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Formation of corneal endothelium is essential for anterior segment development – a transgenic mouse model of anterior segment dysgenesis

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SUMMARY

The anterior segment of the vertebrate eye is constructed by proper spatial development of cells derived from the surface ectoderm, which become corneal epithelium and lens, neuroectoderm (posterior iris and ciliary body) and cranial neural crest (corneal stroma, corneal endothelium and anterior iris). Although coordinated interactions between these different cell types are presumed to be essential for proper spatial positioning and differentiation, the requisite intercellular signals remain undefined. We have generated transgenic mice that express either transforming growth factor α (TGFα) or epidermal growth factor (EGF) in the ocular lens using the mouse αA-crystallin promoter. Expression of either growth factor alters the normal developmental fate of the innermost corneal mesenchymal cells so that these cells often fail to differentiate into corneal endothelial cells. Both sets of transgenic mice subsequently manifest multiple anterior segment defects, including attachment of the iris and lens to the cornea, a reduction in the thickness of the corneal epithelium, corneal opacity, and modest disorganization in the corneal stroma. Our data suggest that formation of a corneal endothelium during early ocular morphogenesis is required to prevent attachment of the lens and iris to the corneal stroma, therefore permitting the normal formation of the anterior segment.

Key words: TGFα, EGF, EGF Receptor, Corneal endothelial cell, Anterior segment dysgenesis, Eye, Transgenic mouse

INTRODUCTION

In mouse, the development of the anterior segment of the eye begins at about embryonic day 11 (E11) when the lens vesicle closes and separates from the surface ectoderm (Pei and Rhodin, 1970). The surface ectoderm restores its continuity and forms the corneal epithelium. Beginning at E12-E13, mesenchymal cells of neural crest origin invade the space underneath the corneal epithelium and anterior to the lens vesicle. By E15, these mesenchymal cells differentiate into corneal stromal fibroblasts and a sheet of corneal endothelial cells lining the posterior (inner) border of the cornea. During this time, a definitive anterior chamber forms, which creates a space between the lens and cornea. By E17, the ciliary body begins to differentiate from neuroectoderm at the base of the iris, and the iris begins to elongate and extend into the space between the lens and cornea. The anterior (outer) iris is composed of neural crest-derived cells, while the posterior (inner) iris is of neuroectodermal origin. Elongation of the iris over the next few days defines the extent of the anterior and posterior chambers. The embryonic origins of the anterior segment have been well defined in avian models (Johnston et al., 1979; Noden, 1986). However, very little is known about the molecular mechanisms that coordinate spatial positioning and differentiation during anterior segment formation.

Various anterior segment disorders have been reported in humans and animals (Waring et al., 1975; Cook, 1989; Williams, 1993). One of the well-studied human anterior segment disorders, Peters' anomaly, is characterized by congenital corneal opacity with underlying defects in the corneal endothelium, Descemet's membrane and corneal stroma (Cook and Sulik, 1988; Lee et al., 1989; Myles et al., 1992). Analogous defects occur in a mouse model of fetal alcohol syndrome (FAS) (Cook et al., 1987). In this mouse model, separation of the lens vesicle from the surface ectoderm is delayed or fails to occur, thereby disrupting the migration of the neural crest mesenchymal cells (Cook et al., 1987). Other anterior segment anomalies in humans include autosomal dominant iridogoniodysgenesis, Axenfeld-Rieger's anomaly (Shields et al., 1985; Mears et al., 1998), congenital endothelial dystrophy (Kenyon, 1975), sclerocornea (Kenyon, 1975), and aniridia (Churchill and Booth, 1996). Based on the fact that the defective tissues of these ocular disorders are mostly composed of neural crest-derived mesenchymal cells, it is hypothesized that anterior segment dysgenesis is due to the abnormal migration or differentiation of these cells.

We and others have previously reported that ectopic expression of TGFα in the lens can alter the migration pattern of the periocular neural crest-derived mesenchymal cells (Decsi et al., 1994; Reneker et al., 1995). In this paper, we describe

additional transgenic families that were generated using the same αA-crystallin promoter to express either TGFα or EGF in the lens. The phenotype of the transgenic mice described herein is milder than that seen in the previously reported TGFα transgenic mice (Decsi et al., 1994; Reneker et al., 1995). In these mildly affected transgenic eyes, the iris and lens are displaced anteriorly within the eye and are often attached to the posterior wall of the cornea, resulting in the loss of the anterior chamber. Developmental studies revealed that an early alteration in the transgenic eyes is the defective differentiation of the corneal endothelium. Our data suggest that interference with corneal endothelial cell differentiation during early ocular morphogenesis initiates a cascade of developmental alterations resulting in anterior segment dysgenesis.

MATERIALS AND METHODS

Construction of the TGFα and EGF minigenes

A lens-specific promoter containing 420 base pairs (bp) from the 5′ end of the mouse αA crystallin gene (Overbeek et al., 1985) was inserted into the polylinker site of plasmid pBluescript KS(–) (Stratagene). To make the human TGFα (hTGFα) minigene, plasmid MTE4RV (Rosenthal et al., 1986) was cut with *Hin*dIII and *Sal*I to release a 1.9 kilobase (kb) fragment, which contains the human TGFα coding sequences followed by human hepatitis B surface antigen 3′ polyadenylation sequences. This fragment was inserted downstream from the αA crystallin promoter to generate the hTGFα minigene (Fig. 1A). The 2.3 kb fragment for microinjection was produced by *Sac*I digestion and was purified by agarose gel electrophoresis and extraction using Geneclean (Bio101 Inc., Vista, CA). The human EGF coding sequences followed by human hepatitis B surface antigen 3′ polyadenylation sequences were excised from plasmid EHED22 (a gift from Axel Ullrich) by digestion with *Eco*RI and were similarly inserted downstream from the αA crystallin promoter to produce the hEGF minigene (Fig. 1A). A 1.3 kb *Sac*I fragment containing the hEGF minigene was isolated for microinjection.

Generation and detection of transgenic mice

Transgenic mice were produced using standard methods of pronuclear microinjection (Taketo et al., 1991). DNA at a concentration of 1 ng/μl was microinjected into the pronuclei of 1-cell stage FVB/N mouse embryos. Surviving embryos were transferred into the oviducts of pseudopregnant ICR female mice and allowed to develop to term.

