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FGF8 dose-dependent regulation of embryonic submandibular salivary gland morphogenesis

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Abstract

FGF8 has been shown to play important morphoregulatory roles during embryonic development. The observation that craniofacial, cardiovascular, pharyngeal, and neural phenotypes vary with *Fgf8* gene dosage suggests that FGF8 signaling induces differences in downstream responses in a dose-dependent manner. In this study, we investigated if FGF8 plays a dose-dependent regulatory role during embryonic submandibular salivary gland (SMG) morphogenesis. We evaluated SMG phenotypes of *Fgf8* hypomorphic mice, which have decreased *Fgf8* gene function throughout embryogenesis. We also evaluated SMG phenotypes of *Fgf8* conditional mutants in which *Fgf8* function has been completely ablated in its expression domain in the first pharyngeal arch ectoderm from the time of arch formation. *Fgf8* hypomorphs have hypoplastic SMGs, whereas conditional mutant SMGs exhibit ontogenic arrest followed by involution and are absent by E18.5. SMG aplasia in *Fgf8* ectoderm conditional mutants indicates that FGF8 signaling is essential for the morphogenesis and survival of *Pseudoglandular* Stage and older SMGs. Equally important, the presence of an initial SMG bud in *Fgf8* conditional mutants indicates that initial bud formation is FGF8 independent. Mice heterozygous for either the *Fgf8* null allele ($Fgf8^{+/N}$) or the hypomorphic allele ($Fgf8^{+/H}$) have SMGs that are indistinguishable from wild-type ($Fgf8^{+/+}$) mice which suggest that there is not only an FGF8 dose-dependent phenotypic response, but a nonlinear, threshold-like, epistatic response as well. We also found that enhanced FGF8 signaling induced, and abrogated FGF8 signaling decreased, SMG branching morphogenesis in vitro. Furthermore, since FGF10 and Shh expression is modulated by *Fgf8* levels, we postulated that exogenous FGF10, Shh, or FGF10 + Shh peptide supplementation in vitro would largely "rescue" the abnormal SMG phenotype associated with decreased FGF8 signaling. This is as expected, though there is no synergistic effect with FGF10 + Shh peptide supplementation. These in vitro experiments model the principle that mutations have different effects in the context of different epigenotypes.

Keywords: FGF8; Submandibular salivary gland; Pharyngeal arch ectoderm; FGF10; Shh; Branching morphogenesis; Craniofacial defects

Introduction

The FGF family includes at least 23 members which have been shown to induce diverse biological processes, including cell proliferation, cell survival, branching morphogenesis, and histodifferentiation. FGF function is mediated by a family of five transmembrane receptors (FGFRs) to potentially activate several different signaling cascades, including the RAS/MAPK, PLC-γ, P13K, and PKC pathways (see reviews, Goldfarb, 2001; Ornitz and Itoh, 2002; Sleeman et al., 2001; Szebenyi and Fallon, 1999). *Fgf8* is one of the most intensively studied members of this gene family. FGF8/FGFR binding provides survival, mitogenic, anti/pro-differentiation, and patterning signals during embryonic development (Chi et al., 2003; Frank et al., 2002; Garel et al., 2003; Macatee et al., 2003; Moon and Capecchi, 2000). FGF8 is required in gastrulation, neural pattern-

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ing, and left–right axis determination (Boettger et al., 1999; Meyers and Martin, 1999; Storm et al., 2003; Sun et al., 1999). Since *Fgf8* null embryos die early in embryogenesis (Meyers et al., 1998; Moon and Capecchi, 2000; Sun et al., 1999), several groups have employed conditional mutagenesis and hypomorphic alleles to study the role of FGF8 signaling during craniofacial, cardiovascular, pharyngeal, limb, and neural development (Abu-Issa et al., 2002; Frank et al., 2002; Garel et al., 2003; Macatee et al., 2003; Meyers and Martin, 1999; Meyers et al., 1998; Moon and Capecchi, 2000; Moon et al., 2000; Storm et al., 2003; Sun et al., 2000; Trumpp et al., 1999). These studies clearly demonstrate that FGF8 signaling plays multiple important morphoregulatory roles during development. The phenotypes of *Fgf8* germline null mutants (Meyers and Martin, 1999; Meyers et al., 1998; Moon and Capecchi, 2000) and *Fgf8* hypomorphic and conditionally mutant mice (Abu-Issa et al., 2002; Chi et al., 2003; Frank et al., 2002; Garel et al., 2003; Lewandoski et al., 2000; Macatee et al., 2003; Moon and Capecchi, 2000; Storm et al., 2003) indicate that in many of its expression domains, FGF8 has unique and required roles that cannot be compensated by other endogenous FGFs, and that FGF8 induces specific downstream signal transduction pathways during embryonic development. Moreover, since craniofacial, cardiovascular, pharyngeal, and neural phenotypes vary with *Fgf8* gene dosage (Abu-Issa et al., 2002; Chi et al., 2003; Frank et al., 2002; Garel et al., 2003; Lewandoski et al., 2000; Macatee et al., 2003; Meyers et al., 1998; Moon and Capecchi, 2000; Storm et al., 2003), FGF8 signaling likely induces dose-dependent differences in downstream pathways and responses.

Embryonic submandibular salivary gland (SMG) development is a well-studied and experimentally accessible exemplification of embryonic epithelial branching (e.g., Gresik et al., 1998; Hardman et al., 1994; Hoffman et al., 2002; Jaskoll and Melnick, 1999; Kashimata and Gresik, 1997; Kashimata et al., 2000a,b; Koyama et al., 2003; Larsen et al., 2003; Melnick et al., 2001a,b,c; Wessells, 1977, see reviews, Jaskoll and Melnick, 2003; Melnick and Jaskoll, 2000), and is therefore an ideal system to investigate whether FGF8 regulates branching morphogenesis, and if it does so in a dose-dependent manner.

Embryonic mouse SMG morphogenesis is initiated with a thickening of the oral epithelium of the mandibular arch around E11 and is best conceptualized in stages (Jaskoll and Melnick, 1999): (1) *PreBud 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 neural-crest derived 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 into the surrounding mesenchyme; (4) *Canalization Stage*: the number of lobes is increased, the presumptive ducts begin to exhibit distinct lumina lined by cuboidal epithelial cells, the mesenchyme is more loosely packed; and (5) *Terminal Bud Stage*: distinct, well-developed lumina are seen in presumptive ducts and terminal buds (presumptive acini). Epithelial cell proliferation is found in all stages, even after well-defined lumen formation in the *Terminal Bud Stage*. By contrast, epithelial apoptosis begins with the onset of lumen formation in the *Canalicular* Stage.

It has become increasingly apparent that morphogenesis of complex organs such as the SMG requires cooperation and coordination of multiple signaling pathways to regulate cell proliferation, quiescence, apoptosis, and histodifferentiation (Davidson et al., 2002, 2003; Gardner et al., 2003; Melnick et al., 2001b). Functional studies by us and others demonstrate that embryonic SMG epithelial cell proliferation, apoptosis, and histodifferentiation are mediated by specific growth factors and cytokines (e.g., TGFα/EGF, TGF-β, FGF10, TNF, IL-6, Eda, Shh) expressed at specific times and locations (Hardman et al., 1994; Hoffman et al., 2002; Jaskoll and Melnick, 1999, in press; Jaskoll et al., 2002, 2003, 2004; Kashimata and Gresik, 1997; Kashimata et al., 2000a,b; Melnick et al., 2001a,b,c; Ohuchi et al., 2000). However, it is also clear that other signaling pathways play key roles during SMG morphogenesis.

