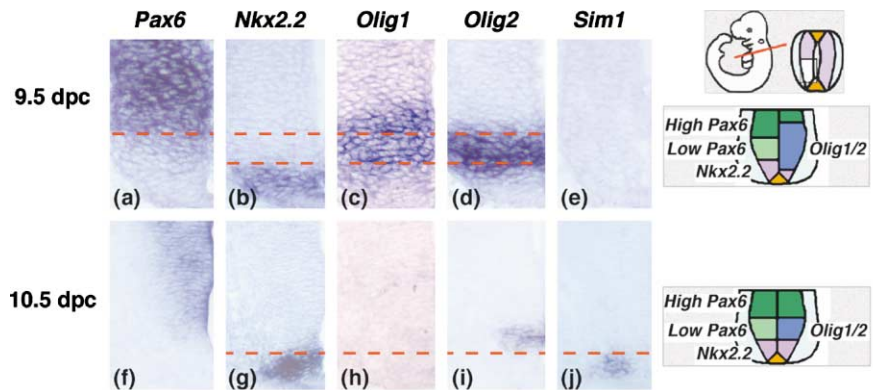
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Figure 1

Dynamic expression of *Olig* genes from 9.5 dpc to 10.5 dpc in the ventral murine spinal cord. Adjacent sections through cervico-thoracic spinal cords of mouse embryos were hybridized in situ with antisense mRNA probes for (a,f) *Pax6*, (b,g) *Nkx2.2*, (c,h) *Olig1*, (d,i) *Olig2*, and (e,j) *Sim1*. The region in the spinal cord shown in this figure is highlighted in the scheme (top right). The expression of *Olig1* and *Olig2* is largely encompassed within the *Pax6*^{low} expression domain at 9.5 dpc (dotted lines in [a]–[d]). Overlap of *Olig* expression with *Pax6*^{high} and *Nkx2.2* expression regions is also observed. At 10.5 dpc, *Olig2* expression is restricted to a (i) relatively small domain, while *Olig1* expression is undetectable in (h) rostral spinal cord. *Sim1* is initially expressed at 10.5 dpc (compare [j] and [e]). *Olig* gene expression relative to *Pax6* and *Nkx2.2* in 9.5 and 10.5 dpc spinal cords is summarized in schemes at the right.



Results

Dynamic expression of *Olig* genes in the embryonic neural tube at stages associated with neurogenesis

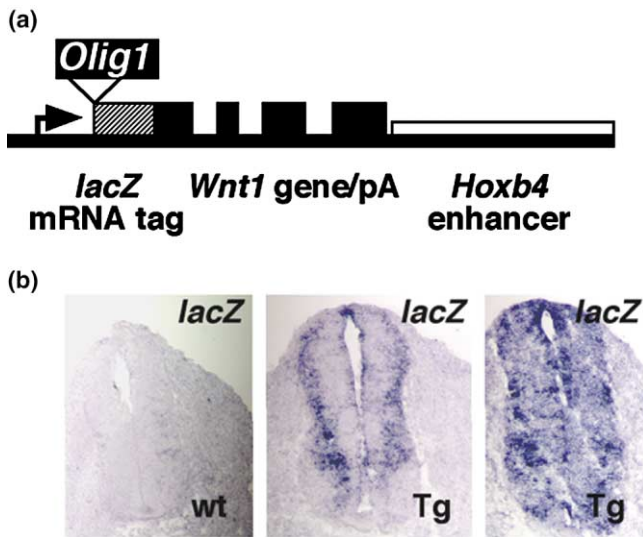
The expression of *Olig* genes has previously been observed as early as 8.5 dpc in the mouse spinal cord, well before the time of oligodendrocyte precursor appearance at 12.5 dpc [19–22]. It has been proposed that Shh activity induces the expression of *Nkx2.2* and represses *Pax6*, and that interactions between *Nkx2.2* and *Pax6* proteins set up the boundary between V3 interneurons and motor neurons in the ventral spinal cord [4, 8]. To identify populations of neural progenitors that express *Olig* genes at early stages of ventral neural tube development, we performed a comparative analysis of *Olig* expression with respect to *Pax6* and *Nkx2.2* between 9.5 and 10.5 dpc of mouse development. As shown in Figure 1, both *Olig1* and *Olig2* are initially expressed in a broad domain of the ventral neural tube. Analysis of adjacent sections tested with *Pax6* and *Nkx2.2* indicated that the *Pax6*^{low} domain encompassed the majority of *Olig1*- and *Olig2*-expressing cells (Figure 1a–d). Additionally, overlap of *Olig* expression within *Pax6*^{high}- and *Nkx2.2*-expressing regions was also apparent (Figure 1a–d). At 10.5 dpc, *Olig2* expression was relatively restricted and appeared to segregate away from *Nkx2.2*- and *Pax6*^{high}-expressing cells (Figure 1f,g,i), while *Olig1* expression in the rostral spinal cord was downregulated (Figure 1h). Contemporaneously, *Sim1*-expressing cells were first observed in a ventral domain of the neural tube adjacent to the floor plate (compare Figures 1e and 1j). In summary, *Olig* expression at 9.5 dpc overlapped that of neuronal progenitors thought to give rise to distinct neuronal subtypes at 10.5 dpc, consistent with possible roles during neurogenesis. Indeed, the absence of *Olig1* expression at 10.5 dpc coincided with the appear-