The potential transgenic mice were screened by Southern hybridization (Robinson et al., 1998). Genomic DNA (10 μg from the tail biopsies) was digested with *Eco*RI or *Sal*I and size fractionated by agarose gel electrophoresis. The DNA was then transferred to a nylon membrane (Zeta-Probe, Bio-Rad) and hybridized overnight to ^{32}P-labeled probes generated from the microinjected DNA using random primers. The membranes were washed to a final stringency of 65°C, 0.1% SDS, 0.1× SSC, and then autoradiographed overnight using Kodak AKR-5 film.

Screening of the TGFα mice was also performed by polymerase chain reaction (PCR) using genomic DNA isolated from mouse tails. An upstream sense primer (Pr4) from the αA crystallin promoter (5′gcattccagctgctgacggt) and a downstream antisense primer (7488) from human TGFα (5′ttcaggagggcgctgggcttctcgt) were used to amplify a 642 bp fragment (see Fig. 1A). PCR amplifications were performed using the conditions described in Reneker et al. (1995).

Histology

Samples for histology were fixed in 10% neutral buffered formalin, paraffin embedded, cut as 5 μm sections, and stained with Hematoxylin and Eosin (H&E). For PAS staining, slides were stained with Schiff's base and counterstained with Hematoxylin. The transgenic mice were initially produced on the albino FVB/N background, but were occasionally bred to pigmented mouse lines (either C57BL/6 or C3H) to facilitate histological studies.

In situ hybridization

To examine transgenic TGFα expression in the eye, the hTGFα cDNA (Rosenthal et al., 1986) was subcloned into pBluescript (Stratagene), then used to generate ^{35}S-labeled sense and antisense riboprobes using an in vitro transcription kit (Promega). In situ hybridizations for EGF receptor (EGFR) expression were performed as described in Reneker et al. (1995). For N-cadherin in situ hybridizations, a 600 bp *Hin*dIII-*Bam*HI fragment from a mouse N-cadherin cDNA clone (gift from Chris Kintner at Salk Institute, San Diego, CA) was subcloned into pBluescript and used to generate riboprobes. In situ hybridization was carried out as previously described (Reneker et al., 1995; Robinson et al., 1995). Mouse TGFα and Pax6 cDNA clones for in situ hybridizations to detect the endogenous gene expression during eye development were obtained from David Lee at the University of North Carolina and Kathleen Mahon at Baylor College of Medicine, respectively.

RESULTS

Transgenic mice expressing TGFα or EGF in the lens

We and others have previously used the mouse αA-crystallin promoter to generate transgenic mice expressing TGFα in the lens (Decsi et al., 1994; Reneker et al., 1995). The TGFα minigene used in the current study contains the same promoter and human TGFα coding sequences but has no introns (Fig. 1A). The 3′ polyadenylation signal is derived from the human hepatitis B surface antigen gene. Two transgenic families, designated OVE44 and OVE45, are described in this report. We also constructed a human EGF minigene with the same layout as the TGFα minigene (Fig. 1A). Three founder mice were generated, but only one of them successfully passed the transgene to its offspring, and family OVE66 was established. The phenotype of the EGF mice is essentially identical to that seen in the TGFα mice (described below).

To assess the expression pattern of the TGFα transgene, in situ hybridizations were performed on embryonic eyes using a ^{35}S-labeled riboprobe specific to the human TGFα sequence (Fig. 1B-G). TGFα transcripts were detected in the elongating primary lens fiber cells as early as embryonic day 11 (E11). Transgene expression remained lens specific and localized to the lens fiber cells in E13 and E15 transgenic eyes (Fig. 1F,G). In the E15 transgenic lens (Fig. 1G), the TGFα mRNA was most abundant in the posterior region of the fiber cells. The human TGFα probe did not detect any endogenous mouse TGFα mRNA in the E11 (Fig. 1B,C), E13 and E15 eyes. In situ hybridizations using a mouse TGFα cDNA probe were also performed on E13, E14 and E15 eyes. A low level of TGFα mRNA was found in the corneal and conjunctival epithelia in the E14 and E15 eyes (data not shown).

Phenotypes and ocular histology of the TGFα and EGF transgenic mice

The TGFα and EGF transgenic founder mice displayed opacification of the cornea (Fig. 2A-D), a characteristic phenotype that was visible upon opening of the eyelids at day

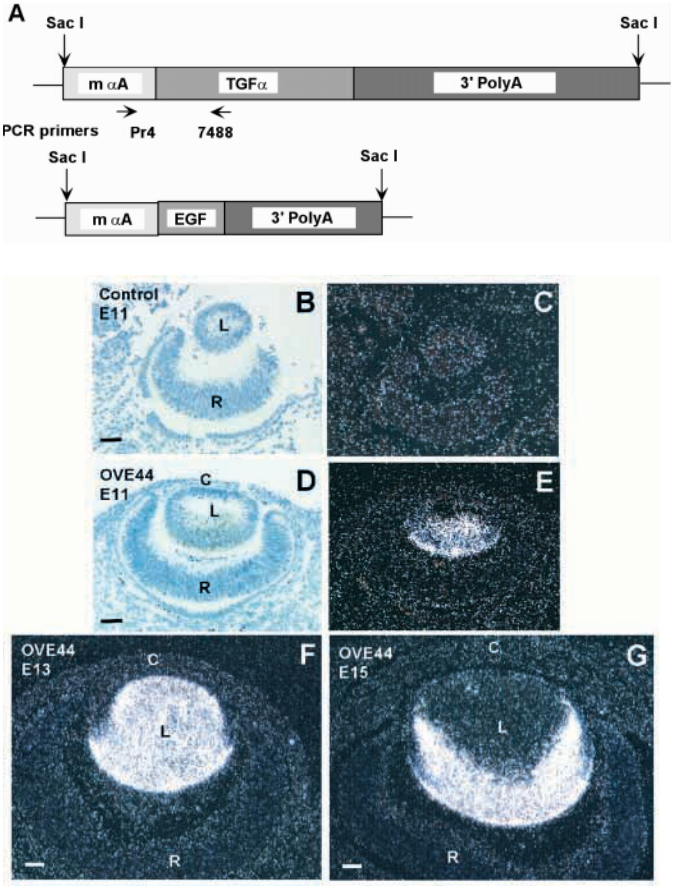

Fig. 1. Transgenic constructs and lens-specific expression of the TGFα transgene. (A) Diagrams of the TGFα and EGF minigenes, showing the mouse αA crystallin promoter (mαA), human TGFα and EGF coding sequences and human hepatitis B surface antigen polyadenylation sequences (3′ poly(A)). PCR primers (Pr4 and 7488) for screening the TGFα transgenic mice are indicated by the arrows. (B-G) In situ hybridizations using a ^{35}S-labeled TGFα riboprobe. (B) Bright-field view of an E11 non-transgenic eye. (C) Dark-field view of the same section, showing no detectable TGFα signal in the non-transgenic eye. (D) Bright-field view of an E11 transgenic eye from family OVE44. (E-G) Dark-field views of E11, E13 and E15 transgenic eyes, showing that transgene expression is localized to the elongating fiber cells of the lens. In all panels, the following abbreviations are used: C, cornea; L, lens; R, retina. Scale bar, 50 μm.

