Ongoing Sonic Hedgehog Signaling is Required for Dorsal Midline Formation in the Developing Forebrain

Monica Hayhurst,[1] Bryan B. Gore,[2,3] Marc Tessier-Lavigne,[3] Susan K. McConnell[1]

[1] Department of Biological Sciences, Stanford University, Stanford, California 94305

[2] Neurosciences Program, Stanford University, Stanford, California 94305

[3] Genentech Inc., 1 DNA Way, South San Francisco, California 94080

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ABSTRACT: The division of the mammalian forebrain into distinct left and right hemispheres represents a critical step in neural development. Several signaling molecules including sonic hedgehog (SHH), fibroblast growth factor 8 (FGF8), and bone morphogenetic proteins (BMPs) have been implicated in dorsal midline development, and prior work suggests that the organizing centers from which these proteins are secreted mutually regulate one another during development. To explore the role of the ventral organizing center in the formation of two hemispheres, we assessed dorsal midline development in *Shh* mutant embryos and in wildtype embryos treated with the SHH signaling inhibitor HhAntag. Collectively, our findings demonstrate that SHH signaling plays an important role in maintaining the normal expression patterns of *Fgf8* and *Bmp4* in the developing forebrain. We further show that FGF8 can induce the expression of *Zic2*, which is normally expressed at the midline and is required *in vivo* for hemispheric cleavage, suggesting that FGF signaling may stimulate dorsal midline development by inducing *Zic2* expression.

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INTRODUCTION

During mammalian brain development, the rostral neural tube bifurcates into the left and right cerebral hemispheres. This critical event in forebrain development involves the action of molecules secreted by at least three signaling centers that are critical for patterning (Furuta et al., 1997; Crossley et al., 2001; Fukuchi-Shimogori and Grove, 2001; Ohkubo et al., 2002). The dorsal signaling center expresses bone morphogenetic proteins (BMPs), which are critical for dorsal midline formation (Furuta et al., 1997; Panchision et al., 2001; Hébert et al., 2002). Wnt proteins are also expressed at the dorsal midline; however, it appears that Wnts primarily regulate hippocampal development and proliferation (Lee et al., 2000). The rostral signaling center expresses several fibroblast growth factors (FGFs), including FGF8, which is important for anterior forebrain development and cortical arealization (Meyers et al., 1998; Fukuchi-Shimogori and Grove, 2001; Hébert et al., 2003a,b; Storm et al., 2003, 2006). The ventral signaling center expresses sonic hedgehog (SHH), which induces

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Correspondence to: S.K. McConnell (suemcc@stanford.edu).

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ventral telencephalic structures (Ericson et al., 1995; Chiang et al., 1996; Shimamura and Rubenstein, 1997; Fuccillo et al., 2004, 2006).

Each of these three signaling centers has also been implicated in the process of hemispheric cleavage. Application of beads coated with either BMP4 or FGF8 is sufficient to induce dorsal midline properties in neural tube explants (Furuta et al., 1997) or *in vivo* (Crossley et al., 2001). Dorsal midline development and hemisphere formation are disrupted, in whole or in part, following mutation of the *Shh* or *Fgf8* genes in mice (Chiang et al., 1996; Meyers et al., 1998; Storm et al., 2006). Although mice with a conditional mutation in *Bmp4* develop a phenotypically normal telencephalon (Hébert et al., 2003a,b), conditional knockouts of the BMP receptor gene *Bmpr1a* disrupt the development of the choroid plexus, the dorsomedial-most forebrain tissue (Hébert et al., 2002), and the dorsal midline fails to form in mice lacking both *Bmpr1a* and *Bmpr1b* (Fernandes et al., in press). These studies suggest that multiple BMPs collaborate in stimulating midline development.

Although the positions of the FGF and BMP organizing centers at the anterior and dorsal midline, respectively, are consistent with a direct role in hemispheric cleavage, the role of SHH in dorsal midline development presents an interesting puzzle. *Shh* is expressed in the ventral telencephalon and is required for establishing ventral cell fates (Ericson et al., 1995; Briscoe and Ericson, 2001; Fuccillo et al., 2006). Curiously, despite the ventral expression of *Shh*, the *Shh* mutant mice also exhibit abnormal dorsal midline development, which ultimately leads to an undivided telencephalon (Chiang et al., 1996). Similar to mice, humans with mutations in *SHH* exhibit a brain disorder known as holoprosencephaly (HPE; Muenke and Beachy, 2000; Wallis and Muenke, 2000). HPE is characterized by the failure of hemispheric cleavage, and in its most severe form a single brain ventricle is present. The ventral localization of *Shh* expression suggests that SHH regulates dorsal midline fates only indirectly.

It is possible that SHH exerts its effects on dorsal midline development by regulating other forebrain signaling centers. Ohkubo et al. (2002) suggested that the patterning centers are set up sequentially, such that the early expression of *Shh* and *Fgf8* helps to establish the *Bmp* signaling center. Evidence for such a "cascade mechanism" has been obtained from the examination of $Shh^{-/-}$ mice, which display a loss of *Fgf8* expression at the rostral midline and expansion of *Bmp4* expression (Ohkubo et al., 2002). The appropriate expression of *Bmps* at the dorsal midline appears to be exquisitely sensitive to the levels of FGF8, as evidenced by *Fgf8* mutant mice. Loss of *Fgf8* in the forebrain results in a loss of *Bmp4* expression (Storm et al., 2006); however, mice hypomorphic for *Fgf8* show a broadening of *Bmp4* expression (Storm et al., 2003). Thus, loss of SHH signaling early in development results in a disregulation of other signaling centers, which, we propose, likely causes the HPE phenotype in dorsal regions. Interestingly, disruption of hedgehog (Hh) signaling within the brain at embryonic Day 9 (E9), using a *Foxg1-Cre* allele to inactivate a conditional allele of *Smoothened*, resulted in severe ventral defects but allowed successful dorsal midline development (Fuccillo et al., 2004). These data suggested that SHH must be required before E9 for normal dorsal midline development. In addition to regulating signaling centers at early stages of forebrain development, SHH may play a role in maintaining signaling centers at later developmental times.

To address the role of SHH in maintaining the FGF8 and BMP4 signaling centers, and to define the critical period during which SHH must be present to support normal dorsal midline formation, we have used the SHH signaling pathway inhibitor HhAntag (Frank-Kamenetsky et al., 2002; Williams et al., 2003; Romer et al., 2004) to interfere with SHH signaling between E9.5 and E11.5. HhAntag inhibits SHH signaling in cultured embryos after 2 days of treatment, as shown by the loss of *Ptc1* expression in the ventral telencephalon. We find that the expression of both *Fgf8* and *Bmp4* in the forebrain is lost in HhAntag-treated embryos, suggesting that SHH is required for the maintenance of these signaling centers after the initial establishment of their appropriate expression domains.

The function of forebrain signaling centers has been further illuminated by recent molecular genetic studies of HPE in humans. Eight known genes (*DispA, Gli2, Shh, Six3, Tdf1, Tgif, Ptc*, and *Zic2*) are associated with familial or sporadic forms of HPE (Belloni et al., 1996; Roessler et al., 1996, 2003; Wallis et al., 1999; Gripp et al., 2000; de la Cruz et al., 2002; Ma et al., 2002; Ming et al., 2002). Most of these genes either encode components of the SHH pathway or are expressed in the ventral telencephalon rather than in the region of the dorsal midline where hemispheric cleavage occurs. However, *Zic2* is expressed in the dorsal forebrain at E9.5 (Nagai et al., 1997) and is thus positioned at the right time and place to play a direct role in dorsal midline development. A second goal of the present experiments was to explore the possibility that *Zic2* expression is regulated by forebrain signaling centers and links ventral determination to the differentiation of the dorsal midline.

EXPERIMENTAL PROCEDURES

Maintenance of Mouse Lines

Shh mutant mice were maintained in a C57BL/6 background and genotyped as previously described by Chiang et al. (1996). Control embryos were wildtype or $Shh^{+/-}$ littermates of $Shh^{-/-}$ mutants.

Whole Embryo Culture

Mouse embryos were cultured from E9.5 to E11.5 using a whole embryo culture incubator (B.T.C. Engineering, Bolton, UK). CD1 mouse embryos were dissected out of the uterus at E9.5. The yolk sac and amnion were opened but left attached. Embryos were then cultured for 2 days with rotation at 37°C in 100% heat-inactivated male rat serum (Harlan Bioproducts, Indianapolis, IN), supplemented with 10 m*M* glucose, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 292 μg/mL L-glutamine (Invitrogen, Carlsbad, CA). The gas mixture supplied to the embryos was 60% O_2/35% N_2/5% CO_2 for the first day and 95% O_2/5% CO_2 for the second day. HhAntag an antagonist of the SHH signaling pathway (Frank-Kamenetsky et al., 2002; Gabay et al., 2003; Williams et al., 2003; Romer et al., 2004) was obtained from Genentech (South San Francisco, CA). On the first day, either 1% DMSO, 50 μM HhAntag in 1% DMSO, or 100 μM HhAntag in 1% DMSO was added to the culture medium. Culture medium was replaced after 24 h and 1% DMSO, 50 μM HhAntag/1% DMSO, or 100 μM HhAntag/1% DMSO was added to the new medium.

Measurement of HhAntag Concentration in Embryos

E9.5 embryos were cultured in media under four conditions: 1% DMSO, 1 μM HhAntag + 1% DMSO, 10 μM HhAntag + 1% DMSO, and 100 μM HhAntag + 1% DMSO. After 2 days in culture, the head of each embryo was isolated, weighed, and frozen in liquid nitrogen. Samples were analyzed by ALTA Analytical Laboratory using liquid chromatography mass spectrometry (LC-MS) and a TurboIonSpray ionization source.

