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Requirement for TGFβ receptor signaling during terminal lens fiber differentiation

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SUMMARY

Several families of growth factors have been identified as regulators of cell fate in the developing lens. Members of the fibroblast growth factor family are potent inducers of lens fiber differentiation. Members of the transforming growth factor β (TGFβ) family, particularly bone morphogenetic proteins, have also been implicated in various stages of lens and ocular development, including lens induction and lens placode formation. However, at later stages of lens development, TGFβ family members have been shown to induce pathological changes in lens epithelial cells similar to those seen in forms of human subcapsular cataract. Previous studies have shown that type I and type II TGFβ receptors, in addition to being expressed in the epithelium, are also expressed in patterns consistent with a role in lens fiber differentiation. In this study we have investigated the consequences of disrupting TGFβ signaling during lens fiber differentiation by using the mouse αA-crystallin promoter to overexpress mutant (kinase deficient), dominant-negative forms of either type I or type II TGFβ receptors in the lens fibers of transgenic mice. Mice expressing these transgenes had pronounced bilateral nuclear cataracts. The phenotype was characterized by attenuated lens fiber elongation in the cortex and disruption of fiber differentiation, culminating in fiber cell apoptosis and degeneration in the lens nucleus. Inhibition of TGFβ signaling resulted in altered expression patterns of the fiber-specific proteins, α-crystallin, filensin, phakinin and MIP. In addition, in an in vitro assay of cell migration, explanted lens cells from transgenic mice showed impaired migration on laminin and a lack of actin filament assembly, compared with cells from wild-type mice. These results indicate that TGFβ signaling is a key event during fiber differentiation and is required for completion of terminal differentiation.

Key words: Lens, Differentiation, TGFβ, TGFβ receptors, Transgenesis, Mouse

INTRODUCTION

Previous studies have provided evidence that members of the fibroblast growth factor (FGF) and transforming growth factor β (TGFβ) growth factor families play important roles in lens biology. Numerous in vitro studies have shown that members of the FGF family influence lens cell survival, proliferation, migration and differentiation, and have led to the hypothesis that a gradient of FGF stimulation in the eye plays a key role in regulating lens differentiation and growth (reviewed by Chamberlain and McAvoy, 1998). This hypothesis is supported by several transgenic studies that used crystallin gene promoters to drive expression of either dominant-negative FGF receptors or members of the FGF family in the differentiating fibers of the lens. Interruption of FGF signaling, via dominant-negative FGF receptors, resulted in fiber cell death by apoptosis (Chow et al., 1995; Robinson et al., 1995a; Stolen and Griep, 2000). Similarly, overexpression of a secreted, self-dimerizing FGFR3, so as to sequester endogenous FGFs in the ocular media, resulted in delayed initiation of fiber differentiation (Govindarajan and Overbeek, 2001). However, overexpression of FGFs (Lovicu and Overbeek 1998; Robinson et al., 1995b; Robinson et al., 1998) resulted in premature and inappropriate differentiation of the anterior epithelial cells.

Members of the TGFβ superfamily have been implicated in various stages of lens and ocular development. Targeted deletion of BMP4 (Furuta and Hogan, 1998) and BMP7 (Dudley et al., 1995; Luo et al 1995; Wawersik et al., 1999) in mice, resulted in failure of lens placode formation, indicating key roles for these molecules in lens induction. TGFβ2-null mice also show various developmental ocular defects (Sanford et al., 1997). However, at later stages of development, members of the TGFβ family have also been shown to disturb normal lens architecture and to induce pathological changes in lens

epithelial cells similar to those seen in forms of human subcapsular cataract. TGFβ induced the formation of spindle-shaped cells that contain α-smooth muscle actin, as well as capsule wrinkling, apoptotic cell death and accumulation of extracellular matrix proteins such as fibronectin, heparan sulfate proteoglycan and type I collagen (Hales et al., 1994; Hales et al., 1995; Liu et al., 1994). All three mammalian isoforms of TGFβ can induce such cataractous changes in vitro but TGFβ2 and TGFβ3 are about tenfold more potent than TGFβ1 (Gordon-Thomson et al., 1998). Overexpression of an active form of TGFβ1 in the lenses of transgenic mice resulted in the formation of similar opacities that also showed the characteristic features of subcapsular cataract including expression of α-smooth muscle actin and extracellular matrix deposition (Srinivasan et al., 1998).

Members of the TGFβ family exert their biological actions by binding to and activating type I (TβRI) and type II (TβRII) receptors, which are transmembrane, serine-threonine kinases. Signal transduction involves binding of the ligand to TβRII, which induces dimerization of TβRII with TβRI, resulting in phosphorylation and activation of TβRI, and subsequent activation of downstream pathways (reviewed by Massagué, 1998; Massagué, 2000). We have demonstrated that lens epithelial and fiber cells express type I and type II TGFβ receptors (de Iongh et al., 2001; Srinivasan et al., 1998). Induction of TGFβ receptor expression in the anterior lens epithelium postnatally coincided with the period when epithelial cells became responsive to the cataractogenic effects of TGFβ. However, both types of receptors were also expressed in developing fibers during lens morphogenesis, and in the transitional zone and cortex of postnatal lenses where fiber cells undergo elongation and differentiation. In addition, exposure of lens epithelial explants to FGF2, which has been shown to induce lens fiber differentiation, resulted in increased TGFβ receptor expression. These results, together with previous studies indicating significant expression of TGFβ in various ocular tissues (Millan et al., 1991; Pelton et al., 1991), particularly the lens (Gordon-Thomson et al., 1998), as well as its presence in the ocular media (Granstein et al., 1990; Kurosaka and Nagamoto, 1994; Lutty et al., 1993), suggested that TGFβ signaling may play a role during lens fiber differentiation (de Iongh et al., 2001). We have investigated the consequences of disrupting TGFβ signaling during lens fiber differentiation by overexpressing mutant (kinase-deficient), dominant-negative forms of either type I or type II TGFβ receptors (Brand et al., 1993; Brand and Schneider, 1995) in the lens fibers of transgenic mice.

MATERIALS AND METHODS

Generation of transgenic mice

Both TβRI and TβRII consist of a secretory signal sequence, an extracellular domain, a transmembrane domain and a serine-threonine kinase domain (Fig. 1A). TβRI additionally has a domain rich in glycine and serine residues (GS domain) that is necessary for signal transduction (Franzén et al., 1995). The polymerase chain reaction (PCR) was used to generate cDNAs that coded for truncated, kinase-deficient forms of TβRI and TβRII (ΔkTβRI and ΔkTβRII).

For ΔkTβRI, a 534 bp fragment of rat Alk5 (R4), incorporating the signal peptide, extracellular domain, transmembrane domain and 13 residues of the intracellular domain, was amplified by PCR from the full-length cDNA (He at al., 1993) using the following primers: sense, 5′-TTGATATCGAATTCGGCACGAGCCG-3′; antisense, 5′-CG-CGGTGGCGGCCGCTGCAGAACTAGTGGATCCTATTC-3′. The antisense primer incorporated three nucleotide insertional mutations that introduce a stop codon after residue 159 (glutamine) in the intracellular domain of rat Alk5 (He at al., 1993) and a *Pst*I site three nucleotides further downstream. The *Eco*RI-*Pst*I fragment was cloned into CPV2 (Reneker et al., 1995), which contains the –366/+46 αA-crystallin promoter, the small t intron and early region polyadenylation sequences of the SV40 virus. A 1.8 kb αA-cryst/ΔkTβRI/SV40 injection fragment (Fig. 1) was isolated from the vector by digestion with *Not*I and microinjected into pronuclei of FVB/N embryos at a concentration of 2 ng/μl.

For ΔkTβRII a 940 bp cDNA that encoded the cytoplasmic kinase domain deletion mutant (Brand et al., 1993) and incorporated *Eco*RI and *Hin*dIII enzyme restriction sites was cloned into CPV2. A 2.2kb αA-cryst/ΔkTβRII/SV40 injection fragment (Fig. 1) was isolated from the vector by digestion with *Kpn*I and *Pvu*I and used for microinjection. Embryos were implanted into pseudopregnant female mice and allowed to develop to term. Potential founder transgenic mice were screened, after weaning, by PCR analysis of genomic DNA isolated from tail biopsies (see below). To establish transgenic lines, founder mice were mated to FVB/N albino mice and lines were maintained by sibling pair matings until mice homozygous for the transgene were generated.

PCR analyses

Transgenic progeny were identified by PCR screening of genomic tail DNA using primers that hybridize specifically to the SV40 sequence of the transgene (Fig. 1). The sense primer (5′-GTGAAGGAA-CCTTACTTCTGTGGTG-3′) hybridizes 5′ to the intron splice site and the antisense primer (5′-GTCCTTGGGGTCTTCTACCTTTCTC-3′) hybridizes 3′ to the intron splice site to yield a 300 bp fragment in PCR reactions. Reactions were carried out for 28-32 cycles using the following conditions: denaturation for 30 seconds at 94°C; annealing for 30 seconds at 60°C; and extension for 60 seconds at 72°C. A final extension was carried out for 2 minutes at 72°C.

