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Endogenous and Ectopic Gland Induction by FGF-10

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FGF-10, a member of the fibroblast growth factor family, is expressed in mesodermally derived cell populations during embryogenesis. During normal ocular development, FGF-10 is expressed in the perioptic mesenchyme adjacent to the Harderian and lacrimal gland primordia. In this report, we provide evidence that FGF-10 is both necessary and sufficient to initiate glandular morphogenesis. Lens-specific expression of FGF-10 was sufficient to induce ectopic ocular glands within the cornea. In addition, lacrimal and Harderian glands were not seen in FGF-10 null fetuses. Based on these results we propose that FGF-10 is an inductive signal that initiates ocular gland morphogenesis. © 2000 Academic Press

Key Words: **FGF-10; cornea; Harderian gland; lacrimal gland; transgenic mice; KGF receptor.**

INTRODUCTION

Epithelial–mesenchymal interactions are involved in many of the inductive events that shape the mammalian embryo. Inductive signals are thought to be provided by diffusible molecules such as fibroblast growth factors (FGFs), bone morphogenetic proteins, Wnts, and Sonic hedgehog (Gilbert, 1994). FGF-10, a member of the FGF family and a mesodermally derived mitogen, has been shown to actively participate in epithelial–mesenchymal interactions during embryogenesis (Hogan and Yingling, 1998; Hogan, 1999). In the limb bud, reciprocal signaling between FGF-10 (expressed in the prospective limb mesoderm) and FGF-8 (expressed in the overlying apical ectodermal ridge) helps to maintain limb outgrowth (Ohuchi *et al.,* 1997; Xu *et al.,* 1998). In the lung, FGF-10 expression is seen in the mesenchyme at sites of branching of the pulmonary endoderm (Bellusci *et al.,* 1997). Targeted deletion of FGF-10 in mice leads to defects in lung development as well as near complete inhibition of limb bud initiation and outgrowth (Sekine *et al.,* 1999; Min *et al.,* 1998), indicating that FGF-10 is a potent mesenchymal signal that can alter the development of adjacent epithelial cells.

In our studies we have examined the role of FGF-10 during ocular development. Several of the FGF family members have been shown to be expressed in the murine eye and surrounding ocular tissues. During embryogenesis, FGF-1 and FGF-2 have been shown to be expressed in the lens (Lovicu *et al.,* 1997; de Iongh and McAvoy, 1993). FGF-3, FGF-5, FGF-11, FGF-12, FGF-13, and FGF-15 are expressed in the retina (Wilkinson *et al.,* 1989; Kitaoka *et al.,* 1994; Smallwood *et al.,* 1996; McWhirter *et al.,* 1997; Govindarajan and Overbeek, unpublished results) and FGF-7 has been detected in perioptic mesenchymal cells (Finch *et al.,* 1995). Studies from our laboratory and others have shown that inappropriate activation or inhibition of FGF signaling pathways in ocular tissues can alter developmental programs and patterning of the eye (Lovicu and Overbeek, 1998; Lovicu *et al.,* 1999; Robinson *et al.,* 1995a,b, 1998; Chow *et al.,* 1995). Overexpression of FGF-1, FGF-3, FGF-4, FGF-8, or FGF-9 in the lens leads to premature differentiation of the lens epithelial cells into fiber cells (Lovicu and Overbeek, 1998; Robinson *et al.,* 1995a,b, 1998). In some instances the fate of surrounding ocular tissues, such as the cornea and the retina, is altered as well (Lovicu and Overbeek, 1998; Lovicu *et al.,* 1999; Robinson *et al.,* 1998; S. Zhao *et al.,* submitted for publication). We examined the role of FGF-10 during ocular development by misexpression of FGF-10 in the lens under the control of the αA-crystallin promoter. Histological and molecular analyses of transgenic eyes revealed that FGF-10 expression was sufficient to induce the formation of ectopic Harderian and lacrimal gland-like structures within the cornea. During normal ocular development, endogenous expression of FGF-10 was found to be elevated in the perioptic mesenchyme near the invaginating Harderian and lacrimal gland

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primordia. Also, FGF-10 null mice were analyzed and these ocular glands were found to be absent. Based on our results we propose a three-component model for normal ocular gland morphogenesis involving (i) the establishment of a field of competence, (ii) initiation of gland outgrowth, and (iii) proliferation and differentiation required for glandular morphogenesis. The results of our studies suggest that FGF-10 is essential for initiation and proliferation.

Our transgenic studies also indicate that FGF-10 fulfills the three criteria that have been previously proposed for inducing signals: (i) expression in an appropriate spatial and temporal pattern, (ii) appropriate activity when ectopically expressed, and (iii) loss of induction upon inactivation of the inducer (Slack, 1993). Thus our results provide a novel molecular framework for ocular gland induction and morphogenesis.

MATERIALS AND METHODS

Construction of the FGF-10 Transgene

The coding region of the rat FGF-10 gene (a gift from Dr. Nobuyuki Itoh) was amplified by PCR and inserted into *Hin*dIII and *Eco*RI restriction sites between the αA-crystallin promoter (Overbeek *et al.,* 1985) and the small t intron/polyadenylation sequences of the CPV2 vector (Reneker *et al.,* 1995). A 1.9-kb fragment was released from the CPV2-FGF10 construct by *Not*I digestion, gel purified using the QiaexII gel extraction kit (Qiagen, Hilden, Germany), and used for microinjection into individual pronuclei of one-cell stage FVB/N mouse embryos at a concentration of 2 ng/μl (in 10 mM Tris–HCl, pH 7.4, and 0.1 mM EDTA). Injected embryos were transferred into pseudopregnant ICR females and allowed to develop to term. Potential FGF-10 transgenic mice were identified by isolating genomic DNA from tail biopsies and screening by PCR, using primers specific to the SV40 portion of the transgene: 5′-GTGAAGGAACCTTACTTCTGTGGTG-3′ (SV40A, Fig. 1) and 5′-GTCCTTGGGGTCTTCTACCTTTCTC-3′ (SV40B, Fig. 1). The PCR cycle conditions were as follows: denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 60 s, for 28 cycles. A final extension step of 72°C for 2 min was included.

Histological Analyses

For routine histology, embryos were obtained from timed pregnancies using FVB/N females that were mated to either homozygous or heterozygous FGF-10 transgenic males. Embryos were delivered by C-section and transgenic offspring were identified by PCR. Heads or eyes of transgenic mice were removed, fixed in 10% formalin, dehydrated, embedded in paraffin, sectioned (5 μm), and used for histological analyses, *in situ* hybridizations, and immunohistochemistry.

FGF-10 Null Fetuses

Heads of E18.5-day-old FGF-$10^{+/-}$ and FGF-$10^{-/-}$ fetuses (kindly provided by Drs. Gerald Cunha and Anne Donjacour) were fixed in 10% formalin, decalcified in 40 mM EDTA, sectioned serially, and used for histological analyses. Heterozygous FGF$10^{+/-}$ mice (Min *et al.,* 1998) were mated and the null offspring identified by their limbless phenotype (Min *et al.,* 1998). Wild-type and heterozygous littermates were identified by a PCR assay of genomic tail DNA. The primers used were as follows: wild-type oligo—5′-CAT TGT GCC TCA GCC TTT CCC-3′, knockout oligo—5′-CAC CAA AGA ACG GAG CCG GTT G-3′, shared oligo—5′-ACT CTT TGG CCT CTA TCT AG-3′. PCR was done separately for the wild-type and knockout primer pairs for 30 cycles with an annealing temperature of 60°C.

