Overexpression of ptc1 Inhibits Induction of Shh Target Genes and Prevents Normal Patterning in the Neural Tube

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INTRODUCTION

The Hedgehog (Hh) pathway is a conserved signaling system that plays an important role in cell–cell communication during development and growth. Hh proteins are autocatalytically cleaved ligands (Lee et al., 1994; Bumcrot et al., 1995; Porter et al., 1996a,b) that can bind to the transmembrane protein Patched (Ptc) (Marigo et al., 1996a; Stone et al., 1996) and induce transcription of target genes in the recipient cell (Hammerschmidt et al., 1997). In vertebrates, Sonic hedgehog (Shh) secreted from axial mesoderm is believed to induce expression of the transcription factor HNF-3β and differentiation of the floor plate in the adjacent neural tube (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994, 1995). Shh and the related proteins Indian hedgehog (Ihh) and Desert hedgehog (Dhh) also organize development of the skeleton (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Vortkamp et al., 1996), limbs (Riddle et al., 1993), gut (Roberts et al., 1995), and germline (Bitgood et al., 1996).

Despite the large number of biological activities attributed to Hh proteins, little is known about how Hh signaling affects Ptc function and subsequently causes changes in gene transcription. Ptc has 12 potential transmembrane domains and a proposed sterol-sensing domain (Goodrich et al., 1996; Carstea et al., 1997; Loftus et al., 1997; Johnson and Scott, 1998). It is related to the Niemann Pick Type C disease gene NPC1 (Carstea et al., 1997; Loftus et al., 1997) which regulates cholesterol transport and homeostasis, but no biochemical function other than Hedgehog binding has been ascribed to Ptc.

One clear biological function of Ptc is the regulation of Hh target gene transcription. Ptc represses transcription of genes that are induced by Hh (Goodrich et al., 1997). Because Hh and Ptc have opposing activities, Hh protein is proposed to bind and inactivate the Ptc protein (Marigo et al., 1996a; Stone et al., 1996). Although some Hh target genes vary, two are nearly always induced: the zinc finger transcription factor Gli1 and ptc1 itself. ptc1 and Gli1 are expressed in cells that receive Hh signals and are induced by ectopic sources of Hh (Marigo et al., 1996a; Stone et al., 1996). Furthermore, ptc1 expression decays in Shh and Dhh mutant mice (Bitgood et al., 1996; Chiang et al., 1996). Conversely, in mouse ptc1 mutants, Gli1 and ptc1...
are ectopically expressed, consistent with the idea that Ptc protein normally represses target gene transcription in cells that do not receive the Hh signal (Goodrich et al., 1997). Gli1 (Kinzler et al., 1987; Walterhouse et al., 1993; Hui et al., 1994) is a vertebrate homolog of Cubitus interruptus (Ci), a transcription factor that appears to be directly responsible for activation and repression of Hh target genes in flies (Forbes et al., 1993; Hooper, 1994; Von Ohlen et al., 1997). Similarly, Gli1 can induce Hh target gene expression in mice (Hynes et al., 1997) and in vitro (Sasaki et al., 1997).

Additional insight into biological roles for ptc1 came with the discovery that germline PTC1 mutations are associated with the human disease basal cell nevus syndrome (BCNS) (Hahn et al., 1996; Johnson et al., 1996). People with BCNS are large and suffer from a number of developmental disorders such as polydactyly and bifid ribs (Gorlin, 1987). In addition, BCNS patients have an increased frequency of the skin tumor basal cell carcinoma (BCC), the deadly brain tumor medulloblastoma, and other proliferative disorders such as jaw cysts (Gorlin, 1987). PTC1 mutations have also been found in a number of sporadic tumors (Gailani et al., 1996; Hahn et al., 1996; Johnson et al., 1996; Undén et al., 1996; Pietsch et al., 1997; Raffel et al., 1997; Vorechovsky et al., 1997; Wolter et al., 1997; Xie et al., 1997). The developmental defects associated with BCNS are believed to arise from PTC1 haplinsufficiency while the tumors generally have both copies of PTC1 inactivated. Mice that are heterozygous for ptc1 display some phenotypes reminiscent of the human disease and are at increased risk for medulloblastoma and other tumors (Goodrich et al., 1997; Hahn et al., 1998). In addition, homozygous ptc1 mutant mice die early in development with severe neural tube defects and an abnormal circulatory system.

