

# Gene expression profiles of mouse submandibular gland development: FGFR1 regulates branching morphogenesis in vitro through BMP- and FGF-dependent mechanisms

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## SUMMARY

Analyses of gene expression profiles at five different stages of mouse submandibular salivary gland development provide insight into gland organogenesis and identify genes that may be critical at different stages. Genes with similar expression profiles were clustered, and RT-PCR was used to confirm the developmental changes. We focused on fibroblast growth factor receptor 1 (FGFR1), as its expression is highest early in gland development. We extended our array results and analyzed the developmental expression patterns of other FGFR and FGF isoforms. The functional significance of FGFR1 was confirmed by submandibular gland organ culture. Antisense oligonucleotides decreased expression of FGFR1 and reduced branching morphogenesis of the glands. Inhibiting FGFR1 signaling with SU5402, a FGFR1 tyrosine kinase inhibitor, reduced branching morphogenesis. SU5402 treatment decreased cell proliferation but did not increase apoptosis. *Fgfr*, *Fgf* and *Bmp* gene expression was localized

to either the mesenchyme or the epithelium by PCR, and then measured over time by real time PCR after SU5402 treatment. FGFR1 signaling regulates *Fgfr1*, *Fgf1*, *Fgf3* and *Bmp7* expression and indirectly regulates *Fgf7*, *Fgf10* and *Bmp4*. Exogenous FGFs and BMPs added to glands in culture reveal distinct effects on gland morphology. Glands cultured with SU5402 were then rescued with exogenous BMP7, FGF7 or FGF10. Taken together, our results suggest specific FGFs and BMPs play reciprocal roles in regulating branching morphogenesis and FGFR1 signaling plays a central role by regulating both FGF and BMP expression.

Supplemental data available on-line

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## INTRODUCTION

The embryonic mouse submandibular salivary gland (SMG) is a classic developmental model system used to study epithelial-mesenchymal interactions, branching morphogenesis, and organogenesis (Bernfield and Banerjee, 1972; Grobstein and Cohen, 1965; Spooner et al., 1989; Spooner and Wessells, 1972). SMG development involves a complex interplay between inductive events, cell migration, proliferation, differentiation, and morphogenesis, and underlying these cellular processes are orchestrated changes in gene expression. There is little information about the genetic regulation of mouse SMG development. The genetic regulation of development in other systems, such as the *Drosophila* salivary gland (Andrew et al., 2000; Bradley et al., 2001; Zhou et al., 2001) and trachea (Metzger and Krasnow, 1999), and the murine lung, pancreas and kidney (Edlund, 2002; Hogan, 1999; Metzger and Krasnow, 1999; Vainio and Lin, 2002),

suggest similar multigene families may regulate development in a tissue-specific manner.

The initiation of mouse SMG development occurs at embryonic day 11 (E11), and by E12, a single epithelial bud forms within a condensed mesenchyme. By E13, clefts form in the epithelial bud, which continues to proliferate and cleft in successive rounds of branching, giving rise to multiple cords and buds by E14 (Jaskoll et al., 2001) (Fig. 1). By E17, morpho-differentiation and lumenization of ducts and terminal buds has occurred. Functional differentiation of the gland begins just before birth and continues on day 1 (D1) after birth when the gland starts to secrete saliva. Most acinar and ductal cell differentiation occurs by D5. The androgen-dependent differentiation of the granular convoluted tubules (GCT) occurs at puberty (Gresik, 1975). The developmental stages are not absolute, and there is a range of developmental variation occurring within an individual gland and between glands from animals in the same litter.

Branching morphogenesis of SMGs in vitro involves EGF signaling (Kashimata and Gresik, 1997; Kashimata et al., 2000; Morita and Nogawa, 1999), integrin  $\alpha 6$ , laminin-1 (Hosokawa et al., 1999; Kadoya et al., 1995), the TNF/TNFR1/IL6 pathways (Melnick et al., 2001b; Melnick et al., 2001c), and FGF7 (Morita and Nogawa, 1999). Fibroblast growth factors (FGFs) and their receptors (FGFRs) are important in many developmental events, including branching morphogenesis in other organs (Martin, 1998; Metzger and Krasnow, 1999; Ornitz, 2000; Spencer-Dene et al., 2001). FGFs are a family of intercellular signaling molecules that contains at least 22 members (Ornitz and Itoh, 2001). The role of BMPs as agonists or antagonists to FGFs has been studied in other developmental systems including tooth formation (Neubuser et al., 1997), lung organ culture (Weaver et al., 2000; Weaver et al., 1999), limb growth (Niswander and Martin, 1993) and kidney development (Piscione et al., 2001).

There are four known FGF receptors, and receptors 1, 2 and 3 each have two isoforms due to alternate splicing in their extracellular domains (Ornitz et al., 1996). Recently, a fifth FGFR has been identified with two splice isoforms but no intracellular kinase domain (Kim et al., 2001; Sleeman et al., 2001). The roles of FGFRs, particularly FGFR1, and FGFs in SMG branching morphogenesis and differentiation are not well defined. Although FGF7 promotes stalk elongation of SMG epithelial explants cultured in the absence of mesenchyme (Morita and Nogawa, 1999), branching morphogenesis in *Fgf7* knockout mice occurs normally (Guo et al., 1996). Abnormal salivary gland phenotypes were reported in mice with a heterozygotic abrogation of *Fgfr2c*-exon 9(IIIc), *Bmp7*-null and *Pax6*-null mice (Jaskoll et al., 2002). Further, mice engineered to lack either the *Fgfr2b* isoform or *Fgf10* do not develop SMGs (De Moerloze et al., 2000; Ohuchi et al., 2000; Sekine et al., 1999). These data show that FGFR2b and FGF10 are necessary for SMG development, but do not provide information on the mechanisms of FGF and FGFR action during branching morphogenesis and gland differentiation.

Complementary DNA (cDNA) arrays generate profiles of gene expression and provide insight into the genetic regulation of complex biological systems. Array analysis has been used to compare gene expression profiles in cells (Rolli-Derkinderen and Gaestel, 2000), tissues (Lemkin et al., 2000), organisms (Galitski et al., 1999), disease states (Selaru et al., 2002) and developmental stages (Hilsenbeck et al., 1999; White et al., 1999), as well as to predict disease prognosis (van 't Veer et al., 2002) and to define the patterns of gene expression during kidney development and maturation (Stuart et al., 2001). We used cDNA arrays to profile gene expression in developing mouse SMGs to investigate the genetic mechanisms involved in gland development. We found that *Fgfr1* is expressed most highly early in gland development. Using organ culture of embryonic submandibular glands, we investigated the biological function of FGFR1 by decreasing *Fgfr1* gene expression and by inhibiting FGFR1 signaling. Analysis of *Fgfr*, *Fgf* and *Bmp* expression downstream of FGFR signaling suggests that FGFR1 signaling regulates the expression of growth factors important for early SMG branching morphogenesis. Our studies begin to define the FGF- and BMP-dependent mechanisms involved in branching morphogenesis in SMG development. FGFs and BMPs play reciprocal roles in regulating branching morphogenesis and

FGFR1 signaling plays a central role by regulating both FGF and BMP expression.

## MATERIALS AND METHODS

### Atlas arrays

Pregnant ICR strain mice were purchased from Harlan Laboratories (Indianapolis, IN). The submandibular/sublingual gland complex, referred to as SMG, were dissected from the embryos at E14 and E17, from the pups at postnatal D1 and D5, and from 3-month old adult mice. The tissue was lysed and homogenized in the denaturing solution from the Atlas Pure Total RNA labeling system (Clontech Laboratories, Inc., Palo Alto, CA) and stored at  $-80^{\circ}\text{C}$ . Total RNA was prepared and 100  $\mu\text{g}$  of each sample was DNase-treated using the Atlas Pure Total RNA labeling system according to the manufacturer's instructions. Radiolabeled probes were generated using 4  $\mu\text{g}$  of DNase-treated total RNA labeled with  $[^{32}\text{P}]\text{dATP}$ . Atlas 1.2 cDNA Expression Arrays, containing 1176 known genes, were hybridized overnight, washed, and exposed to a phosphorimager screen for up to 4 days. The arrays were stripped and reused three times. The arrays were analyzed using Atlas Image 2.0, and three array results at each stage were averaged as recommended (Lee et al., 2000), and analyzed with Atlas Navigator 1.0 software (Clontech Laboratories, Inc., Palo Alto, CA). The three replicate array experiments were normalized at each stage to *Gapd* and *Actb* ( $\beta$ -actin), and then each gene was normalized to itself across the five developmental stages so the results could be presented graphically as profiles of gene expression. *Gapd* and *Actb* were also used to normalize the RT-PCR to confirm the array results. Separately, we either normalized to all housekeeping genes or used global normalization, and obtained similar expression profiles. Genes were excluded if they were detected in only one of the three experiments at a particular developmental stage and were not expressed at least twice in another developmental stage.

### RT-PCR

Array results were confirmed using RT-PCR. cDNA was generated using Advantage RT for PCR Kit, and PCR was performed with either Advantage Plus or Titanium Taq PCR kits using Atlas Primers (all reagents from Clontech Laboratories, Inc., Palo Alto, CA). PCR was performed with a Robo-Cycler (Stratagene, La Jolla, CA). 20 ng of each cDNA was amplified with an initial denaturation at  $95^{\circ}\text{C}$  for 3 minutes, then  $95^{\circ}\text{C}$  for 1 minute, and  $68^{\circ}\text{C}$  for 2.5 minutes for 18, 23, 28, and 33 cycles and a final elongation step of  $68^{\circ}\text{C}$  for 5 minutes. Aliquots (5  $\mu\text{l}$ ) were removed after 18, 23, 28, 33, and 38 cycles and separated on 2% agarose gels. The band intensity was measured with a Stratagene Eagle Eye II (Stratagene, La Jolla, CA). The optimum number of PCR cycles for each primer pair was determined when linear amplification of the product was still occurring as estimated by band intensity. Therefore, the difference in the intensity of the PCR product reflects the difference in the message level at each stage of gland development. The amount of starting cDNA was adjusted so that the band intensity of *Gapd* and *Actb* were equal. The PCR results presented in Figs 3 and 4 were repeated at least twice from a particular cDNA sample, and from at least 2 independent cDNA preparations.

