Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex

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Introduction

The regulation of the numbers of stem and precursor cells during development is of crucial importance for the control of brain size (Rakic, 1995; Caviness et al., 1995). Of the different growth factors reported to affect cell proliferation in the CNS (see Vaccarino et al., 1999; Coffin et al., 1999; Lillien and Raphael, 2000), Sonic hedgehog (SHH) has been recently implicated in cell proliferation and growth of the late embryonic and postnatal dorsal brain (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Weschler-Reya and Scott, 1999; Dahmane et al., 2001; Lai et al., 2003). SHH is expressed in a layer-specific manner by differentiated cells in these structures, weakly in precursor populations, and is proposed to affect Glit cell proliferation in germinal zones (Dahmane et al., 2001). In mice, genetic analyses of the only known mediators of HH signals, the GLI transcription factors, have shown that GLI1 function is redundant (Park et al., 2000), whereas GLI2 and GLI3 show specific defects but also have overlapping functions (Franz, 1994; Ding et al., 1998; Matise et al., 1998; Mo et al., 1997; Theil et al., 1999). The GLI proteins thus act through a partly redundant combinatorial code that interprets HH signals in a context-dependent manner (reviewed by Ruiz i Altaba et al., 2002a). Even though SHH-GLI signaling affects precursor proliferation, it remains unclear whether SHH-GLI signaling affects stem cells in the vertebrate brain.

To address this question, we have investigated the role of SHH-GLI signaling in the developing mouse neocortex, analyzing the mid- and late-gestation brain phenotypes of Shh, Gli2 and Gli3 mutants. The results of these experiments, together with those testing for the function and requirement of SHH signaling in vivo as well as in vitro, carried out under conditions in which self-renewal and multipotentiality can be tested, indicate that the SHH-GLI pathway controls the number of cells with stem cell properties in the developing neocortex. In addition, we find synergism between SHH and EGF signaling. We discuss the implications of these findings for brain development, for the ability to manipulate stem cell lineages and for tumorigenesis.

Materials and methods

Animals and treatments

Tissue from outbred Swiss-Webster mice were used for all in vitro assays unless otherwise specified. The Shh (Chiang et al., 1996), Gli1 (Park et al., 2000) and Gli2 (Mo et al., 1997) mutants from our colony were in this background. Gli3xtj (Johnson, 1967; Hui and Joyner, 1993) mice in a C57 background were obtained from the Jackson laboratory (Bar Harbor, Maine). Octyl-modified recombinant SHH-N proteins were a kind gift of Ontogeny/Curis. Cyclopamine (cyc; Toronto Research Biochemicals) was used at 2.5-10 mg/ml dissolved in ethanol for in vitro experiments. Ethanol alone, at the same doses, was used in untreated, control in vitro samples. For all experiments that involved the use of cyc in vivo, five- to ten-week-old inbred C57/B16/J mice were used. Here, cyc was used at 1 mg/ml, conjugated with 2-Hydropropyl-β-Cyclodextrin (HBC; Sigma; prepared as a 45% solution in PBS) (van den Brink et al., 2001). These mice were injected daily, intraperitoneally (IP), for one week, with HBC alone as a control or with cyc-HBC at 10 mg/kg/day. For the in vivo treatment followed by the preparation of neocortical neurospheres (nsp), pregnant mothers were injected for 5 days with HBC alone or with cyc-HBC at 10 mg/kg/day.