Radioactive *In Situ* Hybridization

Frozen sections were prepared and *in situ* hybridization was performed as described previously (Frantz et al., 1994). A minimum of three mutants and three control embryos were analyzed for each probe for *Shh* mutant analysis. Because the brain morphology is abnormal in *Shh* mutants, each brain was sectioned serially from its rostral-most extent back to the hindbrain. The telencephalon was then located within these serial sections and its identity was confirmed by *in situ* hybridization for forebrain markers including *Foxg1* and *Emx2*. For experiments using HhAntag on cultured embryos, a minimum of three embryos were analyzed for each probe, each treatment condition, and each day *in vitro*. Plasmids used to make probes were kindly provided by John Wozney (*Bmp4*), John Rubenstein (*Foxg1*, *Fgf8*), Juan Botas (*Lhx2*), Frederic Charron (*Ptc1*), Andrew McMahon (*Shh*), and William Blaner (*Ttr*).

BrdU Labeling, Immunohistochemistry, and TUNEL Staining

To assess proliferation in cultured embryos, 50 μM BrdU was added to the culture medium for 1 h. Embryos were then fixed in 4% paraformaldehyde overnight, transferred to 30% sucrose overnight, and frozen in OCT. Sections were cut on a cryostat at 15 μm. BrdU staining was done after antigen retrieval in citrate buffer for 30 min, washing in PBS, and blocking for 1 h in PBS containing 0.2% BSA/0.3% Triton/2% goat serum. Sections were incubated with primary antibodies for 2 h then secondary antibodies for 1.5 h at room temperature. The following antibodies were used at a dilution of 1:500: BrdU (Accurate Chemical, Westbury, NY), Ki67 (BD PharMingen, San Diego, CA), Cy2-labeled donkey anti-mouse (Jackson West Grove, PA), and Cy5-labeled goat anti-rat (Jackson). Propidium iodide (Molecular Probes, Eugene, OR) was added at 1:2000. The labeling index was determined by counting the number of BrdU-positive and Ki67-positive cells in radial segments spanning the cerebral wall from the ventricular surface to the pial surface, then dividing the total number of BrdU-positive cells by the number of Ki67-positive cells in that segment. Immunohistochemistry with Tuj1 (Covance, Princeton, NJ) and ZO1 (Transduction Laboratories, San Jose, CA) was performed on fixed frozen sections using Cy2- and Cy5-conjugated secondary antibodies, as earlier. For TUNEL staining, fixed frozen sections were washed in 1% Tween in PBS, then incubated for 1 h in PBS containing 0.2% BSA/0.3% Triton/2% goat serum, washed with PBS, equilibrated for 10 min in TdT buffer to which dATP, dUTP-biotin (Roche, Indianapolis, IN), and TUNEL enzymes (Roche, Indianapolis, IN) were then added for 1 h at 37°C. Slides were washed in 2 × SSC then in PBS, incubated with Cy3-streptavidin (Jackson) at 1:200 for 45 min, washed in PBS, and coverslipped.

Bead Implantation and Explant Culture of Lateral Telencephalon

FGF8, SHH, and BMP4-coated beads were prepared as previously described (Furuta et al., 1997) using recombinant mouse FGF8b (0.5 mg/mL; R&D Systems, Minneapolis, MN), recombinant SHH (0.05 mg/mL; Genentech), or recombinant human BMP4 (0.5 μg/mL; R&D Systems). Implantation of beads into explants of the lateral telencephalon and digoxigenin *in situ* hybridization were performed as described by Furuta et al. (1997). Plasmids for probe preparation were kindly provided by Elizabeth Grove (*Bmp4*, *Fgf8*, *Msx1*, and *Shh*), Andrew MacMahon (*Gli1*), and Edwin Monuki (*Zic2*).

RESULTS

Shh is Required for the Maintenance of Forebrain Signaling Centers

To explore the role of SHH in patterning the dorsal telencephalon, we performed *in situ* hybridization and compared the expression of *Fgf8*, *Bmp4*, and potential downstream effectors in wildtype and *Shh* embryos. Previous studies showed that *Fgf8* is expressed in the anterior neural ridge (ANR) at the 10 somite stage in *Shh* mutants, but expression is lost by E9.0 (Ohkubo et al., 2002). To ascertain whether *Fgf8* expression recovers later in telencephalic development, *in situ* hybridization was performed on E12.5 *Shh* mutants. Wildtype embryos showed strong expression of *Fgf8* at the anterior midline [Fig. 1(A)], but *Shh* deficient mice exhibited a complete loss of *Fgf8* expression in the telencephalon [Fig. 1(B)]. To assess the functional consequences of this loss, we examined the expression of *Foxg1*, which is induced by FGF8 in the forebrain (Shimamura and Rubenstein, 1997; Ye et al., 1998; Storm et al., 2003, 2006). In contrast to the robust expression of *Foxg1* in the wildtype telencephalon [Fig. 1(C)], $Shh^{-/-}$ mice showed very weak *Foxg1* expression [Fig. 1(D)], which may have been triggered by the earlier expression of *Fgf8* in the ANR (Ohkubo et al., 2002). A previous report suggested that *Foxg1* expression was completely lost in *Shh* mutants (Ohkubo et al., 2002); we presume that we could detect low-level expression because of the higher sensitivity of radioactive (vs. digoxygenin) probes for *in situ* hybridization.

In E9.5 *Shh* mutants, the expression domains of the dorsal midline markers *Bmp4* and *Msx1* expand to encompass the entire telencephalon (Ohkubo et al., 2002). We find similar expression at E12.5, with *Bmp4* and *Msx1* expressed throughout the $Shh^{-/-}$ holosphere [Fig. 1(F,H)]. To ascertain whether this misexpression of *Bmp4* and *Msx1* causes an expansion of dorsal midline fates, we assessed the expression of *Ttr*, which marks the choroid plexus [Thomas et al., 1988; Fig. 1(I)]. *Ttr* expression was not evident in *Shh* mutants [Fig. 1(J)], suggesting that broad expression of *Bmp4* and *Msx1* is insufficient to induce dorsal midline differentiation in ectopic locations. This failure might be due to repressive or antagonistic effects of other gene products that are normally excluded from the dorsal midline. Indeed, *Bmpr1b* and *Emx2*, which are normally present in dorsolateral tissues but excluded from the midline [Suppl. Fig. 1(A,C)],

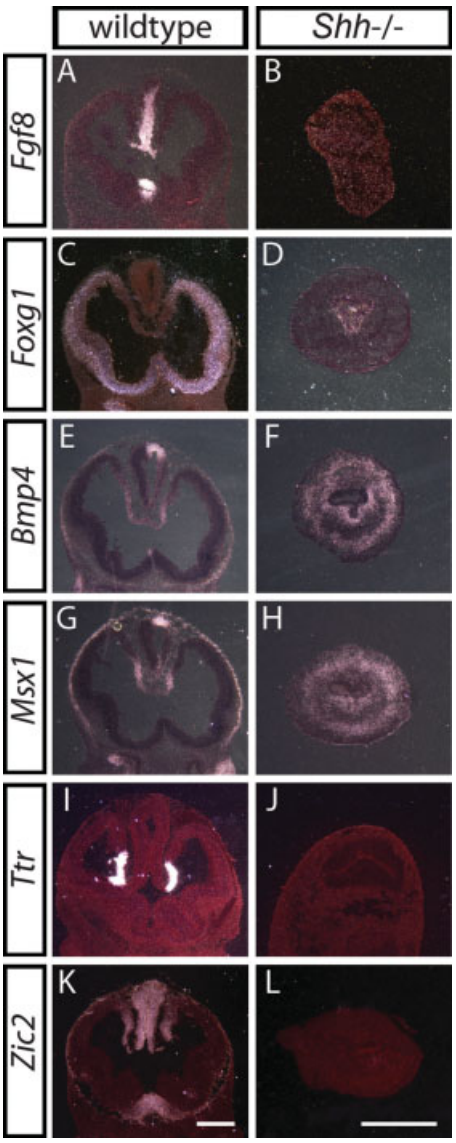

Figure 1 $Shh^{-/-}$ mice show a disregulation of signaling centers in the telencephalon. Gene expression was analyzed by *in situ* hybridization on coronal sections from E12.5 wildtype (A,C,E,G,I,K) and $Shh^{-/-}$ (B,D,F,H,J,L) embryos. (A,B) *Fgf8* is normally expressed at the anterior midline of the telencephalon, but expression is lost in mutants. (C,D) Expression of the FGF8 target gene *Foxg1* encompasses the telencephalon outside of the dorsal midline in contro mice, but is severely reduced in *Shh* mutants. (E,F) *Bmp4* is normally expressed at the dorsal midline at E12.5. In contrast, *Bmp4* expression in *Shh* mutants expands to encompass the holosphere. (G,H) The expression of the BMP4 target *Msx1* closely resembles that of *Bmp4* in both controls and mutants. (I,J) *Ttr* is expressed in the choroid plexus of control embryos, but expression is not present in $Shh^{-/-}$ mice. (K,L) *Zic2* is normally expressed at the midline (both dorsally and ventrally) of the telencephalon, and expression is also visible in the dorsal thalamus in controls, but expression is lost in *Shh* mutants. Scale bars: 0.5 mm.

are ectopically expressed at the dorsal midline of *Shh* mutants [Suppl. Fig. 1(B,D)]. These data suggest that *Bmp4* and *Msx1* expression alone is insufficient to induce dorsal midline fates.