To confirm expression of the transgenes, RT-PCR (Promega, Madison WI) was carried out on RNA isolated from P5 and P21 lenses. Reverse-transcribed first strand cDNA (2 μl) was amplified using the SV40 primers and conditions described above for genomic DNA. Amplification of a correctly spliced transgene mRNA was detected by the presence of a 250 bp fragment after agarose gel electrophoresis.

Histology

Embryonic tissues for histology were obtained by mating superovulated FVB/N female mice with transgenic males. Female FVB/N mice were superovulated by injection with 5 IU of pregnant mare serum gonadotrophin (Lyppard, Sydney, Australia) followed by injection with 5 IU of human chorionic gonadotrophin (Lyppard), 47 hours later. After injection with HCG, mice were placed in a cage with the transgenic male overnight. The presence of a vaginal plug the following morning indicated successful mating (0.5 day gestation; E0.5). Pregnant mice were euthanased by cervical dislocation or CO_2 asphyxiation at various stages of development and conceptuses removed. Embryos, fetal heads or postnatal eyes were fixed in 10% phosphate-buffered formalin for 24-48 hours, dehydrated and embedded in Paraplast paraffin wax (Oxford Labware, St Louis, MO). Tissues were also fixed in 3% glutaraldehyde, post-fixed in 1% osmium tetroxide and stained ‘en bloc’ with 2% uranyl acetate before being embedded in Spurr’s resin.

Preparation of lens epithelial explants

Explants of lens epithelium were prepared from 5-day-old mice as described previously for rat lenses (Hales et al., 1995). After

incubation of lenses for 15 minutes at 37°C and 5% CO_2 in medium 199 (containing 0.1% bovine serum albumin and antibiotics), the capsule with associated epithelium was peeled away from the fibers and pinned, cells facing down, onto laminin-coated Thermanox coverslips (two explants per coverslip) as described previously (Hales et al., 1992). Medium was replaced with fresh culture medium (1 ml/dish) with or without FGF2 at a final concentration of 100 ng/ml. The migratory response of lens epithelial cells was assessed and photographed daily by phase contrast microscopy during the 4-day culture period. After 4 days, explants were fixed for 10 minutes in 1% paraformaldehyde, 0.24% glutaraldehyde in phosphate-buffered saline (PBS) and then rinsed or stored in PBS until stained. To examine the actin filament component of the cytoskeleton, cultures were incubated at room temperature with 1 μg/ml rhodamine-conjugated phalloidin (Sigma, St Louis, MO) in PBS for 1 hour, rinsed extensively with PBS and stained with 5 μg/ml Hoechst dye for 10 minutes at room temperature to label cell nuclei. After washing with PBS, the Thermanox cover slips, with explants attached, were mounted onto 50 mm coverslips, and viewed and photographed with an epi-fluorescence microscope.

In situ hybridization

The expression patterns of the transgene and other endogenous genes were examined by in situ hybridization (ISH) of paraffin sections using ^{35}S-UTP-labeled riboprobes as described previously (Robinson et al., 1995b). Transgene expression was assayed using a riboprobe coding for the SV40 region of the transgene (Fig. 1). cDNAs for hamster αB-crystallin (530 bp) and rat βB2-crystallin (569 bp) were obtained from Dr Nicolette Lubsen (Department of Biochemistry, University of Nijmegen, The Netherlands). A 556 bp cDNA for MIP (Robinson et al., 1998) was obtained from Dr Ana Chepelinsky (National Eye Institute, Bethesda MD).

Immunofluorescence

For intermediate filament (CP49, CP115) and crystallin immunofluorescence, 5-7 μm paraffin sections of embryonic or postnatal eyes were hydrated and incubated for 30 minutes in blocking solution (3% normal goat serum in PBS with 0.1% BSA) to reduce nonspecific staining. Sections were then incubated overnight at 4°C with polyclonal rabbit antibodies specific for CP49/CP115 (1:100) or for either α- or β-crystallin (1:200) in blocking solution. After rinsing in PBS with 0.1% BSA, sections were incubated for 1 hour at room temperature with FITC-conjugated sheep anti-rabbit polyclonal antibody (Silenus/Amrad, Melbourne, Australia), rinsed again and examined by fluorescence microscopy. Polyclonal antibodies against human CP49 (phakinin) and CP115 (filensin) were obtained from Dr Paul Fitzgerald (Cell Biology and Human Anatomy, University of California, Davis, CA). The rabbit polyclonal antibodies for the crystallins have been described previously (McAvoy, 1978).

Bromodeoxyuridine labeling

To examine patterns of cell proliferation the incorporation of 5-bromo-2′-deoxyuridine (BrdU) in cells undergoing S phase was examined using immunofluorescence. Postnatal and pregnant mice at 15 days of gestation were injected with 100 μg/g BrdU, supplemented with 1/10 the concentration of 5-fluoro-2′-deoxyuridine (Sigma), in PBS. One hour after injection, tissues were fixed and embedded in paraffin. Incorporation of BrdU was detected in hydrated sections (7 μm), treated with 1 M HCl for 20 minutes to denature nucleic acids and allow antibody binding. Sections were extensively rinsed in PBS with 0.1% BSA and 0.5% Tween 20 before being incubated for 30 minutes in blocking solution to reduce nonspecific staining. Sections were then incubated overnight at 4°C with a monoclonal antibody against BrdU (Bioclone, Sydney, Australia) diluted 1:100 in blocking solution. Reactivity for BrdU was visualized by incubation for 1 hour at room temperature with FITC-conjugated anti mouse IgG antibody (Silenus), rinsing and examination by fluorescence microscopy.

TUNEL reaction

Paraffin sections were deparaffinized and rehydrated to water and then digested with 0.001% Proteinase K in 0.1 M Tris pH 8, 50 mM EDTA for 15 minutes at room temperature. After rinses in water, sections were incubated for 10 minutes in terminal deoxy-transferase buffer (tdT buffer; 200mM sodium cacodylate, 30 mM Tris pH8, 10 mM CoCl) before being incubated with 30 U/ml terminal transferase (Roche), in tdT buffer containing 4 μM biotin-16-dUTP (Roche) at 37°C for 60 minutes. After washing in 2× saline sodium citrate and PBS, the incorporated biotinylated dUTP was localized by incubation with Cy3-labeled extravidin (Jackson Laboratories) diluted 1:1000 in PBS with 0.1% BSA for 60 minutes at 37°C.

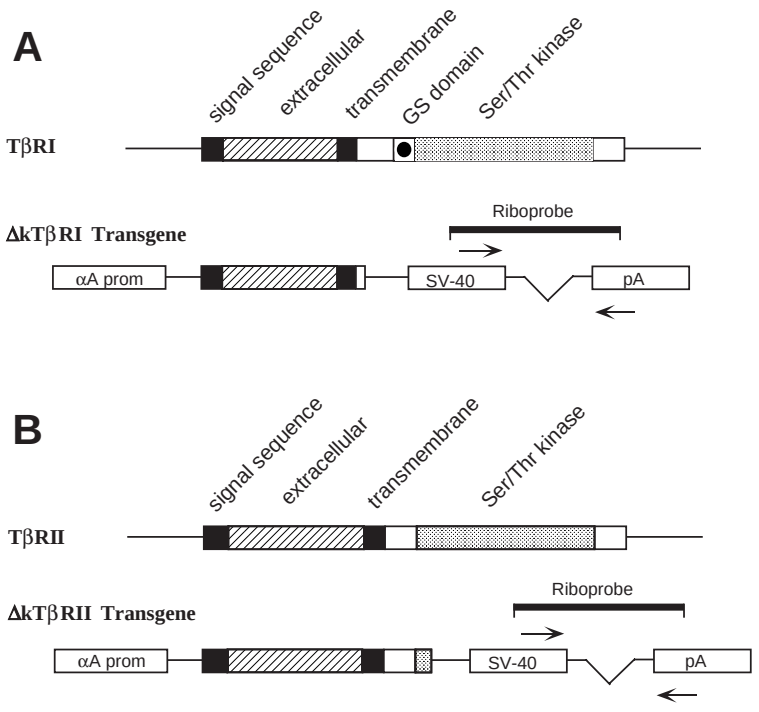

Fig. 1. Dominant-negative TβR transgenes. Diagrams of type I (A) and type II (B) TGFβ receptors showing the secretory, extracellular, transmembrane and intracellular kinase domains of both forms of receptors. In addition, the type I receptor has a glycine-serine rich domain (GS domain). PCR was used to generate truncated, kinase-deficient forms (Δk) of TβRI and TβRII. ΔkTβRI (A) was a 534 bp cDNA that included the signal peptide, extracellular domain, transmembrane domain and 13 residues of the intracellular domain. ΔkTβRII (B) was a 940 bp cDNA, that included the signal peptide, extracellular domain, transmembrane domain and part of the intracellular domain. Both sequences were cloned between the αA-crystallin promoter, and the small t intron and early region polyadenylation sequences of SV40 for microinjection. Transgenic mice were screened with primers specific for the SV40 sequence (arrows) and riboprobes for in situ hybridization were generated from a cDNA (bar) encoding part of the SV40 region of the transgenes.

