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RESEARCH ARTICLE

Sonic Hedgehog Signaling Plays an Essential Role During Embryonic Salivary Gland Epithelial Branching Morphogenesis

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Gene targeting studies indicate that sonic hedgehog (Shh) signaling plays an essential role during craniofacial development. Because numerous mandibular derivatives (e.g., teeth, tongue, Meckel's cartilage) are absent in *Shh* null mice and the embryonic submandibular salivary gland (SMG) develops from the mandibular arch, we postulated that Shh signaling is important for embryonic SMG development. To address this question, we first determined the spatiotemporal distribution of Shh; two transmembrane proteins, patched 1 (Ptc) and Smoothened (Smo), which act as a negative or a positive regulator of the Shh signal, respectively; and the Gli 3 transcription factor, which is downstream of the Shh signal. The epithelial localization of Shh, Ptc, Smo, and Gli 3 suggests that Shh signaling may act within the epithelium in a juxtacrine manner. The SMG phenotype in our embryonic day (E) 18.5 *Shh* null mice can be characterized as "paedomorphic," that is, it fails to progress to ontogenic stages beyond the *Early Pseudoglandular* (~E14). In a complementary set of experiments, we used organ culture to evaluate the effect of enhanced or abrogated Shh signaling on embryonic SMG development in vitro. Paired E13 (*Late Initial Bud* stage) or E14 (*Pseudoglandular* stage) SMGs were cultured in the presence or absence of exogenous Shh peptide supplementation; Shh-supplemented explants exhibit a significant stage-dependent increase in branching morphogenesis compared with control explants. Furthermore, by using cyclopamine, a steroidal alkaloid that specifically disrupts the Shh pathway, to abrogate endogenous Shh signaling in vitro, we found a significant decrease in branching in cyclopamine-treated explants compared with controls, as well as a significant decrease in epithelial cell proliferation. Our results indicate that Shh signaling plays an essential role during embryonic SMG branching morphogenesis. Exogenous FGF8 peptide supplementation in vitro rescues the abnormal SMG phenotype seen in cyclopamine-treated explants, demonstrating that overexpression of a parallel, but related, downstream signaling pathway can compensate for diminished Shh signaling and restore embryonic SMG branching morphogenesis. *Developmental Dynamics 229:722–732, 2004.* © 2004 Wiley-Liss, Inc.

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INTRODUCTION

After a classic initial epithelial–mesenchymal interaction, the mouse neonatal submandibular salivary gland (SMG) is composed of large and small ducts, which terminate in lumen-containing, presumptive acini that express embryonic mucin (Wessells, 1977; Cutler and Gremski, 1991; Gresik et al., 1998; Jaskoll et al., 1998; Kashimata et al., 2000; Melnick and Jaskoll, 2000). Mouse SMG development, beginning around embryonic day 11.5 (E11.5), is best conceptualized

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in stages (Jaskoll and Melnick, 1999; Jaskoll et al., 2002): (1) *Pre-Bud* stage: an initial formation of the primitive SMG knot, a thickening of the oral epithelium adjacent to the developing tongue; (2) *Initial Bud* stage: primitive oral cavity epithelium adjacent to the developing tongue grows down into compact mesenchyme to form a solid, elongated epithelial stalk terminating in a bulb; (3) *Pseudoglandular* stage: the solid cord of epithelium elongates and grows by repeated end-bud branching, giving rise to multiple ductal cords and buds; (4) *Canalization* stage: the number of lobes is increased and the presumptive ducts begin to exhibit distinct lumina lined by cuboidal epithelial cells; and (5) *Terminal Bud* stage: distinct, well-developed lumina are seen in presumptive ducts and terminal buds (presumptive acini), with mucin protein being produced by terminal bud epithelia.

Embryonic SMG morphogenesis requires a complex interplay between cell proliferation, apoptosis, and histodifferentiation, all mediated by growth factors, cytokines, and transcription factors at specific times and places (Hardman et al., 1994; Kashimata and Gresik, 1997; Jaskoll and Melnick, 1999; Kashimata et al., 2000; Melnick et al., 2001a,b; Jaskoll et al., 2001, 2002, 2003). Functional studies in our laboratory and that of others have demonstrated the importance of several signaling pathways, including fibroblast growth factor(FGF)/FGF receptor (FGFR), transforming growth factor-alpha (TGF-α)/epidermal growth factor (EGF)/EGF receptor (EGFR), TGF-β/TGF-β-RII, tumor necrosis factor (TNF)/TNF receptor (TNFR), and Eda/Edar (Hardman et al., 1994; Kashimata and Gresik, 1997; Jaskoll and Melnick, 1999; Kashimata et al., 2000; Melnick and Jaskoll, 2000; Melnick et al., 2001a–c; Jaskoll et al., 2002; De Moerlooze et al., 2000; Ohuchi et al., 2000; Hoffman et al., 2002).What remains to be determined are which additional pathways known to be important for the morphogenesis of other tissues (e.g., tooth, lung, pancreas, kidney, etc) also play essential morphoregulatory roles during embryonic SMG development.

Sonic hedgehog (Shh) is a member of the hedgehog family of signaling molecules that act as an inductive signal during development (see review in McMahon et al., 2003). Shh is a secreted protein involved in cell survival, proliferation, differentiation, and pattern formation in various embryonic tissues. The cellular response to Shh is controlled by two transmembrane proteins, Patched 1 (Ptc) and Smoothened (Smo). Ptc acts as a negative regulator of the Shh signal, whereas Smo is a positive activator (Taipale et al., 2002; see reviews in Ingham and McMahon, 2001; McMahon et al., 2003). In the absence of Shh, Ptc inhibits the activity of Smo to block the downstream signaling cascade. Shh binding to Ptc relieves Smo from its inhibition to initiate a signaling cascade that results in the activation of target genes. The zinc finger family of transcription factors, Cubitus interruptus/Gli, mediates the Shh signal (see reviews in Ingham and McMahon, 2001; McMahon et al., 2003). In vertebrates, there are three Gli proteins (Gli1, 2, and 3) that exhibit distinct yet partially redundant functions. Gli 1 acts as a transcription activator, whereas Gli 2 and Gli 3 can act as activators or suppressors of transcription, depending on the cellular conditions (Ruiz i Altaba et al., 2002). In the absence of Shh signaling, Gli 3 is cleaved and processed to form its repressor form; the Shh signal blocks Gli3 processing and then induces the expression of Gli 3 target genes, including Gli1 (Marigo et al., 1996; Ruiz i Altaba, 1999; Dai et al., 1999). Furthermore, the expression of the Ptc and Smo receptors and Gli 1 are up-regulated by Shh signaling, whereas *Gli3* expression is down-regulated (Marigo and Tabin, 1996; Sasaki et al., 1997, 1999; McMahon et al., 2003).