The defects observed in ptc1 mutants suggest that a balance between Hh and Ptc activities is necessary for normal development. For instance, in BCNS patients there may not be enough Ptc to keep Hh target genes off. Alternatively, as has been observed in flies (Taylor et al., 1993; Chen and Struhl, 1996), Hh ligand may diffuse farther in tissues with less Ptc protein. Also, in vitro experiments in vertebrates suggest that Shh acts as a dose-dependent signal to induce diverse cell types in the neural tube (Martí et al., 1995a; Roelink et al., 1995; Ericson et al., 1997). Perhaps differences in the relative amounts of Shh and Ptc activities lead to distinct cell fate decisions.

To explore the idea that a highly regulated balance in Shh and Ptc activities is necessary for normal development, we examined the effects of Ptc overexpression in transgenic mice. Here we show that excess Ptc attenuates induction of Shh target genes and causes abnormal patterning in the neural tube. These results demonstrate that Ptc is sufficient to oppose Shh signaling and reveal additional roles for Ptc in development and growth.

MATERIALS AND METHODS

Plasmids

449-8. To remove the 5' UTR, a piece of ptc1 was amplified from plasmid M2-1 (Goodrich et al., 1996) using primers PATG and S3. This product was digested and cloned into the AAvrl and XbaI sites of M2-1 to make plasmid 331-2. To remove the 3' UTR, ptc1 was amplified from plasmid M9 (Goodrich et al., 1996) using primers SS and PTGA. This product, which ends at the ptc1 stop codon, was digested and cloned into the Ncol and XbaI sites of M9 to create plasmid 264-6. 331-2 was digested with SacI and EcoRV, filled in with T4 DNA polymerase, and then cloned into the EcoRV site of 264-6, resulting in plasmid 447-1. 447-1 was digested with Bsal to drop repeated sequences and then reclosed to create plasmid 449-8. This plasmid contains the entire ptc1 coding region, minus both 5' and 3' UTRs and including an optimized Kozak consensus sequence for the AUG (Kozak, 1991). All PCR-derived DNA and the cloning junctions were sequenced and found to be correct.

487-1. A HindIII/XbaI fragment containing the second intron of the nestin gene was released from construct F (Zimmerman et al., 1994) and cloned into pTKβ (Clontech). The resulting plasmid contains a lacZ gene under the control of a thymidine kinase basic promoter and the nestin enhancer. Plasmid sequences were removed by digestion with HindIII and SalI prior to microinjection.

499-6. The lacZ gene was removed from plasmid 487-1 by digestion with NotI and replaced with a NotI fragment from 449-8 that contains the ptc1 coding region. Plasmid sequences were removed by digestion with HindIII and SalI prior to microinjection.

Generation of Transgenic Mice

Purified linearized plasmids 487-1 and 499-6 were microinjected into fertilized oocytes from B6CBAF2 mice (Hogan et al., 1994). To test the nestin enhancer, embryos injected with 487-1 were collected and stained with X-gal (Bonnerot and Nicolas, 1993). As previously reported, staining was observed throughout the central nervous system (Zimmerman et al., 1994). Two stable lines were generated from oocytes injected with 499-6: Np1c77 and Np1c118. Transgenic animals were genotyped by Southern blot or PCR. Genomic DNA was digested with EcoRI and probed with the 5' NsiI fragment of the ptc1 cDNA. Due to the high copy numbers in both lines, it proved difficult to determine with certainty whether animals were heterozygous or homozygous for the transgene; the high hybridization signal from the transgene generally obscured the endogenous bands, making it impossible to normalize between samples. PCR was performed with primers H56 and S2 under the following conditions: 94°C 5'; [94°C 30', 52°C 35', 72°C 90']x 25 cycles; 72°C 10'. All transgenic lines were maintained using heterozygous matings. For embryo collections, noon on the day of plugging was called E0.5.