RESULTS

Generation of transgenic mice

To assess the function of TGFβ signaling during lens development we generated transgenic mice that expressed cDNAs encoding truncated forms of TβRI (ΔkTβRI) or TβRII (ΔkTβRII), driven by the αA-crystallin promoter (Fig. 1). Previous studies have shown that this promoter directs expression of transgenes to differentiating fibers in the developing lens as early as E12.5 (Reneker et al., 1995; Robinson et al., 1995a; Robinson et al., 1995b; Robinson et al., 1998; Srinivasan et al., 1998; Lovicu and Overbeek, 1998). Transgenic mice were identified by PCR, using transgene-specific primers. Two independent transgenic founders were identified for each transgene, designated as R20 and R25 (ΔkTβRI) and OVE550 and OVE591 (ΔkTβRII). Transgenic founder mice were mated with FVB/N mice to establish separate transgenic lines and then mated to homozygosity.

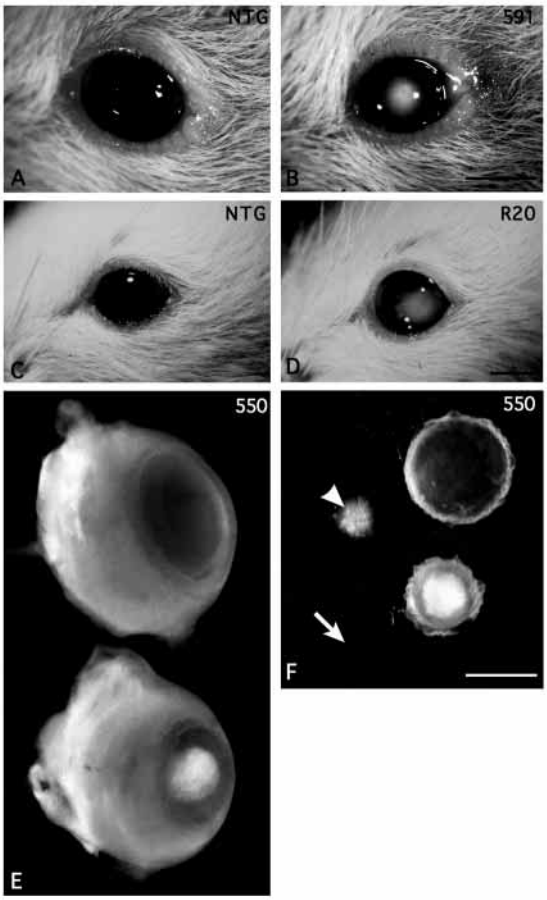

Fig. 2. Phenotype of ΔkTβRI and ΔkTβRII transgenic mice. Photographs showing eyes of non-transgenic (A,C) and transgenic OVE591 (B), R20 (D) and OVE 550 (E,F) mice at P21 (A,B), 8 weeks (C,D) and P5 (E,F). (A) Non-transgenic eye at P21. (B) The ΔkTβRII transgenic (OVE591) eye at P21 is slightly smaller than the non transgenic eye and has a distinct opacity in the lens. (C) Non-transgenic eye at 8 weeks of age. (D) The ΔkTβRI transgenic (R20) eye at 8 weeks is of similar size to the non transgenic but has a distinct opacity in the lens. (E) Dissected eyes from P5 non-transgenic and transgenic ΔkTβRII (OVE550) mice showing the prominent nuclear cataract in the transgenic eye. (F) Further dissection of lenses from the globes clearly revealed the nuclear cataract in the smaller transgenic lens. The clear wild-type lens is able to focus a light source (arrowhead); however, the cataractous, transgenic lens blocks the light (arrow). Scale bar: 3 mm.

The severity of phenotype varied significantly between the different transgenic lines and according to the type of transgene. Both lines bearing the ΔkTβRII transgene (OVE550 and OVE591) exhibited a similar ocular phenotype at eye opening (P14). Distinct bilateral nuclear cataracts were clearly evident in transgenic (Fig. 2B) but not in wild-type mice (Fig. 2A). Dissection of OVE550 eyes at P5 showed that transgenic lenses were smaller (Fig. 2F) than wild type (Fig. 2F). Although wild-type lenses were clear and able to focus a light source (arrowhead, Fig. 2F), the transgenic lenses were characterized by a distinct nuclear opacity that blocked the light path (arrow, Fig. 2F). Examination of eyes at various postnatal ages showed that the cataract was visible at birth (P1) in line OVE550 and at P3 in line OVE591. Of two lines bearing the ΔkTβRI transgene, only R20 showed a significant macroscopic phenotype (although R25 showed some microscopic changes; see later) that became visible between 6 and 8 weeks of age (Fig. 2D) in mice that were homozygous for the transgene. The nucleus of the lens became increasingly opaque until at 8 weeks of age there was a distinct nuclear opacity.

Transgene expression is restricted to differentiating lens fibers

Expression of the transgenes in the lenses of transgenic mice was confirmed by RT-PCR analysis of RNA isolated from P21 mice. Agarose gel electrophoresis of PCR amplifications from lens cDNA using the SV40 primers, identified a correctly spliced transgene mRNA (250 bp) in all transgenic, but not wild-type, lenses compared with a 300 bp fragment amplified from genomic DNA (data not shown). To characterize the pattern of expression of the transgenes in situ hybridization was carried out using an antisense RNA probe complementary to the SV40 sequences (Fig. 1). In situ hybridization analysis was carried out on sections of embryonic (E15) and postnatal (P3 and adult) ocular tissues. In all cases transgene expression was detected exclusively in the lens fiber cells and no detectable transgene expression was detected in the anterior epithelium of transgenic lenses (Fig. 3). In situ hybridization with probes to the extracellular domain of TβRII showed similar patterns of expression as the SV40 probe and also showed that the dominant-negative type II receptor is abundantly overexpressed compared with the wild-type TβRII (data not shown).

Comparison of the two ΔkTβRII lines showed high levels of expression in both, with stronger hybridization signals detected in OVE550 (Fig. 3B) than in OVE591 lenses (Fig. 3D). Of the two ΔkTβRI lines, R20 showed high levels of expression (Fig. 3F), whereas R25 showed only weak expression (data not shown). This analysis indicated that, in general, within both the ΔkTβRI and ΔkTβRII lines, the stronger the transgene expression, the earlier and more severe the phenotype. No specific hybridization was observed in non-transgenic littermates or when the sense riboprobe was used (data not shown). Transgene expression was also detected in cortical fibers of postnatal transgenic lenses at P3 and P120 (data not shown).

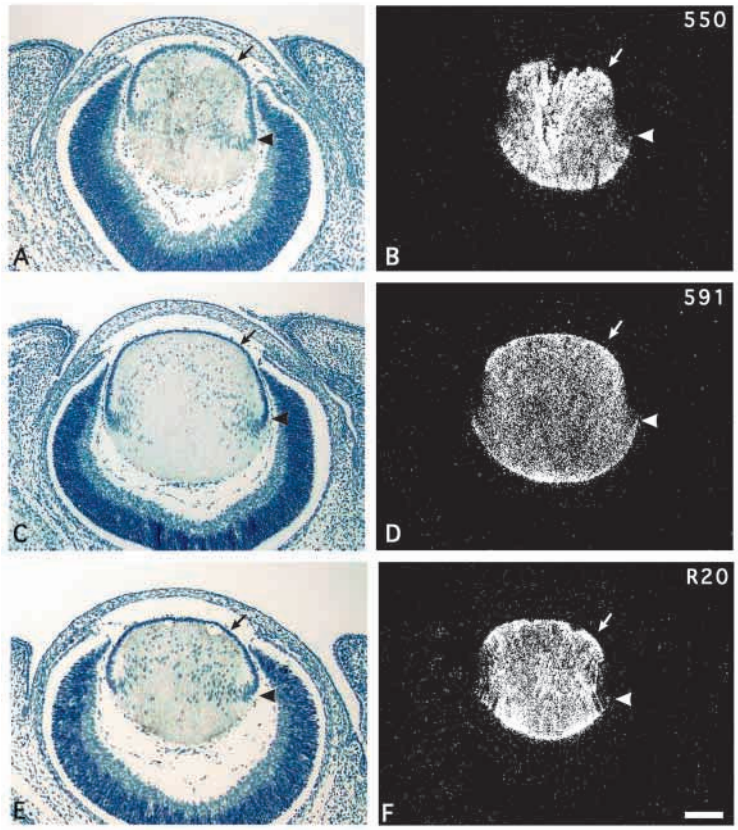

Fig. 3. Fiber-specific expression of transgenes in embryonic lenses. Micrographs showing bright-field (A,C,E) and dark-field (B,D,F) images of in situ hybridization for expression of transgene mRNA in heterozygous E15.5 embryos from lines OVE550 (A,B), OVE591 (C,D) and R20 (E,F). The embryonic pattern of transgene expression was similar in all three lines. There was no detectable expression in the anterior epithelia (arrows). Expression commenced at the equator in early fibers (arrowheads) and was detectable throughout the fiber mass. Strongest expression of the transgene was detected in line OVE550, followed by R20 and OVE591. Scale bar: 100 μm.

Histological analyses of transgenic mice

Histological analyses were carried out on transgenic and non-transgenic lenses using paraffin-embedded tissues, sectioned (7 μm) and stained with Hematoxylin and Phloxine. As lines expressing the ΔkTβRII transgene demonstrated phenotypes that were more severe and occurred earlier during ocular development, these will be described first.