One of the best candidates is the FGF8 signal transduction pathway, shown to be essential for craniofacial, neural, and cardiovascular development (Abu-Issa et al., 2002; Frank et al., 2002; Garel et al., 2003; Macatee et al., 2003; Meyers and Martin, 1999; Meyers et al., 1998; Moon and Capecchi, 2000; Moon et al., 2000; Storm et al., 2003; Sun et al., 2000; Trumpp et al., 1999). Since (1) embryonic SMGs are derived from mandibular epithelia in the first pharyngeal arch (see review, Jaskoll and Melnick, 2003), (2) *Fgf8* hypomorphic and conditional mutant mice have severely malformed mandibles (Abu-Issa et al., 2002; Frank et al., 2002; Macatee et al., 2003; Trumpp et al., 1999), (3) FGF8 signaling is critical for tooth morphogenesis in the first pharyngeal arch (Trumpp et al., 1999; Tucker et al., 1999), and (4) FGF8 and its cognate receptor are present in all stages of embryonic SMG ontogeny (Jaskoll et al., 2002), we postulated that FGF8 signaling plays an important organogenetic role. Additionally, we observed hypoplastic SMGs in *Fgfr2-IIIc* mutant mice (Jaskoll et al., 2002), which appears to be the primary FGF8 receptor in the embryonic SMG (Jaskoll et al., 2002). Since mandibular phenotypes in mouse mutants vary markedly with *Fgf8* dosage, we further postulated that FGF8 regulates embryonic SMG development in a dose-dependent manner.

To address these hypotheses, we evaluated SMG development in *Fgf8* hypomorphic and tissue-specific ablated mutant mice and demonstrated genotype-specific differences in SMG phenotype. In a complementary set of in vitro experiments, we determined that enhanced FGF8 signaling significantly induces, and abrogated FGF8 signaling significantly reduces, SMG branching morphogenesis in vitro. Finally, since FGF10 and Shh are downstream of the FGF8

signal, we sought to rescue the abnormal phenotype due to reduced FGF8 signaling in vitro with exogenous FGF10 and/or Shh peptide supplementation. Our observation that enhanced FGF10, Shh or FGF10 + Shh signaling incompletely restored branching morphogenesis toward normal indicates that the FGF8 signal transduction pathway induces other specific downstream signaling responses that are essential for embryonic SMG development.

Materials and methods

Characterization of Fgf8 mutant mouse SMGs

Fgf8 wild-type and mutant mice were generated using the strategy previously described (Frank et al., 2002; Macatee et al., 2003; Moon and Capecchi, 2000) and their genotypes were confirmed by PCR as previously described (Frank et al., 2002; Moon and Capecchi, 2000). Fig. 1 demonstrates the different alleles used in this study: the $Fgf8^{H}$ hypomorphic allele (Fig. 1A), the $Fgf8^{C}$ nonhypomorphic conditional reporter allele (Fig. 1B), Cre-mediated recombination of the $Fgf8^{C}$ allele to the $Fgf8^{CR}$ allele (Fig. 1C), and the $Fgf8^{N}$ null allele (Fig. 1D). Cre-mediated recombination of the $Fgf8^{C}$ allele to the $Fgf8^{CR}$ allele deletes exon 5 and prevents the production of functional *Fgf8* mRNA. All mutant embryos are in a 75% C57Bl6, 25% SV129 background. Complete characterization of these alleles is previously described (Frank et al., 2002; Macatee et al., 2003).

The *AP2α-IRESCre* driver was constructed by targeting an IRESCre cassette into the 3′ untranslated region of the *AP2α* locus (Macatee et al., 2003). This cassette contains an Internal Ribosomal Entry Site, IRES (Jackson et al., 1990; Jang and Wimmer, 1990), upstream of the Cre recombinase gene (Sauer and Henderson, 1988) and an frt-flanked neomycin phosphotransferase gene. Placing the IRESCre cassette between the stop codon and the endogenous polyadenylation signal allows regulated production of Cre by the *AP2α* locus without interfering with endogenous *AP2α* function. Complete characterization of the function of this Cre driver is detailed in Macatee et al. (2003). This allele was used to drive recombination/inactivation of the nonhypomorphic $Fgf8^{AP}$ allele in the conditional mutant mice so that FGF8 is completely ablated from the first pharyngeal arch ectoderm from the time of formation of the first arch (Macatee et al., 2003).

Fgf8 wild-type and mutant pregnant females were euthanized by cervical dislocation on days 13.5, 15.5, and 18.5 of gestation, the embryos were dissected in cold phosphate-buffered saline (PBS), and the heads were dissected. Tissues were placed in Carnoy's fixative, and embedded in paraplast as previously described (Jaskoll and Melnick, 1999). Serial coronal sections were mounted on gelatin-coated slides and stained with hematoxylin and eosin as previously described (Jaskoll and Melnick, 1999). A minimum of three heads per age for each genotype was analyzed.

Culture system

Timed-pregnant females (strain C57BL/6) were obtained from Harlan (Indianapolis, IN). Embryos were dissected in cold PBS, staged according to Theiler (1989), and E13

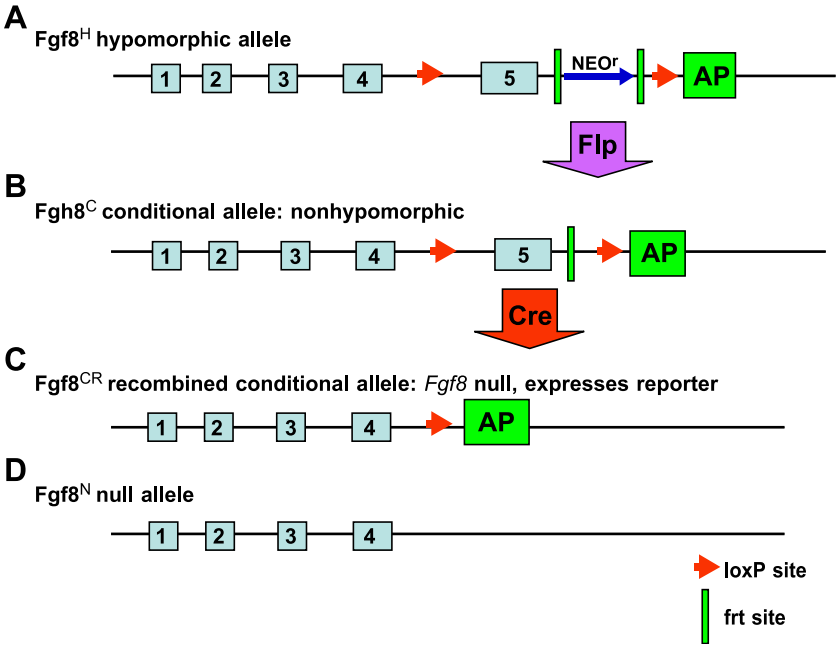

Fig. 1. The different *Fgf8* alleles. (A) The $Fgf8^{H}$ hypomorphic allele. (B) The $Fgf8^{C}$ nonhypomorphic conditional reporter allele. (C) Cre-mediated recombination of the $Fgf8^{C}$ allele to the $Fgf8^{CR}$ allele. (D) The $Fgf8^{N}$ null allele.