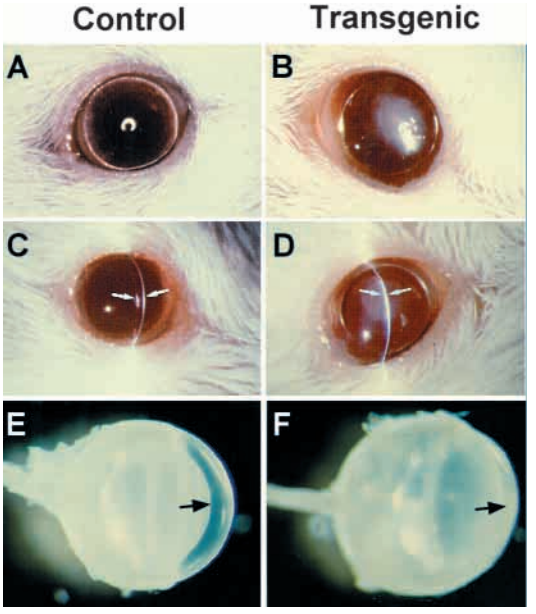

Fig. 2. External appearance and altered architecture of the TGFα transgenic eyes. (A,C,E) Non-transgenic eyes and (B,D,F) transgenic eyes. (A-D) External appearance of the eyes. The corneal opacity in the transgenic eye is readily apparent (B,D). Under slit lamp illumination, the space between the cornea and the lens (white arrows), which represents the anterior chamber, can be seen in the control eye (C). No anterior chamber is apparent in the transgenic eye (D). (E,F) Dissected eyes. The black arrows indicate the anterior surface of the lens. The transgenic eye (F) lacks an anterior chamber and has cloudiness in the cornea.

14 after birth (P14). Slit lamp biomicrography indicated that no anterior chamber was present in the transgenic eyes (Fig. 2D); this was confirmed when the eyes were dissected and observed under low magnification (Fig. 2E,F). Unilateral or bilateral cataracts occurred in about 30% of animals by 3-4 months of age (data not shown). No neovascularization of the cornea was observed in the transgenic eyes.

Ocular histology showed that defects were present in the anterior segments of TGFα and EGF transgenic eyes (Fig. 3). The iris was present but adherent to the posterior surface of the cornea in transgenic eyes (Fig. 3D-H), with complete obliteration of the anterior chamber and elimination of the iridocorneal angle. The ciliary body, normally a convoluted and lobulated structure, was consistently rudimentary in the transgenic eyes (Fig. 3D,G). The lens displaced anteriorly and was often adjacent to or attached to the posterior surface of the cornea in the TGFα transgenic eyes (Fig. 3D). The corneal epithelium, normally 5-7 cells in thickness, was reduced to two cell layers in most transgenic corneas (Fig. 3E,F,H). In addition, the corneal endothelium was either partially formed (indicated by the arrows in Fig. 3E) or completely missing (Fig. 3F,H) in the transgenic mice. After PAS staining for identification of basement membranes, neither Bowman's membrane (made by the corneal epithelium) nor Descemet's membrane (made by the corneal endothelium) were detected in the TGFα transgenic eyes (Fig. 3, compare F and C).

Defects associated with malformation of the corneal endothelium

In transgenic family OVE45, localized regions of intact corneal endothelium were often observed (Fig. 4A, indicated by the arrows). Adhesion of the lens and iris to the cornea occurred specifically in the areas where corneal endothelial cells were not present (Fig. 4A). In the region where corneal endothelial cells had formed (shown in Fig. 4B), the lens and iris did not attach to the cornea and an anterior space between the lens and cornea was formed. Defects in the corneal epithelium and stroma were also found to correlate spatially with the absence of the corneal endothelium (Fig. 5). At P14, the time of eyelid opening, the corneal epithelium in the non-transgenic eye is 5-7 cells in thickness, with 4-6 layers of stratified squamous suprabasal cells and a single layer of basal cells adherent to a basement membrane (Bowman's membrane; Fig. 5A). For the transgenic eyes from family OVE45, in the areas where corneal

