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# *olig2* Is Required for Zebrafish Primary Motor Neuron and Oligodendrocyte Development

**Hae-Chul Park, Amit Mehta, Joanna S. Richardson, and Bruce Appel**[1]
*Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee 37235*

**Oligodendrocytes are produced from the same region of the ventral spinal cord that earlier generated motor neurons in bird and rodent embryos. Motor neuron and oligodendrocyte precursor cells express *Olig* genes, which encode basic helix–loop–helix transcription factors that play important roles in the development of both motor neurons and oligodendrocytes. We found that oligodendrocytes develop similarly in zebrafish embryos, in that they arise from ventral spinal cord and migrate to new positions. Developing primary motor neurons and oligodendrocytes express *olig2* as do neural plate cells that give rise to both primary motor neurons and oligodendrocytes. Loss of *olig2* function prevented primary motor neuron and oligodendrocyte development, whereas *olig2* overexpression promoted formation of excess primary motor neurons and oligodendrocytes. We provide genetic evidence that Hedgehog signaling is required for zebrafish *olig2* expression and oligodendrocyte development. However, *olig2* overexpression did not promote primary motor neuron or oligodendrocyte development in embryos with reduced Hedgehog signaling activity. One possibility consistent with these data is that Hedgehog signaling, partly by inducing *olig2* expression, specifies neural precursor cells that have potential for primary motor neuron or oligodendrocyte fate.** 

***Key Words:*** **zebrafish; oligodendrocyte; glia; motor neuron; spinal cord; neurogenesis; Hedgehog; Olig.**

## INTRODUCTION

During vertebrate development, multipotent neural precursor cells produce many different kinds of neurons and glia. Some types of cells arise from distinct regions of the central nervous system (CNS), reflecting the activity of spatially distributed signaling molecules. For example, a gradient of Sonic hedgehog (Shh) initiates patterning processes that culminate in production of different classes of neurons from precursors located at distinct positions along the dorsoventral axis of the spinal cord (Briscoe and Ericson, 2001). Further cell-type diversity results from production of different cells from a particular region of the CNS. In bird and rodent embryos, a discrete region of the ventral spinal cord produces first motor neurons and then oligodendrocytes, the mylenating glial cell type of the CNS (Richardson *et al.,* 2000). The mechanisms that regulate the switch between motor neuron and oligodendrocyte production are poorly understood.

Recently, several reports showed that *Olig* genes, which encode basic helix–loop–helix (bHLH) transcription factors, are important for motor neuron and oligodendrocyte development. In chick, ventral neural precursor cells, corresponding to the site of origin of motor neurons and oligodendrocytes, express *Olig2,* whereas cells within the same region of mouse and rat embryos express *Olig2* and the closely related *Olig1* gene (Lu *et al.,* 2000; Takebayashi *et al.,* 2000; Zhou *et al.,* 2000, 2001; Mizuguchi *et al.,* 2001; Novitch *et al.,* 2001; Sun *et al.,* 2001). Forced expression of *Olig2* in chick spinal cords produced excess motor neurons (Mizuguchi *et al.,* 2001; Novitch *et al.,* 2001) and premature differentiation of oligodendrocytes in ventral spinal cord, whereas together with *Nkx2.2, Olig2* expression caused ectopic formation of oligodendrocytes (Sun *et al.,* 2001; Zhou *et al.,* 2001). Finally, targeted mutagenesis revealed that *Olig* gene functions are required for development of both motor neurons and oligodendrocytes in mice (Lu *et al.,* 2002; Zhou and Anderson, 2002). It is not yet clear how *Olig*-expressing neural precursors produce both motor neurons and oligodendrocytes.

A complete understanding of motor neuron and oligodendrocyte specification will require knowing the relationship between motor neuron and oligodendrocyte precursor cells

[1] To whom correspondence should be addressed. Fax: (615) 343-6707. E-mail: b.appel@vanderbilt.edu.

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(Richardson *et al.*, 2000). One possibility is that proliferative precursors divide asymmetrically to produce first motor neurons and then oligodendrocytes. Consistent with this are lineage data showing that marked clones that contain motor neurons sometimes include putative oligodendrocytes (Leber *et al.*, 1990). Another possibility is that some ventral neural precursors generate motor neurons and that, later, other precursors produce oligodendrocytes. These precursors could be committed to motor neuron or oligodendrocyte fate or they could have equivalent potential for either fate.

To begin to address these issues, we initiated an investigation of oligodendrocyte development in the relatively simple spinal cord of zebrafish, which has not been described previously. Here, we report on zebrafish *olig2*. We show that proliferative ventral neural precursors and developing primary motor neurons and oligodendrocyte progenitors express *olig2*. By fate mapping, we provide evidence that *olig2*-positive neural plate cells develop as primary motor neurons and oligodendrocytes, indicating that these cell types arise from a common precursor population. Reducing *olig2* function by injection of antisense morpholino oligonucleotides resulted in absence of nearly all primary motor neurons and oligodendrocytes, whereas overexpression of *olig2* produced excess primary motor neurons and oligodendrocytes. Finally, we show that Hedgehog signaling is required for *olig2* expression and oligodendrocyte development in zebrafish but that *olig2* overexpression does not rescue primary motor neuron and oligodendrocyte development in embryos that have reduced Hedgehog signaling. Our data raise the possibility that Hedgehog signaling acts, in parallel, on *olig2* and other factors to specify a common population of neural precursors that have potential for either primary motor neuron or oligodendrocyte fate.

## MATERIALS AND METHODS

### *Embryos*

Embryos were collected from pair matings, raised at 28.5°C, and staged according to hours postfertilization (hpf) and morphological criteria (Kimmel *et al.*, 1995). The $smu^{b641}$ allele is a missense mutation that substitutes arginine for glycine in the predicted second transmembrane domain (Varga *et al.*, 2001).

### *olig2 Cloning*

To clone *olig2,* we designed PCR primers using sequence from an EST, Accession No. AI883483, and amplified a fragment from 26-hpf cDNA. We obtained 5′ and 3′ sequences by RACE using the Marathon cDNA Amplification Kit (Clontech) and Advantage–HF PCR Kit (Clontech). For 5′ RACE, we used an *olig2*-specific primer having the sequence 5′-GTGGCTATTTTAGAGAGCTTGCGCAC-3′, and for 3′ RACE, we used a primer having the sequence 5′-GCGCTGAGGAGGACTCAATGGCTCTAA-3′. To clone *dm20/plp,* we designed PCR primers using sequence from EST clone AW280774 and amplified a product from 72-hpf cDNA. PCR products were cloned by using pPCR-Script Amp SK(+) plasmid (Stratagene). The GenBank Accession No. for the completed *olig2* cDNA sequence is AF442964. The dendrogram shown in Fig. 1A was produced by using the GENETYX-MAC 9.01 program (Software Development Ltd.).

### *Cell Labeling*

We injected one- to two-cell-stage zebrafish embryos with a 2% solution of DMNB-caged fluorescein dextran (Molecular Probes) in 1× Danieau solution. Injected embryos were maintained in the dark at 28.5°C in embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15 mM $KH_2PO_4$, 0.05 mM $NH_2PO_4$, 0.7 mM $NaHCO_3$) until the one- to two-somite stage. Embryos were dechorionated with watchmaker's forceps and mounted in 2% methyl cellulose on bridged coverslips with the neural plate facing upwards. The dye was photoactivated by using pulses of 365-nm light (Serbedzija *et al.*, 1998) generated by a Photonics Micropoint Laser System focused using a 40× objective mounted on a Zeiss Axioskop. Labeled embryos were maintained in the dark at 28.5°C until the appropriate stage for analysis. The live image was obtained by using a Hammamatsu Orca CCD camera. Embryos used for histochemical analyses were fixed in 4% paraformaldehyde.