ance of *Sim1* expression, suggesting the possibility that the formation of *Sim1*-expressing cells might require *Olig* downregulation.

Ectopic expression of *Olig* genes inhibits development of V3 interneurons

In order to test whether overlapping expression of *Olig1* with HD proteins had functional consequences for the determination of neuronal identity, we used a gain-of-function approach employing well-characterized regulatory sequences for *Hoxb4* [23]. The *Hoxb4* regulatory element initially drives expression broadly throughout the dorsoventral axis of the spinal cord from about 9 dpc, but enhancer-driven expression is transient and rapidly downregulated, because activity of β -galactosidase proteins are undetectable by 12.5 dpc [24]. Thus, the characteristics of temporal-spatial expression driven by the *Hoxb4* regulatory element were suitable to test effects of transient ectopic expression of *Olig* genes in the neural tube at early stages before OLP formation. Mouse *Olig1* was cloned upstream of the *Hoxb4* regulatory element (Figure 2a), and transgenic founder embryos were generated at 10.5, 14.5, and 18.5 dpc (Table 1). While we were able to achieve production of at least 10%–12% transgenic animals when they were harvested prenatally, we were unable to recover any founder mice at weaning, suggesting that transgene-driven *Olig1* expression was lethal at some time during the first 3 weeks postpartum (Table 1).

We then examined the consequences of *Olig1* ectopic expression on the development of neuronal progenitors and postmitotic neurons. Transgene expression was observed widely throughout the spinal cord by 10.5 dpc (Figures 2b and 3a,e). The overall pattern in the neural

Figure 2

The generation of transgenic mice misexpressing *Olig1* under the control of the *Hoxb4* regulatory element in the neural tube. **(a)** Full-length rat *Olig1* cDNA was cloned upstream of a *lacZ* mRNA tag and a *Hoxb4* regulatory element. Transgenic mice were genotyped by PCR. **(b)** Expression driven by the *Hoxb4* regulatory element in spinal cords of 10.5 dpc was detected by in situ hybridization using a *lacZ* antisense mRNA probe. Transgene expression in two independent transgenic founders is shown, indicating variation in the levels of transgene expression among founder embryos [25].

tube, as judged by the expression of *Nkx2.2*, *Pax6*, *Nkx6.1*, *Olig2*, *Ngn1*, and *Ngn2*, was unaffected in transgenic animals compared with wild-type littermates (Figure 3b,c,f,g and data not shown). Similarly, markers for somatic motor neurons (*Isl1* and *Lim3*) and V2 interneurons (*Lim3* and *Chx10*) were unaffected in the transgenic embryos (data not shown). We then tested whether there was a late defect of motor neuron and V2 interneuron development in the transgenic neural tube. Similar to results in transgenic animals at 10.5 dpc, at 14.5 dpc, populations of

Table 1**Generation of *Hoxb4-Olig1* transgenic mice.**

Age harvested/genotyped	Number tested	Number transgenic (%)
10.5 dpc	96	14 (14)
14.5 dpc	65	7 (10)
18.5 dpc	64	6 (9)
2–3 weeks postnatal	178	4 (2 ^a)

Transgenic founder embryos were harvested at 10.5, 14.5, and 18.5 dpc and were genotyped by PCR. While at least 9%–14% of the transgenic embryos were obtained at various antenatal stages, no founders were recovered at the time of weaning that transmitted the transgene, suggesting lethality of the *Hoxb4-Olig1* transgene. ^aFounders that did not transmit transgene to progeny, consistent with a high degree of mosaicism.