Shh is Required for the Appropriate Expression of *HPE* Genes

Many of the genes disrupted in human HPE encode components of signaling pathways involved in ventral development (Brown et al., 1998, 2001; Wallis et al., 1999; Gripp et al., 2000; Laflamme et al., 2004). To better understand, how the loss of SHH and disruption of signaling centers affect the expression of other *HPE* genes and ultimately disrupt the dorsal midline development, we examined the expression of *Six3, Tgif*, and *Zic2* in *Shh* mutants. In wildtype animals, *Six3* is expressed in the ventral telencephalon [Suppl. Fig. 1(G)]; expression is lost in *Shh* mutants [Suppl. Fig. 1(H)]. *Tgif1* is normally expressed in the dorsal and ventral midline [Suppl. Fig. 1(I)]; it is weakly expressed throughout holosphere in *Shh* mutants [Suppl. Fig. 1(J)]. Interestingly, although *Tgif* has been implicated in human cases of HPE, mice with a targeted disruption of *Tgif1* do not exhibit an HPE phenotype (Shen and Walsh, 2005). In wildtype animals, *Zic2* is expressed throughout the midline, including the dorsal telencephalon [Fig. 1(K)]. $Shh^{-/-}$ mice show a complete loss of *Zic2* expression in the telencephalon by E12.5 [Fig. 1(L)]. Because mice are hypomorphic for *Zic2* they show HPE (Nagai et al., 2000), the loss of *Zic2* expression in the $Shh^{-/-}$ telencephalon is consistent with the hypothesis that *Zic2* is regulated by SHH signaling to mediate dorsal midline formation. However, the fact that SHH localizes to the ventral telencephalon suggests that this regulation is indirect. We further explore this hypothesis later in this article.

HhAntag Represses SHH Signaling and Induces an HPE Phenotype *In Vitro*

Both the present and previous analyses of *Shh* mutant mice reveal that SHH regulates the *Fgf8* and *Bmp4* signaling centers. However, it is not known whether ongoing SHH signaling is required to maintain these signaling centers during dorsal midline formation. To explore the role of SHH in the maintenance of organizing centers and dorsal midline development, we utilized a specific Hh antagonist called HhAntag to block SHH signaling in wildtype embryos after the establishment of the BMP4 and FGF8 signaling centers. HhAntag was identified in a screen for small molecule antagonists of Hh signaling; it blocks signaling specifically by binding directly to Smoothened (SMO; Frank-Kamenetsky et al., 2002; Gabay et al., 2003; Williams et al., 2003; Romer et al., 2004).

Because, we had access to limited amounts of HhAntag, which precluded treatment of pregnant mothers via oral gavage, we used a whole embryo culture system and applied the drug directly to the developing embryos. E9.5 was chosen as a starting point for the culture period, because at this stage both *Fgf8* and *Bmp4* expression has been established in the forebrain, but the two cerebral hemispheres have not yet cleaved. E9.5 embryos were cultured in heat-inactivated rat serum containing 1% DMSO as a control, or in either a low dose (50 μM) or high dose (100 μM) of HhAntag for 2 days [Fig. 2(A)]. We found that concentrations of <50 μM HhAntag produced no effects on developing embryos (data not shown), whereas the range of $50-100$ μM produced phenotypes reminiscent of those in *Shh* mutants, such as smaller limbs and an unusually shaped tail [Chiang et al., 1996; Fig. 2(B–D)]. Curiously, the concentrations of HhAntag needed to cause phenotypic differences between control and treated cultured embryos were higher than concentrations required in tissue culture or explants to block SHH signaling (Frank-Kamenetsky et al., 2002; Williams et al., 2003; Romer et al., 2004). To ascertain whether the diffusion of HhAntag from the culture medium into the developing brain may have been impeded (for example, by nonneural tissues such as skin and skull), we cultured embryos in HhAntag then measured the amount of drug that actually penetrated into head tissues using LC-MS. These measurements revealed that the heads of mice treated with 1, 10, or 100 μM HhAntag contained only about one-third of the concentration of HhAntag present in culture medium (Table 1). Because whole heads were used for LC-MS analysis, it is possible that the concentration of HhAntag in brain tissue was even lower than that measured from entire sample, since the skin, skull, and meninges may have each further restricted drug penetration into the nervous system. Nevertheless, the effective concentrations of HhAntag in whole heads were roughly within an order of magnitude of those used in previous studies of cultured cells.

Control embryos cultured in 1% DMSO vehicle grew at normal rates *in vitro*; the dorsal midline underwent its characteristic invagination and two discrete hemispheres were obvious after 2 days *in vitro* [DIV2; Fig. 2(C)]. A subset of embryos treated with HhAntag, in contrast, exhibited dramatic morphological differences compared with controls. First, they appeared much smaller in size, particularly at the

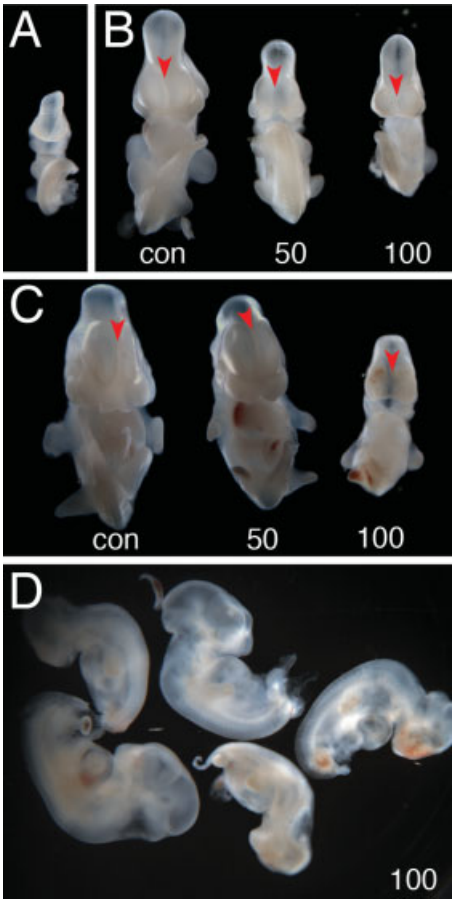

Figure 2 HhAntag induces HPE in cultured whole embryos. (A) Rostral view of a typical E9.5 embryo at DIV0 of a whole embryo culture experiment. (B) At DIV1, control (con) embryos resembled wildtype embryos at E10.5, with telencephalic midline structures beginning to emerge. Embryos treated with 50 μM (50) or 100 μM (100) HhAntag were somewhat smaller than control embryos. (C) At DIV2, control embryos resembled wildtype E11.5 embryos and showed a clear bifurcation of the telencephalon (arrowhead). Embryos treated with 50 μM HhAntag were slightly smaller than controls and had underdeveloped limbs; however, the telencephalic hemispheres appeared well-divided (arrowhead). Embryos treated with 100 μM HhAntag were small, had underdeveloped limbs, and showed an unbifurcated telencephalon that resembled HPE (arrowhead). (D) Embryos treated with 100 μM HhAntag displayed variable phenotypes, even when cultured together.

higher concentration [Fig. 2(B,C)], a defect was also observed in $Shh^{-/-}$ mice (Chiang et al., 1996). Second, although bifurcation of the hemispheres was evident in embryos treated with 50 μM HhAntag, many of those treated with 100 μM HhAntag lacked well-divided cerebral hemispheres [Fig. 2(C)], suggesting that treatment resulted in HPE. However, the phenotypes of embryos treated with 100 μM HhAntag were heterogeneous [Fig. 2(D)]: some showed minor defects such as a slight reduction in size or partially

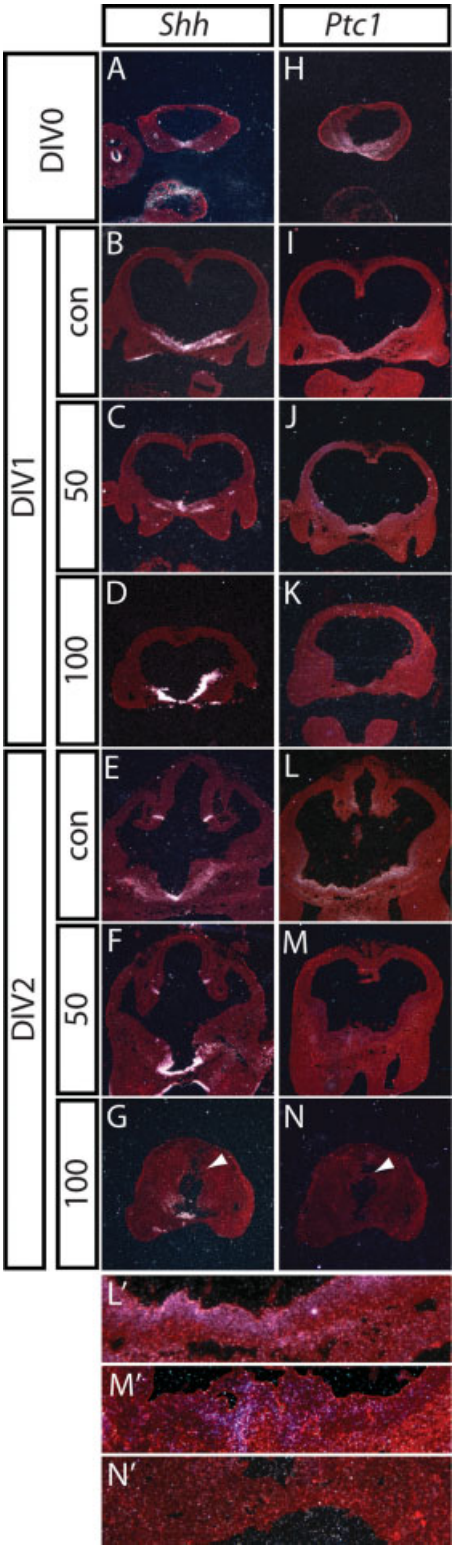

Figure 3

Table 1 E9.5 Embryos Were Cultured in Media for 2 Days Under Three Conditions: 1 μM HhAntag + 1% DMSO, 10 μM HhAntag + 1% DMSO, and 100 μM HhAntag + 1% DMSO

