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TGFα can act as a chemoattractant to perioptic mesenchymal cells in developing mouse eyes

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SUMMARY

Growth factors are believed to play an important role in regulating cell fate and cell behavior during embryonic development. Transforming growth factor α (TGFα), a member of the epidermal growth factor (EGF) superfamily, is a small polypeptide growth factor. Upon binding to its receptor, the EGF receptor (EGFR), TGFα can exert diverse biological activities, such as induction of cell proliferation or differentiation. To explore the possibility that TGFα might regulate cell fate during murine eye development, we generated transgenic mice that express human TGFα in the lens under the control of the mouse αA-crystallin promoter. The transgenic mice displayed multiple eye defects, including corneal opacities, cataracts and microphthalmia. At early embryonic stages TGFα induced the perioptic mesenchymal cells to migrate abnormally into the eye and accumulate around the lens. In situ hybridization revealed that the EGFR mRNA is highly expressed in the perioptic mesenchyme, suggesting that the migratory response is mediated by receptor activation. In order to test this model, the TGFα transgenic mice were bred to EGFR mutant waved-2 (wa-2) mice. We found that the eye defects of the TGFα transgenic mice are significantly abated in the wa-2 homozygote background. Because the EGFR mutation in the wa-2 mice is located in the receptor kinase domain, this result indicates that the receptor tyrosine kinase activity is critical for signaling the migratory response. Taken together, our studies demonstrate that TGFα is capable of altering the migratory decisions and behavior of perioptic mesenchyme during eye development.

Key words: transforming growth factor α, cell migration, perioptic mesenchyme, eye, transgenic mice, development, TGFα, EGF receptor

INTRODUCTION

One major goal in research on vertebrate embryogenesis is to determine the molecular mechanisms that control cell fate and cell behavior during development. Many studies have focused on the role of growth factors as signaling molecules (reviews, Jessell and Melton, 1992; Gurdon, 1992; Snider, 1994). Through interactions with their receptors, growth factors appear to influence the fate of the responding cells by regulating proliferation or differentiation decisions. One well-characterized group of growth factors is the EGF/TGFα family, and their transmembrane tyrosine kinase receptor, EGF receptor (EGFR) (reviews, Partanen, 1990; Adamson, 1990; Massagué and Pandiella, 1993). In addition to EGF and TGFα, other members in this family include amphiregulin (AR) (Shoyab et al., 1989), the schwannoma-derived growth factor (SDGF) (Kimura et al., 1990), and the heparin-binding EGF-like growth factor (HB-EGF) (Higashiyama et al., 1991). Growth factors in this family are secreted upon proteolytic cleavage of transmembrane precursors. In most cell types, the proteolysis is limited and both the soluble growth factors and membrane-anchored precursors are present. The transmembrane precursors can bind to the EGFR and exert biological activity (Wong et al., 1989; Anklesaria et al., 1990). These findings lead to the hypothesis that the soluble factor and transmembrane precursor may have dual functions in vivo (Massagué, 1990). For instance, the diffusible growth factors could stimulate target cells located at a distance while adhesion or homing of responding cells could be mediated by the membrane anchored precursors.

TGFα is perhaps the most intensively studied member of the EGF-family. Its production is often associated with transformation and tumorigenesis (Barton et al., 1991; Derynck et al., 1987; Lee et al., 1991). TGFα is known to be a potent mitogen for fibroblasts and epithelial cells in vitro (Blasband et al., 1990; Rosenthal et al., 1986; McGeady et al., 1989). Overexpression of TGFα in transgenic mice can cause hyperplasia and tumorigenesis in a variety of tissues, including coagulation gland and colon (Sandgren et al., 1990), liver and pancreas (Jhappan et al., 1990), mammary gland (Matsui et al., 1990) and skin (Vassar and Fuchs, 1991).

In addition to its mitogenic effects, TGFα is also implicated in several important biological processes such as induction of cell migration (Barrandon and Green, 1987), wound healing

(Schultz et al., 1987), angiogenesis (Schreiber et al., 1986) and control of cell differentiation (Luetteke et al., 1993a). TGFα is also expressed in a wide array of normal tissues, both prenatally (Rappole et al., 1988; Wilcox and Derynck, 1988) and postnatally (Han et al., 1987; Wilcox and Derynck, 1988). Therefore, TGFα could potentially serve as a signaling molecule that controls cell fate and cell behavior during normal development. Surprisingly modest roles for TGFα in normal development were established by the generation of null mutant mice. It was found that TGFα deficiency in both targeted knock-out mice and in the waved-1 (wa-1) mutation results in abnormal skin, hair and eye development (Luetteke et al., 1993b; Mann et al., 1993).

As an alternative strategy to identify target tissues for TGFα in the eye, we generated transgenic mice that express human TGFα in the lens under the control of the αA crystallin promoter (Overbeek et al., 1985). Our transgenic mice display a phenotype analogous to similar transgenic mice reported by Decsi et al. (1994), including corneal opacities, anterior segment dysgenesis, cataracts, microphthalmia and retinal dysplasia. We report here on studies of embryonic ocular development that clarify the roles of TGFα in specification of normal and transgenic ocular architecture. At early embryonic stages, perioptic mesenchymal cells are induced to migrate to a novel location within the transgenic eye. These cells are normally present periocularly surrounding the optic cup (the future retina). The migratory response in the transgenic mice is mediated through the EGFR, which is highly expressed in the perioptic mesenchymal cells. The altered location of the perioptic mesenchyme initiates a cascade of secondary changes in normal eye development. Our transgenic mice not only demonstrate that TGFα can alter the migratory decisions and behavior of perioptic mesenchyme, but also provide relevant insights into the interactions that specify normal ocular development.

MATERIALS AND METHODS

DNA constructs and generation of transgenic mice

To generate the TGFα transgene, a 600 bp *Bam*HI-*Xmn*I fragment of human TGFα cDNA (Derynck et al., 1984) was inserted into the *Bam*HI-*Sma*I site of crystallin promoter vector 2 (CPV2) which contains the small t intron and early region polyadenylation sequences from the SV40 virus (see Fig. 1A). CPV2 was constructed by the following steps. A 847 bp *Bgl*II-*Bam*HI fragment containing the SV40 small intron and poly(A) sequence was transferred from pSV2-BG (Gorman et al., 1982) into the *Bam*HI site of plasmid Bluescript KS (Stratagene). The *Bam*HI site was then deleted by S1 nuclease digestion and religation by T4 ligase. Next, a 415 bp *Kpn*I-*Sal*I fragment containing the αA crystallin promoter (αA) was transferred from the plasmid pTZ18R+αAP (obtained from Dr. John Hope) into a *Kpn*I-*Sal*I digest of the Bluescript-SV40 poly(A) construct. In order to make the *Pst*I and *Eco*RI sites of the Bluescript polylinker useful for cloning, a 56 bp *Pst*I-*Eco*RI fragment was deleted from the αA promoter region by partial digestion and blunt end ligation to generate the CPV2 plasmid.