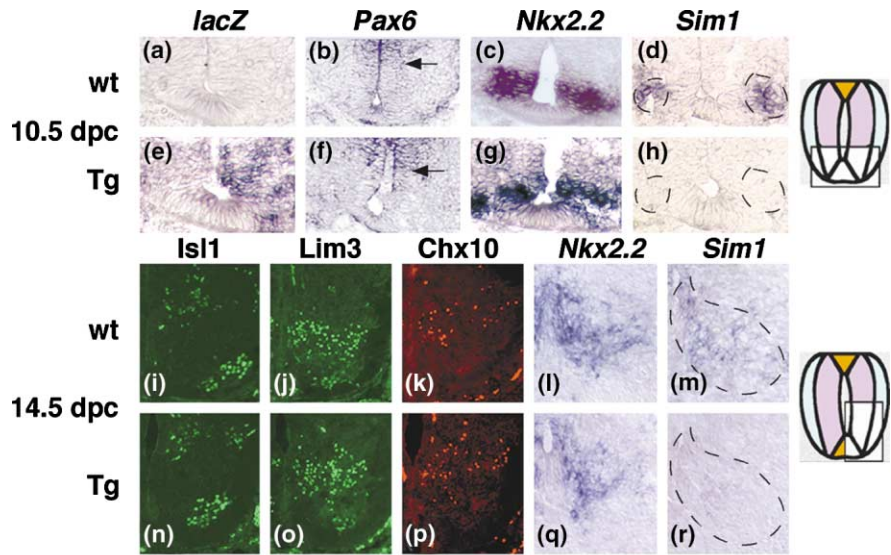
motor neurons and V2 interneurons were readily detected (Figure 3i–k,n–p), suggesting that *Olig1* ectopic expression did not adversely affect the formation of motor neurons and V2 interneurons. In contrast, *Sim1*-expressing cells were absent in 40% (2/5) of the transgenic animals examined at 10.5 dpc (compare Figure 3d with 3h). To test whether *Olig1* ectopic expression caused a delay in the formation of V3 interneurons, we examined transgenic animals at 14.5 dpc. *Sim1* expression was undetectable in 50% (3/6) of the transgenic animals tested at this age (compare Figure 3m with 3r), despite the normal expression of *Nkx2.2* (Figure 3l,q). The finding that 40%–50% of the transgenic founders had the phenotype is consistent with previous findings in transgenic mice using the identical *Hoxb4* A-region regulatory element for gene misexpression in the developing neural tube [24].

These results did not rule out the possibility that V3 interneurons were initially specified but then underwent apoptotic cell death as a result of toxic effects of *Olig1* misexpression. To assess cell death, we performed TUNEL assays on spinal cord sections from wild-type and transgenic founder embryos at 10.5 and 14.5 dpc. However, the numbers of TUNEL-positive cells in the neural tube of transgenic animals did not differ significantly from those of wild-type littermates (Figure 4). Thus, it was unlikely that *Sim1*-expressing cells in *Hoxb4-Olig1* transgenic animals were lost because of apoptosis. Additionally, the absence of *Sim1*-expressing cells in 40% of the transgenic animals at 10.5 dpc argued for a primary failure in the establishment of *Sim1*-expressing cells, rather than a subsequent loss. Together, these results suggest that ectopic expression of *Olig1* in the ventral-most regions of the neural tube inhibited production of *Sim1*-expressing V3 interneurons, but not of V2 interneurons or somatic motor neurons.

Since *Nkx2.2* has been shown to be both necessary and sufficient for the development of *Sim1*-expressing V3 interneurons [4, 8], we considered it likely that the absence of *Sim1*-expressing cells in *Hoxb4-Olig1* transgenic animals resulted from an alteration of cell fate in the *Nkx2.2* progenitors. To determine specific effects of *Olig* expression on *Nkx2.2* functions in the establishment of neural cell fate, we coexpressed *Olig2* and *Nkx2.2* by electroporation in the chick neural tube. As shown (Figure 5a–c), misexpression of *Nkx2.2* was sufficient to induce ectopic *cSim1*-expressing V3 interneurons in 12/15 (80%) of the embryos analyzed, in keeping with previous findings [4]. Interestingly, this effect was completely inhibited by coelectroporation with *Olig2* in 11/11 of the cases analyzed (Figure 5h). These results confirmed observations from the transgenic mice and furthermore extended results to *Olig2*, suggesting that antagonism of V3 interneuron development is a general property of *Olig* proteins.