Gene targeting studies have demonstrated that the Shh signaling cascade is essential for many aspects of mammalian embryogenesis, including neural tube, craniofacial, limb, and kidney development (see review in McMahon et al., 2003). Of particular interest is the absence of most mandibular arch derivatives (e.g., teeth, tongue, Meckel's cartilage) in *Shh* null mice (Chiang et al., 1996). Given that the embryonic submandibular salivary gland (SMG) initially develops as an oral epithelial invagination into the neural crest-derived mesenchyme of the mandibular arch lateral to the developing tongue (Jaskoll et al., 2002; Jaskoll and Melnick, 2002), it was reasonable to postulate that Shh signaling plays an essential role during embryonic SMG development. To address this hypothesis, we determined the spatiotemporal distribution of Shh and key components of its signaling pathway during embryonic SMG development and evaluated the SMG phenotype in *Shh* null mice. In a complementary set of experiments, we analyzed the effect of enhanced or abrogated Shh signaling on embryonic SMG development in vitro. Our results indicate that Shh signaling plays an essential mitogenic role during embryonic SMG development, with Shh acting directly on branching epithelia. Finally, we demonstrate that Shh signaling regulates FGF8 protein expression and that FGF8 peptide supplementation in vitro can rescue the abnormal SMG phenotype seen with abrogated Shh signaling. Our results indicate that enhancement of a downstream signaling pathway (FGF8) can sufficiently compensate for reduced Shh signaling and restore embryonic SMG branching morphogenesis.

RESULTS

To investigate the role of Shh signaling during embryonic SMG development, we first examined the stage- and cell-specific distribution of Shh, the two transmembranous proteins (Ptc and Smo) that mediate the Shh signal, and a member of the Gli family of transcription factors (Gli 3), which is a direct target of Shh signaling. Beginning in the *Early Initial Bud* stage, Shh, Ptc, Smo, and Gli 3 proteins are detected in SMG bud epithelia (Fig. 1A–D). By the *Pseudoglandular* stage, Shh and Ptc are diffusely localized to ductal and terminal bud epithelia (Fig. 1E,F), whereas Smo and Gli 3 are seen in a subset of terminal bud epithelial cells

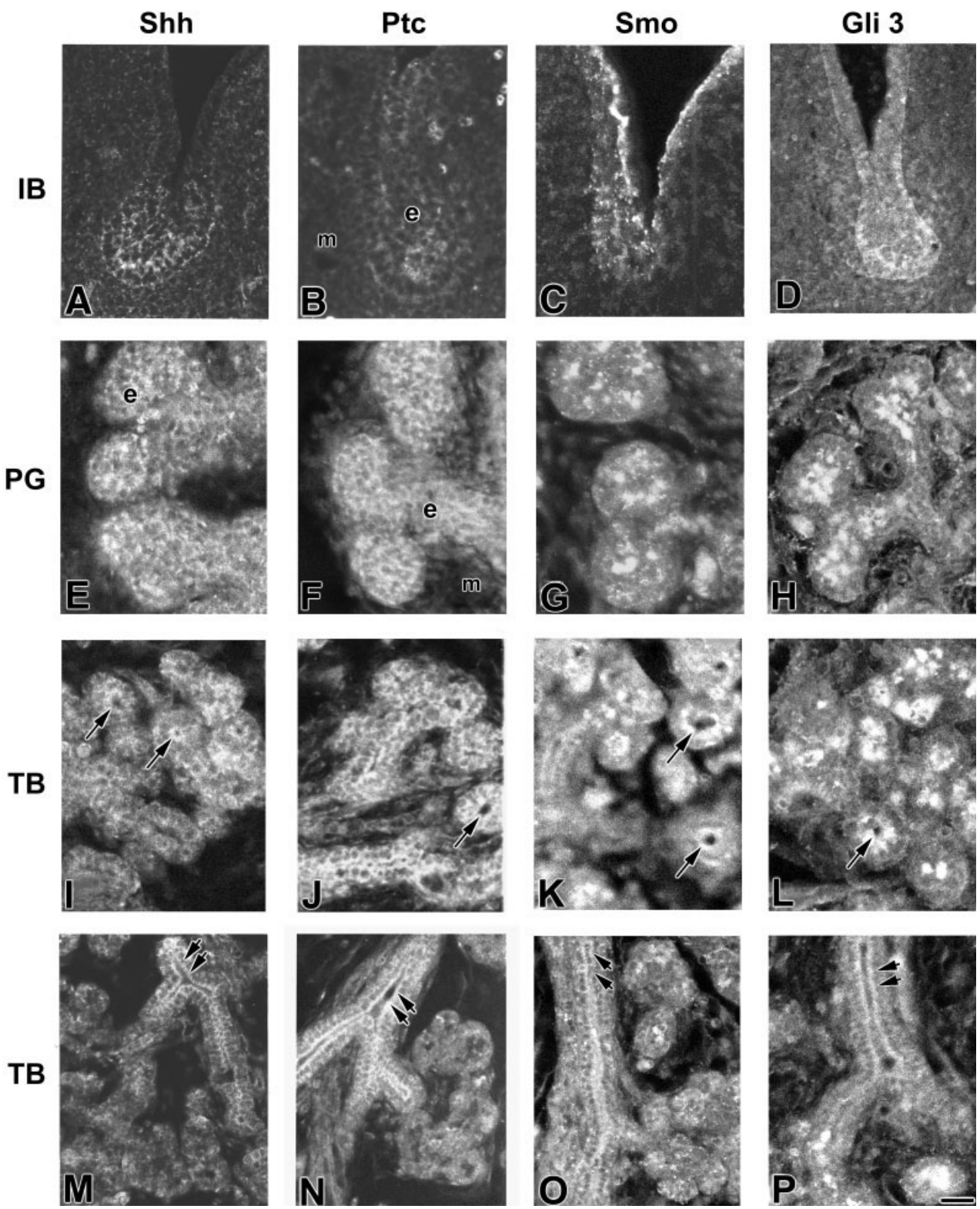

Fig. 1. Epithelial distribution of sonic hedgehog (Shh), Patched (Ptc), Smoothened (Smo), and Gli 3 proteins during embryonic submandibular gland development. A–D: *Initial Bud* (IB) stage. **E–H:** *Pseudoglandular* (PG) stage. **I–P:** *Early Terminal Bud* (TB) stage. e, epithelial cells; arrows, epithelial cells surrounding the forming ductal (double arrows) and terminal bud (arrow) lumina. Scale bar = 50 μm in P (applies to A–P).