In Situ Hybridization

Endogenous ptc1 expression was detected using a 752-bp riboprobe (M21-3' UTR) corresponding to nucleotides 4435-5187 in the 3' UTR. Endogenous and exogenous ptc1 expression was detected using riboprobes from plasmids M2-2 and M2-3 (Goodrich et al., 1996). These two probes yielded identical results and will subsequently be referred to as ptc1 probes. The Shh probe was generated from Hh-16.1 (Echelard et al., 1993). A 1.7-kb riboprobe encom-
passing most of the zinc finger domain was used to detect Gli1 (Hui et al., 1994). The Pax 3 probe corresponded to a 519-bp PstI/HindIII fragment from the 3′ end of the gene (Goulding et al., 1991).

Digoxigenin in situ hybridization and radioactive in situ hybridization of sections were performed and photographed as described (Storm and Kingsley, 1996; Oro et al., 1997).

**Immunohistochemistry**

Immunohistochemistry was performed as described (Ericson et al., 1997) using mouse anti-Nkx2.2 (Ericson et al., 1997) and rabbit anti-Pax6 antibodies (MacDonald et al., 1995).

**Double-Label in Situ Hybridization and Immunohistochemistry**

In situ hybridization was performed as above but with the following modifications. 1:5000 K5 anti-islet antibody (Ericson et al., 1992) was added to the anti-DIG antibody and incubated overnight at 4°C. After several PBS washes, the sections were incubated with secondary antibody in 1% normal goat serum. Depending on the secondary antibody, the signal either was directly visualized using diaminobenzidine as a substrate or was amplified and visualized using the VectaStain ABC system (Vector Laboratories). The slides were then washed in PBS and Buffer B1 and transferred to Buffer B3 for visualization as in the original in situ hybridization protocol. Nuclei were stained with DAPI.

**Histology**

Embryos and postnatal brains were dissected in PBS, fixed overnight in 4% paraformaldehyde, and embedded in paraffin according to standard protocols. Six- to 8-μm paraffin sections were deparaffinized in xylene and rehydrated through a graded alcohol series before staining with hematoxylin (10 min) and eosin (2 min) (Newcomer Supply). Excess stain was removed in water and 0.4% acetic acid water. Sections were dehydrated, cleared in xylene, and mounted in Permount (Sigma).

**Primers**

Primers used included PATG, GCCACCATGCGCTCGCTGTG (Kozak sequence underlined); S3, CAAAGGCCACATCAG; S5, GTTCTGGACGTTGCTG; PTGA, TGAGTTGAGCTGCTCCCC; H56, GTGCTCGAGCAGCGAATTTCAG; and S2, GAAGCTGTTTTAGGGTG.

**RESULTS**

**Defects Caused by Overexpression of Ptc in the Neural Tube**

To gain insight into the antagonistic relationship between Shh and Ptc as well as the role of this pathway in neural development, we overexpressed ptc1 in the developing murine nervous system. The coding region of ptc1 was placed under the control of an enhancer from the nestin gene that drives high-level expression in most central nervous system progenitors beginning at about embryonic day 9 (E9) (Fig. 1A) (Zimmerman et al., 1994). Two viable lines of mice, Nptc77 and Nptc118, were generated by microinjection of embryos with this construct. Both lines have 10 to 20 copies of the transgene and exhibit the same phenotypes. The lines were maintained by matings of transgenic animals.

The nestin enhancer drives high-level, persistent expression of ptc1 in cells throughout the neural tube. In wild-type embryos, ptc1 is expressed in a ventral to dorsal gradient in the ventricular zone of the neural tube (Fig. 1B). We confirmed that ptc1 is overexpressed in the nestin pattern in E10.5, E11.5, and P0 animals from both lines. In E10.5 and E11.5 transgenic embryos, ptc1 is strongly transcribed throughout the anterior neural tube except for the floor plate (Fig. 1C). In progressively more posterior regions of the embryo, ptc1 is overexpressed more highly in the ventral neural tube than in the dorsal neural tube (Fig. 1D). As in wild-type embryos, ptc1 is primarily expressed in cells of the ventricular zone. In a small number of transgenic embryos (2/19 examined), ptc1 transcription was variegated, causing a punctate staining pattern (Fig. 1E).