Figure 3

Ectopic expression of *Olig1* in the neural tube inhibits the development of *Sim1*-expressing V3 interneurons. Adjacent sections through rostral spinal cords of (e–h,n–r) *Hoxb4-Olig1* transgenic embryos and (a–d,i–m) wild-type littermates at (a–h) 10.5 dpc and (i–r) 14.5 dpc were hybridized in situ with antisense mRNA probes for (a,e) *lacZ*, (b,f) *Pax6*, (c,g,i,q) *Nkx2.2*, and (d,h,m,r) *Sim1* and were immunolabeled with antibodies against (i,n) Isl1, (j,o) Lim3, and (k,p) Chx10. Regions in the spinal cords shown in this figure are highlighted in schemes at the right. Note the transgene expression in the spinal cord at (e) 10.5 dpc and the normal expression of markers indicating a dorsal-ventral pattern, such as *Pax6* and *Nkx2.2* (compare [b,f] and [c,g]). Populations of somatic motor neurons (Isl1⁺ and Lim3⁺ cells) and V2 interneurons (Lim3⁺ and Chx10⁺ cells) were comparable in 14.5 dpc transgenic and wild-type spinal cords (compare [i–k] and [n–p]). However, *Sim1*-expressing V3 interneurons were undetectable in transgenics at (d,h) 10.5 dpc and (m,r) 14.5 dpc, despite the intact expression of *Nkx2.2* (compare [c,g] and [l,q]).



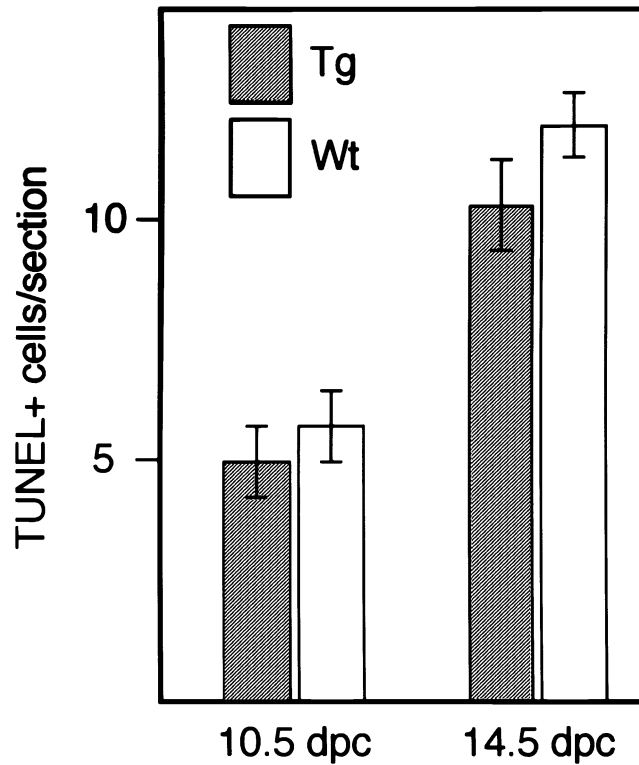
Olig-Nkx2.2 interactions promote development of *Sox10*-expressing cells

The expression of *Olig* genes after 12.5 dpc is tightly linked to cells of the oligodendrocyte lineage [19, 20], and recent work has shown that *Nkx2.2* proteins are expressed in oligodendrocytes of the chick neural tube [26, 27]. Our results indicated that ectopic expression of *Olig* genes inhibited the formation of V3 interneurons but did not lead to increased levels of cell death. One possibility is that, in the presence of *Olig* proteins, *Nkx2.2* progenitor cells might fail to form V3 interneurons, instead becoming oligodendrocytes. In the 12.5 dpc mouse spinal cord, *Olig* expression remained largely confined to a region of *Pax6*^{Low} expression (Figure 6a–d), and *Sox10*-expressing oligodendrocyte precursors were observed within the *Olig* domain (Figure 6e), as previously reported [19, 20]. The dorsal extent of the *Nkx2.2* expression domain overlapped with that of *Olig1* and *Olig2* (compare Figure 6b with 6d), and double immunolabeling indicated that *Olig2* proteins colocalized with *Nkx2.2* in progenitor cells with an apparent nuclear distribution (Figure 6f). In the electroporation experiments, neither *Nkx2.2* nor *Olig2* alone was capable of inducing cells expressing the OLP marker *cSox10* [20, 28] (Figure 5c,f). In distinction, *cSox10* induction was observed in 11/15 (73%) of the embryos that coexpressed *Nkx2.2* and *Olig2* (Figure 6i). No ectopic *cSim1* expression was observed in these 11 embryos, while weak ectopic *cSim1* expression was detected in the remaining 4/15 embryos that failed to show *cSox10* induction. Together, these results suggested that, in the presence of *Olig2*, *Nkx2.2*-expressing progenitors were directed to an alter-