(*Late Initial Bud* Stage) SMG primordia were cultured using a modified Trowell method as previously described (Melnick et al., 2001a). The medium consisted of BGJb (Life Technologies, Rockville, MD) supplemented with 1% BSA, 0.5 mg ascorbic acid/ml, and 50 units penicillin/streptomycin (Life Technologies), pH 7.2, and replicate cultures were changed every other day. *Supplementation studies*: paired E13 SMG primordia were cultured for 3 days in the absence or presence of exogenous FGF8 peptide (200 ng/ml, R&D Systems, Inc.); controls consisted of enriched BGJb alone. *Interruption studies*: paired E13 SMG primordial were cultured for 3 days in the presence of soluble FGFR2-IIIc-Fc chimera (3 or 5 ng/ml; R&D Systems); controls consisted of primordium cultured in IgG-Fc (3 or 5 ng/ml; R&D Systems). Because a notable difference in SMG epithelial branch number is seen between embryos within a given litter and among litters, we calculated the Spooner branch ratios (epithelial bud number on day 3/bud number on day 0) for each explant as previously described (Jaskoll et al., 1994, 2003) and compared the Spooner branch ratios in right and left glands (treated and control) from each embryo. Mean Spooner ratios were determined, the data were arcsin transformed to insure normality and homoscedasticity, and compared by paired *t* test for all embryos studied (Sokal and Rohlf, 1981). In this set of experiments, three to five explants/treatment were analyzed.

Rescue experiments

Paired E13 SMG primordia were cultured in 5 ng/ml FGFR2-IIIc-Fc chimera for an initial period of 3 h and then each pair was cultured in FGFR2-IIIc-Fc or FGFR2-IIIc-Fc + 200 ng/ml FGF10 (R&D Systems) for 3 days ($n = 8$). This FGF10 concentration has been shown in our laboratory to induce a significant increase in branching morphogenesis in E13 + 3 SMGs compared to controls (unpublished). A second set of paired E13 primordia was cultured in 5 ng/ml FGFR2-IIIC-Fc or FGFR2-IIIc-Fc + 2.5 μg/ml Shh peptide (R&D Systems) ($n = 4$). This Shh concentration has been shown to induce a significant increase in branching morphogenesis in E13 + 3 SMGs compared to controls (Jaskoll et al., 2004). A third set of paired E13 primordia was cultured in 5 ng/ml FGFR2-IIIC-Fc or FGFR2-IIIc-Fc + 200 ng/ml FGF10 + 2.5 μg/ml Shh peptide ($n = 7$). A separate set of primordial was set as controls to verify that FGFR2-IIIc-Fc interrupted branching morphogenesis; these controls consisted of E13 primordia cultured in 5 ng/ml IgG-Fc for 3 days ($n = 6$). The explants were collected and mean Spooner ratios determined and compared as described above.

FGF10 and Shh protein expression

FGF10 and Shh protein expression was analyzed in E15.5 control and conditional mutant SMGs by immunohistochemistry using anti-FGF10 and anti-Shh polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) essentially as previously described (Jaskoll et al., 2002, 2004).

Results

Fgf8 gene dosage and salivary gland branching

To delineate the role of FGF8 signaling during embryonic SMG morphogenesis, we initially investigated the SMG phenotype in *Fgf8* hypomorphic and conditional mutant mice. Compound heterozygous mice bearing both the $Fgf8^{H}$ and $Fgf8^{N}$ alleles (genotype $Fgf8^{H/N}$, Abu-Issa et al., 2002; Frank et al., 2002; Moon and Capecchi, 2000) are hypomorphs; they produce less than 50% the amount of *Fgf8* mRNA of wild-type animals (Frank et al., 2002); they are small and exhibit craniofacial abnormalities such as micrognathia and cleft palate, central nervous system malformations, and a host of other congenital defects (Abu-Issa et al., 2002; Frank et al., 2002). The tissue-restricted, conditional mutant mice used in this study are also compound heterozygotes at the *Fgf8* locus; they bear a *non-hypomorphic conditional* allele called $Fgf8^{C}$, and the $Fgf8^{N}$ allele. They also carry the Cre-recombinase driver, AP2α-IRESCre, in which Cre recombinase coding sequences are expressed under control of the AP2α gene; this Cre driver inactivates the *Fgf8* conditional allele in the *ectoderm* of the first pharyngeal arch from the time of arch formation (Macatee et al., 2003). The genotype of these animals is $Fgf8^{C/N}$; AP2α $^{IRESCre/+}$. These conditional mutants display an array of severe craniofacial, cardiovascular, and glandular abnormalities, including a severely hypoplastic mandible (Macatee et al., 2003).

We analyzed E13.5, E15.5, and E18.5 *Fgf8* control ($Fgf8^{+/+}$ or $Fgf8^{C/+}$), hypomorphs ($Fgf8^{H/N}$), and conditional mutant ($Fgf8^{C/N}$; AP2α$^{IRESCre/+}$) SMGs. These pharyngeal arch, *Fgf8* ectoderm-ablated mutants will henceforth be referred to as *Fgf8* conditional mutants (CM). The E13.5 control SMG appears as an elongated solid cord of epithelium terminating in an end-bulb consisting of several branches (i.e., a *Late Initial Bud* Stage) (Figs. 2A, D). In contrast, the E13.5 *Fgf8* hypomorphs exhibit a hypoplastic, branchless SMG bud (compare Figs. 2B, E to 2A, D) which is similar in appearance to an earlier *Initial Bud* Stage (Jaskoll and Melnick, 1999). The E13.5 *Fgf8* conditional mutant SMG is severely hypoplastic (compare Figs. 2C, F to 2A, D), consisting of an extremely small, branchless initial bud similar to the earliest *Initial Bud* Stage (Jaskoll and Melnick, 1999). Interestingly, in all E13.5 conditional mutants analyzed (5/5), the SMG buds laterally from the epithelium instead of ventrally (compare Figs. 2C, F to 1A, D); this abnormal SMG positioning is also frequently (3/5) seen in *Fgf8* hypomorphs (Figs. 2B, E).