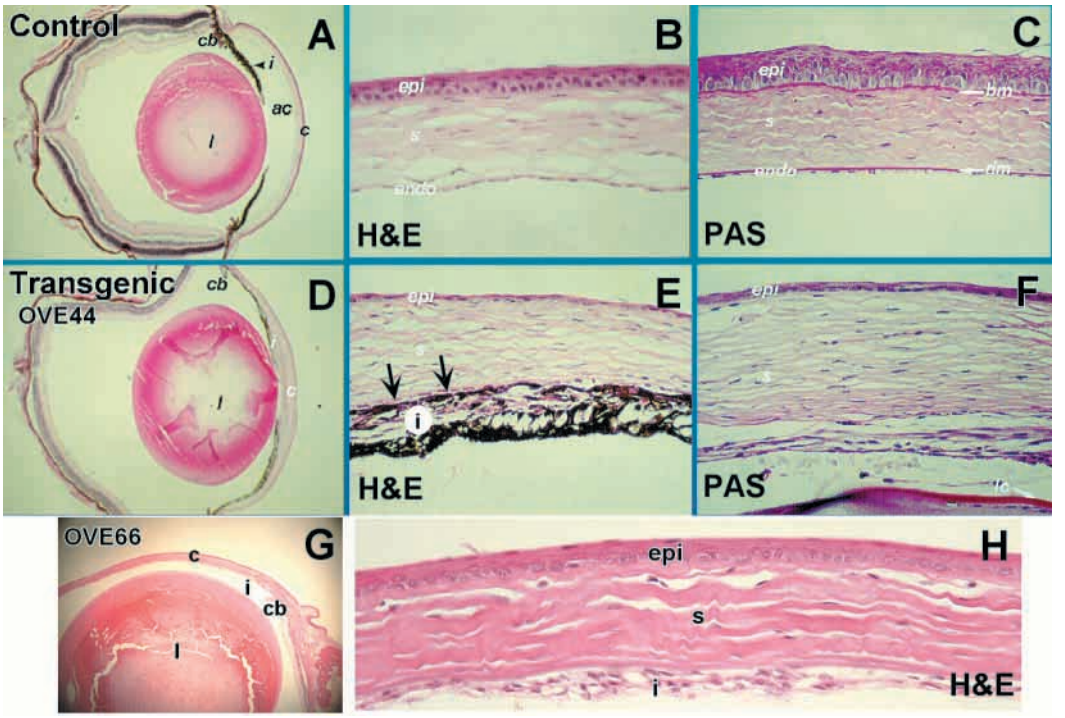

Fig. 3. Ocular histology. (A-C) Non-transgenic eyes; (D-F) TGFα transgenic eyes from OVE44; (G,H) an EGF (OVE66) transgenic eye. (A,D,G) Adult eye histology (H&E stain, magnification, ×40). Defects in the anterior structures of the transgenic eyes (D,G) include reduced curvature of the cornea (c), attachment of the iris (i) to the posterior surface of the cornea with obliteration of the anterior chamber (ac) and elimination of the iridocorneal angle. (B,C,E,F,H) Higher magnification views of the corneas stained with H&E (B,E,H) or PAS (C,F) (magnification , ×400). The transgenic corneas (E,F,H) show a thinning of the corneal epithelium (epi), partial loss (E) or absence (F,H) of the conreal endothelium (endo), and adherence of the iris to the cornea. Neither Bowman's membrane (bm) nor Descemet's membrane (dm) were detected in the transgenic cornea (F). Note: the control eye (A-C) is from an FVB by C57BL/6 mating and the TGFα transgenic eye (D-F) is an FVB by C3H mating. Abbreviations are: ac, anterior chamber; bm, Bowman's membrane; c, cornea; cb, ciliary body; dm, Descemet's membrane; endo, corneal endothelium; epi, corneal epithelium; i, iris; l, lens, s, stroma.

endothelial cells were absent, the corneal epithelium was reduced to a bilayer of cells with no apparent basement membrane (Fig. 5B). The corneal stroma was often enlarged to contain more cells. In adjacent areas of the cornea, where corneal endothelial cells were present, the epithelium was thicker and contained more cell layers, but still lacked a basement membrane structure (Fig. 5C). The structure of the corneal stroma also appeared to be more normal. Our data suggest that proper differentiation and maturation of corneal epithelial and stromal cell may be dependent on the presence of an underlying corneal endothelium.

N-cadherin expression in corneal endothelial cells

In situ hybridizations using a probe specific for the mouse N-cadherin gene revealed that normal corneal endothelial cells express the N-cadherin gene at high levels (Fig. 6). N-cadherin mRNA was not detected in the corneal stroma or in the corneal epithelium (Fig. 6). Therefore, we have used N-cadherin as a molecular marker to identify corneal endothelial cells in the transgenic eyes. In P7 transgenic corneas, the N-cadherin expression pattern confirmed that corneal endothelial cells were present in the area where the lens and iris were not attached to the cornea (indicated by the arrows in Fig. 6B,D). In other regions of the transgenic cornea, N-cadherin expression was either absent or present at a very low level (Fig. 6D). These in situ hybridization results are consistent with the histological results (Fig. 4). We did not observe N-cadherin-positive cells accumulating at the peripheral margin of the cornea, suggesting that a blockage of endothelial cell migration is not the primary cause of the corneal defects in the transgenic eyes.

Histological analysis of the anterior segment development

To further characterize the developmental changes in the TGFα

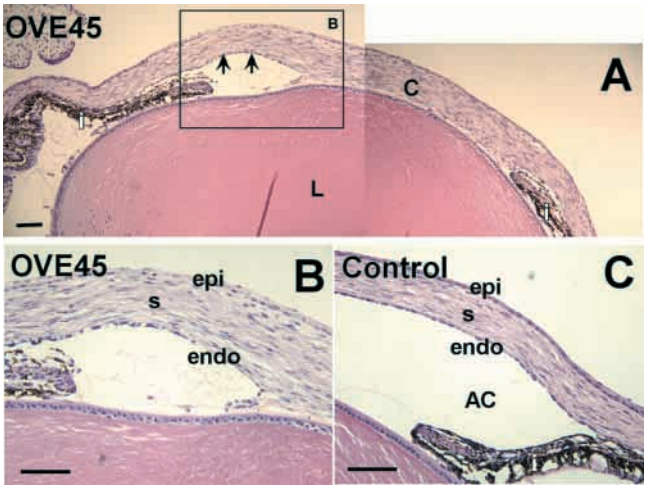

Fig. 4. Localized adhesion of the lens and iris to the cornea. (A,B) In a P6 transgenic eye from OVE45 (H&E staining), adhesion of the iris and lens to the cornea can be seen in the regions where corneal endothelial cells are absent. Arrows indicate the area where corneal endothelial cells can be identified. The boxed region in (A) is shown at a higher magnification in (B). (C) Anterior region of a non-transgenic eye. Abbreviations: AC, anterior chamber; C, cornea; i, iris; L, lens; endo, corneal endothelium; epi, corneal epithelium; s, corneal stroma. Scale bar, 50 μm.