The crystallin-TGFα fragment for microinjection was isolated by *Sac*I digestion, agarose gel electrophoresis and recovery from agarose by Qiaex gel extraction kit (Qiagen). The purified fragment was microinjected into the pronuclei of 1-cell-stage FVB/N mouse embryos (Hogan et al., 1986; Taketo et al., 1991). Injected embryos were transferred into pseudopregnant ICR/HSD females and allowed to develop to term. Potential transgenic mice were screened at 3-4 weeks of age by polymerase chain reaction (PCR) (see below) using genomic DNA isolated from mouse tails.

PCR and RT-PCR analysis

For identification of transgenic mice, PCR was carried out using primers that span the SV40 small intron of the transgene (see Fig. 1A). The sense primer (termed SV40A) was GTGAAGGAACCT-TACTTCTGTGGTG, and the antisense primer (termed SV40B) GTCCTTGGGGTCTTCTACCTTTCTC (Fiers et al., 1978). A 300 bp fragment was amplified by PCR in a 50 μl reaction consisting of 1 μl of tail DNA, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin), 25 μM of each dNTP, 4 μM of each primer, and 2.5 units of Taq polymerase (Perkin-Elmer Cetus). The thermal cycle profile was denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute for 30 cycles. An aliquot of each reaction was analyzed on a 1% agarose gel and visualized by staining with ethidium bromide.

For reverse transcriptase-polymerase chain reaction (RT-PCR), total RNA was isolated from 4-5 eyes using an RNA extraction kit (RNA STAT-60, Test-Test 'B' Inc.). The RT reaction was performed in a 20 μl volume of 1× PCR buffer, 100 μM of each of dNTP, 29 units of RNAguard (Pharmacia), 180 ng random primers (Promega), 1 μg of total RNA, and 200 units of Moloney Murine Leukemia Virus reverse transcriptase (Gibco, BRL) at 37°C for 1 hour. Subsequently 5 μl of each RT reaction were used for PCR amplification using the primers and conditions described above.

In situ hybridizations

In situ hybridizations were performed following the procedure described by Angerer and Angerer (1992). In brief, paraffin sections of formalin-fixed tissues were deparaffinized in xylene, rehydrated with ethanol, treated with 20 μg/ml proteinase K in 50 mM Tris-HCl, pH 7.6, and 5 mM EDTA for 7 minutes at room temperature, and then post-fixed in 4% paraformaldehyde for 15 minutes. The treated sections were prehybridized for 1 hour and then hybridized with ^{35}S-UTP labeled riboprobes (prepared as described below) at 50°C overnight. Sections were washed in FSM (50% formamide, 2× SSC, and 20 mM mercaptoethanol) at 65°C for 1 hour, digested with 6 μg/ml RNase A at 37°C for 30 minutes and then washed again in FSM at 65°C for 1.5 hour. Sections were dipped in Kodak NTB2 liquid emulsion and stored in the dark at 4°C before developing.

To generate the transgene-specific riboprobe, an 840 bp *Eco*RI-*Xba*I fragment which contains the SV40 viral sequences of the transgene was released from the CPV2 plasmid and inserted into *Eco*RI-*Xba*I digested pBluescript KS. The plasmid was then linearized by digestion with either *Eco*RI or *Xba*I. An antisense riboprobe was generated by incubation with T7 RNA polymerase using an in vitro transcription kit (Promega). The DNA template was removed by RNase-free DNaseI (Boehringer Mannheim) treatment for 15 minutes at 37°C. A sense probe was made by incubation with T3 RNA polymerase and used as a negative control.

To generate the riboprobe for the mouse EGFR, a 2.2 kb *Eco*RI fragment of rat EGFR cDNA was transferred from the plasmid pBSIIER1 obtained from Dr. H. Shelton Earp (Petch et al., 1990) into the *Eco*RI site of pBluescript KS (−). The plasmid was linearized by *Hin*dIII digestion and an antisense riboprobe was generated by incubation with T7 RNA polymerase as described above. A sense riboprobe was made by incubation with T3 polymerase after the plasmid was cut by *Bam*HI.

Eye and tissue histology

Tissues were fixed in 10% neutral-buffered formalin at room temperature overnight. Tissues were dehydrated and embedded in paraffin. Sections at 5 μm were stained with hematoxylin and eosin.

RESULTS

Generation of TGFα transgenic mice

To express TGFα in the developing eye, we linked a 359 bp mouse αA-crystallin promoter to the 600 bp human TGFα cDNA (Fig. 1A). The αA-crystallin promoter has been previously shown to direct the lens-specific transgene expression (Overbeek et al., 1985). In order to enhance the expression level of the transgene, a 847 bp DNA segment from the SV40 virus early region which contains the small t antigen intron and early region polyadenylation sequence was placed at the 3′ end of the TGFα cDNA (Fig. 1A). The purified DNA fragment was microinjected into the pronuclei of 1-cell-stage FVB/N mouse embryos. Potential transgenic mice were screened by PCR using genomic DNA isolated from the mouse tails. Five TGFα transgenic founder mice were obtained. Although the transgenic mice opened their eyes at the same time as their non-transgenic litter mates, they all displayed severe eye defects, including corneal opacities, anterior segment dysgenesis, cataracts, microphthalmia and retinal dysplasia (described below in more detail). The founder mice were mated to non-transgenic FVB/N mice to establish separate transgenic lines (line OVE413 to OVE417). Offspring of founder mouse OVE415 showed two distinctive phenotypes suggesting two different sites of integration, which was confirmed by Southern hybridization (data not shown). The different integration sites are designated as OVE415A (microphthalmia) and OVE415B (mild cataracts).

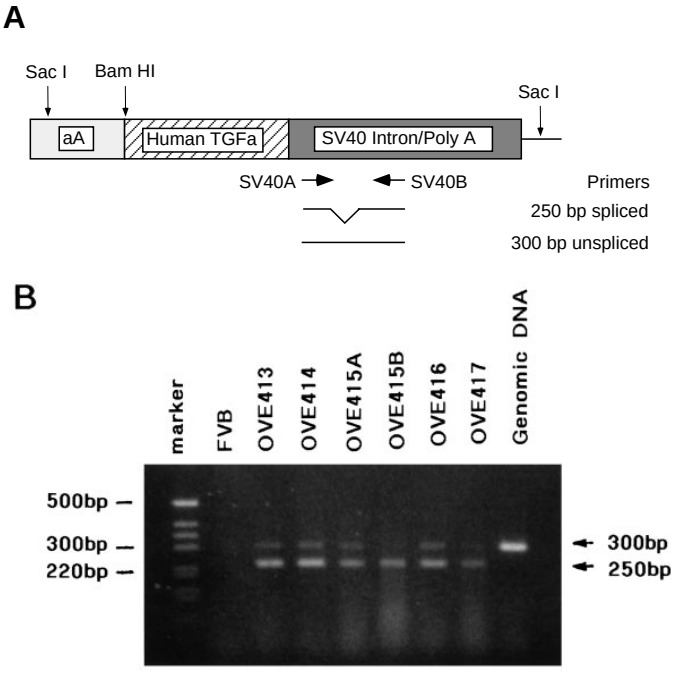

Fig. 1. (A) Schematic drawing of the TGFα construct used for generation of transgenic mice. The PCR primers SV40A and SV40B are indicated below the construct along with the sizes for the amplification bands from DNA (300 bp) and appropriately spliced mRNA (250 bp). (B) Detection of transgene expression by RT-PCR. Total RNA was isolated from the eyes of non-transgenic (FVB) and different transgenic families (OVE413-OVE417). Genomic DNA was isolated from the tail of a transgenic mouse. The molecular marker (1 kb ladder) was obtained from Gibco, BRL.

