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Embryonic Submandibular Gland Morphogenesis: Stage-Specific Protein Localization of FGFs, BMPs, Pax6 and Pax9 in Normal Mice and Abnormal SMG Phenotypes in *FgfR2-IIIc*$^{+/\Delta}$, *BMP7*$^{-/-}$ and *Pax6*$^{-/-}$ Mice

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Key Words

Fibroblast growth factor · BMP7 · Pax6 · Pax9 · Salivary glands · Development · Mouse

Abstract

Embryonic submandibular salivary gland (SMG) initiation and branching morphogenesis are dependent on cell-cell communications between and within epithelium and mesenchyme. Such communications are typically mediated in other organs (teeth, lung, lacrimal glands) by growth factors in such a way as to translate autocrine, juxtacrine and paracrine signals into specific gene responses regulating cell division and histodifferentiation. Using *Wnt1-Cre/R26R* transgenic mice, we demonstrate that embryonic SMG mesenchyme is derived exclusively from cranial neural crest. This origin contrasts to that known for tooth mesenchyme, previously shown to be derived from both neural crest and nonneural crest cells. Thus, although both SMGs and teeth are mandibular derivatives, we can expect overlap and differences in the details of their early inductive interactions. In addition, since embryonic SMG branching morphogenesis is analogous to that seen in other branching organs, we also expect similarities of expression regarding those molecules known to be ubiquitous regulators of morphogenesis. In this study, we performed an analysis of the distribution of specific fibroblast growth factors (FGFs), FGF receptors, bone morphogenetic proteins (BMPs) and Pax transcription factors, previously shown to be important for tooth development and/or branching morphogenesis, from the time of initiation of embryonic SMG development until early branching morphogenesis. In addition, we report abnormal SMG phenotypes in *FgfR2-IIIc*$^{+/\Delta}$, *BMP7*$^{-/-}$ *and Pax6*$^{-/-}$ mice. Our results, in comparison with functional studies in other systems, suggest that FGF-2/FGFR-1, FGF-8/FGFR-2(IIIc) and FGF-10/FGFR-2(IIIb) signaling have different paracrine and juxtacrine functions during SMG initial bud formation and branching. Finally, our observations of abnormal SMGs in

Abbreviations used in this paper

BMP	bone morphogenetic protein
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
SMG	submandibular salivary gland

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BMP7$^{-/-}$ *and Pax6*$^{-/-}$ indicate that both *BMP7* and *Pax6* play important roles during embryonic SMG branching morphogenesis.

Introduction

Mouse embryonic submandibular gland (SMG) morphogenesis is best conceptualized in stages. In the prebud stage, it begins as a thickening of the primitive oral cavity epithelium adjacent to the developing tongue. During the initial bud stage, this thickened epithelium grows down into the first branchial (mandibular) arch mesenchyme to form the initial SMG bud. With continued epithelial proliferation and downgrowth, the SMG primordium becomes a solid, elongated epithelial stalk terminating in a bulb; this SMG primordium is surrounded by condensed mesenchyme. The primordium branches by repeated furcation at the distal ends of successive buds to produce a bush-like structure comprised of a network of elongated epithelial branches and terminal epithelial buds surrounded by loosely packed mesenchyme (the *Pseudoglandular* Stage). These branches and buds hollow out by epithelial cell apoptosis during the *Canalicular* and *Terminal Bud* Stages to form the ductal system and presumptive acini [for details, see Jaskoll and Melnick, 1999; Melnick and Jaskoll, 2000]. Although it has conclusively been shown that SMG morphogenesis is dependent on epithelial-mesenchymal interactions [see reviews, Wessells, 1977; Cutler, 1990], little is known about which molecules are involved in the induction and regulation of early embryonic SMG branching.

Over the last decade, it has become increasingly apparent that many of the same morphogenetic signaling pathways play key inductive roles during the development of different organs. Thus, to gain insight into which growth and transcription factors may be important for SMG initial bud formation and morphogenesis, we turn to other well-studied developing systems [see reviews, Hogan, 1999; Jernvall and Thesleff, 2000; Warburton et al., 2000]. Tooth development is an ideal choice since the initial tooth bud develops in a pattern similar to that seen for the SMG, i.e. as an oral epithelial thickening which grows down into the underlying mandibular mesenchyme, at sites lateral to SMG development [see review, Jernvall and Thesleff, 2000]. During tooth morphogenesis, fibroblast growth factor 8 (FGF-8) and bone morphogenetic protein 4 (BMP-4; and/or BMP-2) antagonistic effects have been shown to regulate early embryonic mandibular arch patterning and tooth bud formation by the induction or inhibition of the expression of transcription factors, including Pax9 [Wang et al., 1999; see reviews, Peters and Balling, 1999; Jernvall and Thesleff, 2000]. Given the similarity in tooth and SMG bud formation and their common mandibular origin, it was reasonable to postulate that FGF-8, BMP-2, BMP-4 and Pax9 may also be important during initial stages of embryonic SMG morphogenesis.

In addition, since embryonic SMG branching morphogenesis is known to be analogous to that seen in other branching organs (e.g., lung, lacrimal gland, kidney), we can investigate factors already identified as important morphoregulators in other branching systems. Observations on lung and kidney development in vivo and in vitro indicate that FGF-7, FGF-10, BMP-4 and BMP-7 are developmentally important [Dudley et al., 1999; Lebeche et al., 1999; Weaver et al., 1999, 2000; see reviews, Hogan, 1999; Kuure et al., 2000; Warburton et al., 2000]. In addition, FGF-10 and Pax6 have been implicated in lacrimal gland development [Makarenkova et al., 2000]. Thus, it can reasonably be postulated that BMP-4, BMP-7, FGF-7, FGF-10 and Pax6 may also play key roles during early embryonic SMG development.

In this study, we compared the spatial distribution of specific FGFs (1–3, 7, 8, 10), FGF receptors (FGFR; 1–4), BMPs (2, 4, 7), Pax6 and Pax9 in early embryonic SMGs, from prior to the overt appearance of the SMG bud (i.e. the *Prebud* Stage) to the *Pseudoglandular* Stage. Our results suggest that cellular interactions occur not only between epithelium and mesenchyme (i.e. epithelial-mesenchymal interactions), but also within the epithelium as well. In addition, our analyses of *FgfR2-IIIc*$^{+/\Delta}$, *BMP7*$^{-/-}$ and *Pax6*$^{-/-}$ SMGs indicate that FGF8/FGFR-2(IIIc), BMP 7, and Pax6 signaling are all essential for embryonic SMG development. The data generated from our studies provides the rationale and framework for future mechanistic studies of related signaling pathways.

Materials and Methods

Tissue Collection

Female B10A/SnSg mice, obtained from Jackson Laboratories (Bar Harbor, Me., USA), were maintained and mated as previously described [Jaskoll et al., 1994] (plug day = day 0 of gestation). Pregnant females were anesthetized on days 11.5–18 of gestation (E11.5–18) with methoxyflurane (Metafane) and euthanized by cervical dislocation. Embryos were dissected in cold phosphate-buffered saline and staged according to Theiler [1989]. E11.5–E13 heads and E14–E15 SMGs were collected and processed for histology.

Transgenic Mice. Wnt1-Cre transgenic mice and the *R26R*-conditional reporter allele have been previously described [Danielian et

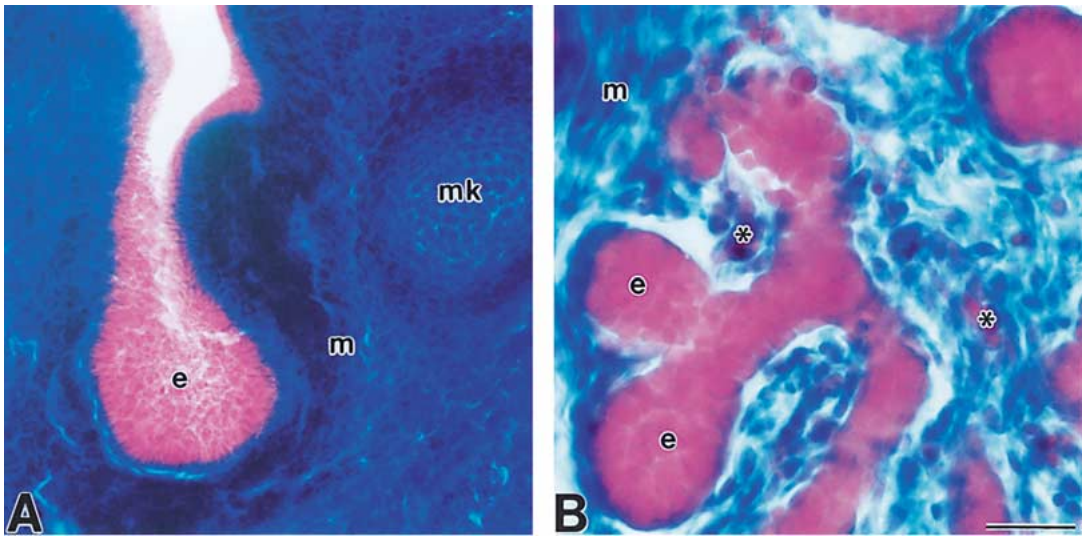


Fig. 1. *Wnt1-Cre/R26R* transgenic mice. **A** *Initial Bud* Stage SMG. Strong *lacZ* expression (blue) is seen in all SMG mesenchymal (m) cells and is absent from SMG epithelium (e). Meckel's cartilage (mk) is also derived from cranial neural crest. **B** *Pseudoglandular* Stage. *LacZ* expression is seen throughout the mesenchymal cell and not in branching epithelia. Blood cells (*) do not exhibit *lacZ* staining, indicating that they are derived from head mesoderm and not from cranial neural crest cells. Bar 50 μm.