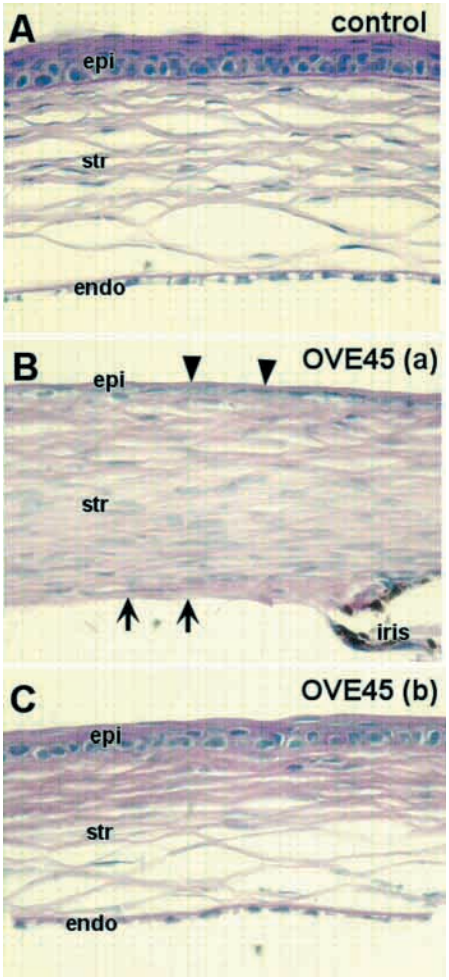

Fig. 5. Defects in corneal epithelium in P14 TGFα transgenic mice (PAS staining, magnification, ×400). (A) Cornea of a P14 non-transgenic eye. The corneal epithelium (epi) is 5-7 cell layers in thickness with flattened squamous apical cells and cuboidal basal cells. Bowman's membrane can be identified by PAS staining. The corneal endothelial cells (endo) have a distinctive appearance and synthesize a basement membrane, Descemet's membrane. (B,C) Cornea of a P14 TGFα transgenic eye from OVE45. (B) Only one or two layers of corneal epithelial cells are present (arrowheads) in areas where corneal endothelial cells are not formed (arrows). Neither Bowman's membrane nor Descemet's membrane is apparent. The corneal stroma in this region is slightly thicker and contains more cells. In regions where corneal endothelial cells have formed (C), stratification of the corneal epithelial cells is apparent, and the corneal stroma is close to normal thickness. Abbreviations: endo, corneal endothelium; epi, corneal epithelium; str, corneal stroma.

transgenic eyes, eyes at E12, E14, E16 and P4 were analyzed (Fig. 7). At E12, there were no significant changes between the control and the transgenic eyes (Fig. 7A,B). The transgenic lens vesicle separated successfully from the surface ectoderm. By E14, mesenchymal cells had migrated in between the surface ectoderm and the lens, to give rise to the presumptive corneal stroma in both control and transgenic eyes (Fig. 7C,D). Formation of the anterior chamber should initiate at this stage in the regions indicated by arrows (Fig. 7C,D). In the E14 transgenic eye, there are excess cells in this region (Fig. 7D). By E16, the anterior chamber had formed in the control eyes

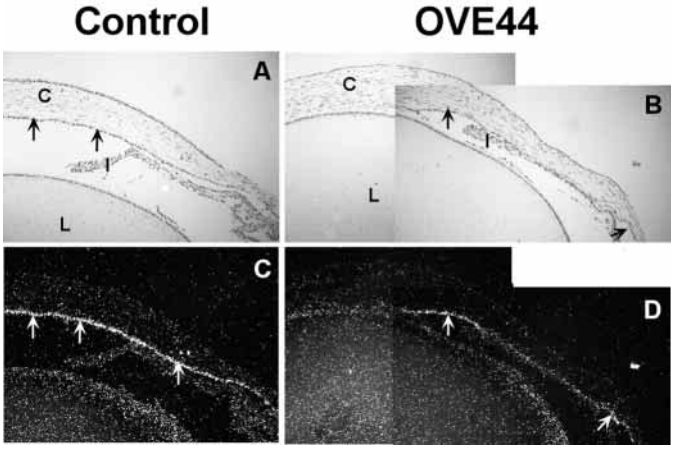

Fig. 6. N-cadherin expression in corneal endothelial cells. N-cadherin mRNA was detected by in situ hybridization with a ^{35}S-labeled riboprobe. Bright-field (A, B) and dark-field (C, D) images of eyes are shown (magnification, ×200). (A,C) Normal corneal endothelial cells (arrows) express high levels of N-cadherin. (B,D) In the transgenic eyes, high levels of N-cadherin mRNA are detected only in the areas where corneal endothelial cells are apparent (indicated by the arrows). Abbreviations: C, cornea; L, lens, I, iris.

and an iridopupilary membrane separated the lens and the cornea (Fig. 7E). Corneal endothelial cells were present at this stage and expressed N-cadherin (Fig. 8A). In contrast, no separation between the lens and the corneal stroma was observed in the transgenic eye (Fig. 7F). The transgenic corneas also did not show detectable N-cadherin expression at this stage (Fig. 8B), indicating a nearly complete inhibition of the formation of corneal endothelium. The transgenic corneal stroma was thickened and hypercellular (Fig. 7F).

In newborn non-transgenic eyes, a high-level of N-cadherin expression was found in the corneal endothelium (Fig. 8C). In contrast, N-cadherin mRNA was either completely absent or only found in some small areas in the transgenic eyes (Fig. 8D, indicated by the arrow). In the area where N-cadherin mRNA was detected, the cornea was separated from the lens and iris.

During postnatal development in non-transgenic eyes, the cells of the iris stay separated from the cells of the cornea and a definitive iridocorneal angle is formed (Fig. 7G). In contrast, in the transgenic eyes, the elongating iris remained attached to the cornea (Fig. 7H).

Expression of EGF receptor (EGFR) in developing eyes

It is known that TGFα and EGF exert their biological activities by activating a cell surface tyrosine kinase receptor, the EGF receptor (EGFR) (Adamson, 1990; Partanen, 1990). Therefore, we examined the expression pattern of EGFR in developing mouse eyes by in situ hybridizations (Fig. 9). In E13 and E14 eyes, EGFR was highly expressed in the migrating mesenchymal cells (arrows in Fig. 9A,B), which give rise to the corneal stroma and endothelium, as well as the periocular tissues such as the choroid and sclera. This result supports the notion that ectopic expression of TGFα in the lens can stimulate these cells and cause extra mesenchymal cells to migrate into the anterior region of the transgenic eyes (Fig. 7D,F). EGFR expression in non-transgenic corneal mesenchymal cells starts to decrease at E15 (Fig. 9E,F), the

stage at which corneal endothelial cells begin to differentiate and express N-cadherin (Fig. 8A). Expression slightly above background was detected in the corneal stroma and endothelium after E16 (Fig. 9G-J). This result suggests that differentiation of corneal endothelial cells may require downregulation of EGFR activity in these mesenchymal cells. Overstimulation of the EGFR may result in a delay or failure in the formation of corneal endothelial cells.

EGFR expression patterns were compared between the non-transgenic and transgenic eyes at E12 (data not shown) and birth (Fig. 9I,J). EGFR expression was detected specifically in the corneal epithelium in both non-transgenic (Fig. 9I) and transgenic (Fig. 9J) eyes. The transgenic eyes did not show significant upregulation or downregulation of EGFR transcription. EGFR expression was not detected in the lens at any of the stages examined (Fig. 9).

DISCUSSION

We and others have previously reported that overexpression of TGFα in the lens can alter the normal developmental fate of the mesenchymal cells that migrate to the eye during ocular morphogenesis (Decsi et al., 1994; Reneker et al., 1995). High levels of TGFα expression in the lens induce periocular mesenchymal cells to migrate into the intraocular region around the lens, resulting in microphthalmia and retinal dysplasia (Reneker et al., 1995). In this paper, we describe transgenic mice expressing either TGFα or EGF in the lens but developing a milder phenotype. The milder phenotypes are likely due to lower levels of transgene expression since the TGFα and EGF constructs reported here did not include any intronic sequences. In these mildly affected TGFα and EGF transgenic eyes, the corneal endothelial cells fail to differentiate properly and the anterior chamber does not form. The absence of a corneal endothelium is associated with, and perhaps causes directly, secondary defects in anterior segment structures, including adhesions of the iris and the lens to the cornea, stromal edema and abnormal differentiation of the corneal epithelium.