Lens-specific transgene expression

Transcription of the TGFα transgene was assayed by RT-PCR using primers that span the SV40 intron (Fig. 1B). Total RNA was isolated from the newborn eyes of different transgenic families (OVE413-OVE417). As predicted (see Fig. 1A), a 250 bp fragment was amplified from the transgenic RNAs (Fig. 1B). We also detected a 300 bp fragment in the RNA extracts from different families. This reflects either the amplification of unspliced transcripts or the presence of genomic DNA contaminating the isolated RNA. The RT-PCR was not carried out quantitatively, so the intensity of each band in Fig. 1B does not necessarily reflect the relative levels of transgene expression.

Because the ocular defects in the TGFα transgenic mice occurred not only in the lens, but also in non-lenticular tissues, we confirmed lens-specific expression of the TGFα transgene by in situ hybridization. The hybridization probe was the SV40 sequences of the TGFα transgene (Fig. 1A). At embryonic day 15 (E15) transgenic mRNA was detected exclusively in the lens (Fig. 2B,D), with the highest levels of mRNA found in the lens fiber cells. This result is consistent with previous studies of the endogenous αA-crystallin expression pattern (Treton et al., 1991). No signal was detected in non-transgenic eyes (Fig. 2A,C). The black layer that surrounds the retina in both non-transgenic and transgenic eyes is the pigmentation of the retinal pigment epithelium (rpe).

TGFα induces the migration of perioptic mesenchyme

To characterize the effects of ectopic TGFα expression on eye development, histological examinations were performed on embryonic eyes. At day E15, the architecture and size of the lenses are relatively normal in the transgenic mice (compare Fig. 3B to Fig. 3A). The earliest distinctive abnormalities in the transgenic mice occur in the anterior portions of the eye (compare Fig. 3D to Fig. 3C). The transgenic eyes exhibit a thicker corneal epithelium and stroma compared to non-transgenic eyes. The corneal epithelial cells are elongated and multi-layered while the stromal cells are more randomly aligned and show increased extracellular spacing (Fig. 3B). In addition, the transgenic mice show delayed closure of the eyelids. Most interestingly, there is an abnormal accumulation of cells in the developing anterior chamber, posterior to the cornea and adjacent to the lens (indicated by the arrows in Fig. 3D). Morphologically these cells resemble the perioptic mesenchymal cells, which are normally positioned periocularly (indicated by the arrows in Fig. 3C) adjacent to the optic cup (the future retina) at this stage. Elevated levels of TGFα in the lens can apparently induce the intraocular migration of perioptic mesenchymal cells in developing eyes.

This intraocular migration of mesenchymal cells continues during prenatal development in TGFα transgenic mice (compare Fig. 3F to Fig. 3E). In the non-transgenic mice, perioptic mesenchymal cells are restricted to a periocular location adjacent to the retina (Fig. 3E, indicated by the arrows). By birth (P0) in the transgenic mice, these mesenchymal cells had migrated to surround the entire lens, occupying the anterior and posterior chambers, as well as the vitreous cavity (Fig. 3F). These cells invade the interior of the eye by migrating both anteriorly over the rim of the retina, and posteriorly through the optic nerve (Fig. 3F). At high magnification, pigment cells can be seen among the invading mes-

enchymal cells. These cells are neural-crest-derived melanocytes, which would normally be located specifically in the uveal regions (iris and choroid). The transgenic mice also exhibit lens and retinal abnormalities, including folding of the neural retina, loss of the vitreous, and vacuolization within the lens.

We also examined the eye histology of TGFα transgenic mice at later stages (Fig. 4). In non-transgenic mice, the perioptic mesenchyme differentiates into distinctive periocular structures, such as choroid and sclera (Fig. 4E). The former consists of blood vessels and pigmented melanocytes, while the later forms fibrous connective tissue. In the transgenic eyes, the development of periocular structures appears to be normal. The perioptic mesenchymal cells that migrate into the eye fail