al., 1998; Soriano, 1999]. Mating *Wnt1-Cre*$^{+/-}$ with *R26R*$^{+/-}$ generated *Wnt-Cre/R26R* mice (double transgenic). Genotypes of the double transgenic embryos and adult animals were determined by PCR as previously described [Chai et al., 2000]. E12.5 and E14.5 heads were collected and stained for β-galactosidase activity as described previously [Chai et al., 2000]. *Pax6* mutant fetuses lacking functional Pax6 protein were produced by intercrossing mice heterozygous for different *Pax6* mutant alleles (*Pax6*$^{Sey/+}$ × *Pax6*$^{Sey\text{-}Neu/+}$) as described by Collinson et al. [2000]. For simplicity the *Pax6*$^{Sey/Sey\text{-}Neu}$ fetuses are designated *Pax6*$^{-/-}$ because both alleles result in truncated, nonfunctional proteins [Hill et al., 1991]. *Pax6*$^{-/-}$ embryos were easily identified by their absence of eyes and shortened snout [Hill et al., 1991]. *BMP7* null mice were produced from intercrosses of mice heterozygous for the *BMP7*^{mlRob} null alleles as described in Dudley and Robertson [1997]. To investigate FgfR2-IIIc function, a Cre/LoxP recombination strategy was used to remove exon 9 of *FgfR2* which is specific for this receptor isoform [Hajihosseini et al., 2001]. Only mice exhibiting complete excision *(FgfR2-IIIc*$^{+/\Delta}$*)* were analyzed. For all transgenic and mutant mice, genotypes were verified by PCR as previously described [Dudley and Robertson, 1997; Collinson et al., 2000; Hajihosseini et al., 2001]. For each genotype, a minimum of 3 SMGs were evaluated and the phenotype observed was consistent for all mice examined.

Histology and Immunochemistry

Embryonic heads and SMGs were fixed in Carnoy's fixative, processed, embedded in low-melting point Paraplast, and immunostained as previously described [Jaskoll and Melnick, 1999]. In all experiments, negative controls were incubated in the absence of primary antibody or with preimmune serum; controls were routinely negative. A minimum of 5 heads or 5 SMGs were evaluated for each stage of development per experimental group. All polyclonal antibodies were purchased from Santa Cruz Biotech (Santa Cruz, Calif., USA).

Fgfr2(IIIc) hemizygotes and their wild-type littermates were collected at E16.5, *BMP 7* null mice and their wild-type littermates were collected at E17.5, and *Pax6*$^{-/-}$ mice and their wild-type littermates were collected at E18.5. The SMGs were dissected and fixed in 10% buffered formalin, and evaluated by hematoxylin and eosin histology.

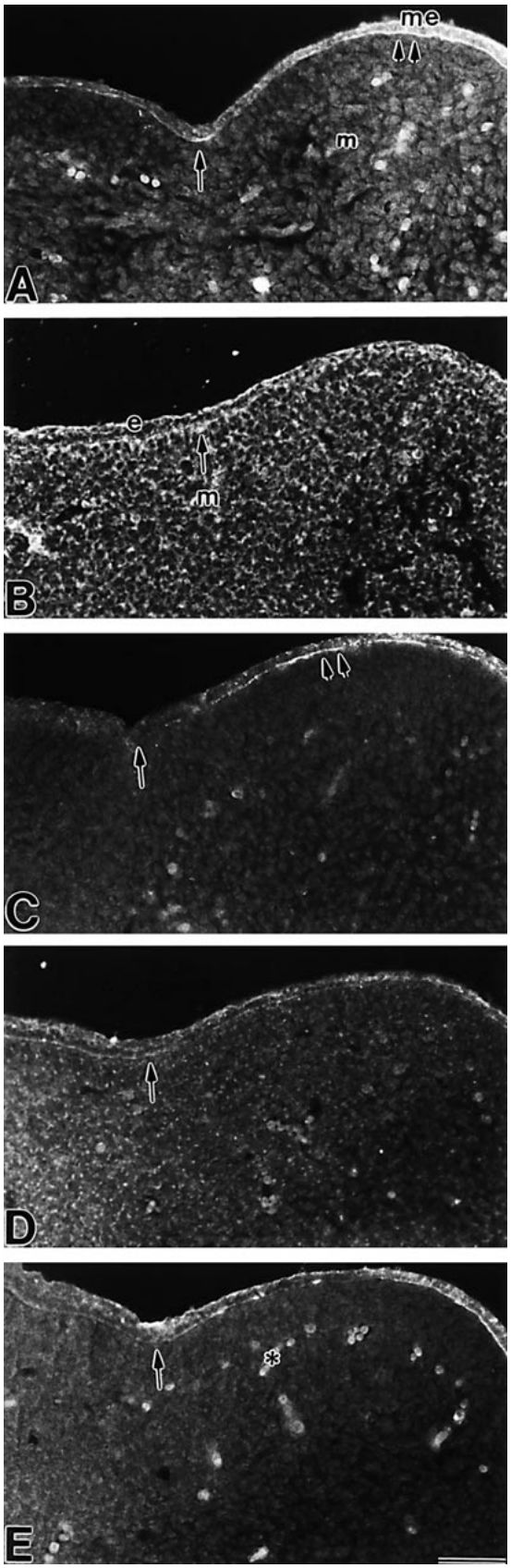
Determination of Cranial Neural Crest Cells

By crossing *Wnt1-Cre* with *R26R* mice, transgenic mice expressing β-galactosidase in migrating neural crest cells were generated [Chai et al., 2000]. E12.5 (early initial bud stage) and E14.5 (*Pseudoglandular* Stage) mouse *Wnt1-Cre/R26R* heads were cryostat-sectioned at 10 μm and stained for β-galactosidase *(lacZ)* activity as previously described [Chai et al., 2000]. Briefly, the tissue sections were incubated in detergent rinse solution for 10 min at 4°C, stained in X-gal solution overnight at room temperature in the dark and counterstained with nuclear fast red and eosin.

Results

Origin of SMG Mesenchyme

To determine the origin of embryonic mouse SMG mesenchyme, we evaluated *Wnt1-Cre/R26R* transgenic mice, in which *lacZ* expression pattern is colocalized with cranial neural crest cells [Chai et al., 2000]. In the *Initial Bud* Stage, the SMG appears as an elongated, solid epithelial stalk with a terminal bulb surrounded by condensed



mesenchyme; all SMG mesenchymal cells strongly express *lacZ* (fig. 1A). By contrast, *lacZ* expression is absent from ectodermally derived SMG epithelium. As development continues into the *Pseudoglandular* Stage, the solid cord of epithelium elongates and branches to form the presumptive ducts and terminal buds now surrounded by loosely packed mesenchyme. As indicated by *lacZ* expression, cranial neural crest-derived ectomesenchymal cells populate the SMG mesenchyme (fig. 1B). No *lacZ* staining is seen in the branching epithelia, confirming its separate ectodermal origin. Based on these results, we conclude that embryonic SMG mesenchyme is entirely derived from cranial neural crest.

Spatiotemporal Distribution of FGFs and Their Receptors

Members of the FGF family have been shown to regulate the morphogenesis of another oral epithelial derivative, teeth [Peters and Balling, 1999; Jernvall and Thesleff, 2000] and branching organs (e.g. lacrimal glands, lungs, mammary glands, kidneys) [Jackson et al., 1997; Kuure et al., 2000; Makarenkova et al., 2000; Warburton et al., 2000]. To identify which FGF ligands and receptors may play functional roles during early embryonic SMG morphogenesis, we determined the spatial distribution of FGF-1 to -3, -7, -8, -10 and receptors 1–4 prior to and during early embryonic SMG morphogenesis. In the prebud stage E11.5 mandibular arch, immunohistochemical analyses demonstrate FGF-7 (fig. 2A) in the medial oral epithelium and basement membrane region, FGF-2 (not shown) and FGF-8 (fig. 2B) throughout oral epithelia and mandibular mesenchyme, and FGF-10 (fig. 2C) in oral epithelium medial to the site of future SMG bud formation. In contrast, FGF-1 and FGF-3 are not found (not shown). At this stage, FGFR-1 and FGFR-2 are immunolocalized (fig. 2D–E) whereas FGFR-3 and FGFR-4 are not (not shown). FGFR-1 is in oral epithelium and mesenchyme (fig. 2D) while FGFR-2 is primarily seen in oral epithelia (fig. 2E).

Fig. 2. Spatial distribution of FGFs and their receptors in the *Prebud* Stage. **A** FGF-7 is localized in oral epithelium medial (me) to the site of future SMG development (arrow) and in the basement membrane region (double arrowheads) of medial epithelium and SMG epithelial thickening. **B** FGF-8 is seen throughout oral epithelia (e) and mandibular mesenchyme (m). **C** FGF-10 is found only in the medial oral epithelium at sites medial to SMG bud formation and in association with the basement membrane region (double arrowheads); it is not found in presumptive SMG bud epithelia. FGFR-1 (**D**) is immunodetected in oral epithelium and lateral mesenchyme while FGFR-2 (**E**) is seen primarily in the epithelium. FGFR-2 is also seen in blood cells (*). Bar 50 μm.

