Transduction of graded Hedgehog signaling by a combination of Gli2 and Gli3 activator functions in the developing spinal cord

Qiubo Lei, Alice K. Zelman, Ed Kuang, Shike Li and Michael P. Matise*

Department of Neuroscience and Cell Biology, UMDNJ/Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA

*Author for correspondence (e-mail: matisemp@umdnj.edu)

Accepted 22 April 2004

Development 131, 3593-3604
Published by The Company of Biologists 2004
doi:10.1242/dev.01230

Summary

The three vertebrate Gli proteins play a central role in mediating Hedgehog (Hh)-dependent cell fate specification in the developing spinal cord; however, their individual contributions to this process have not been fully characterized. In this paper, we have addressed this issue by examining patterning in the spinal cord of Gli2;Gli3 double mutant embryos, and in chick embryos transfected with dominant activator forms of Gli2 and Gli3. In double homozygotes, Gli1 is also not expressed; thus, all Gli protein activities are absent in these mice. We show that Gli3 contributes activator functions to ventral neuronal patterning, and plays a redundant role with Gli2 in the generation of V3 interneurons. We also show that motoneurons and three classes of ventral neurons are generated in the ventral spinal cord in double mutants, but develop as intermingled rather than discrete populations. Finally, we provide evidence that Gli2 and Gli3 activators control ventral neuronal patterning by regulating progenitor segregation. Thus, multiple ventral neuronal types can develop in the absence of Gli function, but require balanced Gli protein activities for their correct patterning and differentiation.

Supplementary data available online

Key words: Sonic hedgehog, Gli genes, Patterning, Cell fate, Spinal cord, Sorting, Mouse, Chick

Introduction

In mammals, the secreted signaling protein Sonic hedgehog (Shh) is both necessary and sufficient to specify ventral cell fates in the developing central nervous system (CNS). The primary mediators of Shh signaling are the Gli zinc-finger-containing transcription factors. Three Gli genes have been identified in vertebrates – Gli1, Gli2 and Gli3. These genes are homologous to the Drosophila Cubitus interruptus (Ci) gene, which is thought to mediate most, if not all, of the known responses to Hedgehog (Hh) signaling in this organism (Methot and Basler, 2001). It is not clear whether an obligatory role for Gli/Ci genes in mediating Hh signaling has been conserved from flies to vertebrates.

The Ci gene encodes a zinc-finger-containing transcription factor with two distinct activities, repression and activation (Aza-Blanc and Kornberg, 1999). Hh signaling controls these dual activities by promoting the formation of a full-length Ci activator protein at the expense of the shorter repressor form that is constitutively generated by partial proteolysis in the absence of Hh (Aza-Blanc et al., 1997). The differential sensitivity of Hh target genes to the two forms of Ci indicates that activation of Hh target genes involves both disinhibition and direct activation (Mueller and Basler, 2000). Similarly, evidence suggests that vertebrate Gli2 and Gli3, but not Gli1, proteins can be cleaved to generate repressor forms, analogous to Ci (Dai et al., 1999; Wang et al., 2000; Aza-Blanc et al., 2000). However, the dual transcriptional activities embodied in Ci appear to be unequally distributed among the three vertebrate Gli proteins. Both gain- and loss-of-function studies indicate that Gli1 and Gli2 are the primary Gli activators that function downstream of Shh signaling, but only Gli2 is required in developing mice (Ruiz i Altaba, 1999; Ding et al., 1998; Matise et al., 1998; Bai et al., 2002). Gli3, however, appears to function as the primary Gli repressor (Wang et al., 2000), although biochemical and genetic evidence suggests that Gli3 can also function as an activator under certain circumstances (Dai et al., 1999; Shin et al., 1999; Motoyama et al., 2003; Bai et al., 2004). A repressor role for Gli2 has not been demonstrated.

All three Gli genes are expressed in spinal cord progenitor cells during early neurogenesis when cell fate specification is occurring (Sasaki et al., 1997; Platt et al., 1997; Lee et al., 1997). However, mouse mutant studies reveal only a limited role for each individual Gli gene in dorsoventral (DV) patterning in the spinal cord. Targeted Gli2 mutant mice lack floor plate (FP) and most V3 cells that develop near the ventral midline, but other ventral cell classes are present in their normal DV positions, except motoneurons (MsNs), which extend across the midline (Ding et al., 1998; Matise et al., 1998). Gli1 mutants have no discernable spinal cord phenotype, even on a Gli2 mutant background (Matise et al., 1998; Park et al., 2000), while loss of Gli3 has only a subtle effect on the position of interneurons that develop in the intermediate region of the spinal cord (Persson et al., 2002).
By contrast, Shh mutants have a severe phenotype including cyclopia and an absence of most ventral cell types along the entire neuraxis (Chiang et al., 1996). In Shh;Gli3 and Smo;Gli3 double mutants, many ventral cells are rescued except FP and V3 interneurons (Litingtung and Chiang, 2000; Wijgerde et al., 2002), indicating that an important function of Hh signaling is to oppose the repressive activities of Gli3 in the ventral spinal cord. In addition, these results, and others (Krishnan et al., 1997), suggest the possibility that a Hh/Gli-independent pathway could mediate some aspects of Shh signaling in the ventral spinal cord. However, as Gli2 is still expressed in Shh;Gli3 and Smo;Gli3 mutants, a Hh-independent role for this factor in generating some ventral cell types cannot be ruled out.