nate cell fate, as evidenced by the expression of *cSox10*. To assess whether the induction of *cSox10* was associated with the acquisition of additional markers of oligodendroglial precursors, all 11 embryos that showed ectopic induction of *cSox10* were further analyzed for *cPDGF α R* expression. Though we failed to detect ectopic expression of *cPDGF α R* in the neural tube (data not shown), it should be noted that our analysis was performed at E4, before the normal commencement of *cPDGF α R* expression at E6–6.5 [27]. Thus, while *Sox10* expression is an indicator of cells that have adopted an alternate cell fate (e.g., versus *Sim1*), such cells do not apparently express additional markers of the oligodendrocyte lineage at E4 in the chick. Together, these findings suggest that *Olig* proteins interact with *Nkx2.2* in the determination of interneuronal versus glial cell fate in the neural tube.

Discussion

The vertebrate CNS is a complex structure consisting of many thousands of distinct neuronal and glial cell types, any one of which may require multiple intercellular and intracellular interactions to generate a functional subtype. Here, we provide evidence for dual *Olig*-*Nkx2.2* functional interactions spanning a period in CNS development when neurons and oligodendrocytes emerge in the mouse embryonic spinal cord. Ectopic expression of *Olig1* in transgenic mice inhibited the development of V3 interneurons, and, similarly, *Olig2* blocked the induction of ectopic V3 interneurons by *Nkx2.2* in the chick neural tube. Our results further indicate that *Olig* bHLH tran-

Figure 4

The absence of V3 interneurons is not due to cell death in *Hoxb4-Olig1* transgenic spinal cords. TUNEL assays were performed on sections from wild-type and *Hoxb4-Olig1* transgenic spinal cords at 10.5 and 14.5 dpc. The numbers of TUNEL-positive cells in the neural tube of transgenic animals (10.5 dpc, [mean ± SD] 5 ± 1.04; 14.5 dpc, 10.4 ± 1.06) and wild-type littermates (10.5 dpc, 6.3 ± 0.95; 14.5 dpc, 13 ± 0.71) were counted. No significant difference was observed between wild-type and transgenics.

scription factors can interact with HD protein Nkx2.2 to promote the formation of *Sox10*-expressing cells. Together, these findings provide the first evidence for a mechanism of interactions between HD- and bHLH-containing transcription factors in modulating the development of progenitors in the developing ventral neural tube.

Regulatory interactions at the borders of the *Olig-Nkx2.2* expression domain

HD proteins in the neural tube have been classified on the basis of repressive or activating regulatory effects of Shh in neural explants [4]. For instance, Shh protein has been shown to induce Nkx2.2 expression at relatively high concentrations, while repressing Pax6 [4, 8]. Previous work has shown that *Shh* is also an essential regulator of early *Olig* expression [19]. While Shh as well other factors (e.g., [29]) likely establish early patterns in the ventral neural tube, the maintenance of neural progenitor domains appears to involve subsequent refinement and

cross-regulatory interactions between transcription factors expressed in adjacent regions.

We observed that *Olig1* expression invaded a dorsal portion of the *Nkx2.2* expression domain at 9.5 dpc. Gain-of-function studies further suggest that, during this phase, Olig proteins may delimit the *Nkx2.2*-expressing progenitor pool that gives rise to *Sim1*⁺ V3 interneurons (Figure 7). By 10.5 dpc, *Olig* expression is restricted to a more dorsal region associated with the production of motor neurons. Interestingly, in mutants homozygous for null mutations of *Nkx2.2*, *Olig1/2* expression is ventralized [30]. Thus, at early stages of neural tube development, Nkx2.2 acts, directly or indirectly, to regulate the *Olig* expression domain. Together, these observations are consistent with a model of complex interactions in which Nkx2.2 proteins repress the expression of *Olig* genes and Olig proteins inhibit the activity of Nkx2.2. We speculate that this may constitute a mechanism whereby these progenitor domains are refined after the initial patterning of the neural tube by organizing factors such as Sonic hedgehog.