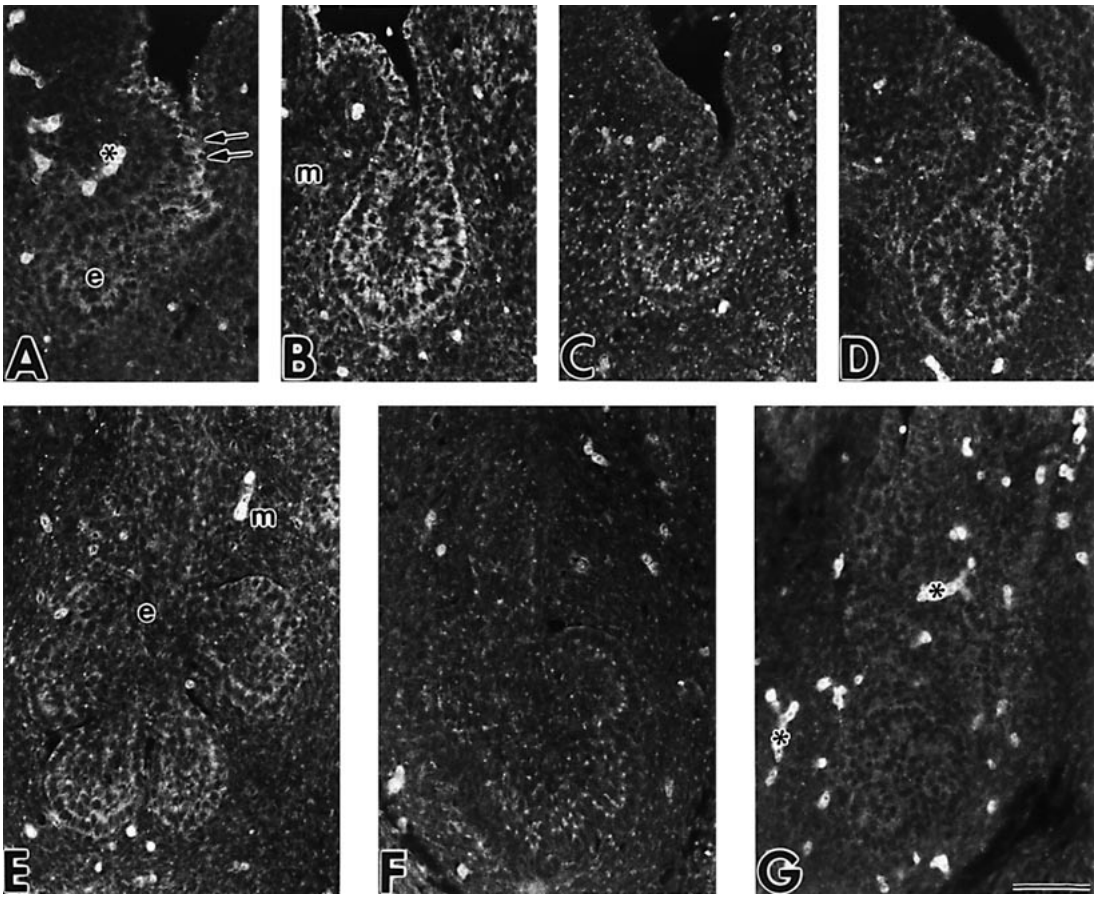


Fig. 3. Spatial distribution of FGFs and their receptors in the *Initial Bud* Stage. **A–D** *Early Initial Bud* Stage. **E–G** *Late Initial Bud* Stage. **A** FGF-7 is primarily seen in SMG bud epithelium (e), with enhanced immunostain being detected in the epithelial stalk (double arrows). **B**, **E** FGF-8 is localized in epithelium (e) and mesenchyme (m). **C**, **F** FGFR-1 is immunolocalized throughout epithelium and mesenchyme. **D**, **G** FGFR-2. In the *Early Initial Bud* Stage (**D**), FGFR-2 is distributed throughout epithelium and mesenchyme; its distribution pattern is similar to that seen for FGF-8. By the *Late Initial Bud* Stage (**G**), FGFR-2 is weakly seen. Note nonspecific immunostaining in blood cells (*). Bar 50 μm.

By the early initial bud stage, we detect FGF-7 in the SMG epithelium, with a more intense immunostain being seen in the SMG epithelial stalk than in the end-bulb (fig. 3A). FGF-8 (fig. 3B) and its receptor, FGFR-2 (fig. 3D), are primarily immunodetected in SMG epithelium. A similar pattern of immunolocalization is seen for FGF-2 (not shown). FGFR-1 (fig. 3C) is found in SMG epithelium and mesenchyme. FGF-10 remains absent. By the *Late Initial Bud* Stage, FGFR-1, FGF-2 and FGF-8 are seen in both the epithelium and mesenchyme (fig. 3E, F, not shown); FGF-7 retains its epithelial distribution (not shown). FGFR-2 is localized only in the epithelium (fig. 3G), with a relative decrease in the FGFR-2 immunostain intensity being seen compared to earlier stages (compare fig. 3D–G). Throughout the initial bud stage, FGF-1, FGF-3, FGF-10, FGFR-3 and FGFR-4 remain absent (not shown).

By the *Pseudoglandular* Stage, there is a marked shift in FGF-7 localization (fig. 4A); FGF-7 is now distributed throughout the mesenchyme and virtually absent from the epithelia. In contrast, FGF-8 (fig. 4B) and FGF-10 (fig. 4C) are seen throughout branching epithelia; a similar pattern of immunolocalization is seen for FGF-2 (not shown). FGFR-1 is primarily seen in branching epithelia and, to a lesser extent, in mesenchyme (fig. 4D); FGFR-2 is weakly immunolocalized in the epithelia (fig. 4E). At this stage, FGFR-4 is detected for the first time in the central regions of terminal bud epithelia (fig. 4F). FGF-1, FGF-3 and FGFR-3 are never immunodetected in early embryonic SMGs.

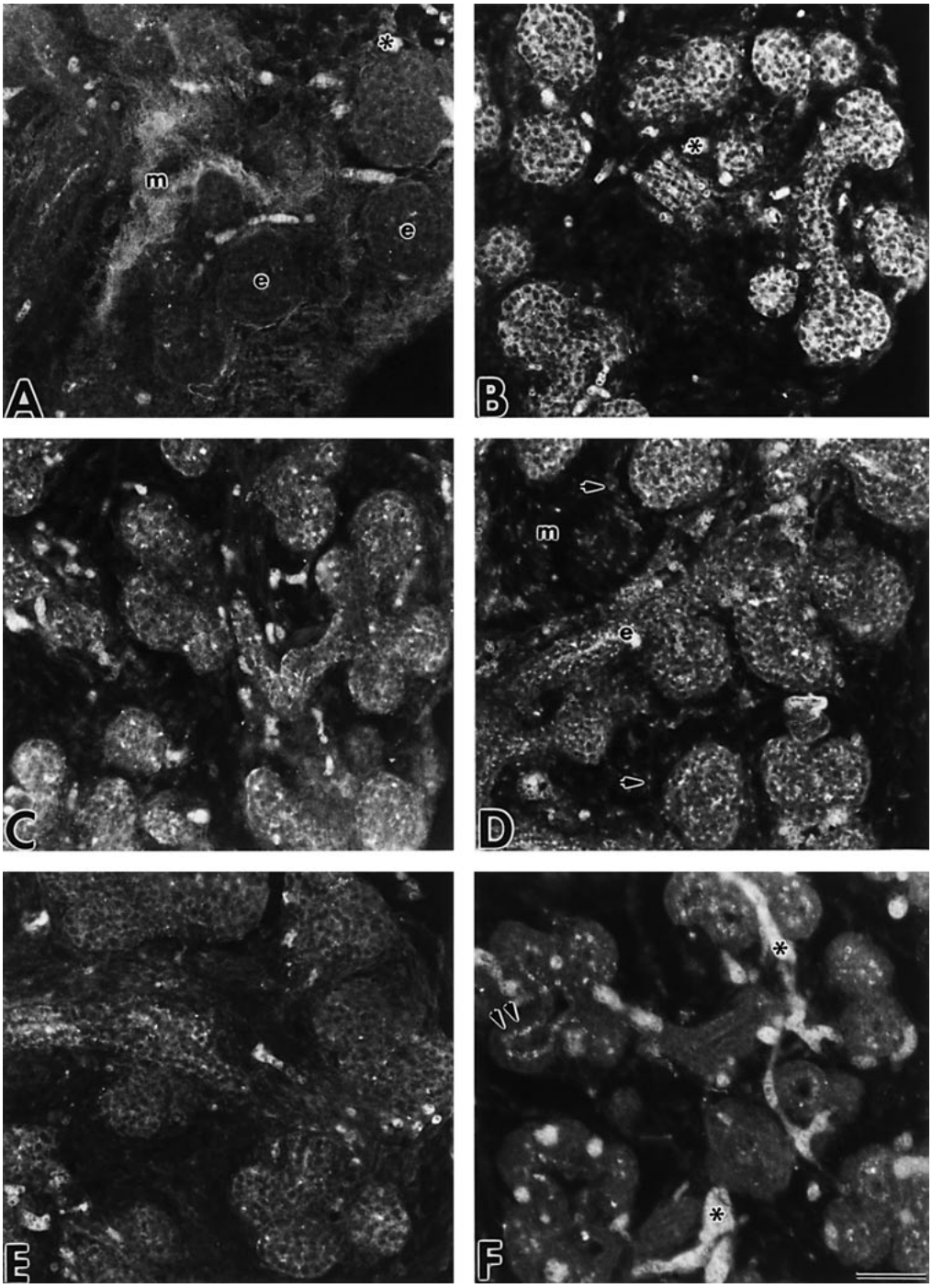


Fig. 4. Spatial distribution of FGFs and their receptors in the *Pseudoglandular* Stage. **A** FGF-7 is primarily seen throughout SMG mesenchyme (m) and is virtually absent from the branching epithelia (e). FGF-8 (**B**) and FGF-10 (**C**) are seen throughout branching epithelia. **D** FGFR-1 is primarily seen in branching epithelia, and to a lesser degree, in mesenchyme (arrowheads). **E** FGFR-2 is weakly distributed throughout branching epithelia. **F** FGFR-4 is seen in the center of epithelial terminal buds (double arrowheads). FGF-7, FGF-8, FGF-10 and FGFR-4 are also seen in blood vessels (*). Bar 50 μm.