In Situ Hybridizations

To analyze expression of the FGF-10 transgene, a ^{35}S-UTP-labeled riboprobe specific to the SV40 sequences of the transgene was made (see Fig. 1). The endogenous expression of FGF-10 was analyzed by using ^{35}S-labeled riboprobes made against the rat FGF-10 cDNA. The FGF-10 antisense probe was synthesized using *Sac*II-digested rat FGF-10 cDNA and SP6 RNA polymerase (Promega) while the sense probe used *Not*I-digested FGF-10 cDNA and T7 RNA polymerase (Promega). The antisense probe for FGF-7 was synthesized using *Sac*I-digested mouse FGF-7 cDNA (kindly provided by Dr. Clive Dickson) and T7 RNA polymerase. *In situ* hybridizations on tissue sections were done using hybridization and washing conditions described previously (Robinson *et al.,* 1995a). The hybridized slides were soaked in Kodak NTB-2 emulsion, dried, and exposed for 3 days at 4°C. Following development and fixation, the slides were counterstained with hematoxylin. For whole-mount *in situ* hybridizations, X-gal-stained tissue samples were washed thrice in PBS and postfixed in 4% paraformaldehyde. Hybridizations were performed using digoxigenin-labeled sense or antisense FGF-10 probes following standard procedures (Wilkinson, 1992). The X-gal staining appears blue and the NBT/BCIP color reaction appears purple.

Immunohistochemistry

DNA replication was examined by BrdU incorporation as described previously (Lovicu and Overbeek, 1998).

Detection of β-Galactosidase Activity

Embryos were obtained from timed pregnancies of homozygous or heterozygous FGF-10 transgenic females mated to heterozygous transgenic males that express the *lacZ* gene under the control of a 5-kb promoter element of *Pax-6* (Williams *et al.,* 1998; Kammandel *et al.,* 1999). Embryos or heads of embryos were collected at appropriate time points during development and fixed for 2 h at 4°C in 0.1 M phosphate buffer containing 2% paraformaldehyde, 0.2% glutaraldehyde. Following fixation, the tissue samples were rinsed thrice at room temperature in 0.1 M phosphate buffer containing 0.01% sodium deoxycholate, 0.02% NP-40, 2 mM $MgCl_2$ and stained overnight at 4°C in an X-gal substrate solution (0.01% sodium deoxycholate, 0.02% NP-40, 2 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-gal in 0.1 M phosphate buffer).

RESULTS

Generation of Transgenic Mice

To study the effects of FGF-10 expression on ocular development, we generated transgenic mice with a rat FGF-10 cDNA linked to the lens-specific αA-crystallin

promoter (Fig. 1G) (Overbeek *et al.,* 1985). This construct was injected into pronuclear stage FVB/N embryos to generate transgenic lines. Five founder animals were identified by PCR and designated OVE1134, 1169, 1170, 1171, and 1172. In transgenic line OVE1172, there were two integration sites (confirmed by Southern blots; data not shown) that segregated in subsequent generations and were designated 1172A and 1172B.

Transgenic mice for four of the FGF-10 integration sites (OVE1172A, 1172B, 1134, and 1171) displayed ocular defects (Fig. 1; Table 1). Eyes of OVE1172A mice were microphthalmic (Figs. 1B and F) while mice from OVE1172B, 1134, and 1171 had eyes of nearly normal size (Figs. 1C, 1D, 1E, and 1F). Corneal opacities and lens cataracts were visible in mice of these transgenic families when the mice first opened their eyes at P14 (postnatal day 14). Mice from families OVE1169 and OVE1170 did not show any ocular abnormalities (data not shown).

FGF-10 Expression in Lens Fiber Cells Induces Formation of Gland-like Structures in the Cornea

Hematoxylin and eosin staining of ocular sections from adult transgenic mice revealed dramatic changes in the architecture of the cornea (Fig. 2). In nontransgenic mice, the cornea is composed of a stratified outer epithelium, an inner monolayered endothelium, and an intervening corneal stroma (Figs. 2A and A′). In transgenic families OVE1172A and OVE1172B (Figs. 2B 2B′, 2C, and 2C′), the corneal stroma was invaded by a well-organized secretory epithelium that had a gland-like morphology. The glands appeared either tubuloalveolar with a wide lumen similar to the Harderian gland (see Fig. 3E) or acinar with a narrow lumen similar to the lacrimal gland (see Fig. 3F). In transgenic family OVE1172A, the cornea displayed both lacrimal (Fig. 2B′, red arrowhead) and Harderian gland-like (Fig. 2B′, green arrowhead) morphologies, while in OVE1172B, predominantly lacrimal gland-like structures were seen. The corneal epithelia in transgenic families OVE1134 and 1171 (Figs. 2D, 2D′, 2E, and 2E′) did not invaginate but instead stratified and formed goblet cell-like structures similar to the goblet cells seen in the normal conjunctiva (Figs. 2D′ and 2E′, arrows).

In adult eyes from all transgenic families, the lenses became vacuolated and the fiber cells were disorganized (Figs. 2B, 2C, 2D, and 2E). In the family OVE1172A, the size of the lens was dramatically reduced, accounting for the microphthalmia. The neural retinas in all the transgenic families appeared to differentiate normally, though the retina in family OVE1172A was usually folded (Fig. 2B), presumably because of the smaller lens.

Levels of Transgene Expression Correlate with Severity of Ocular Phenotypes

To confirm lens-specific expression of the transgene, *in situ* hybridizations were performed on sections of E15 eyes using a riboprobe specific to the transgene (SV40 poly(A) and intronic regions) (Fig. 1). Hybridization signals were seen exclusively in the fiber cells of the lens (Figs. 2G′, 2H′, 2I′, and 2J′), suggesting that the observed changes in corneal architecture were due to a diffusible signal from the transgenic lens and not due to inappropriate expression of the transgene in the cornea. Different levels of transgene expression were seen in the different transgenic families. Transgenic line OVE1172A showed the highest level of expression (Fig. 2G′), OVE 1172B showed an intermediate level of expression (Fig. 2H′), OVE1134 and 1171 showed lower levels of expression (Figs. 2I′ and 2J′), and OVE1169 and 1170 did not show any detectable expression (data not shown).

Development of Endogenous Harderian and Lacrimal Glands in Mice

In order to begin to define the events that initiate normal development of lacrimal and Harderian glands, we examined the ontogeny of these ocular glands histologically. The Harderian gland primordium is clearly present by E15 (Fig. 3A) as an invagination of the conjunctival epithelium into the mesenchyme on the nasal side of the eye. At E18 (Fig. 3B, arrow) and P2 (Fig. 3C, arrow) the Harderian gland consists of tubules and by P7 (Fig. 3D) several distinct lobules can be seen. By P14, the secretory epithelium in the Harderian gland (Fig. 3E) has matured. The termini of the Harderian glands are tubuloalveolar with a distinct lumen (Fig. 3E).

To analyze the origin of the lacrimal gland, a reporter strain expressing the *lacZ* gene under the control of a 5-kb *Pax6* promoter element was used (Williams *et al.,* 1998; Kammandel *et al.,* 1999). This 5-kb element has been shown to be sufficient to drive expression of the *lacZ* gene in the corneal epithelium, lens epithelium, and lacrimal gland (Williams *et al.,* 1998; Kammandel *et al.,* 1999). X-gal

FIG. 1. Eyes of FGF-10 transgenic mice show corneal opacity and cataracts. Representative eyes of 3-month-old nontransgenic (NT) (A and F) and FGF-10 transgenic mice (B–F) are shown. Eyes of OVE1172A mice were microphthalmic (B, F) and eyes of mice from the families OVE1172B (C, F), 1134 (D), and 1171 (E) were of nearly normal size. Eyes of mice from these transgenic families displayed corneal opacity and lens cataracts (B, C, D, E, F). G shows a schematic representation of the FGF-10 transgene. The coding region of rat FGF-10 cDNA was amplified by PCR and inserted between the αA-crystallin promoter (αAp) and an intron and polyadenylation sequence derived from SV40 virus (Reneker *et al.,* 1995). The microinjection fragment was generated by *Not*I digestion. The SV40 sequences were used to make a riboprobe for detection of expression of the transgene. Primers (SV40A and SV40B) used for PCR are indicated. Scale bar, A–F, 2 mm.