BrdU incorporation, histology, immunofluorescence and in situ hybridization

BrdU treatment (20 mg/kg, IP injection), microtome (12 μm) or cryostat (10-18 μm) sections, immunofluorescence, in situ hybridization, and Hematoxylin and Eosin staining was performed as described (Dahmane et al., 2001). BrdU was added to cortical nsp
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Egfr and expected band sizes of 459 bp for cloning assays with size of cloned nsps was counted after one week in culture. For conditioned media documented to yield clonal cultures (Reynolds and assessing clonal sphere colony formation at low cell densities in 1/3 media containing EGF (10 ng/ml) and bFGF [10 ng/ml], or by assays, cells were plated either by dilution at 1 cell/well in 96-well slides without growth factors and incubated for 5-7 days. For cloning differentiation assays, growing nsps were plated at 20,000 cells/well (Nunc) in the presence of EGF and FGF, and grown for 1 week. For otherwise noted. For proliferation assays, nsps were plated at 3000 recombinant, GIBCO) and 10ng/ml of bFGF (Upstate Biotech) unless otherwise stated. Neurosphere medium [Neurobasal Medium (GIBCO), containing N2 tca ta; and mGLI2-2 (reverse), cgg tgc tca tgt gtt tg. Reactions procedures (see Doetsch et al., 2002). Cells were incubated in Neurospheres and Ihh marker-positive cells as a percentage of DAPI-positive cells per ocular grid area at · marker-positive cells as a percentage of DAPI-positive cells per ocular grid area at ×40 magnification (5-12 random areas per experiment, from at least three independent samples).

RT-PCR and genotyping

Conditions and sequences were as described previously ( Dahmane et al., 2001). Other primers were: mGLI2-1 (forward), gca gct ggt gca cta ta; and mGLI2-2 (reverse), cgg tgc tca tgt gtt tg. Reactions conditions were Tm=55°C, producing an 828 bp band for the wild-type allele and a 913 bp band for the Gli2 mutant allele. Primers for Ihh and Dhh were used at Tm=58°C, and gave expected band sizes of 267 bp for Ihh and 311 bp for Dhh (Tekki-Kessaris et al., 2001). Egfr and Enx1/2 primers were used at Tm=55°C and gave the expected band sizes of 459 bp for Egfr, 183 bp for Enx1 and 247 bp for Enx2 (Represa et al., 2001; Yoshida et al., 1997).

Neurosphere

Primary and late embryonic cortical nsps were obtained by standard procedures (see Doetsch et al., 2002). Cells were incubated in neurosphere medium [Neurobasal Medium (GIBCO), containing N2 (GIBCO), 2 mM glutamine, 0.6% (w/v) glucose, 0.02 mg/ml insulin, and 15 mM HEPES] with 10 ng/ml of EGF (human recombinant, GIBCO) and 10ng/ml of bFGF (Upstate Biotech) unless otherwise noted. For proliferation assays, nsps were plated at 3000 cells/well onto polyornithine/laminin coated Lab-Tek chamber slides (Nunc) in the presence of EGF and FGF, and grown for 1 week. For differentiation assays, growing nsps were plated at 20,000 cells/well onto polyornithine/laminin coated (10 mg/ml) Lab-Tek chamber slides without growth factors and incubated for 5-7 days. For cloning assays, cells were plated either by dilution at 1 cell/well in 96-well plates (Nunclon), with 50% conditioned media [50% nsp-defined media containing EGF (10 ng/ml) and bFGF (10 ng/ml)], or by assessing clonal sphere colony formation at low cell densities in 1/3 conditioned media documented to yield clonal cultures (Reynolds and Weiss, 1996; Troppepe et al., 2000; Seaberg et al., 2002). The number and size of cloned nsps was counted after one week in culture. For cloning assays with Gli2−/− and wild-type littermate neuropheres at E18.5, healthy cells from the first or second passage were visually chosen for their round morphology and homogeneous appearance under the microscope, and manually transferred to single wells in 96-well plates (Nunclon). This was to avoid the potential problem of damaged Gli2−/− cells, which are fragile, after dissociation of pelleted neuropheres.