Experiment Number	[HhAntag] in Media (μM)	[HhAntag] in Tissue (μM)	Average [HhAntag] in Tissue (μM)
1	1	0.20	0.31 + 0.23
2	1	0.20	
3	1	0.18	
4	1	0.27	
5	1	0.73	
6	10	3.77	3.79 + 2.12
7	10	6.76	
8	10	2.73	
9	10	1.92	
10	100	43.6	42.74 + 28.58
11	100	53.1	
12	100	13.3	
13	100	86.2	
14	100	17.5	

Each experiment represents one embryo head sample. Samples were analyzed by ALTA Analytical Laboratory using liquid chromatography mass spectrometry (LC-MS) and a TurboIonSpray ionization source to obtain the concentration of HhAntag in the tissue. The average HhAntag concentration was obtained by averaging all experiments for a given condition.

divided hemispheres, while others were quite small with fully fused hemispheres. We therefore wanted to ascertain whether HhAntag successfully blocked Shh signaling in individual embryos.

HhAntag binds directly to the cell surface protein SMO to inhibit Hh signaling (Williams et al., 2003). *Shh* expression itself does not require ongoing Hh signaling (Cordero et al., 2004) and thus should remain unperturbed by HhAntag. As predicted, HhAntag-treated embryos expressed *Shh* normally in the ventral forebrain [Fig. 3(A–G)]. In contrast, the Hh receptor Patched 1 (Ptc1) is transcriptionally regulated by Hh signaling, with high levels of Hh inducing increased *Ptc1* expression (Ingham and Fietz, 1995; McMahon et al., 2003). Thus, the disruption of SHH signaling by HhAntag should result in the downregulation of *Ptc1*, and any variability in the extent of inhibition should be reflected in the level of *Ptc1* expression, in that embryos treated with a high dose of HhAntag should lack *Ptc1* expression, whereas low-dose-treated embryos should show weak expression. Consistent with these predictions, *Ptc1* was expressed very weakly in the ventral telencephalon at DIV1 of embryos treated with 100 μM of HhAntag [Fig. 3(K)]; embryos treated with 50 μM showed higher expression [Fig. 3(J)], and maximal expression was observed in controls [Fig. 3(I)]. At DIV2, *Ptc1* expression remained high in controls [Fig. 3(L,L′)], whereas embryos treated with 50 μM HhAntag showed weak expression [Fig. 3(M,M′)]. *Ptc1* expression was lost completely in 7 of the 16 embryos treated with 100 μM HhAntag [Fig. 3(N,N′)]. These data parallel the variability in penetration seen in embryos treated with 100 μM HhAntag (Table 1), which suggest that when a high dose of antagonist effectively penetrated the head, SHH signaling was silenced efficiently after 2 days.

To assess whether the heterogeneity in phenotypic responses to HhAntag treatment [Fig. 2(D)] was correlated with the extent to which *Shh* signaling was actually disrupted in each embryo, we compared the expression of *Ptc1* in the ventral telencephalon with the phenotype observed in each embryo. In the nine (of 16) HhAntag-treated embryos that exhibited relatively normal development (larger body size, evidence of hemisphere formation), each maintained *Ptc1* expression in the ventral telencephalon, suggesting that *Shh* signaling was not functionally impaired. In contrast, the seven embryos that exhibited smaller bodies and hemispheric fusion also showed a marked decrease in ventral *Ptc1* expression (data not shown), consistent with a successful inhibition of SMO function. These observations support the idea that the range of phenotypic responses to HhAntag results from variable penetration of the drug into developing tissues. Thus, for subsequent analyses of embryos treated with 100 μM HhAntag and analyzed at DIV2, we include only those embryos in which *Ptc1* expression was absent from the ventral telencephalon.

Figure 3 SHH signaling is inhibited in embryos cultured with HhAntag. (A–G) The expression of *Shh* mRNA was normal at all times in culture, regardless of whether embryos were cultured in control medium (con), 50 μM (50), or 100 μM (100) HhAntag. In all cases, *Shh* was expressed at and adjacent to the ventral midline. (H–N) *Ptc1* mRNA expression was used to assess the level of SHH signaling in cultured embryos. (H) *Ptc1* expression at DIV0 was observed in the ventral telencephalon. (I,J,K) At DIV1, *Ptc1* expression in control and 50 μM HhAntag-treated embryos was evident in the ventral telencephalon, but was severely reduced in embryos cultured in 100 μM HhAntag. (L,M,N) At DIV2, control embryos showed strong ventral expression of *Ptc1*, but expression was weak in embryos cultured in 50 μM HhAntag and absent in those cultured in 100μM HhAntag. Arrowheads in (M,N) mark an uneven ventricular surface that was present in some 100 μM HhAntag-treated embryos. (L′,M′,N′) Higher-power views of ventral *Ptc1* expression in control (L′), 50 μM HhAntag (M′), and 100 μM HhAntag (N′)-treated embryos at DIV2. Scale bars: 0.5 mm.

HhAntag Treated Embryos Show Normal Levels of Proliferation and Apoptosis, But Neurons Differentiate Prematurely

During normal development between E9.5 and E11.5, the telencephalon expands rapidly due to the proliferation of progenitors, with little neurogenesis. This produces a relatively thin, balloon-like cerebral wall [Fig. 3(I,J)]. However, in embryos treated at the high dose of HhAntag, the cerebral vesicles failed to expand and the cerebral wall was thicker than in controls [Fig. 3(G,N)], a phenotype was also obvious in $Shh^{-/-}$ mice (Chiang et al., 1996). The smaller overall size of the telencephalon could result from increased cell death or decreased proliferation. To assess apoptotic cell death, we performed TUNEL staining on coronal brain sections. These experiments showed no obvious differences in the numbers of TUNEL positive cells in control vs. 100 μM HhAntag-treated embryos; in both, very few labeled cells (1–3 per section) were apparent in the cortex [Fig. 4(A,B)]. TUNEL positive cells were most noticeably present at the dorsal midline in both conditions, consistent with high levels of BMP4 inducing cell death in this region (Furuta et al., 1997). The absence of obvious increases in apoptosis within the forebrain suggests that increased cell death cannot explain the smaller brain size of HhAntag-treated embryos. We note that these results differ from those in $Shh^{-/-}$ and conditional *Smo* knockout mice, which show significantly increased levels of telecephalic cell death (Dahmane et al., 2001; Fuccillo et al., 2004). It is possible that the timing of HhAntag treatment failed to match the period during which *Shh* signaling is required for cell survival.

Shh mutant mice also show marked decreases in the proliferation of telencephalic progenitors, which likely contributes to their smaller brain size as well (Dahmane et al., 2001). To assess proliferation in HhAntag-treated embryos, the nucleotide analog BrdU was added to the culture medium 1 h before the end of the 2-day culture period. The labeling index was determined by counting the percentage of BrdU-positive cells in the ventricular zone (VZ), which was immunostained for Ki67 to label all cycling cells [Fig. 4(B)]. By limiting the counts to include only actively cycling Ki67-positive cells, changes in the fraction of BrdU-labeled cells directly reveal changes in the length of the cell cycle (Woodhead et al., 2006), with a shorter cell cycle reflected as an increase in the fraction of BrdU-positive cells that also express Ki67. The labeling index in the dorsal telencephalon of controls (48.6 $\pm$ 1.7% SD) did not differ statistically from that in 100 μM HhAntag-treated embryos [49.8 $\pm$ 1.2%; Fig. 4(C)]. Thus, the smaller brain size in the latter is not due to alterations in the length of S-phase. However, Ki67-positive cells encompassed nearly the entire thickness of the telencephalic wall in controls [Fig. 4(B)], suggesting that the vast majority of cells continued to cycle and thus expand the area of the VZ. In embryos treated with 100 μM HhAntag, in contrast, Ki67-positive cells were confined to the region adjacent to the lateral ventricle and were surrounded by a domain that lacked Ki67 [Fig. 4(B)]. These observations raised the possibility that the smaller size of the cerebral vesicles following HhAntag treatment may result from the premature exit of progenitors from the cell cycle.

To ascertain whether the Ki67-negative cells in HhAntag-treated embryos differentiated precociously into neurons, we stained sections for the neuronal marker Tuj1. These experiments revealed an obvious increase in the number of Tuj1-positive cells in HhAntag-treated embryos when compared with controls [Fig. 5(A,B)]. This increase suggests that many VZ progenitor cells underwent terminal mitosis, as indicated by the loss of Ki67 staining, then adopted a neuronal fate prematurely. This behavior is also likely to disrupt the expansion of the cerebral hemispheres by depleting the VZ of progenitors, resulting in a smaller brain size. Interestingly, in a previous study in which the SHH effector *Smo* was disrupted by conditional mutagenesis starting at $\sim$E9.0 (Fuccillo et al., 2004) Tuj1 immunoreactivity in the ventral telencephalon was reduced at E10.5; this was interpreted to reflect perturbations in early telencephalic patterning rather than a direct effect on neurogenesis. The same experiments produced no obvious alterations of Tuj1 staining in the dorsal telencephalon (Fuccillo et al., 2004), suggesting that the precise timing of SMO disruption may be critical in governing differentiation and cell survival.