### *In Situ RNA Hybridization and Immunohistochemistry*

Previously described RNA probes included *is12* (Appel *et al.*, 1995) and *sox10* (Dutton *et al.*, 2001; Pauliny, 2002). *In situ* RNA hybridization was performed as previously described (Hauptmann and Gerster, 2000). For double RNA labeling, the different probes were labeled with digoxygenin and fluorescein. The first probe was detected by using either anti-digoxygenin or anti-fluorescein antibody conjugated to alkaline phosphatase (AP), followed by a color reaction using a solution of NBT/BCIP (Roche Diagnostics) as substrate. The first antibody was stripped by incubating embryos with 0.1 M glycine, pH 2.2. The appropriate secondary antibody was applied and developed by using Fast Red (Roche Diagnostics) as substrate.

For detection of photoactivated fluorescein in hybridized embryos, we first performed *in situ* hybridization using digoxygenin-labeled RNA probes, which we visualized using NBT/BCIP to produce a blue precipitate. After stripping the AP-conjugated anti-digoxygenin antibody by washing with 0.1 M glycine, pH 2.2, we detected photoactivated fluorescein by incubating embryos with AP-conjugated anti-fluorescein antibody and carried out a color reaction using Fast Red.

For immunohistochemistry, the following primary antibodies were used: mouse anti-Is1 [39.4D5, 1:100; Developmental Studies Hybridoma Bank (DSHB)], rabbit anti-phospho-Histone H3 (1:1000; Upstate Biotechnology), and mouse anti-BrdU (G3G4, 1:1000; DSHB). For fluorescent detection of antibody labeling, we used Alexa Fluor 568 goat anti-mouse conjugate (1:200; Molecular Probes). For BrdU/RNA double labeling, *in situ* hybridization was performed first. The stained embryos were immersed in 2 M HCl for 1 h and then incubated with anti-BrdU antibody, followed by incubation with biotinylated goat anti-mouse IgG and then streptavidin–peroxidase (Jackson ImmunoResearch Laboratories,

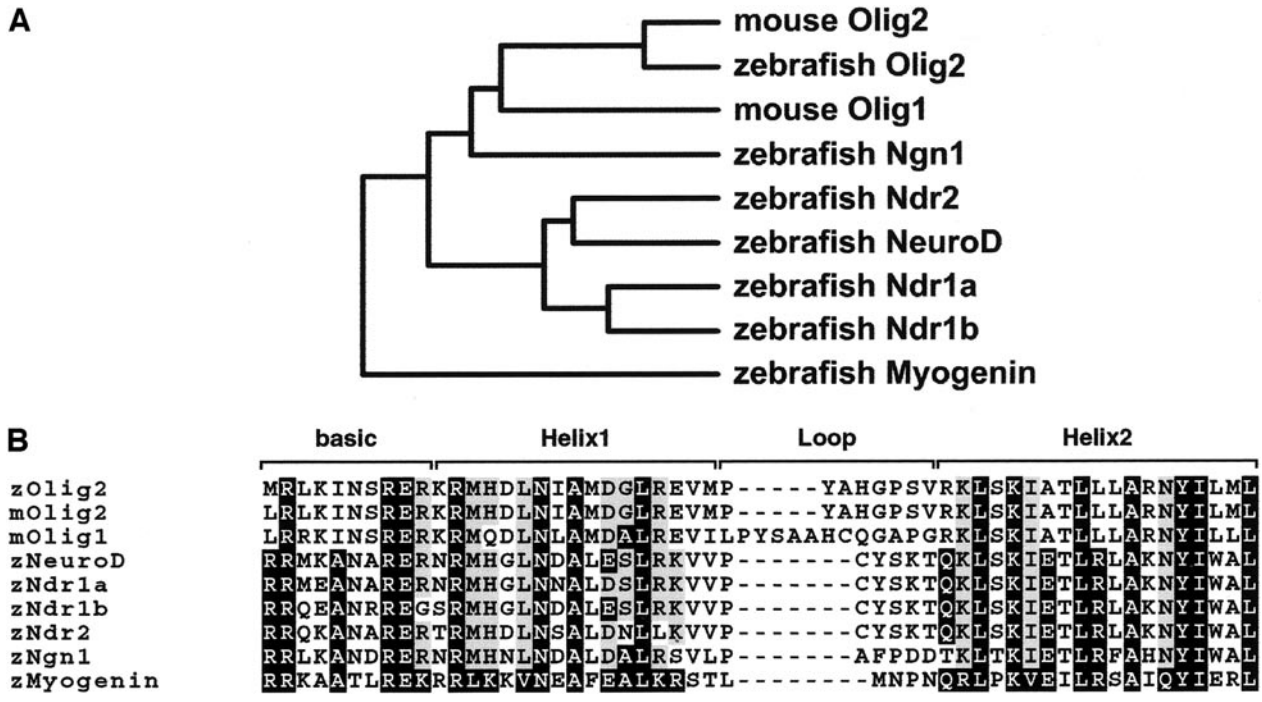


**FIG. 1.** Comparison of zebrafish Olig2 with other bHLH proteins. (A) Dendrogram showing that zebrafish Olig2 is more closely related to mouse Olig2 than mouse Olig1 or other zebrafish bHLH proteins. (B) Amino acid alignment showing sequence conservation between bHLH domains of zebrafish Olig2 and related proteins. Abbreviations: z, zebrafish; m, mouse.

Inc.). Peroxidase activity was used to produce a brown precipitate in the presence of Fast DAB (Sigma).

Embryos for sectioning were embedded in 1.5% agar/5% sucrose and frozen in 2 methyl-butane chilled by immersion in liquid nitrogen. Sections (10 $\mu$m) were obtained by using a cryostat microtome. Whole-mount embryos were cleared in methanol and equilibrated and mounted in 75% glycerol. All images were obtained with a Spot digital camera mounted on a compound microscope and processed by using Adobe Photoshop. Image manipulation was limited to Levels, Hue, and Saturation adjustments.

### *Antisense Morpholino Oligonucleotide and RNA Injections*

An antisense morpholino oligonucleotide having the sequence 5′-CGTTCAGTGCGCTCTCAGCTTCTCG-3′ (Gene-Tools, LLC) was designed to target the *olig2* translation start site. The oligonucleotide was resuspended in 1× Danieau solution and 1–2 ng was injected into the yolk of one- to two-cell-stage embryos. For mRNA injection experiments, a cDNA containing the full-length open reading frame was subcloned into the pCS2 vector (Turner and Weintraub, 1994) and mRNA was produced by using the Message Machine Kit (Ambion). *olig2* mRNA (100–200 pg) was injected into the yolk of one- to two-cell-stage embryos.

### *Cylopamine Treatments*

Cyclopamine (Toronto Research Chemicals, Inc.) was dissolved at 10 mM concentration in ethanol. Embryos, in their chorions, were incubated in cyclopamine diluted to 100 $\mu$M in embryo medium from shield stage (6 hpf) to 22 hpf, when they were fixed in 4% paraformaldehyde.