Results

SHH and EGF signaling synergize to regulate neocortical precursor proliferation

In order to test for a direct effect of SHH signaling on neural stem cells, but lacking the means for the prospective identification of such cells in vivo, we have used neurosphere (nsp) culture to test for stem cell behavior. EGF can sustain nsp growth at late embryonic stages, whereas at earlier stages FGF is required (Reynolds and Weiss, 1996; Tropepe et al., 1999; Martens et al., 2000). Addition of recombinant human SHH (5 nM) to nsp media containing (10 ng/ml) EGF, or EGF plus (10 ng/ml) FGFR2, induced higher levels of Gli1 expression but did not increase cell proliferation of neocortical nsps from mid [embryonic day (E) 15.5] and late (E18.5) gestation stages (not shown). SHH (5nM) alone was also unable to initiate or sustain nsp growth (not shown). The induction of Gli1, however, indicated the ability of nsps to respond to SHH signaling, but proliferation parameters may already have been at a maximum with saturated growth factor levels. E15.5 and E18.5 wild-type cortical nsps were then selected in media containing 10 ng/ml EGF without FGF, which is only required at earlier stages, and were passed and grown at different concentrations of EGF. SHH (5 nM) synergized with EGF at concentrations of between 2.5 and 0.05 ng/ml to promote nsp growth, as measured by BrdU incorporation (Fig. 1A). Similarly, growth of nsps at 1 ng/ml EGF showed a concentration-dependent increase in proliferation by SHH between 1 and 5 nM (Fig. 1B). SHH treatment increased the levels of Gli1 (Fig. 1D). In addition, nsp cultures expressed Shh, as well as Ihh, Dhh and Gli-3, the RNAs of which were also detected in fresh cortical tissue (Fig. 1D; Fig. 2L; and not shown).

SHH signaling is required for normal proliferation and self-renewal of neocortical neuropheres

To address the role of SHH signaling on stem cell self-renewal, we assayed the number of E15.5 wild-type nsp cells able to give rise to secondary nsps in cloning assays. SHH treatment, in the presence of 2.5 ng/ml of EGF, increased the number of derived nsps over that of control by ~5-fold (Fig. 1C). Conversely, treatment with 5 µM cyc, a specific inhibitor of HH signaling (Incardona et al., 1998; Cooper et al., 1998), reduced the number of secondary cloned nsps by 50% (Fig. 1C), and also decreased the level of Gli1 (Fig. 1D).

SHH often acts in a concentration-dependent manner (reviewed by Ingham and McMahon, 2001). Therefore, to test for a concentration-dependent effect of inhibition of HH signaling by cyc, wild-type E15.5 and postnatal day 2 (P2) neocortical EGF-responsive nsps were selected and treated with varying concentrations of cyc. Treated and control nsps were assayed for BrdU incorporation and cloned. Increasing concentrations of cyc lead to a progressive decrease in the level of BrdU incorporation and in the number of secondary clones, as compared with non-treated nsps (Fig. 1E-H). SHH pathway activity thus regulates the size of the pool of nsp cells with self-renewal properties in a concentration-dependent manner.

Pharmacological inhibition of HH signaling in vivo

To test whether an in vivo effect of cyc could be discerned, cyclodextrin-conjugated cyc, or just cyclodextrin (HBC) as control, was injected intraperitoneally, daily, into pregnant mothers (van den Brink et al., 2001) carrying E10.5-E12.5 embryos, and these were collected five days later. By E10.5, the ventral forebrain has already received HH signaling and the eyes have formed. Consequently, cyc treatment did not cause cyclopia and the treated E15.5-E17.5 embryos appeared normal. Analyses of fresh neocortical tissue dissected ~12 hours after the last cyc injection showed that in vivo cyc treatment decreased the expression of Gli1, a loyal marker of SHH signaling (Lee et al., 1997), and abolished that of Egfr (Fig. 1I), whereas expression of Gli2 and Gli3 was unchanged.
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Acute dissociation of neocortical tissue at this time, and its subsequent culture without growth factors, confirmed that in vivo cyc treatment inhibits cell proliferation (Fig. 1J). To expand this finding, we analyzed neocortical cell behavior from mice mutant for \( \text{Shh} \), \( \text{Gli2} \) and \( \text{Gli3} \), as \( \text{Gli1} \) null mice appear normal (Park et al., 2000).

