

Indian Hedgehog b Function Is Required for the Specification of Oligodendrocyte Progenitor Cells in the Zebrafish CNS

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A subset of ventral spinal cord precursors, known as pMN precursor cells, initially generate motor neurons and then oligodendrocyte progenitor cells (OPCs), which migrate and differentiate as myelinating oligodendrocytes in the developing neural tube. The switch between motor neuron and oligodendrocyte production by the pMN neural precursors is an important step in building a functional nervous system. However, the precise mechanism that orchestrates the sequential generation of motor neurons and oligodendrocytes within the common population of pMN precursors is still unclear. The current study demonstrates that Indian Hedgehog b (Ihhb), previously known as Echidna Hedgehog, begins to be expressed in the floor plate cells of the ventral spinal cord at the time of OPC specification in zebrafish embryos. Ihhb loss-of-function analysis revealed that Ihhb function is required for OPC specification from pMN precursors by negatively regulating the proliferation of neural precursors. Finally, results showed that Sonic Hedgehog (Shh) could not replace Ihhb function in OPC specification, suggesting that Ihhb and Shh play separate roles in OPC specification. Altogether, data from the present study suggested a novel mechanism, mediated by Ihhb, for the sequential generation of motor neurons and oligodendrocytes from pMN precursors in the ventral spinal cord of zebrafish embryos.

Introduction

During vertebrate neural development, proliferative neuroepithelial precursors give rise first to neurons and later to glial cells. A particularly good example of this process is seen with a specific subset of ventral spinal cord precursor cells known as pMN precursors, which produce motor neurons during neurogenesis. The production of motor neurons is followed by the generation of oligodendrocyte progenitor cells (OPCs), which form oligodendrocytes, the myelinating cell type of the CNS (Rowitch, 2004). However, the precise mechanism that orchestrates the sequential generation of motor neurons and oligodendrocytes within the common population of pMN precursors is still unclear.

In the developing spinal cord, morphogenetic gradient of Sonic hedgehog (Shh) induces distinct pMN precursor domains

in the ventral spinal cord, and then Shh signaling is required for the generation of motor neuron and OPCs from the pMN precursors (Poncet et al., 1996; Pringle et al., 1996; Lewis and Eisen, 2001; Park et al., 2002). However, it is not clear how Hh signaling instructs the complex sequence of motor neurons and OPC specification that occurs over time during development. The present study shows that Indian Hedgehog b (Ihhb, previously known as Echidna Hedgehog), another member of the Hh signaling pathway, begins to be expressed in the floor plate cells of the ventral spinal cord at the time of OPC specification in zebrafish embryos. An Ihhb loss-of-function study revealed that in the absence of Ihhb function, neural precursors failed to specify OPCs and instead proliferated continuously to generate neurons, indicating that Ihhb function is required for the specification of OPCs from pMN precursors by negatively regulating the proliferation of neural precursors. Finally, we found that Shh could not replace Ihhb function in OPC specification, suggesting that Ihhb and Shh play separate roles in OPC specification. Together, our data suggested a novel mechanism, mediated by Ihhb, for the sequential generation of motor neurons and oligodendrocytes from pMN precursors in the ventral spinal cord of zebrafish embryos.

Materials and Methods

Fish lines and cyclopamine treatment. Wild-type AB, *Tg(olig2:egfp)* (Shin et al., 2003), *Tg(olig2:dsred)* (Kucenas et al., 2008), *Tg(hsp70:gal4)* (Scheer and Campos-Ortega, 1999), *Tg(uas:shha)* (Jung et al., 2011), and *Tg(hsp70:ihhb:mCherry)* zebrafish of either sex were used for this study.

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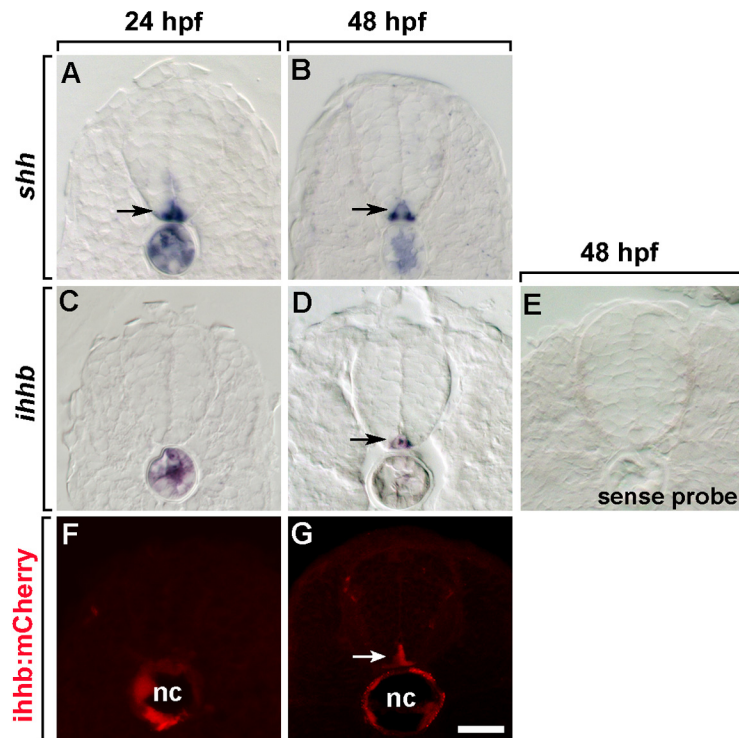