## RESULTS

### *Identification of a Zebrafish olig2 Gene*

We identified a zebrafish homolog of mouse, rat, chicken, and human *Olig* genes within the EST database (see Materials and Methods). Conceptual translation of a cDNA containing the entire open reading frame predicted a protein of 273 amino acids and revealed a bHLH domain. The amino acid sequence of this protein is more similar to that of mouse Olig2 than mouse Olig1 or other zebrafish bHLH proteins (Figs. 1A and 1B). In particular, within the bHLH domains, 53 of 54 amino acids are identical to mouse Olig2. In keeping with zebrafish nomenclature guidelines, we designate the zebrafish gene *olig2*. Thus far, we have not identified other zebrafish *olig* genes by using degenerate PCR.

### *Primary Motor Neuron and Oligodendrocyte Precursor Cells Express olig2*

Using *in situ* RNA hybridization, we first detected *olig2* expression in midgastrula-stage (80% epiboly, 8.5 hpf) em-

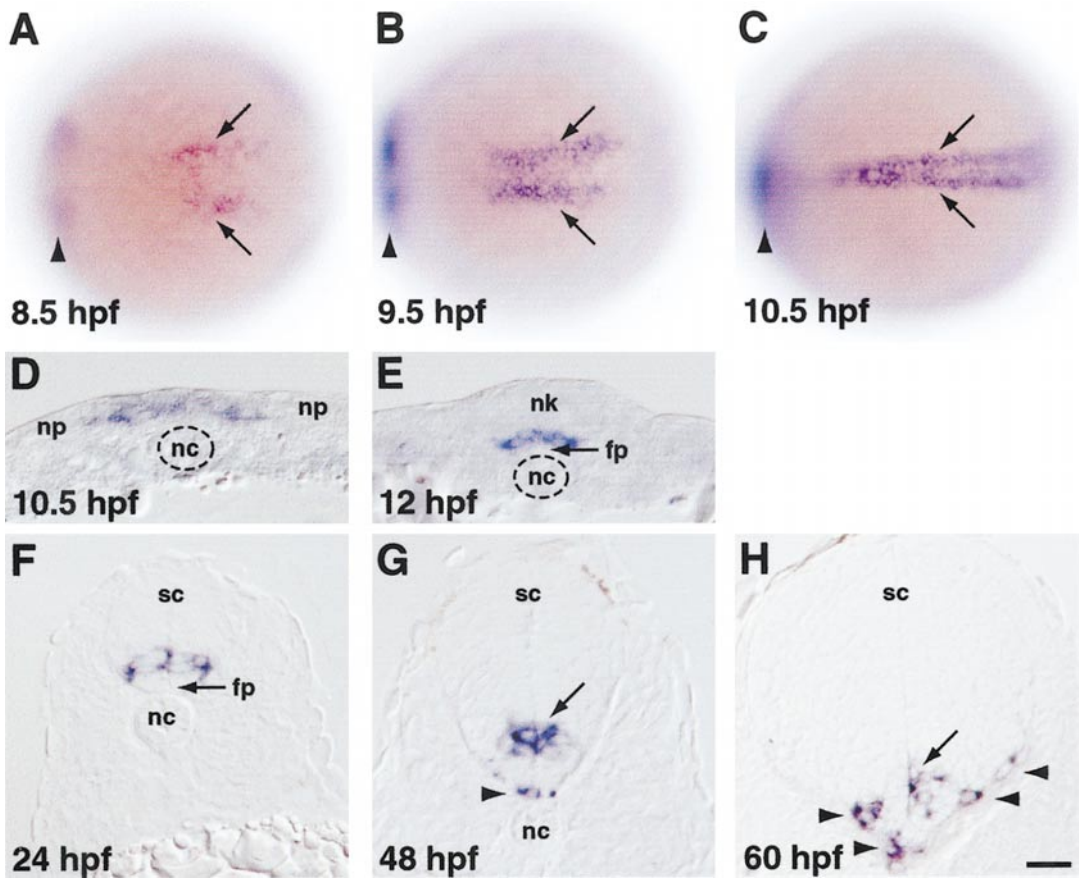


**FIG. 2.** *olig2* expression revealed by *in situ* RNA hybridization. (A–C) Dorsal views, anterior to the left, of whole embryos. Arrowheads indicate expression in prospective ventral diencephelon, which is out of the focal plane. (A) Prospective trunk spinal cord cells begin to express *olig2* at midgastrula stage (arrows). (B) By late gastrula stage, *olig2*-expressing cells form two longitudinal stripes bordering the embryonic midline. (C) At neural plate stage, *olig2*-expressing cells form a single longitudinal domain at the midline. (D–H) Transverse sections, dorsal to the top, through trunk region. (D) Medial neural plate (np) cells, overlying notochord (nc), uniformly express *olig2*. (E) As neurulation proceeds, *olig2*-expressing cells occupy ventral neural keel (nk), overlying floor plate (fp). (F) After neurulation, cells within the ventral portion of the spinal cord (sc) express *olig2*. (G,H) Some *olig2*-expressing cells occupy basolateral positions, associated with the white matter (arrowheads). Scale bar equals 80 $\mu$m for (A–C) and 20 $\mu$m for (D–H).

bryos (Fig. 2A), approximately 1.5 h before the first neurons are born (Myers *et al.*, 1986). Initially, an anterior group of cells in prospective brain and a posterior group of cells clustered near the dorsal midline express *olig2*. By 9.5 hpf, two longitudinal stripes of *olig2* expression are evident within prospective trunk neuroectoderm abutting the dorsal midline (Fig. 2B). The longitudinal stripes border cells that express *shh* (data not shown). By neural plate stage (10.5 hpf), cells that express *olig2* in the developing trunk form a single, broad column within the medial neuroectoderm (Figs. 2C and 2D). During early stages of neurulation, the neural plate converges on the embryonic midline, forming a thickening of the neuroectoderm known as the neural keel (Schmitz *et al.*, 1993). *olig2*-expressing cells occupy the ventromedial portion of the neural keel, which is the prospective ventral spinal cord (Fig. 2E). After completion of neurulation, the *olig2* expression domain forms a narrow stripe just dorsal to the ventral-most cells of the spinal cord (Fig. 2F). At 48 hpf, some *olig2*-expressing cells are clustered around the ventral ventricle and some are basally located in ventral spinal cord (Fig. 2G). By 60 hpf, fewer *olig2*-expressing cells are clustered near the ventral ventricle and more occupy basoventral and basolateral positions, within the white matter (Fig. 2H). Thus, *olig2*-expressing cells appear to disperse from the region of the ventral ventricle to the white matter, consistent with the behavior of oligodendrocyte progenitors.

To determine whether proliferative cells express *olig2*, we probed embryos for *olig2* expression and at the same time identified cells in M phase of the cell cycle using an

