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Shh and Gli3 activities are required for timely generation of motor neuron progenitors

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ABSTRACT

Generation of distinct ventral neuronal subtypes in the developing spinal cord requires Shh signaling mediated by the Gli family of transcription factors. Genetic studies of $Shh^{-/-}$;$Gli3^{-/-}$ double mutants indicated that the inhibition of Gli3 repressor activity by Shh is sufficient for the generation of different neurons including motor neurons. In this study, we show that although ventral neural progenitors are initiated in normal numbers in $Shh^{-/-}$;$Gli3^{-/-}$ mutants, the subsequent appearance of motor neuron progenitors shows a ~20-hour lag, concomitant with a delay in the activation of a pan-neuronal differentiation program and cell cycle exit of ventral neural progenitors. Accordingly, the $Shh^{-/-}$;$Gli3^{-/-}$ mutant spinal cord exhibits a delay in motor neuron differentiation and an accumulation of a ventral neural progenitor pool. The requirement of Shh and Gli3 activities to promote the timely appearance of motor neuron progenitors is further supported by the analysis of $Ptch1^{-/-}$ mutants, in which constitutive Shh pathway activity is sufficient to elicit ectopic and premature differentiation of motor neurons at the expense of ventral neural progenitors. Taken together, our analysis suggests that, beyond its well established dorso-ventral patterning function through a Gli3-derepression mechanism, Shh signaling is additionally required to promote the timely appearance of motor neuron progenitors in the developing spinal cord.

Introduction

Neurogenesis in the vertebrate central nervous system is characterized by a series of coordinated events, involving specification, expansion and differentiation of distinct neuronal progenitor cell types. Among these, regulated differentiation of neuronal progenitors is a prerequisite to control the expansion of neuronal progenitor pools as well as the generation of appropriately numbered post-mitotic neurons (Bertrand et al., 2002). Therefore, tight regulation of the length and duration of neuronal progenitor cell cycles is critical for proper neural tube size regulation and appearance of distinct neuronal subtypes at the correct time and location.

In the developing ventral spinal cord, the expression of homeodomain protein Nkx6.1 highlights the early ventral progenitor population which eventually differentiates into the floor plate, motor neurons and several interneuron subtypes (Briscoe et al., 2000; Sander et al., 2000). Establishment of the Nkx6.1+ progenitor domain is regulated by secreted signaling molecule Sonic hedgehog (Shh) (Briscoe et al., 2000). Mice lacking Shh function display severe neural patterning defects including a lack of most ventral neuronal cell types in the spinal cord (Chiang et al., 1996). However, in $Shh^{-/-}$;$Gli3^{-/-}$ or $Smo^{-/-}$;$Gli3^{-/-}$ double mutants, motor neurons and several classes of ventral interneurons are generated, indicating that inhibiting Gli3 transcriptional repressor activity by Shh signaling is central to the establishment of Nkx6.1+ neural progenitors (Litingtung and Chiang, 2000; Persson et al., 2002; Wijgerde et al., 2002). Indeed, activation of Shh signaling inhibits Gli3 repressor formation by preventing Gli3 proteolytic processing (Litingtung et al., 2002; Wang et al., 2000). The full-length form of Gli3 can also act as a transcriptional activator in certain developmental context. This is demonstrated by the observation that ectopic Hh pathway activation in $Ptch^{-/-}$ mutants is partially dependent on Gli3 function (Motoyama et al., 2003). Moreover, Gli3 apparently shares redundant function with Gli2, another Gli family member, in the development of V3 interneurons (Bai et al., 2004; Lei et al., 2004; Motoyama et al., 2003). Thus, Gli3 is a bifunctional transcription factor and its activity is modulated by Shh signaling.

It has been shown that ventral spinal cord progenitor cells initially express Nkx6.1 at an early somite stage, then subsets of Nkx6.1+ cells express cell-type specific (floor plate, MN progenitor, V3 or V2 interneuron progenitor) transcription factors that eventually dictate their fates (Jeong and McMahon, 2005). This temporal stepwise cell fate determination process appears to be associated with gradual accumulation of Shh ligand in the responding neural progenitors (Chamberlain et al., 2008). Emerging evidence suggests that both the strength and duration of Shh signaling activity affect the final cellular

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response. Specifically, it has been shown in chick spinal cord that prolonged exposure to a defined level of Shh signaling leads to progressively more ventral cell fates (Stamataki et al., 2005; Dessaud et al., 2007). It is therefore important to note that Shh ligand continues to be expressed by the notochord and floor plate well after the completion of spinal dorso-ventral patterning until midgestation, which raises the possibility that Shh signaling may assume an as yet undetermined functional role.

The differentiation of motor neurons in the spinal cord is initiated by the expression of bHLH protein Olig2 within the Nkx6.1 progenitor domain (Mizuguchi et al., 2001; Novitch et al., 2001). Both gain- and loss-of-function studies indicated that Olig2 acts downstream of Nkx6.1 and related Nkx6.2 proteins to promote motor neuron differentiation (Novitch et al., 2001; Vallstedt et al., 2001). Abrogating Olig2 function not only led to lack of motor neuron generation but also prolonged ventral neural progenitor cell proliferation (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). The latter property is associated with the ability of Olig2 to promote cell cycle exit by activating a generic neuronal differentiation program (Mizuguchi et al., 2001; Novitch et al., 2001). Thus, precise regulation of Olig2 expression is critical not only for motor neuron generation but also for the balanced development of ventral progenitor pools. While Shh is capable of inducing Olig2 expression, this is thought to be indirect and mediated through Nkx6.1 expression (Briscoe and Novitch, 2008; Novitch et al., 2003; Rowitch et al., 2002). Moreover, recent studies have identified retinoic acid as an obligate signal in activating Olig2 expression and motor neuron differentiation (Novitch et al., 2003). In this study, we show that the timely appearance of Olig2 expression in the Nkx6.1+ progenitor domain also depends on Shh signaling. The delay of Olig2 expression in $Shh^{-/-};Gli3^{-/-}$ double mutants leads to a defective pan-neuronal differentiation program and accumulation of ventral neural progenitors. Thus, our