\( \text{Shh} \) mutant mice show deficits in cells able to form neurospheres

\( \text{Shh}^{–/–} \) mice die at birth showing overt signs of cyclopia and lacking all ventral CNS cell types (Chiang et al., 1996). Their dorsal-only CNS comprises an \( \text{Emx1}^+ \), \( \text{Tbr1}^+ \) forebrain cortex (Chiang et al., 1996; Dahmane et al., 2001) (Fig. 2A). The \( \text{Shh}^{–/–} \) cortex produced nsp cultures in full media, but these were fewer and smaller than those from wild-type cortices, and contained fewer BrdU+ cells (Fig. 2B-G,M-O). Analyses of gene expression confirmed the loss of \( \text{Shh} \) transcripts in the few \( \text{Shh}^{–/–} \) nsps that formed (representing a small pool of viable cells). A decrease in \( \text{Pit1} \) and \( \text{Dhh} \) expression was detected, whereas the expression of \( \text{Ihh}, \text{Gli1} \) and \( \text{Gli2} \) were unchanged, and the expression of \( \text{Gli3} \) expression was slightly higher (Fig. 3L). These \( \text{Shh}^{–/–} \) nsps expressed nestin (Fig. 2H) and were tripotential, as judged by the ability to differentiate as Tuj1 + neurons, GFAP + astrocytes or O4 + oligodendrocytes (Fig. 2I-K and not shown). Cloning assays showed that \( \text{Shh}^{–/–} \) nsps contain approximately one quarter of the number of nsp-forming stem cells of wild-type nsps at E15.5 (Fig. 2O). At E18.5, there were very few, if any, mutant nsps (Fig. 2O).

\( \text{Gli2} \) mutant mice display a mid and late embryonic dorsal brain phenotype

\( \text{Gli2}^{–/–} \) mice also die at birth, displaying defects in multiple organs (Mo et al., 1997; Ding et al., 1998; Matise et al., 1998). We have found novel dorsal brain phenotypes of \( \text{Gli2}^{–/–} \) mice at mid and late gestation stages in an outbred background. \( \text{Gli2} \) null embryos present a variably penetrant severe phenotype, displaying excencephaly by E13.5 (also seen at E17-E18.5; not shown), and a consistent milder phenotype.
characterized by expanded but thinner telencephalic vesicles, most clearly seen posteriorly, and an overtly reduced tectum and cerebellum (Fig. 3A-C). We have focused here on the non-exencephalic Gli2<sup>–/–</sup> mice. Histological analyses showed that E18.5 Gli2<sup>–/–</sup> telencephalic vesicles have a thinner proliferative zone (an ~30-50% reduction of the vz/svz; Fig. 3D). Gli2<sup>–/–</sup> mice have fewer BrdU<sup>+</sup> precursors in the cortex at mid and late gestation periods (Fig. 1E-G), suggesting defects in neuronal as well as glial cell populations. The decrease is most notable in the deeper proliferative area (the svz). Local variations without a clear pattern in the density of BrdU<sup>+</sup> nuclei were also observed (not shown), indicating an additional degree of neocortical disorder in these mutant mice. TUNEL and activated caspase 3 analyses did not show an increase in apoptosis (not shown).

In the Gli2<sup>–/–</sup> neocortex, Gli1 transcripts were absent, there was a consistent decrease in the expression of mutant Gli2 transcripts, and the expression of Gli3 was diminished (Fig. 3J-O). The expression of Neurod1, which marks neuroblasts, and that of one vz/svz-specific cDNA, clone 53, showed a reduction of neuroblasts and vz/svz cells, respectively, by ~50% in Gli2<sup>–/–</sup> cortex compared with that of wild-type littermates (Fig. 3P-U). Decreases in the number of Neurod<sub>1</sub> cells were detected in the Gli2<sup>–/–</sup> cerebellum, which showed abnormal early foliation and more pronounced posterior defects (Fig. 3H,I), and in the hippocampus (Fig. 3P,O, arrow).