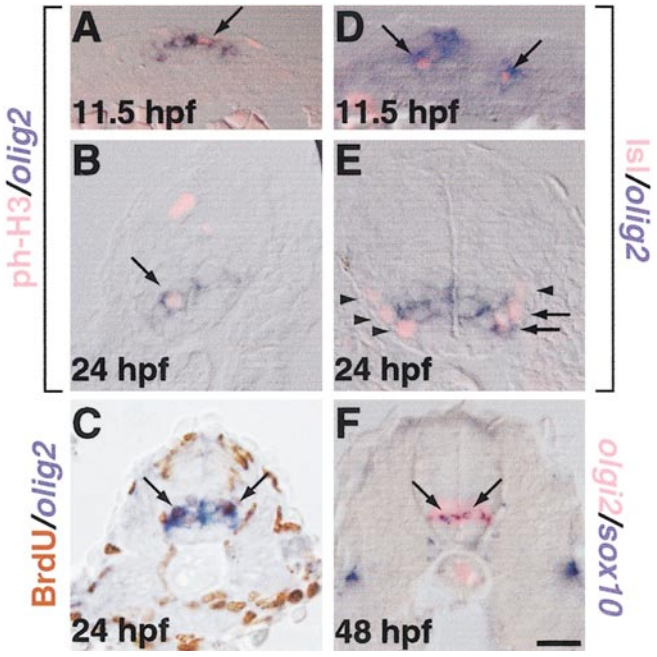


**FIG. 3.** Proliferative neural cells, motor neurons, and oligodendrocytes express *olig2*. (A–F) Transverse sections through trunk regions of embryos probed for *olig2* RNA expression by *in situ* hybridization. (A,B) M-phase cells, labeled by anti-phospho-histone H3 antibody (pink), and (C) S-phase cells, revealed by BrdU incorporation (brown) express *olig2* (blue; arrows). (D) Primary motor neurons, labeled by anti-Is1 antibody (pink, arrows) express *olig2* (blue, arrows). (E) At 24 hpf, some motor neurons express *olig2* (arrows), whereas others do not (arrowheads). (F) At 48 hpf, *olig2*-positive cells (pink) include oligodendrocyte progenitors, marked by *sox10* expression (blue, arrows). Scale bar, 20 $\mu$m.

anti-phospho-histone H3 antibody. Cells that express *olig2* at 11.5 and 24 hpf include M-phase cells (Figs. 3A and 3B). They also include S-phase cells as revealed by incorporation of BrdU (Fig. 3C, and data not shown). By 24 hpf, many zebrafish neural cells have stopped dividing and differentiated as neurons (Appel *et al.*, 2001). Thus, proliferative cells of the ventral spinal cord express *olig2* during zebrafish neurogenesis.

Consistent with their location in medial neural plate, at 11.5 hpf, *olig2*-expressing cells include primary motor neurons, revealed by an anti-Isl antibody (Fig. 3D). At 24 hpf, some, but not all, motor neurons express *olig2* (Fig. 3E). One possible explanation for the latter observation is that motor neurons downregulate *olig2* expression as they differentiate. Motor neurons in chick embryos similarly express and then apparently downregulate *Olig2* expression (Mizuguchi *et al.*, 2001; Novitch *et al.*, 2001).

To investigate whether developing oligodendrocytes express *olig2*, we examined expression of *PDGFRα*, *sox10*, and *dm20/plp*, which are homologs of genes that serve as oligodendrocyte markers for other species (Timsit *et al.*, 1995; Hall *et al.*, 1996; Kuhlbrodt *et al.*, 1998; Zhou *et al.*, 2000). We did not detect ventral spinal cord expression of *PDGFRα* (data not shown), but spinal cord cells express *sox10* and *dm20/plp* at 48 and 72 hpf, respectively, in patterns consistent with the distribution of oligodendrocyte

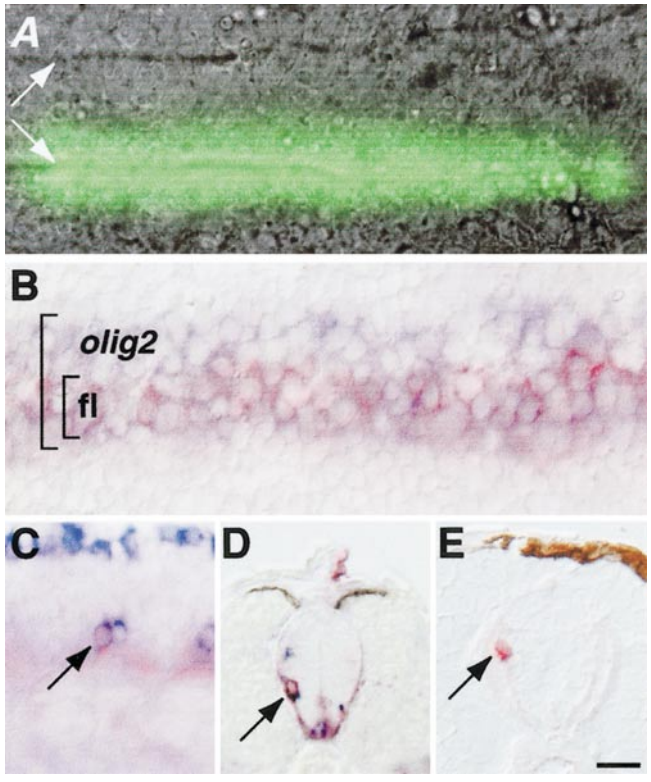


**FIG. 4.** Fate mapping reveals that *olig2*-expressing neural plate cells give rise to primary motor neurons and oligodendrocytes (A) Dorsal view of living 10.5-hpf embryo in which caged fluorescein was photoactivated in a stripe of neuroectoderm overlying one edge of the notochord (notochord boundaries marked by white arrows). (B) Dorsal view of fixed 10.5-hpf embryo in which caged fluorescein had been photoactivated similarly to embryo in (A) and probed for *olig2* RNA expression. Larger bracket indicates *olig2*-expressing cells, which are blue. Smaller bracket indicates cells in which caged fluorescein (fl) was photoactivated, revealed by red staining. Photoactivated cells are within the *olig2* expression domain. (C) Side view of 19-hpf embryo probed for *isl2* expression (blue) to reveal primary motor neurons and processed to reveal photoactivated fluorescein label (red). Photoactivated cell is a primary motor neuron (arrow). (D) Transverse section of 48-hpf embryo processed to reveal photoactivated fluorescein (red) and *sox10* expression (blue). Arrow indicates double-labeled oligodendrocyte progenitor that originated in medial neural plate. (E) Transverse section of 72-hpf embryo processed to reveal photoactivated fluorescein label (red,arrow). Labeled cell occupies dorsal white matter, indicating that it is an oligodendrocyte, which originated in medial neural plate. Scale bar, 20 $\mu$m.

progenitors and mature oligodendrocytes (Fig. 3F; also, see Figs. 6A and 6C) (Pauliny, 2002). At 48 hpf, a subset of *olig2*-positive cells express *sox10* (Fig. 3F); however, *olig2* expression did not persist until 72 hpf, when we first observed *dm20/plp* expression. These observations suggest that oligodendrocyte progenitors express *olig2* transiently as they differentiate.

There are at least two possible interpretations of the above data. First, *olig2*-expressing cells might have the potential to give rise to either primary motor neurons or oligodendrocytes. Second, *olig2*-expressing cells generate motor neurons during early neurogenesis. As motor neuron production ends, nearby cells initiate *olig2* expression and produce oligodendrocytes. To discriminate between these possibilities, we performed fate-mapping experiments. Using the underlying notochord as a landmark, we photoactivated caged fluorescein within the *olig2* expression domain of 10.5-hpf embryos. To check our accuracy, we labeled large numbers of cells within longitudinal domains of the medial neural plate of some embryos, immediately fixed them, and probed for *olig2* expression. In all cases ($n = 20$), the photoactivated fluorescein was restricted to *olig2*-expressing cells (Figs. 4A and 4B). In other embryos, we usually labeled small clusters of two to four cells. In embryos that we allowed to develop until 19 hpf, we found the photoactivated fluorescein in primary motor neurons, marked by *isl2* expression (Fig. 4C). In embryos that we analyzed at 48 hpf, we found photoactivated fluorescein in *sox10*-expressing oligodendrocyte progenitors (Fig. 4D), and in others, analyzed at 72 hpf, we found the label in cells occupying white matter of dorsal spinal cord, consistent with the final location of oligodendrocytes (Fig. 4E). Together, these data indicate that cells that express *olig2* at neural plate stage can develop as primary motor neurons or oligodendrocytes.