Figure 1. *Ihhb* begins to be expressed in the spinal cord at the time when OPCs are specified. **A–G**, Transverse sections of spinal cord, dorsal to the top. **A–E**, RNA *in situ* hybridization with *shh* (**A, B**) and *ihhb* antisense probes (**C, D**), and *ihhb* sense probes (**E**) in wild-type embryos. **F, G**, mCherry expression in the notochord and floor plate cells of *ihhb:mCherry* DNA-injected embryos. Arrows indicate floor plate cells. nc, Notochord. Scale bar, 20 μ m.

For cyclopamine treatment, embryos were incubated in embryo medium (EM) containing 100 μ M cyclopamine (Toronto Research Chemicals) diluted from a 10 mM stock in ethanol at 28.5°C.

Plasmid construction and bacterial artificial chromosome homologous recombination. To produce *Tg(hsp70:ihhb:mCherry)* fish, PCR was used to amplify *ihhb* using a forward primer containing an attB1 site (5'-GGGACAAGTTTGTACAAAAAGCAGGCTTTATGAGACTCTCCACGGCG-3') and a reverse primer containing an attB2 site (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGGATCTCTCAGTTGCCTC-3'). The PCR product containing the attB sites was cloned into a middle-entry vector using the BP reaction of the Gateway system (Invitrogen). A 5' entry clone containing a fragment of the zebrafish heat shock 70 (*hsp70*) promoter and a 3' entry clone containing the *mcherry* were kindly provided by Chi-Bin Chien, University of Utah, Salt Lake City, UT (Kwan et al., 2007). The Gateway LR reactions were performed using LR II clonase with the entry clones, according to the manufacturer's recommendations (Invitrogen).

The bacterial artificial chromosome (BAC) clone for *ihhb* (CH211-237C6) was purchased from GenomeCube to generate *ihhb:mCherry* BAC DNA. Targeting vectors were made by multisite Gateway cloning system (Invitrogen). The 5' entry vector was generated by cloning of PCR product containing 750 bp of left-side homologous arms for *ihhb* into p5E-MCS vector (Kwan et al., 2007) linearized with *Sma*I. A middle entry clone was generated by inserting PCR product including *mcherry* sequence, FRT-Kan-FRT cassette, and I-SceI recognition site. A 3' entry vector contains 700 bp of right-side homologous arms of the *ihhb* coding region. LR reaction was performed to generate final targeting vector using LR II clonase with the three entry clones. Targeting vector was linearized by *Kpn*I/*Bgl*II digestion and electroporated into EL250 cells that harbor *ihhb* BAC clone. The BAC homologous recombination was performed as previously described (Shin et al., 2003).

Bromodeoxyuridine labeling, immunohistochemistry, and in situ RNA hybridization. Embryos were labeled with bromodeoxyuridine (BrdU; Roche) by incubating them for 20 min on ice in a solution of 10 mM BrdU and 15% dimethyl sulfoxide in EM. The embryos were then placed in

EM, incubated for 20 min at 28.5°C, and fixed using 4% paraformaldehyde. Embryos were processed for immunohistochemistry, treated for 1 h with 2 M HCl, and then processed for anti-BrdU immunohistochemistry. For immunohistochemistry, we used the following primary antibodies: rabbit anti-Sox10 (Park et al., 2005), rabbit anti-GFP (1:500, Abcam), mouse anti-BrdU (G3G4, 1:1000, Developmental Studies Hybridoma Bank), mouse anti-Is1 (39.4D5, 1:100, Developmental Studies Hybridoma Bank), and mouse anti-HuCD (16A11 1:20, Invitrogen). Alexa Fluor 488- and 568-conjugated secondary antibodies were used for fluorescence detection (1:500, Invitrogen). *In situ* RNA hybridization was performed with previously described RNA probes including *ihhb* (Currie and Ingham, 1996), *shh* (Krauss et al., 1993), *olig2* (Park et al., 2002), and *ngn1* (Blader et al., 1997).

Morpholino and RNA injection. A previously described morpholino oligonucleotide (MO) for *ihhb* (*ihhb* MO, 5'-ATGAGACTCTCCACGGCGGGCGCTCCTC-3') (Lewis and Eisen, 2001) and scrambled MO (5'-CCTCCTACCTCAGTTACAATTTATA-3') were purchased from Gene Tools, LLC. The MOs were dissolved in 1 \times Danieau solution at a concentration of 20 μ g/ μ l and diluted further with distilled water, and then 5 ng of MOs were injected into one-cell stage zebrafish embryos. For RNA injection, 50 pg of *olig2* RNA was injected into one-cell embryos.