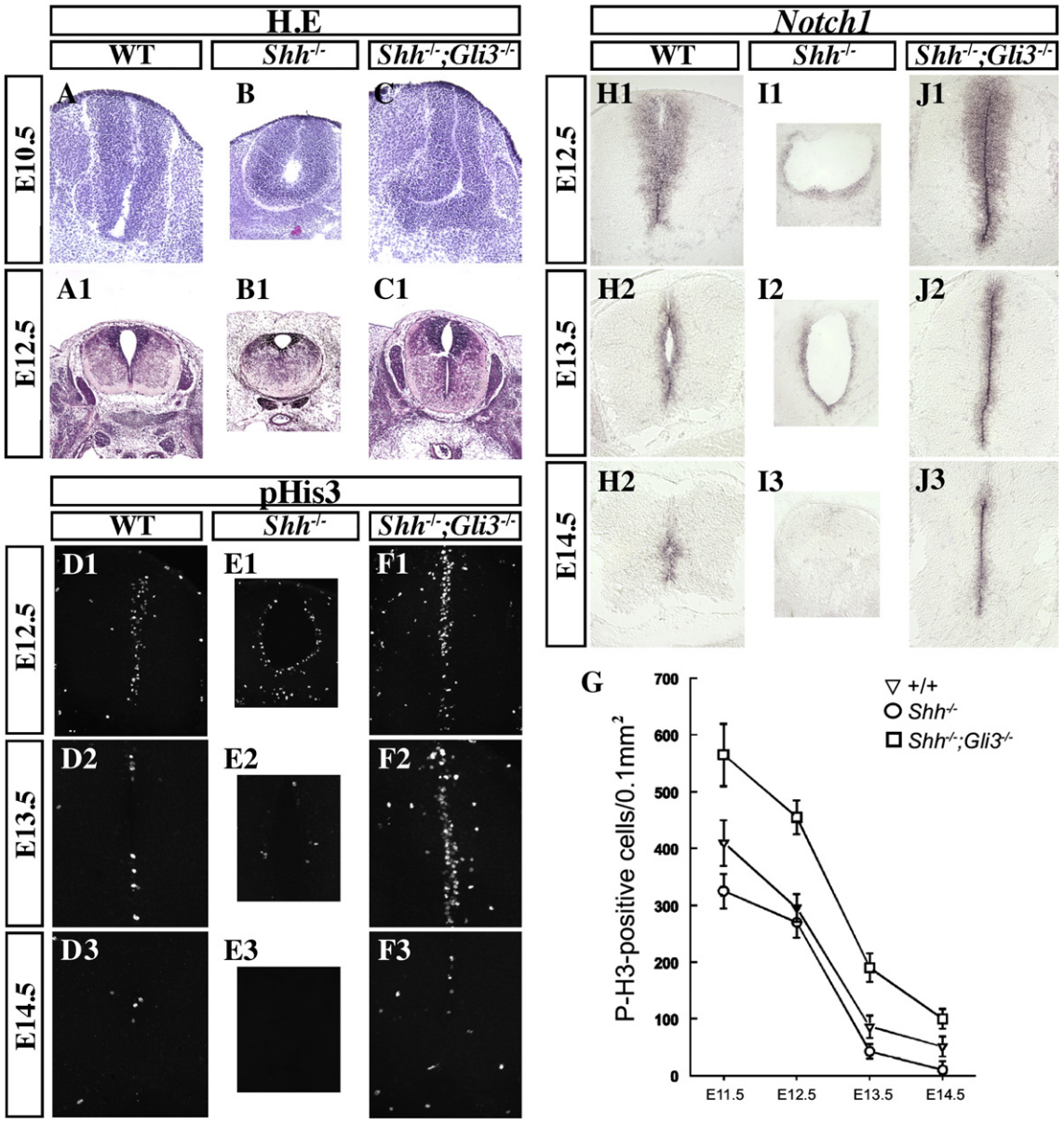

Fig. 1. Increased cell numbers and augmented mitotic activity in $Shh^{-/-};Gli3^{-/-}$ mutant spinal cord. (A–C, A1–C1) H&E-stained cross-sections of E10.5 and E12.5 wildtype (A, A1), $Shh^{-/-}$ (B, B1) and $Shh^{-/-};Gli3^{-/-}$ (C, C1), showing a larger spinal cord size in $Shh^{-/-};Gli3^{-/-}$ mutant. (D1–D3, E1–E3, F1–F3) Distribution of pHis3 protein in wildtype (D1–D3), $Shh^{-/-}$ (E1–E3), and $Shh^{-/-};Gli3^{-/-}$ (F1–F3) embryos at various stages in the lumbar region. Note that there are more mitotic pHis3+ cells in the $Shh^{-/-};Gli3^{-/-}$ mutant spinal cord ventricular zone compared with wildtype and $Shh^{-/-}$ (G). (H1–H3, I1–I3, J1–J3) Neuronal progenitor marker *Notch1* in wildtype(H1–H3), $Shh^{-/-}$ (I1–I3) and $Shh^{-/-};Gli3^{-/-}$(J1–J3). Note the consistent expansion of *Notch1* expression domains in $Shh^{-/-};Gli3^{-/-}$ mutant spinal cord.