In paraffin-embedded sections of P1 lenses, distinct deviations from normal lens development were detected in the OVE550, but not the OVE591 line (Fig. 4A,B). Non-transgenic and OVE591 lenses exhibited normal patterns of lens growth with a monolayer of cuboidal epithelium covering the anterior surface, elongating young fibers cells at the equator and outer cortex and mature fiber cells in the center of the lens (Fig. 4A). In the cortical fibers, the positioning of nuclei within fibers results in the formation of the characteristic 'bow zone' and in the central fibers there is rapid loss of cell nuclei from the most mature fibers (Fig. 4A). By contrast, P1 eyes from OVE550 mice were significantly smaller than wild-type eyes (inset, Fig. 4A). The lenses showed marked disruption of the central fibers, and also modest changes in the anterior epithelium (Fig. 4B). The anterior epithelium was partially multi-layered, particularly in regions overlying disrupted fiber cells (upper inset, Fig. 4B). The young lens fibers in the transitional zone and outer cortex appeared to have differentiated relatively normally, but the formation of the bow zone was disturbed with most nuclei having migrated in a posterior rather than anterior direction. The most marked abnormality was the disruption of the inner cortical and nuclear fibers (asterisk, Fig. 4B). The nuclei of these fibers were pyknotic (lower inset, Fig. 4B), showed a more scattered distribution and persisted into the lens nucleus, indicating disruption of nuclear positioning within fibers and also of subsequent fiber denucleation. The central nuclear fibers were degenerate and appeared to be displaced anteriorly (asterisk, Fig. 4B).

In P21 lenses the fiber degeneration was very pronounced in both OVE550 and OVE591 lines. In contrast to the normal epithelial monolayer and tightly packed, concentric layers of secondary fiber cells found in the nontransgenic lens (Fig. 4C,D), the morphology of the transgenic (OVE591) lens was clearly disrupted (Fig. 4E,F). The nuclear and inner cortical fibers appear to have degenerated and there was a plaque of multilayered epithelial cells at the anterior pole (inset, Fig. 4E). At higher magnification, cortical fibers at the lens equator were less densely packed and the 'bow zone' was less distinct (Fig. 4F). In the inner cortical fibers there was marked accumulation of eosinophilic material (arrows, Fig. 4F). These fibers were disordered, frequently swollen and contained pyknotic nuclei. These pyknotic nuclei persisted in the degenerate nuclear fibers (arrowheads, Fig. 4F).

As the macroscopic phenotype in the R20 line was not apparent until 6-8 weeks of age, we examined lenses from adult (P120) mice. The non-transgenic lens (Fig. 4G) showed normal arrangement of the anterior epithelium, differentiating fibers in the transitional and bow zones, and the uniformly stained mass of denucleated mature fiber cells that form the lens nucleus. There is a clearly demarcated zone of fiber denucleation where denucleated fibers lie adjacent to nucleated fibers (inset, Fig. 4G). The lack of distinctly pyknotic nuclei or nuclear fragmentation in this region suggests fiber denucleation occurs rapidly. By contrast, the transgenic lens exhibited accumulation of eosinophilic material in the cortical fibers (arrows, Fig. 4H), and pyknotic nuclei and nuclear fragments were clearly evident in the eosinophilic fiber cells (inset, Fig. 4H). Severe disruption of the lens nucleus coincided with the occurrence of a nuclear opacity (see Fig. 2). Examination of the morphology of lenses from another line of mice (R25), which did not show any macroscopic evidence of cataract but did show weak expression of the transgene, showed similar accumulation of eosinophilic material in the cortical fibers but no evidence of nuclear fiber degeneration (not shown).

Developmental analysis

As the ΔkTβRII lines showed the most striking phenotype we concentrated on their analysis in the remainder of the study. To carry out a more detailed morphological analysis of the progression of the lens phenotype in the OVE550 line, ocular tissues from embryonic (E18) and postnatal (P2 and P21)

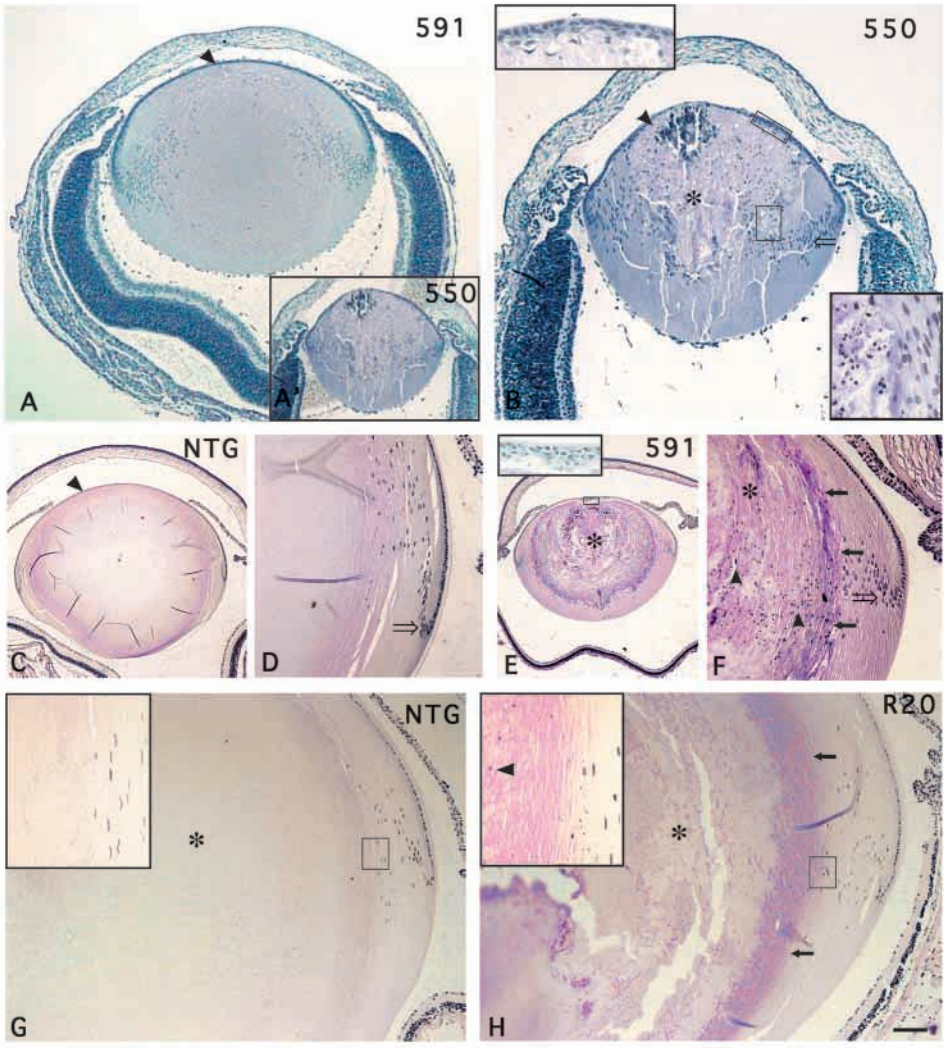

Fig. 4. Histology of transgenic eyes. Hematoxylin and Phloxine-stained sections of non-transgenic (C,D,G) compared with transgenic OVE591 (A,E,F), OVE550 (A′,B) and R20 (H) eyes. (A) At P1, transgenic OVE591 (A) eyes and lenses showed normal morphology and were similar in size to the non-transgenic (not shown). However the OVE550 eyes and lenses were markedly smaller (A′) and showed disrupted morphology. (B) Higher magnification view of the OVE550 eye showing the distinctly abnormal morphology. The anterior epithelium, which is normally a monolayer (A, arrowhead), was multi-layered (upper inset) and, while fibers in the transitional zone and outer cortex appeared relatively normal, the bow zone was abnormal with aberrant posterior positioning of nuclei (open arrow) within the fiber cell. There was marked disruption of inner cortical and nuclear fibers (*) with evidence of pyknotic nuclei (lower inset). (C,D) At P21, non-transgenic eyes and lenses showed normal morphology with a normal monolayer of epithelial cells (C, arrowhead), tightly packed, concentric layers of secondary fiber cells and a distinct 'bow zone' (D, open arrow). (E,F) The OVE591 transgenic lenses were slightly smaller then wild type and there was distinct degeneration of inner cortical and nuclear fibers (*) and multilayering of the adjacent epithelium at the anterior pole (E, inset). Cortical secondary fibers were less densely packed and the 'bow zone' was disrupted (F, open arrow). In the inner cortical fibers there was accumulation of eosinophilic material (arrows), and evidence of fiber swelling and pyknotic nuclei that persisted in the degenerate nuclear fibers (arrowheads). (G) Normal lens morphology of non-transgenic lenses at P120, showing the normal arrangement of anterior epithelium, differentiating fibers in the transitional and bow zones, and the uniformly stained mass of denucleated mature fiber cells that form the lens nucleus (asterisk). (H) The transgenic R20 lens shows accumulation of eosinophilic material in the cortical fibers (arrows) and disruption of the lens nucleus (asterisk). Pyknotic nuclei were also evident in the inner cortical fibers (arrowhead, inset). Scale bar: 100 μm in A,A',D,F; 50 μm in B,G,H; 200 μm in C,E; 25 μm in other insets.

animals were collected, embedded in resin, sectioned (1 μm) and stained with Toluidine Blue.