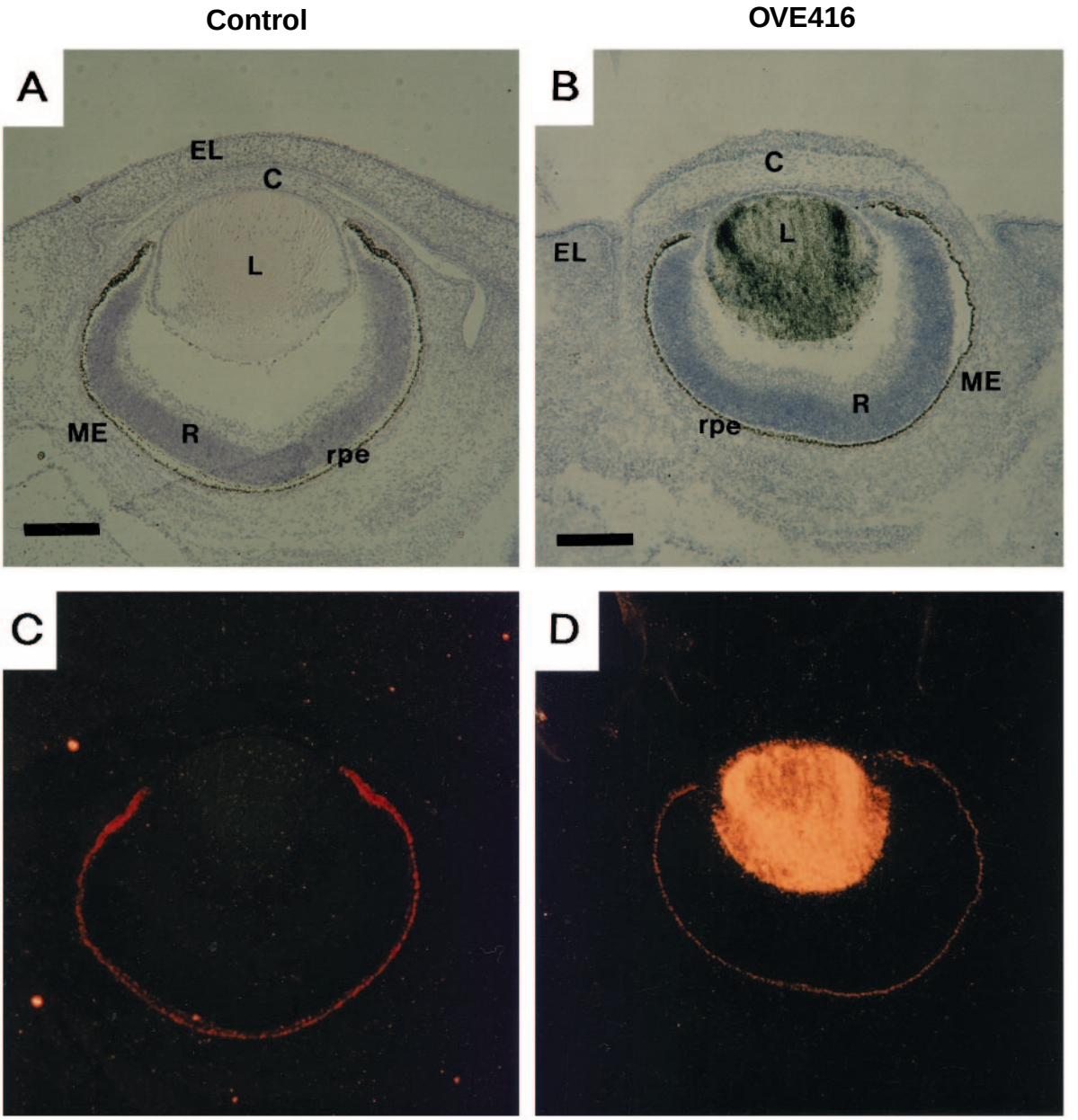

Fig. 2. Lens-specific expression of the TGFα transgene. Transgenic mice from line OVE416 were mated to non-transgenic C57BL/6 mice. Pregnant females were killed and the E15 embryonic heads were removed and processed for in situ hybridization. Eye sections from non-transgenic mice (A,C) and transgenic mice (B,D) were hybridized to an ^{35}S-labeled antisense riboprobe containing the SV40 viral sequences of the transgene. Emulsions were exposed for 2 days at 4°C. After developing, the sections were counterstained with hematoxylin. Sections were visualized with bright-field (A,B) and dark-field (C,D) microscopy, respectively. Note that the transgenic mRNA is found exclusively in the lens of the transgenic eye (B,D). The black layer surrounding the retina is the pigmentation of the retinal pigment epithelium (rpe). At E15, the eyelid is closed over the front of the eye in non-transgenic (A), but not transgenic (B) eyes. The transgenic cornea shows thicker epithelial and stromal layers. Additionally the stromal cells are more randomly oriented and are loosely arranged in the transgenic cornea at E15 (shown at higher magnification in Fig. 3). Abbreviations: EL (eyelid), C (cornea), L (lens), R (retina), ME (mesenchyme), rpe (retinal pigment epithelium). Scale bar represents 200 μm.

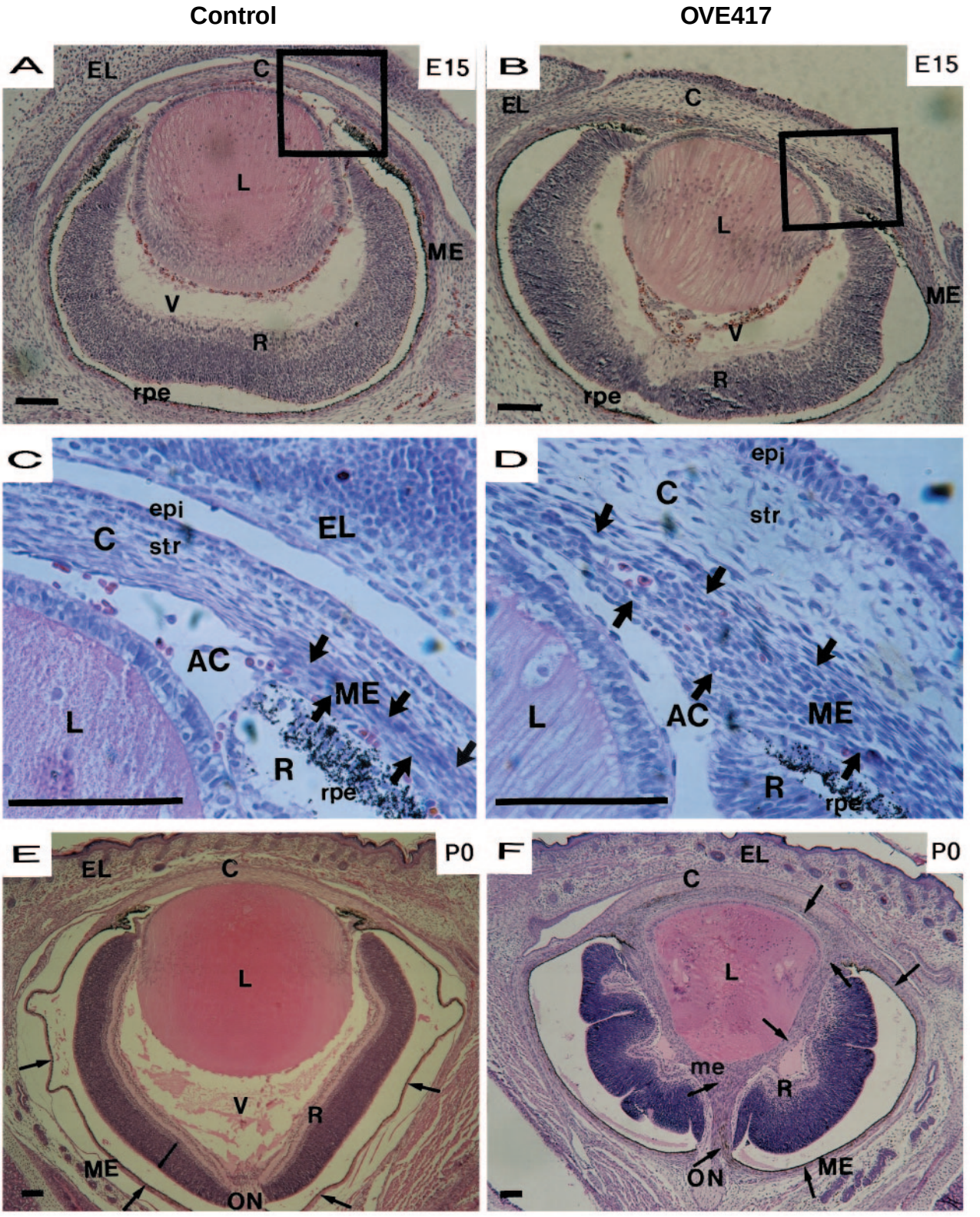

Fig. 3. Developmental eye histology. Non-transgenic FVB (control) or transgenic (OVE417) mice were mated to non-transgenic C57BL/6 mice. Embryos at E15 (A-D) or newborns (E-F) were taken for standard eye histology. (C,D) Enlargements of the boxed regions in A,B, respectively. Abbreviations are: EL (eyelid), C (cornea), L (lens), R (retina), ME (mesenchyme), AC (anterior chamber), V (vitreous), ON (optic nerve), epi (corneal epithelium), str (corneal stroma), rpe (retinal pigment epithelium). The arrows indicate the mesenchyme that is normally present periocularly (surrounding the optic cup). In the transgenic eyes, the perioptic mesenchymal cells migrate into the interior of the eye through the anterior chamber and along the optic nerve (D,F) and accumulate as a multicellular corona surrounding the transgenic lens. Scale bar represents 100 μm.

to develop into the normal periocular structures of choroid and sclera. Instead, they differentiate into several distinctive layers of cells (Fig. 4F).