The dramatic increase in ptc1 RNA production in transgenic lines matches the pattern reported for nestin enhancer activity. Enhancer-driven transcription normally initiates in the ventral forebrain, midbrain, and hindbrain of E9 embryos and expands posteriorly and dorsally over the next 2 days of development (Zimmerman et al., 1994). The enhancer continues to drive expression in neural progenitors throughout development. Similarly, ptc1 expression persists in the postnatal cerebellum of transgenic animals (data not shown). In summary, in Nptc animals, after neural tube closure, ptc1 is expressed at high levels throughout neurogenesis and throughout the neural tube, except for the floor plate.

Nptc animals exhibited dramatic neural defects as embryos, pups, and adults. Of 95 embryos collected between E10.5 and E11.5, 13 (13.7%) had obviously abnormal hindbrain (data not shown). In summary, in Nptc animals, after neural tube closure, ptc1 is expressed at high levels throughout neurogenesis and throughout the neural tube, except for the floor plate. In Nptc offspring, the most severe phenotype was observed in Nptc118 line. In the Nptc118 line, which had a slightly more severe phenotype, the average litter size from E10.5 to E11.5 was 8.16 (n = 6 litters), but only an average of 4.7 (n = 7 litters) animals were alive at weaning. In addition, dying pups or carcasses were often observed at the day of birth. Hematoxylin and eosin staining of brain sections from P0 pups revealed defects in 5/8 (62.5%) transgenic animals. In the least affected animals, the ven-
tricles were enlarged and the corpus callosum appeared slightly disorganized (Figs. 2C and 2D). One animal, which was clearly dying at the time of sacrifice, had fused ventricles and major defects in the corpus callosum. Here, neural processes failed to properly cross the midline, instead growing in circles and trapping cell bodies (Fig. 2E).

Neural deficiencies were also observed in animals that survived the perinatal period. Two 2-week-old animals (of...
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55 closely followed) were much smaller than their siblings and died before weaning. One of these was obviously hydrocephalic. Hydrocephaly was also observed in 4 animals after weaning; 3 additional animals were found dead at 3 months of age but a cause of death could not be established due to deterioration of tissues. To better assess the incidence of hydrocephaly, brains from 24 transgenic animals were collected at 6 to 8 weeks of age. Of these, 4 (16.6%) had excess fluid and obviously deformed brains. Histological analysis of one of these brains showed extensive thinning of the cortex, fused ventricles, and massive necrosis (data not shown). Hydrocephaly has never been observed in wild-type animals of the colony. Other transgenic animals survive and reproduce without exhibiting obvious phenotypes.

Since some of the problems observed in newborn animals and hydrocephalic adults may be secondary to earlier developmental abnormalities, subsequent studies focused on the effects of Ptc overexpression in the embryo.

Excess Ptc Decreases Expression of ptc1 and Gli1 in the Neural Tube

Hh and Ptc proteins have opposing activities: Hh induces expression of target genes that are otherwise kept off by Ptc. An apparently universal target is ptc1 itself. Hh seems to establish a highly regulated feedback system by directing production of its own antagonist. In wild-type neural tubes, ptc1 is therefore expressed at high levels close to the floor plate, which produces Shh, but at low levels in the dorsal neural tube (Fig. 1; Goodrich et al., 1996). Paradoxically, high levels of ptc1 transcription correspond to low levels of Ptc function, due to the inactivation of Ptc by the Shh ligand (Goodrich et al., 1997). Conversely, ptc1 is expressed weakly where the protein is most active. In Nptc animals, the feedback loop is circumvented because expression of ptc1 is under the control of the nestin enhancer. In transgenic embryos, Ptc is produced in excess relative to Shh. We tested whether this imbalance interferes with the effectiveness of the Shh signal.

Two good indicators of Shh signaling are the transcriptional inductions of ptc1 and Gli1. To distinguish endogenous ptc1 from ptc1 produced from the transgene, we used a probe from the ptc1 3′ UTR, which is not included in the construct (Fig. 1A). In 5/11 E10.5 embryos examined, endogenous ptc1 expression was significantly decreased in the neural tube, while expression in the surrounding mesoderm was unaffected (Figs. 3C and 3D). Weaker effects were observed in five other transgenic embryos (n = 5 wild-type littermates for comparison). Gli1 expression was even more severely affected (obvious decrease in six of six embryos; n = 3 wild-type embryos for comparison) (Figs. 3E and 3F).