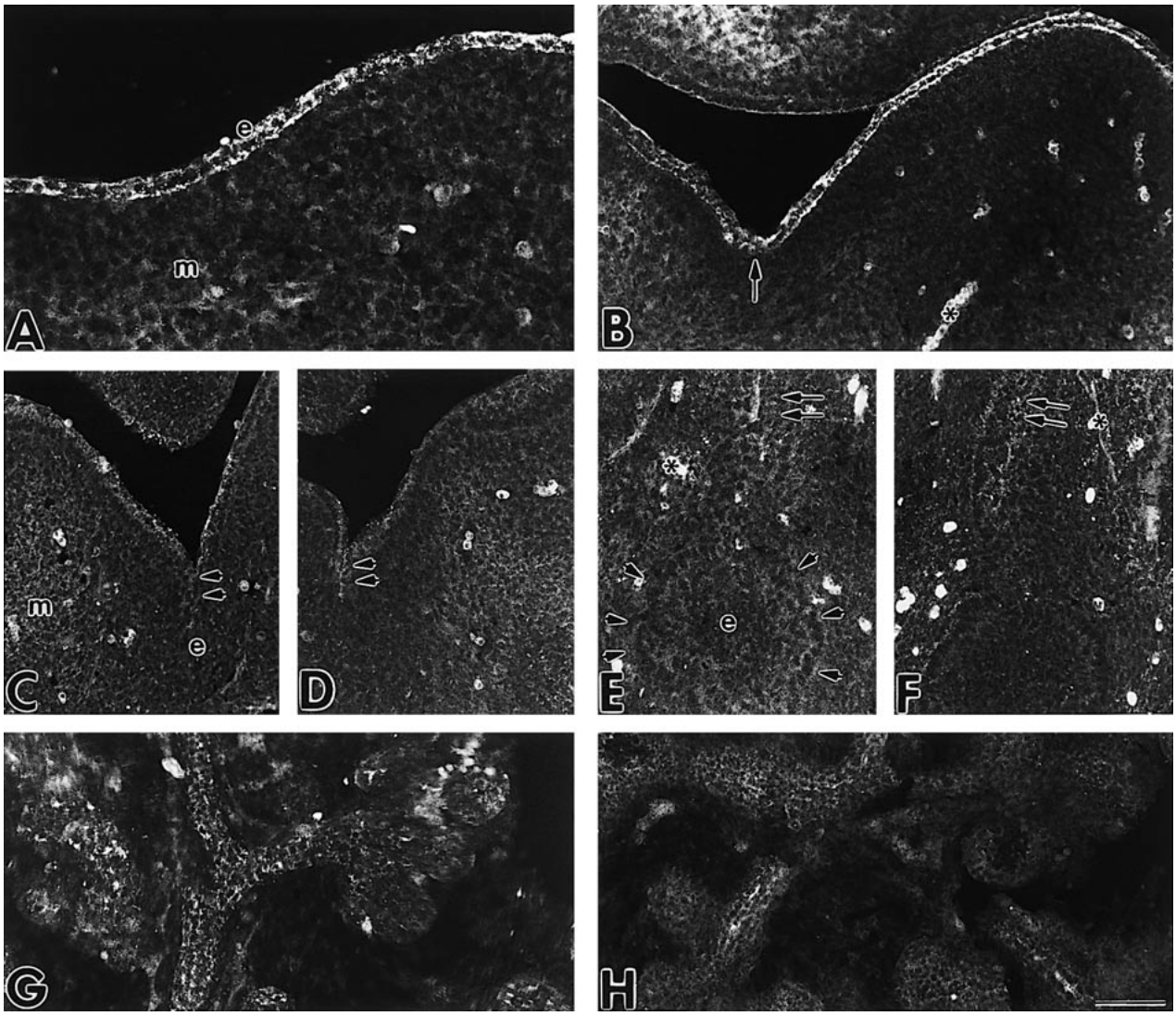


Fig. 5. Spatiotemporal distribution of BMPs. **A**, **B** BMP-4 immunolocalization in the *Prebud* Stage. BMP-4 is initially seen throughout oral epithelium (e) and, to a lesser extent, in lateral mesenchyme (m). With the appearance of the SMG epithelial thickening (arrow, **B**), BMP-4 is primarily found in medial oral epithelium and presumptive SMG epithelium, and to a lesser extent, in lateral epithelium and mesenchyme. **C**, **D** *Early Initial Bud* Stage. BMP-4 (**C**) and BMP-2 (**D**) are primarily immunolocalized in oral epithelium and epithelial stalk (double arrowheads), and to a lesser extent, in lateral mesenchyme; BMP-4 and BMP-2 are relatively absent from the SMG epithelial bud (e). **E**, **F** *Late Initial Bud* Stage. BMP-4 (**E**) is immunolocalized throughout epithelial stalk (double arrows) and end-bulb (arrowheads) as well as in mesenchyme. At this stage, BMP-2 (**F**) is seen in the epithelial stalk (double arrows). **G**, **H** The *Pseudoglandular* Stage. BMP-4 (**G**) and BMP-7 (**H**) exhibit similar patterns of epithelial distribution. Note nonspecific immunostain in blood cells (*). Bar 50 μm.

BMP-2, BMP-4 and BMP-7 Immunolocalization in Embryonic SMGs

BMPs are members of the TGF-β superfamily of secreted signaling molecules shown to be important for tooth and pulmonary morphogenesis [Peters and Balling, 1999; Wang et al., 1999; Jernvall and Thesleff, 2000; Warburton et al., 2000]. Thus, we also determined their spatiotemporal distribution in early embryonic SMGs (fig. 5). In the *Prebud* Stage mandible, BMP-4 protein is localized throughout the oral epithelia, and to a lesser extent, in the mesenchyme (fig. 5A); BMP-2 and BMP-7 proteins are not detected (not shown). With the appearance of the initial SMG epithelial thickening (fig. 5B) and the initial bud (fig. 5C), BMP-4 is seen in the epithelia,