Together, these studies suggest a model whereby graded Shh signaling controls the balance between Gli activator and repressor activities in progenitor cells along the DV axis, and predicts that the summation of Gli activities at specific Shh concentrations (and DV levels) will control distinct cell fates, ultimately by regulating expression of progenitor fate determinants (Stone and Rosenthal, 2000; Jacob and Briscoe, 2003). However, a number of issues remain unresolved. First, it is unclear which specific cell fates and progenitor determinants are controlled by the different Gli activities. For example, while it has recently been demonstrated that many ventral class II determinants are sensitive to Gli3 repression (Persson et al., 2002; Meyer and Roelink, 2003), whether and how specific Gli activator activities are involved in controlling the expression of these factors in spinal cord DV patterning has not been resolved. Second, it is unclear whether Gli3 plays a positive role in mediating Hh signaling. Indeed, it has recently been shown that loss of Gli2 or Gli3, but not Gli1, can reverse aspects of the Ptc1−/− mutant phenotype in which the Hh pathway is constitutively activated (Bai et al., 2002; Motoyama et al., 2003), suggesting that they both possess the ability to transduce Hh signaling as activators in vivo. Finally, it is uncertain whether the Hh-dependent establishment of neuronal patterning and ventral cell fates requires all Gli protein functions. Resolving this issue is the rate-limiting step in determining the contribution of Hh-independent pathways to ventral cell fate specification and patterning.

In this study, we have addressed these issues using two approaches. First, we generated mouse embryos lacking both Gli2 and Gli3 genes and analyzed spinal cord development during early neurogenesis. As Gli1 is not expressed in double homozygotes, all Gli protein activities are absent in these mice, providing an opportunity to address the requirement for all three vertebrate Gli factors and their combined activities in mediating Hh signaling in the developing spinal cord. To complement these studies, we employed gain-of-function experiments in chick embryos to study the individual transcriptional activities of Gli2 and Gli3. Our results show that motoneurons and three ventral interneuron subclasses are generated in Gli2;Gli3 mutants, except floorplate and V3 cells, but strikingly these cells develop as intermingled populations. Furthermore, we show that Gli3 contributes activator functions to ventral neuronal patterning, playing a redundant role with Gli2. The similarities of these results to previously published studies (Litingtung and Chiang, 2000; Wijgerde et al., 2002), indicate that Gli proteins mediate all of the patterning functions of Hh in the developing spinal cord. We also show that activation of the Shh pathway in chick dorsal neural tube cells using Gli2 and Gli3 activator constructs, as well as expression of Shh, elicits cell clustering. Together, these results indicate both distinct and partially overlapping roles for Gli2 and Gli3 activator in patterning and cell fate specification in the ventral spinal cord downstream of Shh signaling, and suggest an important role for these activities in establishing or maintaining the segregation of ventral progenitors in discrete pools.

Materials and methods

Generation of Gli2−/−;Gli3xt/xt double mutant mouse embryos

Gli2−/−;Gli3xta double-mutant embryos were generated by crossing mice heterozygous for a targeted mutation in Gli2 (Mo et al., 1997) with the extra-toes (Xt) mutant mice containing an intragenic deletion in the Gli3 gene (Hui and Joyner, 1993). All lines were maintained on a Swiss Webster background. Double-homozygous embryos, identified using PCR as previously described (Mo et al., 1997), were collected for analysis at embryonic day (E) 9.5 (n=7), E10.5 (n=11) and E11.5 (n=2).

In ovo electroporation in chicken embryos

cDNAs encoding amino acids 280-1544 of mouse Gli2 and 345-1596 of mouse Gli3 were cloned into the bi-cistronic expression vector pCIG (Sasaki et al., 1999; Megason and McMahon, 2002). An ATG codon was added in frame upstream of both constructs, and was confirmed by sequencing. For co-electroporation of these constructs with ptcΔloop2, the GFP was removed and ptcΔloop2 cDNA (Briscoe et al., 2001) was subcloned into pCIG. Five 25 V pulses were delivered for 50 mseconds at 1 second intervals at HH stages 12-14. Embryos were sacrificed 24 or 48 hours later and processed for analysis. At least 10 embryos were analyzed for each set of experiments. Noon on the day when vaginal plugs were detected was designated E0.5.

Immunohistochemistry, RNA in-situ hybridization and RT-PCR

Embryo collection, antibody staining and RNA in situ hybridization was performed as described (Matise et al., 1998). Antibodies used were mouse anti-BrdU (Sigma), cyclin D1 (Upstate Biotechnology), Shh, Nkx2.2, Hb9/Mnr2, Isl1, Pax6, Pax7, Foxa2 (DSHB), Gata3, Jag1 (Santa Cruz), Mash1 and Ngn1 (D. Anderson); rabbit anti-phosphorylated caspase 3 (Idun Pharmaceuticals), Dbx1, Dbx2, Nkx6.1 (T. Jessell), Chx10 (K. Sharma) and Olig2 (H. Takebayashi); and guinea-pig anti-Nkx2.9, Nkx6.2 (J. Ericson) and Evx1 (T. Jessell). Fluorochrome-conjugated secondary antibodies were obtained from Jackson ImmunoResearch or Molecular Probes. RNA in situ probes were mouse Pch1 (M. Scott), chicken Pch1 and Pch2 (C. Tabin). RT-PCR was performed as described using primers specific for mouse Gli1 (Park et al., 2000).

Quantification of neurons and precursors was performed by averaging counts from wild-type and double-homozygous embryos. At least four sections for each set of markers were counted in three to eight different embryos at similar anterior-posterior levels.

Results

Expression of Shh target genes in Gli2−/−;Gli3xt/xt mutant embryos

During early neurogenesis, the expression of Gli2 and Gli3 overlaps extensively in the spinal cord ventricular zone (VZ), although by E10.5, after neural tube closure, Gli3 becomes repressed in the ventral most region with only weak levels persisting in motoneuron progenitors (Sasaki et al., 1997; Platt et al., 1997; Lee et al., 1997) (Fig. 1A,B). To determine the
Fig. 1. Defects in Shh signaling in Gli2−/−;Gli3vt/vt mutant embryos.
(A,B) Widespread and overlapping Gli2 and Gli3 expression in the ventral spinal cord at E10.5. Gli2 expression extends further ventral than Gli3 at this stage (yellow bars mark similar DV position).
(C–J) Shh protein expression in the notochord and floorplate (FP). (C,D) Wild-type embryos express Shh in the notochord and FP.
(E,F) In Gli2−/− mutants, FP expression of Shh is selectively lost (arrowhead), while notochord expression is retained. (G,H) In Gli3vt/vt mutants, Shh expression in the notochord and FP is similar to that in wild type. (I,J) Gli2−/−;Gli3vt/vt mutants do not show Shh expression in FP (arrowhead) but notochord expression is similar to Gli2−/− mutants.