Neocortical cells from Gli2 mutant mice show compromised neurosphere-forming abilities

Gli2<sup>–/–</sup> neocortices gave rise to nsps (Fig. 4A-D), containing Nestin<sup>+</sup> cells (Fig. 4F) that were tripotential (Fig. 4G-I). However, at late embryonic stages mutant nsps progressively became smaller, more delicate, and showed more blebbing than wild-type nsps (Fig. 4A-D,J,K). Gli2<sup>–/–</sup> nsps decreased in numbers during culture and, after a few passes (~6), they were rare, and all died soon after. The Gli2<sup>–/–</sup> nsps surviving at passage ~2-4 lacked Gli1 expression and showed downregulation of Ihh and Dhh expression. Shh expression was unchanged, whereas Gli3 and Ptc<sub>h1</sub> expression was reduced (Fig. 4E). The expression of Egfr was also reduced. Cloning assays in the presence of EGF and FGF showed that there was an ~10-fold decrease in the number of Gli2<sup>–/–</sup> cells able to form secondary nsps, as compared with wild-type cells (Fig. 4L,M).

Gli3 mutant mice do not form stable neurosphere cultures

Gli3 mutant (X<sup>i</sup>) mice die at birth. Those that are not exencephalic have a severely reduced cortex (Franz et al., 1994; Theil et al., 1999) (Fig. 5A), and we have used these for our analyses. Primary cultures of E18.5 Gli3<sup>–/–</sup> neocortices in full nsps media yielded transiently forming clumps that
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rapidly degenerated, whereas nsp cultures from wild-type siblings formed stable nsp clusters (Fig. 5E,F). In one out of six cases, we were able to obtain a small number of nsps that survived up to 4 passes. Instead of nsps, E18.5 Gli3–/– primary cultures normally yielded isolated attached cells with differentiated morphology that expressed neuronal antigens (Fig. 5E,F). After one week, a time when newly formed nsps are clearly visible in the wild-type control, there were almost no Gli3 null nsps, and the cells with differentiated morphologies died soon after. At E15.5, the first nsps were detected after one week in both wild-type and mutant cultures, and these were able to be passed but most died in the first pass (Fig. 5B,C). The fraction of nsps that survived were tripotential (not shown). Most of these nsps, however, died after subsequent passes. The context-dependent function of GLI3 was further suggested by the finding that Gli3–/– tectal nsps could be easily grown and passed (not shown). Neocortical Gli3–/– E15.5 nsps expressed Emx2 (Fig. 5D), but lacked Emx1 (Theil et al., 1999) and Gli3, confirming their provenance. These cells had unchanged levels of Gli2, but expression of Gli1 was absent, and that of Shh and Ihh were drastically reduced. By contrast, expression of Dhh was enhanced. Expression of Egfr and Ptch1 was slightly reduced.

Discussion

The results we present here indicate that HH signaling represents an endogenous mechanism regulating the behavior
of cells with stem cell properties in the developing mouse neocortex. The lack of specific, prospective neural stem cell markers, and the finding that nsp's can derive from both bona fide stem cells and amplifying precursors that acquire stem cell properties through EGF signaling in vitro (Doetsch et al., 2002), make a strict correlation between in vivo stem cells and cells that can form nsp's difficult. However, the effects of manipulating HH-GLI signaling on self-renewal (as revealed in nsp cloning assays) allow the investigation of the role of HH-GLI signaling in regulating stem cell properties.