Fluorescence-activated cell sorting. Approximately 300 wild-type and *ihhb* MO-injected 2 days postfertilization (dpf) *Tg(olig2:egfp)* embryos were used to isolate GFP⁺ cells by fluorescence-activated cell sorting (FACS). Cell dissociation and FACS were performed as previously described (Chung et al., 2011) using a FACSariaII (Becton Dickinson).

RNA extraction and quantitative reverse transcription-PCR. Embryos were collected and homogenized in TRIzol solution (Invitrogen) to purify total RNA. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed using a LightCycler (Roche) in a reaction mixture containing 1 μ l of PCR-amplified total cDNA as a template, 0.2 M 5' and 3' PCR primers, 0.8 μ l of MgCl₂, and 1 μ l of LightCycler Fast Start DNA Master SYBR Green I (Roche). The following oligonucleotide primers were used for qRT-PCR: *cdkn1c* forward (F) (5'-CAAGAATCCGAGGGAGTCCC-3'); reverse (R) (5'-GTTCATCCTGCTTCGACTCC-3'); *cyclinEF* (5'-AGGCTCTCGACATCAAGGA-3'); *R* (5'-TCGAGTCTTATGCCAATGCTT-3'); *cdk2* F (5'-GGCCTGGAGTTACATCAATGC-3'); *R* (5'-CCCCTCTTGCCAAGTTGATCT-3'); β -*actin* F (5'-TAGTCATTCCAGAAGCGTTTACC-3'); and *R* (5'-TACAGAGACACCCTGGCTTACAT-3').

Results

Ihhb signaling is required for the specification of OPCs in the ventral spinal cord

In previous studies, we have shown that the zebrafish Hh-related genes, *shh* and *tiggy-winkle* hedgehog (*twhh*), are expressed in the floor plate cells of the ventral spinal cord, and that their function is required for the induction and maintenance of *olig2* expression, which is crucial for motor neuron and oligodendrocyte development in the pMN precursor domain of the spinal cord (Park et al., 2004). We also have shown previously that overexpression of *olig2* failed to rescue defects in OPC specification in embryos treated with cyclopamine, a specific inhibitor of the Hedgehog signaling pathway (Chen et al., 2002), suggesting that Hh signal-