We first examined Gli1 expression as its transcription is under the direct control of Shh (Bai et al., 2002). In Gli2−/− mutants, Gli1 expression is greatly reduced (Ding et al., 1998; Matise et al., 1998), indicating that its expression in the spinal cord depends primarily on Gli2. Notably, no Gli1 expression was seen in Gli2−/−;Gli3vt/vt mutants using both RNA in situ hybridization and RT-PCR (data not shown). Thus, both Gli3 and Gli2 are necessary for Shh-dependent activation of Gli1.

In wild-type embryos, Shh expression is first seen in notochord precursors and then later in prospective floorplate (FP) cells in the midline of the neural plate. At E10.5, after neural tube closure, Shh expression is seen in both the FP and notochord (Fig. 1C,D). In Gli2−/− mutant embryos that lack a FP, Shh is detected only in the notochord (Fig. 1E,F) (Ding et al., 1998; Matise et al., 1998), while Gli3vt/vt mutants are indistinguishable from wild type (Fig. 1G,H). In Gli2−/−;Gli3vt/vt mutants, Shh was analyzed in spinal cords of Gli2−/−;Gli3vt/vt mutants using both RNA in situ hybridization and RT-PCR (data not shown). Thus, both Gli3 and Gli2 are necessary for Shh-dependent activation of Gli1.

Expression of Gli2 and Gli3 activators mediates Hh patterning
3595

In wild-type embryos, Shh expression is first seen in notochord precursors and then later in prospective floorplate (FP) cells in the midline of the neural plate. At E10.5, after neural tube closure, Shh expression is seen in both the FP and notochord (Fig. 1C,D). In Gli2−/− mutant embryos that lack a FP, Shh is detected only in the notochord (Fig. 1E,F) (Ding et al., 1998; Matise et al., 1998), while Gli3vt/vt mutants are indistinguishable from wild type (Fig. 1G,H). In Gli2−/−;Gli3vt/vt mutants, Shh was detected in the notochord at all axial levels examined, but as in Gli2 mutants the FP did not form and no Shh expression was seen in the ventral midline (Fig. 1J).

Pitchl expression is a reliable indicator of Shh signaling as its transcription is derepressed by Shh (Goodrich and Scott, 1998). In wild-type embryos, Pitchl expression is strong in VZ cells near the ventral midline and weaker in the ventral VZ, but is undetectable in the dorsal VZ (Fig. 1K). In Gli2−/− mutants, weak Pitchl expression was seen in the ventral VZ, but the strong expression adjacent to the FP was not detected, probably owing to the absence of most V3 cells in these embryos (Fig. 1L). By contrast, Gli3vt/vt mutants showed strong ventral expression adjacent to the FP as in wild type, but weak ectopic expression was also detected in the dorsal VZ (Fig. 1M), suggesting that Gli3 normally represses Pitchl transcription here. Interestingly, in Gli2−/−;Gli3vt/vt double mutants, low levels of uniform Pitchl expression were detected in a pattern that is essentially a composite of the Gli2 and Gli3 single mutant patterns (Fig. 1N). By contrast, Pitch2, which is expressed only at low levels in the neural tube (Motoyama et al., 1998), was not affected in double mutants (data not shown). These results show that Pitchl can be transcribed at basal levels in the absence of Gli activity but requires both Gli2 and Gli3 to establish its normal pattern of expression in the spinal cord.

We noted overgrowth of the neuroepithelium in the thoracic regions of some Gli2−/−;Gli3vt/vt mutants, suggesting a proliferative defect (n=3/11 embryos at E10.5; see Fig. S1 at http://dev.biologists.org/supplemental). This was accompanied by an increase in cell cycle markers in affected areas, but was not confined specifically to dorsal or ventral regions (see Fig. S1 at http://dev.biologists.org/supplemental). The majority of Gli2−/−;Gli3vt/vt mutants did not exhibit this phenotype, and cell proliferation was similar to wild type (see Fig. S1 at http://dev.biologists.org/supplemental). This finding indicates that loss of both Gli2 and Gli3 lead to sporadic defects in cell proliferation independent of patterning defects, which were confined to the ventral spinal cord.

Absence of V3 interneurons in Gli2−/−;Gli3vt/vt mutant embryos

V3 interneurons that express Sim1 develop adjacent to the FP and derive from progenitors expressing Nkx2.2 and Nkx2.9
proteins (Fig. 2A,B) (Ericson et al., 1997; Briscoe et al., 1999). These cells are greatly reduced in number in the thoracic and lumbar spinal cord of Gli2−/− mutant embryos (Fig. 2C,D) (Ding et al., 1998; Matise et al., 1998), while Gli3−/− mutants are indistinguishable from wild type (data not shown). In Gli2+/−;Gli3−/− double mutants, Nkx2.2, Nkx2.9 and Sim1 were not detected at any stage in either region (Fig. 2E,F). Thus, in the absence of both Gli2 and Gli3, V3 interneurons are not specified.