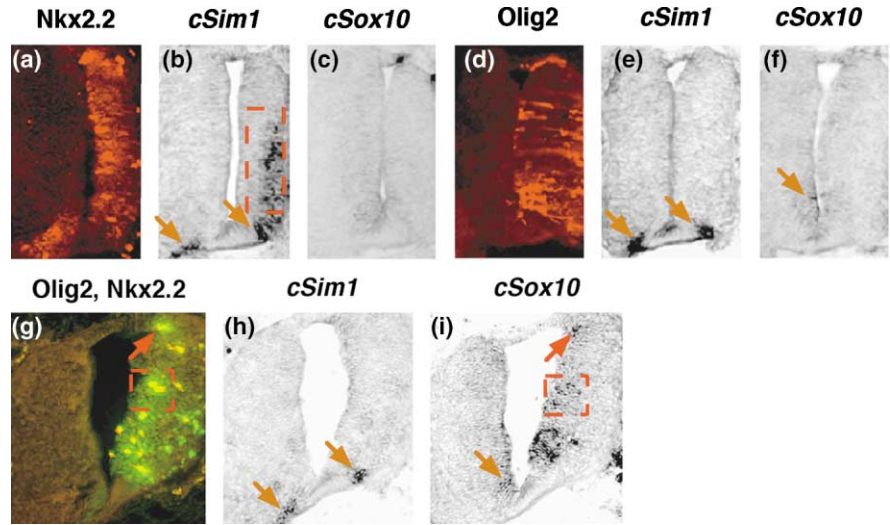
Recent work has indicated that cell fate specification by certain HD proteins relies on the presence of a conserved eh1 motif, which mediates a transcription repressor function by interacting with Gro/TLE corepressors [31]. It is unclear whether Olig effects are dominant to Nkx2.2 and act, for example, to block its normal transcriptional activity. Direct physical interactions between the class A bHLH transcription factor, Pan1, and HD protein Pitx1 in vitro have been described [32]. We have observed that Olig proteins are distributed in the cytoplasm of cos7 cells but acquire nuclear distribution when cotransfected with *Nkx2.2* (T. Sun and D. Rowitch, unpublished data). Further work will be required to establish the nature of putative Olig-Nkx2.2 transcriptional regulatory interactions.

Olig functions that promote oligodendrocyte precursor development are complex and require additional factors

The specification of neurons and glia occurs over a protracted period in the mouse CNS. Neurons initially differentiate as early as 9.5 dpc in the spinal cord, whereas oligodendrocyte precursors first emerge from the ventricular zone at 12.5 dpc. We have previously shown that *Olig1* can promote oligodendrocyte precursor development in dispersed rat neuroepithelial cultures [19]. To further analyze whether *Olig* genes are sufficient for the production of oligodendrocyte precursors within the developing neural tube, we overexpressed *Olig2* and *Olig1* in the chick and mouse spinal cord, respectively. We found induction of *Sox10*-expressing cells under conditions of *Olig2-Nkx2.2* coexpression, but not when *Olig2* was expressed alone. These findings provide evidence that additional factors are required to interact with Olig proteins during oligodendroglial specification. Indeed, we have observed colocalization of *Olig2* and Nkx2.2 proteins in progenitor cells

Figure 5

Coexpression of *Olig2* and *Nkx2.2* in the chick neural tube blocks induction of *Sim1*-expressing V3 interneurons and results in the formation of *Sox10*-expressing cells. Expression constructs containing cDNAs of *Nkx2.2* and *Olig2* were electroporated into the neural tube of stage-10–12 chick embryos, and 48 hr later, embryos (stage 21–23) were harvested for analysis. Adjacent sections were immunolabeled with antibodies against ([a], in red) rabbit polyclonal *Nkx2.2* and ([d, g], in red) *Olig2* and ([g], in green) mouse *Nkx2.2* and were hybridized in situ with probes for chick (b,e,h) *cSim1* and (c,f,i) *cSox10*. Ectopic expression was directed to the right half of the neural tube, and the contralateral (nonelectroporated) side served as a control. (a–c) Ectopic expression of *Nkx2.2* induces *cSim1* expression (The square indicates ectopic *Sim1* expression, and the brown arrows indicate endogenous *Sim1*), but not *cSox10* expression. Ectopic *cSim1* expression was detected in 12/15 (80%) of the embryos that expressed *Nkx2.2*. (d–f) Ectopic expression of *Olig2* does not induce *cSox10* expression. However, (g–i) coelectroporation of *Nkx2.2* and *Olig2* induces (red arrow in [i]) *cSox10* expression, but not (h) *cSim1* expression, in contrast with



results above (c) and (f). The red box in (g) and (i) highlights an area of coexpression associated with ectopic *Sox10*-expressing cells. *cSox10* induction was observed in 11/15 (73%) of the embryos that coexpressed *Nkx2.2* and *Olig2*, and ectopic *cSim1* expression was not detected in these 11

embryos (though endogenous *cSim1* expression was detectable). Note that *cSox10* expression is located in the ventricular zone, whereas *cSim1* is expressed in postmitotic cells in the mantle layer of the spinal cord. Brown arrows indicate regions of endogenous gene expression in the panels.