Our data suggest that cells with stem cell properties increase their numbers in response to higher than normal endogenous HH levels; normal HH levels equate to homeostasis, and reduction of HH signaling below a critical threshold decreases their numbers. In this case, our present and previous data (Dahmane et al., 2001) suggests that HH signaling affects both precursors and neocortical stem cells, although definitive proof requires the prospective identification of stem cells in vivo and their direct analyses in terms of response to SHH-GLI function, which is not yet feasible. The scenario we propose parallels the multiple effects of SHH signaling on adjacent cell types in other parts of the brain (Dahmane and Ruiz i Altaba, 1999), and the separate effects of SHH signaling on the differentiation of a single cell type at different times (Ericson et al., 1996). Our results are also consistent with the finding that Smoothened, a crucial component of the HH-GLI pathway, is expressed in populations enriched for SOX2+ neural stem cells, as well as in derived nsp's from the midgestation mouse telencephalon (D’Amour and Gage, 2003). It remains possible that the smaller size of nsp's that we observe after inhibiting SHH-GLI function could also reflect changes in cell identity or cell populations, as wild-type nsp's of different sizes can express partially different subsets of genes (Suslov et al., 2002) and SHH might also affect precursor maturation (Viti et al., 2003). However, the loss of virtually all nsp's from E18.5 Shh and Gli3 null mice suggest the general requirement of the HH-GLI pathway.

The phenotype of Gli2, Gli3 and Shh mutant mice is likely to include a decrease in the number of neocortical cells with stem cell properties. The existence of severe (exencephalic) and milder Gli2 and Gli3 null phenotypes indicates that genetic modifiers affect GLI function. Functional compensation between GLI3 and GLI2 may alleviate the phenotypes, and also explain the maintained response of Gli2–/– neocortical explants to SHH (data not shown). Nevertheless, such compensation is likely to be only partial as Gli2–/– nsp's are severely affected, and Gli2 and Gli3 regulate gene expression differently. For example, loss of either one results in lower Ihh and to a lesser extent Dhh in Gli2–/– cells. Hprt is shown as a control.

(F-I) Expression of nestin in precursors of Gli2–/– nsp's (F), of TuJ1 in neurons (G), of GFAP in astrocytes (H) and of O4 in oligodendrocytes (I) after differentiation. Nuclei were counterstained with DAPI. (J-M) Quantification of nsp size at E15.5 (J) and E18.5 (K). An average of 20 nsp's from two independent experiments is shown (E15.5, P<0.05; E18.5, P<0.001). Given their rarity at E18.5, single Gli2–/– nsp's were measured. (L,M) Quantification of nsp's obtained in cloning assays. Three independent experiments are shown for E15.5 (P<0.001) and one for E18.5. Scale bar: 75 μm for A-D; 10 μm for F-I.
Hedgehog-GLI signaling in neocortical stem cells with low doses of EGF, and that high doses of EGF cannot efficiently induce nsp cultures from Shh−/− mice, indicates that SHH acts on EGF-responsive cells and that it is unlikely to be only a stem cell survival factor. It is possible that the few nsp's observed from Shh null mice derive from the complementary action of IHH and DHH. How the EGF and SHH pathways interact is not clear, but it is interesting to note that neocortical precursors express EGFR, and that changes in EGFR expression have been associated with changes in progenitor cell behavior (see Burrows et al., 1997). This raises the possibility that changes in the responsiveness to EGF signaling could modulate or alter the response to SHH signaling or vice versa. Here we provide evidence that SHH signaling may synergize with EGF signaling in the brain through its regulation of Egfr expression, paralleling its regulation in the early neural tube and fly brain (Amin et al., 1999; Viti et al., 2003).

