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 Ptc1−/− 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.

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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 Ptc1−/− 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...
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

![Figure 1](image_url)
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 what extent neural differentiation is affected. At E9.5, we detected 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). 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.

The defective cell cycle exit, accompanied by a consequential expansion of neural progenitor pools, suggests that neuronal differentiation is compromised in Shh−/−;Gli3−/− mutants. 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 dependent mechanism (Novitch et al., 2003). The delay in neuronal differentiation occurs similarly in 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), few Isl1+ motor neurons were present in Shh−/−;Gli3−/−.

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).
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
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

Disproportionate 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.

Fig. 5. 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 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+ progenitors 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 neuroepithelial 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 (Ruiu 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 interruptus (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 Ptch 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 naive 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-IsI1/2 (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-GK67 (Novoceastra 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 MxS1 (gift of Dr. R.