### SMG organ culture

SMGs dissected from either E12 or E13 ICR mice were cultured on Whatman Nucleopore Track-etch filters (13 mm, 0.1  $\mu\text{m}$  pore size, VWR, Buffalo Grove, IL) at the air/medium interface. The filters were floated on 200  $\mu\text{l}$  of DMEM/F12 in 50 mm glass-bottom microwell dishes (MatTek, Ashland, MA). The medium was supplemented with 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, 150  $\mu\text{g}/\text{ml}$  vitamin C, and 50  $\mu\text{g}/\text{ml}$  transferrin. Six SMGs were cultured on each filter at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2/95\%$  air atmosphere. Glands were photographed after approximately 2, 20, and 44 hours and the number

of end buds was counted at each timepoint. Each experiment was repeated at least three times.

Antisense oligonucleotides and controls directed to FGFR1 were designed and manufactured by Biognostik, Gottingen, Germany. FITC-labeled oligonucleotide uptake into the mesenchyme and epithelial buds was detected with confocal microscopy analysis at 20 and 44 hours. The oligonucleotides (2  $\mu$ M) were added at the beginning of each experiment, and the media and oligonucleotides (1  $\mu$ M) were replaced after 44 hours of culture. RT-PCR was used to determine the decrease in gene expression.

SU5402 (Calbiochem, La Jolla, CA) was a gift from Dr F. Unda, Universidad del Pais Vasco, Spain. SU5402, a FGFR1 tyrosine kinase inhibitor that does not inhibit EGF, PDGF, or the insulin receptor (Mohammadi et al., 1997), was added to the culture media at the beginning of the experiment. Control glands were cultured with an equal volume of the vehicle DMSO.

### Detection of cell proliferation and apoptosis

After 20 hours of culture, the glands were incubated with 10  $\mu$ M BrdU (BrdU Labeling and Detection Kit 1, Roche Molecular Biochemicals, Indianapolis, IN) for 90 minutes at 37°C. Then the medium was replaced with PBS, and after 20 minutes at 37°C, the glands were fixed in 50 mM glycine in 70% ethanol, pH 2.0, for 20 minutes at -20°C. The anti-BrdU antibody was incubated for 2 hours at 37°C, and the secondary antibody was incubated for 1 hour at 37°C. Immunofluorescence was examined using a Zeiss LSM 510 microscope.

Apoptosis was detected by TUNEL staining using an In Situ Cell Death Detection Kit, TMR-red (Roche, Indianapolis, IN) as described in the manufacturer's instructions, except that glands were fixed for 60 minutes and permeabilized for 10 minutes. Apoptosis was also detected using the Vybrant Apoptosis Assay Kit (Molecular Probes, Eugene, OR) that uses YO-PRO-1 dye and propidium iodide to distinguish apoptotic and necrotic cells. Glands were also stained with peanut lectin conjugated to either FITC or Rhodamine (Vector Laboratories Inc., Burlingame, CA) to stain epithelial cells, and an anti-perlecan antibody (Chemicon, Temecula, CA) to stain the basement membrane and mesenchyme cells. The fluorescent BrdU and apoptosis staining were quantitated using the MetaMorph image analysis program (Universal Imaging Corp, Downingtown, PA). The fluorescent pixels from all optical sections of each gland were measured and expressed as a ratio of the total pixel area of the gland. At least five glands per condition were used for quantitation and the experiments were repeated three times.

### Localization of gene expression in E13 glands

E13 SMGs with sublingual glands removed were treated with 1.6 U/ml Dispase 1 (Roche, Indianapolis, IN) at 37°C for 20 minutes. The mesenchyme and epithelium were separated with fine forceps and pools of RNA enriched in mesenchyme or epithelium were prepared using the RNAqueous<sup>TM</sup>-4PCR kit with DNase treatment (Ambion, Inc., Austin, TX). All purified RNA was checked for DNA contamination by PCR with *Gapd* primers. RT-PCR was performed as described above, using 10 ng of cDNA per reaction.

### Real time PCR

For analysis of gene expression, at least six E13 SMGs were cultured with either 5  $\mu$ M SU5402 or an equal volume of DMSO for 2, 6 or 20 hours. RNA was prepared as described above and TaqMan<sup>TM</sup> reverse transcription reagents (Applied Biosystems, Foster City, CA) were used to make cDNA. Real time PCR was performed using Clontech primers and SYBR Green PCR Master Mix and a TaqMan<sup>TM</sup> 7700 thermocycler (both from Applied Biosystems, Foster City, CA). The PCR conditions were the same as described above, and aliquots of the PCR reactions were analyzed by gel electrophoresis to confirm that a single product of the expected size was amplified. The reactions were run in triplicate, the experiment repeated three times, and the results were combined to generate the graphs.

### Addition of exogenous growth factors and rescue of SU5402-mediated inhibition of branching morphogenesis with FGFs

A range of concentrations of exogenous FGF1 (1-500 ng/ml), FGF2 (1-500 ng/ml), FGF7 (1-500 ng/ml), FGF10 (10-2500 ng/ml), BMP7 (1-1000 ng/ml), and BMP4 (50-1000 ng/ml), were added to glands and cultured for 20 hours.

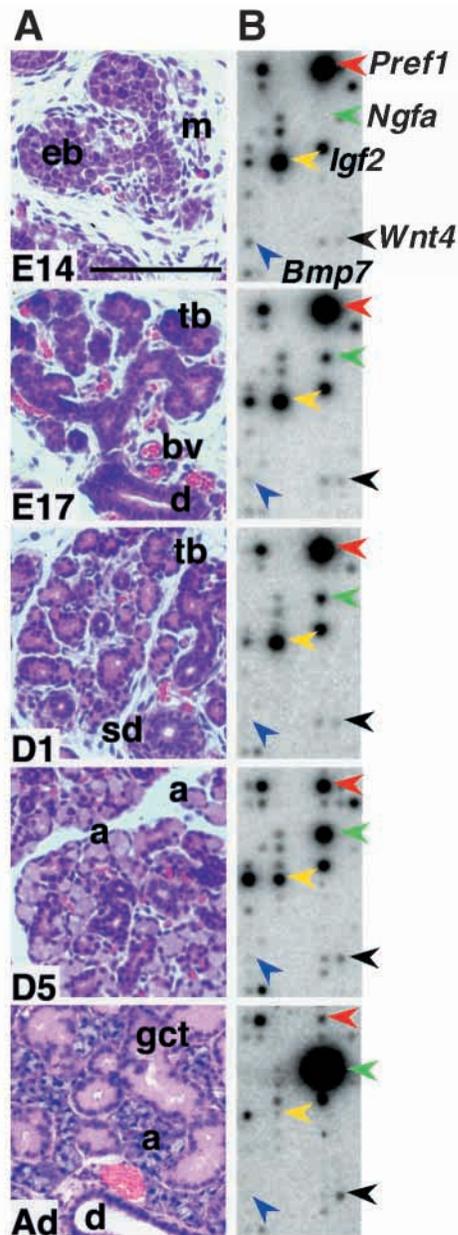
E13 gland rudiments were cultured with 1.5  $\mu$ M SU5402, the concentration required to give ~50% inhibition of branching. The rudiments were incubated with the inhibitor for 30 minutes and then FGF1 (1, 20, 100, or 200 ng/ml), FGF7 (50, 100, 200, 500, 1000 ng/ml), FGF10 (100, 500, 1000, 2500 ng/ml), or BMP7 (20, 50, 100, 200, 500 ng/ml), alone or in combination, were added to the cultures (all growth factors were purchased from R&D Systems, Minneapolis, MN).

## RESULTS

### Atlas array results

Five different stages of gland development were used for gene profiling (Fig. 1A). The Atlas 1.2 filter contains 1176 known genes, and 468 (40%) are present in at least one developmental stage. A section of each array is shown as an example (Fig. 1B), highlighting five genes with developmentally regulated expression patterns. The 468 genes were clustered into eight groups with similar expression profiles (Fig. 2A). We selected 10 examples from each gene cluster (Fig. 2B) based on information from other experimental systems or their potential role in gland development. The entire gene lists with the signal intensity for each gene are presented in the Supplementary Table (<http://dev.biologists.org/supplemental/>).

Cluster Group 1 contains 91 genes that are expressed highly at early stages of gland development. We are interested in this group because they are highly expressed during branching morphogenesis before cell differentiation occurs. Group 2 contains 198 genes that are highly expressed at postnatal day 5. Some Group 2 genes have a biphasic profile with another peak in expression at E17. These may play a role in postnatal morphogenesis and secretory cell differentiation, and be involved in late embryonic gland development. Similar patterns of gene expression may occur during embryonic and postnatal gland morphogenesis. Group 3 contains 49 genes that are expressed highly after birth and increase in expression after D1. This group may be involved in postnatal development and secretory function of the gland. Group 4 contains 21 genes that are expressed highly at D1. These genes may be involved in the initiation of secretory cell function or be involved in ductal morphogenesis and differentiation. Group 5 contains 34 genes with increasing expression during development. Group 6 contains 21 genes that are highly expressed in adult glands. These genes may be involved in either the exocrine or endocrine secretory functions of the adult gland, although, the major known secreted salivary proteins are not on the array filter. Group 7 contains 27 genes with increased expression between postnatal day 1 and 5 and which levels off or decreases, in the adult gland. These genes may be involved in development and homeostasis of the adult gland. Group 8 contains 27 genes with a level profile of gene expression, and both *Gapd* and *Actb* fall within this group.



**Fig. 1.** (A) Developmental stages of mouse SMGs used for array analysis. eb, epithelial bud; m, mesenchyme; tb, terminal bud; d, duct; bv, blood vessel; a, acini; sd, striated duct; gct, granular convoluted tubule. (B) A portion of the Atlas 1.2 cDNA arrays shows five examples of developmentally regulated genes during gland formation.

### Confirmation of array results by RT-PCR

We used RT-PCR to confirm the array results for 13 genes including *Gapd* and *Actb* as housekeeping genes (Fig. 3). The array results are presented as individual graphs of the expression profile (Fig. 3A) together with the RT-PCR result (Fig. 3B). In general, the array results are confirmed by the RT-PCR. In our experience with Atlas arrays, if a gene is barely detectable, but is reproducible, it will be detected by RT-PCR. Since we are interested in Group 1 genes, with high expression early in development, we also confirmed the expression profiles of six other Group 1 genes (not included in Fig. 2B),

including *Fgfr1*, *Bmb7*, *Igf2*, *Igf2r*, *Pref1* and *necdin (Ndn)* (Fig. 3B). In addition, the expression profiles of *Bmp1*, a gene most highly expressed on postnatal D5, FZ6, a member of the Frizzled family of WNT receptors that increases gradually during development, *Ngfa* and *Wnt4*, both highly expressed in adult glands, and *Tmsb4x* (thymosin B4), a gene whose expression gradually increases then levels off in the adult gland, were all confirmed by RT-PCR (Fig. 3).