The wild-type lens at E18 showed the normal progression of epithelial-fiber cell differentiation at the lens equator (Fig. 5A). In the transgenic (OVE550) lens, there was distinct evidence of disruption of this process (Fig. 5B). In the germinative zone of the epithelium, occasional stellate cells were present between the fibers and the epithelium (white arrowhead, Fig. 5B). In the transitional zone, where fibers commence elongation and differentiation, the cell nuclei appeared to be more densely packed and condensed (arrowhead, Fig. 5B) than nuclei in the wild-type lens. Elongation of these fibers appeared attenuated; their apical ends did not reach the epithelial layer as in the wild-type lens (Fig. 5A). Instead they formed a junction with the lateral surfaces of primary fibers (small arrow, Fig. 5B). In the cortex, the fiber cells were poorly aligned and the positioning of nuclei within the fiber cell cytoplasm appeared more random than in the wild-type lens, leading to a less well defined 'bow zone' (large arrow, Fig. 5B). In the center of the lens, particularly in the anterior region of the fiber mass, the fibers became increasingly disorganized and degenerate (asterisk, Fig. 5B).

In the postnatal lenses (P2), the fiber degeneration in the lens nucleus was more evident (Fig. 5D). Initial differentiation in the transitional zone of the transgenic lens appeared normal (Fig. 5D). In the cortex, fibers also appeared to elongate, differentiate and became concentrically aligned, similar to that seen in wild-type lenses. However, in some lenses elongation was incomplete and, as with E18, fibers did not meet the anterior epithelium (data not shown). In the inner cortex, fibers appeared to swell and degenerate rapidly (arrows, Fig. 5D) and as a result the central nucleus of the lens appeared as a large, acellular vacuole, containing amorphous cellular debris. The epithelium that overlies the degenerate fibers was often

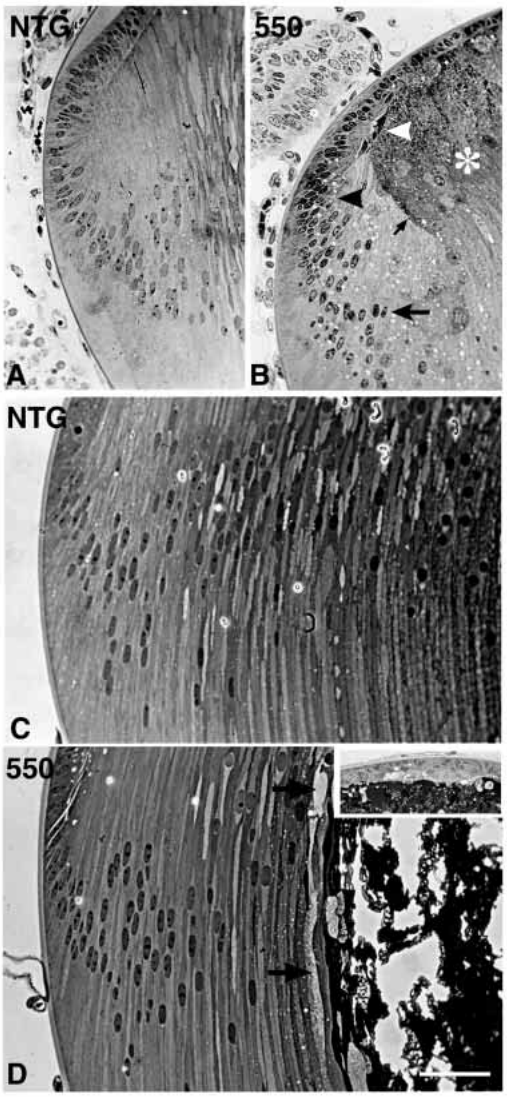

Fig. 5. Histology of transgenic lenses. Toluidine Blue stained resin sections (1 μm) of non-transgenic (A,C) and transgenic OVE550 (B,D) lenses. (A) Equatorial region of an E18 wild-type lens, showing the normal sequence of events of epithelial cell elongation and differentiation into fibers. (B) Equatorial region of the transgenic (OVE550) lens, showing disrupted fiber differentiation. There is an accumulation of fiber cell nuclei (black arrowhead) in the transitional zone. Fibers in this region and the cortex have not elongated fully and do not contact the epithelial layer. Instead they have formed junctions with the lateral surfaces of the nuclear fibers (large arrow). Abnormal stellate cells (white arrowhead) were present between fibers and epithelial cells in the germinative zone. Disrupted positioning of nuclei (small arrow) in cortical fibers results in loss of the bow zone. In the lens nucleus the fibers are grossly disorganized and degenerate (asterisk). (C) Equatorial region of the P2 wild-type lens showing normal fiber cell elongation and terminal differentiation. (D) Equatorial region of the P2 transgenic (OVE550) lens showing more marked disruption of terminal fiber differentiation. Initial differentiation in the transitional zone and cortex appeared similar to the wild type. However, in the inner cortex, fibers appeared to swell and degenerate rapidly (arrows), forming a large, central, cellular debris-filled vacuole. The anterior epithelium, overlying the degenerate fibers was multi-layered (inset). Scale bar: 100 μm in A-D; 50 μm in inset.

multilayered (inset Fig. 5D). The P21 lenses were similar to the P2 lens, except that the central acellular vacuole was larger (not shown). While lenses from the other line (OVE591) showed a similar phenotype, it was slightly less severe in that central fiber degeneration was not observed until P3 and the aberrant distribution of nuclei in cortical fibers was less apparent.

Fiber cell apoptosis

The condensed, pyknotic nuclei in degenerating fibers of the transgenic lenses morphologically resembled nuclei undergoing apoptosis. To determine whether ΔkTβRII expression induced fiber cell apoptosis we used Hoechst dye staining and TUNEL reaction on sections from both wild-type and transgenic lenses at P2. We used OVE550 lenses for this because of the abundance of pyknotic nuclei. In wild-type lenses, Hoechst dye staining revealed the normal monolayer of epithelial cells and the 'bow-like' distribution of fiber nuclei at the equator (Fig. 6A). Whereas the wild-type lens fibers often showed a low-level of background fluorescence after TUNEL reaction, intensely TUNEL-positive nuclei were not observed in wild-type lenses (Fig. 6B). In the transgenic lens, Hoechst labeling revealed multilayering of the anterior epithelium and inappropriate posterior nuclear positioning in the cortical fibers leading to formation of an abnormal 'bow zone' (Fig. 6C). Coincident with the region in which the fibers became swollen and started to degenerate, the fiber nuclei became more condensed and pyknotic and were increasingly located more anteriorly in the cytoplasm (boxed region, Fig. 6C; arrows Fig. 6E). Many of these condensed, pyknotic nuclei were intensely TUNEL-positive (Fig. 6D,F) indicating these fibers were undergoing apoptotic changes. Occasional aberrantly migrating TUNEL-positive nuclei were also observed in close association with the posterior capsule (arrowheads, Fig. 6C,D) in and posterior to the transitional zone. Occasional TUNEL-positive nuclei were also detected in the multilayered anterior epithelium of the transgenic lenses (arrows, Fig. 6D).

Cell proliferation

TGFβ has been shown to affect regulation of the cell cycle, most typically as an anti-mitogen (Hocevar and Howe, 1998). As apoptosis can be a result of abnormal cell cycle progression (Chen et al., 2000) we examined, by BrdU incorporation, whether there were changes in patterns of cell proliferation in transgenic lenses prior to the occurrence of fiber degeneration. Similar patterns of BrdU-labeled nuclei were detected in the anterior epithelia in both wild-type and transgenic (OVE591) lenses at E15.5 and at P1, just before the appearance of degenerative changes in the fibers (data not shown). BrdU-labeled nuclei were not detected in the fibers of either wild-type or transgenic lenses (data not shown). These results indicate that expression of ΔkTβRII did not significantly affect cell cycle progression in epithelial or fiber cells at the ages examined.

Effects on fiber differentiation

To examine more closely the effects of the dominant-negative receptor on lens fiber differentiation we examined several major differentiation markers, including crystallins (lens specific proteins), MIP (a water channel protein) and filensin and phakinin (intermediate filament proteins).