Ongoing SHH Signaling is Required for Dorsal Midline Formation and Maintenance of Forebrain Patterning Centers

In mice in which *Smo* was conditionally disrupted starting at $\sim$E9.0, the dorsal midline formed normally, leading the authors to conclude that SHH signaling must be required before E9.0 to pattern the dorsal telencephalic midline (Fuccillo et al., 2004). In contrast, here we find that culturing whole embryos for 2 days with HhAntag between E9.5 and E11.5 severely disrupts dorsal midline formation, whereas

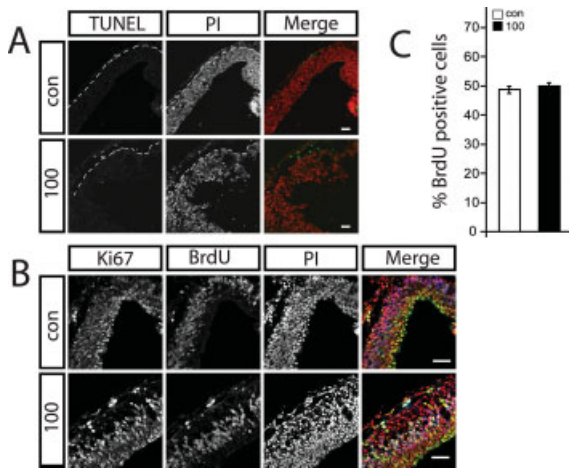

Figure 4 Embryos cultured in HhAntag showed normal levels of apoptosis and of BrdU incorporation by cycling cells in the telencephalon. (A,B) Brain sections were stained with propidium iodide (PI, red) to label cell bodies. (A) TUNEL staining (green) marked apoptotic cells, which were present primarily in the region outside of the telencephalon (the boundary between the telencephalon and epidermis is marked by dashed line) and at the dorsal midline (not shown) of both control (con) and 100μM HhAntag (100)-treated embryos. No differences in the extent or location of TUNEL-positive cells were observed following treatment with HhAntag. (B) Ki67 immunostaining (green) was used to identify proliferating cells, and pulse labeling with BrdU (blue) identified cells in S-phase. A domain of Ki67-negative cells was apparent near the pial surface of embryos treated with 100 μM HhAntag. (C) The fraction of Ki67-positive cells that also incorporated BrdU was assessed from sections similar to those in (B) ($n = 5$ control and 3 HhAntag-treated embryos). The resulting labeling index revealed no significant differences in BrdU incorporation between control and HhAntag-treated embryos. Scale bars: 50 μm.

culturing them with vehicle (1% DMSO) had no effect. Although there was no morphological evidence of hemispheric cleavage at the start of the culture period [Fig. 3(A,H)], the midline was invaginated dorsally at DIV1 in control embryos [Fig. 3(B,I)] and clearly delineated into two discrete cerebral hemispheres at DIV2 [Fig. 3(E,L)]. 100 μM HhAntag blocked dorsal midline formation either partially [Fig. 3(D)] or completely [Fig. 3(G,K,N)]. Intermediate effects were seen with 50 μM HhAntag [Fig. 3(C,E,F,J,K,L,M)], in which midline invagination was less pronounced than in controls. The difference between our results and those from *Smo* conditional knockouts (Fuccillo et al., 2004) is puzzling, but may have arisen because the latter method requires a turnover of both *Smo* mRNA and protein before the consequences of a genetic loss of function can be seen, whereas HhAntag is expected to block ongoing SHH signaling immediately.

As discussed earlier, it seems unlikely that SHH directly induces dorsal midline formation; instead, we hypothesize that SHH acts indirectly by regulating the expression of other signaling molecules such as FGF8 and BMPs in the anterior and dorsal midline regions of the telencephalon. Previous studies demonstrated that the FGF8 and BMP signaling centers have been established by E9.5 (Furuta et al., 1997; Maruoka et al., 1998), the time at which HhAntag treatment was initiated. This was confirmed by *in situ* hybridization, which revealed the expression at E9.5 of *Fgf8* at the anterior midline, both dorsally and ventrally [Fig. 6(A)], and of *Bmp4* at the dorsal midline [Fig. 7(A)].

Fgf8 expression was assessed in HhAntag-treated and control embryos, and the latter showed strong expression of *Fgf8* throughout the 2-day culture period [Fig. 6(B,E)]. In contrast, embryos treated with

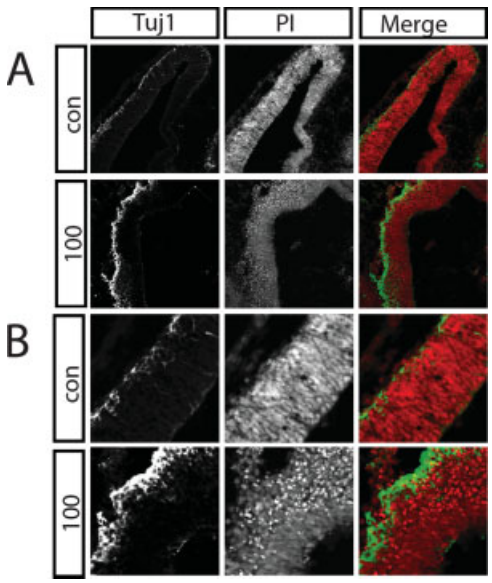

Figure 5 Premature neuronal differentiation in embryos treated with HhAntag. Brain sections were stained with propidium iodide (PI, red) to label cell bodies and TuJ1 antibodies (green) to identify differentiating neurons. (A) Tuj1 staining revealed that few cells have differentiated into neurons in control (con) embryos at DIV2, and the magnified view in (B) shows that staining was confined to a sparse population of cells adjacent to the pial surface. In contrast, the domain of TuJ1 immunoreactivity in embryos treated with 100 μM HhAntag (100) was much broader than that in controls, suggesting that the Ki67-negative cells shown in Figure 4 have differentiated prematurely into neurons. Scale bars: 50 μm.

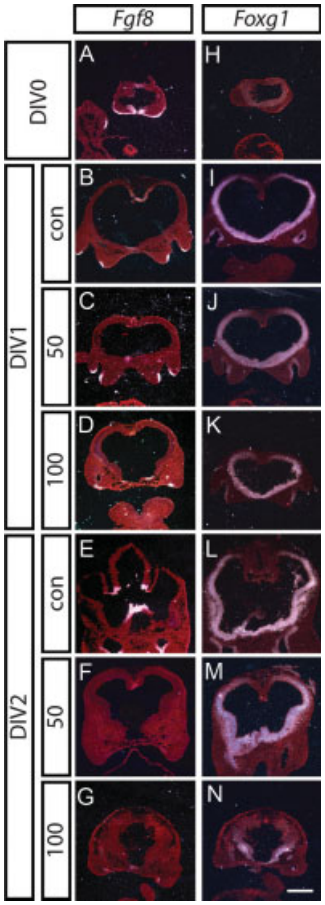

Figure 6 Inhibition of SHH signaling with HhAntag results in a loss of *Fgf8* and diminution of *Foxg1* expression in the telencephalon. *In situ* hybridization was performed for *Fgf8* (A–G) and *Foxg1* (H–N) mRNAs on coronal sections from embryos cultured in control medium (con), 50 μM HhAntag (50), or 100 μM HhAntag (100). (A) At DIV0, *Fgf8* expression was already established at the anterior midline of the telencephalon. (B,E) *Fgf8* expression in control embryos intensified over the 2 days of culture. (C,D,F,G) *Fgf8* expression was markedly diminished at DIV1 upon treatment with either 50 μM or 100 μM HhAntag (C,D) and was lost in most embryos treated by DIV2 (F,G), although very weak expression was sometimes observed at the ventral midline (G). (H) At DIV0, *Foxg1* was expressed at low levels in the developing telencephalon. (I,J,K) Strong expression of *Foxg1* was observed in all cultured embryos at DIV1. (L,M) *Foxg1* expression remained robust in embryos cultured in control medium or 50 μM HhAntag. (N) In contrast, *Foxg1* was expressed weakly in the telencephalon following a 2-day exposure to 100 μM HhAntag. Scale bar: 0.5 mm.

either dose of HhAntag showed a progressive reduction in *Fgf8* expression, evidenced by weak *Fgf8* signal in the anterior midline at DIV1 [Fig. 6(C,D)] and a complete loss of expression by DIV2 [Fig. 6(F,G)]. Hence, continuous SHH signaling is required to maintain the previously established *Fgf8* signaling center. To examine the functional consequences of the loss of FGF8, we assessed *Foxg1* expression, which is normally induced by FGF8 (Shimamura and Rubenstein, 1997). No obvious alterations in *Foxg1* expression were observed in embryos treated with 50 μM HhAntag [Fig. 6(J,M)] or at DIV1 following treatment with 100 μM HhAntag [Fig. 6(K)]. However, at DIV2, the latter displayed a decreased but visible *Foxg1* signal in dorsal telencephalon [Fig. 6(N)] relative to controls [Fig. 6(L)]. Collectively, these data suggest that early, transient expression of FGF8 may have been sufficient to induce the continued (albeit reduced) expression of *Foxg1* throughout most of the forebrain, but that continued FGF8 expression is probably required to maintain wild-type levels of *Foxg1*.