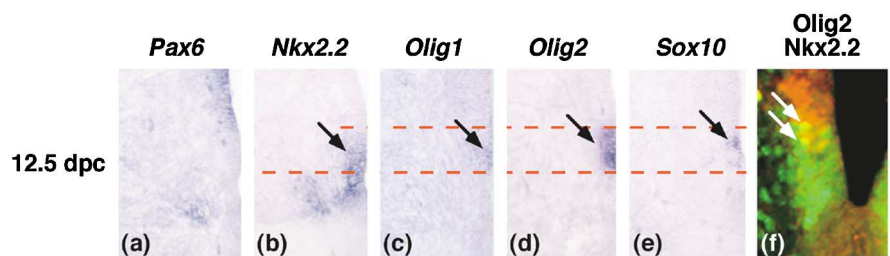
at 12.5 dpc, consistent with possible interactions that regulate oligodendrocyte fate. It should be noted, however, that such interactions would only contribute to the production of a portion of OLPs in the mouse neural tube, because the overlap of *Nkx2.2* and *Olig* proteins was seen in a relatively small number of cells.

A further complication in dissecting requirements for oligodendrocyte specification is the temporal restriction on the formation of oligodendrocyte precursors in the mammalian CNS. We have observed colocalization of *Olig2* and *Nkx2.2* proteins in neural tube progenitors at 9.5 dpc, well before the emergence of OLP (T. Sun and D.

Rowitch, unpublished data). Similarly, coexpression of *Olig2* and *Nkx2.2* resulted in the induction of *Sox10*-expressing cells, but we failed to detect the expression of additional markers of oligodendrocyte precursors at E4 in the chick neural tube. In the rat optic nerve, *Id4* appears to regulate the timing of oligodendrocyte precursor differentiation [33]. The factors that regulate the timing of oligodendrocyte development in the spinal cord, however, remain poorly understood. Indeed, we cannot rule out the possibility that *Olig*-*Nkx2.2* protein expression might establish a novel type of self-renewing neural progenitor that gives rise to a neuronal daughter cell prior to 12.5 dpc and an OLP thereafter (Figure 7). In any case, *Olig*

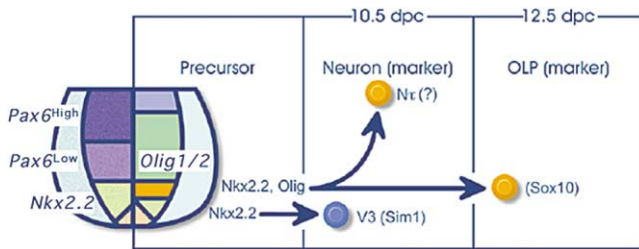
Figure 6

Overlapping expression of *Olig1/2* and *Nkx2.2* at the time of oligodendrocyte precursor emergence. Adjacent sections through cervico-thoracic spinal cords of mouse embryos at 12.5 dpc were hybridized in situ with antisense mRNA probes for (a) *Pax6*, (b) *Nkx2.2*, (c) *Olig1*, (d) *Olig2*, and (e) *Sox10*. The region in the spinal cord that is shown in this figure is highlighted in the scheme (Figure 1, top right). Expression of *Olig1* and *Olig2* is largely encompassed within the *Pax6*^{low} expression domain at 12.5 dpc (dotted lines in [b]–[e]). Overlap of *Olig* expression with *Nkx2.2* expression regions is also observed. (f) *Olig2* proteins overlapped



Nkx2.2 with an apparent nuclear distribution in a subset of *Nkx2.2*-expressing neural progenitor cells (yellow pseudocolor and white arrows indicate double-labeled cells).

The domain of *Olig* and *Nkx2.2* expression is comparable to that giving rise to oligodendrocyte precursors, indicated by (e) *Sox10* expression.