Alterations in motoneuron and ventral interneuron patterning and number in Gli2−/−;Gli3−/−;Gli3xt/xt embryos

Our previous analysis of Gli2−/− mutant embryos showed that MNs are present in similar numbers as in wild type, but extend across the ventral midline (Ding et al., 1998; Matise et al., 1998). As both Gli2 and Gli3 are expressed in MN progenitors in the mouse spinal cord (Fig. 1A,B), it is possible that they are both required for the development of this cell type. Indeed, a recent study reported absence of MNs in Gli2−/−;Gli3−/− mutant embryos at E9.5 (Motoyama et al., 2003). However, our analysis of later stage (E10.0-11.5) double mutant embryos revealed that MNs are present in both thoracic and lumbar regions, but in significantly reduced numbers, compared with wild type (Fig. 3A-C). This reduction was not due to increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants is not due solely to depletion of pMN cells. In addition, this decrease was not correlated with upregulation of cyclin D1 or downregulation of Ngn1 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

Abnormal MN differentiation and intermingling of MN, V1 and V2 progenitors in Gli2−/−;Gli3−/−;Gli3xt/xt mutants

To study the reduction in MN numbers in double mutant embryos, we examined the expression of MN progenitor (pMN) factors. In Gli2−/−;Gli3−/− mutants, Olig2+ pMN cells were shifted ventrally in both thoracic and lumbar regions (Fig. 3L-P). Interestingly, in thoracic regions a higher numbers of pMN cells were seen in Gli2−/−;Gli3−/− mutants compared with wild type (~20% increase), while in lumbar regions there were fewer (~15% decrease) (Fig. 3N; n=21 sections scored in three embryos; data not shown). Thus, the reduction in postmitotic MNs generated in Gli2−/−;Gli3−/− mutants is not due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

We next examined Pax6, which shows weak expression in pMN cells and strong levels in p2, p1 and p0 progenitor cells (Fig. 3Q) (Ericson et al., 1997). In Gli2 mutants, Pax6 is repressed in the ventral pMN domain, a finding that could be explained by reduced but persistent Nkx2.2/Nkx2.9 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants is not due solely to depletion of pMN cells. In addition, this decrease was not correlated with upregulation of cyclin D1 or downregulation of Ngn1 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

Abnormal MN differentiation and intermingling of MN, V1 and V2 progenitors in Gli2−/−;Gli3−/−;Gli3xt/xt mutants

To study the reduction in MN numbers in double mutant embryos, we examined the expression of MN progenitor (pMN) factors. In Gli2−/−;Gli3−/− mutants, Olig2+ pMN cells were shifted ventrally in both thoracic and lumbar regions (Fig. 3L-P). Interestingly, in thoracic regions a higher numbers of pMN cells were seen in Gli2−/−;Gli3−/− mutants compared with wild type (~20% increase), while in lumbar regions there were fewer (~15% decrease) (Fig. 3N; n=21 sections scored in three embryos; data not shown). Thus, the reduction in postmitotic MNs generated in Gli2−/−;Gli3−/− mutants is not due solely to depletion of pMN cells. In addition, this decrease was not correlated with upregulation of cyclin D1 or downregulation of Ngn1 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

We next examined Pax6, which shows weak expression in pMN cells and strong levels in p2, p1 and p0 progenitor cells (Fig. 3Q) (Ericson et al., 1997). In Gli2 mutants, Pax6 is repressed in the ventral pMN domain, a finding that could be explained by reduced but persistent Nkx2.2/Nkx2.9 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants is not due solely to depletion of pMN cells. In addition, this decrease was not correlated with upregulation of cyclin D1 or downregulation of Ngn1 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

We next examined Pax6, which shows weak expression in pMN cells and strong levels in p2, p1 and p0 progenitor cells (Fig. 3Q) (Ericson et al., 1997). In Gli2 mutants, Pax6 is repressed in the ventral pMN domain, a finding that could be explained by reduced but persistent Nkx2.2/Nkx2.9 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

We next examined Pax6, which shows weak expression in pMN cells and strong levels in p2, p1 and p0 progenitor cells (Fig. 3Q) (Ericson et al., 1997). In Gli2 mutants, Pax6 is repressed in the ventral pMN domain, a finding that could be explained by reduced but persistent Nkx2.2/Nkx2.9 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

We next examined Pax6, which shows weak expression in pMN cells and strong levels in p2, p1 and p0 progenitor cells (Fig. 3Q) (Ericson et al., 1997). In Gli2 mutants, Pax6 is repressed in the ventral pMN domain, a finding that could be explained by reduced but persistent Nkx2.2/Nkx2.9 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

We next examined Pax6, which shows weak expression in pMN cells and strong levels in p2, p1 and p0 progenitor cells (Fig. 3Q) (Ericson et al., 1997). In Gli2 mutants, Pax6 is repressed in the ventral pMN domain, a finding that could be explained by reduced but persistent Nkx2.2/Nkx2.9 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.

We next examined Pax6, which shows weak expression in pMN cells and strong levels in p2, p1 and p0 progenitor cells (Fig. 3Q) (Ericson et al., 1997). In Gli2 mutants, Pax6 is repressed in the ventral pMN domain, a finding that could be explained by reduced but persistent Nkx2.2/Nkx2.9 expression in pMN cells, nor increased MN apoptosis in Gli2−/−;Gli3−/− mutants (see Fig. S1 at http://dev.biologists.org/supplemental). These findings suggest that the large reduction in the number of MNs in Gli2−/−;Gli3−/− mutants in both thoracic and lumbar regions could be due to a delay or decrease in the progression of motoneuron progenitors to post-mitotic neurons.
p0 cells (Pierani et al., 1999), was confined to its normal intermediate domain, consistent with the localization of V0 interneurons to this region in Gli2^{−/−};Gli3^{xt/xt} mutants (data not shown).

As Dbx2 is expressed in multiple progenitor domains, we examined Jag1 expression to further define the identity of ectopic Dbx2 cells in the ventral spinal cord of Gli2^{−/−};Gli3^{xt/xt} mutants. Jag1 is expressed specifically in the p1 and pd6 domains.
domains but is excluded from the p0 domain (Fig. 4E) (Matise and Joyner, 1997). In Gli2+/−;Gli3xt/xt mutants, only the ventral domain of Jag1 that marks the p1 domain was expanded ventrally (Fig. 4F). No cells were seen that co-expressed these two factors was not seen. (E,F) In wild-type embryos, Jag1 is expressed between Mash1 domains, but is excluded from the p0 domain. In Gli2+/−;Gli3xt/xt mutants, only the ventral Jag1 expression domain in p1 progenitors has expanded ventrally, while the more dorsal domain, as well as the intervening Jag1-negative domain, were similar to wild type. Co-expression of Jag1 and Mash1 was not detected.