### *Primary Motor Neuron and Oligodendrocyte Development Require olig2 Function*

To test whether *olig2* is required in zebrafish for primary motor neuron and oligodendrocyte development, we injected one- to two-cell-stage embryos with antisense morpholino oligonucleotides (MO), which, when targeted to the translation start sequence, act as translation inhibitors (Heasman *et al.*, 2000; Nasevicius and Ekker, 2000).To examine primary motor neurons, we probed embryos for *isl2* RNA expression, which normally is detected in one or two primary motor neurons per hemisegment and dorsal Rohon–Beard sensory neurons (Fig. 5A) (Appel *et al.*, 1995). The number of primary motor neurons was dramatically reduced in most embryos injected with *olig2* MO (Fig. 5B; Table 1). Rohon–Beard neurons and *lim1*-expressing interneurons appeared normal in these embryos, demonstrating that *olig2* MO did not interfere with development of these neurons that arise outside the *olig2* expression domain (Figs. 5B and 5E). Additionally, coinjection of *olig2* mRNA with *olig2* MO suppressed the loss of primary motor neuron phenotype, indicating that the *olig2* MO is specific for its intended target (Table 1). Thus, zebrafish primary motor neurons require *olig2* function.

By contrast, one- to two-cell-stage embryos injected with synthetic mRNA encoding full-length Olig2 protein developed, on average, a 1.7-fold excess of *isl2*-expressing primary motor neurons (Table 1). Up to five *isl2*-expressing primary motor neurons developed in each hemisegment of injected embryos, and these were restricted to the ventral spinal cord (Fig. 5C). More dorsally located Rohon–Beard sensory neurons and interneurons were unaffected (Figs. 5C and 5F). We conclude that *olig2* can promote primary motor neuron development but only within the ventral spinal cord.

We also tested the consequences of reduced or excess *olig2* function for oligodendrocyte development. The number of oligodendrocytes, marked by either *sox10* or *dm20/plp* expression, was greatly reduced in most embryos injected with *olig2* MO (Figs. 6A–6D; Table 1). By contrast, embryos injected with *olig2* mRNA had a 1.5-fold increase in the number of *dm20/plp*-expressing cells (Fig. 6E; Table 1). Taken together, these data show that *olig2* is required for development of both primary motor neurons and oligodendrocytes and that ectopic *olig2* expression can promote specification of primary motor neuron and oligodendrocyte fate.

### *Hedgehog Signaling Is Required for olig2 Expression and Oligodendrocyte Development*

During late gastrulation and neural plate stages, *olig2*-expressing neuroectodermal cells are close to prospective floor plate cells, which express *shh* (data not shown). *syu* mutant embryos, which are deficient for Shh (Schauerte *et al.*, 1998), expressed *olig2* at lower than normal levels in brain but at apparently equivalent levels to wild type in spinal cord (data not shown). However, zebrafish midline cells express two additional *hedgehog* homologs: *echidna hedgehog* in notochord cells and *tiggywinkle hedgehog* in floor plate cells (Ekker *et al.*, 1995; Currie and Ingham, 1996). Expression of the latter two genes may partially compensate for loss of *syu* function in mutant embryos. Mutation of *smoothened* (*smu*), which encodes a coreceptor for Hedgehog proteins, eliminates most Hedgehog signaling (Barresi *et al.*, 2000; Chen *et al.*, 2001; Varga *et al.*, 2001). Trunk expression of *olig2* is nearly absent in 10.5-hpf *smu* mutant embryos (Fig. 7B) and entirely absent in 24-hpf mutant embryos (Fig. 7D). By contrast, prospective ventral diencephelon expression of *olig2* appears normal in 10.5-hpf *smu* mutant embryos, although it is greatly reduced by 24 hpf (data not shown). *smu* mutant embryos entirely lack hindbrain and spinal cord oligodendrocytes, marked by *dm20/plp* expression (Fig. 7F) and most motor neurons (Chen *et al.*, 2001; Lewis and Eisen, 2001), consistent with the requirement of *olig2* function for oligodendrocyte and motor neuron development.

### *olig2 Is Not Sufficient to Promote Primary Motor Neuron and Oligodendrocyte Development in the Absence of Hedgehog Signaling*

The above observations raise the possibility that *olig2* is the principal mediator of Hedgehog activity for specification of primary motor neurons and oligodendrocytes. If so, *olig2* overexpression should restore primary motor neuron and oligodendrocyte development to embryos that have reduced levels of Hedgehog signaling. As an *in vivo* test of this prediction, we injected *olig2* mRNA into embryos that were treated with cyclopamine, which inhibits Hedgehog signaling (Cooper *et al.*, 1998; Incardona *et al.*, 1998). Cyclopamine-treated embryos that were not injected with *olig2* mRNA had fewer than normal anterior trunk primary motor neurons and few, if any, posterior trunk and tail primary motor neurons ($n = 62$ embryos) (Figs. 8A and 8B). On average, these embryos had 11.1 *isl2*-positive primary motor neurons along the entire length of one side of the spinal cord ($n = 12$ embryos). Cyclopamine-treated embryos injected with *olig2* mRNA had more primary motor neurons in anterior trunk than noninjected embryos (Fig. 8C), which indicates that our injections produced Olig2 overexpression, but no increase in primary motor neurons in posterior trunk and tail relative to noninjected embryos ($n = 85$ embryos) (Fig. 8D). On average, these embryos had 14.6 *isl2*-positive primary motor neurons along the entire length of one side of the spinal cord ($n = 12$ embryos). This represents a 1.3-fold increase in the number of primary motor neurons compared with noninjected embryos. Notably, the increase in primary motor neuron number occurred entirely within anterior trunk, where primary motor neurons developed in cyclopamine-treated, noninjected embryos. We observed very similar results when we injected *olig2* mRNA into *smu* mutant embryos and forskolin-treated embryos, which also blocks Hedgehog signaling (data not shown). We interpret these data to mean that, under our experimental conditions, cyclopamine reduced, but did not eliminate, Hedgehog signaling in anterior trunk, but nearly abolished it in posterior trunk and tail. *olig2* overexpression promoted primary motor neuron formation in anterior trunk, where some Hedgehog signaling remained, but could not in posterior trunk and tail, where Hedgehog signaling was absent.

We also tested the ability of *olig2* overexpression to rescue oligodendrocyte development in Hedgehog signaling-deficient embryos. Spinal cord expression of *sox10* was entirely absent from embryos treated with cyclopamine ($n = 12$ embryos) (Fig. 8E), indicating that cyclopamine treatment effectively blocked oligodendrocyte development. Embryos injected with 100–200 pg *olig2* mRNA, which promotes formation of excess oligodendrocytes in wild-type, untreated embryos (Fig. 6E; Table 1), and incubated in cyclopamine similarly failed to express *sox10* in spinal cord ($n = 12$ embryos) (Fig. 8D). From these data, we conclude that the ability of *olig2* to promote primary motor neuron and oligodendrocyte development *in vivo* is dependent on Hedgehog signaling.

## DISCUSSION

### *Cells That Express olig2 Give Rise to Both Primary Motor Neurons and Oligodendrocytes*

Gene expression patterns that mark prospective primary motor neurons in zebrafish show that cells fated to become primary motor neurons are interspersed with other cells (Appel *et al.*, 1995). The fates of cells nearby primary motor neurons are unknown as are the mechanisms that specify them for different fates. In chick and rodent embryos, oligodendrocytes appear to arise from the same region that, at an earlier developmental stage, produced motor neurons. Evidence for this comes from comparison of gene expression patterns that mark developing motor neurons and oligodendrocytes. For example, cells that express *PDGFRα* and *Sox10* first appear in ventral spinal cord, at the dorsoventral level of the origin of motor neurons (Pringle *et al.*, 1996; Zhou *et al.*, 2000). More recently, *Olig2* was shown to be expressed continuously during the period of motor neuron and oligodendrocyte generation in precursors of those cell types (Mizuguchi *et al.*, 2001; Novitch *et al.*, 2001; Sun *et al.*, 2001; Zhou *et al.*, 2001).