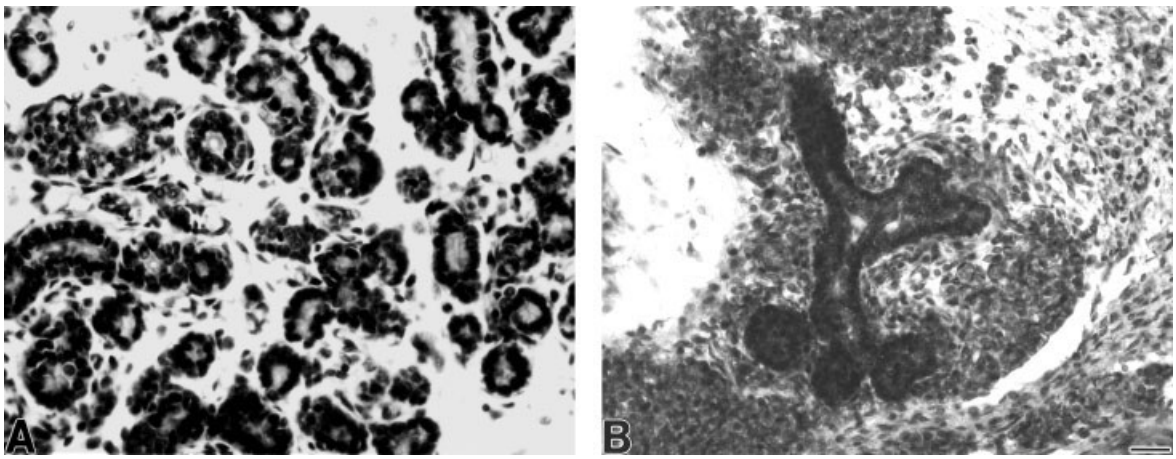

Fig. 2. The sonic hedgehog (*Shh*) null mouse submandibular gland (SMG) is severely paedomorphic. **A:** Embryonic day (E) 18.5 wild-type *Shh*$^{+/+}$ SMG. **B:** *E18.5 Shh*$^{-/-}$ SMG. The E18.5 *Shh*$^{-/-}$ SMG consists primarily of undifferentiated epithelium with few branches surrounded by undifferentiated mesenchyme. Scale bar = 50 μm in B (applies to A,B).

(Fig. 1G,H). With lumen formation in the *Canalicular* and *Terminal Bud* stages, Shh, and Ptc are widely co-distributed on ductal and terminal bud epithelia (Fig. 1I,J,M,N). In ductal epithelia, Smo and Gli 3 proteins are immunodetected in a pattern similar to Shh and Ptc (Fig. 1O,P); in terminal bud epithelia, Smo and Gli 3 proteins predominantly localize to cells surrounding forming lumina (Fig. 1K,L). Gli 1 distribution is similar to that seen for Gli 3 (data not shown).

SMG Phenotype in *Shh* Null Mice

To determine whether Shh signaling is essential for embryonic SMG development, we analyzed the SMG phenotype in *Shh* null homozygous (*Shh*$^{-/-}$) mice and compared it with that seen in their wild-type (*Shh*$^{+/+}$) littermates (Fig. 2). Our microscopic examination of E13.5 and E15.5 *Shh*$^{-/-}$ mice reveals a tiny, dysplastic SMG remnant (data not shown) that, by E18.5, is a slightly larger but undifferentiated dysplastic gland. In the E18.5 wild-type mouse, normal *Terminal Bud* stage SMGs are found; these glands are characterized by loosely packed mesenchyme surrounding ductal and terminal bud epithelia, which exhibit distinct lumina (Fig. 2A). In contrast, the severely abnormal *Shh*$^{-/-}$ SMG consists primarily of undifferentiated epithelium composed of very few branches surrounded by undifferentiated, condensed mesenchyme (Fig. 2B). This abnormal phenotype, having some similarity to that seen normally in the much earlier *Pseudoglandular* stage (~E14; see Fig. 1, Jaskoll and Melnick, 1999), indicates that Shh signal transduction plays an essential role during in vivo embryonic SMG development. In essence, the null phenotype is "paedomorphic," failing to progress to later ontogenic stages.

Enhanced Shh Signaling In Vitro Induces Embryonic SMG Branching Morphogenesis

To investigate the functional role of Shh, we used our well-defined organ culture system to investigate the effect of enhanced Shh signaling on embryonic SMG development. Paired E13 (*Late Initial Bud* stage) or E14 (*Pseudoglandular* stage) SMG primordia were cultured in the presence or absence of 2.5 μg/ml Shh peptide. Because a notable difference in SMG branch number is seen among littermates, we compared the number of terminal buds in right and left glands (treated and control) from each embryo. Spooner ratios (end bud number/initial bud number) were determined for each explant, the data were then arcsin transformed, and the mean ratios compared by paired *t*-test. Shh supplementation induced a significant stage-dependent increase in branching morphogenesis (Fig. 3A,B,E). Specifically, Shh supplemented E13+3 and E14+2 explants exhibit a highly significant ~28% ($P < 0.002$) and ~60% ($P < 0.01$) increase, respectively, in branching morphogenesis compared with controls (Fig. 3E). Moreover, this ~twofold difference between E13+3 and E14+2 Spooner ratios is highly significant ($P < 0.005$). Because a greater response to Shh supplementation was seen in E14 primordia, we then conducted a dose-response study using E14+2 SMG primordia; no significant difference was seen between 0.5 μg/ml and 2.5 μg/ml Shh supplementation (Fig. 3E).