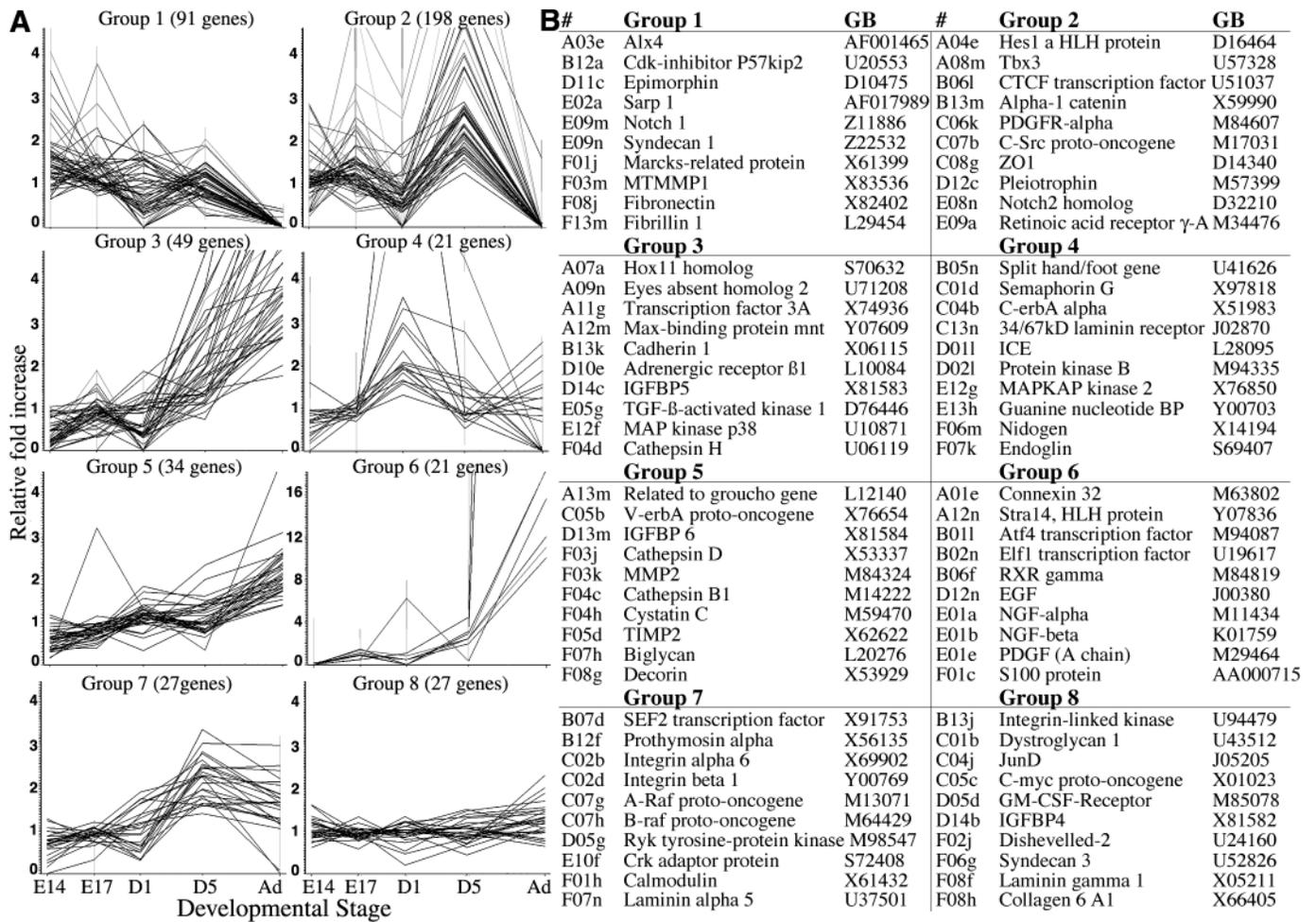
### *Fgfr* and *Fgf* expression are developmentally regulated during SMG development

We focused on the *Fgfrs* and *Fgfs*, which are important in many developmental events, and extended the array results by including cDNAs from E12 and E13 in our RT-PCR analysis. We also extended our RT-PCR analysis to include the *Fgfr1* and *Fgfr2b* and *c* splicing isoforms, and *Fgfr3*, *Fgfr4* (Fig. 4A), and additional FGFs (Fig. 4B) that were not present on the arrays. RT-PCR analysis of the *Fgfr* isoforms reveals distinct developmental regulation of the different receptors (Fig. 4A). The highest expression occurs from E13-E14 with another increase in expression at postnatal D5 for all isoforms except *Fgfr3*. At E13, the gland is undergoing the first round of clefting and then through D1 it undergoes multiple rounds of branching morphogenesis. At E12, when the gland is a single epithelial bud, it begins to undergo cell proliferation, and *Fgfr1b*, *Fgfr2b*, *Fgfr3* and *Fgfr4* are all expressed. At D5, where postnatal growth and secretory cell differentiation are occurring, there is also increased expression of *Fgfr1c*, *Fgfr2b* and *Fgfr4*. In the adult, the most detectable isoforms expressed are *Fgfr1c* and *Fgfr2b*. *Fgfr3* is barely detected by RT-PCR compared with the other *Fgfr* isoforms and requires more PCR cycles.

The expression patterns of *Fgfs* reveal significant developmental regulation of the different isoforms. Analysis of *Fgf1*, 2, 3, 7, 8, 10 and 13 expression at different stages of SMG development is shown (Fig. 4B). *Fgf1* is expressed at all developmental stages but is most highly expressed at postnatal D5 and adult. *Fgf2* has a biphasic pattern of expression being most highly expressed at E13 and then again at postnatal D5. *Fgf3* expression is present highest at E12, E13, and E14 with another peak at D5. *Fgf7* and *Fgf10* show increased expression at E13, when branching morphogenesis begins to occur. *Fgf8* is expressed at E12, E13, and E14. *Fgf13* is expressed highest at E13, but is expressed at a similar level in all other stages except adult.

### Antisense oligonucleotides to *Fgfr1* decrease branching morphogenesis of E12 SMGs

We next focused on *Fgfr1* and tested the functional importance of this receptor in an organ culture assay. Antisense oligonucleotides to *Fgfr1* decrease branching morphogenesis of cultured E12 glands by ~50% compared to an oligonucleotide control (Fig. 5A,B). FITC-labeled oligonucleotides were used to monitor antisense uptake after 20 (data not shown) and 44 hours (Fig. 5C). Confocal microscopy sections show uptake in both mesenchyme and epithelium, with the mesenchyme showing greater uptake. The decrease in *Fgfr1* gene expression with antisense treatment was measured by RT-PCR after 20 hours. The antisense oligonucleotides recognize a sequence in both *Fgfr1* splicing isoforms, however, RT-PCR analysis with isoform-specific



**Fig. 2.** (A) The expression data from the Atlas arrays were clustered into eight groups of genes with similar expression profiles using Atlas Navigator software (Clontech Laboratories, Inc., Palo Alto, CA). A total of 468 genes were identified in at least one stage of SMG development. The number of genes in each cluster is written in parentheses. (B) Ten examples from each group are listed in order of their Atlas array coordinates (#). The table also includes their common name and a GenBank accession number (GB).

primers shows a ~75% reduction in message level of *Fgfr1c* (Fig. 5D) and ~25% reduction in message level of *Fgfr1b*, as compared to control glands. The greater decrease in *Fgfr1c* expression may be due to the greater uptake of oligonucleotide by the mesenchyme. These data demonstrate *Fgfr1* expression levels are important during branching morphogenesis of SMGs in vitro.

**Inhibition of FGFR1 signaling decreases SMG branching morphogenesis**

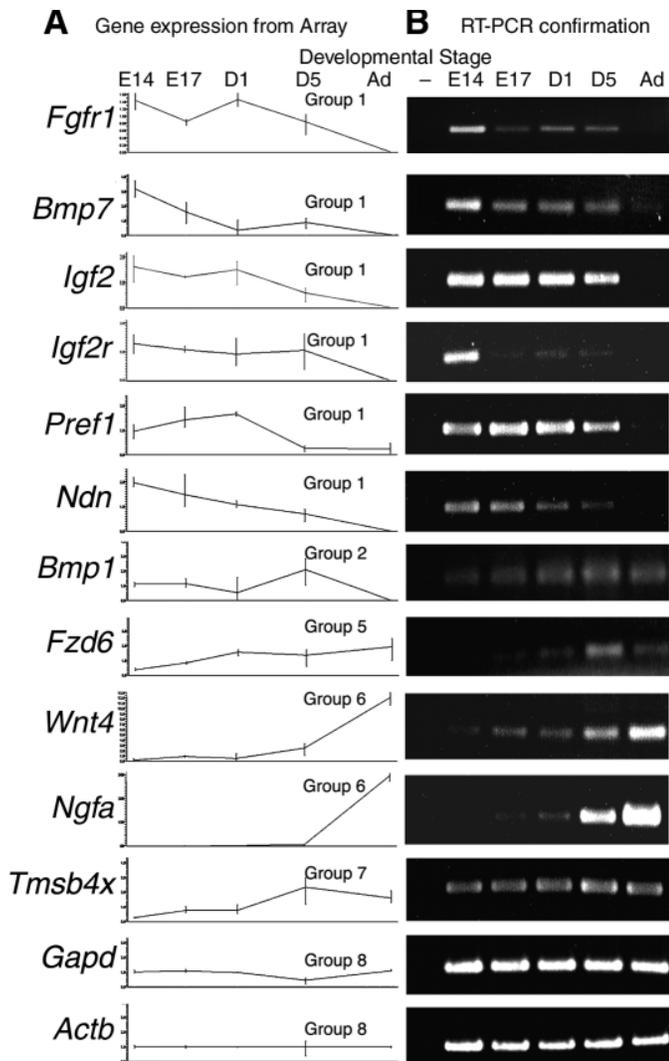
SU5402 inhibits FGFR1 signaling and decreases branching morphogenesis of E12 (data not shown) and E13 SMGs in a dose-dependent manner (Fig. 6A). After 20 hours of treatment, the epithelial buds remain small and do not enlarge and cleft. After 44 hours, the gland forms epithelial finger-like projections, although the duct of the gland lengthens and undergoes lumen formation (Fig. 6A, arrowhead). A range of concentrations (2.5-25  $\mu$ M) inhibits branching (Fig. 6B), at 1  $\mu$ M a partial inhibition is seen, and at lower concentrations, 0.5, 0.1 and 0.02  $\mu$ M, no effect on branching is apparent. The effect of SU5402 is reversible, as the glands resume branching

if it is washed out after 20 hours (data not shown). These data demonstrate that FGFR1 signaling is required for epithelial buds to enlarge and undergo further clefting during SMG branching morphogenesis.