We showed here that, in zebrafish, cells of the medial neural plate and ventral spinal cord express *olig2* from midgastrula stage to at least 60 hpf. Double-labeling experiments revealed that proliferative cells express *olig2* during the time that motor neurons are born. During early stages of neurogenesis, only a subset of *olig2*-positive cells express a motor neuron marker. Later, a subset of *olig2*-positive cells express an oligodendrocyte progenitor marker. These observations are consistent with two different possibilities concerning the relationship of primary motor neuron and oligodendrocyte precursors. First, neural precursors that express *olig2* produce first primary motor neurons and then oligodendrocytes. Alternatively, precursor cells that express *olig2* early in neurogenesis produce primary motor neurons. Later, a different, nearby population of precursors turn on *olig2* transcription and produce oligodendrocytes. Our fate-mapping data, which indicate that neural plate cells that uniformly express *olig2* give rise to both primary motor neurons and oligodendrocytes, are most consistent with the first scenario. This raises the possibility that specification of primary motor neuron and oligodendrocyte development requires a choice for one or the other fates.

### *olig2 Function Is Required for Primary Motor Neuron and Oligodendrocyte Development*

We used antisense morpholino oligonucleotides to perform loss of *olig2* function tests in zebrafish. Injected embryos lacked nearly all primary motor neurons and oligodendrocytes, showing that *olig2* function is absolutely

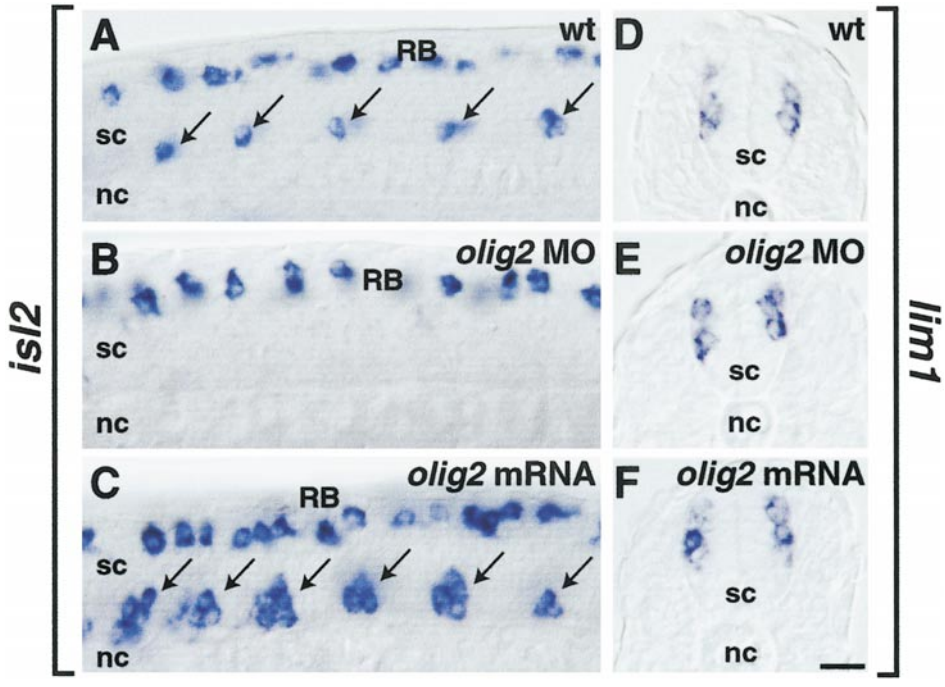


**FIG. 5.** *olig2* promotes primary motor neuron development. (A–C) Side views of 20-hpf embryos hybridized with *isl2* probe. (A) Normal distribution of one to two primary motor neurons per ventral spinal cord (sc) hemisegment (arrows) and dorsal Rohon–Beard neurons (RB). (B) Embryo injected with *olig2* MO lacked primary motor neurons but had Rohon–Beard neurons. (C) Embryo injected with *olig2* mRNA. Excess primary motor neurons developed in ventral spinal cord (arrows), whereas Rohon–Beard neurons appeared normal. Apparent differences in Rohon–Beard cell number reflect normal variation and difference in depth of optical section. (D–F) Transverse sections of embryos hybridized with *lim1* RNA probe. *lim1*-expressing cells appeared normal in embryos injected with *olig2* MO (E) or *olig2* mRNA (F). Scale bar, 20 $\mu$m for all panels.

required for their development. These data are consistent with recent reports describing mouse embryos lacking *Olig1* and *Olig2* functions resulting from targeted mutagenesis (Lu *et al.*, 2002; Zhou and Anderson, 2002). Together, these observations show that Olig functions are required for development of motor neurons and oligodendrocytes in both anamniote and amniote embryos.

Conversely, we found that, in zebrafish, overexpression of *olig2* alone by mRNA injection into cleavage-stage embryos produced a nearly equal increase in the number of

**TABLE 1**

Effect of *olig2* MO and mRNA Injections on Primary Motor Neurons and Oligodendrocytes

| | Primary motor neurons | | | | Oligodendrocytes | | | |
|---|---|---|---|---|---|---|---|---|
| | Decrease | Increase | No change | Total embryos | Decrease | Increase | No change | Total embryos |
| *olig2* MO | 87% | 0% | 13% | 93 | 93% | 0% | 9% | 121 |
| *olig2* mRNA | 0% | 78%[a] | 22% | 140 | 0% | 69%[b] | 31% | 88 |
| *olig2* MO + mRNA | 28% | 17% | 55% | 88 | | | | |

*Note.* Embryos were injected with 1-2 ng *olig2* MO or 100–200 pg *olig2* mRNA. Percentile numbers represent the fraction of total embryos analyzed for the experiment.

[a] Primary motor neurons were counted in 14 hemisegments of 10 wild-type embryos and 10 embryos injected with *olig2* mRNA. Injected embryos had a 1.7-fold increase of primary motor neurons compared with wild type ($P < 0.00000001$ by Student's *t* test).

[b] Oligodendrocytes were counted in 20 sections taken from each of 6 wild-type and 6 embryos injected with *olig2* mRNA. Injected embryos had a 1.5-fold increase of oligodendrocytes compared with wild type ($P < 0.00001$ by Student's *t* test).