Abrogated Shh Signaling In Vitro Decreases Embryonic SMG Branching Morphogenesis

Because supplementation studies are not entirely probative of the morphoregulatory role of endogenous signaling, we inhibited endogenous Shh signaling with cyclopamine, an antagonist that specifically binds to Smo to subvert the Shh signal transduction pathway (Chen et al., 2002). Cyclopamine has been used previously to successfully interrupt endogenous Shh signaling in vitro (Kim and Melton, 1998; Taipale et al., 2000; Hall et al., 2003; Mistretta et al., 2003). In this set of experiments, paired E13+3 or E14+2 SMG primordia were cultured in control or cyclopamine-supplemented (5 μM or 10 μM) medium and Spooner ratios were determined as described

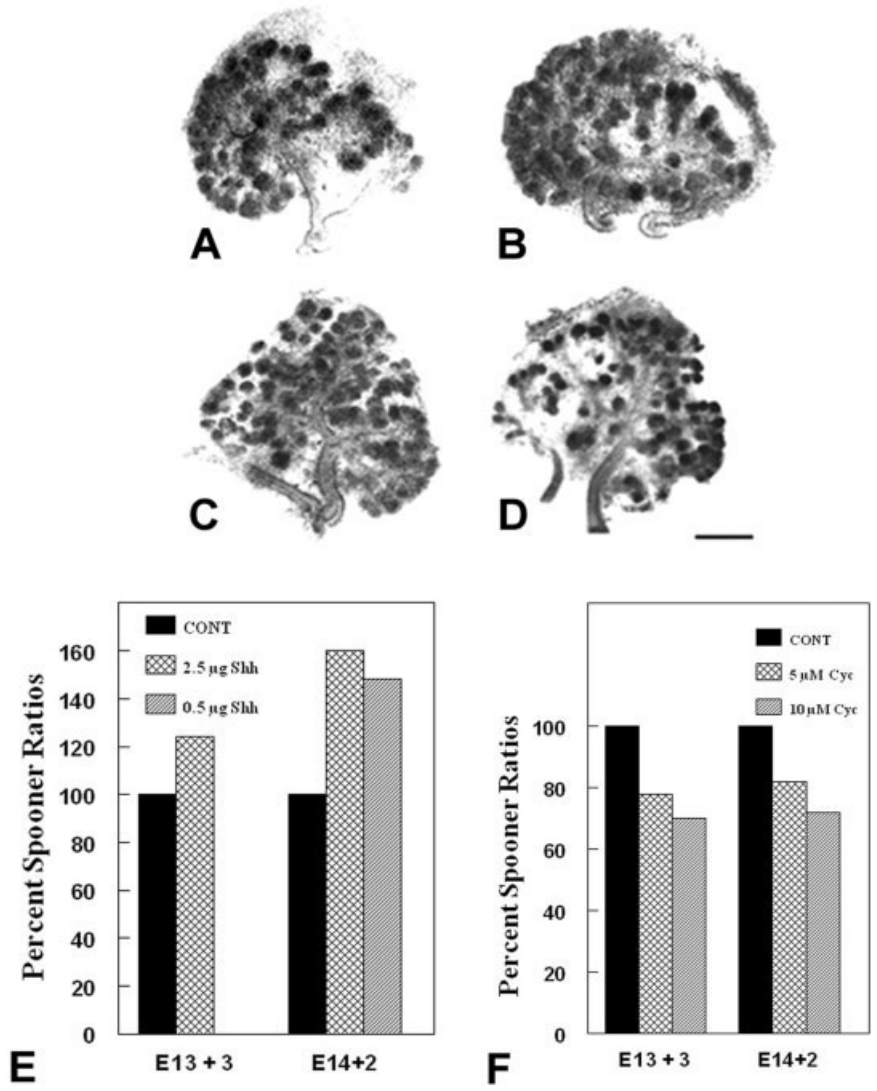

Fig. 3. **A,B:** Enhanced sonic hedgehog (Shh) signaling induced embryonic submandibular gland (SMG) branching morphogenesis. Paired embryonic day (E) 13 + 3 (*Late Initial Bud* stage) SMGs were cultured in the absence (A) or presence (B) of 2.5 μg/ml Shh peptide. **C,D:** Cyclopamine-mediated inhibition of Shh signaling significantly decreased branching morphogenesis. Paired E13+3 SMGs were cultured in the presence (D) or absence (C) of 10 μM cyclopamine supplementation. E,F: Quantitative analysis of branching morphogenesis with enhanced or abrogated Shh signaling *in vitro*. **E:** E13 + 3 or E14+2 SMG primordia were cultured in the absence or presence of 2.5 μg/ml Shh supplementation (E13 + 3, n = 6; E14 + 2, n = 4) or 0.5 μg/ml Shh supplementation (E14 + 2, n = 3), and Spooner ratios were determined. **F:** Quantitative analysis of branching morphogenesis in cyclopamine (Cyc) -treated explants. E13+3 or E14+2 SMG primordia were cultured in the presence or absence of 5 μM (E13+3, n = 6; E14+2, n = 5) or 10 μM (E13+3, n = 4; E14+2, n = 5) cyclopamine and Spooner ratios were determined. CONT, control. Scale bar = 35 μm in D (applies to A–D).

above. A significant ~26% ($P < 0.01$) and ~24% ($P < 0.02$) reduction in branching morphogenesis was seen in E13+3 and E14+2 cyclopamine-treated explants, respectively, compared with controls (Fig. 3C,D,F). We did not find significant dose-dependent or stage-dependent differences with cyclopamine treatment.

Given the notable decrease in SMG branch number in *Shh* null mice and cyclopamine-treated explants, as well as prior reports that Shh signaling induces cell proliferation (Kenney and Rowitch, 2000; Barnes et al., 2001; Lowry et al., 2002; Hall et al., 2003), we postulated that the cyclopamine-mediated decrease in branching morphogenesis is due to a reduction in epithelial cell proliferation. Thus, we cultured E14+3 SMG primordia in the presence or absence of 10 μM cyclopamine and determined the epithelial cell proliferation index (the number proliferating cell nuclear antigen (PCNA) -positive cells/total number of cells) for each treatment. Cyclopamine-treated explants exhibit a significant 45% decrease ($P < 0.01$) in epithelial cell proliferation, indicating that endogenous Shh signaling modulates embryonic SMG epithelial cell proliferation.

Effect of Enhanced or Interrupted Shh Signaling on Gli 3

The Shh signal transduction pathway is largely mediated through Gli. Of particular note is the demonstration that Shh signaling directly inhibits the processing of Gli 3 into its repressor form and simultaneously down-regulates *Gli3* gene expression (Marigo et al., 1996; Lee et al., 1997). Because little is known about the relationship between Shh signaling and its downstream effectors during embryonic SMG development and Gli 3 is a downstream target of Shh (Marigo et al., 1996; Lee et al., 1997), we enhanced or interrupted Shh signaling in vitro and determined the cell-specific distribution of Gli 3 protein in E14 + 3 explants. Shh-supplemented SMGs exhibit a marked decrease in immunodetectable Gli 3 protein within terminal bud epithelia compared with control (compare Fig. 4A with B). In contrast, a substantial increase in Gli3 protein was seen with cyclopamine-mediated inhibition of Shh signaling in vitro (compare Fig. 4C with B).