Since HhAntag treatment causes a loss of *Fgf8* expression that mirrors that seen in *Shh* mutants, we hypothesized that these embryos would experience an expansion of *Bmp4* expression, as in *Shh* mutants

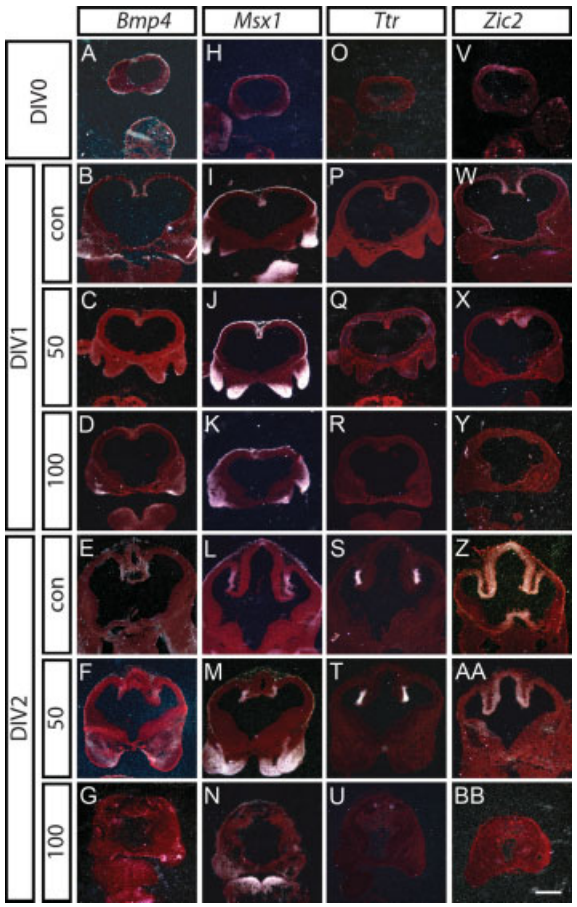

Figure 7

(Ohkubo et al., 2002). Surprisingly, this was not the case. At DIV1, control embryos expressed *Bmp4* at the telencephalic dorsal midline [Fig. 7(B)], as did embryos treated with either dose of HhAntag [albeit at somewhat lower levels than in the controls; Fig. 7(C,D)]. After 2 days of exposure to 50 μM HhAntag, *Bmp4* continued to be expressed in a domain similar to that in controls, but at reduced levels [Fig. 7(E,F)]. However, in embryos treated with 100 μM HhAntag, *Bmp4* expression was lost completely from the dorsal midline [Fig. 7(G)]. Thus, in contrast to the expansion of *Bmp4* expression in *Shh* mutants, *Bmp4* expression was abrogated by treatment with a high dose of HhAntag. To ascertain whether the precise timing of interference with SHH signaling is responsible for this difference, we initiated whole embryo cultures and HhAntag treatment a day earlier, at E8.75. However, in these experiments neither the *Bmp4* nor *Msx1* expression domains were expanded, instead a loss of expression was observed for both genes (data not shown). Thus, it appears that SHH signaling must be disrupted before E8.75 to mimic the expansion of *Bmp4* and *Msx1* expression observed in $Shh^{-/-}$ mice.

To ascertain whether the loss of *Bmp4* expression in HhAntag-treated embryos altered the expression of BMP target genes, we assessed the expression of *Msx1* (Furuta et al., 1997). In control embryos, *Msx1* showed a normal pattern of expression first at the dorsal midline [Fig. 7(I)] and later in the cortical hem [Fig. 7(L)], overlapping with that of *Bmp4* [Fig. 7(B,E)]. In embryos cultured from E9.5 to E11.5 in 50 μM HhAntag, *Msx1* expression was comparable with that in controls [Fig. 7(I,J,L,M)]. However, 100 μM HhAntag caused a marked reduction of *Msx1* expression after DIV1 [Fig. 7(K)] and the expression was lost at DIV2 [Fig. 7(N)], consistent with the loss of *Bmp4* expression in these embryos. To ascertain whether the loss of *Bmp4* and *Msx1* expression disrupted the production of differentiated dorsomedial fates, we assessed the expression of the choroid plexus marker *Ttr*. Both controls and embryos treated with 50 μM HhAntag displayed *Ttr* expression at DIV2 [Fig. 7(S,T)], consistent with the normal onset of *Ttr* expression at E11.5 *in vivo* (Thomas et al., 1988). Interestingly, despite the loss of *Bmp4* and *Msx1* expression, embryos treated with 100 μM HhAntag showed weak *Ttr* expression in two faint domains on either side of the dorsal midline [Fig. 7(U)]. These data suggest that earlier expression of *Bmp4* and *Msx1* before or during the first day of HhAntag treatment was sufficient to weakly induce choroid plexus differentiation, but that continued *Bmp4* and *Msx1* expression is required for the production of a normal dorsal midline. Collectively, these studies suggest that SHH signaling is required for the normal maintenance of the BMP signaling center after its initial establishment at the dorsal midline. Furthermore, our data are consistent with previous work (Chiang et al., 1996; Golden et al., 1999; Panchision et al., 2001) suggesting that either the expansion or loss of *Bmp4* can result in HPE.

Figure 7 HhAntag treatment disrupts the expression of *Bmp4* and other dorsal midline markers in the developing telencephalon. *In situ* hybridization for (A–G) *Bmp4*, (H–N) *Msx1*, (O–U) *Ttr*, and (V–BB) *Zic2* mRNA was performed on coronal sections from embryos cultured in control medium (con), 50 μM HhAntag (50), or 100 μM HhAntag (100). (A) At DIV0, *Bmp4* expression was apparent at the dorsal midline. (B–D) At DIV1, *Bmp4* was expressed at the dorsal midline in controls, but expression was weak in embryos treated with 50μM or 100μM HhAntag. (E–G) At DIV2, *Bmp4* expression at the dorsal midline remained apparent in controls and embryos exposed to 50 μM HhAntag. However, 100 μM HhAntag treatment resulted in a loss of *Bmp4* expression at the midline. (B,H) At DIV0, *Msx1* expression was evident in a pattern similar to that of *Bmp4*. (I,J,L,M) Expression of *Msx1* at the dorsal midline appeared comparable in control and 50μM HhAntag treated embryos at DIV1 and DIV2. (K,N) In embryos treated with 100 μM HhAntag, *Msx1* expression appeared weaker than controls at DIV1 was lost at DIV2. (O–R) *Ttr*, a marker of the dorsal midline, was not expressed at DIV0 or DIV1 in any embryos. (S,T) *Ttr* was expressed strongly by differentiating choroid plexus cells at the dorsal midline of control and 50μM HhAntag-treated embryos at DIV2. (U) Treatment with 100 μM HhAntag resulted in markedly lowered levels of *Ttr* expression, which marked two small domains on either side of the midline at DIV2. (V) Weak midline expression of *Zic2* was evident at DIV0. (W,X,Z,AA) *Zic2* was expressed at the dorsal midline of embryos exposed to control medium or 50 μM HhAntag at both DIV1 and DIV2. (Y,BB) *Zic2* expression was barely detectable at DIV1 and absent at DIV2 in embryos treated with 100 μM HhAntag. Scale bar: 0.5 mm.

Temporal Requirements for SHH Signaling

The earlier experiments demonstrate that HhAntag treatment for 2 days can phenocopy many of the effects on dorsal midline formation of germline mutations in *Shh*. To ascertain whether removing SHH signaling during only 1 of the 2 DIV is sufficient to disregulate the *Fgf8* and *Bmp4* signaling centers, we cultured embryos for 2 DIV as earlier, but

treated with 100 μM HhAntag during only the first or the second DIV. Unfortunately, it appeared that SHH signaling was not blocked completely under these conditions: although *Ptc1* expression was reduced compared with control embryos, we still observed a moderate level of *Ptc1* mRNA in the ventral telencephalon [Suppl. Fig. 2(A–C)]. This partial repression of SHH signaling did not obviously disregulate genes important for telencephalic patterning; the expression patterns of *Shh*, *Fgf8, Foxg1, Bmp4, Msx1*, and *Zic2* all appeared similar to those in control embryos, regardless of the timing of HhAntag treatment [Suppl. Fig. 2(D–U)]. These experiments imply either that SHH signaling works at a threshold in an "all or none" fashion to establish and maintain the *Bmp4* and *Fgf8* signaling centers during midline formation, or that a single day's disruption of SHH was insufficient to abrogate its patterning activities.

Zic2 Expression is Lost in HhAntag-Treated Embryos

In considering how SHH acts at a distance to induce the dorsal midline, Zic2 is an interesting candidate effector since mutations in *Zic2* cause HPE both in humans (Brown et al., 1998, 2001) and mice (Chiang et al., 1996). We showed earlier that *Shh* is required for the induction of *Zic2* expression. To determine if the maintenance of *Zic2* expression requires SHH signaling, we performed *in situ* hybridization on embryos treated with HhAntag. *Zic2* is already expressed at the dorsal midline at E9.5 in controls [Fig. 7(V)], and this pattern extends and strengthens to encompass the ventral midline over 2 days in culture [Fig. 7(W,Z)]. Embryos treated with 50 μM HhAntag continued to express *Zic2* at the dorsal midline [Fig. 7(X,AA)] in a pattern similar to that in controls. However, embryos treated with 100 μM HhAntag showed very weak expression of *Zic2* at DIV1 [Fig. 7(Y)], and by DIV2 *Zic2* expression at the midline was lost completely [Fig. 7(BB)]. Thus, *Zic2* appears quite sensitive to the disruption of SHH signaling.