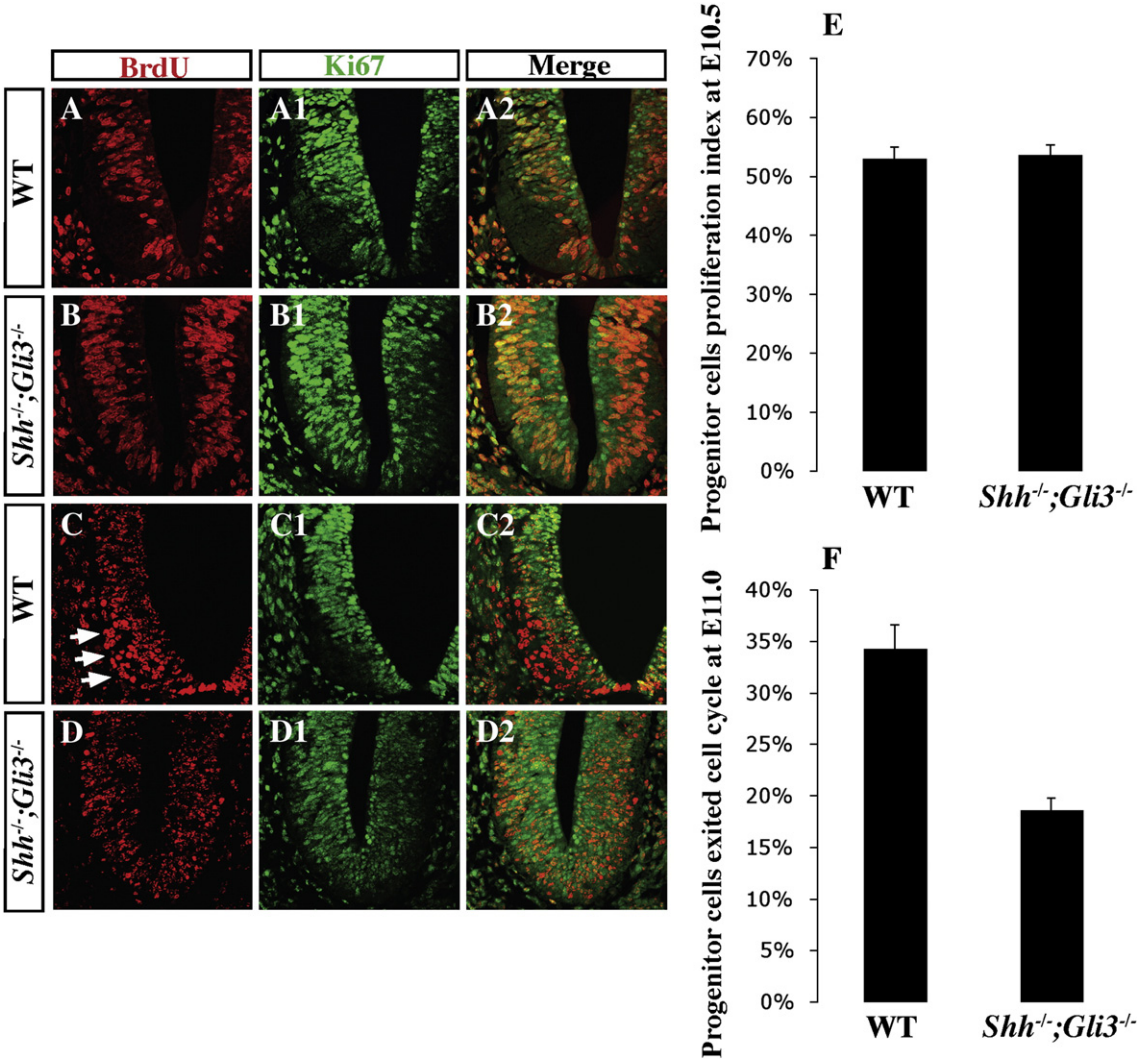

Fig. 2. Defective progenitor cell cycle exit in $Shh^{-/-};Gli3^{-/-}$ spinal cords. (A–A2, B–B2) Distribution of Ki67+ (green) and 1-hour pulse BrdU+ (red) cells in wildtype (A–A2), $Shh^{-/-};Gli3^{-/-}$ (B–B2) at E10.5 in the lumbar region of the spinal cord. Histogram (E) shows progenitor cell proliferation index (BrdU+ versus total Ki67+ cells). (C–C2, D–D2) Distribution of Ki67+ (green) and 24-hour pulse BrdU+ (red) cells in wildtype (C–C2), and $Shh^{-/-};Gli3^{-/-}$ (D–D2) at E11.0 in the lumbar region of the spinal cord. Histogram (F) shows the percentage of progenitor cells (BrdU+;Ki67− versus total BrdU-positive cells) that exited the cell cycle during the 24-hour period. Notice that approximately twice as many wildtype progenitors leave the cell cycle compared to those of $Shh^{-/-};Gli3^{-/-}$ mutants. ($p<0.001$, Student's *t*-test).

study uncovers a crucial role for Shh signaling in the balancing of these two cellular processes by regulating Olig2 expression during a critical period of neurogenesis.

Results

$Shh^{-/-};Gli3^{-/-}$ mutant spinal cords contain a larger pool of neuronal progenitor cells that maintain their proliferative potential

Since we observed an enlarged spinal cord size in $Shh^{-/-};Gli3^{-/-}$ mutants after E10.5 and throughout midgestation, compared with wildtype and $Shh^{-/-}$ mutants (Figs. 1A–C, A1–C1), we determined the neural progenitor cell proliferation pattern by examining the distribution of mitotic cells using phosphorylated-Histone 3 (pHis3) antibody. We found a significant increase in the number of mitotic cells in $Shh^{-/-};Gli3^{-/-}$ mutant spinal cord compared to wildtype and $Shh^{-/-}$ mutant spinal cords from E11.5 to E14.5 (Figs. 1D–D3, E–E3, F–F3, G), suggesting that removing both Shh and Gli3 functions increase the duration of spinal cord progenitor cell proliferation. The Gli3 single mutant spinal cord mitotic activity is comparable to that of the wildtype (data not shown), consistent with the minor patterning defects observed near the dorsal–ventral boundary of the spinal cord (Persson et al., 2002). As neurogenesis proceeds, neural progenitors become restricted in the progressively smaller ventricular zone within the expanding spinal cord. The transmembrane receptor Notch1 is a critical component in regulating neuronal cell fate and *Notch1* expression marks the neuronal progenitor cell population (Ohtsuka et al., 1999; Weinmaster, 2000). We found that the domain containing *Notch1*-expressing progenitor cells was expanded in $Shh^{-/-};Gli3^{-/-}$ mutants compared with wildtype or $Shh^{-/-}$ mutants from E12.5 to E14.5 (Figs. 1H1–H3, I1–I3, J1–J3). This expanded *Notch1*-expressing progenitor domain is consistent with the increased number of mitotic cells in $Shh^{-/-};Gli3^{-/-}$ mutants. Therefore, we conclude that enhanced mitotic cell numbers in $Shh^{-/-};Gli3^{-/-}$ mutants are caused by either increased mitotic rate or delayed progenitor cell cycle exit or both.