FGF8 Rescues Cyclopamine-Treated Explants

In a further attempt to understand how Shh exerts its stimulatory effects on SMG branching morphogenesis, we focused our attention on FGF8, a putative target of Shh signaling shown to be essential for embryonic SMG and other facial development (Trumpp et al., 1999; Aoto et al., 2002; Frank et al., 2002; Jaskoll,

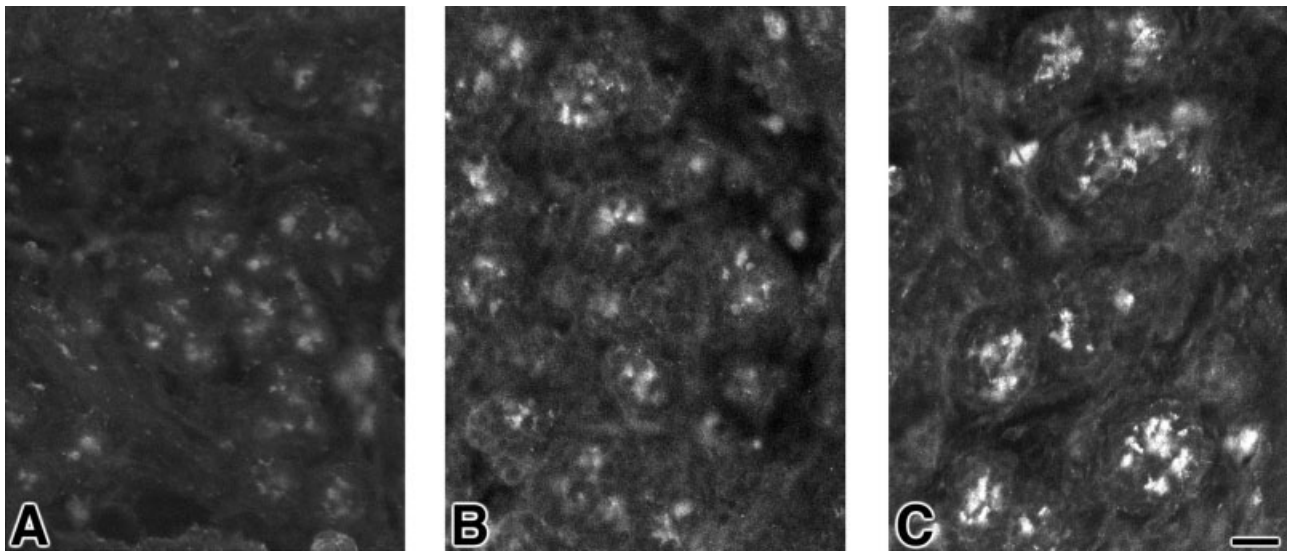

Fig. 4. Shh signaling regulates immunodetectable Gli 3 protein in embryonic submandibular glands (SMGs) in vitro. **A:** Embryonic day (E) 14+3 SMG primordium cultured in 2.5 μg/ml sonic hedgehog peptide. **B:** E14+3 SMG primordium cultured in control medium. **C:** E14+4 SMG primordium cultured in 10 μM cyclopamine. Scale bar = 50 μm in C (applies to A–C).

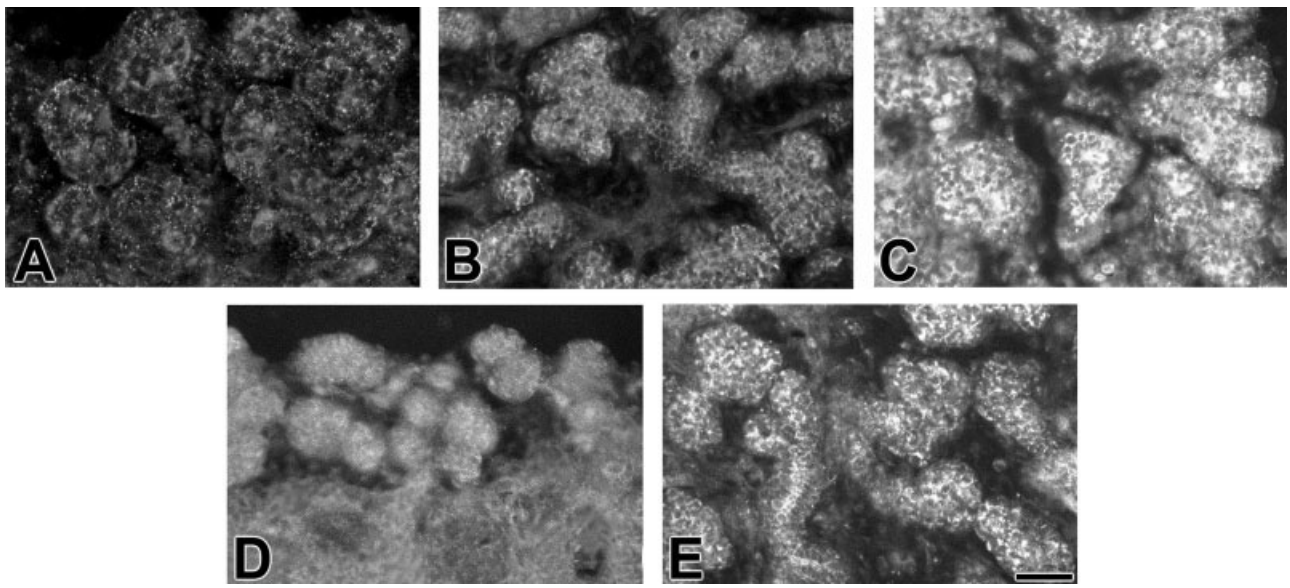

Fig. 5. Regulation of immunodetectable fibroblast growth factor 8 (FGF8) and sonic hedgehog (Shh) in vitro. A–C: FGF8 protein immunolocalization. **A:** Cyclopamine-treated (10 μM) embryonic day (E) 14+3 submandibular gland (SMG). **B:** Control E14 + 3 SMG. **C:** Shh-supplemented (2.5 μg/ml Shh peptide) E14 + 3 SMG. D,E. Shh protein immunolocalization. **D:** Control E14 + 3 SMG. **E:** FGF8 supplemented (200 ng/ml) E14 + 3 SMG. Scale bar = 50 μm in E (applies to A–E).