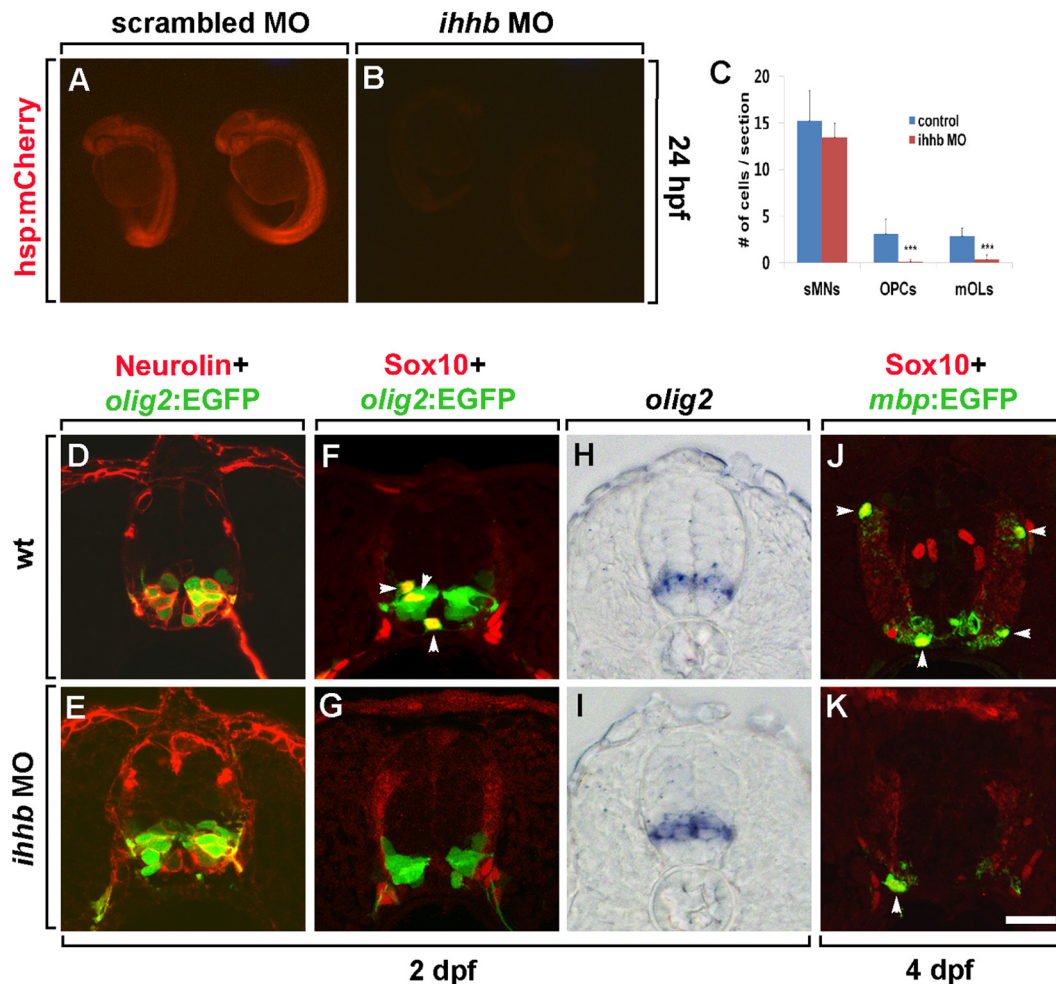


Figure 2. *Ihhb* function is required for the specification of OPCs. **A, B**, Scrambled MO-injected control (**A**) and *ihhb* MO-injected (**B**) *Tg(hsp70:ihhb-mcherry)* embryos heat shocked at 20 hpf. **C**, Quantification of motor neurons (sMNs), OPCs, and mature oligodendrocytes (mOLs) in wild-type and *ihhb* MO-injected embryos (** $p < 0.001$). Data were obtained from 15 sections from each of 6 control and 6 *ihhb* MO-injected embryos. **D–K**, Transverse sections of spinal cord, dorsal to the top. Wild-type (**D, F**) and *ihhb* MO-injected (**E, G**) *Tg(olig2:egfp)* embryos were labeled with anti-Neurolin (**D, E**) and anti-Sox10 (**F, G**) antibodies. **H, I**, *In situ* RNA hybridization of wild-type (**H**) and *ihhb* MO-injected (**I**) embryos with an *olig2* probe. **J, K**, Wild-type (**J**) and *ihhb* MO-injected (**K**) *Tg(mbp:egfp)* embryos were labeled with anti-Sox10 antibody. Arrowheads indicate *olig2:EGFP*⁺/*Sox10*⁺ OPCs in **F** and *mbp:EGFP*⁺/*Sox10*⁺ mature oligodendrocytes in **J** and **K**. Scale bar, 20 μ m.

ing might play an additional role in OPC specification in parallel with the maintenance of *olig2* expression in the pMN precursor domain (Park et al., 2002). In contrast to *shh* and *twhh*, *ihhb* has been shown to be expressed exclusively in the notochord and to be required for the formation of muscle pioneer cells in developing somites (Currie and Ingham, 1996).

To explore the function of Hh signaling in OPC specification, we first investigated the expression of *shh* and *Ihhb* in the developing spinal cord using *in situ* RNA hybridization. As shown in Figure 1, *shh* was expressed in the notochord and floor plate cells throughout neurogenesis [24 hours postfertilization (hpf)] and oligodrogenesis (48 hpf), although its expression decreased at 48 hpf (Fig. 1A, B). *Ihhb* was expressed exclusively in notochord cells and was not detected in the spinal cord at 24 hpf; however, floor plate cells began to express *Ihhb* at 36 hpf (data not shown) and this expression was clearly detectable in floor plate cells and notochord cells at 48 hpf (Fig. 1C, D) but was not detectable with a sense probe for *ihhb* (Fig. 1E). To confirm *Ihhb* expression in the spinal cord, an *ihhb:mcherry* BAC DNA, which expresses mCherry under the control of the *ihhb* promoter, was generated using *Escherichia coli*-based homologous recombination (Shin et al., 2003). Consistent with the *in situ* RNA hybridization data, embryos injected with *ihhb:mcherry* BAC DNA expressed

mCherry in the notochord only at 24 hpf but in the notochord and floor plate cells at 48 hpf (Fig. 1F, G), suggesting that *Ihhb* might play a role in OPC specification.

To investigate *Ihhb* function in oligodendrocyte development, we used an antisense MO against *ihhb* (*ihhb* MO), which was previously reported to block *ihhb* expression (Lewis and Eisen, 2001). The specificity of the *ihhb* MO was further confirmed by injecting it into *Tg(hsp70:ihhb-mcherry)* zebrafish embryos, which expressed an mCherry-tagged *Ihhb* protein by heat shock 70 promoter. When the embryos were heat shocked at 39°C for 30 min, a very low level of mCherry expression was detected (Fig. 2B), whereas scrambled MO-injected control embryos displayed robust mCherry expression (Fig. 2A), demonstrating that the *ihhb* MO inhibited *ihhb* expression efficiently.