HH signaling in defined brain domains may be involved in maintaining niches in which stem cells exist and proliferate. Such niches may be defined, in part, by a critical concentration range at which SHH acts, possibly cooperating with EGF. This idea is consistent with the finding that Shh and GLI genes are expressed in other stem cell niches, such as the adult forebrain subventricular zone (SVZ) (V.P., N. Dahmane, D. Lim, A. Álvarez-Buylla and A.R.A., unpublished) and the dentate gyrus (Dahmane et al., 2001), and with the requirement of HH signaling in the subgranular layer of the hippocampus (Lai et al., 2003), in the SVZ (V.P., D. Lim, N. Dahmane, P. Sánchez, Y. Gitton, A. Álvarez-Buylla and A.R.A., unpublished) and for somatic stem cell proliferation in the fly ovary and brain (Zhang and Kalderon, 2001; Park et al., 2003). Together, these findings suggest an unexpected general role of SHH-GLI signaling in the control of the behavior of stem cell lineages throughout the brain and in different species. How some niches, such as the developing neocortex, are extinguished in adulthood, while others persist is unclear but it is possible that alterations in SHH-GLI pathway activity might underlie these changes.

How SHH signaling is integrated with other niche factors is also not known. For example, in the adult SVZ, a niche of persistent stem cell-derived neurogenesis (see Doetsch et al., 1999), BMP signaling inhibits neuronal differentiation and promotes gliogenesis (Lim et al., 2000), whereas endogenous SHH signaling enhances neurogenesis and the number of cells with stem cell properties (V.P., D. Lim, N. Dahmane, P. Sánchez, Y. Gitton, A. Álvarez-Buylla and A.R.A., unpublished). Here, ependymal cells secrete the BMP antagonist noggin, thereby forming a niche in which neurogenesis may occur (Lim et al., 2000). In the developing neocortex, noggin inhibits whereas BMPs enhance differentiation (Li et al., 1998; Li and LoTurco, 2000; Mabie et al., 1999), and BMPs are also proposed to inhibit the transition of stem cells to an EGF-responsive state (Lillien and Raphael, 2000). In the neocortex, as in the SVZ and neural tube (Liem et al., 1995), BMPs and SHH may also therefore act in opposite manners. This raises the possibility that the synergism we report here between EGF and SHH signaling may take place, not only directly through the activation of Egfr by SHH, but also indirectly through the inhibition of BMP signaling. This may be supported by the finding that activation of ERKs by EGF leads to the phosphorylation of SMAD1 in the linker region, rendering it unable to respond to activation by activated BMP receptors (Kretzchmar et al., 1997) and thus silencing BMP signaling. The antagonism between SHH and BMP signaling, possibly also integrated at the level of SMAD-GLI interactions (Liu et al., 1998), may thus underlie many aspects of stem cell and neurogenic niches, being a common target for regulators of stem cell properties and neurogenesis.

The present findings support a crucial role of SHH signaling...
in building the vertebrate brain, by modulating its size through the regulation of the number of cells with stem cell properties, in addition to controlling progenitor proliferation (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; WescLER-Reya and Scott, 1999; Dahmane et al., 2001; Lai et al., 2003). Overall or local changes in SHH signaling, or its reception, during evolution may have contributed to the evolving sizes and shapes of the brain, including the expansion of the neocortex in primates, of the tectum in birds and of the cerebellum in electrosensitive fish. The price for such plasticity may be tumorigenesis. Our data, together with previous results on the involvement of the SHH-GLI pathway in tumorigenesis (reviewed by Ruiz i Altaba et al., 2002b), suggest that the many cancers that arise from constitutive HH signaling in various tissues, such as brain, skin, muscle and lung, may derive from cells with stem cell properties that inappropriately maintain an active response to HH signaling, with continued signaling being required for tumor maintenance. In the brain, the synergism between EGF and SHH signaling raises the possibility that enhancement or inappropriate activation of either pathway, such as through EGFR amplification or upregulation of GLI1 function, could give stem cells an advantage to initiate cancer.

In contrast to tumor development resulting from unregulated activity, the controlled modulation of SHH and EGF signaling in vitro and in vivo is likely to lead to the development of protocols to increase the number of cells with stem cell properties in an effort to ameliorate the effects of degenerative diseases.

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