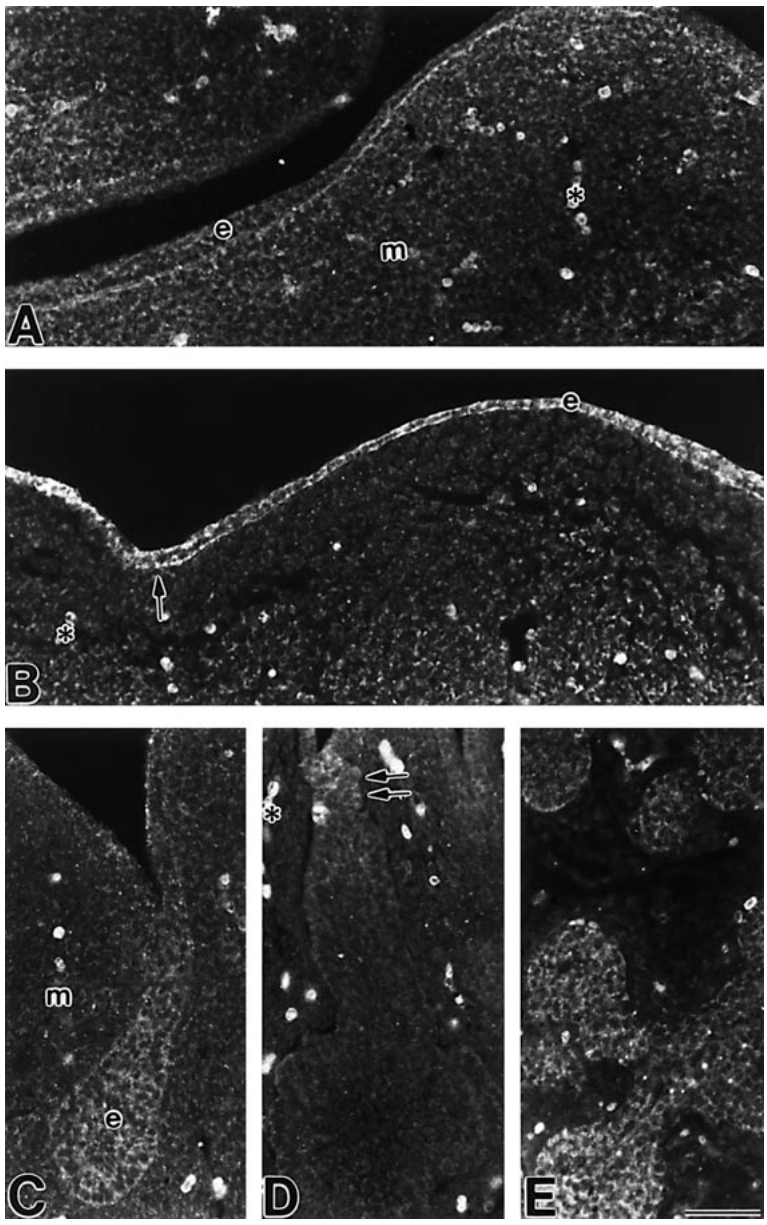


Fig. 6. Spatiotemporal distribution of Pax9. **A, B** *Prebud* Stage. **A** In the early mandibular arch, Pax9 is immunolocalized in oral epithelium (e), adjacent mesenchyme (m), and the basement membrane region. **B** With the appearance of SMG epithelial thickening (arrow), Pax9 is primarily seen in oral epithelium and in ventral mandibular mesenchyme. **C** In the *Early Initial Bud* Stage, Pax9 is seen in the epithelial bud and is relatively absent from mesenchyme. **D** In the *Late Initial Bud* Stage, Pax9 is only weakly immunodetected in the epithelial stalk (double arrows). **E** In the *Pseudoglandular* Stage, Pax9 is distributed throughout branching epithelia. Note the nonspecific immunostaining in blood cells (*). Bar 50 μm.

and to a lesser extent, in mandibular mesenchyme lateral to the site of SMG formation. In the *Early Initial Bud* Stage, both BMP-4 (fig. 5C) and BMP-2 (fig. 5D) distribution is primarily restricted to embryonic SMG epithelial stalk. By the *Late Initial Bud* Stage, BMP-4 is seen throughout SMG epithelial stalk, end-bulb and condensed mesenchyme (fig. 5E). BMP-2 is primarily seen in stalk epithelium (fig. 5F). BMP-7 is not detected until the *Pseudoglandular* Stage. At this stage, the localization patterns of BMP-4 (fig. 5G), BMP-2 (not shown) and BMP-7 (fig. 5H) proteins show remarkable similarities, all being distributed throughout branching epithelia.

Pax9 and Pax6 Immunolocalization

Vertebrate *Pax* genes are related to the *Drosophila* paired-rule gene, *paired,* which encodes a protein with two DNA binding domains, a paired domain and a paired-like homeodomain [reviewed in Noll, 1993; Mansouri et al., 1999]. Pax9, one member of this family of transcription factors, has been shown to be essential for mandibular tooth development [Peters and Balling, 1999]. Thus we investigated Pax9 protein's spatial and temporal distribution (fig. 6). As shown in figure 6A, Pax9 is immunodetected in the *Prebud* Stage mandibular oral epithelium and adjacent mesenchyme. With the appear-

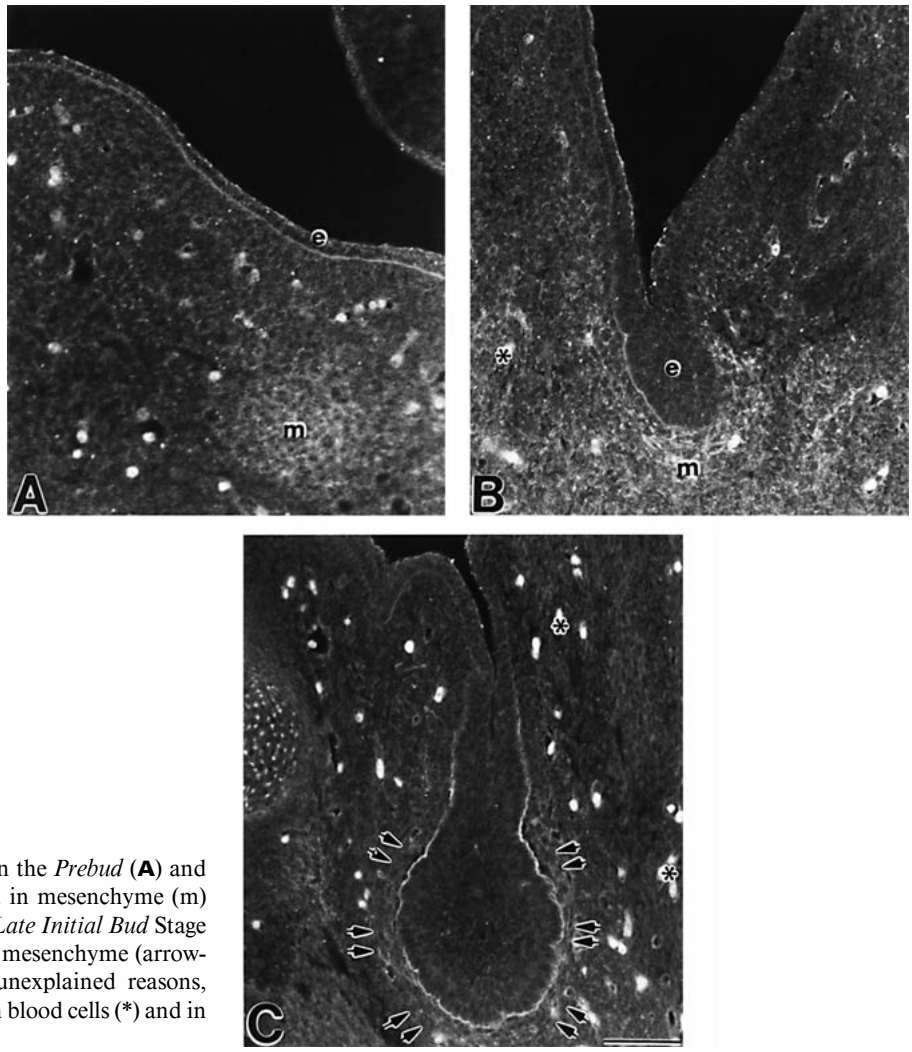


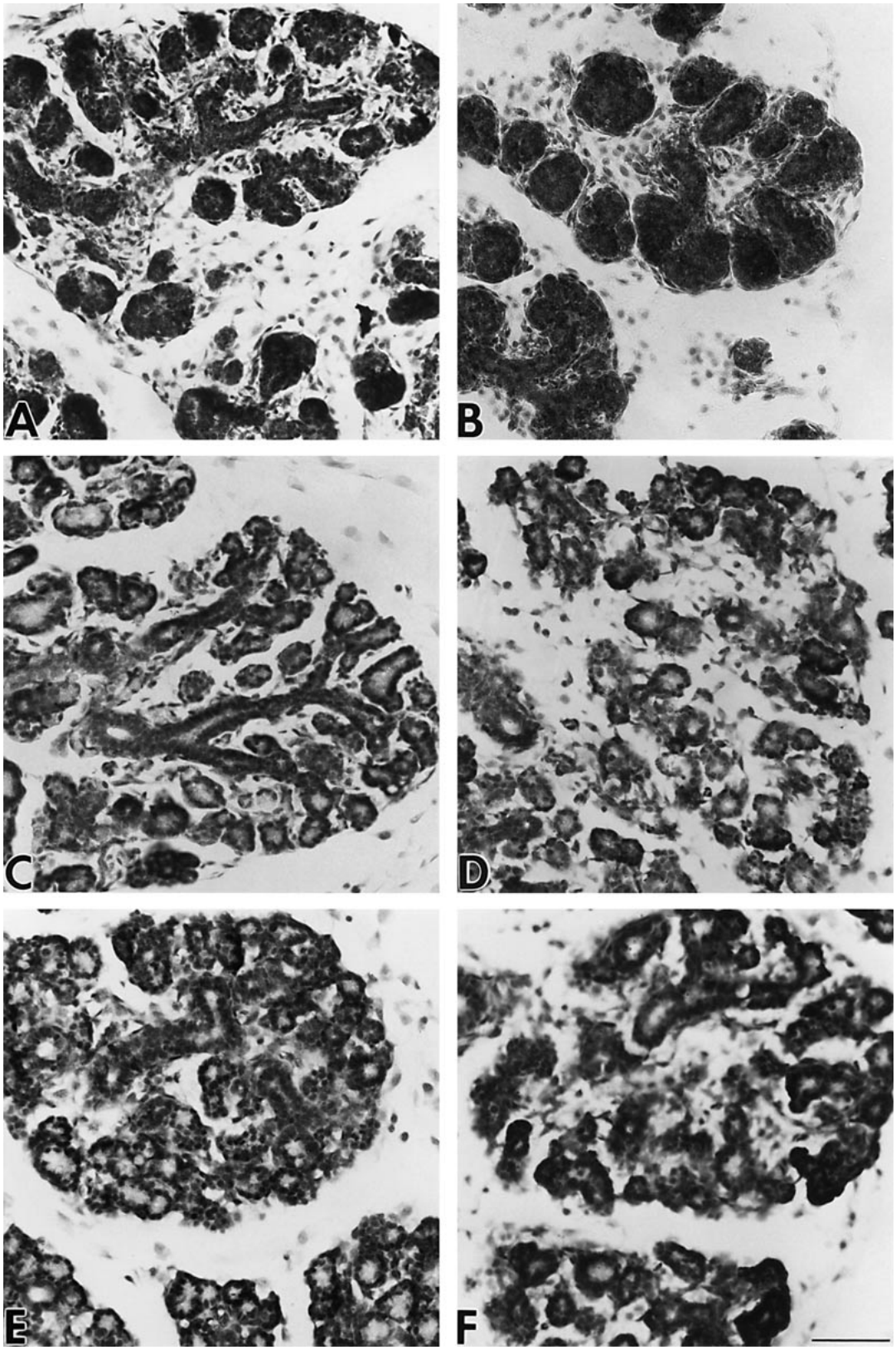
Fig. 7. Spatiotemporal distribution of Pax6. In the *Prebud* (**A**) and *Early Initial Bud* (**B**) Stages, Pax6 is localized in mesenchyme (m) and is absent from the epithelium (e). By the *Late Initial Bud* Stage (**C**), Pax6 protein is weakly seen in condensed mesenchyme (arrowheads) surrounding the epithelial bud. For unexplained reasons, there is nonspecific trapping of immunostain in blood cells (*) and in the basement membrane region. Bar 50 μm.