Next, *Ihhb* loss-of-function was tested by injecting the *ihhb* MO into *Tg(olig2:egfp)* embryos. Since *ihhb* is first expressed in the spinal cord at 36 hpf, when secondary motor neurons (sMNs) are already specified, embryos injected with the *ihhb* MO showed normal development of EGFP⁺/Neurolin⁺ sMNs (Fig. 2C–E). However, anti-Sox10 antibody labeling of the spinal cord section revealed that *ihhb* MO-injected *Tg(olig2:egfp)* embryos failed to specify EGFP⁺/*Sox10*⁺ OPCs at 2 dpf (Fig. 2C, F, G), indicating that *Ihhb* function is required for OPC specification. Interest-

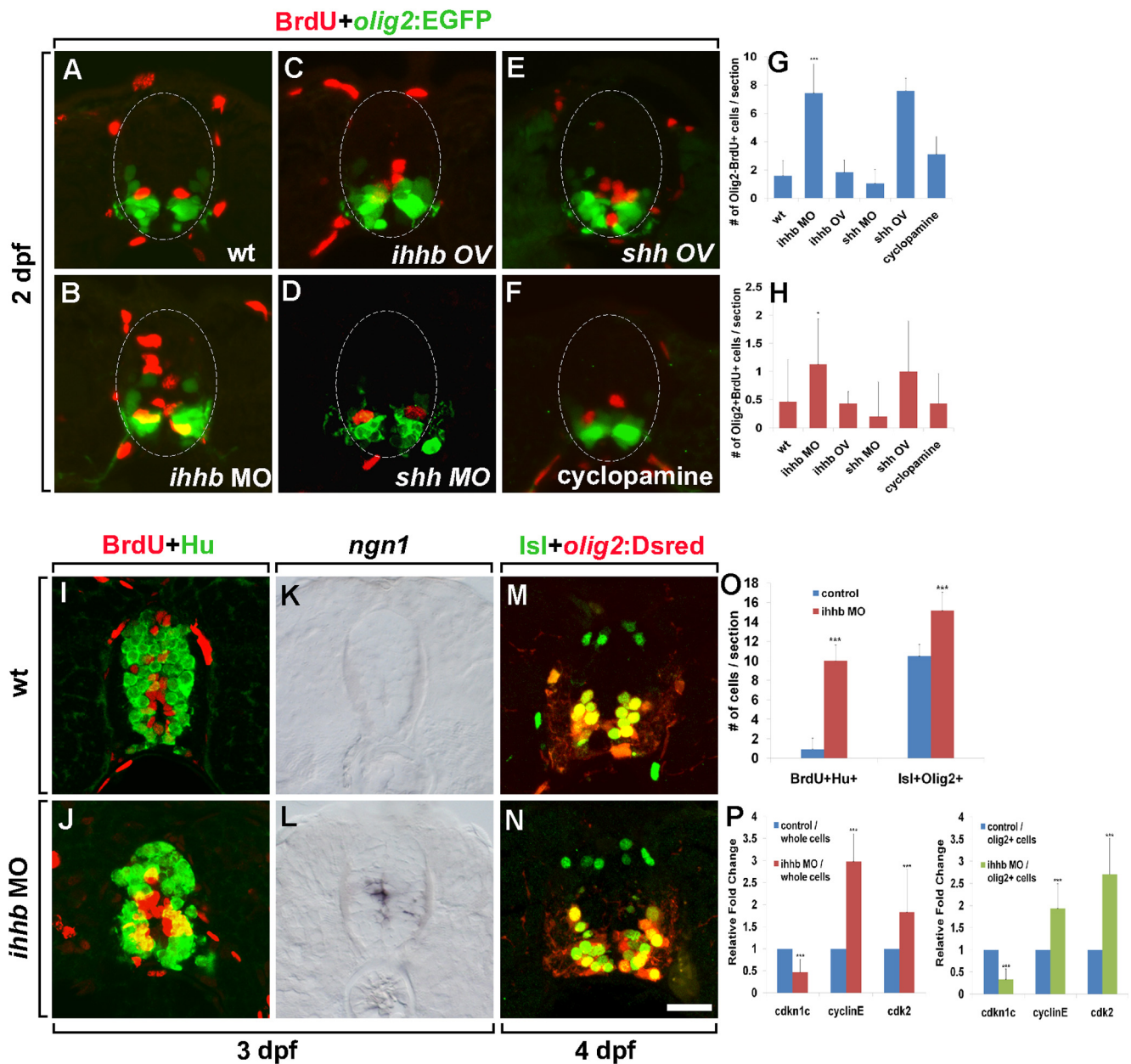


Figure 3. Ihhb function is required for neurogenesis-oligodendrogenesis switching by regulating precursor proliferation. **A–F**, Transverse sections of the spinal cord of *Tg(olig2:egfp)* embryos, dorsal to the top. Wild-type (**A**), *ihhb* MO-injected (**B**), heat-shocked *Tg(hsp70:ihhb-mcherry);Tg(olig2:egfp)* (**C**), *shh* MO-injected (**D**), heat-shocked *Tg(hsp70:gal4;uas:shh);Tg(olig2:egfp)* (**E**), and cyclopamine-treated (**F**) *Tg(olig2:egfp)* embryos were treated with BrdU for 20 min at 2 dpf, and labeled with anti-BrdU antibody. Dashed circles outline the spinal cord. **G, H**, Quantification of BrdU⁺/*olig2:EGFP*[−] cells (**G**) and BrdU⁺/*olig2:EGFP*⁺ cells (**H**) ($***p < 0.001$) (**G**) and BrdU⁺/*olig2:EGFP*⁺ cells ($*p < 0.05$) (**H**). OV, Overexpression. Data were obtained from 15 sections from each of 6 control and 6 MO-injected embryos. **I–N**, Transverse sections of the spinal cord, dorsal to the top. (**I, J**) Wild-type (**I**) and *ihhb* MO-injected (**J**) embryos were incubated with BrdU for 20 min at 2 dpf, cultured for one more day, and then labeled with anti-BrdU and anti-Hu antibodies at 3 dpf. **K, L**, *In situ* RNA hybridization of wild-type (**K**) and *ihhb* MO-injected (**L**) embryos with a *ngn1* probe. **M, N**, Labeling of wild-type (**M**) and *ihhb* MO-injected (**N**) *Tg(olig2:dsred)* embryos with anti-Isl antibody at 4 dpf. **O**, Quantification of BrdU⁺/Hu⁺ cells from **I** and **J**, and Isl⁺/*olig2:Dsred*⁺ cells from **M** and **N**. Data were obtained from 15 sections from each of 6 control and 6 *ihhb* MO-injected embryos. **P**, Real-time RT-PCR analysis to compare the expression of cell cycle regulators in wild-type, *ihhb* MO-injected embryos and *olig2:EGFP*⁺ cells FACS-isolated from wild-type and *ihhb* MO-injected *Tg(olig2:egfp)* embryos. Data are shown as the average fold change relative to the wild-type control and are normalized to β -actin expression. The results are presented as the mean \pm SD from three independent experiments ($***p < 0.001$). Scale bar, 20 μ m.