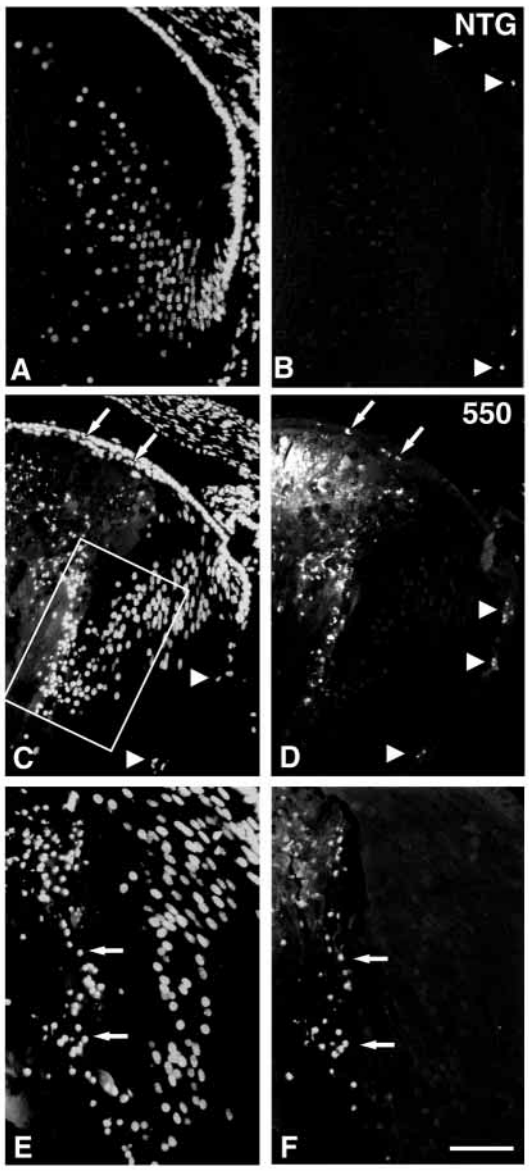

Fig. 6. Fiber cell apoptosis. Hoechst dye staining (A,C,E) and TUNEL reaction (B,D,F) on sections of P2 non-transgenic (A,B) and transgenic OVE550 (C-F) lenses at P2. (A) In non-transgenic lenses Hoechst staining revealed the normal monolayer of epithelial cells and the 'bow-like' distribution of nuclei in the lens fibers. (B) No TUNEL-positive cells were seen in non-transgenic lenses. Occasional labeled cells (arrowheads) were detected in cornea and blood vessels lining the inner limiting membrane of the retina. (C) Hoechst staining of transgenic lens revealed a multilayered anterior epithelium and disrupted formation of the 'bow zone'. In the inner cortex, nuclei appear to be positioned abnormally within the fiber cells and formed an aberrant 'bow zone'. Many of these fiber nuclei were condensed and pyknotic (boxed). Occasional Hoechst-stained nuclei, representing aberrantly migrating epithelial cells, were found localized along the posterior capsule (arrowheads). (D) TUNEL reaction revealed labeling of the pyknotic nuclei in the inner cortex and nucleus of the lens and also occasional labeled cells in the multilayered epithelium (arrows). TUNEL-positive nuclei were also observed at the equator and in the cells that had migrated posteriorly along the posterior capsule (arrowheads). (E) Higher magnification view of boxed region in C, showing condensed and pyknotic nuclei (arrows) of inner cortical fibers. These fiber nuclei also showed an abnormal distribution pattern within the fiber cells, resulting in an abnormal 'bow zone'. (F) TUNEL reaction of the same region shown in E, showing that the pyknotic nuclei are TUNEL positive (arrows). Scale bar: 100 μm in A-D; 65 μm in E,F.

α-crystallin

α-crystallins are expressed in both epithelial and fiber cells, but strongly upregulated during fiber differentiation. As TGFβ can downregulate αA-crystallin promoter activity (Ueda et al., 2000) we examined whether inhibition of TGFβ signaling affected α-crystallin expression by in situ hybridization and immunohistochemistry in P1 wild-type and transgenic (OVE550 and OVE591) lenses. To allow direct comparison of expression levels, adjacent sections for each sample were processed under identical conditions. In the wild-type lens (Fig. 7A,D) strong expression of αB-crystallin was detected in anterior epithelium and cortical fibers. However, expression declined in the nuclear fibers (Fig. 7D). In the OVE591 lenses at P1 (Fig. 7B,E) no overt phenotypic changes were evident (compare Fig. 7A with 7B). Similar expression was detected in the lens epithelium as in the wild-type lens, but expression in the cortical and nuclear fibers appeared to be stronger in the transgenic (Fig. 7E) than in the wild-type (Fig. 7D) lens. In the OVE550 transgenic line at P1 (Fig. 7C,F) the phenotypic changes in the epithelium and fibers were clearly evident (Fig. 7C). There was considerably stronger expression of αB-crystallin in the multilayered anterior epithelium and in the fibers of the transgenic lens (Fig. 7F) than was observed in the anterior epithelium of the non-transgenic control (Fig. 7D). However, expression of αB-crystallin was absent in the degenerate nuclear fibers (Fig.7F, asterisk). Similar patterns of α-crystallin protein expression were observed by immunohistochemistry (not shown).

β-crystallin

As β-crystallins are lens fiber-specific proteins, often used as early markers of fiber differentiation, in situ hybridization was used to determine whether ΔkTβRII affected β-crystallin expression. βB2-crystallin was only expressed in fiber cells of P1 wild-type and transgenic lenses (Fig. 7G-I). Similar levels and patterns of expression were observed in the wild-type and OVE591 lines (Fig. 7G,H), with strong expression detectable in the outer cortical fibers but little or no expression in inner cortical or nuclear fibers. In the OVE550 line, there was increased expression of βB2-crystallin in the outer cortical fibers and strong expression was also present in the inner cortical and nuclear fibers (Fig. 7I). However, in the degenerate nuclear fibers, expression of βB2-crystallin was absent (Fig. 7I, asterisk). Similar patterns of β-crystallin protein expression were observed by immunohistochemistry (not shown).

Lens major intrinsic protein (MIP)

MIP is a member of the aquaporin family that is expressed specifically in lens fibers (Yancey et al., 1988). Mutation of the MIP gene has been shown to be associated with nuclear opacity formation and fiber degeneration in the 'cataract Fraser' mouse (Shiels and Bassnett, 1996). We examined whether ΔkTβRII affected MIP expression by in situ hybridization in wild-type and transgenic (OVE591, OVE550) lenses at P1. In the wild-type lens (Fig. 8A,D), MIP was not expressed in the epithelium, although expression was strong at the apical ends of the fibers

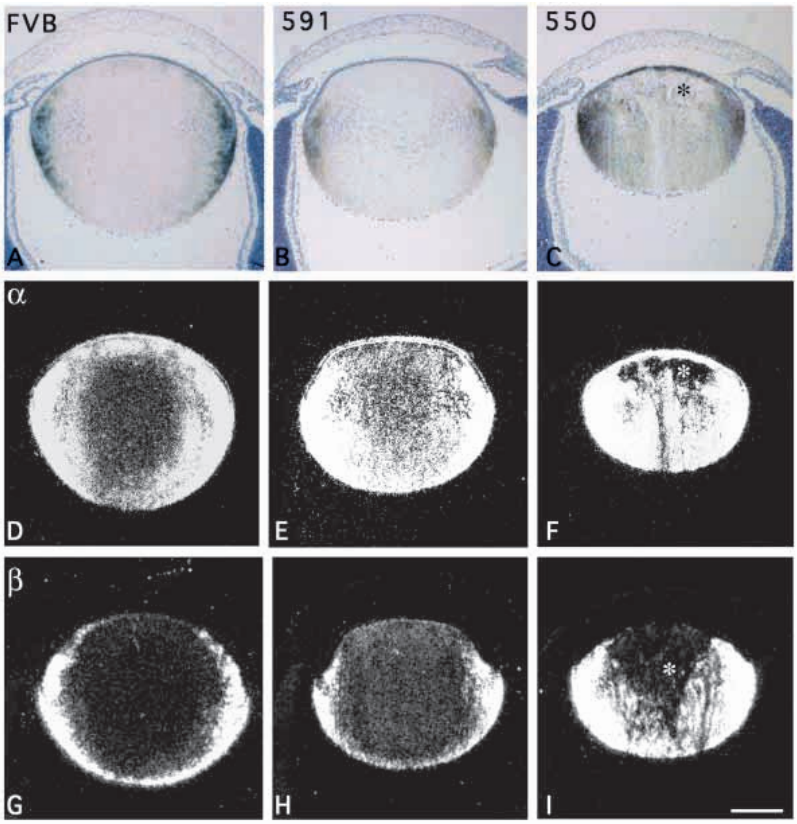

Fig. 7. Crystallin expression. Expression of αB-crystallin (D-F) and β-crystallin (G-I) in non-transgenic (A,D,G) and transgenic OVE591 (B,E,H) and OVE550 (C,F,I) lenses at P1. (A-C) Hematoxylin-stained bright-field images of D-F showing fiber degeneration has not yet occurred in OVE591 (B) lens but is clearly evident (*) in OVE550 (C) lens. (D) In wild-type lens, αB-crystallin was strongly expressed in the anterior epithelium and in cortical fibers, but little or no expression was detected in nuclear fibers. (E) In the OVE591 lens, a similar level of expression was detected in the lens epithelium but increased expression was detected in the cortical and nuclear fibers. (F) In the anterior epithelium and in the cortical fibers of the OVE550 lens, there was stronger expression of αB-crystallin compared with the non-transgenic control, but expression was absent in the degenerate nuclear fibers (*). (G) Expression of β-crystallin in wild-type lenses was restricted to cortical fiber cells. (H) The OVE591 lens showed similar level and pattern of expression to the wild type. (I) The OVE550 lens line showed increased expression in the outer cortical fibers that persisted to the inner cortical and nuclear fibers, but expression was absent in the degenerate nuclear fibers (*). Scale bar: 240 μm.