The lens and retinal defects in the transgenic mice become more dramatic postnatally. In non-transgenic eyes, a layer of cuboidal epithelial cells covers the anterior aspect of the lens. The epithelial cells normally elongate into lens fiber cells at the bow region (as indicated by the open arrows in Fig. 4A,C).

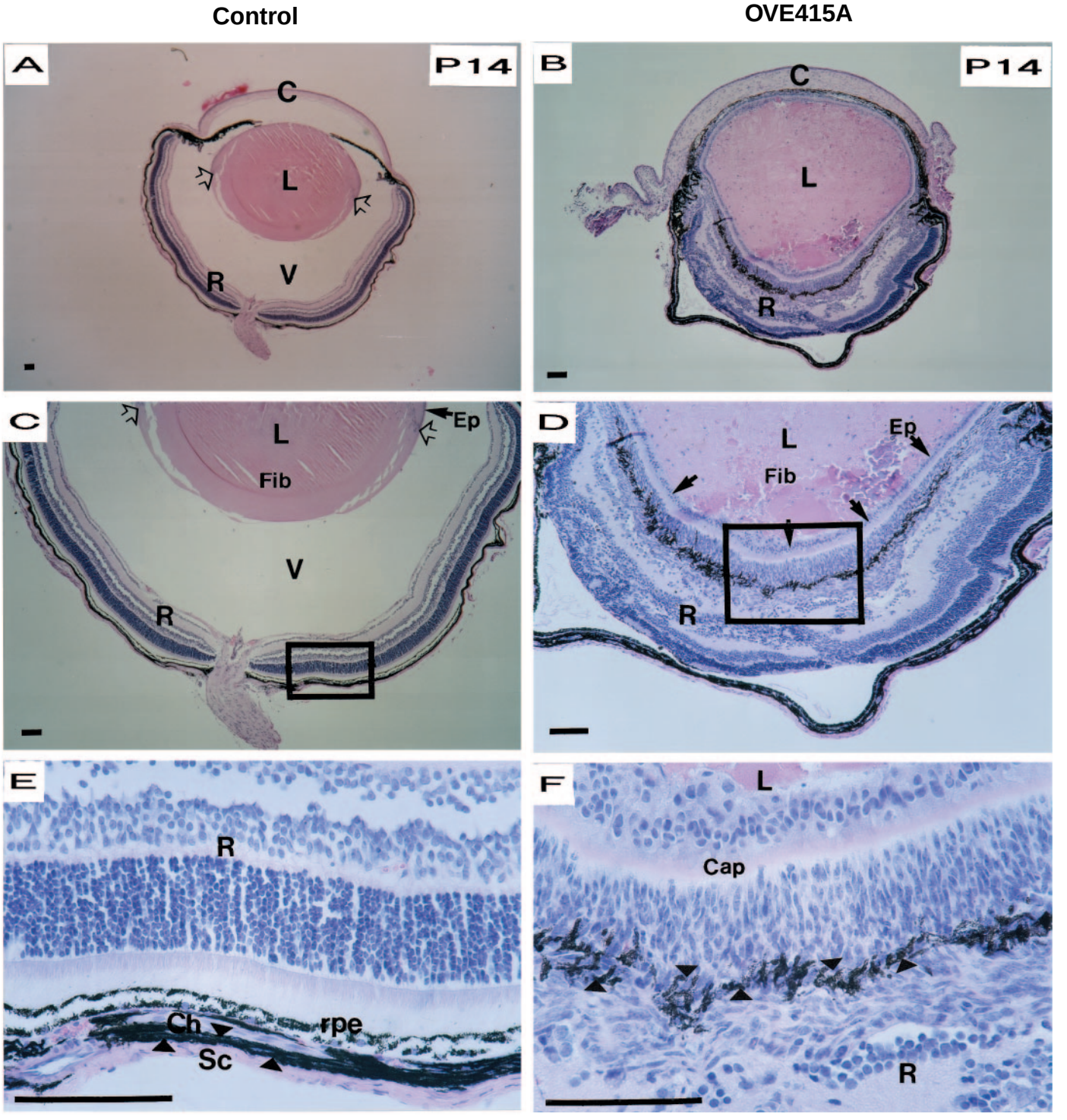

Fig. 4. Eye histology at postnatal day 14 (P14). Eye histology is shown for a non-transgenic (A,C,E) and a transgenic (B,D,F) mouse (OVE415A). (E,F) Enlargements of the boxed regions in C,D, respectively. Abbreviations are: C (cornea), L (lens), R (retina), V (vitreous), Ch (choroid), Sc (sclera), Cap (lens capsule), Ep (lens epithelium), Fib (lens fiber cells). Open arrows in A,C indicate the lens bow region where the elongation of the lens epithelial cells begins. Arrows in D indicate the expansion of the lens epithelial cells to the posterior of the lens. Arrowheads indicate pigmented melanocytes in eyes of control (E) and transgenic (F) mice. Scale bar represents 100 μm. Note that the anterior chamber and vitreous are absent in the transgenic eye. The transgenic retina becomes disorganized and dysplastic.

In the TGFα transgenic mice (Fig. 4B), the accumulation of mesenchymal cells around the lens is accompanied by a unique posterior expansion of the lens epithelial cells (indicated by the arrows in Fig. 4D). Cuboidal lens epithelial cells eventually circle the entire lens, indicating that the signal(s) that induce fiber cell differentiation at equatorial and posterior parts of the lens have been inhibited.

Localization of EGFR mRNA in normal and transgenic eyes

To assess whether the intraocular invasion of mesenchymal cells might be mediated by the EGF receptor (EGFR), we examined the embryonic ocular expression of the EGFR mRNA by in situ hybridization (Fig. 5). EGFR mRNA is highly expressed in the perioptic mesenchyme surrounding the retina in both non-transgenic and transgenic eyes at day E15 (open arrows in Fig. 5). No detectable EGFR mRNA was found in the rpe (indicated by asterisks in Fig. 5E,F). In the transgenic eyes, the perioptic mesenchyme appears to be thicker toward the anterior portion of the eye, presumably due to the anterior migration induced by the elevated levels of TGFα in the lens. The expression of EGFR mRNA is also detected postnatally in the intraocular mesenchy-