Neuronal progenitor cells in $Shh^{-/-};Gli3^{-/-}$ spinal cord remain proliferative for a longer duration

To determine whether the proliferation rate is altered in $Shh^{-/-};Gli3^{-/-}$ mutants, we measured the proliferative index of E10.5 spinal cord at the hindlimb level, by counting the proportion of BrdU-labeled neuronal progenitors after a 1-hour BrdU pulse. The total number of

progenitor cells was identified with an antibody against Ki67, a cell cycle marker. We found that the relative proliferative indices (BrdU+ versus Ki67+ cells) of wildtype and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants were comparable (Figs. 2A–A2, B–B2, E), although the number of proliferative cells in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants are increased by almost 80% in the ventral spinal cord (Supplementary Fig. 1). This result indicated that *Shh*$^{-/-}$;*Gli3*$^{-/-}$ neural progenitor cells, although presented at larger number, have similar proportions of cells in S-phase and proliferated at a rate that was comparable to wildtype. Next, we examined whether *Shh*$^{-/-}$;*Gli3*$^{-/-}$ neural progenitors were defective in exiting the cell cycle promptly after S-phase thus contributing to the increased mitotic cell numbers. In order to determine the percentage of neural progenitor cells that have exited the cell cycle to begin differentiation, we injected BrdU into wildtype and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants at E10.0, and then determined, 24 h after BrdU injection at E11.0, the proportion of cells with BrdU labeling that no longer expressed Ki67 versus total BrdU-positive cells. BrdU signals in actively dividing progenitor cells appear punctate due to BrdU signal dilution upon active cell division and segregation into daughter cells (Chenn and Walsh, 2002). In contrast, strong and uniform BrdU signals were found in post-mitotic cells that were labeled with BrdU followed immediately by cell cycle exit. These signals were observed more frequently in the wildtype than *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants, noticeably in the mantle zone of motor column (Fig. 2, compare C–C2 with D–D2). We found an approximately two-fold increase in the number of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ cells that failed to exit cell cycle when compared to wildtype (Figs. 2C–C2, D–D2, F). We conclude that Shh and Gli3 functions, by regulating cell cycle exit in the spinal cord, are essential to the timely reduction of the neuronal progenitor pool. This finding suggests that Shh and Gli3 function is not only required for proper patterning, but are also indispensable in the early regulation of neuronal progenitor expansion in the developing spinal cord.

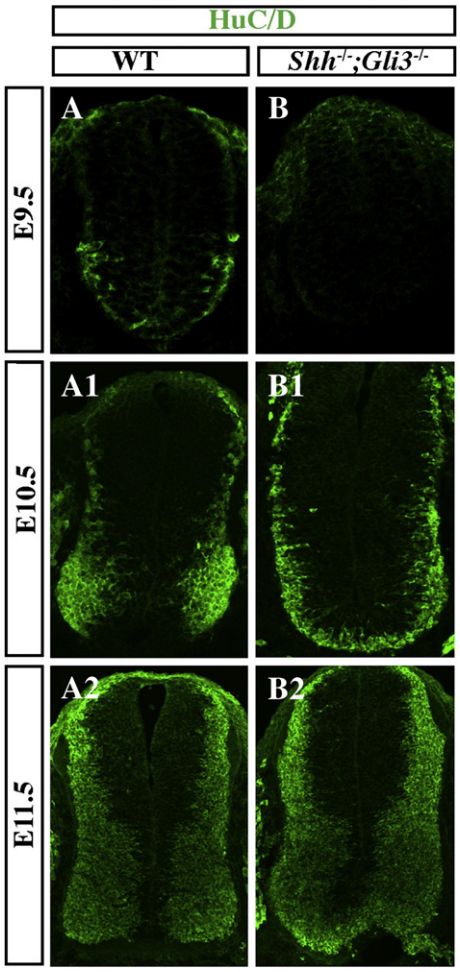

Fig. 3. (A–A2, B–B2) Reduced neuronal differentiation in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutant spinal cord. Distribution of HuC/D proteins in wildtype (A–A2), *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (B–B2) at various stages in the lumbar region of the spinal cord. HuC/D+ differentiating neurons are present in the mantle zone of wildtype spinal cord at E9.5 (A), a stage when we did not observe any apparent HuC/D+ cells in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants (B). Delayed neuronal differentiation was apparent in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants at E10.5, particularly in the presumable motor neuron region (B1) comparing to that of wildtype (A1). Comparable neuronal differentiation was observed between wildtype and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ spinal cords at E11.5 (A2 and B2).

The defective cell cycle exit, accompanied by a consequential expansion of neural progenitor pools, suggests that neuronal differentiation is compromised in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants. Next, we used the HuC/D antibody, a pan-neuronal marker, to determine to what extent neural differentiation is affected. At E9.5, we detected an emerging expression of HuC/D in the outer mantle zone in wildtype spinal cord (Fig. 3A). However, HuC/D-expressing neurons could not be readily detected in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants (Fig. 3B). At E10.5, a significant number of early neuronal progenitor cells have already differentiated in wildtype, occupying large portions of the spinal neuroepithelium (Fig. 3A1). In contrast, HuC/D-expressing neurons in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants were significantly less abundant, especially in the domain where motor neurons normally develop (Fig. 3B1, see below), suggesting that motor neuron differentiation is delayed in the mutant. This is not due to a terminal block in differentiation, as significant numbers of neurons are detected in the mantle zone of E11.5 *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos (Figs. 3A2, B2). Our findings underscore the essential role of Shh and Gli3 activities in promoting neural progenitor cell cycle exit in the developing spinal cord.