FGF8 can Induce Zic2 Expression

The upstream regulators of *Zic2* expression are unknown. Although *Zic2* expression is reduced in HhAntag-treated embryos, it is unlikely that SHH directly induces *Zic2* expression, given their nonoverlapping expression patterns. Two pieces of evidence suggest that either BMP4 or FGF8 might directly regulate *Zic2* at the dorsal midline. First, the expression

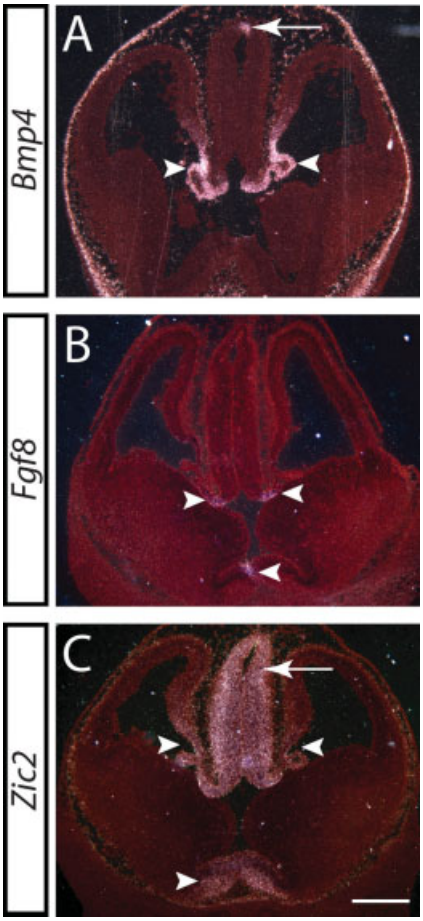

Figure 8 *Bmp4*, *Fgf8*, and *Zic2* mRNAs are expressed in the dorsal midline. *In situ* hybridization was performed on coronal sections from E12.5 wildtype embryos. (A) *Bmp4* is expressed at the dorsal midline of both the telencephalon (arrowheads) and diencephalon (arrow). (B) *Fgf8* is expressed in the dorsal and ventral anterior midline (arrowheads). (C) The domain of *Zic2* expression overlaps with that of *Bmp4* at the telencephalic dorsal midline (arrowheads), and lies adjacent to that of *Fgf8* at the ventral and dorsal midline. The pattern of *Zic2* expression in the diencephalon is broader than that of either *Bmp4* or *Fgf8*, but is not under study here. Scale bar: 0.5 mm.

of *Bmp4* and *Fgf8* overlaps at least in part with that of *Zic2* (Fig. 8). *Bmp4* and *Zic2* are both expressed in the most medial regions of the developing hippocampal field, choroid plexus, and eminentia thalami (which connect the telencephalon and diencephalon), although *Zic2* expression extends further to encompass the dorsal thalamus whereas *Bmp4* expression remains restricted to the roofplate [Fig. 8(A,C)]. *Fgf8* and *Zic2* expression overlaps within both the ventral and dorsal midline regions of the telencephalon, although the *Zic2* expression domain is broader than

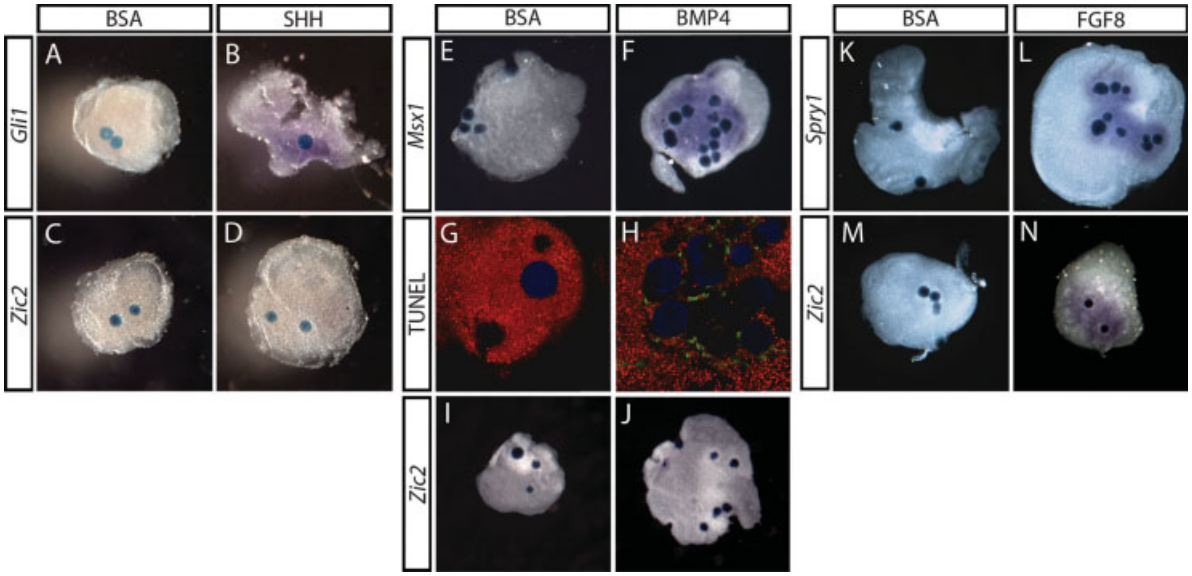

Figure 9 FGF8 induces *Zic2* expression in lateral telencephalic explants. Explants from E10.5 embryos were cultured in the presence of beads coated with BSA, 0.05 mg/mL SHH, 0.5 μg/mL BMP4, or 0.5 mg/mL FGF8. (A,B) Beads coated with SHH induced a halo of *Gli1* mRNA expression (purple) surrounding the beads, while beads coated with BSA did not. (C,D) Neither BSA nor SHH induced the expression of *Zic2* mRNA. (E,F) Beads coated with BMP4 induced a halo of *Msx1* mRNA expression (purple), while beads coated with BSA did not. (G,H) Similarly, BMP4-coated beads, but not those coated in BSA, induced an increase in cell death as assessed by TUNEL staining (green); propidium iodide staining is shown in red. (I,J) Neither BSA nor BMP4 induced the expression of *Zic2*. (K,L) Beads coated with FGF8, but not those coated with BSA, induced the expression of *Spry1* mRNA (purple) in lateral telencephalic explants. (M,N) FGF8-coated beads strongly induced the expression of *Zic2* (purple), but no induction was seen with BSA.

that of *Fgf8* [Fig. 8(B,C)]. Second, *Zic2* expression is lost in HhAntag-treated embryos, coincident with the loss of *Fgf8* and *Bmp4* expression (Figs. 6 and 7). These observations raise the possibility that either BMP4 or FGF8 might directly induce the expression of *Zic2*.

To test this, beads coated with either SHH, BMP4, or FGF8 proteins were implanted into tissue explants from the E9.5 lateral telencephalon (which does not normally express *Bmp4*, *Fgf8*, *Shh*, or *Zic2*), and explants were cultured for 1 day before assessing *Zic2* expression. To eliminate the possibility that SHH may directly induce *Zic2* expression, we coated beads with SHH and first confirmed that these beads induce the expression of the SHH target gene *Gli1* [Fig. 9(B)], whereas BSA-coated beads did not [Fig. 9(A)]. We then evaluated the expression of *Zic2* in response to beads coated with either BSA or SHH, and found that neither successfully induced the expression of *Zic2* in telencephalic explants [Fig. 9(C,D)], as anticipated.

To address the possibility that *Zic2* might be induced by BMP4, we first performed two positive control experiments to confirm that beads coated with BMP4 were biologically active. Previous studies by Furuta et al. (1997) showed that BMP4-coated beads induce *Msx1* expression and cause high levels of apoptosis in the region adjacent to the beads. *In situ* hybridization on our explants revealed that *Msx1* was induced in a halo surrounding beads coated with BMP4 but not BSA [Fig. 9(E,F)], and TUNEL staining revealed high levels of apoptosis surrounding BMP4-coated beads [Fig. 9(H)]. We then assessed the expression of *Zic2*, but we did not observe any induction of *Zic2* adjacent to either BMP-coated or control beads [Fig. 9(I,J)]. These data suggest that BMP4 does not directly induce *Zic2*.

We then performed a similar experiment using beads coated with FGF8. As a positive control, we demonstrated that FGF8-coated beads induced the expression of *Sprty1* in lateral telencephalic explants [Fig. 9(L)], confirming that FGF8 can induce this downstream target gene in an ectopic location (Minowada et al., 1999). We then assessed *Zic2* expression in similar experiments, and found that *Zic2* was expressed robustly in the area surrounding FGF8-

coated beads but not beads coated with BSA [Fig. 9(M,N)]. These data show that FGF8 can induce *Zic2* expression in the forebrain, and suggest that FGF8 expression at the anterior midline may be responsible for the adjacent patterns of *Zic2* expression *in vivo*.

DISCUSSION

A substantial body of evidence suggests that SHH signaling plays a critical role in patterning the embryonic forebrain (Chiang et al., 1996; Crossley et al., 2001; Ohkubo et al., 2002; Fuccillo et al., 2004). Not only does SHH locally regulate the development of ventral forebrain structures, but it is also required for the development of the dorsal telencephalic midline (Chiang et al., 1996; Ohkubo et al., 2002). The latter role has presented a puzzle, since SHH is expressed at a distance from the dorsal midline and is unlikely to act there directly. Our data suggest that SHH signaling is required for the normal maintenance of additional signaling centers in the brain and ultimately for the expression of downstream effectors such as *Zic2*. Here, we have explored the role of and temporal requirements for SHH signaling in regulating the expression of *Fgf8*, *Bmp4* and *Zic2* in the telencephalon.