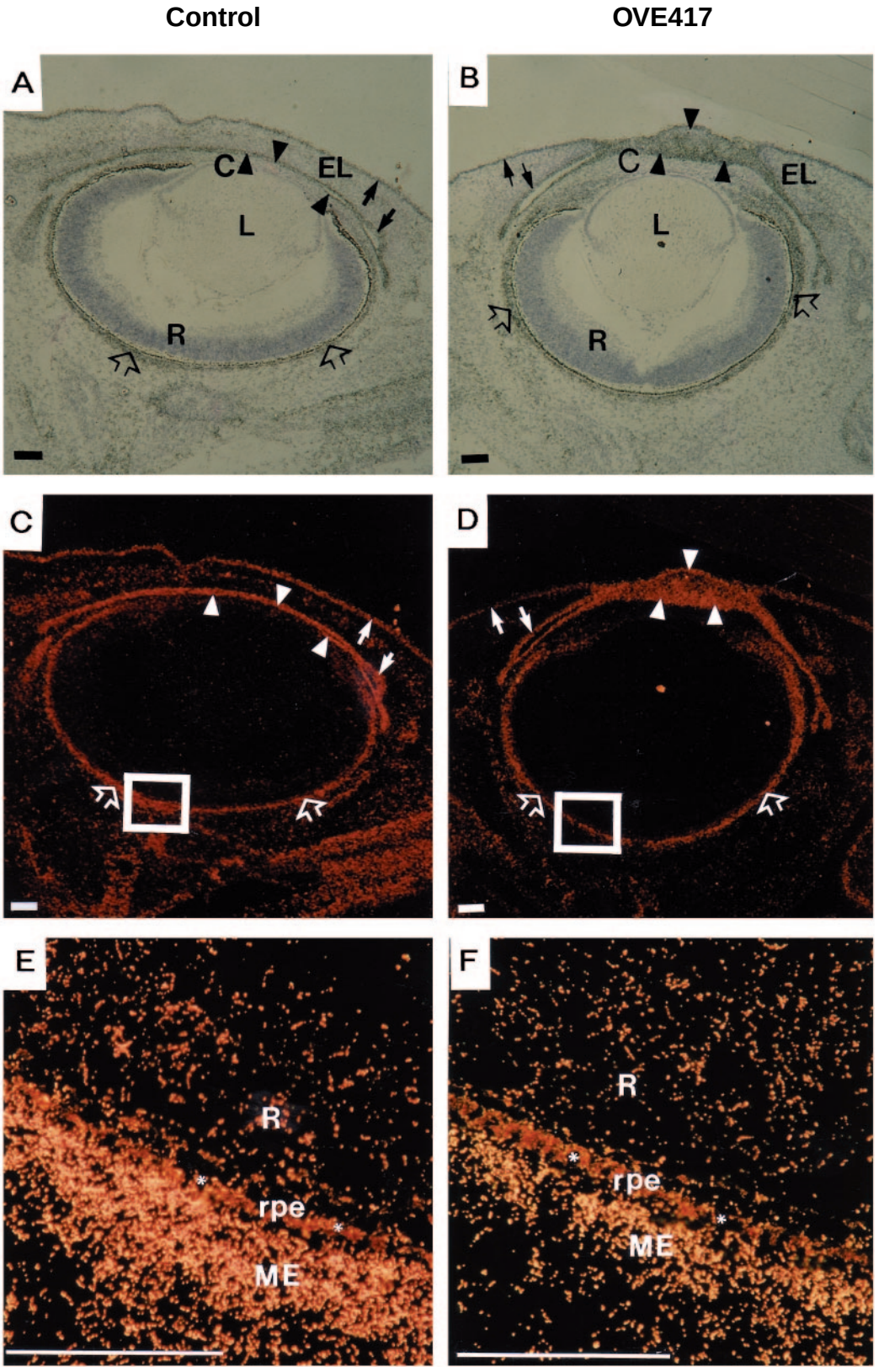

Fig. 5. Localization of EGFR mRNA in mouse eyes at day E15. Non-transgenic (Control) and transgenic mice from OVE417 were mated to non-transgenic C57BL/6 mice. Embryonic heads were removed at day E15 and processed for in situ hybridization. Eye sections of a non-transgenic (A,C) and a transgenic (B,D) embryo were hybridized to a 2.2 kb ^{35}S-labeled riboprobe encoding to the extracellular domain of rat EGFR. Emulsions were exposed for 1 week at 4°C. Sections were visualized with bright-field (A,B) and dark-field illumination (C,D,E,F). (E,F) Enlargements of the boxed regions in C,D, respectively. Abbreviations are given in the legend of Fig. 2. EGFR mRNA is localized to perioptic mesenchyme (open arrows), corneal epithelium (arrowheads), and inner and outer eyelid epithelium (small arrows), but not in the rpe (asterisks). Scale bar represents 100 μm.

mal cells surrounding the lens (data not shown). The fact that EGFR mRNA is present in perioptic mesenchyme supports the hypothesis that overexpression of TGFα in the lens results in the abnormal intraocular migration of these EGFR-expressing cells.

In addition to the perioptic mesenchyme, EGFR mRNA was also localized in tissues derived from the embryonic surface ectoderm, including the inner and outer eyelid epithelium (arrows in Fig. 5) and corneal epithelium (arrowheads in Fig. 5), but not in the lens. The high expression of EGFR mRNA in the corneal epithelium correlates with the corneal hyperplasia in the transgenic mice (Fig. 3B,D). The eyelids appear normal in newborn transgenic mice, although embryonic closure of the eyelids and fusion of lid folds are delayed (Fig. 3B), probably due to the hyperplasia of the corneal epithelium.

It has been reported recently that waved-2 (wa-2) mice carry a point mutation in the EGFR kinase domain (Luetteke et al., 1994). Homozygous wa-2 mice exhibit a phenotype of wavy hair and curly whiskers, similar to TGFα-deficient mice. In order to test whether the activation of EGFR is essential for the intraocular mesenchymal cell migration, we bred TGFα transgenic mice to the wa-2 mutants. TGFα transgenic mice of OVE416 were cross-bred to homozygous wa-2 mice. The offspring, which were transgenic for TGFα and heterozygous for wa-2, were mated to each other to produce TGFα transgenic mice that were homozygous for the wa-2 mutation. Eye defects of these mice were significantly abated relative to the control TGFα transgenic mice (Fig. 6). Histological examination revealed that TGFα/wa-2 mice still develop anterior