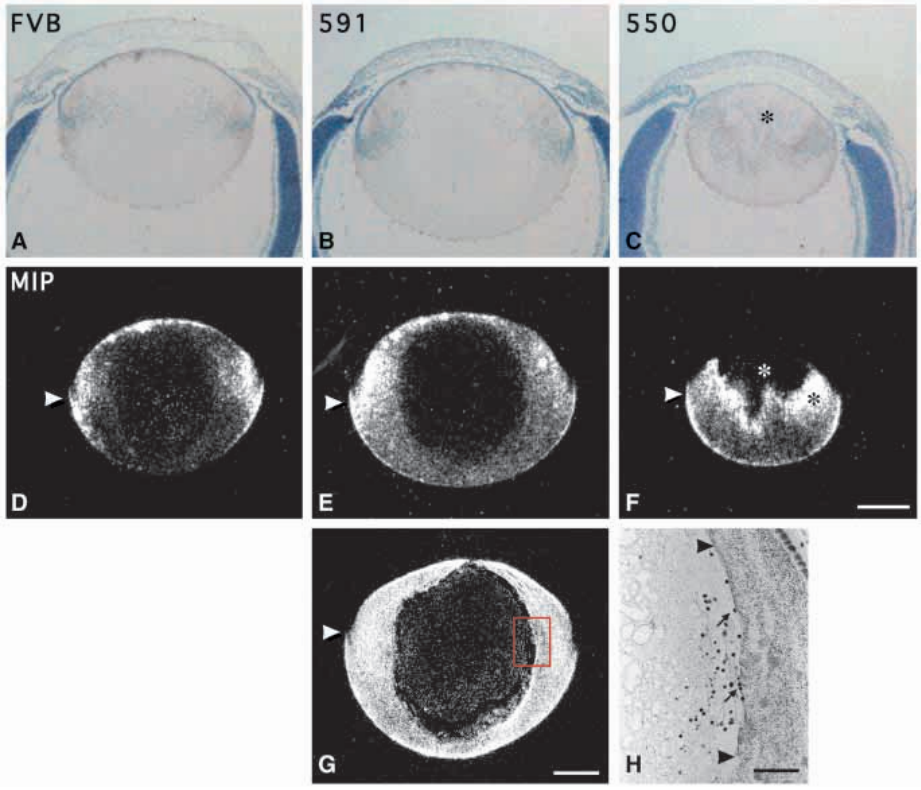

Fig. 8. MIP expression. Expression of MIP in non-transgenic (A,D) and transgenic OVE591 (B,E,G,H) and OVE550 (C,F) lenses at P1 (A-F) and P3 (G,H). (A-C) Bright-field images of D-F showing wild-type (A) OVE591 (B) and OVE550 (C) lenses. (D) In the wild-type lens, expression of MIP was initiated in the transitional zone (arrowhead) and strongest in the outer cortical fibers. Expression decreased in the inner cortical fibers and was absent from the nuclear fibers. (E) Similarly, in the OVE591 lens there was initiation of expression in the transitional zone (arrowhead) but there was enhanced expression of MIP in the cortical fibers compared with the wild-type lens. (F) In the OVE550 lens, MIP expression commenced in the transitional zone and was increased in the cortical fibers, particularly in fibers (black asterisk) adjacent to the degenerative zone (white asterisk). No expression was detectable in the degenerated nuclear fibers (white asterisk). (G) In the OVE591 lens at P3, when fiber degeneration became apparent, MIP expression was initiated normally in the transitional zone but was strongly expressed in the cortical fibers, particularly in the regions preceding the zone of degeneration where fiber cells were swollen and disorganized (see Fig. 5D). Expression ceased abruptly in the degenerative zone (boxed). (H) Higher magnification bright-field view of the boxed region in G. Loss of MIP expression signal (arrowheads) in the cortical fibers precedes the occurrence of the nuclear pyknosis (arrows). Scale bar: 225 μm in A-F; 250 μm in G; 50 μm in H.

where they abut the epithelium. Marked expression was detected in fibers of the transitional zone (arrowhead, Fig. 8D) and outer cortex. Expression decreased in the inner cortical fibers and was absent from the nuclear fibers (Fig. 8D). Similarly in transgenic (OVE591 and OVE550) lenses, expression commenced in the transitional zone (arrowheads, Fig. 8E,F). However, in both transgenic lines expression of MIP was enhanced and extended further into the cortical fibers (Fig. 8E,F). The increased expression was most noticeable in the OVE550 lens, with markedly increased expression in fibers (black asterisk, Fig. 8F) immediately adjacent to the degenerate nuclear fibers (white asterisk, Fig. 8F) that showed no expression. Similarly increased expression of MIP was found in the OVE591 line at P3 when the aberrant lens phenotype became apparent (Fig. 8G). A strikingly sharp decline in MIP expression was evident in the region where the inner cortical fibers start to degenerate. At high magnification the loss of hybridization signal (arrowheads, Fig. 8H) in the cortical fibers precedes the occurrence of the nuclear pyknosis (arrows, Fig. 8H).

Intermediate filaments

The intermediate filaments, filensin (CP115) and phakinin (CP49), are key markers for fiber differentiation (Prescott et al., 1996; Sandilands et al.,1995). Immunofluorescence was used to examine the expression of these proteins in wild-type and transgenic lenses. As OVE591 showed a major deterioration in the structure of the fibers between P1 and P3, we concentrated our analysis on these stages.

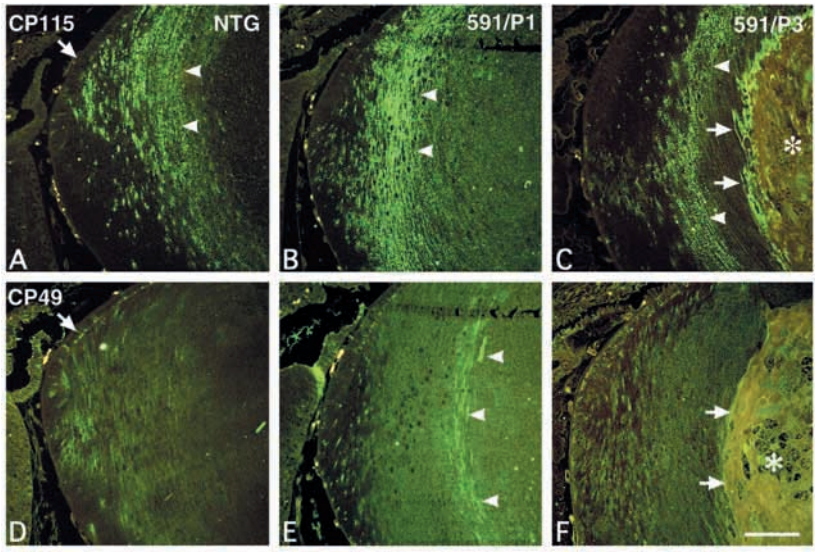

Fig. 9. Expression of the intermediate filaments filensin (CP115) and phakinin (CP49). Immunofluorescence for CP115 (A-C) and CP49 (D-F) in wild-type (A,C) and OVE591 (B,C,E,F) lenses at P1 (A,B,D,E) and P3 (C,F). (A) Patchy reactivity for CP115 was detected in fiber cells of the non-transgenic lens at P1, particularly the inner cortical fibers (arrowheads). No reactivity was present in the epithelium (arrow) or lens nucleus. (B) Increased reactivity for CP115 was present in the inner cortical fibers (arrowheads) and also the nucleus of the transgenic OVE591 lens at P1. (C) At P3 in the OVE591 lens, the central fibers are degenerate (asterisk). Distinct reactivity for CP115 was present in a band of cortical fibers (arrowheads) and in fibers that adjoin the zone of degeneration (arrows). (D) At P1, CP49 was localized diffusely in fibers of the wild-type lens and was also detected along apico-lateral membranes of the epithelial cells (arrow). (E) Increased reactivity for CP49 was detected in fibers of the OVE591 lens at P1, particularly in a band of fibers in the deeper cortex (arrowheads). (F) At P3, the fibers that were highly reactive for CP49 at P1 (E) have degenerated. Only occasional cells at the edge of the degenerate fiber region (asterisk) show intense reactivity for CP49 (arrows). Scale bar: 100 μm.

Filensin (CP115)

Using the antiserum raised against human CP115, patchy reactivity was detected in early fiber cells of the non-transgenic lens at P1, particularly in the anterior parts of the fibers (Fig. 9A). This reactivity increased and became more homogeneous in the inner cortical fibers (arrowheads, Fig. 9A) but no reactivity was detectable in the lens nucleus or in the anterior epithelial cells. In the transgenic lens at P1, degeneration of the central fibers has not yet commenced but there were distinct differences in the pattern of CP115 localization. In the early fiber cells there appeared to be increased reactivity for CP115, and in the inner cortical fibers there was markedly increased reactivity, evident as an intense band (arrowheads, Fig. 9B). Faint, diffuse reactivity persisted in the lens nucleus. By postnatal day 3 there is frank degeneration of the central fiber cells (asterisk, Fig. 9C) of the OVE591 lens. While there was weak reactivity for CP115 in the early and outer cortical fibers, the distinct band of reactivity in deeper cortical fibers was still evident (arrowheads) followed by a band of no reactivity in the inner cortex. However, the swollen fibers that just precede the zone of degeneration showed intense staining for CP115 (arrows, Fig. 9C), particularly along their membranes.