ance of the initial SMG epithelial thickening (fig. 6B), Pax9 primarily displays an epithelial distribution; it is also found in ventral mandibular mesenchyme. By the early initial bud stage (fig. 6C), Pax9 is seen in SMG epithelium. By the *Late Initial Bud* Stage, weak Pax9 immunostain is found in the SMG stalk, being virtually absent from the SMG end-bulb (fig. 6D). By the *Pseudoglandular* Stage, Pax9 is seen throughout branching epithelia (fig. 6E).

Pax6, a second member of this transcription factor family, has been shown to be involved in FGF-mediated branching morphogenesis [Makarenkova et al., 2000]. Thus, we also determined the immunolocalization pattern of Pax6 protein; Pax6 distribution markedly differs from that of Pax9 (compare fig. 7 to fig. 6). Pax6 is immunolocalized to mesenchyme but not into epithelium (fig. 7A–C). By the *Pseudoglandular* Stage, no Pax6 immunostain is seen in the SMG mesenchyme (not shown).

Abnormal SMG Phenotypes in FgfR2-IIIc$^{+/\Delta}$, BMP7$^{-/-}$ and Pax6$^{-/-}$ Mice

FGF-8 has been shown to be essential for the development of mandibular arch derivatives [Trumpp et al., 1999]. Although alternatively spliced forms of FGF-8 bind to FGFR-4 and specific isoforms of FGFR-2 and FGFR-3 [i.e. FGFR-2(IIIc) and FGFR-3(IIIc)] [MacArthur et al., 1995], the absence of immunodetectable FGFR-3 as well as the relatively late appearance of FGFR-4 (fig. 4F) indicate that FGF-8 likely transmits its signal by binding to the type 2-IIIc receptor. Therefore, to begin to delineate a role for FGF8/FGFR-2(IIIc) signal transduction during embryonic SMG development, we compared the SMG phenotype in E16.5 *FgfR2-IIIc$^{+/\Delta}$* hemizygous mice to that in wild-type littermates (fig. 8A, B). *FgfR2-IIIc$^{+/\Delta}$* mice exhibit hypoplastic SMGs characterized by a substantial decrease in epithelial branching


A
B
C
D
E
F

and fewer lumina compared to wild-type glands (compare fig. 8B to A). Thus, we conclude that FGF-8/FGFR-2(IIIc) signal transduction is essential for embryonic SMG branching morphogenesis.

Gene targeting experiments had demonstrated the importance of BMP-4 and BMP-7 during tooth, lung and kidney embryonic development [Dudley and Robertson, 1997; Furuta and Hogan, 1998; Dudley et al., 1999]. However, since BMP-4 null mice die prior to the appearance of embryonic SMGs [Furuta and Hogan, 1998], we could only evaluate the SMG phenotype in BMP-7 null mice. As shown in figure 8C and D, an abnormal phenotype is seen in E17.5 *BMP7*$^{-/-}$ SMGs compared to *BMP7*$^{+/+}$ glands; *BMP7*$^{-/-}$ SMGs (fig. 8D) exhibit disorganized mesenchyme and a decrease in epithelial branches and fewer lumina than *BMP7*$^{+/+}$ SMGs (fig. 8C). This indicates that BMP-7 signaling is important during embryonic SMG development.

Finally, we investigated whether Pax6, previously shown to be essential for lacrimal gland development [Makarenkova et al., 2000], plays a key role during embryonic SMG development. We compared E18.5 *Pax6*$^{-/-}$ mouse SMGs to their wild-type littermates (*Pax6*$^{+/+}$) and demonstrate that *Pax6*$^{-/-}$ mutant mouse SMGs are hypoplastic and exhibit disorganized mesenchyme (compare fig. 8F to E). Our results indicate that Pax6 is also essential for normal embryonic SMG development.

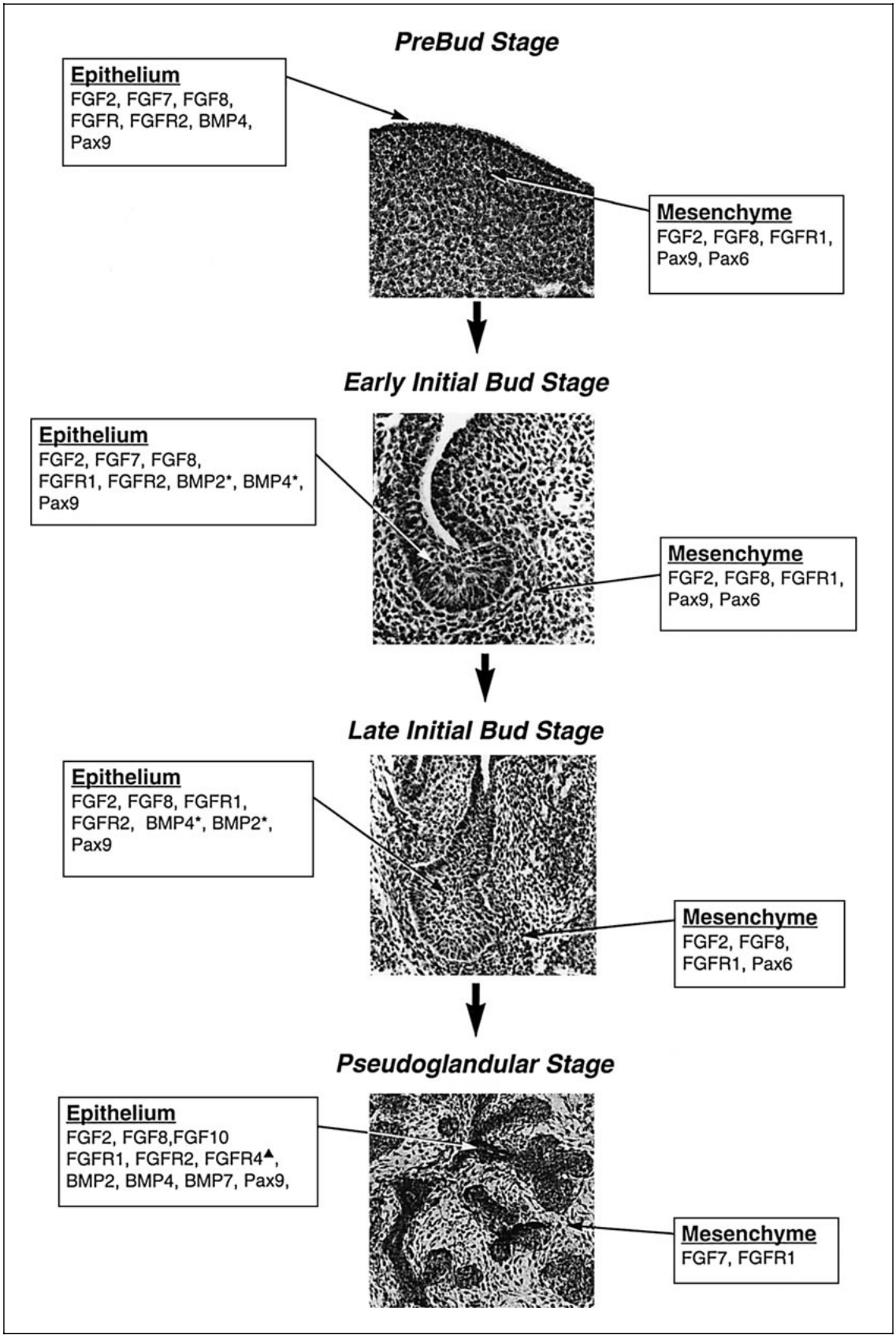
Discussion

The embryonic SMG develops as an invagination of the oral epithelium into undifferentiated mandibular mesenchyme. Numerous avian studies have previously shown that cranial neural crest cells migrate into and proliferate within the mandibular arch to form the majority of mesenchyme, termed ectomesenchyme [Noden, 1983; Le Douarin et al., 1993]. One definitive study on mammalian neural crest branchial arch derivatives has just been reported using the *Wnt1-Cre/R26R* transgenic mouse [Chai et al., 2000]. The determination that mammalian mandibular mesenchyme consists of both cranial neural crest-derived and nonneural crest-derived mesenchymal cells raised the possibility that the origin of SMG mesenchyme may not be exclusively neural crest. To address this question, we analyzed *Initial* Bud and *Pseudoglandular* Stage SMGs in *Wnt1-Cre/R26R* transgenic mice. Our observation of *lacZ* expression in all embryonic SMG mesenchyme clearly demonstrates that it is derived exclusively from cranial neural crest. By contrast, tooth mesenchyme is neural crest- and nonneural-crest-derived [Chai et al., 2000]. Therefore, although we might reasonably expect overlap in the details of early inductive interactions for both teeth and SMGs, differences also likely exist. In addition, since branching morphogenesis in different organs (e.g. lung, SMG, lacrimal gland) is known to be analogous, we also expect similarities of expression of those morphoregulatory factors found more ubiquitously. In this paper, we report the localization patterns for specific FGFs, FGFRs, BMPs and Pax transcription factors previously shown to be important for tooth development and/or branching morphogenesis (fig. 9). Their cell-specific distributions appear to be unique to the early embryonic SMG.