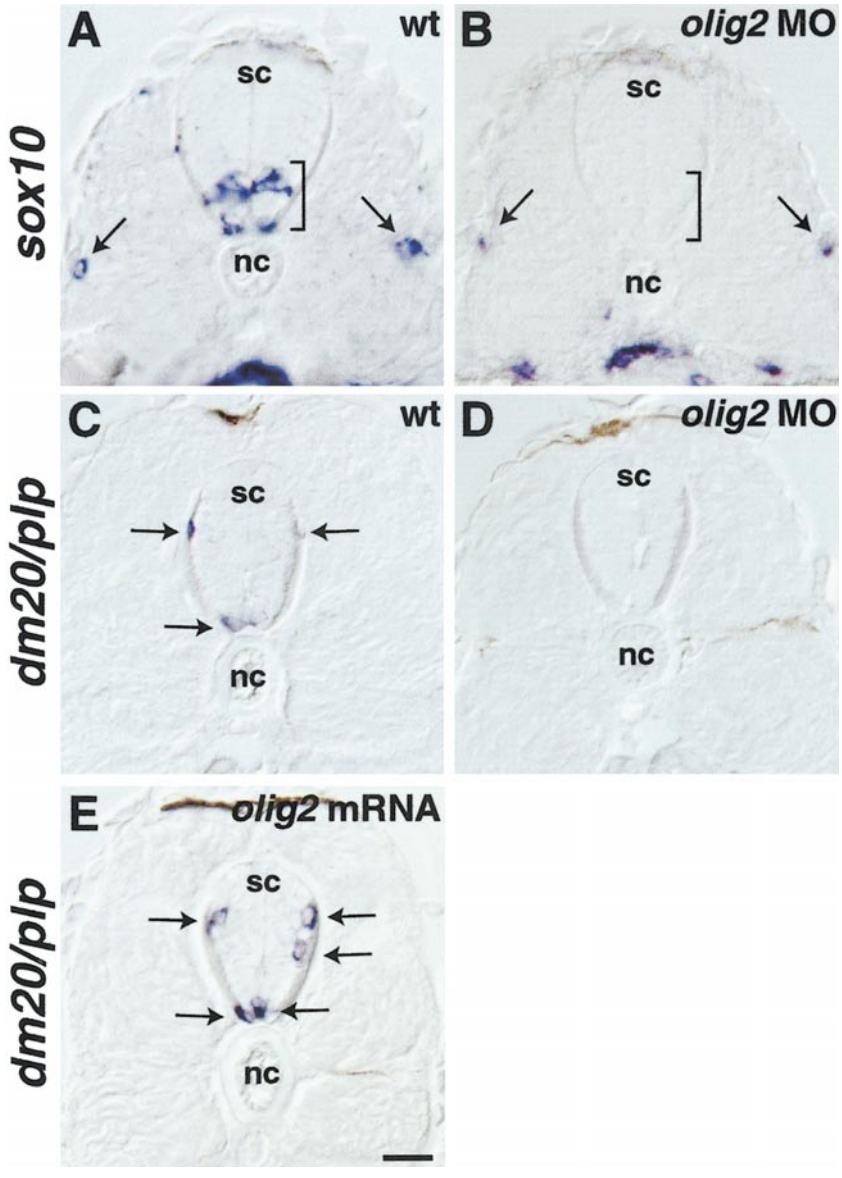


**FIG. 6.** *olig2* promotes oligodendrocyte development. (A, B) Transverse sections through trunk spinal cord (sc) of 48-hpf embryos hybridized with *sox10* RNA probe. Brackets indicate ventral spinal cord and arrows point to *sox10* expression in lateral line. Spinal cord expression of *sox10* was absent from embryo injected with *olig2* MO, whereas lateral line expression was normal (B). (C–E) Transverse sections of 72-hpf embryos hybridized with *dm20/plp* RNA probe. Arrows indicate *dm20/plp*-expressing oligodendrocytes. Embryos injected with *olig2* MO did not express *dm20/plp* (D), whereas those injected with *olig2* mRNA had a small but significant increase in the number of *dm20/plp*-expressing cells (E) (see Table 1). Scale bar, 20 $\mu$m.

primary motor neurons and oligodendrocytes (1.7-fold and 1.5-fold excess, respectively). This method of overexpression typically results in distribution of the injected mRNA throughout the embryo. Nevertheless, excess primary motor neurons formed only in the ventral spinal cord. Similarly, overexpression of *Olig2* alone in chick spinal produced a large excess of motor neurons, mostly in ventral spinal cord (Mizuguchi *et al.*, 2001; Novitch *et al.*, 2001) and, when electroporated directly into ventral spinal cord, premature development of oligodendrocytes (Zhou *et al.*, 2001). When *Olig2* was overexpressed in combination with *Nkx2.2*, prematurely differentiating oligodendrocytes appeared to develop at ectopic positions in dorsal spinal cord, suggesting that the ability of *Olig2* to promote formation of different cells types depends on the combined function of other transcription factors (Sun *et al.*, 2001; Zhou *et al.*, 2001).

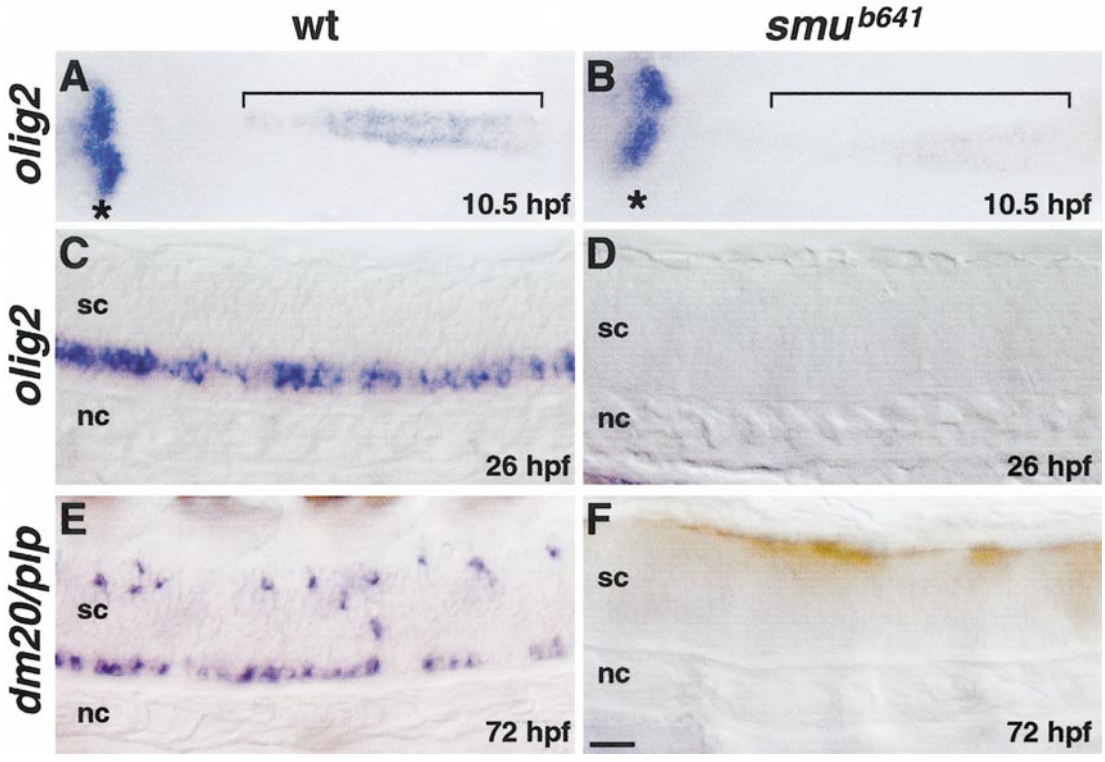


**FIG. 7.** Hedgehog signaling is required for *olig2* expression and oligodendrocyte development. (A, B) Dorsal views, anterior to the left, of flat-mounted embryos hybridized with *olig2* probe. Brackets indicate *olig2* expression in prospective spinal cord cells. *smu* mutant embryos express *olig2* at a much lower level (B) compared with wild type (A). Asterisks mark *olig2* expression in prospective ventral diencephelon, which appears normal in mutant embryos. (C, D) Side views of 26-hpf embryos, anterior to the left. Spinal cord cells of *smu* mutant embryos do not express *olig2*. (E, F) Side views of 72-hpf embryos, anterior to the left. Spinal cord oligodendrocytes, marked by *dm20/plp* expression, do not develop in *smu* mutant embryos. Scale bar, 40 $\mu$m (A and B) and 20 $\mu$m (C–F).