Olig2 expression in pMN cells is flanked by Mash1 expression in p3 and p2 cells (Fig. 5A). In Gli2+/− mutants, the ventral domain of Mash1 is absent, but the pMN and p2 domains segregate normally (Fig. 5B). By contrast, in Gli2+/−;Gli3xt/xt double mutants, Mash1+ (p2) and Olig2+ (pMN) cells were intermingled throughout the ventral region, but no cells were found that co-express these factors (Fig. 5C). Thus, pMN and p2 progenitors are intermingled but distinct in Gli2+/−;Gli3xt/xt mutants.

In wild-type embryos at E10.5, Nkx6.2 expression in the VZ marks p1 and a small number of p0 progenitors, as well as a population of postmitotic MNs (Vallstedt et al., 2001) (Fig. 5D). In Gli2+/− mutants, expression of Nkx6.2 in the p1 and p0 domains was not altered, but expression persisted in postmitotic MNs, which extended across the midline (Fig. 5E).
In Gli2\(^{-/-}\);Gli3\(^{xt/xt}\) mutants, VZ cells expressing Nkx6.2 extended into the ventral spinal cord, but no cells co-expressing Olig2 and Nkx6.2 were detected (Fig. 5F), showing that p1 and pMN progenitors are intermingled but separate in these embryos.

These data show that in the absence of Gli activities, pMN, p2 and p1 progenitors are generated in the ventral spinal cord and maintain their distinct identities, despite developing as intermingled populations (summarized in Fig. 5G-I).

**Dominant Gli2 and Gli3 activator proteins induce ventral and repress dorsal cell fates**

Our analysis of Gli2\(^{-/-}\);Gli3\(^{xt/xt}\) mutants shows that Gli2 and Gli3 play redundant roles in the ventral spinal cord. However, loss-of-function studies do not permit us to address which specific Gli activity – activation or repression – is involved, because all are absent in double mutants.

To investigate this, we assayed the expression of ventral HD and bHLH fate determinants in the spinal cord of chick embryos after transfection, shortly after neural tube closure, of dominant constitutive Gli2 and Gli3 activator constructs. To generate these, we deleted the region encoding the N-terminal repressor domains of Gli2 and Gli3 (Sasaki et al., 1999; Pearse et al., 1999; Murone et al., 2000; Dunaeva et al., 2003) to generate Gli2\(^{\Delta N}\)-term and Gli3\(^{\Delta N}\)-term (see schematic, Fig. 6), and cloned these constructs into a bi-cistronic expression vector (Megason and McMahon, 2002) that also encoded GFP.

Transfection of either Gli2\(^{\Delta N}\)-term or Gli3\(^{\Delta N}\)-term shortly after neural tube closure did not induce ectopic Shh expression (Fig. 6A,A',B,B'). This result is consistent with transplant studies that have defined a critical period for the induction of FP by notochord or ectopic Shh that ends prior to neural tube closure (Dodd et al., 1998).

We found that Gli2\(^{\Delta N}\)-term transfections induced cell-autonomous expression of multiple ventral markers, including Foxa2 (FP,V3), Nkx2.2 (V3), and (weakly) Nkx 6.1 (p3, pMN, p2) (Fig. 6C,E,G). By contrast, Gli3\(^{\Delta N}\)-term only induced Nkx 2.2 in these experiments (Fig. 6F). No effect was seen on Ngn2 or Nkx6.2 expression for either construct (data not shown). Both Gli2\(^{\Delta N}\)-term and Gli3\(^{\Delta N}\)-term were equally capable of repressing Pax6 and Pax 7 expression (Fig. 6I-L'). These results show that dominant Gli2 and Gli3 activator constructs...
are capable of inducing expression of a limited set of ventral Class II fate determinants, with Gli2ΔN-term showing a broader capability compared with Gli3ΔN-term, whose activity appears to be confined primarily to the induction of Nkx2.2.

Interestingly, ~1/3 of Gli2ΔN-term transfected embryos also exhibited an expansion of the neuroepithelium on the transfected side (n=7/20 embryos), which resulted in a unilateral increase in the expression of cell cycle markers (Fig. 6A,C,E; data not shown). Gli3ΔN-term transfections, however, did not alter the size or shape of the neuroepithelium.

We next assayed whether Gli2ΔN-term and Gli3ΔN-term activator constructs could influence the transcription of Ptc1 and Ptc2. Mis-expression of both factors upregulated strong Ptc2 expression (Fig. 6N,P), while Gli2ΔN-term was more efficient than Gli3ΔN-term in inducing Ptc1 (Fig. 6M,O). These results suggest that Ptc1 and Ptc2 are differentially responsive to Gli2 and Gli3 activators.

To rule out the possibility that Gli2ΔN-term or Gli3ΔN-term induced the expression of Shh at low levels that escaped detection, we co-transfected these constructs with ptc-Aloop2, a dominant inhibitor of Shh signaling (Briscoe et al., 2001). On its own, ptc-Aloop2 can block Nkx2.2 and activate Pax7 expression (see Fig. S2 at http://dev.biologists.org/supplemental) (Briscoe et al., 2001). Co-transfection of Gli2ΔN-term or Gli3ΔN-term with ptc-Aloop2 overcame these effects and instead activated Nkx2.2 and repressed Pax7 expression, and for all markers examined co-transfection results were similar to Gli2ΔN-term or Gli3ΔN-term alone (see Fig. S2 at http://dev.biologists.org/supplemental; data not shown). These results show that alterations in gene expression after transfections with Gli2ΔN-term and Gli3ΔN-term activator constructs cannot be explained by the secondary induction of Shh, but instead are the result of these factors acting in a ligand-independent cell-autonomous manner to induce target gene expression.