FGF/FGFR Signaling

FGFs are a large family of at least 23 secreted growth factors which have conclusively been shown to induce diverse biological processes, including cell proliferation, ductal branching and histodifferentiation [Hogan, 1999; Jernvall and Thesleff, 2000; Warburton et al., 2000]. The actions of the FGFs are mediated by the FGFRs, a family of four single pass transmembrane receptors with ligand-induced tyrosine kinase activity [Orr-Urtreger et al., 1991; Ornitz et al., 1996; Igarashi et al., 1998]. All four receptors share a similar protein structure, consisting of two or three immunoglobulin-like domains on the extracellular side of the cell membrane linked to a split tyrosine kinase domain in the cytoplasm. Receptors 1–3 have alternate spliced forms of the receptor, whereas receptor 4 does not; this creates receptor isoforms with quite different ligand-binding specificities [Ornitz et al., 1996; Igarashi et al., 1998]. Although FGF-1 is a universal ligand which activates all receptors [Ornitz et al., 1996], the absence of FGF-1 indicates that it does not play a role during early embryonic SMG development. In addition, the absence of FGF-3, a FGFR-1(IIIb) and FGFR-2(IIIb) ligand indicates that FGF-3-mediated signal transduction is also not involved.

Fig. 8. Analysis of *FgfR2-IIIc*$^{+/\Delta}$, *BMP7*$^{-/-}$, and *Pax6*$^{-/-}$ transgenic mice. **A**, **B** Comparison of E16.5 *FgfR2-IIIc*$^{+/\Delta}$ hemizygous SMG (**B**) to its wild-type littermate (**A**). The relatively large appearance of terminal buds in *FgfR2-IIIc*$^{+/\Delta}$ SMGs compared to wild-type controls indicates a decrease in epithelial branching (hypoplasia). In addition, there is almost no lumen formation in the terminal buds of *FgfR2-IIIc*$^{+/\Delta}$ mice. **C**, **D** Comparison of E17.5 *BMP7*$^{-/-}$ SMG (**D**) to its *BMP7*$^{+/+}$ littermate (**C**). *BMP7*$^{-/-}$ SMGs exhibit disorganized mesenchyme and fewer epithelial branches (hypoplasia) compared to control glands. **E**, **F** Comparison of E18.5 *Pax6*$^{-/-}$ (**F**) and *Pax6*$^{+/+}$ (**E**) SMGs. *Pax6*$^{-/-}$ SMGs exhibit mesenchymal disorganization and fewer epithelial branches (hypoplasia) compared to their wild-type littermates. Bar 50 μm.


PreBud Stage
Epithelium
FGF2, FGF7, FGF8,
FGFR, FGFR2, BMP4,
Pax9
Mesenchyme
FGF2, FGF8, FGFR1,
Pax9, Pax6
Early Initial Bud Stage
Epithelium
FGF2, FGF7, FGF8,
FGFR1, FGFR2, BMP2*, BMP4*,
Pax9
Mesenchyme
FGF2, FGF8, FGFR1,
Pax9, Pax6
Late Initial Bud Stage
Epithelium
FGF2, FGF8, FGFR1,
FGFR2, BMP4*, BMP2*,
Pax9
Mesenchyme
FGF2, FGF8,
FGFR1, Pax6
Pseudoglandular Stage
Epithelium
FGF2, FGF8,FGF10
FGFR1, FGFR2, FGFR4▲,
BMP2, BMP4, BMP7, Pax9,
Mesenchyme
FGF7, FGFR1

We report the spatial distribution of FGFR prior to and during early embryonic SMG development (fig. 9). FGFR-1 (both isoforms) is localized in both epithelium and mesenchyme, whereas FGFR-2 (both isoforms) primarily exhibits an epithelial localization. Interestingly, FGFR-4 is only seen at later embryonic stages in the epithelial terminal buds and FGFR-3 is absent. Our localization of FGFR-1 and FGFR-2 proteins are similar to those previously reported for FGFR-1 and FGFR-2 transcripts [fig. 7, Orr-Urtreger et al., 1991].

Gene targeting experiments have clearly demonstrated the importance of FGFR-1 and FGFR-2 in early development [Deng et al., 1994; Xu et al., 1998, 1999]. In contrast, *Fgfr3* null mice are viable and only exhibit skeletal dysplasias of the long bones [Deng et al., 1994] and *Fgfr4* null mice are developmentally normal [Weinstein et al., 1998]. The evaluation of FGFR-1 role during organogenesis has been difficult due to the early embryonic lethality of the *Fgfr1* null mutation. The role of FGFR-2 has now been addressed in mutant mice expressing a soluble dominant-negative *Fgfr2* [Celli et al., 1998], a *Cre*-mediated excision of *FgfR2-IIIb* [De Moerlooze et al., 2000], or *Cre*-mediated excision of *FgfR2-IIIc* (present study). Significantly, submandibular glands are absent in these mutant mice [Celli et al., 1998; De Moerlooze et al., 2000]. Although SMG buds form in *FgfR2-IIIb* mutant mice, extensive cell death in the E13.5 (*Late Initial Bud* Stage) SMG results in the absence of the gland by E14.5 (the *Pseudoglandular* Stage) [De Moelooze et al., 2000; Spencer-Dane, pers. commun.]. To explain this distinct SMG phenotype, we must focus our attention on those members of the FGF family which bind with high affinity to this receptor (namely FGF-1, -3, -7 and -10). Both FGF-7 and FGF-10 are immunolocalized in embryonic SMGs (fig. 9) while FGF-1 and FGF-3 are absent. Thus FGF-7 and FGF-10 ligand binding to FGFR-2(IIIb) induces this signaling pathway during embryonic SMG development.

In the *Prebud* and *Initial Bud* Stages (fig. 9), FGF-7 is the only FGFR-2(IIIb) ligand detected. The codistribution of FGF-7 and FGFR-2 primarily in the epithelium suggests that FGF-7 is primarily a juxtacrine factor which transduces its mitogenic signal within the epithelium (table 1). In addition, our demonstration of FGFR-2 in branching epithelia and FGF-7 in the mesenchyme in the *Pseudoglandular* Stage suggests that FGF-7 acts in a paracrine manner to likely mediate epithelial-mesenchymal interactions at this later stage. FGF-10, another FGFR-2(IIIb) ligand, is found in epithelia at this stage, suggesting that FGF-10 acts as a juxtacrine factor (table 1). This pattern markedly differs from that seen in other branching organs, i.e. lungs, in which FGF-10 and FGFR-2(IIIb) are found in epithelia or mesenchyme, respectively.

Importantly, the presence of an initial SMG bud in *FgfR2-IIIb* transgenic mice indicates that FGF/FGFR-2(IIIb) signal transduction is not required for bud formation [De Moerlooze et al., 2000; Spencer-Dane, pers. commun.]. Equally important, the absence of SMGs in E14.5 transgenic mice [De Moerlooze et al., 2000] indicates that FGF/FGFR-2(IIIb) signaling is *essential* for *Pseudoglandular* Stage SMG development. Since *Fgf7* null mice exhibit no abnormalities in the adult salivary gland [Guo et al., 1996], another FGFR-2(IIIb) ligand must transduce the critical FGF/FGFR-2(IIIb) signal required for SMG development. Our finding of FGF-10, but not FGF-1 or FGF-3, in the *Pseudoglandular* SMG epithelia suggests that FGF-10/FGFR-2(IIIb) binding is the likely candidate. The absence of SMGs in E19.5 *Fgf10* null mice [Ohuchi et al., 2000] supports this conclusion. However, given (1) the relatively late appearance of FGF-10 begin-

Table 1. Putative FGF/FGFR interactions during early embryonic SMG morphogenesis

	Stages		
	Prebud	Initial Bud	Pseudoglandular
FGF-2/FGF-R1[a]	paracrine juxtacrine	paracrine juxtacrine	paracrine juxtacrine
FGF-7/FGFR-2	juxtacrine	juxtacrine	juxtacrine paracrine
FGF-10/FGFR-2[b]	–	–	juxtacrine
FGF-8/FGFR-2[b]	juxtacrine	paracrine juxtacrine	juxtacrine
FGF-8/FGFR-4	–	–	juxtacrine

[a] FGFR-1 null mutation is embryolethal; thus, it is unclear whether FGFR-1 signal transduction is important for embryonic SMG development.