**SU5402 inhibits cell proliferation and does not cause epithelial apoptosis**

BrdU incorporation into SU5402-treated E13 glands reveals a decrease in epithelial cell proliferation after 20 and 44 hours (Fig. 7A-D,I). Epithelial cell proliferation in the control gland is concentrated in the terminal buds (Fig. 7B), with less labeling detected in the mesenchyme. SU5402 treatment dramatically decreases epithelial cell proliferation and has less effect on the mesenchyme. Total BrdU labeling was quantitated at 20 and 44 hours (Fig. 7I). These data demonstrate that FGFR1 signaling affects epithelial cell proliferation during SMG branching morphogenesis.

Apoptosis was measured to determine if the decrease in branching with SU5402 treatment was mediated by an increase in apoptosis. Apoptosis of mesenchyme cells at the edges and on the surface of the glands in culture (Fig. 7E-H) was detected

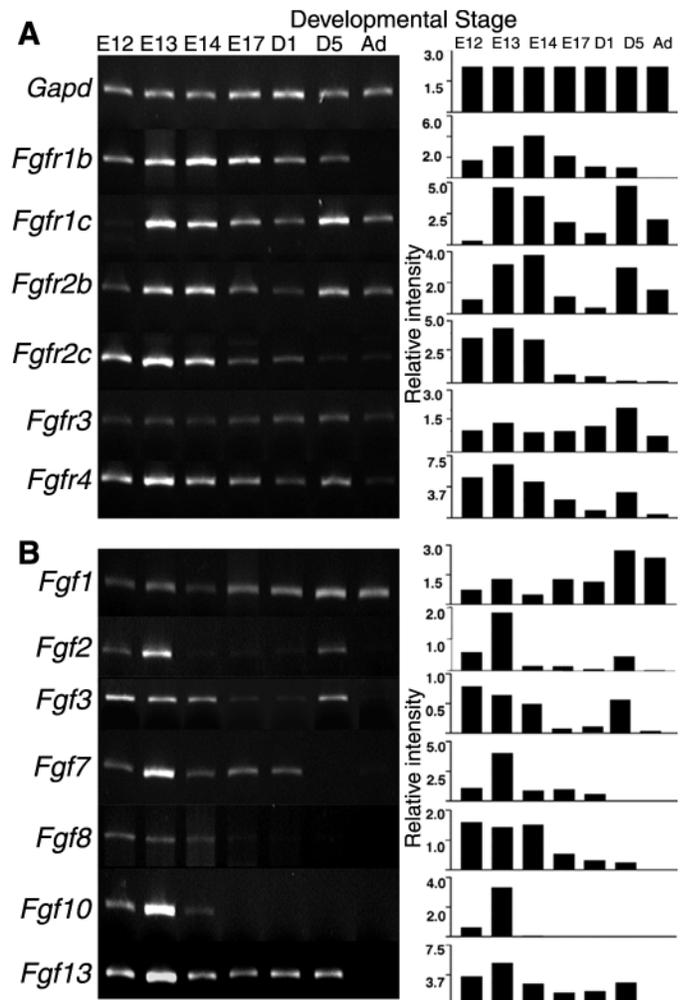


**Fig. 3.** The expression profiles of several developmentally regulated genes were confirmed using RT-PCR. (A) The profile of gene expression of the selected genes from the Atlas 1.2 arrays is shown with relative pixel density on the y-axis. The profiles of gene expression were obtained using Atlas Navigator 1.0 software as described for Fig. 2. (B) The RT-PCR results confirm the profile of gene expression. The cDNAs were normalized to expression levels of *Gapd* and *Actb* ( $\beta$ -actin) for the PCR.

by both TUNEL staining (Fig. 7E-H) and the Vybrant apoptosis kit (data not shown). SU5402 treatment does not cause a significant increase in apoptosis at 20 or 44 hours, although there is a slight increase in apoptosis after 44 hours.

#### ***Fgfr*, *Fgf* and *Bmp* gene expression was localized to either epithelium or mesenchyme**

E13 submandibular glands were micro-dissected into epithelium and mesenchyme and analyzed by RT-PCR (Fig. 8A). The enzymatic separation is not absolute and the pools of RNA are enriched for each cell type, however, our findings are in good agreement with previously published data on *Fgfr* and *Fgf* expression. At E13, *Fgfr1b* and *2b* are expressed in the epithelium and *Fgfr1c*, *2c*, *3* and *4* are in the mesenchyme. *Fgf2*, *3*, *7* and *10* are all expressed in the mesenchyme

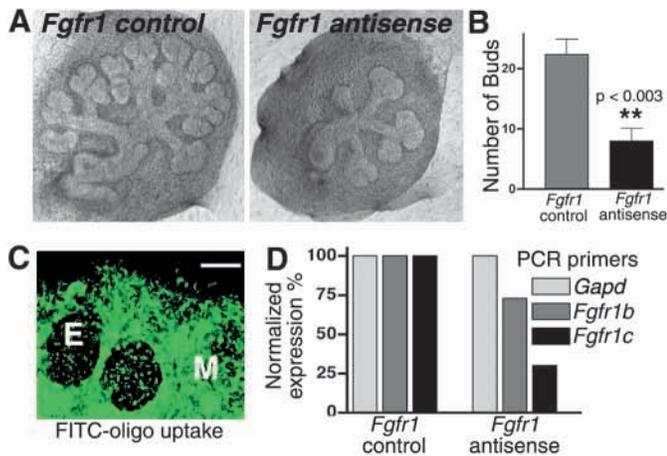


**Fig. 4.** FGFR isoforms and FGFs are developmentally regulated during SMG formation. RT-PCR analysis of FGFR family isoforms (A) and ligands (B) during submandibular gland development is shown with quantitation of the changes shown in the bar graph. The results include receptor/ligand isoforms not present on the Atlas arrays and cDNA from E12 and E13 mouse SMGs. The number of PCR cycles was optimized for each gene so that no products were saturated. Differences in expression levels were quantitated.

while *Fgf1*, *8* and *13* are expressed in both epithelium and mesenchyme. *Bmp1*, *3* and *7* are all expressed in both mesenchyme and epithelium, although *Bmp7* is more abundant in the epithelium. *Bmp2* and *Bmp4* are expressed in the mesenchyme. These data provide information on the localization of gene expression at E13 and help interpret our organ culture experiments.

#### **SU5402 treatment modulates gene expression of *Fgfrs*, *Fgfs* and *Bmps***

Expression of *Fgfs*, *Fgfrs* and *Bmps* was analyzed by real time PCR at 2, 6 and 20 hours of SU5402 treatment, to investigate the molecular mechanism by which SU5402 inhibits branching. Glands treated with SU5402 for 2 and 6 hours look identical to control glands and the first round of branching in culture has not begun (Fig. 6A), but by 20 hours,

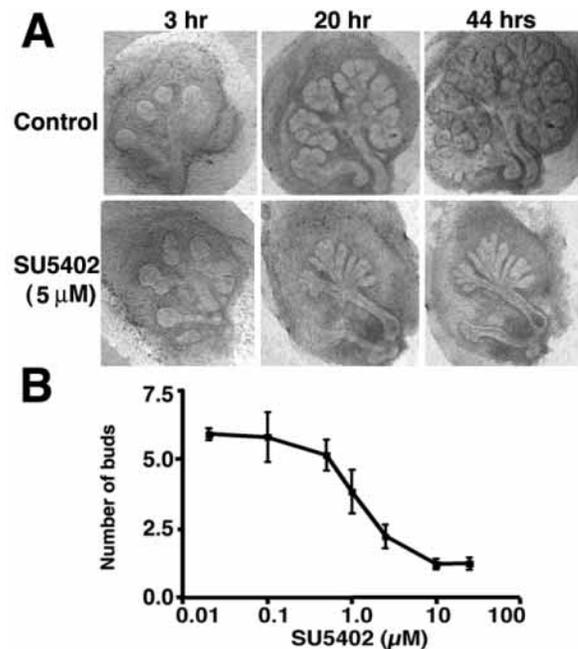


**Fig. 5.** (A) Antisense oligonucleotides to *Fgfr1* decrease branching morphogenesis of E12 SMGs; a representative photograph of each group is shown. (B) At least 6 glands from each group at each timepoint were photographed and the number of terminal buds was counted. An unpaired *t*-test was used to compare branching. There was a significant decrease in the number of buds in the antisense-treated glands (\*\* $P < 0.003$ ). (C) Antisense oligonucleotide uptake into the mesenchyme (M) and the epithelial bud (E) was detected at 44 hours in a 7  $\mu\text{m}$  optical section taken by confocal microscopy. (D) Antisense oligonucleotides to *Fgfr1* decrease *Fgfr1c* expression by ~75% and *Fgfr1b* expression by ~25%. The expression of *Fgfr1b* and *Fgfr1c* were normalized to expression levels of *Gapd*.

morphological changes are apparent (Fig. 6A). After 2 hours of SU5402 treatment there was an increase in the transcription of *Fgfr1b* and *1c* (Fig. 8B), suggesting that inhibition of FGFR1 signaling results in up-regulation of receptor expression via an autocrine feedback mechanism. However, by 6 hours the expression of *Fgfr1b* and *1c* drop below control levels. After 6 hours of SU5402 treatment, there was also decreased expression of *Fgf1*, *Fgf2*, *Fgf3* and *Bmp7*, and after 20 hours of SU5402 treatment, there is still decreased expression of *Fgfr1b*, *Fgf1*, *Fgf3* and *Bmp7* (Fig. 8B), suggesting that these are important regulators of branching morphogenesis. There is also increased expression of *Fgf2*, *Fgf7*, *Fgf10* and *Bmp4* at 20 hours, suggesting these are downstream and may be indirect targets of FGFR1 signaling. Some of the genes had only minor (<1.5 fold) or no changes in gene expression at all time points, including, *Fgfr2b*, *Fgf8*, *Fgf13*, *Bmp1* (all in Fig. 8B) *Fgfr3*, *Fgfr4*, *Bmp2* and *Bmp3* (data not shown). *Fgfr2c* expression increased at all times with SU5402 treatment, which may suggest FGFR1 signaling regulates *Fgfr2c* transcription. These data suggest that *Fgf* and *Bmp* expression, downstream of FGFR1 signaling, regulate branching morphogenesis.