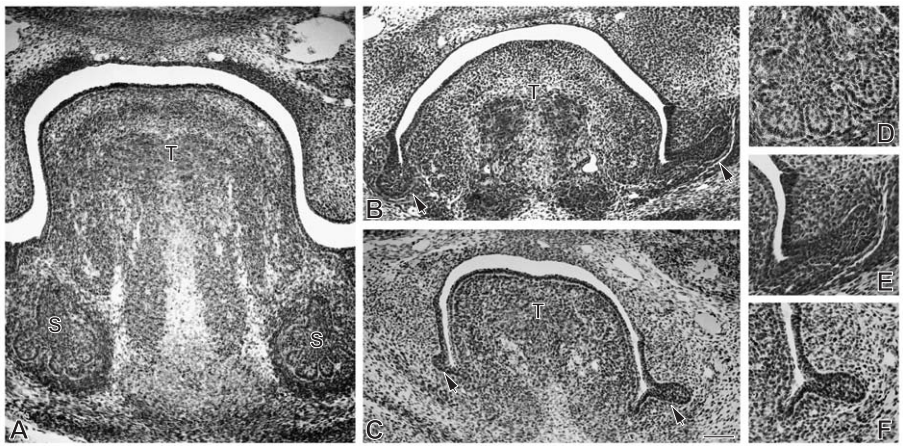

Fig. 2. Abnormal SMG phenotypes in E13.5 *Fgf8* hypomorphic and conditional mutants. (A) E13.5 *Fgf8* control SMG bud. (B) E13.5 *Fgf8* hypomorphic mutant SMG buds (arrows). (C) E13.5 *Fgf8* conditional mutant SMG buds (arrows). (D–F) Higher magnifications of control (D), hypomorphic (E), and conditional mutant (F) SMGs. In the control mouse (A, D), *Late Initial Bud* Stage SMGs (S) with branches in the end-bulb epithelium are seen in the mandible ventrolateral to the tongue (T). The *Fgf8* hypomorphic SMG (B, E) is small and exhibits no branches. In contrast, the conditional mutant gland (C, F) consists of an extremely small epithelial bud composed of disorganized cells. These conditional mutant glands bud laterally from the oral epithelium; this abnormal bud position is also frequently seen in hypomorphic mutants (see right gland in B and E). Coronal sections of control and mutant heads are shown. Bar, A–C, 50 μm; D–F, 25 μm.

With progressive development, extensive epithelial cell proliferation and branching morphogenesis occurs, so that by E15.5, the SMG is characterized by extensive epithelial branching and early ductal luminization (*Canalicular* Stage) (Fig. 3A). The hypomorphic gland undergoes branching but only progresses to the *Early Pseudoglandular* Stage (Fig. 3B); these glands are hypoplastic compared to controls, displaying fewer branches and no ductal lumina (compare Figs. 3B to A). Interestingly, the conditional mutant gland has failed to progress beyond its *Early Initial Bud* Stage and presents as a single, small epithelial bud (Fig. 3C). This severely abnormal phenotype indicates that branching morphogenesis did not occur in the complete absence of FGF8 in the oral epithelium.

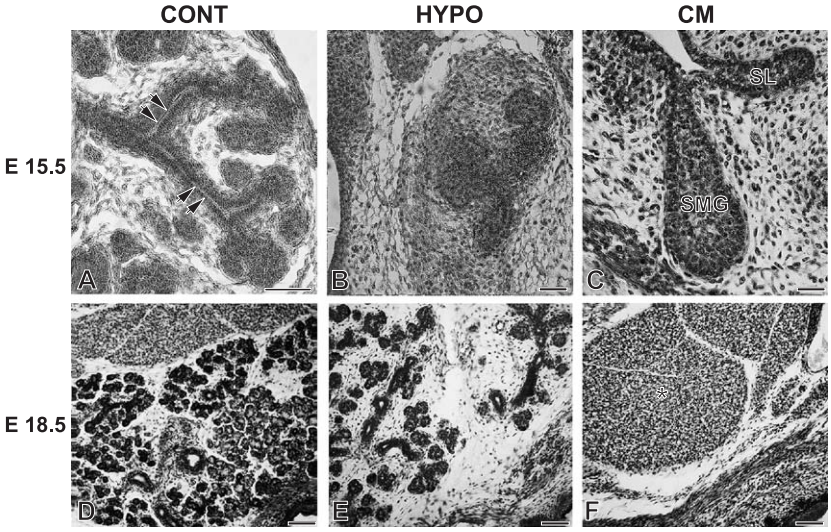

Fig. 3. SMG hypoplasia and aplasia in *Fgf8* hypomorphic and conditional mutant mice. (A–C) E15.5 SMGs. (A) E15.5 *Fgf8* control (CONT) SMG. (B) E15.5 *Fgf8* hypomorphic (HYPO) SMG. (C) E15.5 *Fgf8* conditional mutant (CM) SMG. The E15.5 control SMG (A) is at the *Canalicular* Stage, the stage at which extensive epithelial branches and presumptive ductal lumina (arrow heads) are seen. The *Fgf8* hypomorphic SMG (B) is small, with fewer branches and no ductal lumina (an *Early Pseudoglandular* Stage SMG). The *Fgf8* conditional mutant SMG (C) remains as a single epithelial bud and retains its "initial-bud-like" morphology. (D–F) E18.5 SMGs. (D) E18.5 *Fgf8* control mice exhibit *Late Terminal Bud* Stage SMGs consisting of ducts and terminal buds displaying distinct lumina. (E) E18.5 hypomorphic mutant SMGs are characterized by fewer terminal buds displaying small or absent lumina (*Late Canalicular/Early Terminal Bud* Stage SMG). (F) No SMGs are seen in E18.5 *Fgf8* conditional mutant mice; rather, undifferentiated muscle (*) is found in the site normally occupied by the SMG. SM, submandibular gland; SL, sublingual gland. Bar: A and B, 50 μm; C, 30 μm; D–F, 50 μm.

By E18.5, the control SMG has achieved the *Terminal Bud* Stage, the stage at which distinct, well-developed lumina are seen in presumptive ducts and proacini (Fig. 3D). The hypomorphic gland retains its hypoplastic phenotype; it is characterized by decreased branching morphogenesis and smaller lumina compared to control glands (compare Figs. 3E to D). Surprisingly, no SMGs are found in E18.5 conditional mutants (24/24) (Fig. 3F); instead we detect undifferentiated muscle in the sites normally occupied by the SMG. Our observation of hypoplasia and aplasia in E18.5 *Fgf8* hypomorph and conditional mutants, respectively, indicate that FGF8 signaling is essential for branching morphogenesis and epithelial cell survival during later stages of SMG development.

Finally, to determine if a lesser reduction in *Fgf8* expression affects embryonic SMG morphogenesis, we investigated the SMG phenotype in 18.5 $Fgf8^{+/H}$ and $Fgf8^{+/N}$ mutant mice. These mice express progressively decreasing amounts of functional *Fgf8* mRNA (Frank et al., 2002). They are indis-