Shh$^{-/-}$;*Gli3*$^{-/-}$ *mutant spinal cords contain a larger pool of Nkx6.1+ but Olig2− progenitors at E9.5*

Motor neuron progenitors are among the first groups of progenitors to emerge from the ventral spinal cord (Altman and Bayer, 1984). Several studies indicated that Olig2 acts downstream of Nkx6.1 and related Nkx6.2 proteins to promote motor neuron differentiation (Novitch et al., 2001; Vallstedt et al., 2001). We, therefore, decided to examine whether appearance of Nkx6.1 and Olig2 expressions were affected in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutant spinal cord. At E9.5, we found no significant difference in the number and spatial distribution of Nkx6.1+ progenitors between wildtype and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants (Figs. 4A, B), suggesting that wildtype and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutant spinal cords possess comparable number of proliferative ventral progenitors. However, few Olig2+ motor neuron progenitors and no Isl1+ motor neurons were detected in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutant spinal cords (Fig. 4D), whereas abundant Olig2+ cells and a smattering of Isl1+ cells were detected in wildtype (Fig. 4C). We found no evidence of Olig2 expression prior to E9.5 in the mutant, whereas its expression occurred as early as E8.75 in the wildtype (Fig. 5, data not shown). At E10.5, we found a large number of Olig2+ cells in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ spinal cord, suggestive of Shh-independent motor neuron progenitor specification (Fig. 4D1). Therefore, in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants, Olig2 expression and motor neuron differentiation subsequently occur, albeit delayed, presumably via a Shh-independent but retinoic acid-dependent mechanism (Novitch et al., 2003). The delay in neuronal differentiation appears to be specific to motor neurons. We have determined that other neurons, such as V2 (Supplementary Fig. 2), are generated in a comparable fashion in the spinal cord of the wildtype and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants, although we cannot rule out a subtle difference between the WT and the mutant embryos.

While an appreciable amount of Nkx6.1+ progenitors already differentiated into Isl1+ motor neurons in the wildtype spinal cord (Fig. 4A1), very few Isl1+ motor neurons were present in *Shh*$^{-/-}$;

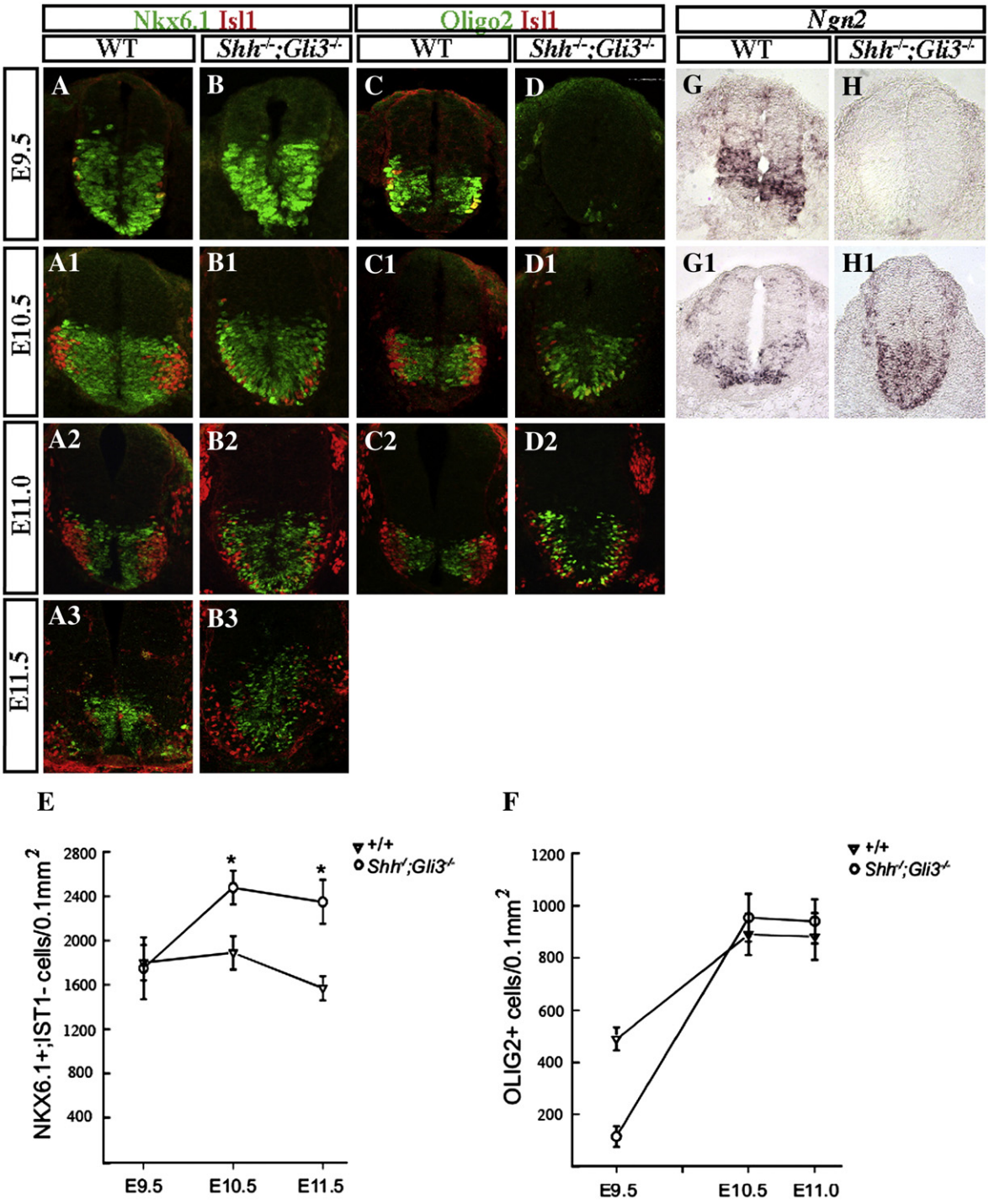

Fig. 4. Increased motor neuron progenitor pool and delayed motor neuron differentiation in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ spinal cord. (A–A3, B–B3) Distribution of Nkx6.1 and Isl1 proteins in wildtype (A–A3) and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (B–B3) embryos in the lumbar region at various stages. Note the expanded expression of Nkx6.1 in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ spinal cord from E10.5 to E11.5. (C–C2, D–D2) Distribution of Olig2 and Isl1 proteins in wildtype (C–C2) and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (D–D2) embryos in the lumbar region at various stages. Note the delayed appearance of Olig2+ and Isl1+ cells in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ spinal cord. (E and F) Quantification of Nkx6.1+;Isl1− (E) and Olig2+ (F) cells ($p<0.05$, Student's *t*-test). (G–G1, H–H1) *Ngn2* expression in wildtype (G–G1) and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (H–H1) spinal cord at E9.5 (G, H) and E10.5 (G1, H1) in the lumbar region.