Loss of ptc1 and Gli1 transcripts was most prominent in the anterior region of the embryo, probably reflecting early activation of the nestin enhancer in the midbrain and hindbrain (Fig. 1; Zimmerman et al., 1994). The neural tubes shown in Fig. 3, for example, are at the level of the heart; much less reduction in ptc1 and Gli1 expression can be seen at the level of the hindlimbs (data not shown). Since both ptc1 and Gli1 are normally highly expressed long before the stages we examined, these changes in gene expression are unlikely to be caused by any developmental delay in transgenic embryos. These results suggest that Shh target gene expression is either not effectively induced or maintained in the presence of excess Ptc in the embryonic neural tube.

Overexpression of Ptc Dorsalizes the Neural Tube

As indicated by ptc1 and Gli1 expression, excess Ptc appears to interfere either directly or indirectly with Shh signaling in the neural tube. Shh has been studied extensively as an inducer of floor plate development, but it has been difficult to study other, later roles in the nervous system. In Shh mutant embryos, for instance, no floor plate forms so it is impossible to know whether subsequent patterning errors are due to the absence of the floor plate or the absence of Shh per se (Chiang et al., 1996). Based on in vitro experiments in chick and marker gene expression in mouse, the floor plate and motor neurons appear to be induced early in development, before neural tube closure (Sasaki and Hogan, 1993; Echelard et al., 1993; Yamada et al., 1993). In Nptc embryos, the nestin enhancer initiates ectopic Ptc expression—reducing effective Shh signaling—after neural tube closure and thus after induction of the floor plate and motor neurons (Zimmerman et al., 1994). Furthermore, the transgene is not expressed in the floor plate (Figs. 1C and 3B). Nptc embryos therefore provide a unique opportunity to study the role of this pathway after the floor plate forms and in intermediate and dorsal cell populations.

Cell culture experiments have shown that graded amounts of Shh regulate expression of key transcription factors and therefore generate diverse types of neurons at specific dorsal–ventral levels in the neural tube (Marti et al., 1995a; Roelink et al., 1995; Ericson et al., 1997). In vitro, small amounts of Shh protein inhibit expression of dorsal marker Pax 3 and intermediate marker Pax 6. Inhibition of Pax 6 expression has the indirect effect of relieving repression of Nkx2.2, which is subsequently induced in, and lateral to, the floor plate. Pax 3, Pax 6, and Nkx2.2 positive progenitors differentiate into specific kinds of neurons along the dorsal–ventral axis.

Changes in the expression of Pax 3 and Pax 6 in Nptc embryos reveal a role for the Ptc in regulating cell diversity after formation of the floor plate. Floor plate development occurs normally as assessed by expression of Shh (Figs. 4A and 4B) in E10.5–E11.5 transgenic embryos. In addition, staining with an α-islet antibody suggests normal development of islet-positive motor neuron progenitors, although we cannot rule out subtle changes in the motor neuron number or pool identities (Figs. 4A and 4B). In the dorsal neural tube, however, there is a marked expansion of Pax 3 transcription in some transgenic embryos (6/15) such that...
The ventral boundary of Pax 3 expression is abnormally close to the dorsal boundary of islet-positive cells compared to expression in wild-type littermates (n = 5) (Figs. 4A and 4B). Less dramatic expansions were seen in four other transgenic embryos. In two embryos, ectopic Pax 3 expression occurred in a discrete patch on one side of the neural
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A defining characteristic of the Shh-Ptc pathway is the antagonistic relationship between the Shh and Ptc proteins. Here we show that excess levels of Ptc lead to misregulation of Shh target genes and subsequent abnormal development of the neural tube. In addition, we demonstrate that Ptc itself is sufficient to delay or prevent Shh target gene transcription.

A Balance between Shh and Ptc Is Necessary for Normal Development

This work further confirms the hypothesis that Shh and Ptc have opposing activities and provides evidence that an imbalance between these activities leads to patterning changes in the neural tube. In flies, ubiquitous expression of Hh induces the same target genes that are derepressed in a ptc mutant embryo (Ingham, 1993; Tabata and Kornberg, 1994). Conversely, excess Ptc in flies prevents expression of Hh target genes even in the presence of Hh signal (Schuske et al., 1994; Johnson et al., 1995). We have found that a similar relationship exists in mice. Increased levels of Ptc prevent transcription of Gli1 and ptc1 itself, two genes that are normally induced by Shh in the ventral neural tube.