Taken together, our gene expression analyses, fate mapping, and functional tests support the idea that *olig2* function is required for primary motor neuron and oligodendrocyte development from a common population of precursor cells. This raises the possibility that *olig2* specifies neural precursors with potential to develop either as primary motor neurons or oligodendrocytes. Previously, we showed that embryos with a mutation of *deltaA* have excess primary motor neurons, indicating that Delta–Notch signaling is required to limit the number of ventral neural precursors that develop as primary motor neurons (Appel *et al.*, 2001). Thus, specification of primary motor neurons and oligodendrocytes could require a mechanism that mediates a cell fate choice among *olig2*-expressing neural precursors.

### *Hedgehog Signaling Establishes a Motor Neuron and Oligodendrocyte Precursor Population, in Part, through olig2*

Hedgehog signaling is required for motor neuron and oligodendrocyte development in zebrafish and mice (Chiang *et al.*, 1996; Orentas *et al.*, 1999; Alberta *et al.*, 2001; Chen *et al.*, 2001; Lewis and Eisen, 2001; Nery *et al.*, 2001; Spassky *et al.*, 2001; Tekki-Kessaris *et al.*, 2001; this paper) and it is also required to promote *Olig* gene expression (Lu *et al.*, 2000; this paper). Furthermore, *Olig* gene functions are required for motor neuron and oligodendrocyte development (Lu *et al.*, 2002; Zhou and Anderson, 2002; this paper). These observations raise the possibility that Olig proteins are the downstream effectors of Hedgehog signaling for specification of motor neurons and oligodendrocytes. Consistent with this, *Olig1* expression in cultured cells promoted expression of an oligodendrocyte lineage marker in the presence of reagents that block Hedgehog signaling (Alberta *et al.*, 2001). However, the ability of *Olig2* overexpression to promote motor neuron development *in vivo* is limited to ventral spinal cord (Mizuguchi *et al.*, 2001; Novitch *et al.*, 2001). Additionally, the prematurely arising oligodendrocyte progenitors that develop in embryos that overexpress *Olig2* appear to originate within the *Nkx2.2* expression domain of ventral spinal cord (Zhou *et al.*, 2001). These observations suggest that *Olig* genes can promote motor neuron and oligodendrocyte development only in cells in which Hedgehog signaling is

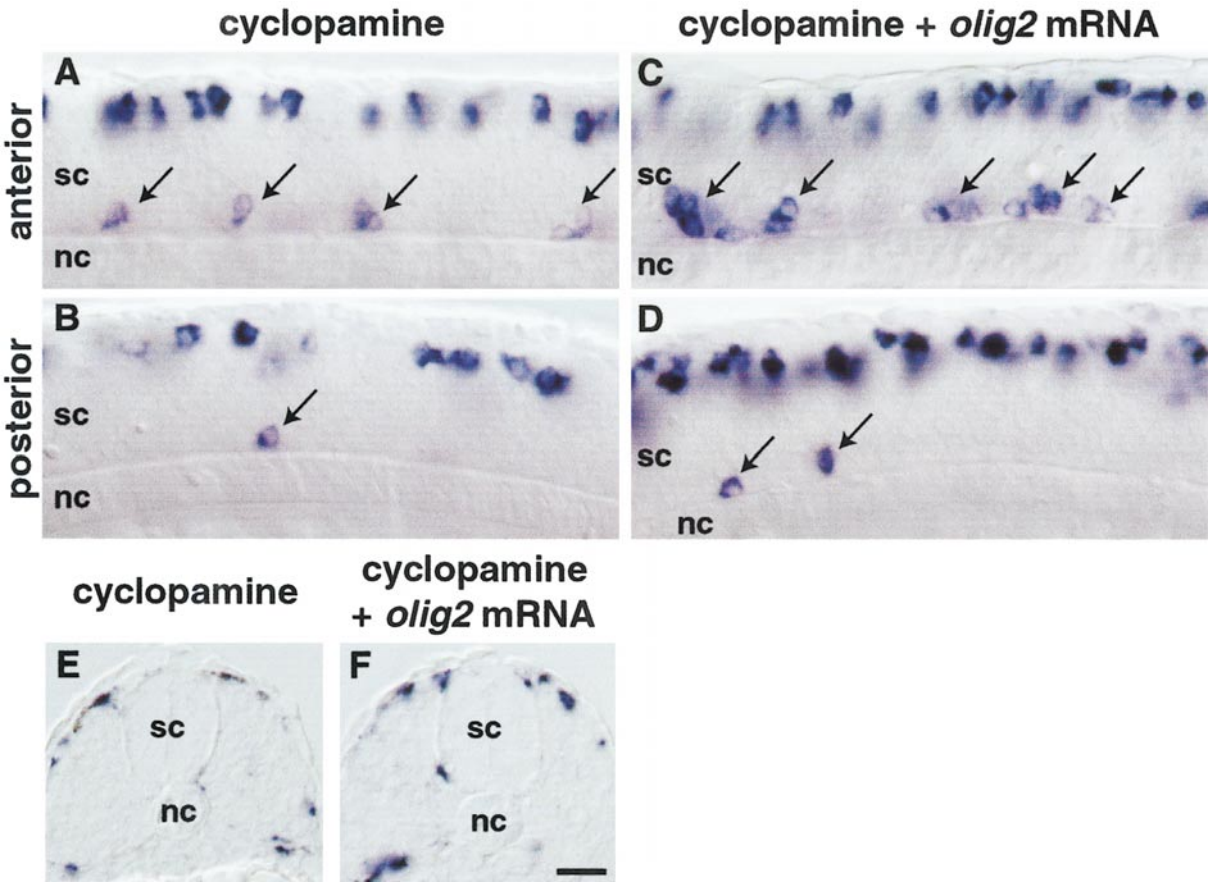


**FIG. 8.** *olig2* requires Hedgehog signaling to promote primary motor neuron and oligodendrocyte development. (A–D) Side views of 22-hpf embryos hybridized with *isl2* probe. (A) Anterior and (B) posterior trunk spinal cord (sc) of embryo treated with cyclopamine. Slightly fewer *isl2*-positive primary motor neurons developed in anterior trunk relative to wild type (compare Fig. 8A with Fig. 5A), whereas few primary motor neurons developed in posterior trunk (B). (C) Anterior and (D) posterior trunk spinal cord of embryo injected with *olig2* mRNA and treated with cyclopamine. Slightly more primary motor neurons developed in anterior trunk (C) compared with noninjected embryos (A), whereas primary motor neurons rarely developed in posterior trunk and tail (D), similar to noninjected embryos (B). (E, F) Transverse sections of 48-hpf embryos hybridized with *sox10* probe. Spinal cord cells did not express *sox10* in cyclopamine-treated embryos (E) or in *olig2*-injected embryos treated with cyclopamine (F). Scale bar, 20 $\mu$m.

active. We found that *olig2* overexpression could not effectively rescue primary motor neuron and oligodendrocyte development in embryos that had abnormally low levels of Hedgehog signaling. These results are most consistent with the idea that, *in vivo*, *olig2* can promote primary motor neuron and oligodendrocyte development only within the context of active Hedgehog signaling. One possible explanation for this is that Hedgehog signaling acts in parallel on *olig2* and other factors required for primary motor neuron and oligodendrocyte development.

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