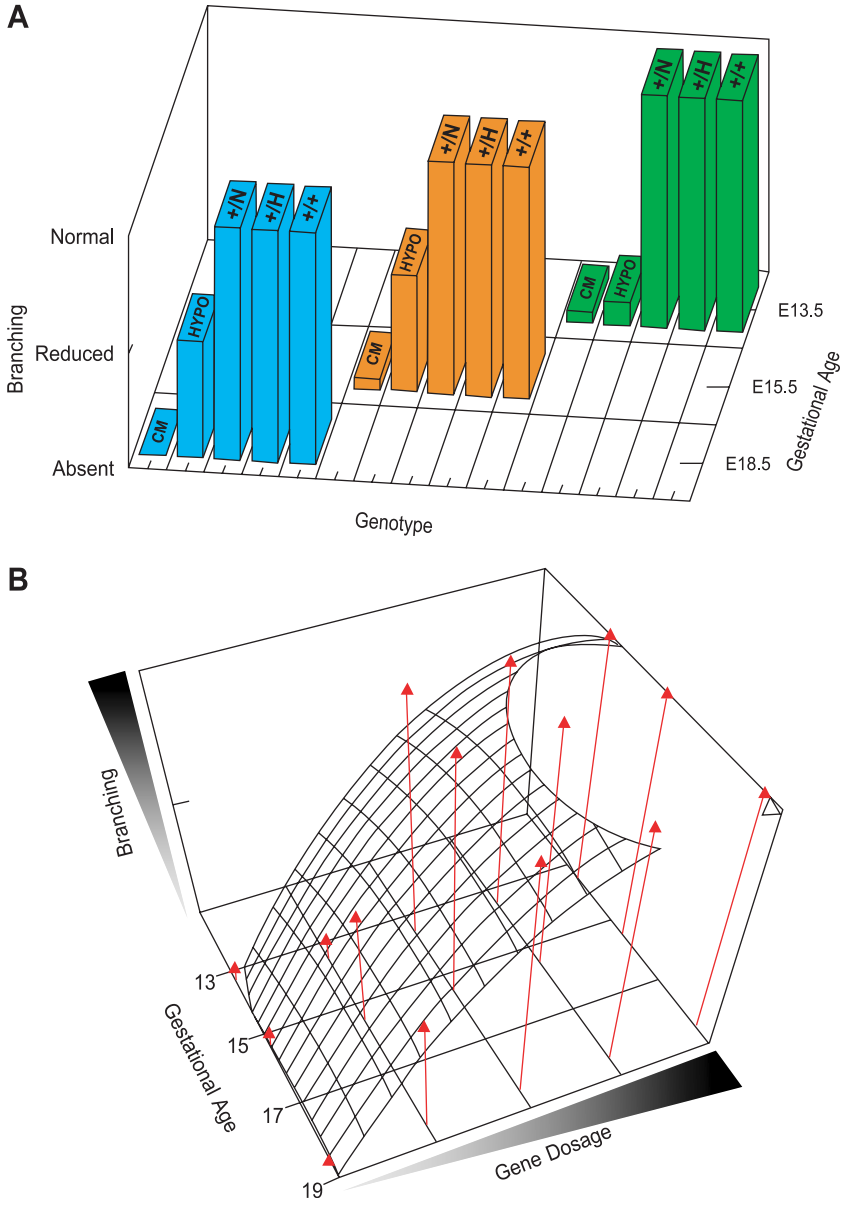

Fig. 4. *Fgf8* gene-dosage and phenotypic effect. (A) Normal branching morphogenesis is seen in E13.5, E15.5, and E18.5 wild-type (+/+) mice and in heterozygous (+/H or +/N) mice which express *Fgf8* transcript amounts greater than or equal to 50% seen in wild-type mice. Reduction of *Fgf8* mRNA in hypomorphic (HYPO; $Fgf8^{H/N}$) mice to less than 50% of wild-type mice results in no branching in E13.5 SMGs and reduced branching in E15.5 and E18.5 glands. The absence of *Fgf8* mRNA in first arch ectoderm in conditional mutants (CM; $Fgf8^{C/N}$; $AP2\alpha^{IRESCre/+}$) results in no SMG branching morphogenesis in E13.5 and E15.5 SMGs and, on day 18.5, SMG aplasia. (B) A graphical representation of the phenotypic surface that derives from the interaction of declining *Fgf8* gene dosage and the evolving epigenome.

tinguishable from wild-type mice throughout development (Moon and Capecchi, 2000; Anne Moon, unpublished data) and both $Fgf8^{+/N}$ and $Fgf8^{+/H}$ SMGs are normal (data not shown).

Taken together, our data indicate that there is a dose-dependent phenotypic response (Fig. 4A). Heterozygous mice with *Fgf8* transcript levels equal to or greater than 50% of wild-type mice (Frank et al., 2002) exhibit normal branching morphogenesis in E13.5, E15.5, and E18.5 SMGs. By contrast, an expression of *Fgf8* transcript levels at less than 50% of wild-type mice, as seen in hypomorphs (Frank et al., 2002), results in no branching morphogenesis in E13.5 SMGs and reduced branching morphogenesis in E15.5 and older SMGs. Finally, the absence of *Fgf8* expression in the ectoderm of the first pharyngeal arch of conditional mutant (CM) mice (Macatee et al., 2003) results in no branching morphogenesis in E13.5 and E15.5 SMGs and SMG aplasia in E18.5.

Epithelial branching is a multifactorial trait, largely dependent upon a series of interrelated genetic circuits through which morphogenesis is realized (i.e., its epigenotype). The functional epigenome changes with advancing gestational age. A phenotypic surface in three-dimensional state space can be utilized to visualize the interaction of variable single gene dosage within an evolving epigenetic context (see review, Nijhout, 2003). A "first pass" graphical visualization of the *Fgf8*-associated phenotypic landscape is depicted in Fig. 4B. Semi-quantitative estimates of branching and gene dosage were derived from the experimental data explicated above. These variable relationships display a trend which is approximately described by a second-order polynomial model with two independent variables

$$z = -1.57 + (0.299x) + (0.301y) + (0.003xy) + (-0.088x^2) + (-0.009y^2)$$

where z is the dependent variable, branching, and x and y are the independent variables, gene dosage and gestational age, respectively. The shape of the phenotypic surface (Fig. 4B) reveals that *Fgf8* loss of function is ultimately epistatic to the epigenome under normal physiologic conditions (i.e., no other gene mutations nor untoward environments). The epistasis of declining *Fgf8* function is a nonlinear emergent property of the complete epigenotype.

Enhanced and abrogated FGF8 signaling in vitro

To further investigate the functional role of FGF8, we used our well-defined organ culture system (Jaskoll et al., 1994, 2003, 2004; Melnick et al., 2001a,b,c) to analyze the effect of enhanced or decreased FGF8 signaling on embryonic SMG development. Paired E13 (*Late Initial Bud* Stage) SMG primordia were cultured in the absence or presence of 200 ng/ml FGF8 peptide for 3 days. Exogenous FGF8 peptide supplementation induced a significant ~36% ($P < 0.05$) increase in branching (Spooner ratios) compared to controls (Figs. 5A, B, E).

We then conducted in vitro interruption studies. Although alternate-spliced forms of FGF8 have been shown to bind with high affinity to FGFR2-IIIc, FGFR3-IIIc, and FGFR4 (MacArthur et al., 1995; Ornitz, 2000), the absence

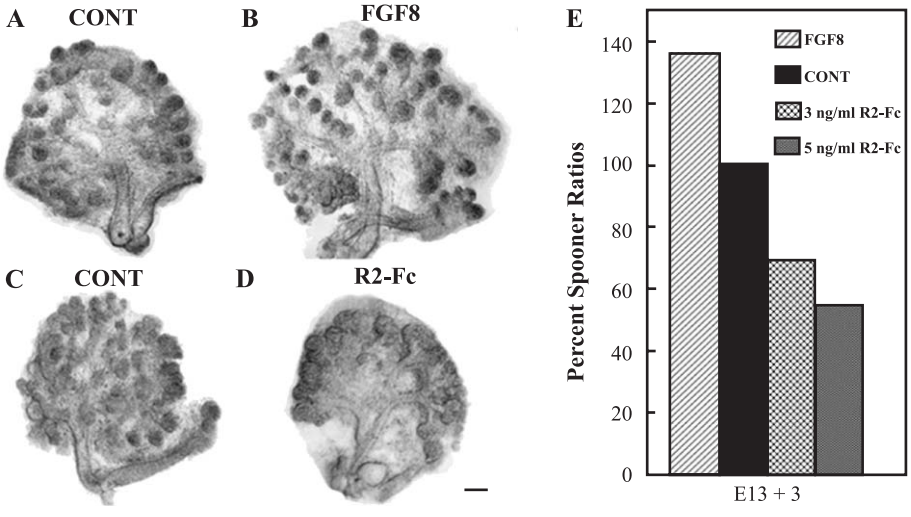

Fig. 5. Enhanced or abrogated FGF8 signaling modulates embryonic SMG branching morphogenesis in vitro. (A, B) Enhanced FGF8 signaling. Paired E13 SMG primordia were cultured for 3 days in the absence (A) or presence (B) of 200 ng/ml FGF8 supplementation. FGF8 supplementation induced a significant increase in bud number compared to controls. (C, D) Abrogated FGF8 signaling. Paired E13 SMG primordia were cultured for 3 days in (C) 5 ng/ml IgG-Fc (Control) or (D) FGFR2-IIIc-Fc (R2-Fc). FGFR2-IIIc-Fc supplementation resulted in a significant decline in branching morphogenesis compared to control. Bar, 75 μm. (E) A comparative representation of the percent change in Spooner branching ratios associated with different treatments relative to each control. A minimum of three explants/treatment were evaluated.