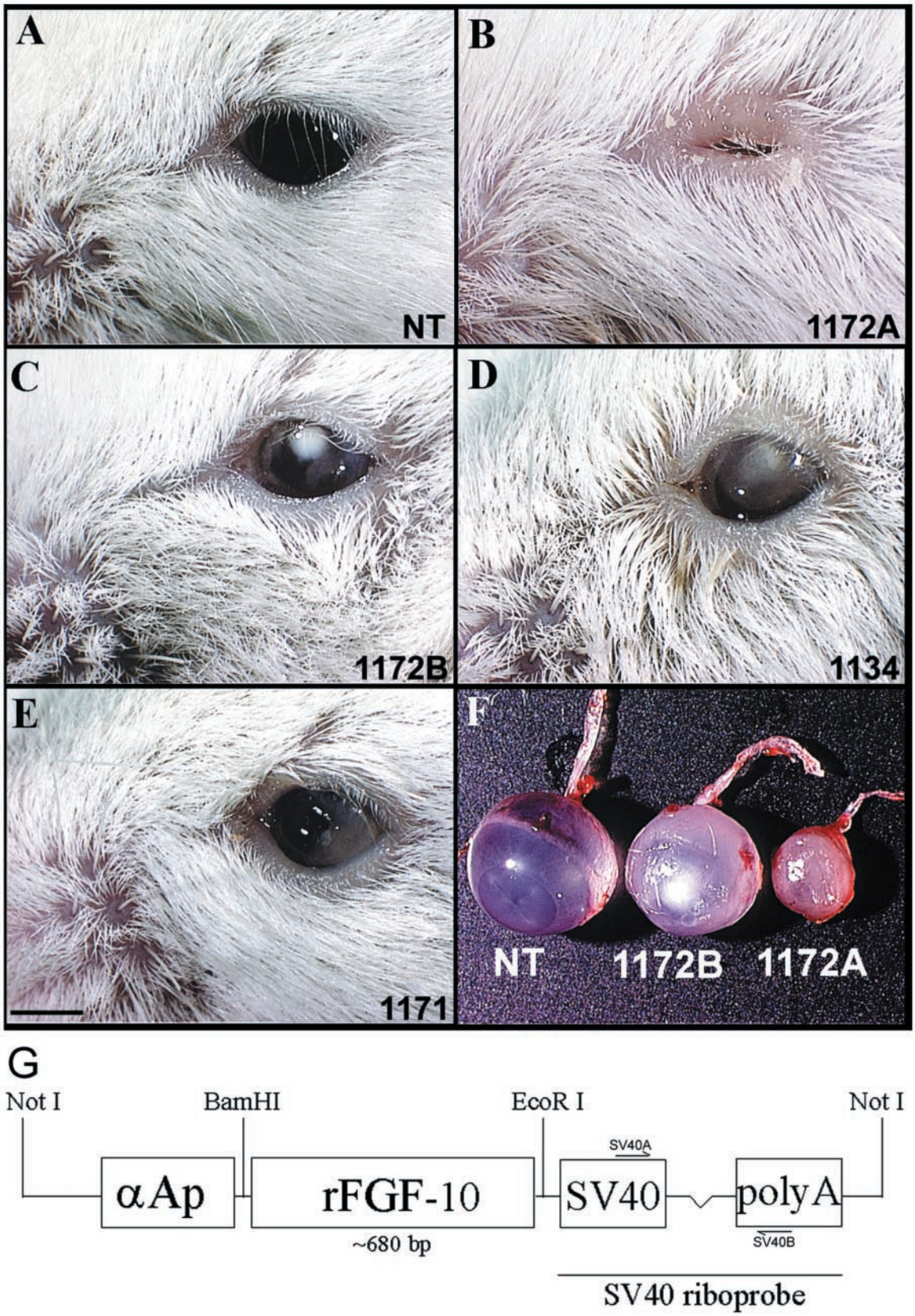


TABLE 1
Ocular Phenotypes of FGF-10 Transgenic Families

Family	E15	Newborn	Adult
OVE 1172A	Overproliferation and invagination of corneal epithelium.	Immature glands in the cornea, elongation of lens epithelial cells.	Microphthalmic eye, lens cataracts, corneal opacity, gland-like structures in the corneal epithelium.
OVE 1172B	Normal	Overproliferation of corneal epithelium.	Lens cataracts, corneal opacity, gland-like structures in the corneal epithelium.
OVE 1134	Normal	Normal	Lens cataracts, corneal opacity, goblet cell-like structures in the corneal epithelium.
OVE 1171	Normal	Normal	Lens cataracts, corneal opacity, goblet cell-like structures in the corneal epithelium.
OVE 1169	Normal	Normal	Normal
OVE 1170	Normal	Normal	Normal

staining of embryos isolated at various ages revealed that the lacrimal gland primordium arises laterally in the orbit on the temporal side and begins as an outgrowth of the epithelium near the fornix of the eyelid at E13.5 (Fig. 3G). The lacrimal duct grows and extends in a ventral-posterior direction toward the ear and branches appear by E15.5 (Figs. 3H and 3I). By E17.5 (Fig. 3J) the duct has undergone extensive branching in the exorbital portion of the lacrimal gland (Fig. 3K). Also, by E17.5, a short branch can be seen growing from the main duct close to the eye (Fig. 3J, arrow). More branches can be seen by E18.5 (Fig. 3K, arrows) and these branches grow ventrally into the eye socket to form the intraorbital portion of the lacrimal gland (Fig. 3L). The lacrimal glands are mature by P14 (Fig. 3F) when the mice open their eyes.

Endogenous Expression Pattern of FGF-10

To assess whether FGF-10 is relevant to the normal development of Harderian and lacrimal glands, expression of FGF-10 in or around the gland primordia was examined by *in situ* hybridizations. The hybridizations for FGF-10 were done using riboprobes made from the rat FGF-10 cDNA. For section hybridizations, the probes were labeled with ^{35}S-UTP and for whole-mount hybridizations, the probes were labeled with digoxigenin–UTP. Some of the whole-mount *in situ* hybridizations were performed on X-gal-stained embryos. The X-gal staining appears blue (Figs. 4A and 4A′, green arrowhead) and positive signals from the whole-mount *in situ* hybridizations appear purple. FGF-10 expression was seen in the mesenchymal cells surrounding the lacrimal glands (Figs. 4A and 4A′, black arrow). As a negative control, the FGF-10 sense riboprobe was used and no hybridization was seen in the mesenchymal cells (data not shown). Expression of FGF-10 in the limb mesenchyme was used as a positive control (data not shown).

In situ hybridizations performed on ocular sections of E14 embryos showed a low level of expression of FGF-10 in the mesenchymal cells near the Harderian gland primordium (Fig. 4B, red arrowhead). At E15, FGF-10 was expressed specifically in the mesenchyme adjacent to the invaginating gland rudiment (Figs. 4C′ and 4D′). As lens-specific expression of FGF-7 has also been shown to induce gland formation in the cornea (Lovicu *et al.*, 1999), expression of FGF-7 was examined using sections adjacent to those used for the FGF-10 *in situ* hybridizations. FGF-7, in contrast to FGF-10, was expressed at low levels in the perioptic mesenchymal cells and did not show localized expression (Figs. 4E′ and 4F′).