Figure 7

Interactions between homeodomain protein Nkx2.2 and Olig bHLH transcription factors at distinct phases of ventral neural tube development. A model for the regulation of V3 interneuron and oligodendrocyte precursor development through Olig-Nkx2.2 interactions in the mouse neural tube. *Sim1*-expressing V3 interneurons are normally produced from Nkx2.2 neuronal progenitors at 9.5–10.5 dpc. The development of V3 interneurons is apparently antagonized by Olig1 and/or Olig2 proteins at 9.5 dpc. In contrast, Olig-Nkx2.2 interactions at 12.5 dpc are sufficient to generate *Sox10*-expressing oligodendrocyte precursors. We cannot rule out that, during the intervening period (<12.5 dpc), Olig-Nkx-expressing progenitor cells divide and give off other neuronal progeny. Additional factors are involved in temporal restrictions on Olig function in neural cell fate regulation.

gene dosage is unlikely to explain the timing of OLP emergence, because overexpression of *Olig1* at early stages under the control of *Hoxb4* regulatory sequences resulted only in modest increases in OLP number (T. Sun and D. Rowitch, unpublished data). We conclude that cell-intrinsic [33] or environmental restrictions on cellular competence modulate Olig functions in the establishment of oligodendrocyte precursors.

Conclusions

Our results suggest that bHLH-HD interactions constitute an important general mechanism underlying cellular diversification in the vertebrate spinal cord. In particular, our results indicate that interactions between Olig and Nkx2.2 proteins can inhibit V3 interneuron development and can promote the formation of alternate cell types, including cells expressing *Sox10*. It is likely that such interactions are relevant at the border of the Olig-Nkx2.2 expression domain when refinement of progenitor boundaries is taking place. Olig functions in neural cell fate specification are likely to be context dependent and reliant on interactions with other transcription factors.

Materials and methods

Generation and genotyping of transgenic mice

Rat *Olig1* [19] was cloned into the EcoRV site of the mammalian expression vector, WEXP3C [34]. The *Wnt-1* enhancer was removed by BglII digestion, and *Hoxb4* regulatory sequences were cloned downstream of the poly A sequence, to create the *Hoxb4-Olig1* transgene. Transgenic mice were produced by standard pronuclear microinjection of purified DNA. Transgenic founders were identified by PCR to amplify a 250-bp lacZ Tag segment (lacZ-F: 5'-TTTAACGCCGTGCGCTGTTCG-3';

lacZ-R: 5'-GATCCAGCGATACAGCGCGTC-3'. PCR conditions: 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min; for 35 cycles).

In ovo electroporation and analysis of chick embryos

Mouse *Nkx2.2* and *Olig2* full-length cDNAs were cloned into pcDNA3.1/Myo-His(B) (Invitrogen), and *Olig2* was also cloned into pFLAG-CMV (Sigma) vectors. Hamburger and Hamilton (HH) stage-10–12 chick embryos were electroporated unilaterally (five 50-ms pulses at 25V) with cDNAs (3 μg/μl) using an ECM830 electro-squareporator (BTX). For coelectroporation, equal amounts (3 μg/μl) of *Nkx2.2* and *Olig2* cDNAs were mixed before electroporation. Embryos were analyzed after 48 hr of incubation (HH stages 21–23).

Tissue preparation and in situ hybridization

Mouse embryos were collected at different ages according to the assumption that mating occurred at midnight. Embryos were fixed in 4% paraformaldehyde/PBS and cryosectioned at 15–17 μm. In situ hybridization was performed as a modified protocol [18], and detailed instructions are available upon request. Digoxigenin (DIG)-labeled antisense mRNA probes for *Olig1*, *Olig2*, *Pax6*, *Nkx2.2*, *Nkx6.1*, *Ngn1/2*, *Sim1*, *Sox10*, *cPDGFαR*, *cSim1*, and *cSox10* were prepared by in vitro transcription.

TUNEL assays and immunohistochemistry

Apoptotic cell death was detected using a Fluorescein in situ apoptosis detection kit (Intergen). Total numbers of dead cells within at least ten separate transverse spinal cord sections were counted per animal analyzed. For immunohistochemistry, mouse and chick embryos were fixed in 4% paraformaldehyde/PBS and were cryosectioned. The following primary antibodies were used at the indicated dilutions: Isl1 (1:50, DSHB), Lim3 (1:100, DSHB), Chx10 (1:5000, gift from C. Cepko, Harvard Medical School), mNkx2.2 (1:500, DSHB), rNkx2.2 (1:5000, gift from T. Jessell, Columbia University), Olig1 (1:300, [19]), Olig2 (1:3000, [21]), and Myc (1:200, Invitrogen). The secondary antibodies that were used were anti-mouse IgG FITC (1:100, Jackson) and anti-rabbit IgG Cy3 (1:200, Jackson). Photomicrographic images were collected on a Nikon E600 microscope and SPOT I digital camera.

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