Gli3$^{-/-}$ in spite of a large Nkx6.1+ progenitor pool (Fig. 4B1). This might be explained by ~20-hour delay in the emergence of Olig2+ motor neuron progenitors in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ (Fig. 4). In agreement, we observed an approximately 30% and 40% increase in Nkx6.1+; Isl1− progenitors in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ at E10.5 and E11.5, respectively (Figs. 4A1–A3, B1–B3, E), suggesting that a larger population of Nkx6.1+ progenitor cells remained undifferentiated for a longer duration in the double mutant.

Differentiation of neural progenitors in the vertebrate central nervous system requires the coordinated activation of programs that both promote pan-neural differentiation and direct neuronal subtype identity. Recent evidence suggests that proneural genes not only direct generic neuronal differentiation but are also engaged in neuronal subtype specifications (Bertrand et al., 2002). Notably, Neurogenin2 (Ngn2), a bHLH protein, has been shown to promote pan-neural differentiation and is required for proper generation of

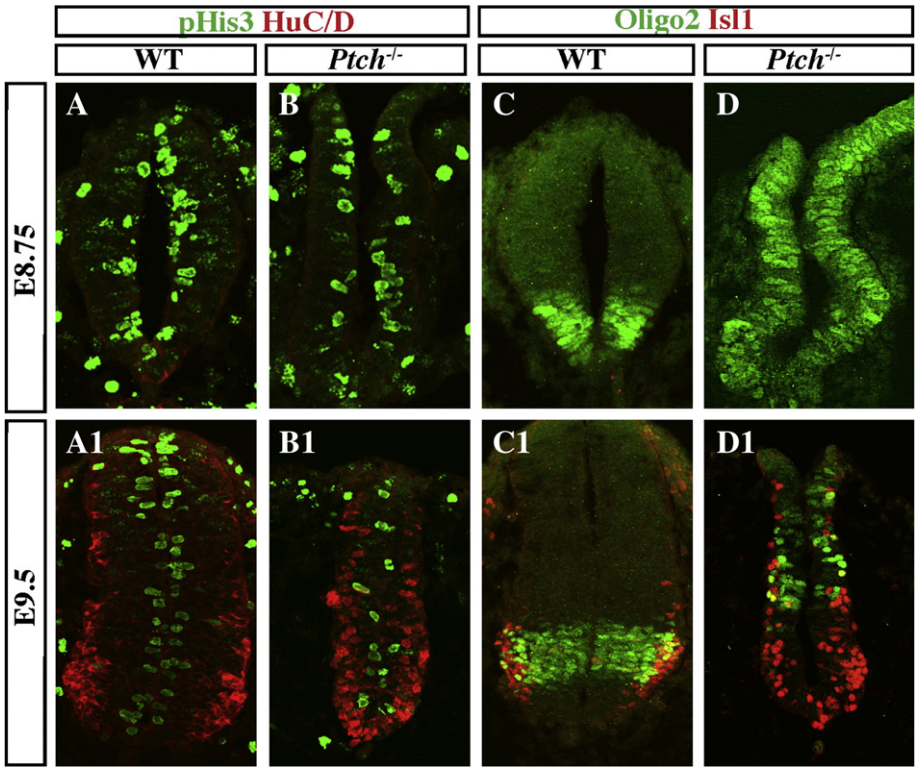

Fig. 5. Depletion of neuronal progenitors and premature differentiation in *Ptch1*$^{-/-}$ mutant spinal cord. (A, A1, B, B1, C, C1, D, D1) Distribution of pHis3, HuC/D, Olig2 and Isl1 proteins in wildtype (A, A1, C, C1) and *Ptch*$^{-/-}$ (B, B1, D, D1) embryos at E8.75 (A–D) and E9.5 (A1–D1) in the brachial region. Note reduced mitotic cells with HuC/D expression encompassing a large portion of the spinal cord in *Ptch*$^{-/-}$ mutants. Similarly, motor neurons are generated at the expense of progenitor cells in *Ptch*$^{-/-}$ mutants.

motor neurons (Scardigli et al., 2001). Furthermore, *in ovo* electroporation study in chick has shown that Olig2 is capable of inducing *Ngn2* expression (Mizuguchi et al., 2001; Novitch et al., 2001), which is necessary for progenitor cell cycle exit (Bertrand et al., 2002). We therefore examined whether *Ngn2* expression was also delayed that could account for the *Shh*$^{-/-}$;*Gli3*$^{-/-}$ phenotype. *Ngn2* expression normally initiates at E9.5 encompassing the Olig2+ domain (Figs. 4G, G1), in contrast, *Ngn2* expression in the *Shh*$^{-/-}$;*Gli3*$^{-/-}$ spinal cord did not occur until E10.5 (Figs. 4H, H1), a stage at which a large number of Olig2+ cells were generated (Fig. 4F). Thus, the delay in *Ngn2* expression is consistent with the delay in Olig2 expression and underscores the indispensable role of Shh and Gli3 activities in promoting a timely cell cycle exit and maintaining a normal pool of progenitor cells in the spinal cord.