Ectopic Gland-like Structures in Transgenic Corneas Express Pax-6

To examine if the ectopic gland-like structures that form in the corneas of FGF-10 transgenic mice express Pax-6, mice from transgenic family OVE1172A were crossed with the Pax-6 *lacZ* transgenic mice used previously to describe the ontogeny of the lacrimal glands. Embryos that resulted from these matings were harvested at E17.5, stained with X-gal, and sectioned. *lacZ*-positive cells were seen in both the ectopic gland-like structures that formed in the cornea

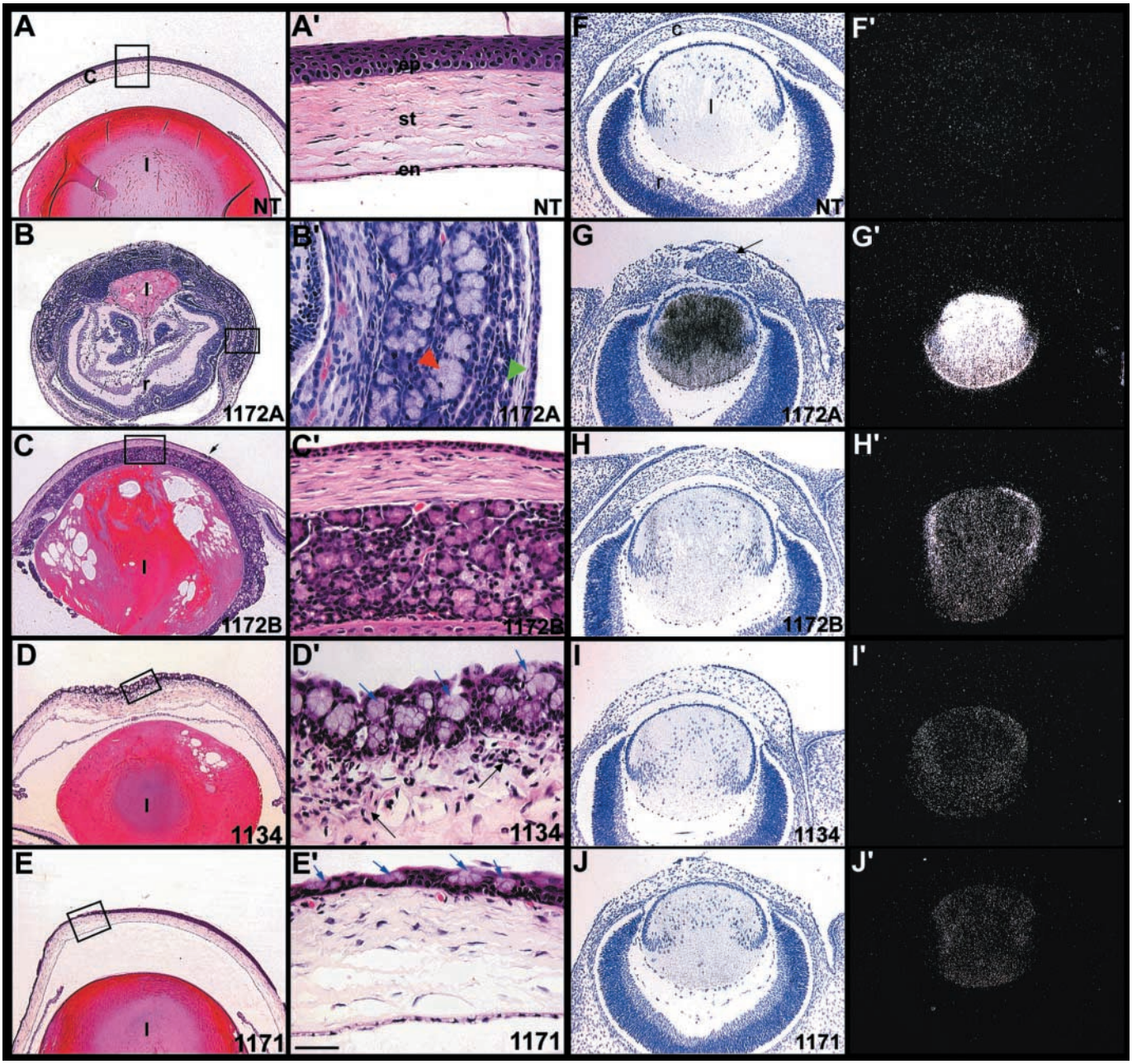
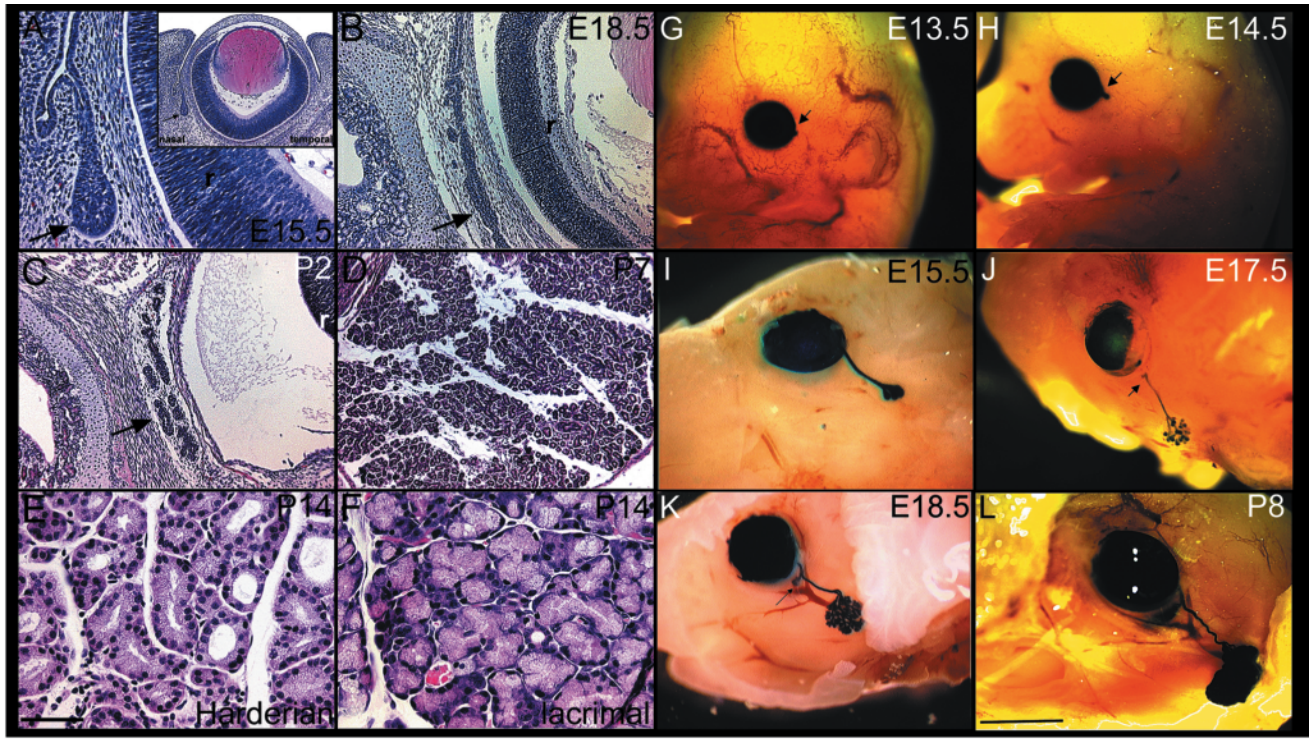


FIG. 2. Expression of FGF-10 in the lens induces ectopic gland formation in the cornea. (A–E) Eyes from adult transgenic mice were sectioned and stained with hematoxylin and eosin. (A′, B′, C′, D′, and E′) Higher magnifications of boxed portions in A, B, C, D, and E, respectively. In nontransgenic mice (A and A′), the cornea (c) is composed of a stratified epithelium (ep) and a monolayer of corneal endothelium (en) enclosing the stroma (st). In transgenic families OVE1172A (B and B′) and OVE1172B (C and C′), the corneal epithelium has invaded the underlying stroma and differentiated into glandular structures. The arrow in C marks a point of invagination of the corneal epithelium. Regions of the gland appear either tubuloalveolar with a wide lumen (B′, green arrowhead) resembling the Harderian gland (see Fig. 3E) or acinar with a narrow lumen (B′, red arrowhead) resembling the lacrimal gland (see Fig. 3F). The corneal epithelium in families OVE1134 and 1171 did not invaginate but showed distinct goblet cell-like structures (D′ and E′, blue arrows) analogous to the normal conjunctiva. The corneal stromal cells are disorganized in OVE1134 (D′, black arrows) and the cornea has become vascularized in all of the transgenic families shown. The lenses (l) of the transgenic mice were vacuolated. (F–J′). *In situ* hybridizations were done on sections of E15 eyes using a ^{35}S-labeled SV40 riboprobe. (F, G, H, I, and J) Bright-field images of ocular sections from nontransgenic (NT) and OVE1172A, 1172B, 1134, and 1171 mice, respectively. (F′, G′, H′, I′, and J′) The corresponding dark-field images. Transgene expression is lens-specific and the levels of transgene expression correlate well with the severity of the ocular phenotypes. In OVE 1172A, the invagination of the corneal epithelium can be seen by E15 (G, arrow). Abbreviations: r, retina; l, lens; c, cornea. Scale bar, A–E, 320 μm; A′–E′, 40 μm; F–J, F′–J′, 160 μm.