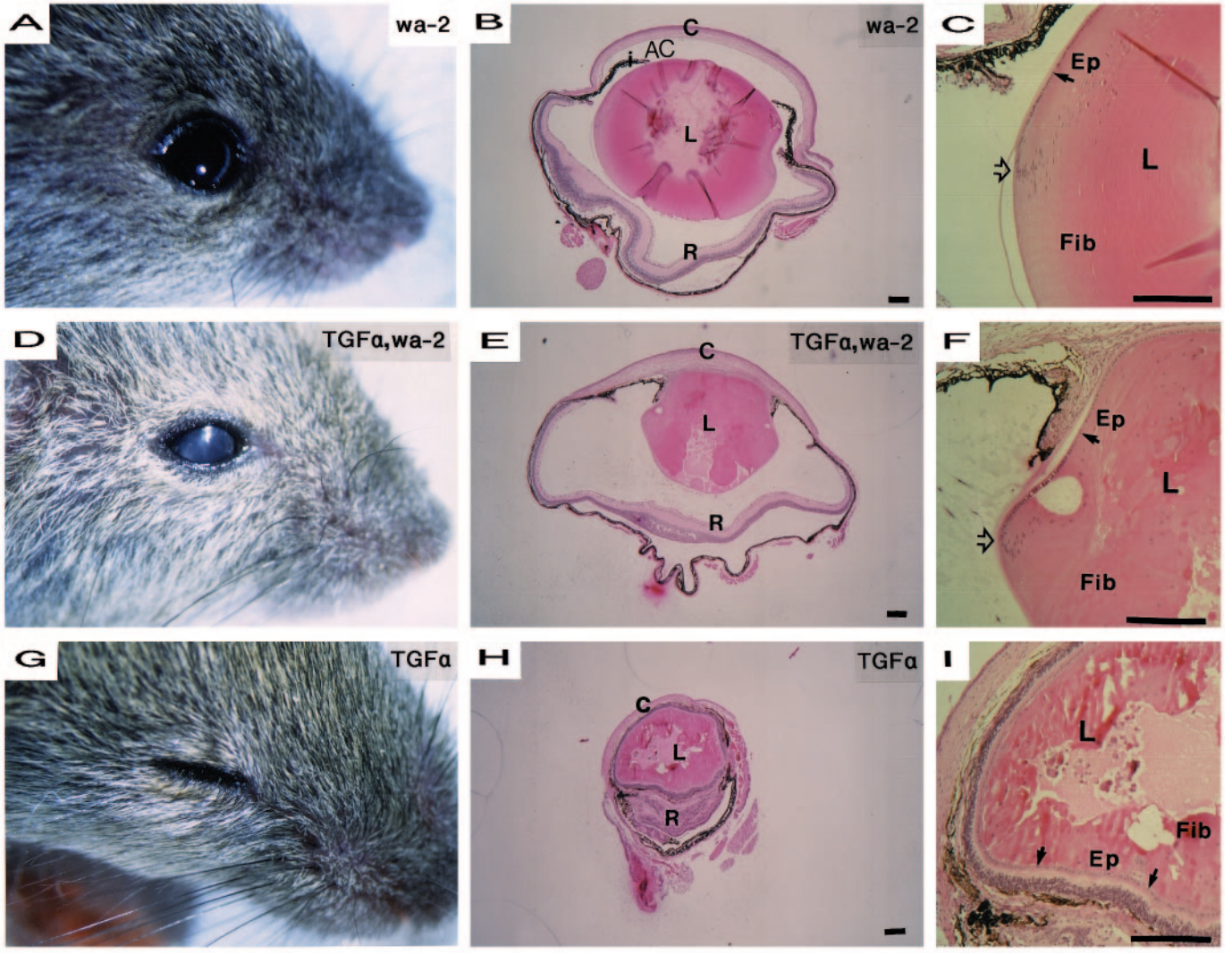

Fig. 6. Eye appearance and ocular histology of homozygous wa-2 mice either not transgenic or transgenic for CPV2-TGFα, and of TGFα transgenic mice at postnatal day 25 (P25). TGFα transgenic mice were cross-bred to wa-2 homozygous mice. Offspring that were transgenic for TGFα and heterozygous for wa-2 were mated to each other to obtain TGFα transgenic mice on a wa-2 homozygous background (wavy hair). Representative mice and ocular histology are shown. (C,F,I) Portions of the lenses in B,E,H, respectively, at higher magnification. Abbreviations are: C (cornea), L (lens), R (retina), AC (anterior chamber), i (iris), Ep (lens epithelium), Fib (lens fiber cells). Scale bar represents 200 μm. Note that, in the TGFα/wa-2 mice, the intraocular invasion of perioptic mesenchyme does not occur (compare F to I). The eyes still show anterior segment dysgenesis (F), so that the anterior chamber and corneal endothelial cells are absent and the lens is attached to the posterior surface of the cornea. However, the TGFα/wa-2 mice maintain a vitreous region, and show elongation of lens epithelial cell (indicated by arrows) at the bow region of the lens (indicated by open arrows).

segment dysgenesis, including loss of the anterior chamber, and attachment of iris and lens to the cornea. However, the accumulation of mesenchymal cells around the lens was significantly diminished (Fig. 6F). Because the EGFR mutation in wa-2 mice causes a reduction in receptor tyrosine kinase activity (Luetteke et al., 1994), this result suggests that the receptor tyrosine kinase activity is critical for the migratory response. When the accumulation of perioptic mesenchyme around the lens is eliminated in TGFα/wa-2 mice, elongation of the lens epithelial cells occurs appropriately at the bow region (Fig. 6F), suggesting that the signals that control the lens cell differentiation are not blocked in these mice.

DISCUSSION

Transgenic mice that express human TGFα in the developing lens were generated. By in situ hybridization, we show that the transgene is expressed in a lens-specific fashion and is readily detectable by E15. Even though expression of the transgene appears to be lens-specific, the transgenic mice exhibit alterations in the architecture of multiple different components of the eye. These alterations include the intraocular accumulation of perioptic mesenchyme, absence of corneal endothelium (data not shown), anterior segment dysgenesis, loss of vitreous and retinal disorganization. Although both EGF and TGFα are known to induce premature eyelid opening in mice (Cohen, 1962; Smith et al., 1985), the transgenic mice open their eyes at the same age as their non-transgenic litter mates.

TGFα induces the intraocular migration of perioptic mesenchyme

Even though the TGFα transgenic mice display ocular defects in both lenticular and non-lenticular tissues, the initial developmental changes are mostly restricted to EGFR-expressing cells. For example, the hyperplasia of the embryonic corneal epithelium correlates with the expression of EGFR mRNA in these cells. The most striking finding is the peri-lenticular accumulation of mesenchymal cells in the transgenic eyes. These mesenchymal cells invade the interior of the eye from both an anterior direction (over the anterior rim of the retina and rudimentary iris) and posteriorly through the optic nerve head. The developmental histology indicates that TGFα produced in the lens induces an intraocular migration of mesenchymal cells that are normally restricted to a perioptic location. In other words, TGFα functions as a chemoattractant for these cells. Since EGFR mRNA is highly expressed in these mesenchymal cells, and since the eye defects were substantially abated when mice were made homozygous for a mutation in the EGFR, the migratory response appears to be caused by TGFα activation of its receptor.

We have considered several other possible explanations for the abnormal intraocular accumulation of mesenchymal cells. One possibility is that TGFα stimulates hyperproliferation of perioptic mesenchymal cells. In this case, TGFα would act as a mitogen, not a chemoattractant. Although TGFα transgenic mice do not develop ocular tumors, we can not rule out the possibility that TGFα may function as a survival and/or mitogenic factor for the perioptic mesenchymal cells. However, a mitogenic effect of TGFα alone is not sufficient for the alteration in ocular location of these mesenchymal cells or the intraocular invasion through the optic nerve. Another possible explanation for the abnormal presence of cells around the lens is that it is caused by the hyperproliferation of corneal stromal cells or lens epithelial cells. Since the lens capsule remains intact in the transgenic mice, the cells outside the lens are not derived from lens epithelial cells. Our in situ hybridizations show that normal perioptic mesenchymal cells express the EGFR while normal corneal stromal cells do not. Since the intraocular mesenchymal cells in transgenic mice express EGFR, we feel that our data supports the proposal that ectopic TGFα expression in the lens can attract some of the mesenchymal cells to migrate to a novel intraocular location.

Mice harboring the wa-2 mutation develop skin and eye abnormalities similar to those in TGFα-deficient mice (Luetteke et al., 1994). The wa-2 allele encodes an EGFR protein with a point mutation in the kinase domain. The wa-2 mutation impairs receptor tyrosine kinase activity but leaves the binding affinity intact (Luetteke et al., 1994). When TGFα is expressed in the wa-2 homozygote background, the intraocular migration of mesenchyme is significantly inhibited (Fig. 6), suggesting that the receptor tyrosine kinase activity is critical for signaling the migratory response. The TGFα transgenic/wa-2 homozygote mice still develop abnormalities associated with anterior segment dysgenesis and attachment of the lens to the cornea, perhaps reflecting the fact that the EGFR in the wa-2 mice can still bind to TGFα.