Disproportionate depletion of neuronal progenitors in Ptch1$^{-/-}$ *embryonic spinal cord*

The inability of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants to restore the timely appearance of Olig2 expression suggests that Shh signaling is additionally required to promote Olig2 expression and motor neuron differentiation. To further support our observation, we examined *Ptch1*$^{-/-}$ mutants, in which Gli3 repressor activity is abolished but Shh pathway is constitutively activated (Goodrich et al., 1997; Huangfu and Anderson, 2005). Soon after neural tube closure at E8.75, we began to observe Olig2 expression in the ventral spinal cord of both the wildtype and *Ptch1*$^{-/-}$ mutants (Figs. 5C, D). As previously reported (Jeong and McMahon, 2005), strong Olig2 expression is detected along the dorso-ventral axis of the *Ptch1*$^{-/-}$ spinal cord (Fig. 5D). At E9.5, we found that while Olig2+ progenitors continued to expand in the ventricular zone with a few Isl1+ motor neurons in the wildtype spinal cord, in contrast, only a small number of Olig2+ progenitors were maintained in *Ptch1*$^{-/-}$ mutants (compare Figs. 5C1 and D1). In fact, the region where a large number of motor neurons were detected in *Ptch1*$^{-/-}$ mutants coincided with the absence of Olig2+ progenitors (compare Figs. 5C1 and D1). These results suggested that excessive Shh signaling can lead to premature depletion of Nkx6.1+ ventral progenitor pools. Indeed, we observed that the number of mitotic cells was significantly reduced and that most Nkx6.1+ progenitors are post-mitotic in E9.5 *Ptch1*$^{-/-}$ spinal cord (comparing Figs. 5A1 and B1, data not shown). As expected, a larger proportion of the *Ptch1*$^{-/-}$ spinal cord expressed differentiation marker HuC/D (compare Figs. 5A1 and B1). The depleted pool of neuronal progenitors in E9.5 *Ptch1*$^{-/-}$ embryos is unlikely due to a smaller or defective founder population as the number of mitotic cells was comparable to wildtype at E8.75 (Figs. 5A, B). In fact, the number and distribution of Olig2+ neuronal progenitors are significantly larger in *Ptch1*$^{-/-}$ than wildtype spinal cords (Figs. 5C, D) and

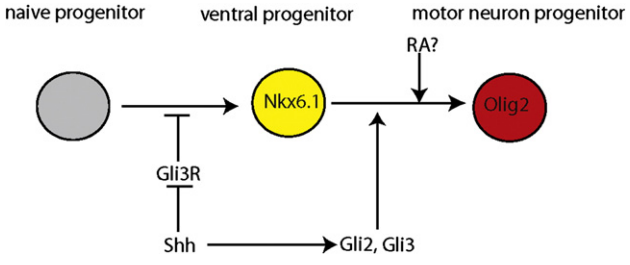

Fig. 6. Two phases of Shh signaling function in the control of motor neuron differentiation. Establishment of the Nkx6.1 ventral progenitor domain from naïve neural progenitors is largely mediated by a derepression mechanism involving Shh-mediated antagonism of Gli3R activity. Additionally, the timely appearance of Olig2 motor neuron progenitors requires additional Shh signaling. In the absence of Shh signaling, there is delayed Olig2 induction compensated later presumably by retinoic acid signaling. Hence, Shh remains essential for regulating the proper timing of ventral neural progenitor expansion and differentiation.

expectedly, the Isl1+ motor neuron population was noticeably relatively larger in *Ptch1*$^{-/-}$ compared with wildtype, given the reduced size of the *Ptch1*$^{-/-}$ spinal cord. Thus, neuronal differentiation occurred at the expense of Nkx6.1+ progenitor expansion in *Ptch1*$^{-/-}$ mutants, which also might explain the reduced size of *Ptch1*$^{-/-}$ spinal cord (Fig. 5). These results indicate that Shh pathway activation in the absence of Gli3R activity is sufficient to promote progenitor cell cycle exit, and further support the critical role of Shh signaling in promoting the timely appearance of Olig2 expression which is not observed in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutant spinal cord.

Discussion

One of the most striking phenotypes of the *Shh*$^{-/-}$;*Gli3*$^{-/-}$ spinal cord is the enlarged ventral neural epithelium, exemplified by the frequent appearance of a gyrification pattern due to enhanced cell proliferation (Fig. 1, Supplementary Fig. 1). Although a similar phenotype has also been reported in *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ double mutant spinal cord at E10.5 (Bai et al., 2004), the mechanism by which the loss of Gli activators and repressor functions leads to enhanced progenitor cell proliferation remains unclear. In this study, we analyzed cell cycle kinetics of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants and found that enhanced ventral progenitor cell proliferation in the spinal cord is not due to an increased proliferation rate per se, but due to a delay in cell cycle exit.

Nkx6.1 is the earliest known transcription factor that is expressed in the ventral neuronal progenitors (Qiu et al., 1998; Briscoe et al., 2000). Previous studies have shown that motor neurons originate from uncommitted Nkx6.1+ progenitors through the induction of lineage-restricted Olig2 motor neuron progenitors. Genetic analysis of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ or *Smo*$^{-/-}$;*Gli3*$^{-/-}$ mutant spinal cords indicated that Shh signaling is not required for the generation of Olig2 motor neuron progenitors, providing that Nkx6.1 progenitor domain is restored (Litingtung and Chiang, 2000; Persson et al., 2002; Wijgerde et al., 2002). Thus, it is generally thought that Shh promotes motor neuron generation through the establishment of the Nkx6.1 progenitor domain (Briscoe and Novitch, 2008; Novitch et al., 2003; Rowitch et al., 2002). Our analysis of *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutant spinal cords uncovered a requirement for Shh signaling in the timely appearance of Olig2 expression and ventral progenitor cell cycle exit, beyond the establishment of the Nkx6.1 progenitor domain. Although Gli3R is also lost in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants, several lines of evidence indicated that it is unlikely that Gli3R activity would contribute to the appearance of Olig2 expression. First, Gli3 expression becomes restricted to the dorsal spinal cord by the time Olig2 expression appears in the ventral spinal cord (Ruiz i Altaba, 1998; Sasaki et al., 1997). Second, ectopic expression of Gli3R has been shown to inhibit Olig2 expression and motor neuron differentiation in chick spinal cord (Meyer and Roelink, 2003; Persson et al., 2002) and third, as shown in this study, Shh pathway activation in the absence of Gli3R activity is sufficient to drive Olig2 expression at the expense of Nkx6.1 progenitors (Fig. 5). In addition, we observed no evidence that the delay in Olig2 expression in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants resulted from the ectopic activation of Bmp signaling that normally antagonizes Shh pathway activity (Supplementary Fig. 3).