Activation of the Shh pathway in dorsal cells elicits cell clustering

The non-uniform expression of induced Ptc1 and Ptc2 by Gli2 and Gli3 activators, and the expansion of the neuroepithelium elicited by Gli2 activator transfections indicate that these factors might possess activities that are not directly related to control of progenitor fate determinant gene expression. To study this, we examined the behavior of transfected cells within the Pax7 domain in the dorsal spinal cord. In these experiments, we were guided by similar studies in Drosophila wing imaginal discs which showed that manipulation of the Hh pathway in anterior and posterior compartment cells could modify cell affinities between Hh transducing and non-transducing cells (Dahmann and Basler, 2000). In addition, recent experiments in mice provided evidence for a similar function of the Hh pathway in spinal cord patterning. In this study, Hh signaling was blocked in ventral cells by generating chimeras with Smo<sup>−/−</sup> ES cells, and it was found that these cells ‘clumped’ together in the ventral spinal cord (Wijgerde et al., 2002). We reasoned that if the Shh pathway functioned in part to segregate ventral from dorsal cells, then activating the pathway in dorsal cells should also elicit similar clustering behaviors.

We first transfected Shh alone into the spinal cord and monitored Nkx2.2 and Pax7 expression to identify cells in which the pathway has or has not been activated, respectively. Not unexpectedly, ectopic Shh induced widespread, almost uniform expression of Nkx2.2, and repressed Pax7 in both cell-autonomous and non-autonomous manners (Fig. 7A,B,D). In this experiment, Nkx2.2-expressing cells did not segregate from one another or from dorsal cells. As a control for these experiments, we transfected GFP alone, which did not elicit cell clustering or alterations in either Nkx2.2 or Pax7 expression (Fig. 7C).

We next co-transfected Shh with ptc-Aloop2 to block autocrine Shh signaling in transfected cells but permit non-cell autonomous induction of Nkx2.2 expression (Fig. 7H). Efficient cell-autonomous blockage of autocrine Shh signaling was indicated by expression of Pax7 (indicating pathway is off) in GFP+ transfected cells (Fig. 7G). In these experiments, induced, untransfected cells expressing Nkx2.2 frequently segregated from transfected (but non-transducing) cells in the dorsal spinal cord (Fig. 7E,F; n=23 sections, 5/7 embryos). Significantly, clustering was not evident in ventral cells induced to express Nkx2.2 (7E,F; n=8).

These results show that activation of the Shh pathway in dorsal cells can elicit cell clustering. To determine whether Gli activators mediated this effect, we transfected Gli2ΔN-term or Gli3ΔN-term constructs and examined transfected embryos that exhibited broad transfections extending into the dorsal spinal cord. In these experiments, we found that in a majority of sections examined (n=13/17 sections, 10 embryos), transfected cells that did not show Pax7 expression were found in clusters, surrounded by non-transfected Pax7+ cells (Fig. 7L), although ventrally located transfected cells were distributed more uniformly (n=14 sections; data not shown; Fig. 7M). Similar results were obtained at the lowest Gli2ΔN-term concentration that induced Nkx2.2 expression (n=3; data not shown). Thus, cell clustering elicited by activation of the Shh pathway in dorsal neural tube cells appears to be mediated by Gli2 and/or Gli3 activator activities (Fig. 7M,N).

To determine whether cell clustering that results from activation of the Shh pathway is mediated by induction of Nkx2.2, we transfected this factor alone and assayed the position of transfected cells in the dorsal spinal cord. In contrast to Shh or Gli2/Gli3 activator transfections, Nkx2.2-transfected cells did not segregate from non-transfected cells in the dorsal spinal cord in any case examined (n=8 sections, three embryos; data not shown). These results show that cell clustering induced by Shh-pathway activation is independent of Nkx2.2 induction in chick neural progenitors.

Discussion

In this report, we addressed the requirement for Gli protein activities in mediating Shh-dependent patterning and cell fate specification in the developing vertebrate spinal cord. Our analysis of mouse mutants lacking both Gli2 and Gli3 genes shows that these factors function redundantly in the specification of V3 interneuron and in DV patterning of multiple ventral neuronal classes. As Gli1 is not transcribed in double homozygotes, all possible repressor or activator forms of Gli proteins are absent. We found that MN, V2, V1 and V0 cells were generated in altered numbers in Gli2;Gli3 double mutants, and MN, V2 and V1 neurons and their precursors were expanded into the ventral midline but retained their role in the specification of V3 interneuron.
Gli2 and Gli3 activators mediate Hh patterning

Individual identities despite developing as intermingled populations. We show that Gli2 and Gli3 activator activities are sufficient and required to induce Nkx2.2 expression in V3 interneuron precursors. Furthermore, we show that N-terminally truncated forms of both Gli2 and Gli3 could activate a limited set of ventral genes, but that they differ in their ability to do so, suggesting that intrinsic functional differences between these proteins contribute to their unique roles in mediating Shh signaling. Finally, we show that activation of the Shh pathway in dorsal cells elicits their segregation from non-transducing dorsal cells. Together, these results raise the possibility that Shh/Gli signaling functions in neuronal patterning by controlling progenitor cell segregation.

**Unique and redundant roles for Gli2 and Gli3 activators in patterning ventral cells**

Our current analysis of neuronal specification in Gli2 single and Gli2;Gli3 double mutants indicates that Gli3 is partially redundant with Gli2 for the expression of Gli1 and the generation of V3 cells. In Gli2 mutants, Gli1 expression is
severely reduced and the FP and most V3 cells are absent (Matise et al., 1998), whereas in Gli2;Gli3 mutants Gli1 expression and V3 cells are completely absent. Our transfection studies, furthermore, show that dominant activator forms of both Gli2 and Gli3 are capable of inducing ectopic expression of the V3 marker Nkx2.2 throughout the neural tube. Together with previous studies (Briscoe et al., 1999; Aza-Blanc et al., 2000; Bai and Joyner, 2001; Bai et al., 2004), these results demonstrate that Gli2 and Gli3 activators function redundantly to define the p3 progenitor domain and specify V3 interneurons by controlling expression of Nkx2.2 and perhaps Gli1.