During early eye morphogenesis, after the lens vesicle is detached from the surface ectoderm, neural crest mesenchymal cells migrate in from the margin of the optic cup to form anterior segment components (Pei and Rhodin, 1970; Hay, 1979; Johnston et al., 1979). In the chick, the mesenchymal cells from a first wave of migration differentiate to form corneal endothelium (Dublin, 1970; Bard et al., 1975). A second wave of cells gives rise to the corneal stromal fibroblasts, and cells of a third wave contribute to the stromal portion of the iris (Bard and Hay, 1975; Johnston et al., 1979). In the mouse, there appears to be a single influx of mesenchymal cells, which differentiate into both the corneal endothelium and the stromal fibroblasts (Dublin, 1970; Pei and Rhodin, 1970). It is still unclear whether the corneal endothelial cells in the mouse eye are specified prior to their arrival in the eye, or whether they are simply induced to become endothelium based on their location at the inner surface of the cornea.

The onset of N-cadherin expression in the corneal endothelium at around E15 suggests that differentiation of

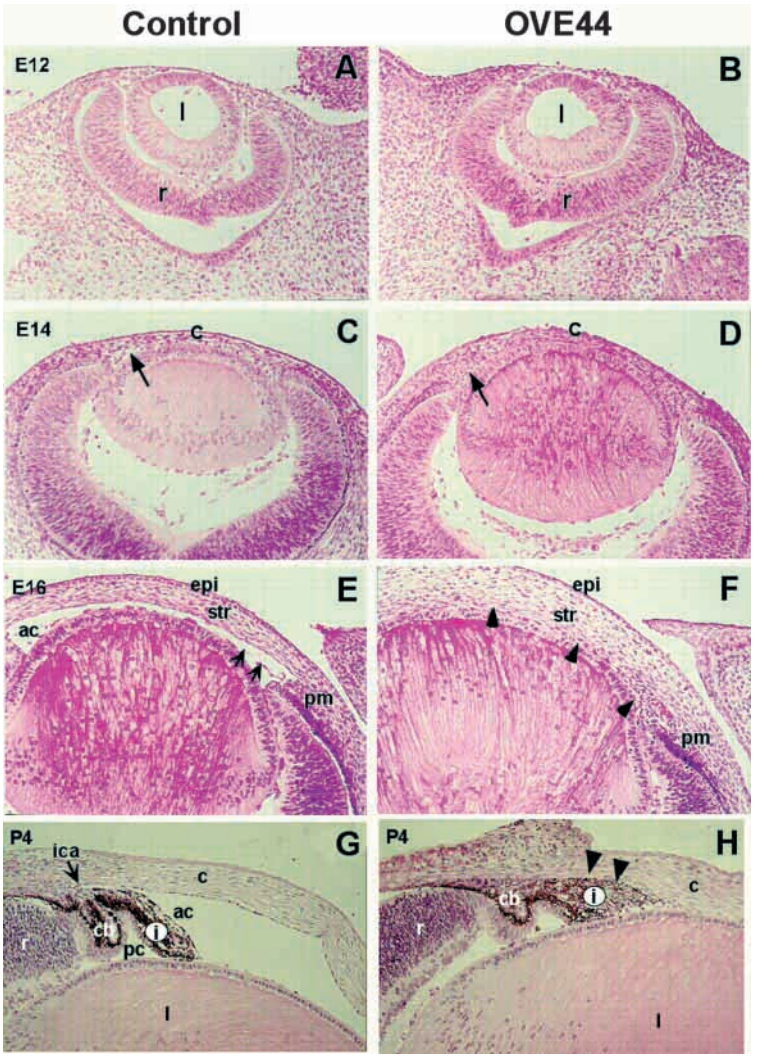

Fig. 7. Development of the anterior segment defects. Left panels are control and right panels are TGFα transgenic eyes from OVE44 (H&E staining, magnification, ×200). At embryonic day 12 (E12), there are no apparent differences between the control (A) and transgenic (B) eyes. By E14, mesenchymal cells have migrated in between the surface ectoderm and the lens vesicle, to give rise to the primitive corneal stroma (C). In the transgenic eye (D), extra cells accumulate in the space where the anterior chamber normally develops (indicated by the arrows in C and D). At E16, the anterior chamber (ac) has formed in the control eye (E). An iridopupillary membrane (arrows) separates the lens and cornea. In the transgenic eye (F), no anterior chamber is formed (arrowheads). The corneal stroma (str) is thicker, somewhat disorganized, and hypercellular in the transgenic eye at E16. In the P4 control eye (G), a layer of corneal endothelial cells is present and the iris is separated from the cornea, such that a definitive iridocorneal angle (ica) is formed. The iris separates the anterior chamber (ac) from the posterior chamber (pc). In the transgenic eye at P4 (H), a definitive corneal endothelial layer is missing and the iris elongates with partial or complete adherence to the cornea (arrowheads). Abbreviations are: ac, anterior chamber; c; cornea; cb, ciliary body; epi, corneal epithelium; i, iris; ica, iridocorneal angle; l, lens; pc, posterior chamber; pm, periocular mesenchyme; r, retina, str, corneal stroma.