Since there is no apparent change in Shh expression in transgenic embryos, simply changing the relative amounts of Shh and Ptc appears to alter the output of the pathway. For instance, excess Ptc dorsalizes and ectopic Shh ventralizes the neural tube (this work and Echelard et al., 1993). Overexpression of Ptc also leads to developmental defects such as spina bifida and hydrocephaly. Thus, a balance between Shh and Ptc appears to be critical for normal development. Ptc could also act independently of Shh, either by interfering with signaling by another unidentified Hh ligand or through a parallel, Hh-independent pathway. While it is possible that Ptc acts completely independently to control cell division or cell death and that the changes in Shh target gene expression are secondary to a much more basic role for Ptc in neural morphogenesis, the lack of reproducible changes in the area of the spinal cord despite consistent changes in the target genes Gli1 and Pax 6 makes this unlikely.

If Ptc acts primarily to oppose Shh signaling in the neural tube, this result can be interpreted in three ways. First, the increased amounts of Ptc protein may bind more Shh protein, thereby sharpening the Shh gradient. In this model, the primary function of Ptc is to bind Shh. Since Shh antibodies do not normally reveal a gradient of Shh protein (Martí et al., 1995b; Roelink et al., 1995), we cannot directly test this hypothesis. Second, perhaps there is a larger amount or concentration of active Ptc protein, since normal levels of Shh protein cannot inhibit an increased number of Ptc molecules. In this model, the absolute number of active Ptc molecules determines whether target genes are expressed. Third, excess Ptc could both limit the range of the Shh signal by binding and change the output of the signal by...
actively inhibiting target gene transcription. In favor of this interpretation, excess Ptc in the fly imaginal disc causes patterning and growth defects when expressed only in the posterior compartment, where Hh is produced, but the phenotype is more severe when Ptc levels are increased throughout the wing disc (Johnson et al., 1995). In the first case, Ptc presumably limits the diffusion of Hh into the anterior compartment, where target genes are normally expressed, but has no effect on transcription of these genes in the posterior compartment, where they are not normally expressed. In the second case, Ptc interferes with Hh diffusion and in addition actively inhibits transcription of target genes in the anterior compartment, thereby causing a greater effect. These two functions of the Ptc protein have been experimentally separated in the fly with a mutant Ptc protein that can bind Hh but cannot efficiently inhibit transcription (Chen and Struhl, 1996).

The Hh Pathway Resists Subtle Changes in the Level of Ptc Protein

A balance between Shh and Ptc appears to be rigorously maintained by the Hh signaling pathway itself. In fact, in both flies and mice, it has been difficult to interrupt the Hh–Ptc feedback loop. Initial reports suggested that overexpression of ptc in flies, while sufficient to rescue a ptc mutant, has no substantial effect on target gene transcription or the arrangement of cell types in the larval cuticle (Ingham et al., 1991; Sampredo and Guerrero, 1991). Similarly, we have found that overexpression of ptc1 in transgenic mice often has no drastic phenotypic effect.

Two features of the pathway help to explain the negative results observed in both flies and mice. First, Ptc protein is quite unstable, with very little protein residing at the plasma membrane (Ingham et al., 1991; Capdevila et al., 1994a). Second, Hh is a potent signal and may not be limiting in normal embryos. To overcome the effects of the Hh signal, Ptc protein must be present in great excess and maintained at that level, even in the presence of rapid protein turnover. In flies, a persistently high level of Ptc was accomplished by repeated induction of ptc or by using the GAL4–UAS system (Schuske et al., 1994; Johnson et al., 1995). In the present work, stable and high levels of Ptc were achieved by using the nestin enhancer, which is very strong and drives enduring expression from neural tube closure until the end of neurogenesis. Even so, development occurred fairly normally in the majority of transgenic animals, further testament to the intrinsic resilience of this pathway. Since Hh itself ultimately controls the level of Ptc protein, self-regulation within the pathway may allow reestablishment of a proper balance.