Both Gli2 and Gli3 contain an N-terminal repressor domain that physically interacts with Su(Fu), a negative regulator of the Shh pathway (Pearse et al., 1999; Murone et al., 2000; Dunavea et al., 2003). Previous studies in transgenic mice showed that misexpression in the dorsal midbrain of Gli2 and Gli3 proteins lacking this N-terminal domain, but retaining the DNA-binding zinc finger and C-terminal activation domains (Yoon et al., 1998), could activate ectopic expression of Foxa2/Hnf3β (Sasaki et al., 1999). Interestingly, we found that misexpression of N-terminally-deleted Gli2 and Gli3 activators in chick spinal cords using in ovo electroporation elicited different outcomes. Gli2 activated expression of Foxa2 (Hnf3β), Nkx2.2 and Nkx6.1, as well as inducing an expansion of the neuroepithelium, while Gli3 was only capable of inducing Nkx2.2. The striking similarity of these activities to the differential requirements for Gli2 and Gli3 in specifying ventral cell fates, as revealed by our analysis of Gli2;Gli3 double mutants, suggests that these might reflect functional differences in the capacity of Gli2 and Gli3 to activate Shh target genes. However, as N-terminally-truncated forms of Gli2 and Gli3 are not detected in situ, we cannot rule out the possibility that the different responses to Gli2AN-term and Gli3AN-term in our transfection experiments do not reflect true differences in the activities of these proteins in vivo. Nevertheless, these results suggest that intrinsic functional differences between Gli2 and Gli3 could play a role in differentiating the cellular responses to Shh.

We found that Ptc1 and Ptc2 are differentially responsive to constitutive Gli2 and Gli3 activators. Taken together with previous studies showing that a Gli3 repressor can downregulate Ptc1 and Ptc2 transcription in the neural tube (Persson et al., 2002), it is likely that Ptc1 and Ptc2 are regulated by both Gli activator and repressor activities in the developing spinal cord. In this regard, the Ptc genes appear analogous to dpp in flies, the expression of which is controlled by both activator and repressor forms of Ci (Muller and Basler, 2000).

Obligatory role for Gli proteins in mediating Hh-dependent neural patterning in the ventral spinal cord

Studies in Drosophila have shown all of the known responses to Hh signaling require the activities of Ci (Methot and Basler, 2001). In Smo;Gli3 mutants, which are in theory incapable of responding to all Hh proteins, many ventral cell types that are lost in Shh mutants are rescued, but some populations are mixed in the ventral spinal cord (Wijgerde et al., 2002). However, as Gli2 is still present in these mice as its transcription is independent of Shh signaling (Bai et al., 2002), it was not possible to conclude that all Gli protein activities are required for this potential function. Results from the present study reveal numerous phenotypic similarities between Smo;Gli3 and Gli2;Gli3 mutants, and provide support for the idea that Gli protein activities are required for all the known patterning functions of Hh signaling in the developing ventral spinal cord.

Role of Gli proteins in mediating graded Shh signaling in the developing spinal cord

From our studies and those from other laboratories, a clear picture has emerged of the individual roles of Gli protein activities in mediating cellular responses to graded Hh signaling in the developing spinal cord. All three Gli factors contribute positive functions to the transduction of the Shh signal in the ventral spinal cord, with Gli2 and Gli3 playing the predominant roles to transduce the initial Shh signal and Gli1, which is induced by Gli2 and Gli3, making a minor contribution. Thus, all three Gli proteins participate as activators in the induction of FP and V3 interneurons, while Gli2 and Gli3 activators are also required for normal motoneuron differentiation and V2-V0 interneuron patterning and number, but not specification. Gli3 serves as the primary Gli repressor that must be inhibited to allow normal ventral cell fate development, but this activity also appears to be required for normal V2-V0 interneuron development independent of Hh signaling (Persson et al., 2002).

Available evidence does not, however, definitively exclude the possibility that a Gli2 repressor could play a minor Hh-independent role in the spinal cord. The increase in V1 interneurons in thoracic regions of Gli2;Gli3 double mutants compared with normal embryos could be due to the removal of a repressive function for Gli2 that normally serves to limit the number of these cells. Interestingly, the situation in Smo;Gli3 embryos (that might retain Hh-independent Gli2 repressor activity) appears to be the converse of this, with slightly more V1 cells in lumbar, but not thoracic, regions (Wijgerde et al., 2002). These findings suggest that if Gli2 does function as a repressor in vivo, this activity is redundant with Gli3 and its role differs along the rostrocaudal axis of the spinal cord. However, no direct evidence has been provided to date demonstrating the presence of an active, truncated repressor form of Gli2 in the developing spinal cord. Alternatively, as it has been shown that V1 and V0 cells can be induced by retinooids (Pierani et al., 1999), it is also possible that the normal formation of these cell types involves a correct balance or integration of both Shh and retinoid signaling pathways, and that this balance is differentially affected in mouse Gli2;Gli3 and Smo;Gli3 mutant backgrounds.