### Exogenous FGFs and BMPs have different morphological effects on submandibular gland epithelium

Exogenous growth factors were added in different doses for 20 hours (Fig. 9A). FGF1 had the least effect and there was no increase in the number of buds (Fig. 9A). In contrast, FGF2 treatment decreased the size and number of the buds, and FGF7 and FGF10 both increase the size of the buds and the width of the duct adjacent to the terminal bud, resulting in the

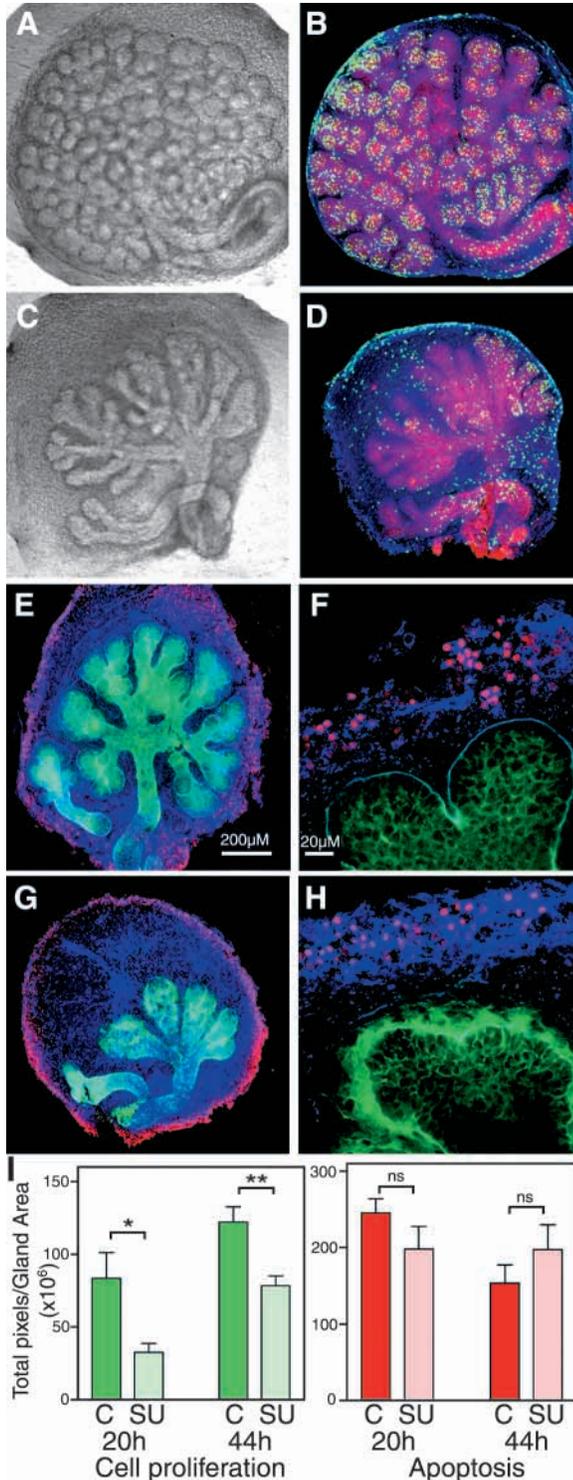


**Fig. 6.** An inhibitor of FGFR1 tyrosine phosphorylation, SU5402, inhibits branching morphogenesis of E13 SMGs. (A) A time-course of SU5402 treatment reveals that at 20 hours (T20) the epithelial buds do not enlarge and cleft. The major duct continues to enlarge and undergo lumen formation (arrowhead). (B) Dose response of SU5402 treatment at 20 hours. The number of buds is expressed as a ratio of the number of buds at 20 hours/the number of buds at 2 hours (T20/T2).

appearance of deep clefts in the epithelium. FGF7 also stimulates elongation of the main ducts (Fig. 9A). Exogenous BMP4 and BMP7 have opposite effects on the glands: BMP7 increases the number of buds and BMP4 inhibits the number of buds and further branching. Glands treated with BMP4 for 44 hours have similar morphology to SU5402-treated glands (Fig. 9A). Taken together with the increase in *Bmp4* expression after 20 hours of SU5402 treatment (Fig. 8B), these data suggest increased expression of BMP4 may be responsible for the morphology of SU5402-treated glands. Thus, BMP4 and FGF2 decrease the size and number of buds, while FGF7 and FGF10 stimulate bud size and BMP7 increases the number of buds. These data suggest that FGFs and BMPs are involved in reciprocal networks that regulate branching morphogenesis.

### FGF7, FGF10 and BMP7 rescue the SU5402-mediated inhibition of branching morphogenesis

Based on the previous experiments we rescued SU5402-treated glands by adding downstream targets of FGFR1 signaling. Different doses of commercially available FGF1, FGF2, FGF7, FGF10, BMP4 and BMP7, were added alone and in combination, to glands treated with 1.5  $\mu\text{M}$  (IC50 for number of buds) of SU5402 (Fig. 9B,C). Active recombinant FGF3 is not available. FGF7 (500 ng/ml), FGF10 (500 ng/ml), and BMP7 (100 ng/ml), but not FGF1, FGF2 and BMP4, were able to rescue the glands (Fig. 9B), further suggesting that FGF7, FGF10 and BMP7 are important regulators of branching. These growth factors did not have an additive effect of

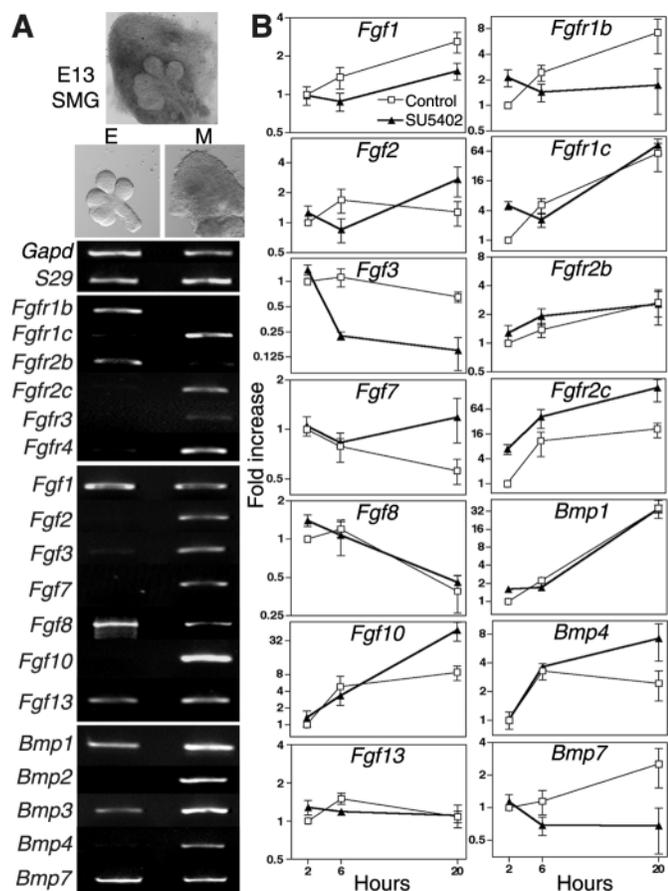


stimulating branching when combined at their maximal dose. If FGF1, FGF2 or BMP4 were added in combination with BMP7, FGF7 or FGF10, they decreased the rescue of branching (data not shown). Taken together with the results of adding exogenous growth factors, these data suggest that both BMP7 and FGFR2b signaling (via FGF7 and FGF10) are downstream of FGFR1 signaling.

**Fig. 7.** SU5402 treatment decreases epithelial cell proliferation but does not cause apoptosis of the epithelial buds. (A–D) BrdU incorporation at 44 hours was detected with fluorescently labeled BrdU. The proliferating cell nuclei appear as green punctate spots. Peanut lectin-rhodamine stains the epithelium and anti-perlecan stains mesenchyme and the basement membrane blue. The images in B, D, E and G are compressed stacks of optical sections through the entire gland. (A) Light micrographs of control gland. (B) Control gland shows BrdU labeling concentrated on the epithelial buds and at the periphery of the mesenchyme. (C) Light micrographs of SU5402-treated gland. (D) SU5402 treatment results in less BrdU labeling on the epithelial buds. Proliferating cells are still apparent in the mesenchyme. (E–I) SU5402 treatment does not cause increased apoptosis at 20 or 44 hours. Apoptosis of mesenchyme cells at the edges and on the surface of the glands in culture was detected with TUNEL staining in red. FITC-peanut lectin stains the epithelium green and anti-perlecan stains mesenchyme and the basement membrane blue. (E) Low power whole-mount view of control gland cultured for 20 hours shows red apoptotic nuclei in the mesenchyme. (F) Higher power section showing apoptotic nuclei in the mesenchyme, but none are detected in the epithelium. (G) Low power whole-mount view of gland treated for 20 hours with SU5402. (H) Higher power section showing apoptotic nuclei in the mesenchyme. (I) The fluorescent BrdU and apoptosis staining were quantitated using the MetaMorph image analysis program. The total fluorescent pixels were expressed as a ratio of the area of the gland. At least 5 glands/condition were used for quantitation and the experiments were repeated three times. \* $P < 0.05$ , \*\* $P < 0.01$ , ns, not significant.