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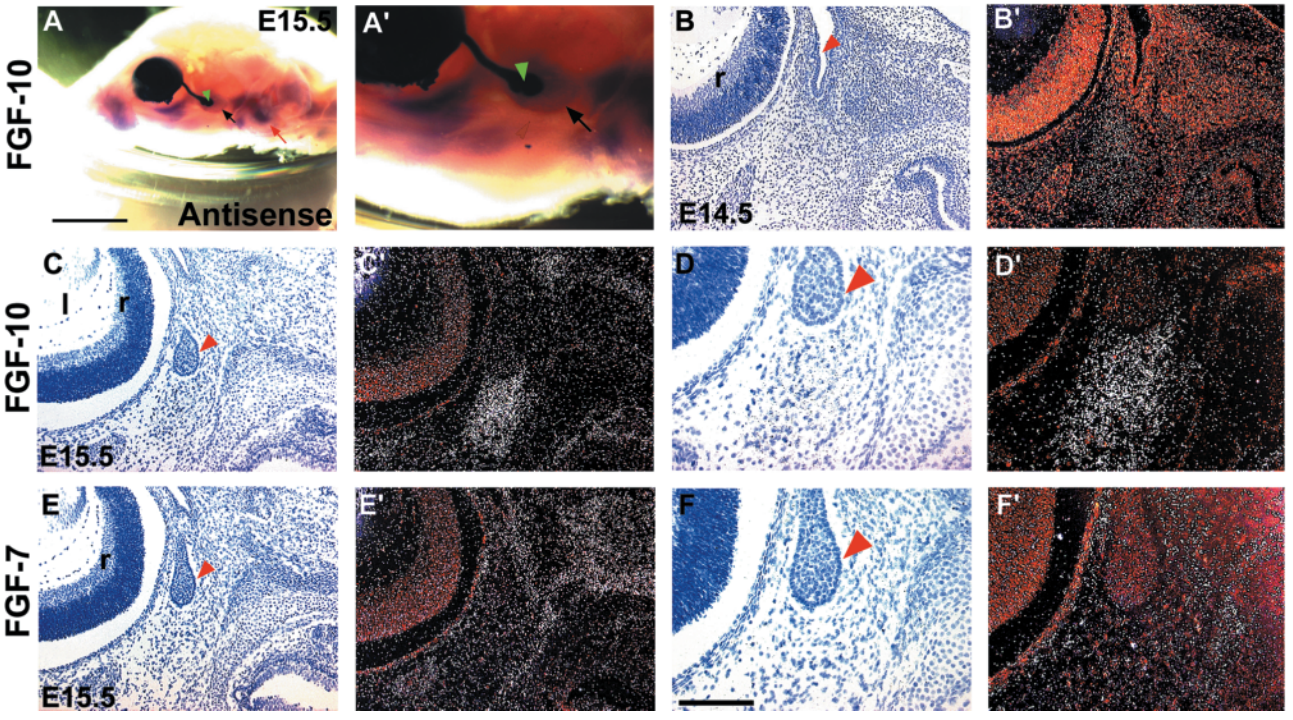

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FIG. 3. Development of the endogenous Harderian and lacrimal glands in mice. Sections of eyes at E15 (A), E18 (B), P2 (C), P7 (D), and P14 (E and F) were stained with hematoxylin and eosin. The Harderian gland primordium is distinctive by E15 (A, arrow). It begins as an invagination of the conjunctival epithelium on the nasal side of the eye (A, inset). At E18 (B, arrow) and P2 (C) the Harderian gland appears as nonluminated tubules and by P7 (D), distinct lobules can be seen. By P14, the differentiation of the glandular epithelium in both the Harderian (E) and the lacrimal glands (F) is mature and the glands are histologically distinct. The Harderian glands (E) are tubuloalveolar with distinct lumina while the lacrimal glands (F) are acinar. (G–L) X-gal staining of embryos that express *lacZ* under the

(Figs. 5B and 5B′, red arrowhead) and the endogenous lacrimal gland primordium (Figs. 5C and 5C′, red arrowhead). Cells of the ectopic gland that were closest to the lens epithelium were often not *lacZ* positive (Fig. 5B′, black arrowhead). This is consistent with the fact that cells forming the Harderian gland lose expression of Pax-6 (Figs. 5D and 5D′, black arrowhead) and adult transgenic mice from family OVE1172A exhibit both Harderian and lacrimal gland-like structures (Figs. 2B and 2B′).

FGF-10 Induces Proliferation of the Corneal Epithelium

To test if the invagination of the corneal epithelium in FGF-10 transgenic mice involved cell proliferation, sections of E15 eyes from FGF-10 transgenic family OVE1172A (Fig. 5F) were assayed for BrdU incorporation. The cells of the invaginated corneal epithelium in OVE 1172A (Fig. 5F) showed increased BrdU incorporation compared to corneal epithelial cells in nontransgenic mice (Fig. 5E). Increased BrdU incorporation can also be seen postnatally in the invaginating corneal epithelium of family OVE1172B (Fig. 5G). BrdU incorporation is seen mainly at the distal tips of the invaginating epithelium (Figs. 5F and 5G, arrowheads), similar to the proliferation pattern seen in the normal Harderian gland primordium (Fig. 5H, arrowhead). These results suggest that the inward growth of the gland primordia involves cell proliferation in response to the FGF-10 signal.

FGF-10 Null Mice Lack Ocular Glands

To test whether FGF-10 is necessary for development of the endogenous lacrimal and Harderian glands, FGF-10 null fetuses were analyzed histologically. As FGF-10 null mice show perinatal lethality, sections of E18.5 and P0 mice were analyzed. Serial sections from wild-type and FGF-$10^{-/-}$ mice were stained with hematoxylin and eosin. Intact Harderian glands were found in ocular sections of wild-type controls (Fig. 6A) but were not detectable in FGF-$10^{-/-}$mice (Fig. 6B). Similarly, lacrimal glands were present in wild-type controls (Fig. 6C) but absent in FGF-$10^{-/-}$mice (Fig. 6D). These results reveal that FGF-10 is essential for formation of both the lacrimal and the Harderian gland rudiments.