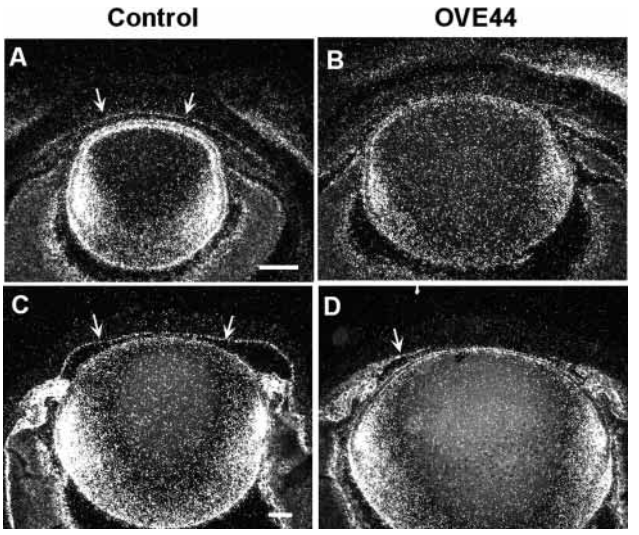

Fig. 8. Expression of N-cadherin in developing eyes. (A,C) N-cadherin expression in E15 (A) and newborn (C) non-transgenic eyes. Arrows indicate the expression of N-cadherin in the developing corneal endothelial cells. N-cadherin is also expressed in the lens and ganglion cells of the retina. (B,D) N-cadherin expression in E15 (B) and newborn (D) TGFα transgenic eyes. N-cadherin mRNA was usually not detected in the transgenic corneas. A small area at the peripheral region of the newborn cornea (arrow in D) shows the presence of N-cadherin mRNA. Eyes in C and D are pigmented. Scale bar, 100 μm.

these cells occurs after arrival of the mesenchymal cells in the eye. N-cadherin belongs to a family of Ca^{2+}-dependent cell adhesion proteins (Takeichi, 1991; Kemler, 1992). Binding between cadherin molecules on adjacent cells leads to the formation of intercellular junctions and the establishment of strong cell-cell adhesion. Cadherins are thought to play important roles in cell fate determination and tissue morphogenesis by linking cellular adhesion to intracellular signaling events (Gumbiner, 1996; Vleminckx and Kemler, 1999). It is possible that expression of N-cadherin in the corneal mesenchymal cells at around E15 initially creates the architectural and structural environment necessary to establish the corneal endothelial layer. Continuous expression of N-cadherin in the corneal endothelium may contribute to the assembly of a tightly adherent cell sheet so that a selective permeability barrier is formed at the posterior surface of the cornea. One of the normal functions of the corneal endothelium is to regulate hydration of the corneal stroma. An additional function may be to establish a 'non-sticky' surface on the inner surface of the cornea. Without this protective cell layer, the iris and the lens adhere to the exposed extracellular matrix of the corneal stroma.

At present, we do not have a straightforward molecular explanation for how TGFα and EGF inhibit differentiation and N-cadherin expression in the corneal endothelium. Our data indicate that mesenchymal cells still migrate into the corneal region but fail to differentiate into mature corneal endothelial cells in the transgenic eyes. One possible explanation is that TGFα and EGF directly inhibit endothelial cell differentiation, and that the other defects in the anterior segment are a consequence of the absence of the endothelium. The fact that TGFα and EGF transgenic mice have similar phenotypes

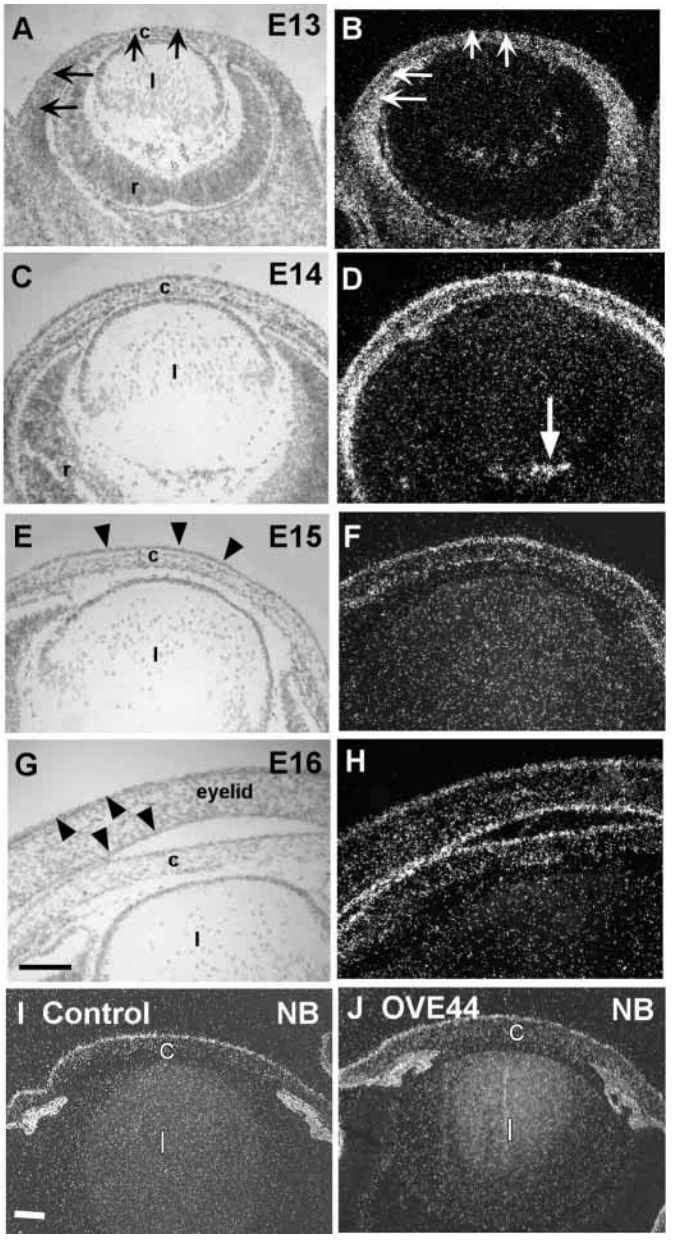

Fig. 9. Expression of EGFR during eye development in non-transgenic (A-I) and OVE44 transgenic (J) eyes. (A,C,E, G) Bright-field and (B,D,F,H-J) dark-field views of ^{35}S in situ hybridizations. At E13 (A,B) and E14 (C,D), EGFR is highly expressed in the migrating mesenchymal cells (arrows in A and B), which form the corneal stroma and the perioptic mesenchyme. The expression of EGFR in the corneal stroma and endothelium is downregulated after E15 (E-J). EGFR mRNA is also found in the surface ectodermal tissues (arrowheads), which include corneal epithelium, and inner and outer eyelid epithelium, but not in the lens. The expression pattern of EGFR in the corneal epithelium is similar between the non-transgenic (I) and transgenic (J) newborn eyes. EGFR mRNA is also detected in some cells of the hyaloid vasculature system (big arrow in D). The signal in the iris in I and J is due to pigmented cells, and the haze in the lens is an artifact. Scale bar, 100 μm.