## DISCUSSION

We generated gene expression profiles of discrete stages of mouse SMG development to provide a molecular basis to investigate branching morphogenesis. Selection of genes for further study can be based on the timing and level of developmental regulation, localization of the gene or protein product within the gland, whether the gene is salivary-tissue specific, or whether it has a known or potentially novel function in the SMG. We focused on genes that are highly expressed early in development when branching morphogenesis occurs. Genes in this group, included *Fgfr1* and *Bmp7*, which are important in development, but the mechanisms by which they influence SMG development have not been determined. Other genes from Group 1 that will be interesting to study include *Alx4*, *Pref1* and *Ndn*. ALX4 is a transcription factor and the *Alx4*-null mouse has craniofacial clefting defects (Qu et al., 1997; Qu et al., 1999). *Pref1* is the most highly expressed gene on the arrays at E14, E17 and D1. PREF1 is a transmembrane protein containing six EGF-like repeats, which are highly homologous to Delta/Notch-family members which participate in cell fate choices during differentiation (Bachmann et al., 1996; Tornehave et al., 1996; Kaneta et al., 2000). NDN a nuclear factor related to the rb family, has growth-suppressing functions. Mutations in *Ndn* are associated with Prader-Willi Syndrome, and *Ndn*-null mice have a hypothalamic deficiency (Gerard et al., 1999; Muscatelli et al., 2000). Group 7 genes have increasing expression during development and may be important in gland development. For example, laminin  $\alpha 5$  (LAMA5) is a laminin chain isoform in laminin 10 in SMG basement membranes. *Lama5*-null mice have defects in kidney and lung development (Miner et al., 1998; Miner and Li, 2000; Nguyen et al., 2002).



**Fig. 8.** (A) *Fgfr*, *Fgf* and *Bmp* gene expression was localized to either epithelium or mesenchyme. E13 submandibular glands ( $n=20$ ) were micro-dissected into epithelium (E) and mesenchyme (M) and the light micrographs show a typical gland before and after separation. Gene expression was analyzed by RT-PCR with GAPDH and S29 as controls. The experiment was repeated three times with similar results. (B) SU5402 treatment modulates gene expression of FGFRs, FGFs and BMPs. Expression of FGFs, FGFRs and BMPs was analyzed by real time PCR at 2, 6, and 20 hours of SU5402 treatment. E13 SMGs ( $n=6$ ) were cultured with either 5  $\mu$ M of SU5402 (black triangle) or an equal volume of DMSO (white square) for 2, 6 or 20 hours. Real time PCR was performed using SYBR Green PCR Master Mix and a TaqMan<sup>TM</sup> 7700 thermocycler. The results are expressed as fold increase in gene expression compared to the control glands after 2 hours of culture. The reactions were run in triplicate, the experiment repeated three times, and the results were combined to generate the graphs.

We extended our array data by including E12 and E13 glands in the RT-PCR analysis of the developmental expression of *Fgfrs* and *Fgfs*. At E12, the SMG is an epithelial stalk with a single bud, and the sublingual gland is a small epithelial bud, both within a condensed mesenchyme. At E13, the SMG begins to undergo the first round of clefting, and with epithelial cell proliferation, the first round of branching, while the sublingual gland forms an epithelial stalk with a single bud. The major feature of the developmental regulation of the *Fgfrs* and *Fgfs* is the increased expression at E13, when branching morphogenesis begins. A similar increase in expression appears again at D5 when acinar cell differentiation is

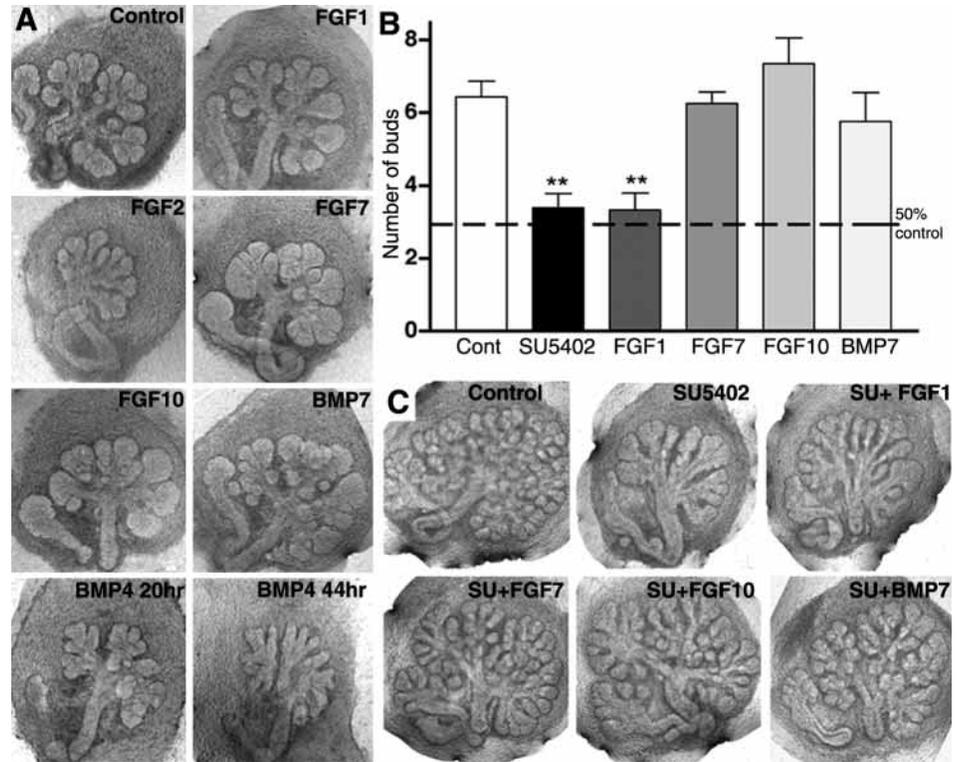
occurring. Similar patterns of gene expression may be conserved and used at different stages of development.

Data from knockout mice provides information about the role of FGFs in SMG development. The *Fgf10*-null (Ohuchi et al., 2000; Sekine et al., 1999) and the *Fgfr2b*-null mice have multi-organ agenesis, including absence of the salivary glands (De Moerloozee et al., 2000). The role of FGF10 and FGFR2b during branching morphogenesis cannot be determined in the knockout models where agenesis of the salivary glands occurs. The known ligands for FGFR2b are FGF1, FGF3, FGF7 and FGF10. Abnormal SMG phenotypes were recently reported in mice with a heterozygotic abrogation of *Fgfr2c*, *Bmp7*-null, and *Pax6*-null mice (Jaskoll et al., 2002). *Fgfr2c*-hemizygotic SMGs (at E16.5) have decreased branching and lumen formation; the mice also have lobulation defects of liver and lungs and fewer nephrons in their kidneys (Hajihosseini et al., 2001). The *Bmp7*-null (at E17.5) and *Pax6*-null (at E18.5) SMGs also have decreased epithelial branching and disorganized mesenchyme (Jaskoll et al., 2002).

We focused on FGFR1 because of its increased expression at early stages of SMG development and because little is known about its role in branching morphogenesis. We used antisense oligonucleotides to decrease *Fgfr1* levels, and a chemical inhibitor of FGFR1 signaling to identify an important role for FGFR1 in regulating branching morphogenesis. Multiple downstream signaling pathways are activated after FGFR stimulation in different experimental systems, including the p42/p44 MAP kinase (Chikazu et al., 2000), p38 MAP kinase (Mehta et al., 2001), PI3 kinase (Chen et al., 2000), and Src family kinases (Landgren et al., 1995; Yayon et al., 1997). The most well-characterized signaling pathway involved in branching morphogenesis of embryonic SMGs involves EGF receptor phosphorylation, which is inhibited by PD98059, an inhibitor of MEK1 phosphorylation (Kashimata et al., 2000). PD98059-mediated inhibition of branching is not as dramatic as SU5402 (data not shown), suggesting that MAP kinase-independent pathways are downstream of FGFRs. Herbimycin, a broad tyrosine kinase inhibitor, also inhibits branching morphogenesis (data not shown). Intracellular signaling through FGFR1, which is inhibited by SU5402, is different from FGFR2-mediated signaling (Rosenthal et al., 2001). In these studies, a FGF2-stimulated calcium channel influx was blocked by the tyrosine kinase inhibitor lavendustin, but was not blocked by either SU5402 or herbimycin A.

SU5402 inhibits branching morphogenesis by decreasing epithelial cell proliferation. SMG branching morphogenesis involves a clefting event that is independent of epithelial cell proliferation (Nakanishi et al., 1987; Spooner et al., 1989). However, for continued rounds of branching to occur, proliferation-mediated expansion of the bud is required for complete formation of new clefts and lobules (Bernfield and Banerjee, 1982). Therefore, SU5402 inhibits epithelial proliferation but clefting still occurs in the bud. Without further epithelial proliferation and bud expansion no further branching occurs. However, SU5402 did not induce apoptosis of the epithelial bud or in ducts undergoing lumen formation. We did not detect epithelial cell apoptosis by either TUNEL staining or with YO-PRO-1 dye/propidium iodide staining, which stains apoptotic/necrotic cells in unfixed glands. However, we detected apoptosis in mesenchymal cells. We presume the mesenchyme apoptosis is a result of the culture conditions at

**Fig. 9.** (A) Exogenous FGFs and BMPs modulate SMG morphology. A range of concentrations of exogenous growth factors were added to glands and cultured for 20 hours. An example of each is shown, FGF1 (100 ng/ml), FGF2 (100 ng/ml), FGF7 (200 ng/ml), FGF10 (500 ng/ml), BMP7 (100 ng/ml), and BMP4 (100 ng/ml), were added to glands and cultured for 20 hours. The gland with BMP4 is also shown after 42 hours in culture, and resembles a SU5402-treated gland. (B) FGF7, FGF10 and BMP7 rescue SMGs treated with SU5402 (1.5  $\mu$ M ~ IC50). The addition of FGF7 (500 ng/ml), FGF10 (500 ng/ml), or BMP7 (100 ng/ml) rescues SMGs treated with SU5402 (1.5  $\mu$ M=IC 50). FGF1 (100 ng/ml) is not able to rescue the glands. The number of buds=(T42/T2), the number of buds at 42 hours/the number of buds at 2 hours. (C) An image of a gland from each group is shown after 44 hours of culture.



the air-medium interface because apoptosis was not detected in E13 glands immediately after dissection. Others have detected extensive epithelial cell apoptosis in E15 submandibular glands cultured for 2 days with SN50, a peptide that inhibits NF- $\kappa$ B nuclear translocation. Apoptosis was increased 10 fold over control peptide-treated glands and almost 50% of the cells underwent apoptosis (Melnick et al., 2001a).