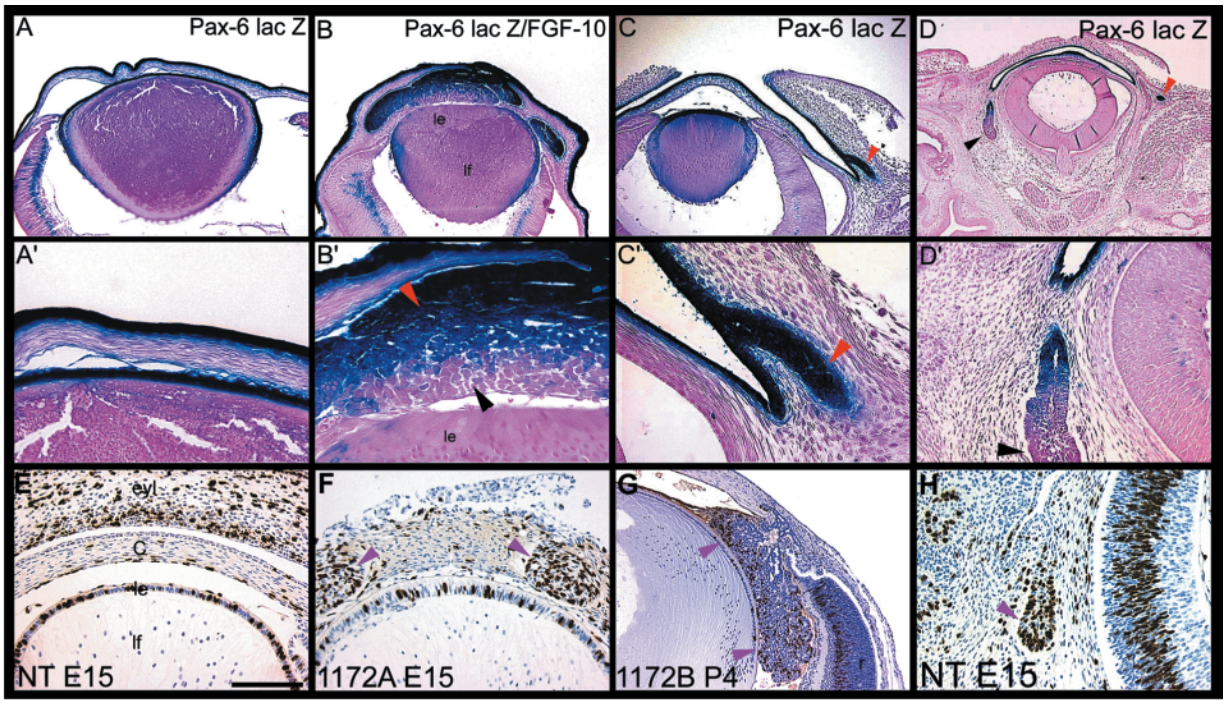
DISCUSSION

Our studies show that FGF-10 expression is both necessary and sufficient to induce lacrimal and Harderian glands. Four lines of transgenic mice that express FGF-10 in their lens fiber cells were generated and the corneal architecture in all these mice was affected. The corneal epithelia in the two families with a higher level of transgene expression differentiated into lacrimal and Harderian gland-like structures.

Inductive signals that initiate epithelial morphogenesis remain undefined for many mammalian tissues. Nonetheless, criteria for a factor to be considered an inductive signal have been proposed. First, the factor needs to be expressed at the appropriate time and location; second, misexpression of the molecule in ectopic locations should be sufficient to alter the fate of the surrounding tissues and induce the formation of the predicted structure(s); and third, in the absence of the signal, the organ that is induced by the molecule should fail to form (Slack, 1993). Our studies, in combination with the studies of Makarenkova *et al.* (2000), indicate that FGF-10 may fulfill all of these criteria for both lacrimal and Harderian glands. *In situ* hybridizations show that FGF-10 is expressed in the mesenchymal cells around the Harderian and lacrimal gland primordia at E15, when organogenesis of both these glands is initiated. Our transgenic studies show that misexpression of FGF-10 in the lens

control of a 5-kb promoter element of *Pax-6.* The lacrimal gland primordium arises on the temporal side of the eye at E13.5 (G, arrow). During the next 5 days, the duct grows in a ventral-posterior direction toward the ear and undergoes extensive branching (H, I, J, and K). At E17.5 and E18.5, secondary branches can be seen to have arisen close to the eye (J and K arrows). These will later form the intraorbital portion of the lacrimal gland. By P8 (L), the intraorbital and the exorbital portions of the lacrimal gland are distinct. Abbreviations: r, retina; l, lens. Scale bar, B, C, D, 250 μm; A, 125 μm; E, F, 62.5 μm; G, H, I, J, K, L, 2 mm.

FIG. 4. Analysis of FGF-10 and FGF-7 expression in the Harderian and lacrimal gland primordia. (A and A′) An FGF-10 whole-mount *in situ* hybridization on an E15.5 embryo. (A′) A higher magnification of A. The whole-mount *in situ* hybridization was done on an X-gal-stained embryo using a digoxigenin-labeled FGF-10 riboprobe. The X-gal stain is blue and the alkaline phosphatase staining for detection of the digoxigenin-labeled FGF-10 riboprobe is purple. Expression of FGF-10 (black arrow) can be seen in the mesenchymal cells around the budding lacrimal gland (green arrowhead) at E15.5 (A and A′). The corresponding sense control did not show any positive signal (data not shown). The red arrow points to FGF-10 expression in the inner ear. (B through H and B′ through H′) Bright- and dark-field images, respectively, of *in situ* hybridizations on tissue sections that were done using ^{35}S-labeled FGF-10 (B–D′) or FGF-7 (E and F′) riboprobes. Weak FGF-10 expression was detected in the perioptic mesenchyme at E14 (B′), but no expression was detected at the site of the initial Harderian gland bud (B, red arrowhead). Expression of FGF-10 was upregulated (C′ and D′) specifically in the mesenchymal cells adjacent to the gland primordium (C and D, red arrowhead). Low levels of FGF-7 expression were detected in the mesenchymal cells around the eye (E and E′) but expression was not localized to or upregulated in the mesenchymal cells near the elongating bud (F and F′). Panels D, D′, F, and F′ are higher magnifications of panels C, C′, E, and E′ respectively. Scale bar, A, 500 μm; A′, 200 μm; B, B′, C, C′, E, E′, 250 μm; D, D′, F, F′, 125 μm.



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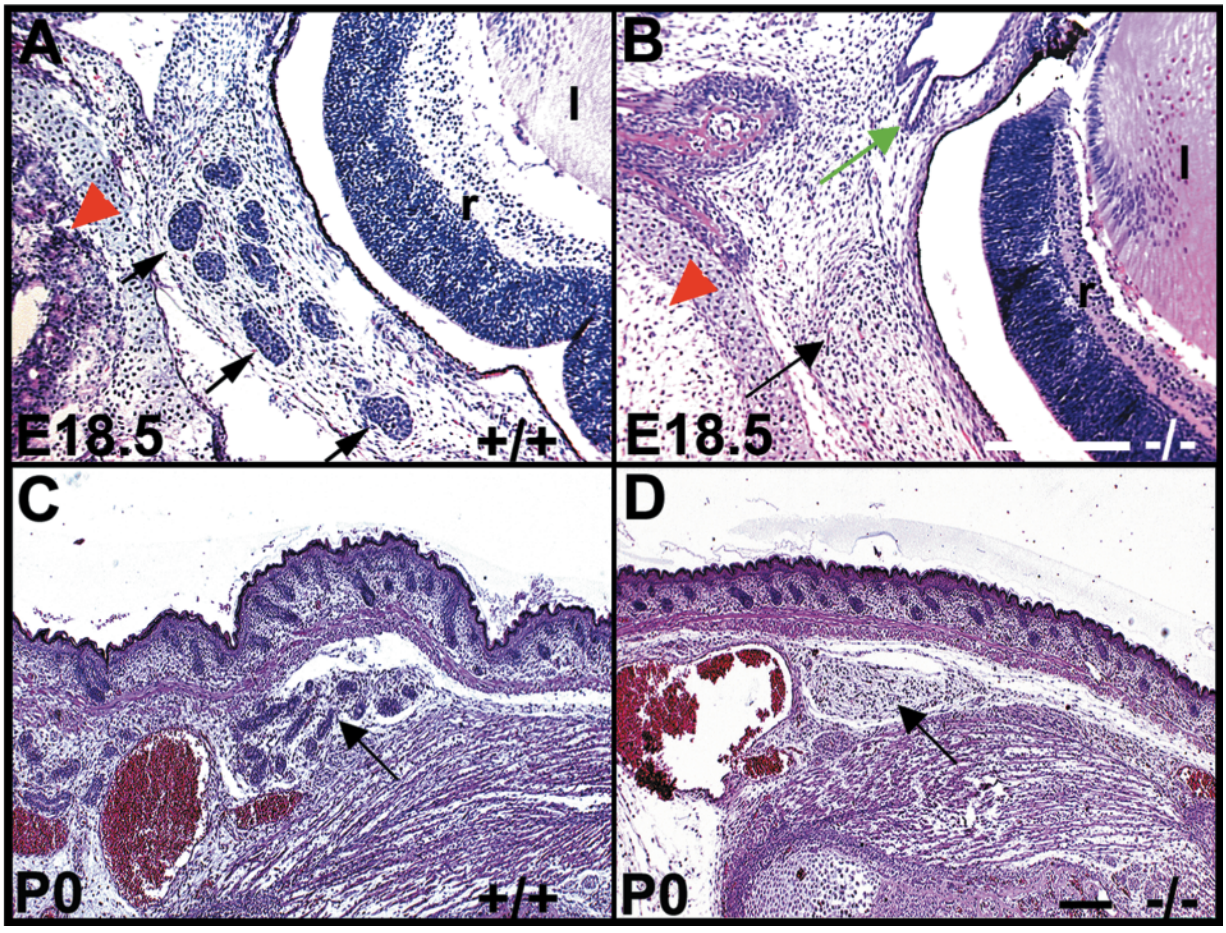