Melnick and Moon, unpublished observations) and a positive regulator of *Shh* expression (Moon and Capecchi, 2000). First, we determined if Shh signaling regulates FGF8 protein expression during embryonic SMG development in vitro. Shh supplementation induces a substantial increase in immunodetectable FGF8 protein in E14+3 explants compared with controls (compare Fig. 5C with B); with cyclopamine treatment, FGF8 protein is barely immunodetectable (compare Fig. 5A with B). In turn, FGF8 supplementation up-regulates Shh protein expression (Fig. 5D,E). Thus, we next postulated that FGF8 peptide supplementation could rescue the abnormal SMG phenotype seen in cyclopamine-treated explants and restore branching morphogenesis. For this rescue experiment, we preincubated paired E13 SMG primordia in 10 μM cyclopamine supplementation for 3 hr, and then cultured the paired explants in 10 μM cyclopamine with or without 200 ng/ml FGF8 peptide supplementation for 3 days; controls consisted of E13 SMG primordia concurrently cultured in BGJb for 3 days. Comparing mean Spooner ratios, FGF8 supplementation induced a significant 58% increase ($P < 0.01$) in branching morphogenesis relative to cyclopamine

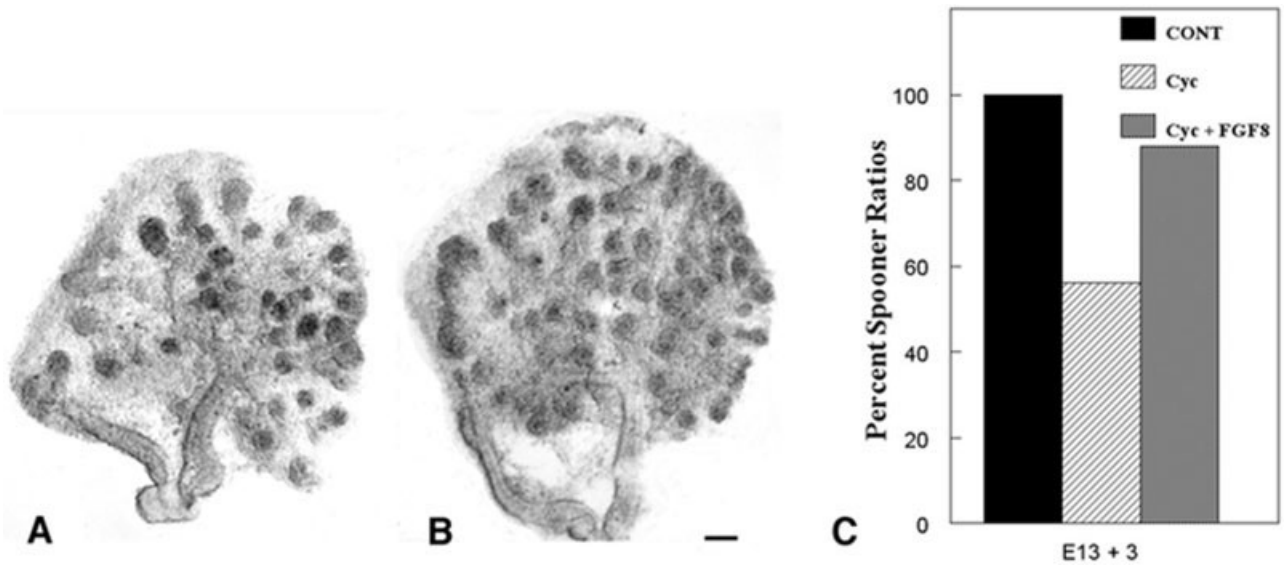

Fig. 6. Fibroblast growth factor 8 (FGF8) supplementation rescues cyclopamine (Cyc) -treated explants. **A,B:** Paired embryonic day (E) 13 submandibular gland (SMG) primordia were preincubated in 10 μM cyclopamine for 3 hr; paired explants were then incubated in 10 μM cyclopamine with (B) or without (A) 200 ng/ml FGF8 for 3 days. n = 3. **C:** Quantitative analysis of SMG explants cultured in control, cyclopamine, or cyclopamine + FGF8 supplemented medium. Scale bar = 35 μm in B (applies to A,B).

treatment alone (Fig. 6); there is no significant difference between cyclopamine + FGF8 and controls ($P > 0.06$). This indicates that enhanced FGF8-mediated signaling can compensate for decreased Shh signaling and restore epithelial branching in vitro.

DISCUSSION

The epithelial localization of Shh and its receptors (Ptc, Smo) clearly suggests the involvement of Shh signaling in SMG epithelial branching morphogenesis and histodifferentiation. Although recent localization studies by McMahon and colleagues (Gritli-Linde et al., 2002) have shown that Shh signaling can occur both at short and long distances from Shh-producing cells, the present study suggests that Shh signaling may occur entirely within the SMG epithelium. As such, the SMG distribution patterns for components of the Shh signaling cascade markedly differ from those seen in other branching organs (i.e., lung, kidney, pancreas, prostate gland; Miller et al., 2001; Lamm et al., 2002; Yu et al., 2002; Wang et al., 2003). In these organs, Shh is localized to the epithelia, whereas Ptc, Smo, and/or Gli are mainly found in the adjacent underlying mesenchyme; it seems the mesenchyme and not the epithelium is the primary target of Shh signaling. Thus, it appears that Shh signaling mediates epithelial–mesenchymal interactions to promote pulmonary, kidney, and prostatic branching morphogenesis and that Shh acts as a paracrine factor in these branching organs (Bellusci et al., 1997; Miller et al., 2001; Lamm et al., 2002; Yu et al., 2002; Chuang and McMahon, 2003; Weaver et al., 2003).

It is unclear why such notable cell-specific differences in the distribution of the Shh pathway components are seen in embryonic SMGs compared with other branching organs. One possible explanation is that the mechanism of SMG branching morphogenesis is unique among branching organs. Unlike the embryonic lung, kidney, pancreas, and prostate gland in which the mesenchymal cells proliferate and form the parenchyma, almost no proliferation is seen in the SMG mesenchyme and the mesenchymal tissue becomes less dense with each successive developmental stage (Jaskoll and Melnick, 1999; Melnick and Jaskoll, 2000). Moreover, as embryonic SMG morphogenesis progresses, ductal and terminal epithelial cell proliferation and cavitation (apoptosis) results in the formation of the differentiated gland (see reviews in Melnick and Jaskoll, 2000; Jaskoll and Melnick, 2003). By contrast, embryonic lung, kidney, pancreas, and prostate exhibit a defined lumen within the initial epithelial bud; thus, only epithelial cell proliferation and not apoptosis is required for organogenesis. Studies in our laboratory suggest that, beginning in the *Pseudoglandular* stage, the SMG mesenchyme and its extracellular matrix components are primarily for structural support and preventing terminal epithelial anoikis (see discussion in Melnick and Jaskoll, 2000).The nearly exclusive epithelial localization of *protein* components of other important signaling pathways (e.g., TGF/EGF/EGFR, FGF/FGFR, TNF/TNFR, Eda/Edar) in *Pseudoglandular* stage and older SMGs supports the hypothesis that post-bud morphogenesis is largely due to epithelial–epithelial, not epithelial–mesenchymal, interactions (see reviews in Melnick and Jaskoll, 2000; Jaskoll and Melnick, 2003). This finding would be markedly different from other branching organs and may reflect its far older evolutionary lineage.