ingly, *ihhb* MO-injected embryos showed normal levels of *olig2* expression, as revealed by *in situ* RNA hybridization with *olig2* (Fig. 2I), indicating that in contrast to Shh, Ihhb is not required for the maintenance of *olig2* expression. Consistent with loss of Sox10⁺ OPCs, *ihhb* MO-injected *Tg(mbp:EGFP)* embryos, which express EGFP in mature oligodendrocytes (Jung et al., 2010), show a dramatically reduced number of Sox10⁺/*mbp:EGFP*⁺ mature oligodendrocytes (Fig. 2C, J, K).

Ihhb function is required for neurogenesis-oligodendrogenesis switching by regulating precursor proliferation

A previous study has shown that *ihh* is required for the cell cycle exit and differentiation of colonic epithelial cells in the mammalian gut (van den Brink et al., 2004), suggesting that Ihhb function might be involved in the regulation of neural precursor proliferation during oligodendrogenesis. To test this idea, we used thy-

midine analog BrdU. The spinal cord of control *Tg(olig2:egfp)* embryos exhibited many BrdU⁺ proliferating neural precursors during neurogenesis at 24 hpf (data not shown) but showed few BrdU⁺ proliferating neural precursors during oligodendrogenesis at 2 dpf (Fig. 3A). Interestingly, *Tg(olig2:egfp)* embryos injected with *ihhb* MO exhibited a number of proliferating cells similar to that of wild-type embryos at 24 hpf (data not shown) but showed a dramatic increase in the number of both BrdU⁺/*olig2:EGFP*⁺ and BrdU⁺/*olig2:EGFP*⁻ cells at 2 dpf, after *ihhb* expression begins in the spinal cord (Fig. 3B,G,H). These results indicate that inhibition of Ihhb function caused defects in cell cycle regulation, which resulted in increased proliferation of neural precursors throughout the spinal cord.

qRT-PCR revealed that the expression of cell cycle components, including *cdk2* and *cyclin E*, was increased, while the expression of cyclin-dependent kinase inhibitor 1c (*cdkn1c*), a cell cycle inhibitor for neural precursors in the zebrafish CNS (Park et al., 2005), was decreased throughout the spinal cord and in FACS-purified *olig2:EGFP*⁺ cells from *ihhb* MO-injected embryos at 2 dpf (Fig. 3P). The spinal cords of *Tg(hsp70:ihhb-mcherry);Tg(olig2:egfp)* embryos showed a similar amount of proliferation of neural precursors after heat shock induction of Ihhb (Fig. 3C,G,H), indicating that overexpression of Ihhb alone does not induce cell cycle arrest in the remaining neural precursors at 2 dpf. Interestingly, the number of BrdU⁺ proliferating neural precursors in the spinal cords of *shh* MO-injected embryos was decreased (Fig. 3D,G,H), but overexpression of Shh in heat-shocked *Tg(hsp70:gal4;uas:shh);Tg(olig2:egfp)* embryos caused a dramatic increase in BrdU⁺ proliferating precursors throughout the spinal cord at 2 dpf (Fig. 3E,G,H). This suggests that in contrast to Ihhb, Shh has a stimulatory effect on cell cycle progression during oligodendrogenesis. Embryos treated with cyclopamine showed a similar number of proliferating neural precursors to wild-type embryos at 2 dpf (Fig. 3F–H).