6

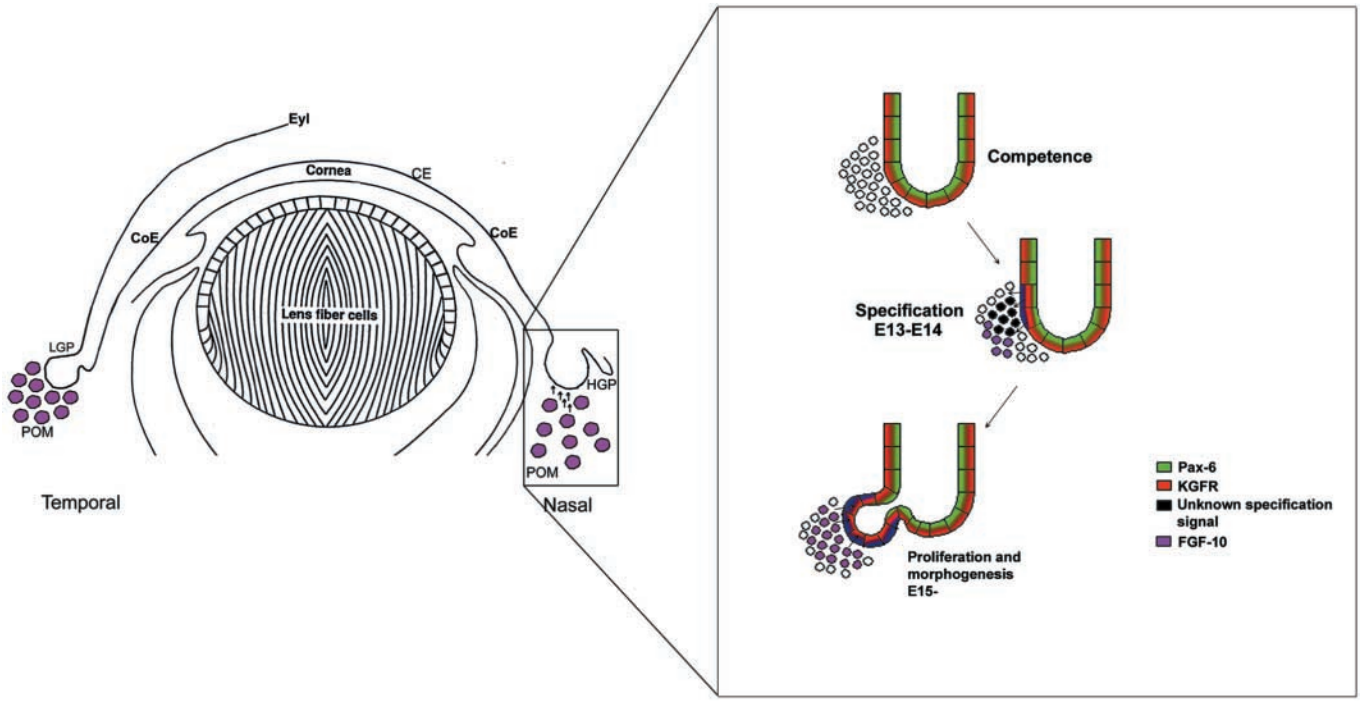

FIG. 7. A three-component model for ocular gland induction. An initial signal (e.g., Pax-6 expression) (colored green) specifies the field of competence within the surface epithelium (which expresses KGFR, colored red). Subsequently, specific clusters of cells are predicted to synthesize localized instructive signals (colored black) that work in combination with FGF-10 (colored magenta), synthesized by the perioptic mesenchymal cells (POM) to initiate and determine the locations of the preglandular buds (colored blue). These localized signals have not been confirmed, but are predicted to be different between the lacrimal and the Harderian glands. During the initiation process, the perioptic mesenchymal cells upregulate expression of FGF-10 to stimulate proliferation and morphogenesis of lacrimal (LGP) and Harderian gland primordia (HGP). Enhanced FGF-10 expression may occur in response to signals from the newly initiated preglandular buds (arrows from cells colored blue). FGF-10 binds to and activates the KGFR (FGFR2IIIb) (colored red), to stimulate the proliferation and inward growth of the glandular primordia. Ectopic expression of FGF-10 in the lens is sufficient to induce proliferation and inward growth of the corneal epithelium (CE) followed by glandular differentiation, thereby establishing the competence of the corneal epithelium and the sufficiency of FGF-10 to serve as an inductive signal. Abbreviations: Eyl, eyelid; CoE, conjunctival epithelium; CE, corneal epithelium.

FIG. 5. Pax-6 expression and proliferation of epithelial cells in the ectopic glands of FGF-10 transgenic mice. Embryos from matings of FGF-10 transgenic family OVE1172A to the Pax-6 *lacZ* transgenic family were harvested at E17.5, stained with X-gal, and sectioned. Embryos transgenic for Pax-6 *lacZ* alone showed X-gal staining in the corneal and lenticular epithelial cells (A and A′). *lacZ*-positive cells were seen in the ectopic glandular structures that form in the cornea of FGF-10 transgenic mice (B and B′, red arrowhead), similar to the endogenous lacrimal gland primordium (C, C′, and D, red arrowhead). Some cells of the ectopic gland, closest to the lens epithelium, were not *lacZ* positive (B′, black arrowhead). By analogy, the ingressing cells of the endogenous Harderian gland were found not to express *lacZ* (D and D′, black arrowhead). (E–H) Assays for BrdU incorporation. The cells of the invaginated corneal epithelium in OVE 1172A (F, magenta arrowheads) show increased BrdU incorporation compared to the cells in the nontransgenic corneal epithelium (E). (G) BrdU incorporation in a P4 eye from an OVE 1172B mouse. BrdU incorporation was elevated in the distal tips of the invaginating epithelium (magenta arrowheads), similar to the pattern seen in nontransgenic (NT) Harderian gland primordium (magenta arrowhead, H). Abbreviations: l, lens; le, lens epithelium; lf, lens fibers; c, cornea; eyl, eyelid; r, retina. Scale bar, C, D, 500 μm; A, B, G, 250 μm; E, F, 125 μm; A′–D′, H, 62.5 μm.

FIG. 6. Absence of ocular glands in FGF-10 null mice. (A though D) Sections from wild-type (A and C) and FGF-$10^{-/-}$ (B and D) mice. Serial sections were stained with hematoxylin and eosin, then analyzed. Harderian glands were seen in ocular sections of wild-type (A, black arrows) mice but were absent in FGF-$10^{-/-}$ (B) mice. The black arrow in B points to where the endogenous Harderian glands are normally present. Lacrimal glands were also present in the wild-type mice (C, black arrow) but absent in FGF-$10^{-/-}$ mice (D, black arrow). The invagination of the conjunctival epithelium in B (green arrow) marks the border of the third eyelid. Serous glands were present adjacent to the nasal epithelium in the wild-type mice (A, red arrowhead) but were not seen in FGF-$10^{-/-}$ mice (B, red arrowhead). Abbreviations: l, lens; r, retina. Scale bar, A, B, 250 μm; C, D, 100 μm.

fiber cells can alter the fate of the nearby corneal epithelial cells and can lead to the formation of ectopic Harderian and lacrimal glands. FGF-10 null mice lack both lacrimal and Harderian glands. Also, specific inhibition of an FGF-10 receptor (KGFR) in the conjunctival epithelium inhibits formation of the lacrimal gland (Makarenkova *et al.,* 2000). Taken together these results suggest that FGF-10 is both necessary and sufficient for initiation of organogenesis for the two glands.