of FGFR3 and FGFR4 at critical stages of SMG development (Jaskoll et al., 2002) indicates that FGF8 likely transmits its signal through FGFR2-IIIc. Thus, we interrupted FGF8 signaling by adding exogenous soluble FGFR2-IIIc-Fc chimera to the culture medium to competitively bind *endogenous* FGFR2-IIIc ligands; controls consisted of IgG-Fc supplementation. This exogenous receptor/ligand binding methodology has previously been used successfully to interrupt FGFR2 signaling (Qiao et al., 2001). E13 SMG primordia were cultured in different concentrations of FGFR2-IIIc-Fc chimera (3 or 5 ng/ml) or IgG-Fc (3 or 5 ng/ml) for 3 days and mean Spooner rations were determined. Treatment with exogenous FGFR2-IIIc-Fc resulted in a significant dose-dependent decrease in Spooner branch ratios compared to controls (Figs. 5C, D, E). Specifically, a 31% ($P < 0.01$) and 45% ($P < 0.02$) decrease in Spooner ratios were seen in the presence of 3 and 5 ng/ml FGFR2-IIIb-Fc chimera, respectively. These results mimic that of the in vivo mutants reported above.

Exogenous FGF10 and/or Shh supplementation in vitro restores the abnormal SMG phenotype toward normal

Although previous studies have demonstrated that FGF8 regulates *Fgf10* and *Shh* expression in the developing embryo (Frank et al., 2002; Macatee et al., 2003; Moon and Capecchi, 2000; Moon and Reichert, unpublished), it was uncertain if FGF8 regulates FGF10 and Shh protein expression in embryonic SMGs. Thus, we analyzed the cell-specific distribution of FGF10 and Shh protein in E15.5 control and conditional mutant mouse SMGs. As shown in Fig. 6, we see a notable decrease in immunodetectable FGF10 and Shh protein in E15.5 *Fgf8* conditional mutant SMGs compared to controls. These results indicate that FGF8 regulates FGF10 and Shh protein expression in the developing SMGs as well.

We then postulated that the abnormal SMG phenotypes of *Fgf8* hypomorphs and conditional mutants result, at least in part, from perturbation of downstream signaling pathways involving FGF10 and/or Shh. To test this hypothesis, we conducted a series of in vitro rescue experiments. We first focused on FGF10 since FGF10 signaling has been shown to play a critical morphoregulatory role during embryonic SMG development (Ohuchi et al., 2000; Revest et al., 2001). Paired E13 SMG primordia were initially cultured in 5 ng/ml FGFR2-IIIc-Fc for 3 h, and then the paired explants were cultured in 5 ng/ml FGFR2-IIIc Fc, with or without 200 ng/ml FGF10 peptide, for 3 days; controls consisted of E13 SMG primordia cultured in 5 ng/ml IgG Fc. Spooner ratios were determined and differences analyzed by paired *t* test. FGF10 supplementation induced a significant 58% ($P < 0.05$) increase in branching compared to FGFR2-IIIc-Fc treatment alone (Figs. 7A, B, G).

We then sought to use exogenous Shh peptide supplementation to rescue the abnormal SMG phenotype associ-

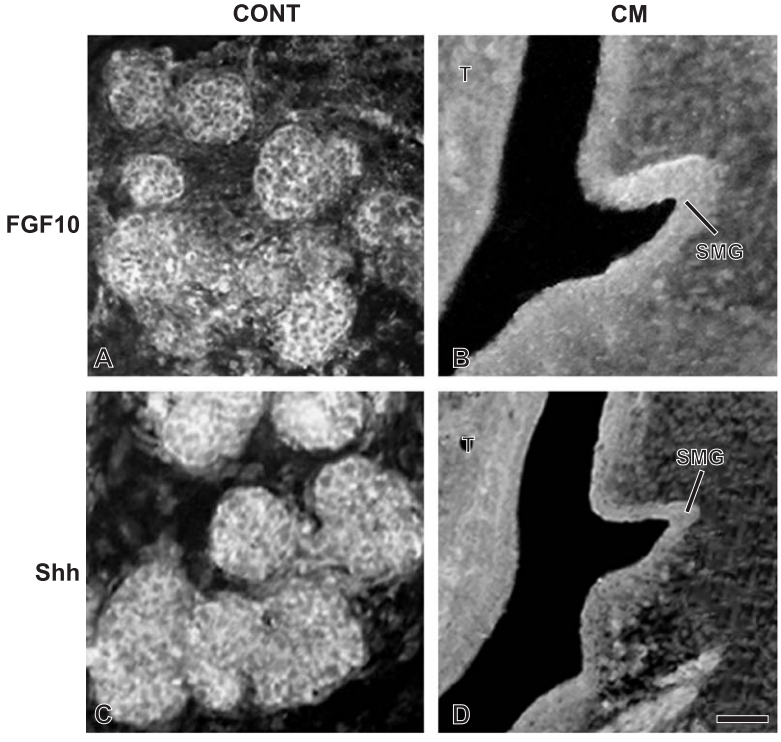

Fig. 6. *Fgf8* conditional mutant SMGs exhibit a marked decrease in FGF10 and Shh protein expression. (A, B) FGF10 immunolocalization: (A) E15.5 control (CONT) SMG. (B) E15.5 conditional mutant (CM) SMG bud. (C, D) Shh immunolocalization: (C) E15.5 control SMG. (D) E15.5 conditional mutant SMG bud. Note that the E15.5 *Fgf8* conditional mutant SMG appears as a small, single, primitive epithelial primordium budding laterally (B, D). T, tongue. Bar, 50 μm.