We localized the expression of *Fgfrs*, *Fgfs* and *Bmps*, to either the epithelium or the mesenchyme by RT-PCR. Recently, the stage-specific localization of FGFR, FGF and BMP proteins in the developing submandibular gland was reported (Jaskoll et al., 2002). These immunohistochemical results are generally in agreement with our PCR data in terms of which FGFRs, FGFs and BMPs are present in the glands. Since antibodies to FGFRs do not distinguish the receptor isoforms, our PCR data provide additional information about receptor isoform localization at E13. The localization of the *Fgfr1* and *r2* splicing isoforms are consistent with in situ data, showing at early stages the 'b' isoforms are in the epithelium and the 'c' isoforms are in the mesenchyme (Orr-Urtreger et al., 1993; Peters et al., 1992).

We analyzed gene expression after SU5402 treatment to identify downstream targets. Our results show that FGFR1 signaling directly regulates multiple receptors and ligands, and begins to define the sequence of transcriptional regulation and the molecular mechanisms involved in SMG branching morphogenesis. A surprising finding was that SU5402 treatment for 2 hours results in an increase in *Fgfr1b* and *1c* transcription, presumably via autocrine feedback. Therefore, an initial response to blocking FGFR1 receptor phosphorylation is to increase the receptor transcription. SU5402 also increased *Fgfr2c* transcription. It is not known if

this is a direct effect of FGFR1 signaling or a direct effect on FGFR2c signaling.

The first round of SMG branching in culture starts within 6-9 hours, and after 6 hours of SU5402 treatment there is decreased gene expression of *Fgfr1b*, (in the epithelium), *Fgf1*, *Fgf2*, *Fgf3* (all in the mesenchyme), and *Bmp7* (mainly in epithelium) suggesting these genes may be important mediators of branching. FGF1 binds to all FGFRs and exogenous FGF1 does not rescue SU5402-treated glands or stimulate branching in vitro. FGF2 binds to FGFR1 and 2c isoforms, and exogenous FGF2 decreases the size of epithelial buds. Therefore FGF2 expression may be localized at sites where bud expansion does not occur. FGF3 binds to FGFR1b and 2b, and has the greatest decrease in expression with SU5402 treatment. Also, *Fgfr1b* expression decreases with SU5402 treatment, suggesting that the FGF3/FGFR1b interaction may be important for branching. Interestingly, the SU5402-treated glands could be rescued with FGF7 and FGF10, which bind similar receptors as FGF3 (FGFR1b and 2b), and may functionally compensate for the decrease in FGF3 expression. Also, after 20 hours of SU5402 treatment there is increased expression of the *Fgf7* and *Fgf10* genes (both in the mesenchyme). Taken together, these data suggest the endogenous increase of FGF7 and FGF10 expression after 20 hours of SU5402 treatment is too late, or not high enough, to rescue the glands.

The role of BMPs as agonists or antagonists to FGFs has been studied in other developmental systems. During neural cell development, SU5402 increases *Bmp4* and *Bmp7* and suppresses *Fgf3* gene expression (Wilson et al., 2000). FGF and BMP signaling pathways have antagonistic interactions during tooth formation (Neubuser et al., 1997). In embryonic lung organ culture, BMP4 from the endoderm and FGF10 from

the mesenchyme play opposing roles in mediating branching morphogenesis of the lung (Weaver et al., 2000; Weaver et al., 1999). FGF4 and BMP2 were shown to have opposite effects on limb growth (Niswander and Martin, 1993). BMP7 increases epithelial cell proliferation during kidney development, although at high doses it had the opposite effect (Piscione et al., 2001). *Bmp7* has a similar expression profile to *Fgfr1* in our array analysis. Furthermore, BMP7 increases the number of SMG buds when added exogenously, its expression is decreased 6 hours after SU5402 treatment, and most importantly it was able to rescue SU5402-treated glands (Fig. 9B). Our data suggest that *Bmp7* expression downstream of FGFR1 signaling is important for branching morphogenesis and provides a mechanism by which crosstalk between FGF and BMP signaling occurs. Our studies show opposite roles for BMP4 and BMP7 during submandibular gland development. Exogenous BMP4 inhibits branching and the glands appear similar to SU5402-treated glands. Interestingly, there is increased *Bmp4* expression after 20 hours of SU5402 treatment. Taken together, these data suggest that *Bmp4* expression is indirectly regulated by FGFR1 signaling and that it plays a negative regulatory role on branching. Reciprocal patterns of *Fgf* and *Bmp* expression may define and regulate areas of epithelial bud expansion, cleft initiation, and/or progression. Our data suggest that FGF7, FGF10, and BMP7 have positive regulatory roles on the number and size of epithelial buds, whereas FGF2 and BMP4 play negative regulatory roles, and potentially decrease epithelial cell proliferation and may define the sites of cleft formation.

We conclude that FGFR1c signaling in the mesenchyme and FGFR1b expression levels and signaling in the epithelium are important for branching. Taken together, with the results using *Fgfr2b*-null mice (De Moerlooze et al., 2000), it is likely that both FGFR1 isoforms and 2b are involved in SMG branching morphogenesis. FGFR1 signaling may be upstream of FGFR2 by directly regulating *Fgf1* and *Fgf3* expression, and indirectly regulating FGF7 and FGF10 expression. Branching morphogenesis of SMGs involves proliferation, migration, cleft formation, duct elongation, and differentiation; therefore, FGFR isoforms, FGFs, and BMPs may have different functions in these processes in both the mesenchyme and the epithelium.

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## REFERENCES

- Andrew, D. J., Henderson, K. D. and Seshiah, P. (2000). Salivary gland development in *Drosophila melanogaster*. *Mech. Dev.* **92**, 5-17.
- Bachmann, E., Krogh, T. N., Hojrup, P., Skjodt, K. and Teisner, B. (1996). Mouse fetal antigen 1 (mFA1), the circulating gene product of *mdk*, *pref-1* and *SCP-1*: isolation, characterization and biology. *J. Reprod. Fertil.* **107**, 279-285.
- Bernfield, M. and Banerjee, S. D. (1982). The turnover of basal lamina glycosaminoglycan correlates with epithelial morphogenesis. *Dev. Biol.* **90**, 291-305.
- Bernfield, M. R. and Banerjee, S. D. (1972). Acid mucopolysaccharide (glycosaminoglycan) at the epithelial-mesenchymal interface of mouse embryo salivary glands. *J. Cell. Biol.* **52**, 664-673.
- Bradley, P. L., Haberman, A. S. and Andrew, D. J. (2001). Organ formation in *Drosophila*: specification and morphogenesis of the salivary gland. *BioEssays* **23**, 901-911.
- Chen, Y., Li, X., Eswarakumar, V. P., Seger, R. and Lonai, P. (2000). Fibroblast growth factor (FGF) signaling through PI 3-kinase and Akt/PKB is required for embryoid body differentiation. *Oncogene* **19**, 3750-3756.
- Chikazu, D., Hakeda, Y., Ogata, N., Nemoto, K., Itabashi, A., Takato, T., Kumegawa, M., Nakamura, K. and Kawaguchi, H. (2000). Fibroblast growth factor (FGF)-2 directly stimulates mature osteoclast function through activation of FGF receptor 1 and p42/p44 MAP kinase. *J. Biol. Chem.* **275**, 31444-31450.
- De Moerlooze, L., Spencer-Dene, B., Revest, J., Hajhosseini, M., Rosewell, I. and Dickson, C. (2000). An important role for the IIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development* **127**, 483-492.
- Edlund, H. (2002). Pancreatic organogenesis – developmental mechanisms and implications for therapy. *Nat. Rev. Genet.* **3**, 524-532.
- Galitski, T., Saldanha, A. J., Styles, C. A., Lander, E. S. and Fink, G. R. (1999). Ploidy regulation of gene expression. *Science* **285**, 251-254.
- Gerard, M., Hernandez, L., Wevrick, R. and Stewart, C. L. (1999). Disruption of the mouse *necln* gene results in early post-natal lethality. *Nat. Genet.* **23**, 199-202.
- Gresik, E. W. (1975). The postnatal development of the sexually dimorphic duct system and of amylase activity in the submandibular glands of mice. *Cell Tissue Res.* **157**, 411-422.
- Grobstein, C. and Cohen, J. (1965). Collagenase: effect on the morphogenesis of embryonic salivary epithelium in vitro. *Science* **150**, 626-628.
- Guo, L., Degenstein, L. and Fuchs, E. (1996). Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.* **10**, 165-175.
- Hajhosseini, M. K., Wilson, S., de Moerlooze, L. and Dickson, C. (2001). A splicing switch and gain-of-function mutation in *Fgfr2-IIIc* hemizygotes causes Apert/Pfeiffer-syndrome-like phenotypes. *Proc. Natl. Acad. Sci. USA* **98**, 3855-3860.
- Hilsenbeck, S. G., Friedrichs, W. E., Schiff, R., O'Connell, P., Hansen, R. K., Osborne, C. K. and Fuqua, S. A. (1999). Statistical analysis of array expression data as applied to the problem of tamoxifen resistance. *J. Natl. Cancer Inst.* **91**, 453-459.
- Hogan, B. L. (1999). Morphogenesis. *Cell* **96**, 225-233.
- Hosokawa, Y., Takahashi, Y., Kadoya, Y., Yamashina, S., Nomizu, M., Yamada, Y. and Nogawa, H. (1999). Significant role of laminin-1 in branching morphogenesis of mouse salivary epithelium cultured in basement membrane matrix. *Dev. Growth Differ.* **41**, 207-216.
- Jaskoll, T., Chen, H., Min Zhou, Y., Wu, D. and Melnick, M. (2001). Developmental expression of survivin during embryonic submandibular salivary gland development. *BMC Dev. Biol.* **1**, 5.
- Jaskoll, T., Zhou, Y. M., Chai, Y., Makarenkova, H. P., Collinson, J. M., West, J. D., Hajhosseini, M. K., Lee, J. and Melnick, M. (2002). 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. *Cells Tissues Organs* **170**, 83-98.
- Kadoya, Y., Kadoya, K., Durbej, M., Holmval, K., Sorokin, L. and Ekblom, P. (1995). Antibodies against domain E3 of laminin-1 and integrin alpha 6 subunit perturb branching epithelial morphogenesis of submandibular gland, but by different modes. *J. Cell Biol.* **129**, 521-534.
- Kaneta, M., Osawa, M., Sudo, K., Nakauchi, H., Farr, A. G. and Takahama, Y. (2000). A role for *pref-1* and *HES-1* in thymocyte development. *J. Immunol.* **164**, 256-264.
- Kashimata, M. and Gresik, E. W. (1997). Epidermal growth factor system is a physiological regulator of development of the mouse fetal submandibular gland and regulates expression of the alpha6-integrin subunit. *Dev. Dyn.* **208**, 149-161.
- Kashimata, M., Sayeed, S., Ka, A., Onetti-Muda, A., Sakagami, H., Faraggiana, T. and Gresik, E. W. (2000). The ERK-1/2 signaling pathway is involved in the stimulation of branching morphogenesis of fetal mouse submandibular glands by EGF. *Dev. Biol.* **220**, 183-196.
- Kim, I., Moon, S., Yu, K., Kim, U. and Koh, G. Y. (2001). A novel fibroblast growth factor receptor-5 preferentially expressed in the pancreas(1). *Biochim. Biophys. Acta* **1518**, 152-156.
- Landgren, E., Blume-Jensen, P., Courtneidge, S. A. and Claesson-Welsh, L. (1995). Fibroblast growth factor receptor-1 regulation of Src family kinases. *Oncogene* **10**, 2027-2035.
- Lee, M. L., Kuo, F. C., Whitmore, G. A. and Sklar, J. (2000). Importance