Nature of FGF-10 Induction

Although our studies suggest that FGF-10 is both necessary and sufficient for ocular gland induction, one question remains unanswered: How does FGF-10 initiate the formation of two distinct ocular glands (as well as a variety of other organ precursors)? One possibility is that autonomous programming within the nasal and temporal conjunctival epithelial cells prespecifies the subsequent fates of their glandular derivatives. An alternative or additional possibility is that local mesenchymal cells provide ancillary signals, in addition to FGF-10, that help to specify the different glands. Based on the fact that the mesenchymal inductive signals are often instructive, we predict that the specification signals will be provided by the mesenchyme. Since FGF-10 is involved in the development of multiple tissues such as ocular glands, limbs, and lungs, we predict that FGF-10 will play a consistent role in these different tissues while tissue specific factors provide alternative, collaborative signals. These considerations suggest that the main function of FGF-10 during development may be to stimulate proliferation and/or to initiate the morphogenesis of organ rudiments.

However, the phenotypes of the transgenic mice suggest an alternative possible mechanism for the specification of ocular gland identity. In the mice, higher levels of FGF-10 expression seem to correlate with the induction of Harderian glands and lower levels with the induction of lacrimal glands. Both lacrimal and Harderian glands were seen in the transgenic family (1172A) with higher levels of FGF-10 expression, and only lacrimal glands were seen in the other transgenic family (1172B) with lower levels of expression. Also, in family 1172A, the glandular cells closest to the lens, which we predict are exposed to the highest concentration of FGF-10, were not *lacZ* positive, indicating that they are differentiating as Harderian glands. In contrast, the glandular cells farthest away from the lens were *lacZ* positive, perhaps due to stimulation by a lower concentration of FGF-10. These observations are correlations at the moment, but they hint at the possibility that different thresholds of KGFR stimulation may contribute to, or even specify, alternative glandular programs of differentiation.

FGF-10 Initiates Glandular Morphogenesis through Activation of the KGFR

FGF-10 can bind to and activate the IIIb splice versions of both FGFR2 (KGFR) and FGFR1 (Lu *et al.,* 1999). KGFR is expressed in the corneal and conjunctival epithelium and the invaginating gland rudiments by E15 (Makarenkova *et al.,* 2000). This correlates well with the expression of FGF-10 in the adjacent perioptic mesenchymal cells surrounding the gland rudiments. The IIIb isoform of FGFR1 has also been shown to be expressed in the corneal epithelium (Lovicu and Overbeek, unpublished results) and therefore the possibility that FGF-10 binds to and activates FGFR1IIIb cannot be ruled out. However, it appears that stimulation of the KGFR, and not FGFR1IIIb, is necessary and sufficient for initiation of glandular morphogenesis based on the following: (i) lens-specific expression of FGF-7, which is thought to signal through only the KGFR, also leads to ectopic gland induction in the cornea (Lovicu *et al.,* 1999); (ii) lens-specific expression of FGF-4, which can signal through FGFR1IIIb, does not lead to formation of glands in the cornea (Lovicu and Overbeek, 1998); and (iii) specific inhibition of the KGFR in explant cultures can prevent formation of lacrimal glands (Makerenkova *et al.,* 2000). Also, previous studies of ectopic expression of FGFs in the lens indicate that only those FGFs that can activate the KGFR, such as FGF-3 and FGF-7, can lead to gland induction in the cornea (Robinson *et al.,* 1998; Lovicu *et al.,* 1999).

Formation of Goblet Cells

In two of the transgenic families, FGF-10 did not induce lacrimal or Harderian glands but instead initiated the formation of goblet cell-like structures. Normally, goblet cells are found in the conjunctiva but not in the central cornea. Therefore it seems possible that FGF-10 may have a role in initiating goblet cell differentiation in the conjunctiva. Initiation of differentiation is likely to be through activation of the KGFR as FGF-7 transgenic mice (mentioned above) also have goblet cells in the central cornea (Lovicu *et al.,* 1999). As the levels of transgene expression in the FGF-10 transgenic families with goblet cells are lower than in the families with glands in their corneas, it is possible that weaker stimulation of the KGFR results in the formation of goblet cells due to activation of only a subset of the signaling pathways downstream of the KGFR. However, it should be noted that the differentiation of goblet cell-like structures occurs in conjunction with changes in the corneal stroma, so the appearance of the goblet cells may be an indirect consequence of the corneal changes.

A Three-Component Model for Ocular Gland Induction

The results from this study and the work of Makarenkova *et al.* (2000) have led us to propose a model for ocular gland induction (Fig. 7) that involves three components: (i) establishment of a field of competence, (ii) specification of cell fate by an instructive signal, and (iii) proliferation and morphogenesis. Pax-6 (Fig. 7, colored green) and KGFR (Fig. 7, colored red) appear to specify the field of competence

from which the ocular glands can arise. Presumably, signal transduction pathways downstream of the KGFR help determine the response of the surface epithelial cells to FGF-10 stimulation. Previous studies have shown that Pax-6 is a competence factor required for specification of other organ rudiments such as the lens and nasal placodes (Grindley *et al.,* 1995; Quinn *et al.,* 1996; Altmann *et al.,* 1997).

The second component would be specification signals that determine the location of the glandular rudiments and perhaps also glandular cell fates. FGF-10 is an essential part of this initiation system. It is possible that there are other signals that work in concert with FGF-10 to specify the fate of the gland rudiments. If so, these signals are likely to be different for lacrimal and Harderian glands. These signals could be either diffusible or membrane bound and could be either positive or negative factors. During the initiation process there is localized upregulation of FGF-10 (Fig. 7, colored magenta) in a subset of mesenchymal cells.

The third component is the morphogenesis of each gland. The conjunctival epithelial cells respond to FGF-10 stimulation with increased proliferation and expansion toward the FGF-10-producing cells. As the gland rudiments grow inward they initiate their differentiation programs: the Harderian glands lose expression of Pax-6 and the lacrimal glands maintain expression of Pax-6. The gland rudiments then mature and acquire their distinctive morphologies.

Conserved Morphogenetic Codes in Epithelial–Mesenchymal Interactions?

Our studies show that FGF-10 is involved in ocular gland morphogenesis. Previous studies have shown that FGF-10 is also involved in lung and limb bud development (Martin, 1998; Bellusci *et al.,* 1997). Ectopic expression of a dominant-negative KGFR in transgenic mice inhibited not only lung and limb bud morphogenesis, but also formation of the salivary glands, thyroid glands, and other organ rudiments (Celli *et al.,* 1998). Also, targeted deletion of the KGFR leads to severe limb and lung defects with abnormal development of the pituitary and salivary glands, inner ear, skin, and teeth (De Moerlooze *et al.,* 2000). Budding in lungs and limbs is initiated in roughly similar ways. In the lung, FGF-10 is expressed in the splanchnic mesenchyme surrounding the ventrolateral foregut (Bellusci *et al.,* 1997) and in the limb bud, FGF-10 is expressed in the lateral plate mesoderm to initiate limb bud outgrowth (for review, see Martin, 1998). In an analogous fashion, Branchless (an FGF ortholog in *Drosophila*) is expressed in cell clusters around the tracheal sacs and has been shown to control branching events through activation of Breathless, its cognate receptor (Metzger and Krasnow, 1999). The role of FGF-10 in initiating lacrimal and Harderian gland organogenesis is consistent with the notion that a conserved morphogenetic code may be employed to mediate epithelial–mesenchymal interactions in different developmental contexts (Hogan, 1999). Elucidation of the mechanism by which a general signal such as FGF-10 overlaps with tissue-specific signals to determine terminal cell fate remains a goal for future research.

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