The results of our studies are consistent with a model in which continued SHH signaling is required for hemisphere formation by maintaining FGF8 expression at the anterior midline. First, analysis of *Shh* mutants and embryos treated with the Hh signaling antagonist HhAntag reveal that SHH signaling is required for maintenance of the FGF8 and BMP signaling centers in the forebrain. Interestingly, although the expression of *Bmp4* expands in *Shh* knockout mice, expression was lost following treatment with HhAntag. Second, we show that a loss of SHH signaling after E9.5 results in a failure of dorsal midline formation, which resembles HPE. Third, we show that FGF8 can induce the expression of *Zic2*, suggesting that the loss of *Zic2* expression observed in $Shh^{-/-}$ mice and HhAntag-treated embryos may result from the loss of FGF8 at the anterior midline. Our results are consistent with and expand on previous experiments showing that implantation of FGF8-coated beads into the developing chick forebrain can induce a sulcus with features of an ectopic rostral midline (Crossley et al., 2001). Collectively these data suggest that SHH signaling plays a crucial and ongoing role in maintaining the *Fgf8* and *Bmp4* signaling centers, which ultimately pattern the dorsal midline by regulating the expression of effector genes such as *Zic2* and *Msx1*.

SHH Signaling is Required for Ongoing Maintenance of the FGF8 and BMP4 Signaling Centers

Previous studies (Ohkubo et al., 2002) and data presented here have shown that *Fgf8* and *Bmp4* expression are altered in *Shh* mutants. The ability of HhAntag to disrupt SHH signaling provided an opportunity to investigate whether SHH plays an ongoing role in the maintenance of the FGF8 and BMP4 signaling centers. We found that telencephalic expression of *Fgf8* was also lost in embryos cultured in HhAntag for 2 days starting at E9.5, suggesting that SHH is required for the ongoing maintenance of *Fgf8* expression.

We anticipated that HhAntag treatment would result in an expansion of *Bmp4* expression, similar to that seen in *Shh* mutants (Ohkubo et al., 2002). The expanded domain of *Bmp4* expression in *Shh*-deficient mice may result from the loss of *Fgf8*, which normally induces and is required for the expression of *Foxg1* (Shimamura and Rubenctein, 1997; Storm et al., 2006). Normally, *Foxg1* and *Bmp4* show complementary patterns of expression (Furuta et al., 1997), and loss of function mutations in *Foxg1* allow *Bmp* gene expression to expand into the lateral telencephalon (Dou et al., 1999, 2000), suggesting that FOXG1 represses the expression of *Bmps*. By this logic, the loss of *Fgf8* and downregulation of *Foxg1* expression in *Shh* mutants may allow *Bmp4* expression to expand laterally. Surprisingly, in the present experiments, 100 μM HhAntag caused not only in a loss of *Fgf8* but also a loss of *Bmp4* expression, which was accompanied by decreased expression of the BMP4 target *Msx1*. However, in our studies, *Foxg1* mRNA expression was relatively normal at DIV1 and reduced but still detectable at DIV2, suggesting that FGF signaling before E9.5 was sufficient to induce *Foxg1* and thus repress the expansion of *Bmp4* that occurs in *Shh* and *Foxg1* mutants.

Previous studies employing a hypomorphic allele of *Fgf8* revealed that neither *Bmp4* nor *Msx1* expression was detected in the hypomorphs (Storm et al., 2003, 2006), which showed a reduction but not a complete loss of *Foxg1* (Storm et al., 2006), similar to the results of HhAntag treatment. In contrast, the conditional deletion of *Fgf8* in the telencephalon using *Foxg1-Cre* resulted in a broadening of the midline domain of *Bmp4* expression (Storm et al., 2003) and a loss of *Foxg1* expression (Storm et al., 2006). These studies suggest that the dosage of *Fgf8* can

affect the level and pattern of *Bmp4* expression, possibly by differentially affecting the expression of *Foxg1*. Interestingly, both the present and previous data (Chiang et al., 1996; Golden et al., 1999; Panchision et al., 2001) suggest that either the expansion or loss of *Bmp4* can accompany a failure of hemispheric cleavage and result in HPE.

The finding that SHH signaling is required after E9.5 for dorsal midline formation and cleavage of the forebrain into two discrete cerebral hemispheres contrasts with a previous report in which dorsal midline development proceeded normally following the conditional knockout of *Smo*, a mediator of SHH signaling (Fuccillo et al., 2004). Although the *Smo* allele was thought to be recombined on or before E9, and the development of ventral structures was severely disrupted by E10, dorsal midline development and hemispheric bifurcation proceeding surprisingly normally in these mice. These observations led Fuccillo et al. (2004) to conclude that SHH signaling is required before E9 to pattern the dorsal telencephalic midline. One difference between this and the present study is that HhAntag is expected to disrupt Hh signaling immediately, whereas the genetic approach used by Fuccillo et al. (2004) requires the turnover of *Smo* mRNA and protein before SHH signaling is blocked completely. It is plausible that inefficient recombination or perdurance of SMO protein may have delayed a disruption of SHH signaling for some period of time after recombination of the *Smo* allele. Alternatively, because Cre-mediated recombination proceeds in a ventral to dorsal gradient in *Foxg1-Cre* mice (Fuccillo et al., 2004), the loss of *Smo* in dorsolateral structures may have lagged somewhat behind that in more ventral areas (although a ROSA26 reporter revealed complete recombination in dorsal structures by E9.5). Because the effects of the conditional loss of *Smo* on *Fgf8* and *Bmp4* expression were not examined, we are uncertain as to how to account for the differences between the previous and present studies.

We cannot exclude the possibility that HhAntag has off-target effects that may have affected the expression of *Fgf8* and *Bmp4*, or that Hh family members such as SHH, IHH or DHH signal through other moieties that bypass SMO. Biochemical studies of HhAntag have revealed that it binds specifically to SMO (Frank-Kamenetsky et al., 2002) and does not affect other developmentally regulated signaling pathways (Williams et al., 2002); no off-target effects have been reported to date. It appears unlikely that IHH or DHH plays important roles in forebrain development, since their expression in the developing telencephalon has not been observed. Finally, it is thought that all three HH ligands (SHH, IHH, and DHH) signal specifically through SMO, making it unlikely that these molecules would activate other pathways when SMO signaling is inhibited

Zic2 and HPE

Zic2 is normally expressed in both dorsal and ventral regions of the telencephalic midline, and mutations in *Zic2* in mouse (Nagai et al., 2000) and humans (Brown et al., 1998, 2001) result in HPE. Our studies reveal that *Zic2* expression in the murine forebrain is lost following disruption of SHH signaling, and that *Zic2* expression in lateral telencephalic explants can be induced by FGF8. These data raise the possibility that *Zic2* serves as a key mediator between ventrally localized Shh signaling, the ongoing maintenance of *Fgf8* expression, and the formation of dorsal midline structures in the telencephalon. Our data are consistent with the hypothesis that FGF8 normally induces *Zic2* expression *in vivo*, and that the requirement for *Shh* in dorsal midline development reflects its role in maintaining *Fgf8* expression at the anterior midline. This model may explain the common failure of dorsal midline development observed in patients with mutations in either *Zic2* or *Shh*.

The ventrally restricted expression of *Shh* makes it unlikely that SHH regulates *Zic2* expression directly. Furthermore, the results of overexpressing *Shh* in zebrafish embryos and chick spinal cord suggest that SHH inhibits *Zic* gene expression in the ventral neural tube (Rohr et al., 1999; Aruga et al., 2002). Finally, our studies reveal that SHH cannot induce *Zic2* expression in telencephalic explants *in vitro*. Thus, it seems likely that SHH affects *Zic2* expression in the telencephalon by regulating other signaling centers in the brain, and the expression patterns of FGF8 and BMP4 suggested them as candidate regulators. Indeed, overexpression of BMP4 or BMP7 induces ectopic expression of *Zic1* in the chick spinal cord (Aruga et al., 2002). In contrast, evidence from zebrafish and Xenopus embryos suggests that BMPs repress *Zic* gene expression, since implantation of BMP4-coated beads (Grinblat et al., 1998) or injection of *Bmp2* RNA (Rohr et al., 1999) into the brain downregulates *Zic1*. Our studies failed to support the view that BMPs are positive inducers of *Zic2* expression, since BMP4-coated beads successfully promoted the expression of *Msx1*, a known target of BMP4, but failed to induce *Zic2*. Furthermore, *Zic2* expression is maintained in *Bmpr1a; Bmpr1b* double mutant mice, suggesting that BMP signaling is not required for *Zic2* expression *in vivo* (Fernandes et al., in press).

In contrast, we found that beads coated with FGF8 induced robust *Zic2* expression in explants of the lateral telencephalon. Taken in conjunction with the fact that *Zic2* is normally expressed adjacent to the domain of *Fgf8* expression in both the dorsal and ventral telencephalon, these data suggest that FGF8 induces *Zic2 in vivo*, which would account for the downregulation of *Zic2* following the loss of *Fgf8* expression in both $Shh^{-/-}$ mice HhAntag-treated embryos. This hypothesis may also explain the failure of dorsal midline development in *Fgf8* hypomorphic mice (Storm et al., 2006), which exhibit a severe form of HPE. Finally, we suggest that the ability of FGF8-coated beads to trigger the formation of an ectopic midline following implantation into the lateral telencephalon (Crossley et al., 2001) may be mediated by the induction of *Zic2*.

Summary

Collectively, these studies provide a potential mechanism by which SHH is required at a distance for dorsal midline formation and the eventual establishment of two discrete cerebral hemispheres during development. We propose that SHH is required for the maintenance of the FGF8 signaling center at the anterior midline, which in turn induces the expression of *Zic2*. A loss of SHH leads to a loss of *Fgf8*, thus disrupting the expression of *Zic2* at the dorsal midline at a critical time when the cerebral hemispheres are in the process of bifurcation. The data presented here, in conjunction with published studies of mutations in *Fgf8* and *Zic2*, are consistent with the possibility that Zic2 is a major downstream effector of dorsal midline formation during forebrain development.

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