- of replication in microarray gene expression studies: statistical methods and evidence from repetitive cDNA hybridizations. *Proc. Natl. Acad. Sci. USA* **97**, 9834-9839.
- Lemkin, P. F., Thornwall, G. C., Walton, K. D. and Hennighausen, L.** (2000). The microarray explorer tool for data mining of cDNA microarrays: application for the mammary gland. *Nucleic Acids Res.* **28**, 4452-4459.
- Martin, G. R.** (1998). The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* **12**, 1571-1586.
- Mehta, P. B., Robson, C. N., Neal, D. E. and Leung, H. Y.** (2001). Keratinocyte growth factor activates p38 MAPK to induce stress fibre formation in human prostate DU145 cells. *Oncogene* **20**, 5359-5365.
- Melnick, M., Chen, H., Min Zhou, Y. and Jaskoll, T.** (2001a). The functional genomic response of developing embryonic submandibular glands to NF-kappaB inhibition. *BMC Dev. Biol.* **1**, 15.
- Melnick, M., Chen, H., Zhou, Y. and Jaskoll, T.** (2001b). Embryonic mouse submandibular salivary gland morphogenesis and the TNF/TNF-R1 signal transduction pathway. *Anat. Rec.* **262**, 318-330.
- Melnick, M., Chen, H., Zhou, Y. M. and Jaskoll, T.** (2001c). Interleukin-6 signaling and embryonic mouse submandibular salivary gland morphogenesis. *Cells Tissues Organs* **168**, 233-245.
- Metzger, R. J. and Krasnow, M. A.** (1999). Genetic control of branching morphogenesis. *Science* **284**, 1635-1639.
- Miner, J. H., Cunningham, J. and Sanes, J. R.** (1998). Roles for laminin in embryogenesis: exencephaly, syndactyly, and placental pathology in mice lacking the laminin alpha5 chain. *J. Cell Biol.* **143**, 1713-1723.
- Miner, J. H. and Li, C.** (2000). Defective glomerulogenesis in the absence of laminin alpha5 demonstrates a developmental role for the kidney glomerular basement membrane. *Dev. Biol.* **217**, 278-289.
- Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J.** (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.
- Morita, K. and Nogawa, H.** (1999). EGF-dependent lobule formation and FGF7-dependent stalk elongation in branching morphogenesis of mouse salivary epithelium in vitro. *Dev. Dyn.* **215**, 148-154.
- Muscattelli, F., Abrous, D. N., Massacrier, A., Boccaccio, I., le Moal, M., Cau, P. and Cremer, H.** (2000). Disruption of the mouse Necdin gene results in hypothalamic and behavioral alterations reminiscent of the human Prader-Willi syndrome. *Hum. Mol. Genet.* **9**, 3101-3110.
- Nakanishi, Y., Morita, T. and Nogawa, H.** (1987). Cell proliferation is not required for the initiation of early cleft formation in mouse embryonic submandibular epithelium in vitro. *Development* **99**, 429-437.
- Neubuser, A., Peters, H., Balling, R. and Martin, G. R.** (1997). Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. *Cell* **90**, 247-255.
- Nguyen, N. M., Miner, J. H., Pierce, R. A. and Senior, R. M.** (2002). Laminin alpha 5 is required for lobar septation and visceral pleural basement membrane formation in the developing mouse lung. *Dev. Biol.* **246**, 231-244.
- Niswander, L. and Martin, G. R.** (1993). FGF-4 and BMP-2 have opposite effects on limb growth. *Nature* **361**, 68-71.
- Ohuchi, H., Hori, Y., Yamasaki, M., Harada, H., Sekine, K., Kato, S. and Itoh, N.** (2000). FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. *Biochem. Biophys. Res. Commun.* **277**, 643-649.
- Ornitz, D. M.** (2000). FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *BioEssays* **22**, 108-112.
- Ornitz, D. M. and Itoh, N.** (2001). Fibroblast growth factors. *Genome Biol.* **2**, 3005.
- Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G. and Goldfarb, M.** (1996). Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem.* **271**, 15292-15297.
- Orr-Urtreger, A., Bedford, M. T., Burakova, T., Arman, E., Zimmer, Y., Yayon, A., Givol, D. and Lonai, P.** (1993). Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). *Dev. Biol.* **158**, 475-486.
- Peters, K. G., Werner, S., Chen, G. and Williams, L. T.** (1992). Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development* **114**, 233-243.
- Piscione, T. D., Phan, T. and Rosenblum, N. D.** (2001). BMP7 controls collecting tubule cell proliferation and apoptosis via Smad1-dependent and -independent pathways. *Am. J. Physiol. Renal Physiol.* **280**, F19-33.
- Qu, S., Niswender, K. D., Ji, Q., van der Meer, R., Keeney, D., Magnuson, M. A. and Wisdom, R.** (1997). Polydactyly and ectopic ZPA formation in Alx-4 mutant mice. *Development* **124**, 3999-4008.
- Qu, S., Tucker, S. C., Zhao, Q., deCrombrugge, B. and Wisdom, R.** (1999). Physical and genetic interactions between Alx4 and Cart1. *Development* **126**, 359-369.
- Rolli-Derkinderen, M. and Gaestel, M.** (2000). p38/SAPK2-dependent gene expression in Jurkat T cells. *Biol. Chem.* **381**, 193-198.
- Rosenthal, R., Thieme, H. and Strauss, O.** (2001). Fibroblast growth factor receptor 2 (FGFR2) in brain neurons and retinal pigment epithelial cells act via stimulation of neuroendocrine L-type channels (Ca(v)1.3). *Faseb J.* **15**, 970-977.
- Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N. et al.** (1999). Fgf10 is essential for limb and lung formation. *Nat. Genet.* **21**, 138-141.
- Selaru, F. M., Zou, T., Xu, Y., Shustova, V., Yin, J., Mori, Y., Sato, F., Wang, S., Olaru, A., Shibata, D. et al.** (2002). Global gene expression profiling in Barrett's esophagus and esophageal cancer: a comparative analysis using cDNA microarrays. *Oncogene* **21**, 475-478.
- Sleman, M., Fraser, J., McDonald, M., Yuan, S., White, D., Grandison, P., Kumble, K., Watson, J. D. and Murison, J. G.** (2001). Identification of a new fibroblast growth factor receptor, FGFR5. *Gene* **271**, 171-182.
- Spencer-Dene, B., Dillon, C., Fantl, V., Kerr, K., Petiot, A. and Dickson, C.** (2001). Fibroblast growth factor signalling in mouse mammary gland development. *Endocr. Relat. Cancer* **8**, 211-217.
- Spooner, B. S., Bassett, K. E. and Spooner, B. S., Jr** (1989). Embryonic salivary gland epithelial branching activity is experimentally independent of epithelial expansion activity. *Dev. Biol.* **133**, 569-575.
- Spooner, B. S. and Wessells, N. K.** (1972). An analysis of salivary gland morphogenesis: role of cytoplasmic microfilaments and microtubules. *Dev. Biol.* **27**, 38-54.
- Stuart, R. O., Bush, K. T. and Nigam, S. K.** (2001). Changes in global gene expression patterns during development and maturation of the rat kidney. *Proc. Natl. Acad. Sci. USA* **98**, 5649-5654.
- Tornehave, D., Jensen, C. H., Teisner, B. and Larsson, L. I.** (1996). FA1 immunoreactivity in endocrine tumours and during development of the human fetal pancreas; negative correlation with glucagon expression. *Histochem. Cell Biol.* **106**, 535-542.
- Vainio, S. and Lin, Y.** (2002). Coordinating early kidney development: lessons from gene targeting. *Nat. Rev. Genet.* **3**, 533-543.
- van 't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T. et al.** (2002). Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**, 530-536.
- Weaver, M., Dunn, N. R. and Hogan, B. L.** (2000). Bmp4 and Fgf10 play opposing roles during lung bud morphogenesis. *Development* **127**, 2695-2704.
- Weaver, M., Yingling, J. M., Dunn, N. R., Bellusci, S. and Hogan, B. L.** (1999). Bmp signaling regulates proximal-distal differentiation of endoderm in mouse lung development. *Development* **126**, 4005-4015.
- White, K. P., Rifkin, S. A., Hurban, P. and Hogness, D. S.** (1999). Microarray analysis of Drosophila development during metamorphosis. *Science* **286**, 2179-2184.
- Wilson, S. I., Graziano, E., Harland, R., Jessell, T. M. and Edlund, T.** (2000). An early requirement for FGF signalling in the acquisition of neural cell fate in the chick embryo. *Curr. Biol.* **10**, 421-429.
- Yayon, A., Ma, Y. S., Safran, M., Klagsbrun, M. and Halaban, R.** (1997). Suppression of autocrine cell proliferation and tumorigenesis of human melanoma cells and fibroblast growth factor transformed fibroblasts by a kinase-deficient FGF receptor 1: evidence for the involvement of Src-family kinases. *Oncogene* **14**, 2999-3009.
- Zhou, B., Bagri, A. and Beckendorf, S. K.** (2001). Salivary gland determination in Drosophila: a salivary-specific, fork head enhancer integrates spatial pattern and allows fork head autoregulation. *Dev. Biol.* **237**, 54-67.