Since the data described above demonstrated that neural precursor proliferation increased without OPC specification in the Ihhb-deficient spinal cord, next we performed a BrdU pulse-chase experiment to test whether proliferating precursors generated neurons instead of OPCs in the absence of Ihhb function during oligodendrogenesis. After the incubation of wild-type and *ihhb* MO-injected embryos with BrdU for 20 min at 2 dpf during oligodendrogenesis, the embryos were cultured for one more day and then labeled with an anti-BrdU antibody together with anti-Hu antibody to label postmitotic neurons. In wild-type embryos, few BrdU⁺/Hu⁺ neurons were observed (Fig. 3I,O), while *ihhb* MO-injected embryos show an increased number of BrdU⁺/Hu⁺ postmitotic neurons in the spinal cord at 3 dpf (Fig. 3J,O), indicating that in the absence of Ihhb function, proliferating spinal precursors continuously generated neurons during oligodendrogenesis. Consistent with this, the expression of *ngn1*, a basic-helix-loop-helix transcription factor that is required for neurogenesis, was maintained at significant levels in the spinal cords of *ihhb* MO-injected embryos (Fig. 3L)

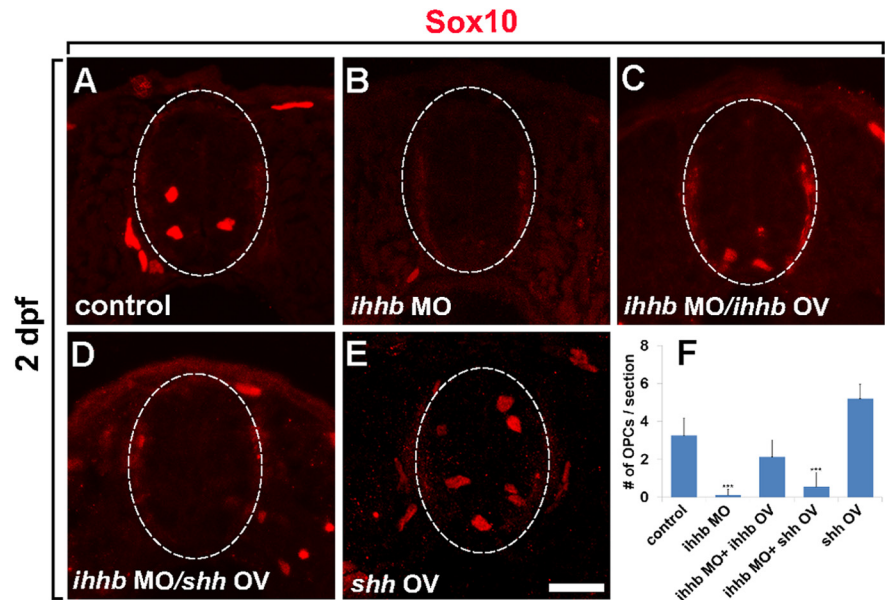


Figure 4. Shh cannot replace Ihhb function in OPC specification. **A–E**, Transverse sections of the spinal cord, dorsal to the top. Wild-type (**A**), *ihhb* MO-injected (**B**), *ihhb* MO-injected *Tg(hsp70:ihhb-mcherry)* (**C**), *ihhb* MO-injected *Tg(hsp70:gal4;uas:shh)* (**D**), and noninjected *Tg(hsp70:gal4;uas:shh)* (**E**) embryos were heat shocked twice at 24 and 36 hpf, and labeled with anti-Sox10 antibody at 2 dpf. OV, Overexpression. Dashed circles outline the spinal cord. **F**, Quantification of Sox10⁺ OPCs (****p* < 0.001). Data were obtained from 20 sections from each of 6 control and 6 *ihhb* MO-injected embryos. Scale bar, 20 μ m.

but was downregulated in wild-type embryos at 3 dpf (Fig. 3K). As a consequence of continuous neurogenesis, an increased number of Isl⁺/*olig2:Dsred*⁺ motor neurons was observed at 4 dpf (Fig. 3M,O) in the spinal cords of *ihhb* MO-injected *Tg(olig2:Dsred)* embryos. Together, these data suggest that Ihhb regulation of neural precursor proliferation is required for the switch from neurogenesis to oligodendrogenesis.