Phenotypic Variation in Nptc Animals

A notable feature of Nptc overexpression is the wide amount of phenotypic variation within each Nptc line, ranging from early embryonic lethality to no visible effect at all. Although transcription of the transgene is occasionally variegated (Fig. 1E), in general the amount of ptc1 expressed from the transgene is consistent within each line (data not shown). A second possibility is that insertion of the transgene interrupted another gene that somehow modifies the effect of excess Ptc. This is also unlikely since the same range of defects was observed in two independent transgenic lines. Third, since these animals were created on a hybrid background, there may be polymorphisms in additional genes that suppress or enhance the phenotype. Finally, some of the variation may be due to differences between embryos with one and two copies of the transgenic array. Due to difficulties in definitively identifying heterozygote and homozygote embryos (see Materials and Methods), we cannot comment on dose-dependent effects of Ptc overexpression. It is likely that multiple factors, including the Ptc dosage and the genetic background, affect the ultimate phenotype.

The nature of the Shh–Ptc signaling cascade itself may also contribute to the observed variation. During normal development a balance between Shh and Ptc is established by a feedback loop that adjusts the amount of Ptc whenever the relative amounts of Shh and Ptc are changed. In Nptc animals, although expression of the transgene is not under the control of Shh or Ptc, Shh still regulates expression of the endogenous ptc1 gene (Fig. 3) and hence can still change the total amount of Ptc protein. If cells assume distinct cell fates depending on the relative amount of Ptc activity, then any changes in the final amount of active Ptc protein may lead to very different outcomes. Similar variation has been observed in hypomorphic alleles of fly ptc or when ptc is overexpressed in the wing imaginal disc (Phillips et al., 1990; Capdevila et al., 1994b; Johnson et al., 1995). Furthermore, ectopic expression of both Pax 3 and Pax 6, which are expanded in Nptc embryos, causes variable phenotypes with low penetrance (Schedl et al., 1996; Tremblay et al., 1996), suggesting that other signaling systems may compensate for forced changes in the neural tube.

Does Shh Act as a Morphogen in the Neural Tube?

Hh is proposed to act as a morphogen to pattern the fly dorsal cuticle (Heemskerk and DiNardo, 1994), but it has been difficult to demonstrate a direct role for graded Shh signaling in the neural tube. If Shh is indeed a morphogen, then it should act in a dose-dependent fashion, the protein should be found in the responding cells, and the effects should be independent of secondary signaling proteins. Our results provide additional evidence for the first two criteria, but do not address the lingering problem of what other signals might also be acting.

Excess Ptc causes predictable changes in gene expression that support the idea of a dose-dependent Shh signal. In vitro, twofold changes in the concentration of Shh regulate the expression of three transcription factors—Pax 3, Pax 6, and Nkx2.2 (Ericson et al., 1996, 1997). Depending on
which of these transcription factors are expressed, progenitors develop as specific types of motor neurons or interneurons at defined locations in the neural tube. Overexpression of Ptc causes an expansion of Pax 3 RNA and of high-level Pax 6 protein, both of which can be repressed by Shh in vitro. Since Shh and Ptc have opposite effects on the same target genes, a simple model is that cell fates are determined by the relative amount of Shh and Ptc activity. The balance is changed in the presence of excess Ptc, consistent with the idea that different concentrations of Shh normally lead to different levels of Ptc activity and therefore different target gene expressions. Although Ptc overexpression at least partially dorsalizes the neural tube, we cannot resolve whether individual ventral cells assume new fates, whether a population of dorsal progenitors divides and expands ventrally, or whether dorsal progenitors survive in abnormally ventral positions. Cells could become confused and express both dorsal and ventral markers, but the expansion of the dorsal marker Pax 3 appears to be coordinated with a change in the level of Pax 6 protein in more ventral cells.