Our results also reveal a redundant role for Gli protein activities in normal motoneuron differentiation. We found that in mice lacking all Gli activities, postmitotic motoneurons were present in greatly reduced numbers relative to their precursors, which were either near normal in number or elevated (in thoracic regions). This defect is not explained by inappropriate up-regulation of cyclin D1 or downregulation of Ngn2 in motoneuron precursors (see Fig. S1 at http://dev.biologists.org/supplemental; Q.L and M.P.M., unpublished). One possibility is that in the absence of regulated Gli protein activities, motoneuron progenitors do not respond normally to differentiation cues that are transmitted to cells during or shortly after their terminal mitosis.
Gli2 and Gli3 activator activities contribute to Shh/Gli control of neuronal patterning by regulating progenitor segregation

Our analysis of neuronal progenitor markers in Gli2;Gli3 mutant spinal cord reveals that p1 and p2 cells that develop in the ventral spinal cord are mixed with each other and pMN progenitors. We show that these progenitors maintain distinct identities despite being mislocalized in the ventral spinal cord. As a result, neural patterning is similarly disrupted, with V2, V1 and MNs forming as intermingled, rather than discrete, populations. Within the context of current models, the simplest interpretation of these results is that, in the absence of Gli activities, many ventral precursors are incapable of responding normally to graded Shh signaling, resulting in the random specification of progenitors and the generation of similarly intermingled neuronal progeny. However, another interesting explanation could also account for these results. Lineage tracing studies indicate extensive intermixing of spinal cord progenitors prior to neurogenesis (Erskine et al., 1998). It is therefore conceivable that the mechanisms involved in specifying positional identity in the spinal cord function in part by restricting this intermixing. This function has been proposed for HD progenitor fate determinants, the expression of which is initiated at the onset of neurogenesis and which function to refine domains by mutual cross-repression (Briscoe et al., 2000). However, with the exception of Nkx2.2, our results show that Gli-mediated Shh signaling is not required for the induction or cross-repressive functions of most ventral HD determinants but rather is needed to organize their normal expression into distinct DV domains.

Furthermore, our gain-of-function studies in chick embryos suggest a role for the Shh/Gli signaling in establishing progenitor pool segregation in the spinal cord that is independent of their regulation of HD determinants. In these experiments, we activated the Shh pathway in dorsal cells using overexpression of ligand or N-terminally truncated Gli2/Gli3 activator proteins. In both cases, cells in which the pathway was activated were segregated from non-transducing dorsal cells when assayed 24 hours after transfection. As cell clustering cannot be induced by Nkx2.2 (the only HD factor consistently induced in these assays), it appears that distinct Gli target genes mediate this effect.

A role for Hh signaling in controlling progenitor segregation has also been proposed based on recent studies in Smo<sup>−/−</sup> mouse mutants (Wijgerde et al., 2002). In this study, Hh signaling was blocked in ventral cells by generating chimeras with Smo<sup>−/−</sup>-ES cells, and it was found that progenitors derived from these cells ‘clumped’ together in the ventral spinal cord. However, as Smo<sup>−/−</sup> mutant cells in chimeras are present throughout embryogenesis, it is unclear whether Smo (or Hh signaling) is required in these cells during neurogenesis (when cell fate specification is occurring) or at some earlier time point. In addition, any Gli proteins present within Smo<sup>−/−</sup> mutant cells are likely to be repressors, so whether Gli activators, or Gli proteins in general, also participate in the hypothesized role of Hh signaling in progenitor segregation was not addressed. Our results establish that the opposite manipulation (activating the pathway in dorsal cells) has a similar outcome. Together, these results demonstrate that altering Shh pathway status in cells relative to their neighbors, either positively (our study) or negatively (Wijgerde et al., 2002), can induce segregation. Furthermore, cell segregation defects are only seen in mouse mutants in which all Gli activator activities are absent (Smo<sup>−/−</sup> chimeras, Smo;Gli3 and Gli2;Gli3 mutants). Together, these results illustrate a crucial role for Gli activators, either on their own or in balance with Gli repressors, in controlling progenitor segregation in the neural tube.

It is not immediately obvious from our studies how Gli activator-induced cell clustering in the dorsal spinal cord is related to their normal role ventrally. Pathway-activated cells in the ventral spinal cord did not form clusters, even at 20-fold lower Gli2 activator concentrations or when co-transfected with Gli3 repressor (Q.L. and M.P.M., unpublished). This could be due to a requirement for co-factors [such as Fu or Su(Fu)] that are known to regulate Gli activity through binding to the N-terminal region deleted from our constructs (Murone et al., 2000), or to other modulators of Gli activator activity that function specifically in the ventral spinal cord.

Our studies do not address the mechanisms that are responsible for Shh/Gli-mediated progenitor segregation. In principle, regulation of either differential cell adhesion or proliferation rates, or some combination of the two, could be involved. In Drosophila wing imaginal discs, Hh signaling in anterior (A) cells controls their segregation from posterior (P) cells at the AP border (Tepass et al., 2002). Inactivation of Hh or Ci result in mutant cells exhibiting sorting behavior characteristic of P cells in which Hh signaling is normally blocked by En (Dahmann and Basler, 2000). Phenotypically similar results were obtained by manipulating cadherin levels, providing a potential link between Hh signaling and the control of differential cell affinity (Dahmann and Basler, 2000). The striking similarities in fly and vertebrate phenotypes that result from manipulating Hh/Ci/Gli signaling suggests that the mechanisms that control differential cell segregation downstream of Hh signaling are conserved through evolution.

We thank Michael Garber and Erin Sciacchetano for technical assistance; David Anderson, Tom Jessell, Jane Johnson, Susan Morton, Andy McMahon, Kamal Sharma and Hirohide Takebayashi for providing antibodies; Matt Scott and Cliff Tabin for in situ probes; Andy McMahon for pCIG vector; Alex Joyner for Gli2 and Gli3 cDNAs; and Gary Struhl for ptc<sub>D</sub>cDNAs; and Gary Struhl for ptc<sub>D</sub>oop2 cDNA. We also thank Guy Pastena, Rikki Racela, Elena Stoico and Ye He for preliminary data. Thanks to Kenny Campbell, Doug Epstein and Randy McKinnon for comments on the manuscript. This work was supported by a March of Dimes Basil O’Connor Starter Scholar Award (to M.P.M.) and a grant from the NSF (IBN 0131264).

References