During normal eye development, the perioptic mesenchyme develops into the periocular choroid and sclera. The choroid consists of blood vessels and pigmented melanocytes. Sclera contains mostly fibroblasts, which form a fibrous connective tissue to support the eye. In the transgenic mice, the architecture of the choroid and sclera are relatively normal. The intraocular mesenchymal cells do not differentiate normally, probably due to the absence of an appropriate environment. However, the mesenchymal cells do form a characteristic pattern around the lens. Pigmented melanocytes are sandwiched between two distinctive populations of cells. Mesenchymal cells with oblong nuclei are found adjacent to the lens. Cells with a more elongated spindle shape are found in the outer layer of cells. Interestingly the timing of appearance of these cell populations is correlated with the normal development of the periocular choroid and sclera.

Aberrant mesenchyme disrupts normal inductive interactions

The abnormal accumulation of intraocular mesenchyme is correlated with a unique change in the polarity of the lens, which may be caused by a physical block to normal positional signaling within the eye. During normal lens development, cuboidal epithelial cells cover the anterior surface of the lens and these cells are induced to differentiate into lens fiber cells at the lens equator (Piatigorsky, 1981). It has been hypothesized that the retina releases factors that specify this polarity of lens development (Coulombre and Coulombre, 1963; Yamamoto, 1976; McAvoy and Chamberlain, 1990; Robinson et al., 1995). In our transgenic mice, the accumulation of mesenchymal cells around the lens results in an expansion of the lens epithelial cells to the posterior of the lens. When the intraocular migration of mesenchymal cells is eliminated in the TGFα/wa-2 mice, the lens epithelial cells continue to elongate at the bow region. These results support the model that normal

lens cell differentiation requires some form of regular inductive communication between lens and retina/vitreous. The accumulation of peri-lenticular mesenchymal cells would block the pathway for communication, resulting in the loss of lens epithelial cell differentiation. A subsequent or associated degeneration of lens fiber cells leads to the microphthalmic eyes seen at later stages in the transgenic mice.

The cause of the retinal disorganization in our transgenic mice is unclear. It has been reported that endogenous TGFα and EGFR are expressed in the developing retina (Anchan et al., 1991; Lillien and Cepko, 1992). It is possible that overexpression of TGFα in the lens and invasion of EGFR-expressing mesenchymal cells in the transgenic eye perturb the normal interactions of this growth factor and its receptor in the retina. Alternatively, defects in the organization of the vitreous or inappropriate signals from the peri-lenticular mesenchyme may contribute to the retinal deterioration.

Role of TGFα (or EGF-like growth factors) and EGFR in normal ocular development

During early eye development, at about day E11.5, condensation of perioptic mesenchyme begins at the anterior region of the optic cup, then extends posteriorly and finally surrounds the optic nerve (Tripathi et al., 1991). These mesenchymal cells are believed to be derived from migrating neural crest cells. The molecular signals that control the destinations of the perioptic mesenchyme have not been identified. Results from our transgenic mice indicate that TGFα (or EGF-related growth factors) may play a role in specifying the normal migration of these cells. We propose that, during normal ocular morphogenesis, the primitive retinal pigment epithelium (rpe) may release EGF-like growth factors that form a morphogen gradient to guide the positioning of the perioptic mesenchyme. The outer coat of the eye is established when these mesenchymal cells surround the optic cup (the future retina) and subsequently differentiate into choroid and sclera. This hypothesis is supported by the report that endogenous TGFα is expressed in the rpe of E16 rat eyes (Lillien and Cepko, 1992). However, the perioptic mesenchyme is still present in TGFα-deficient (Mann et al., 1993; and Luetteke et al., 1993) and EGFR-mutant (wa-2) mice. Since the ocular defects in the TGFα-deficient mice are variable and not confined to a specific cell type, TGFα may play more than one role in the specification of ocular morphogenesis. In addition, there may be redundant or overlapping pathways that control certain aspects of ocular development including migration and differentiation of the perioptic mesenchyme. Such pathways might function either through the use of homologues of TGFα (for instance EGF), or through the use of alternative signalling pathways. Perioptic mesenchymal cells may have the capacity to recognize more than one chemotactic signal, so that an alternative signal may compensate to some extent when the EGFR-mediated signalling pathway is lost or modified. Since the wa-1 and wa-2 mutants both show ocular defects, redundant pathways cannot fully substitute for the EGFR-mediated developmental signals.

The effects of EGF-like growth factors on corneal cells have been investigated previously (Tsutsumi et al., 1967; Schultz et al., 1992). In vitro studies demonstrated that EGF can stimulate the proliferation and migration of corneal cells (Grant et al., 1992). Both EGF and TGFα as well as their receptor (EGFR) have been found in the cornea (Wilson and Lloyd, 1991; Khaw et al., 1992; Wilson et al., 1992). The TGFα knock-out mice often develop inflammation in corneal stroma and their corneal epithelium is thinner (Mann et al., 1993). In our transgenic mice, we found that overexpression of TGFα in the lens causes hyperplasia of the EGFR-expressing embryonic corneal epithelium (see Fig. 3B). These results suggest that elevated levels of TGFα can stimulates proliferation of the corneal epithelial cells. These observation are consistent with the possibility that normal development, turnover, and/or wound healing in the corneal epithelium are controlled by TGFα and EGFR via autocrine or paracrine mechanisms.

Mechanisms of cell migration in embryonic development

Embryonic development requires integrated and coordinated cell and tissue migration. The molecular signals that guide pathways of cellular migration and specify cellular destinations represent fundamental components of normal development that are still poorly defined. Growth factors and their receptors are likely to be involved in specifying certain migratory pathways. Mutations at either the steel (*Sl*) or white spotting (*W*) loci in mice affect the development of several migratory cell lineages, including primordial germ cells, neural crest-derived melanocytes, hematopoietic stem cells and mast cells (Russell, 1979). *Sl* encodes a growth factor (referred to as steel factor, c-kit ligand, stem cell factor, or mast cell growth factor) while *W* encodes the cognate c-kit tyrosine kinase receptor (Geissler et al., 1988; Chabot et al., 1988; Flanagan and Leder, 1990; Williams et al., 1990; Witte, 1990). Alternative splicing gives rise to both cell-associated and soluble forms of steel factor that are biologically active (Anderson et al., 1990; Flanagan et al., 1991). In addition to mitogenic activity, c-kit activation by steel factor elicits migratory and adhesive responses in mast cells in vitro (Meininger et al., 1992; Kinashi and Springer, 1994).

In this report, we provide evidence that TGFα can also act as a chemoattractant and can induce perioptic mesenchymal cells to migrate to a new destination. We propose that secreted TGFα can act as a diffusible chemoattractant for perioptic mesenchymal cells while membrane-associated proTGFα would be predicted to be ineffective as a chemoattractant. This hypothesis can be tested by lens-specific expression of a mutant TGFα, which remains membrane-associated (Wong et al., 1989).

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