Functional Role of Shh Signaling During Embryonic SMG Development

Our in vitro studies demonstrate that Shh functions as a mitogen to pro-

mote SMG epithelial cell proliferation and branching morphogenesis. This finding is supported by previous reports that Shh signaling is an important mitogen during the development of the neural tube, lung, kidney, and prostate gland (Bellusci et al., 1997; Lamm et al., 2002; Yu et al., 2002; McMahon et al., 2003; Guerrero and Ruiz i Altaba, 2003; Thibert et al., 2003). Although the exact mechanism of the mitogenic effect during embryonic SMG development is unclear, Shh likely stimulates cell proliferation by promoting entry into the S-G(2) proliferative stage of the cell cycle, inducing cyclin B1 nuclear translocation, opposing $p21^{cip1}$ growth inhibitory effects, and/or up-regulating *Nmyc* (Fan and Khavari, 1999; Kenney and Rowitch, 2000; Gritli-Linde et al., 2000; Barnes et al., 2001; Lowrey et al., 2002; Kenney et al., 2003; Oliver et al., 2003). Because Schneider et al. (2000) have demonstrated smaller minor salivary glands in *Nkx3.1* null mice, and Nkx3.1 is downstream of the Shh signal, the SMG phenotype may also be mediated by Nkx3.1 expression. Future studies are needed to delineate the precise mechanism involved in Shh-mediated embryonic SMG morphogenesis.

Of interest, there is a significant stage-specific difference in Shh-stimulated branching. *Pseudoglandular* (E14) stage SMG primordia cultured in the presence of Shh supplementation exhibit an ~twofold increase in branching morphogenesis compared with *Initial Bud* (E13) stage primordia. Tissue responsiveness to Shh may change as development proceeds due to the presence of considerably more *endogenous* Ptc receptors in older glands By contrast, no stage-specific or dose-dependent differences in cyclopamine treatment were seen. Because cyclopamine interrupts *endogenous* Shh signaling by binding directly to the Smo receptor (Chen et al., 2002), our results suggest that the lower concentration of cyclopamine used was sufficient to bind most (if not all) endogenous Smo in both *Initial Bud* and *Pseudoglandular* stage SMGs.

Most surprisingly, we found a SMG in *Shh* null mice. Given that (1) $Shh^{-/-}$ mice are characterized by cyclopia, holoprosencephaly, and the virtual absence of mandibular derivatives (i.e., Meckel's cartilage, tongue, teeth) (Chiang et al., 1996), (2) Shh is essential for neural crest cell survival (Ahlgren et al., 2002), and (3) the SMG initial bud develops as an invagination of the oral epithelium into the underlying neural crest-derived mesenchyme of the mandibular arch (Jaskoll and Melnick, 1999; Jaskoll et al., 2001), it was reasonable to predict the absence of a SMG in E18.5 *Shh* null mice. Instead, the null gland is "paedomorphic," failing to progress beyond a stage resembling the *Early Pseudoglandular*.

The explanation for the presence of SMG primordia in the absence of other mandibular derivatives in $Shh^{-/-}$ mice most likely lies in temporal differences in tissue-specific dependence on Shh signaling. For example, several laboratories have elegantly demonstrated the dependence of tooth development on Shh signaling from the outset (Hardcastle et al., 1998; Dassule et al., 2000; Cobourne et al., 2001; Gritli-Linde et al., 2002). By contrast, the presence of very low levels of immunodetectable Shh and components of its signaling cascade in the *Initial Bud* stage suggest that embryonic SMG bud initiation is far less dependent (if at all) on the Shh pathway. Most likely, other signaling pathways, possibly even other hedgehog proteins, regulate initial bud formation. Subsequent stages of SMG appear to be far more dependent on Shh signaling, as evidenced by the ontogenic arrest of E18.5 *Shh* null SMGs.

By analogy, recent studies have clearly shown that Shh signaling plays markedly different roles during prostatic initial bud formation and subsequent ductal differentiation during the embryonic and postnatal periods, respectively (Podlasek et al., 1999; Lamm et al., 2002; Wang et al., 2003). Shh signaling induces embryonic prostate gland epithelial branching, while inhibiting it in the postnatal gland. It is also important to note that Shh signaling targets different cell populations (i.e., epithelium or mesenchyme), as well as elicits markedly different biologic effects, in different developing tissues (present study; Bellusci et al., 1997; Kim and Melton, 1998; Gallego et al., 2002; Yu et al., 2002; Wang et al., 2003). Although Shh signaling promotes morphogenesis in the embryonic SMG (present study), lung (Bellusci et al., 1997; Pepicelli et al., 1998), kidney (Yu et al., 2002), and prostate (Lamm et al., 2002; Wang et al., 2003), it inhibits pancreatic organogenesis (Apelqvist et al., 1997; Kim and Melton, 1998; Hebrok et al., 1998; Kawahira et al., 2003), gastric gland cell proliferation (Van Den Brink et al., 2001), and postnatal prostate ductal branching (Wang et al., 2003). Future studies are needed to delineate which target genes downstream of the Shh signal pathway are important for such temporal and tissue-specific responses.