Since our data suggested that Shh and Ihhb appear to play different roles in cell cycle regulation and *olig2* induction in neural precursors during oligodendrogenesis, we tested whether impaired OPC specification due to *ihhb* MO injection could be recovered by overexpression of Ihhb and Shh. First, *ihhb* expression was induced at a high level by heat shocking *Tg(hsp70:ihhb-mcherry)* embryos twice at 24 hpf and 36 hpf after injection of the *ihhb* MO, followed by embryo analysis. Although the data described above showed that the *ihhb* MO could interfere with transgenic *ihhb:mcherry* expression during early development, we reasoned that sufficiently high expression of the transgene should produce transcripts in excess of the *ihhb* MO as the MO becomes diluted during development. As expected, these embryos partially rescued Sox10⁺ OPC production (Fig. 4C,F) compared with *ihhb* MO-injected transgenic embryos without heat shock (Fig. 4B). However, in contrast to the increased number of Sox10⁺ OPCs in noninjected *Tg(hsp70:gal4;uas:shh)* embryos (Fig. 4E,F), transgenic embryos injected with the *ihhb* MO and heat-shocked twice at 24 hpf and 36 hpf failed to rescue OPC production (Fig. 4D,F), indicating that Shh could not compensate for the loss of Ihhb function in OPC specification. Together, these data suggested that Ihhb and Shh play different roles in OPC specification.

Discussion

In the present study, we reported that *ihhb* expression begins in the floor plate cells of the ventral spinal cord at the time when OPCs are specified, and that Ihhb function is required for OPC specification. Ihhb negatively regulates the proliferation of spinal

precursors, partly by regulating the expression of *cdkn1c*, a negative regulator of the cell cycle. These data suggested a novel mechanism, mediated by Ihhb, for the sequential generation of motor neurons and oligodendrocytes from pMN precursors in the ventral spinal cord. Interestingly, loss-of-Ihhb function caused increased proliferation of neural precursors throughout the spinal cord, indicating that the effect of Ihhb is not restricted to the *olig2*-expressing pMN domain but is a general effect on the cell cycle in spinal cord precursors. These data suggest the possibility that Ihhb function is required for the generation of glial cells throughout the spinal cord, including oligodendrocytes and astrocytes; however, we could not confirm this hypothesis because there is no available marker specific for astrocytes in zebrafish.

Recently, it has been shown that the phosphorylation state of *olig2* regulates the proliferation of neural progenitors in the developing forebrain (Li et al., 2011) and the motor neuron-oligodendrocyte fate switch in the developing spinal cord (Sun et al., 2011). In the developing spinal cord, phosphorylation of Olig2 is required for motor neuron specification, but subsequent dephosphorylation of Olig2 triggers oligodendrocyte fate specification (Li et al., 2011). Since our results show that ihhb function is required for the motor neuron-oligodendrocyte fate switch, these studies suggest the possibility that Ihhb function might be involved in regulation of the phosphorylation state of Olig2 or in regulation of cofactor expression, which is required to activate oligodendrocyte specification and repress motor neuron development.

Our data also suggested that both Shh and Ihhb are required for OPC specification but that they have separate functions. Ihhb is required for the cell cycle inhibition of the spinal precursors involved in OPC specification, while Shh has a stimulatory effect on the cell cycle progression and maintenance of *olig2* expression. These data conflict with previous studies, which have shown that Shh signaling from grafted notochord or floor plate promotes the generation of pMN-derived OPCs in the spinal cord (Poncet et al., 1996; Pringle et al., 1996). One possible explanation is that grafted notochord and floor plate cells can generate both Shh and Ihh, and could thus contribute to generate oligodendrocyte lineage cells together. A similar mechanism for the regulation of proliferation versus differentiation involving separate roles for Shh and Ihh also has been shown in the regulation of colonic epithelium differentiation. Shh was overexpressed in adenomas of the colon and exogenous Shh promoted cell proliferation in colonocytes (Douard et al., 2006), whereas Ihh was required for the differentiation of mature colonocytes (van den Brink et al., 2004), suggesting that the regulation of proliferation versus differentiation by Shh and Ihh is widely used during development.

A precise mechanism for the regulation of differentiation by Shh and Ihh binding through the patched (Ptch) receptor has not yet been proven. A previous study, which showed that the Ihh and Shh proteins have similar structures and biological properties (such as a similar affinity for the Hh-binding proteins) but different potencies (Pathi et al., 2001), may provide a basis for the competitive inhibition of Shh. Ihh may behave as a potent competitive inhibitor of Shh for binding to the Ptch receptor, and the relative expression levels of Ihh and Shh may be involved in maintaining a precise balance between the proliferation and differentiation of spinal precursors. However, more direct evidence is needed to reveal the precise mechanism and the roles played by Ihh and Shh in OPC specification.

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