The observed changes in Pax 3 and Pax 6 raise new questions about when and how Shh patterns the neural tube. In vitro, one of the earliest actions for Shh appears to be inhibition of dorsal markers, including Pax 3 (Ericson et al., 1996). Subsequently, floor plate and motor neuron markers are induced in a dose-dependent fashion. Yet, in the presence of excess Ptc, we see changes in the expression of Pax 3 in the absence of changes to floor plate (indicated by Shh RNA) and motor neurons (indicated by Islet protein). Why are the changes in Pax 3 expression limited to the dorsal neural tube? Shh inhibits Pax 3 expression for an extended period of time, first close to the floor plate and then further away. Perhaps by the time Ptc levels are sufficient to block Shh signaling, Pax 3 expression is permanently repressed in the most ventral cells, but not in the more intermediate cell populations. Given the apparent continued need for Shh signaling after the initial induction of cell fates, increased Ptc levels may interfere with the maintenance, but not the initiation, of Pax 3 repression. In this case, Pax 3 is initially turned off by Shh, but then is reexpressed in cells that still require effective Shh signaling. Both possibilities raise the interesting idea that intermediate cell populations are patterned after ventral cell populations or that these cells respond differently to Shh than the ventral explant cells used for the in vitro experiments. The ultimate fate of the affected cells remains to be determined. Excess Ptc may delay rather than prevent the expression of appropriate genes. Subtle changes in motor neurons or other cell populations cannot be assessed without additional markers.

The effects of excess Ptc also provide indirect evidence for long-range action of Shh. Whether Shh protein acts directly on cells outside of the floor plate is difficult to determine. First, Shh protein, which is tightly associated with the cell surface, has not been detected outside of the floor plate (Martí et al., 1995b; Roelink et al., 1995). However, function-blocking antibodies reveal a requirement for Shh in motor neuron induction (Ericson et al., 1996). We have found that overexpression of Ptc inhibits expression of Gli1 and ptc1, even far away from the floor plate. Taken together with previous evidence that both Shh and Ptc regulate Pax 3 and Pax 6 and that Ptc opposes Shh activity (Goodrich et al., 1997), the simplest interpretation of these results is that Shh protein normally reaches the cells that express these target genes. This implies that, in vivo, Shh protein has a range of at least as far as the ventral border of Pax 3. Alternatively, Ptc overexpression might affect only the cells closest to the floor plate and somehow interfere with production of a different signal that independently regulates expression of Gli1, ptc1, Pax 3, and Pax 6. However, there is no evidence for such a signal. If Shh protein does in fact travel as far as the ptc1 expression pattern implies, then it will be important to determine how the protein gets there and why it is so difficult to detect.

**Roles for Shh and Ptc in Neural Tube Patterning**

Very little is known about Shh and Ptc activities in the nervous system outside of the developing spinal cord. However, the other phenotypes observed in N ptc animals might also be caused by a forced imbalance between Shh and Ptc. For example, the fusion of ventricles and the midline defects observed in some newborn N ptc pups are reminiscent of holoprosencephaly (HPE), a disorder that arises in people who are heterozygous for Shh (Roessler et al., 1996). HPE-like phenotypes are also caused by ectopic expression of Pax 3 throughout the neural tube (Tremblay et al., 1996). Indeed, Pax 3 is ectopically expressed in the ventral neural tube of N ptc embryos (Fig. 4). While there are many potential causes for the hydrocephaly seen in N ptc adults, it is interesting to note that mice mutant for apolipoprotein B have a 50% reduction in plasma cholesterol levels and exhibit a number of neural defects including hydrocephaly (Homanics et al., 1995). A cholesterol moiety is added to Hh proteins during autoproteolysis and may be important for their function (Lee et al., 1994; Bumcrot et al., 1995; Porter et al., 1996a,b). In addition, two teratogens, cyclopamine and jervine, are sterols and can inhibit induction of Shh target genes in neural explants (Cooper et al., 1998; Incardona et al., 1998). Further analysis of the N ptc neural phenotypes may reveal Hh-independent roles for Ptc or additional, unexpected roles for Shh or other Hh family members.

Although the details remain a mystery, the Hh-Ptc pathway clearly has an essential role in human development and disease. We have found that an imbalance in Shh and Ptc leads to cell fate changes and subsequently abnormal development in the murine nervous system. Similar misregulation of the Hh pathway is associated with two human genetic disorders, holoprosencephaly and basal cell nevus syndrome, and also with sporadic cancer. Understanding how this balance is achieved has important implications for future treatment of human birth defects and cancer.
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REFERENCES