FGF8 Can Rescue the Abnormal SMG Phenotype in Cyclopamine-Treated Explants

FGF8 provides mitogenic, survival, and anti/pro-differentiation signals during embryonic development (Szebenyi and Fallon, 1999; Goldfarb, 2001; Frank et al., 2002). Recent studies of mice with *Fgf8* deficiency have shown that FGF8 has unique and required functions during the development of murine craniofacial structures, pharyngeal arch derivatives, SMGs, heart, and lung (Trumpp et al., 1999; Frank et al., 2002; Abu-Issa et al., 2002; Jaskoll, Melnick and Moon, unpublished observations). Because *Shh* null mutants display a substantial decline in *Fgf8* transcript expression in the developing face and neural tube (Aoto et al., 2002), it was reasonable to postulate that FGF8 is downstream of the Shh signal. However, the up-regulation of *Fgf8* expression in $Gli3^{-/-}$ mutant and $Gli3^{-/-}/Shh^{-/-}$ double-mutant mice suggests that Shh was not essential for *Fgf8* induction (Aoto et al., 2002). Given these contradictory results, we first determined whether FGF8 protein expression in developing embryonic SMGs is regulated by Shh signaling. Our observation of a marked increase in FGF8 protein in Shh-supplemented explants and a marked decline in Shh-interrupted explants led us to conclude that

Shh signaling directly or collaterally regulates FGF8 protein expression. Based on these results, we then postulated that FGF8 peptide supplementation could rescue the abnormal SMG phenotype seen in cyclopamine-treated glands. FGF8-supplemented explants exhibit a significant 58% increase in branching morphogenesis compared with cyclopamine treatment alone. Importantly, it appears that enhancement of a parallel, but related, key downstream signaling pathway can compensate for decreased Shh signaling and restore branching morphogenesis. What remains to be determined is the functional relationship between the Shh signal transduction pathway and other key downstream signaling pathways and how these pathways are integrated during development. The clear identification of pathway cross-talk will inform our understanding of embryonic SMG morphoregulation in particular and organogenesis in general.

EXPERIMENTAL PROCEDURES

Tissue Collection

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, ME), were maintained and mated as previously described (Jaskoll and Melnick, 1999); plug day = day 0 of gestation. Pregnant females were killed by cervical dislocation on embryonic days 12–18 (E12–E18). Embryos were dissected in cold phosphate-buffered saline (PBS) and staged according to Theiler (1989). E12 and E13 heads or E14–E18 SMGs were collected, fixed in Carnoy's fixative, dehydrated through graded alcohols, cleared in xylene, and embedded in low-melting-point paraplast.

Immunolocalization

To determine the cell-specific distribution of Shh, Ptc, Smo, and Gli 3 in SMG development, we evaluated E12–E18 SMGs by immunohistochemistry essentially as previously described (Jaskoll and Melnick, 1999) using affinity-purified peptide-specific polyclonal antibodies to Shh (Shh H-160 (sc-9024)), Ptc (patched G-19 (sc-6149); patched C-20 (sc-6147); patched H-267 (sc-9016)), Smo (Smo C-17 (sc-6367) and Smo-N-19 (sc-6366)), and Gli-3 (Gli3-C20 (sc6154)) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For Ptc, we confirmed its distribution pattern by using three different antibodies; these antibodies are specific for Ptc1 and does not cross-react with Ptc2. For Smo, we confirmed its distribution pattern by using two different antibodies. Controls consisted of preimmune serum or no primary antibodies. For FGF8 protein immunolocalization in vitro, anti-FGF8 (FGF-8-N-19 (sc6958)) antibody was purchased from Santa Cruz Biotechnology, Inc.

Characterization of *Shh* Null SMGs

Shh$^{+/-}$ mice were purchased from Jackson Laboratories (Stock Shh, #JR3318) and mated. The resulting genotypes were verified by polymerase chain reaction as previously described (Carlsson and Mahlapuu, 2002). *Shh* wild-type and null mice were fixed in 10% buffered formalin, and the microscopic anatomy of *Shh* wild-type and null mice was determined by routine hematoxylin and eosin staining. A minimum of three mice per genotype were evaluated.

Culture System

E13 (*Late Initial Bud* stage) or E14 (*Pseudoglandular* stage) SMG primordia were cultured by using a modified Trowell method for up to 3 days as previously described (Melnick et al., 2001a,b,c). The medium consisted of BGJb (Life Technologies, Rockville, MD) supplemented with 1% bovine serum albumin, 0.5 mg of ascorbic acid/ml and 50 units of penicillin/streptomycin (Life Technologies), pH 7.2, and replicate cultures were changed every other day. For supplementation studies, paired E13 or E14 SMG primordia were cultured in the presence or absence of exogenous Shh peptide (0.5 μg/ml or 2.5 μg/ml, R&D). Cyclopamine, a steroid alkaloid previously shown to block Shh signaling (Cooper et al., 1998; Incardona et al., 1998; Taipale et al., 2000; Chen et al., 2002), was used to abrogate Shh signaling. For these interruption studies, 250 μM cyclopamine was dissolved in 100 μl of 95% ethanol + 900 μl of BGJb and then diluted in enriched BGJb described above to yield a 5 μM or 10 μM concentration. Similar cyclopamine concentrations have been used to disrupt tongue and prostate gland development (Hall et al., 2003; Mistretta et al., 2003; Wang et al., 2003). Paired E13 or E14 SMG primordia were cultured in 5 μM or 10 μM cyclopamine; controls consisted of enriched BGJb alone. Spooner branch ratios (end bud number/initial bud number) were determined for each explant as previously described (Melnick et al., 2001a,b,c); the data were arcsin transformed to ensure normality and homoscedasticity (Sokal and Rohlf, 1981). Because a notable difference in SMG branch number is seen among litter mates, we compared the number of terminal buds in right and left glands (treated and control) from each embryo by paired *t*-test for all embryos studied. In each culture experiment, we evaluated three to six explants per treatment. For the rescue experiment, paired E13 SMG primordia were cultured in 10 μM cyclopamine for an initial period of 3 hr and then cultured in cyclopamine with or without 200 ng/ml FGF8 peptide (R&D) for 3 days. The explants were collected, and mean Spooner ratios were determined and compared as described above.

To determine whether different levels of Shh signaling regulates Gli 3 and FGF8 protein expression, E14 SMG primordia were cultured in control, Shh-supplemented (2.5 μg/ml), or cyclopamine-supplemented (10 μM) medium (n = 3). Because embryonic SMG morphogenesis progresses substantially slower in vitro than in vivo, we extended the culture period to 3 days to allow for more extensive branching morphogenesis. To determine whether FGF8 supplementation upregulates Shh protein expression, E14 SMG primordia were cultured for 3 days in the presence or absence of exogenous FGF8 peptide (200 ng/ml, R&D Systems; n = 3).

Cell Proliferation Assay

The cell proliferation index was determined as previously described (Melnick et al., 2001a). Briefly, E14 + 3 control and cyclopamine (10 μM) -treated explants were sectioned, incubated with anti-PCNA using the Zymed mouse PCNA kit (South San Francisco, CA), and counterstained with hematoxylin. In this experiment, the cytoplasm appears blue and PCNA-positive cells appear brown. Two sections per explant and three explants per group were photographed at ×400. Cell proliferation is quantitated as the ratio of PCNA-positive epithelial cells/total epithelial cells. The data were arcsin transformed, and the means ratios were compared by *t*-test.

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