suggests that the corneal defects result from abnormal activation of the EGFR during development. It is clear that the corneal mesenchymal cells express EGFR at E13-E14 (Fig. 9),

and can presumably be stimulated through this receptor. The endogenous function of this receptor for cell fate determination in the cornea remains unresolved. TGFα knock-out mice do have multiple eye defects including corneal inflammation and anterior segment dysgenesis (Luetteke et al., 1993; Mann et al., 1993). However, by in situ hybridizations, we were unable to detect significant endogenous TGFα or EGF expression in the embryonic eye (data not shown). Since endogenous TGFα and EGF are not expressed at significant levels in the eye, the lens-specific expression of TGFα or EGF in the transgenic mice is expected to cause inappropriate stimulation of the EGFR in the cells of the E12-E14 corneal mesenchyme. This stimulation may be sufficient to inhibit the endogenous system for specification and differentiation of endothelial cells, perhaps by causing the mesenchymal cells to lose their competence to respond to other inductive signals. Other possible explanations for the defects of corneal endothelial formation include the following: (1) abnormal stimulation of the EGFR in the corneal mesenchymal cells may prevent the production of secondary signals that are required for endothelial cell precusors to migrate to the inner surface of the cornea, (2) TGFα and EGF may stimulate the EGFR in the corneal epithelium and may thereby interfere with the capacity of the epithelium to produce a signal that is required for endothelial cell differentiation, and (3) excess mesenchymal cells accumulating in the anterior chamber of the transgenic eye may block or disrupt an endogenous inductive signal for corneal endothelial differentiation, or perhaps by interfering with the formation of the iridopupillary membrane.

At present, very little is known about the molecular signals that regulate formation of a corneal endothelium. Previous studies on avian models have indicated that the lens plays an inductive role in corneal endothelial formation (Zinn, 1970; Bard et al., 1975). One possibility is that TGFβ2 may specify corneal endothelial cell differentiation during eye development. TGFβ2 is present in aqueous humor (Cousins et al., 1991; Ie et al., 1994), and TGFβ2 mRNA is detected in the ciliary and iris epithelia as well as in the equatorial lens in rat eyes (Gordon-Thomson et al., 1998). Corneal endothelial cells express TGFβ receptor types I, II and III (Joyce and Zieske, 1997; Srinivasan et al., 1998). Furthermore, TGFβ2 knock-out mice have multiple developmental defects in the eye, and many of the affected tissues are neural crest derivatives (Sanford et al., 1997). Corneal mesenchymal cell migration is impaired and the corneal endothelium seems to be absent in the knock-out mice (Chia-Yang Liu and Winston Kao, personal communication). These results together suggest that TGFβ2-signaling plays an essential role in corneal morphogenesis. Moreover, it is known that TGFβ signaling is mediated through SMAD proteins (Derynck et al., 1998; Kretzschmar and Massague, 1998). Recently, it has been demonstrated that mitogenic growth factors such as EGF can negatively regulate TGFβ signaling (Kretzschmar and Massague, 1998). Based on this evidence, we speculate that, in the TGFα and EGF transgenic mice, overstimulation of EGFR in the corneal mesenchymal cells may inhibit the TGFβ/SMAD signaling pathway which is essential for corneal endothelial specification and differentiation.

Our transgenic mice show an interesting correspondence between the presence or absence of corneal endothelium and the maturation of the corneal epithelium after eyelid opening. The maturation and stratification of corneal epithelium occurs around the time of eyelid opening (at about P14). The epithelium becomes 5-7 cell layers in thickness, containing 4-6 layers of superficial squamous cells and a layer of basal cuboidal epithelial cells. In the TGFα transgenic eyes, the corneal epithelium is irregular in thickness. In the areas where underlying endothelial cells are absent, the epithelial layer fails to stratify and differentiate properly (see Fig. 5). In the areas where endothelium is formed, the overlying epithelial layer looks relatively more differentiated. This observation still needs to be confirmed with molecular markers. Since the corneal epithelial cells express EGFR, the epithelial changes may also reflect direct stimulation by the transgenic TGFα, which may diffuse more efficiently through the cornea in regions where the endothelial cells are missing. Postnatal expression of TGFα or EGF in transgenic corneas could potentially help elucidate whether the effect is direct or indirect.

Recently, null mutations in the mouse gene encoding Mf1, a forkhead transcription factor, were found to cause congenital hydrocephalus and defects in anterior segment development, including failure of the cornea to separate from the lens and failure of the corneal endothelium to differentiate (Kume et al., 1998; Kidson et al., 1999). Mf1 is the murine homologue of human FKLH7. Mutation in FKHL7 are known to cause autosomal dominant defects in anterior segment development (Axenfeld-Reiger anomaly) (Mears et al., 1998; Nishmura et al., 1998). Mutations in an unrelated transcription factor, Pitx2, also result in anterior chamber defects in both humans and mice (Semina et al., 1996; Gage and Camper, 1997; Lu et al., 1999). Since Mf1 and Pitx2 are expressed in periocular and corneal mesenchyme prior to differentiation of the corneal endothelium, it is tempting to speculate that stimulation of these mesenchymal cells by TGFα or EGF may disrupt the pattern of expression or the activity of one (or both) of these transcription factors.

The ocular phenotypes of the TGFα and EGF transgenic mice also have features in common with Peters' anomaly in humans, which is characterized by a central corneal opacity with absence of both corneal endothelium and Descemet's membrane in the affected area (Lee et al., 1989; Myles et al., 1992). Adhesion of the iris and/or anterior lens capsule to the posterior surface of the central cornea has been described in Peters' anomaly (Kenyon, 1975; Cook and Sulik, 1988; Myles et al., 1992). Several lines of evidence suggest that Peters' anomaly can occur as a result of mutations in human Pax6 gene (Hanson et al., 1994; Churchill and Booth, 1996). Therefore, we examined the expression patterns of Pax6 in non-transgenic and TGFα transgenic eyes at E15, P1, P7 and P21 by in situ hybridization (data not shown). No differences in Pax6 expression were detected. In both Peters' anomaly and a mouse model of fetal alcohol syndrome (Cook et al., 1987), development of the lens vesicle and separation of the vesicle from the surface ectoderm appear to be delayed. As a result, the persistent lens stalk is thought to interfere with the normal migration of corneal mesenchymal cells. In the TGFα and EGF transgenic mice, separation of the lens vesicle from the surface ectoderm appears to be normal (Fig. 7A,B). Therefore, these mice show that anterior segment defects resembling Peters' anomaly can occur without apparent alterations in either Pax6 expression or lens vesicle formation. Instead, our studies suggest that the corneal

endothelium plays an essential role in anterior segment development and that absence of the corneal endothelium can lead to anterior segment dysgenesis. The transgenic mice can serve as a valuable model for exploring the sequential interactions required for anterior segment morphogenesis.

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