[b] Gene targeting experiments indicate that these pathways are essential during embryonic SMG development [Celli et al., 1998; Trumpp et al., 1999; De Moerlooze et al., 2000; Ohuchi et al., 2000; present study].

Fig. 9. Schematic representation of SMG development prior to and during early embryonic development. The important cell-specific distribution patterns for FGFs, FGFRs, BMPs, Pax6 and Pax9 are shown in the boxes. * = Epithelial stalk localization. FGFR-4▲ is only found in the centers of epithelial terminal buds.

ning in the *Pseudoglandular* Stage, (2) the high binding affinity of FGF-10 to FGFR-2(IIIb), and (3) the presence of initial SMG bud but the absence of *Pseudoglandular* Stage SMGs in *FgfR2-IIIb* mutant mice, it is reasonable to conclude that FGF-10/FGFR-2(IIIb) signal transduction is not *essential* for initial SMG bud formation.

Further, FGF-8 has also been shown to be a key signaling molecule for mandible and tooth development [Neubuser et al., 1997; Trumpp et al., 1999]. *Fgf8;Nes-cre* mutant mice exhibit severe craniofacial abnormalities, including absent Meckel's cartilage, no teeth and microglossias [Trumpp et al., 1999]. Since FGF-8 exhibits several alternative splice forms which activate different receptors [FGFR-2(IIIc), FGFR-3(IIIc) and FGFR-4)] [MacArthur et al., 1995], we used a polyclonal antibody which identified all FGF-8 isoforms to demonstrate the spatial distribution of FGF-8. We report FGF-8 in oral epithelium and adjacent mesenchyme in the *Prebud* and *Initial Bud* Stages (fig. 9); this distribution pattern is consistent with previous observations [MacArthur et al., 1995]. Analyses of FGF-8 receptor's distribution demonstrates FGFR-2 primarily in epithelia, FGFR-3 being absent, and FGFR-4 only at later stages. The localization patterns for FGF-8 and FGFR-2 suggest that FGF-8 ligand and FGFR-2(IIIc) interact in both a juxtacrine and a paracrine manner (table 1). In contrast, the later epithelial localization of FGF-8 and FGFR-2 in the *Pseudoglandular* Stage suggests that FGF-8/FGFR-2(IIIc) signaling occurs within the epithelia. Further, FGF-8 likely also binds in a juxtacrine manner to FGFR-4, seen at this stage in the central region of epithelial terminal buds (table 1). Significantly, we report hypoplastic SMGs in E16.5 *FgfR2-IIIc*$^{+/\Delta}$ hemizygous mice. Based on our results, we conclude that FGF-8/FGFR-2(IIIc) signal transduction is *essential* for branching morphogenesis.

Nevertheless, it is quite perplexing that *FgfR2-IIIc*$^{+/\Delta}$ hemizygotes do not exhibit a more severely abnormal SMG phenotype since other exocrine glands (e.g. exorbital lacrimal glands) fail to develop in these mice [Hajihosseini et al., 2001]. The most likely explanation is that, through a splice switch, *FgfR2-IIIb* is expressed instead of *FGFR2-IIIc*; this substantial elevation in FGFR2-IIIb expression in *FgfR2-IIIc*$^{+/\Delta}$ hemizygous mice likely compensates for the decline in FGFR-2(IIIc) expression [Hajihosseini et al., 2001]. Significantly Hajihosseini et al. [2001] detected a substantial increase in FgfR2-IIIb transcript expression in *FgfR2-IIIc*$^{+/\Delta}$ hemizygous brains and cranial sutures. Given that FGFR2-IIIb signaling is essential for SMG development [De Moerlooze et al., 2000], the relatively mild abnormality of *FgfR2-IIIc*$^{+/\Delta}$ hemizygous SMGs suggests that SMG cells that would normally primarily express FGFR-2(IIIc) likely now express FGFR-2(IIIb) and are responsive to other FGF ligands, including FGF-10. Further studies are needed to address this question.

Finally, we compared the FGFR-1 distribution to that of several known ligands, namely FGF-1, FGF-2 and FGF-3 (fig. 9). The codistribution of FGFR-1 and FGF-2 in embryonic SMG epithelia and mesenchyme, as well as the absence of immunodetectable FGF-1 and FGF-3 indicate that FGF-2/FGFR-1 binding likely transduces the signal (table 1). Although targeted disruption of *Fgfr1* indicates that it is required for early postimplantation growth [Deng et al., 1994; Xu et al., 1999], gene targeting experiments suggest that FGF-2 is not essential for development since *Fgf2* null mice are viable and only exhibit mild defects [Miller et al., 2000; Montero et al., 2000]. Future studies are needed to address whether FGFR-1 is important for early embryonic SMG morphogenesis.

FGF-8, BMPs and Pax9

FGF-8 and BMPs (BMP-4/2) have been identified as key epithelial signaling molecules required for early embryonic mandibular arch patterning and tooth formation [Neubuser et al., 1997; Trumpp et al., 1999; Tucker et al., 1999; Jernvall and Thesleff, 2000]. FGF-8 induces and BMP-4 inhibits Pax9 expression in mesenchyme adjacent to the oral epithelium in E10.5 or younger mandibles; this antagonistic function of FGF-8 and BMP-4 is critical to normal tooth development. Unlike in tooth buds, we report the coincident distribution of FGF-8, BMP-4 and Pax9 in oral epithelium and FGF-8 and Pax9 in adjacent mesenchyme in the *Prebud* Stage (fig. 9). These localization patterns suggest that FGF-8 and BMP 4 do not regulate Pax9 expression in a manner similar to the tooth. Although *Pax9* null mice lack teeth and pharyngeal pouch derivatives (e.g. thymus, parathyroid glands) and exhibit severe craniofacial skeletal abnormalities, their SMGs are normal [Peters et al., 1998]. This suggests that Pax9 regulation by FGF-8 and BMP-4 is not likely to be a key factor in embryonic SMG development.

Interestingly, *BMP7*$^{-/-}$ SMGs exhibit an abnormal phenotype, with disorganized mesenchyme and decreased epithelial branching. Our results are consistent with the previous observation of abnormal lacrimal glands and kidneys in *BMP7*$^{-/-}$ mice [Dudley and Robertson, 1997; Dudley et al., 1999; unpubl. results]. However, given the relatively late appearance of BMP-7 during embryonic SMG development, it is somewhat surprising that *BMP7*$^{-/-}$ SMGs are so severely affected. These results indicate that BMP-7 must play a key role during em-

bryonic SMG branching morphogenesis and that other BMPs, such as BMP-4 or BMP-2, could not compensate for the absence of BMP-7 signaling. What remains unclear is which key signaling pathways are downstream of the BMP-7 signal.

Pax6 and Early Embryonic SMG Morphogenesis

Pax6 is a member of the Pax transcription factor family previously shown to play a critical role during craniofacial development [Grindley et al., 1995; Callaerts et al., 1997; Chapouton et al., 1999; Mansouri et al., 1999; Collinson et al., 2000]. The homozygous *Pax6*$^{-/-}$ mutant mouse lacks functional Pax6 protein, and dies around birth with no eyes or nasal structures [Hill et al., 1991]. It has other craniofacial abnormalities [Kaufman et al., 1995] as well as defects in the forebrain [Schmahl et al., 1993; Caric et al., 1997], cerebellum [Engelkamp et al., 1999], pituitary [Kioussi et al., 1999] and pancreas [St-Onge et al., 1997]. Heterozygotes survive but have small eyes and abnormal lacrimal gland development [Makarenkova et al., 2000]. In this paper, we demonstrated the cell-specific distribution of Pax6 protein in the mesenchyme of *Prebud* and *Initial Bud* Stage SMGs (fig. 9). This distribution pattern markedly differs from that seen in the developing brain and eye, in which Pax6 is primarily seen in optic and neural epithelia [Grindley et al., 1995; Duncan et al., 2000]. Given our observation of an abnormal SMG phenotype in *Pax6*$^{-/-}$ mice, we conclude that Pax6 is important for embryonic SMG branching morphogenesis. To explain this abnormal phenotype, we must focus on those genes known to be downstream of Pax6. Although several *Pax6*-regulated genes, including cell adhesion molecules, have been identified [Stoykova et al., 1997; Meech et al., 1999; Duncan et al., 2000; Pratt et al., 2000], which of these or other *Pax6* downstream genes yet to be identified are important for embryonic SMG morphogenesis remains to be determined.

Conclusions

Our studies demonstrate the distribution of specific FGFs, FGFRs, BMPs and Pax transcription factors prior to and during early embryonic SMG development. Although similar FGF- and BMP-mediated signaling pathways exist in early stages of odontogenesis, lung morphogenesis and SMG development, the cell-specific distributions substantially differ between organs. Our data, together with other functional studies in SMGs and other systems, suggest that FGF-2/FGFR-1, FGF-8/FGFR-2(IIIc), and FGF-10/FGFR-2(IIIb) signal transduction have important paracrine and/or juxtacrine functions during SMG initial bud formation and branching morphogenesis. In addition, the marked overlaps in FGF family members' localization patterns also suggest redundancy in their functions. Finally, our analysis of *BMP7* and *Pax6* mutant mice indicates that both BMP-7 and Pax6 play essential roles during embryonic SMG branching morphogenesis. Further studies are needed to delineate the precise role of each factor during embryonic SMG development.

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