Our finding that Shh signaling is required to promote Olig2 expression indicates a critical role for regulating the normal timing of ventral neural progenitor expansion and differentiation, and it is consistent with the function of Olig2 in orchestrating cell identity and cell cycle for motor neuron differentiation (Mizuguchi et al., 2001; Novitch et al., 2001). Shh signaling may exert this novel function when dorso-ventral patterning is being determined or after completion of dorso-ventral patterning as Shh ligand continues to be produced by the floor plate and notochord. We favor the latter possibility since previous neural explant studies in chick have shown that abrogating Shh function at late stage prior to the appearance of post-mitotic motor neurons blocked motor neuron differentiation, suggesting that Shh acts late into the final progenitor cell division in driving motor neuron differentiation (Ericson et al., 1996). Thus, Shh signaling activates the motor neuron differentiation program by establishing an Nkx6.1+ ventral progenitor domain primarily mediated by a derepression mechanism involving Shh-mediated inhibition of Gli3 repressor activity, and thereafter by promoting Olig2 expression requiring additional Shh signaling including Gli3 activator activity (Fig. 6). This biphasic requirement of Shh signaling for motor neuron differentiation appears to employ different gene regulatory mechanisms. This is highlighted by the observation that the generation of Nkx6.1+ ventral progenitors, but not the timely appearance of Olig2+ progenitors, is restored in *Shh*$^{-/-}$;*Gli3*$^{-/-}$ mutants. These two modes of gene regulation by Shh signaling in the spinal cord parallel the activation of Hh target genes in *Drosophila* imaginal discs by cubitus interuptus (Ci), the *Drosophila* counterpart of vertebrate *Gli* genes. In this system, activation of Dpp expression is mediated by blockade of Ci repressor (CiR) activity in response to low threshold Hh concentration (Methot and Basler, 1999), whereas the expression of Ptc requires additional ongoing Ci activator activity stimulated by an intermediate to high-level Hh concentration (Methot and Basler, 2001). Interestingly, different threshold concentrations of Shh has been shown to elicit distinct cellular states in chick neural explant studies. For example, low threshold concentration of Shh is sufficient to convert naïve neural plate cells into ventralized neural progenitors, whereas an intermediate concentration threshold is required to induce motor neuron generation from ventral progenitors (Ericson et al., 1996). Thus, it is possible that a similar concentration-sensing mechanism may explain the different gene regulatory mechanisms operating at different stages of motor neuron generation.

Methods

Animals

The generation and identification of *Shh* and *Gli3* mutant mice were performed as described (Litingtung and Chiang, 2000). *Ptch1*$^{-/-}$ (Goodrich et al., 1996) mutants and *RARE-LacZ* (Rossant et al., 1991) mice were obtained from the Jackson Laboratories.

BrdU incorporation assay, TUNEL and Immunohistochemistry

BrdU assay was performed as described (Litingtung and Chiang, 2000). Immunofluorescence was performed on 15 μm cryosections as described (Litingtung and Chiang, 2000). The following primary antibodies were used: monoclonal anti-Isl1/2 (Tsuchida et al., 1994), mouse anti-HuC/D (Molecular Probes, Eugene, Oregon), rabbit anti-phosphohistone 3 (Upstate Biotechnology, Lake Placid, New York), rabbit anti-Olig2 (Takebayashi et al., 2000), rabbit anti-Nkx6.1 (Madsen et al., 1997), rabbit anti-Ki67 (Novocastra Lab, Newcastle, United Kingdom). Secondary antibodies were anti-mouse, anti-rabbit, or anti-sheep Alexa 488 or 568 fluorescent conjugates (Molecular Probes, Eugene, Oregon). Stained sections were visualized using Olympus BX60 fluorescence microscope (Olympus, Melville, NY), and images were captured using the MagnaFire CCD camera (Olympus, Melville, NY). Some fluorescent images were obtained using a LSM510 META laser scanning confocal microscope (Carl Zeiss, Germany). Apoptotic cell death was determined by using the Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labeling (TUNEL) analysis.

Transcript detection

In situ hybridizations were performed as described (Litingtung et al., 1998). The *Notch1* (gift of Dr. T. Gridley) and *Msx1* (gift of Dr. R.

Mason) cDNAs were used as templates for synthesizing digoxygenin-labeled riboprobes.

Statistical analyses and quantifications

To assess differences among groups, statistical analyses were performed using a one-way analysis of variance (ANOVA) with Microsoft Excel (Microsoft Corporation) and significance accepted at $p<0.05$. Results are presented as mean +/− SEM.

For quantifying the number of pHis3+ cells, total pHis3 cell numbers were counted and normalized to the total spinal cord size in WT, *Shh*$^{-/-}$, or *Shh*$^{-/-}$;*Gli3*$^{-/-}$. At least five sections were randomly chosen for cell counting for each genotype, and the statistic result shown in Fig. 1 represents the average results from three independent experiments. For quantifying progenitor cell proliferation index, E10.5 WT and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos were injected with BrdU for 1 h and then processed to retrieve paraffin sections at the hindlimb region. The percentage of total number of BrdU+ cells/total number of Ki67+ cells was considered as the progenitor cell proliferation index. For quantifying progenitor cells that have exited the cell cycle from E10.0 to E11.0, WT and *Shh*$^{-/-}$;*Gli3*$^{-/-}$ embryos were injected with BrdU and harvested 24 h later. Paraffin sections at the hindlimb region were collected. The percentage of total number of BrdU+;Ki67− cells/total number of BrdU+ cells was considered cells that have exited cell cycle from E10.0 to E11.0. At least five sections were randomly chosen for cell counting for each genotype, and statistical result shown in Figs. 2E and F represents the average results from three independent experiments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.05.539.

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