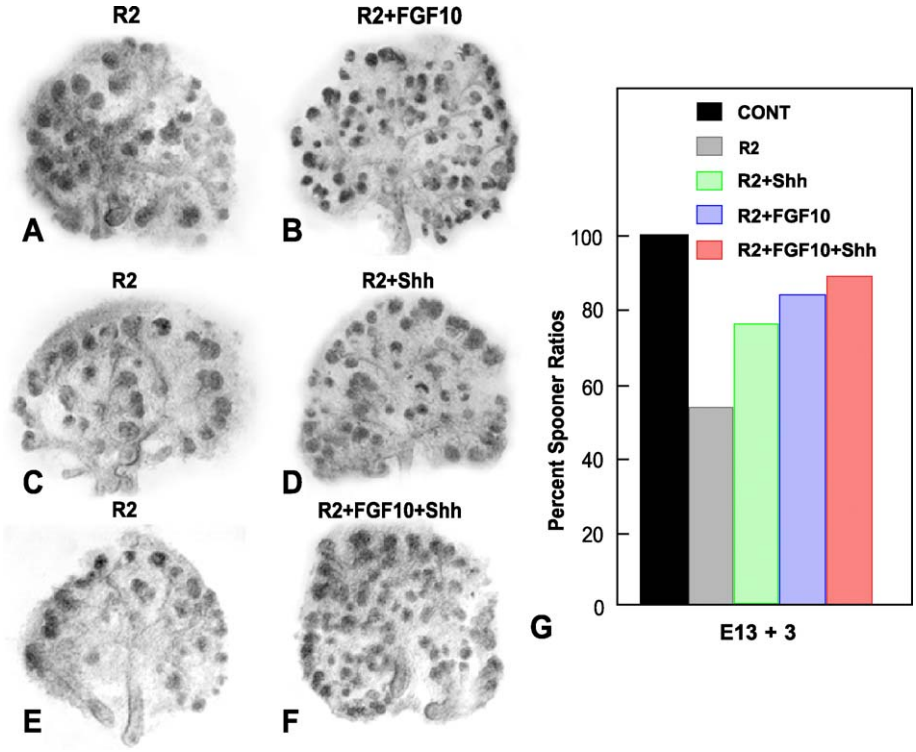

Fig. 7. FGF10 and/or Shh peptide supplementation in vitro restores branching morphogenesis toward normal. Paired E13 embryonic SMGs were initially incubated for 3 h in FGFR2-IIIc-Fc chimera and then cultured for 3 days in FGFR2-IIIc-Fc (A, C, E), FGFR2-IIIc-Fc + 200 mg/ml FGF10 (B), FGFR2-IIIc-Fc + 2.5 μg/ml Shh (D), or FGFR2-IIIc-Fc + 200 mg/ml FGF10 + 2.5 μg/ml Shh (F). Representative control and treated explants are shown for each set of experiments. Bar, 75 μm. (E) A comparative representation of the percent branching restoration associated with different treatments relative to each control. R2 = FGFR2-IIIc-Fc. FGF10 rescue: $n = 8$; Shh rescue: $n = 4$; FGF10 + Shh rescue: $n = 7$ and IgG-Fc controls, $n = 6$.

ated with abrogated FGF8 signaling since Shh has also been shown to play an essential role during embryonic SMG development (Jaskoll et al., 2004). Paired E13 primordia were cultured in 5 ng/ml FGFR2-IIIc-Fc, with or without 2.5 μg/ml Shh peptide, for 3 days as described above. Shh-supplemented explants exhibit a significant 44% ($P < 0.05$) increase in branching morphogenesis compared to FGFR2-IIIc-Fc treatment alone (Figs. 7C, D, G). Shh-supplemented explants do not significantly differ from FGF10-supplemented explants ($P > 0.5$).

Finally, to determine if FGF10 and Shh act synergistically, we attempted to rescue the SMG phenotype with abrogated FGF8 signaling with a combination of exogenous FGF10 + Shh peptide supplementation. Paired E13 primordia were cultured in 5 ng/ml FGFR2-IIIc-Fc, with or without 200 ng/ml FGF10 + 2.5 μg/ml Shh as described above. Combined FGF10 + Shh supplementation induced a significant 68% ($P < 0.001$) increase in branching morphogenesis compared to FGFR2-IIIc-Fc treatment alone (Figs. 7E, F, G). Interestingly, the combined FGF10 + Shh peptide supplementation was no more effective than FGF10 peptide supplementation alone ($P > 0.5$) or Shh peptide supplementation alone ($P > 0.2$). Thus, there appears to be no synergism between FGF10 and Shh.

It is important to note that exogenous FGF10 or Shh peptide supplementation significantly restored branching to 84% or 76% of the level seen in controls, respectively. Combined FGF10 + Shh supplementation restored branching to about 89% of control. This incomplete compensation for decreased FGF8 signaling suggests that FGF8 signaling induces additional critical and specific downstream pathways during SMG organogenesis.

Discussion

It is well established that members of the FGF family play key roles during embryogenesis as they mediate cell proliferation, survival, and/or apoptosis (see reviews, Goldfarb, 2001; Ornitz and Itoh, 2002). Recent studies of mice with FGF8 reduction or conditional inactivation during murine development have shown that FGF8 signaling is essential for the development of the face and mandible, cardiovascular and nervous systems, and pharyngeal arch-derived structures such as the thymus and parathyroids (Abu-Issa et al., 2002; Chi et al., 2003; Frank et al., 2002; Garel et al., 2003; Macatee et al., 2003; Storm et al., 2003).

Of particular interest is the observation that FGF8 regulates mandibular morphogenesis in a dose-dependent manner. The complete ablation of FGF8 in mandibular arch epithelia in *Fgf8* conditional mutants results in more severe craniofacial malformation than seen in the hypomorphic

mouse. The availability of these *Fgf8* hypomorphic and conditional mutant mice provide an opportunity to begin to dissect the relative role of *Fgf8* gene dosage during salivary gland (SMG) morphogenesis.

In this study, we investigated the SMG phenotype in *Fgf8* hypomorphic and conditional mutants with progressive development. Our results indicate that FGF8 signaling is essential for normal embryonic SMG development and that the extent of its development is dependent on *Fgf8* gene dosage. We found that SMG development is unaffected in *Fgf8* mutants heterozygous for the null ($Fgf8^{+/N}$) or hypomorphic ($Fgf8^{H/+}$) allele which have been shown to express decreased levels of Fgf8 transcript compared to control (Frank et al., 2002; Moon and Capecchi, 2000). However, a reduction of *Fgf8* expression in hypomorphic ($Fgf8^{H/N}$) mutants to *less than* 50% of wild-type mice (Frank et al., 2002) results in a hypoplastic gland. Importantly, the complete elimination of *Fgf8* expression from first pharyngeal arch (mandibular) ectoderm in conditional mutants ($Fgf8^{C/N}$; AP2α$^{IRESCre/+}$) results in early ontogenic arrest, involution and absence of the SMGs by E18.5.

Thus, a *Fgf8* gene dosage effect on embryonic SMG development is clearly demonstrated in this study (summarized in Fig. 4A). Similar *Fgf8* dose-dependent effects on midbrain and cerebellar morphogenesis were seen in E17.5 *Fgf8* knockdown and tissue-specific knockout mice (Chi et al., 2003). Deletion of midbrain and cerebellar structures increased in severity with further progressive loss of *Fgf8* function, culminating in the absence of these neural structures with the complete elimination of *Fgf8* function in conditional null mutants.

SMG hypoplasia and aplasia in E18.5 *Fgf8* hypomorphic and conditional mutants, respectively, indicates that FGF8 signaling plays an important morphoregulatory and prosurvival role during embryonic SMG morphogenesis. Specifically, the absence of SMGs in E18.5 *Fgf8* conditional mutant mice indicates that FGF8 signaling is essential for initial epithelial branching and for subsequent *Pseudoglandular* Stage and older SMG development. The functional presence of endogenous levels of other FGF/FGFR signaling pathways (e.g., FGF10/FGFR2-IIIb) and other parallel pathways (e.g., EGF/EGFR, IGF-II/IGF-1R) could not prevent the hypoplastic development of hypomorphic SMGs nor the complete death of embryonic SMG cells in the *Fgf8* conditional mutants. Equally important, the presence of an initial SMG bud in *Fgf8* conditional mutants indicates that FGF8 signal transduction is *not* required for early initial bud formation.

FGF signaling potentially activates several key pathways, including the RAS/MAPK, PLC-γ, and P13 kinase (P13K) pathways (see reviews, Boilly et al., 2000; Jaskoll and Melnick, in press; Ornitz, 2000; Ornitz and Itoh, 2002; Powers et al., 2000). The components of the PLC-γ, P13K, and PKC signaling pathways are found in all stages of embryonic SMG development (Koyama et al., 2003; Larsen et al., 2003). Moreover, inhibition of RAS/MAPK or P13K signaling significantly reduced SMG branching morphogenesis in vitro (Kashimata et al., 2000a; Koyama et al., 2003; Larsen et al., 2003), whereas inhibition of PKC modestly increased branching (Koyama et al., 2003). Although the importance of the RAS/MAPK, P13K, PLC-γ, and PKC during embryonic SMG branching morphogenesis has been demonstrated, it is presently unclear which signaling cascades are specifically downstream of the FGF8 signal during embryonic SMG development. Our demonstration of a marked decrease in epithelial branching in *Fgf8* hypomorphic and conditional mutant mice, as well as the dose-dependent downregulation of epithelial branching morphogenesis in vitro, suggest that FGF8 acts as a mitogenic factor during embryonic SMG branching morphogenesis and epithelial cell proliferation. Furthermore, our observation of SMG aplasia in E18.5 conditional null mutants indicates that FGF8 also acts as a survival factor during later stages of development. Future studies are needed to fully delineate which pathways are downstream of the FGF8 signaling pathway at various stages of embryonic SMG development.

Nevertheless, previous studies have demonstrated that *Fgf10* and *Shh* expression are altered by modulated FGF8 signaling (Aoto et al., 2002; Frank et al., 2002; Moon and Capecchi, 2000). Here we show that abrogated FGF8 signaling downregulates FGF10 and Shh protein expression in the developing embryonic SMG. Knowing that both FGF10 and Shh signaling play critical morphoregulatory roles during embryonic SMG branching development (Jaskoll et al., 2004; Ohuchi et al., 2000; Revest et al., 2001), we postulated that exogenous FGF10 and/or Shh peptide supplementation could restore branching morphogenesis to the normal level. Using soluble FGFR2-IIIc-Fc chimera to bind endogenous FGF8 ligand, we designed in vitro FGF8 abrogation studies which could then be used to assess if FGF10 and/or Shh peptide supplementation could restore branching morphogenesis to the level seen in control glands (Fig. 7).

For several reasons, we had previously concluded that abnormal SMGs in *Fgfr2-IIIc* mutant mice are due to decreased FGF8/FGFR2-IIIc signaling (Jaskoll et al., 2002). First, although FGF8 isoforms have been shown to bind with high affinity to FGFR2-IIIc, FGFR3-IIIc, or FGFR4 (MacArthur et al., 1995; Ornitz, 2000; Ornitz et al., 1996), the absence of FGFR3 and FGFR4 from *Initial Bud*, *Pseudoglandular*, and *Canalicular* Stage SMGs indicates that FGF8 likely mediates its signal through FGFR2-IIIc (Jaskoll et al., 2002). Second, although several investigators have suggested that FGF8 signals through FGFR1, Chellaiah et al. (1999) have clearly demonstrated that FGF8 does not bind to FGFR1. Third, although other FGFs can also bind to FGFR2-IIIc, the absence of *Fgf9* transcripts from embryonic SMGs (Colvin et al., 1999), the normal SMG phenotype in *Fgf4* null mice (A. Moon, E.J. Park, L. Francis, unpublished), and the relatively normal phenotype in *Fgf2* and *Fgf6* null mice (Fiore et al., 1997; Miller et al.,

2000), indicates that FGF2, FGF4, FGF6, and FGF9 (the other known FGFR2-IIIc ligands) do not appear critical to embryonic SMG development. Consequently, if FGFR2-IIIc-Fc disturbs SMG morphogenesis in vitro, it is primarily because FGF8 signaling has been diminished (not because other FGF signaling has been diminished).

As expected, variable concentrations of FGFR2-IIIc chimera demonstrate a significant dose-dependent decrease in branching morphogenesis. FGF10 supplementation of FGF8 abrogated explants induced a significant increase in SMG branching and restored the gland to 84% of normal. This result is consistent with FGF10 peptide supplementation in vitro being able to stimulate SMG branching morphogenesis (Hoffman et al., 2002; Jaskoll and Melnick, unpublished data). The observation of SMG aplasia in *Fgf10* null (Ohuchi et al., 2000) and *Fgf8* conditional mutant mice (present study) suggests that FGF8 and FGF10 probably elicit some of the same downstream targets, although through different receptors (i.e., FGF8 binds to FGFR2-IIIc and FGF10 binds to FGFR2-IIIb). Conversely, our finding that enhanced FGF10-mediated signaling in vitro incompletely compensated for decreased FGF8 signaling suggests that, during embryonic SMG development, the FGF8 signal transduction pathway induces specific and unique downstream responses different from those mediated by FGF10 signaling. There is other evidence that different FGF receptors may mediate different downstream signaling cascades (see reviews Boilly et al., 2000; Goldfarb, 2001; Ornitz and Itoh, 2002; Powers et al., 2000).

Shh supplementation of FGF8 abrogated explants also induced a significant increase in branching morphogenesis and restored branching morphogenesis to 76% of normal. On the basis of this result, we again conclude that enhancement of a single downstream target is insufficient to completely compensate for decreased FGF8 signaling. There was no synergy between FGF10 and Shh. Finally, it is interesting that exogenous FGF8 peptide supplementation is reciprocally able to rescue the abnormal SMG phenotype seen with abrogated Shh signaling in vitro (Jaskoll et al., 2004). Such is to be expected of separate, but related, motifs in a larger genetic-regulatory network which is integrated to form a functional module (Oltvai and Barabasi, 2002).

These "rescue experiments" allow us to further grasp the advantage of deriving a quantitative mathematical model of the phenotypic landscape related to *Fgf8* gene dosage within an epigenetic context (Fig. 4B). To be sure, the epigenome is multidimensional, with as many orthogonal axes as there are independent variables (Nijhout, 2003). Nevertheless, even a simple three-dimensional model (Fig. 4B) permits us to intuitively understand that the epistasis of declining *Fgf8* function is a nonlinear emergent property of the complete epigenotype, and to conceptualize with some accuracy as to how this epistasis might disappear in a context of an altered epigenotype containing a nonphysiologic challenge (e.g., a simultaneous gain of function mutation in a related gene). The in vitro rescue experiments reported here demonstrate that increased FGF10 or Shh signaling precludes the epistasis of decreased FGF8 signaling. They model the principle that mutations have different effects in the context of different epigenotypes (Buchner et al., 2003; Nadeau, 2003). We have designed future in vivo knock-out/knock-in (*Fgf8/Fgf10* or *Fgf8/Shh*) experiments to more fully explore this important issue.

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