Phakinin (CP49)

In the wild-type lens at P1, CP49 was expressed diffusely in fiber cells of the transitional zone and deeper cortex, with little or no reactivity detected in the lens nucleus (Fig. 9D). Reactivity was also detected at the apical surfaces of epithelial cells (arrow). In the OVE591 lens at P1, before the manifestation of fiber degeneration, there was a marked increase of diffuse CP49 reactivity in the cortical and nuclear fibers (Fig. 9E). In addition, a band of fibers in the deeper cortex showed particularly intense accumulation of phakinin (arrowheads, Fig. 9E). This band of fibers, with increased phakinin reactivity, was situated deeper in the cortex than the band of fibers that showed increased filensin reactivity (compare Fig. 9B,E), approximately in the region where fiber degeneration subsequently occurs at P3. Examination of transgenic OVE591 lenses at P3 (Fig. 9F) showed that, while there was increased diffuse reactivity in the cortical fibers compared with the wild type, the band of intensely staining fibers was no longer evident and the region occupied by this band of fibers (Fig. 9F) was degenerate. Only cells at the edge of the zone of degeneration showed distinct reactivity for phakinin (arrows, Fig. 9F).

Effect of ΔkTβRII expression on lens cell migration and actin filament assembly

Inhibition of TGFβ signaling in the lens by overexpression of truncated TGFβ receptors affected major events during lens

fiber differentiation. In addition to the fiber degeneration in the lens nucleus, there was impaired movement/elongation of fiber cells in the transitional and cortical zones, particularly in OVE550 lenses (Fig. 5B), with altered expression of α-crystallin and MIP as well as intermediate filament proteins (filensin and phakinin). To further investigate the effects of transgene expression on lens cell behavior, we examined responses of transgenic and wild-type epithelial cells to FGF2 in a lens explant migration assay. Lens epithelial explants from 5-day-old wild-type and transgenic (OVE550) lenses were established on a laminin-coated substratum (Hales et al., 1992) and cultured with or without a fiber-differentiating dose of FGF2. In this system, FGF2 increases the rate of epithelial cell migration (Hales et al., 1992), increases transcriptional activity of the αA crystallin promoter (Ueda et al., 2000), and increases the expression of TβRI and TβRII (de Iongh et al., 2001). Previous studies have shown that lens epithelial cells express abundant TGFβ2 mRNA (Gordon-Thomson et al., 1998) and release significant quantities of TGFβ2 (~0.7 ng/ml; Allen et al., 1998) into the culture medium. As addition of exogenous active TGFβ can result in lens epithelial cells undergoing an epithelial-mesenchymal transition (Gordon-Thomson et al., 1998; Hales et al., 1994; Hales et al., 1995; Liu et al., 1994), we did not add exogenous TGFβ to these cultures.

Fig. 10 shows the migration responses of wild-type and transgenic (OVE550) lens epithelial cells after 4 days of culture. Both wild-type and transgenic epithelial cells, cultured without FGF-2, showed a migration response with regions of cells observed to migrate from the capsule onto the laminin substratum. Once cells were on the substratum they extended lamellae and pseudopodia (arrowheads, Fig. 10A,B) and were observed to migrate away from the edge of the explant (arrows, Figs 10A,B). However, in the presence of a fiber-differentiating dose of FGF2, the cultures from wild-type and transgenic lenses produced completely different responses. In cultures from the wild-type lenses there was an augmented migratory response (Fig. 10C), similar to responses observed in rat lens epithelial cells (Hales et al., 1992; Hales et al., 1994). Staining of the cells with rhodamine-conjugated phalloidin revealed prominent stress fibers of filamentous actin particularly in the lamellae and ruffled leading edges of the actively migrating cells (arrowheads Fig. 10E). By contrast, addition of FGF-2 to the transgenic explants resulted in marked inhibition of cell migration (Fig. 10D,F), accompanied by an absence of stress fibers as shown by phalloidin labeling of filamentous actin (arrowheads Fig. 10F). Thus, transgenic lens cells lose the ability to migrate on laminin when TGFβ signaling is impaired.

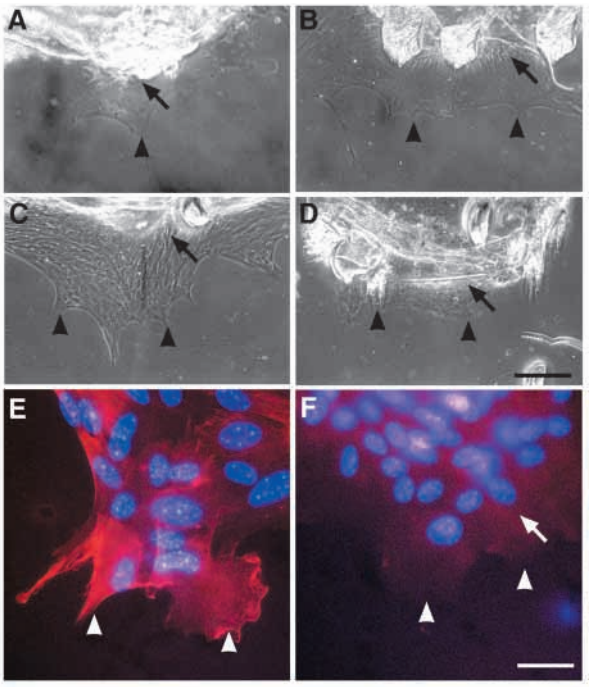

Fig. 10. Migration response on a laminin substratum. Phase contrast (A-D) and fluorescence images, showing labeling of filamentous actin and nuclei (E,F), of migrating non-transgenic (A,C,E) or transgenic OVE550 (B,D,F) lens epithelial cells cultured for 4 days on laminin without FGF2 (A,B) or with 100 ng/ml FGF2 (C-F). (A) Wild-type lens epithelial cells (arrowheads), cultured without FGF2, migrated from the capsule (arrow) onto the laminin substratum. (B) Transgenic (OVE550) lens epithelial cells (arrowheads) showed a similar migration response away from the explant edge (arrow), when cultured without FGF2. (C) In the presence of a fiber-differentiation dose of FGF2, wild-type epithelial cells showed an augmented migratory response (arrowheads). (D) Transgenic (OVE550) epithelial cells showed inhibition of cell migration when cultured with FGF2, with very few cells (arrowhead) migrating from the edge of the capsule (arrow). (E) Staining of filamentous actin with phalloidin-rhodamine, in wild-type cells cultured with FGF-2, revealed labeling of stress fibers (red) in the actively migrating cells (arrowheads). Cell nuclei have been stained with Hoechst dye (blue). (F) Phalloidin-rhodamine staining of FGF2-treated transgenic (OVE550) cells showed a complete absence of stress fibers in epithelial cells (arrowheads) that have remained in close association with the capsule (arrow). Scale bar: 100 μm in A-D; 20 μm in E,F.

DISCUSSION

TGFβ is a pleiotrophic growth factor that can elicit a wide range of responses such as proliferation, differentiation, apoptosis or extracellular matrix deposition, depending upon the cell type. This context-dependent responsiveness to TGFβ is highlighted by the broad range of effects that have been described for experiments involving inhibition of TGFβ signaling using dominant-negative receptors. For example, ectopic expression of dominant-negative TβRII in the epidermis results in loss of cell cycle control and enhanced tumorigenicity (Go et al., 2000). In liver it inhibits fibrosis (Qi et al., 1999); in mammary gland (Joseph et al., 1999) and lung (Zhao et al., 1998) it induces aberrant epithelial branching; and in muscle it inhibits myogenic differentiation (Filvaroff et al., 1994).

A role for TGFβ signaling in lens fiber differentiation has been suggested previously by studies that show immunoreactivity for both TβRI and TβRII in the differentiating fibers of the transitional zone and cortex of the rat lens (de Iongh et al., 2001). Furthermore, in vitro studies have shown that FGF2, which induces fiber differentiation in vitro (reviewed by Chamberlain and, McAvoy, 1998; McAvoy et al, 1999), also induces enhanced TGFβ receptor reactivity in differentiating epithelial explants of postnatal lenses (de Iongh et al., 2001). In this study we have addressed the putative

role of signaling via TGFβ receptors in lens fiber differentiation by expressing truncated, dominant-negative type II and I TGFβ receptors in lenses of transgenic mice using the αA-crystallin promoter.

Similar to previous studies using this promoter (Lovicu and Overbeek, 1998; Reneker et al., 1995; Robinson et al., 1995a; Robinson et al., 1995b; Srinivasan et al., 1998), lens fiber-specific expression of the transgenes was shown, as early as E15.5, by in situ hybridization, using transgene-specific riboprobes. Expression persisted until adulthood. Consistent with TGFβ receptors being transmembrane receptors, morphological changes were restricted to the lens. Other ocular tissues showed relatively normal morphology. Severe lens defects were present in two transgenic lines expressing ΔkTβRII and less severe defects were observed in a line expressing ΔkTβRI. The severity of the phenotypes was related to the level of transgene expression, as well as to the type of dominant-negative receptor expressed. This is consistent with previous findings by Lovicu and Overbeek (Lovicu and Overbeek, 1998), who showed similar variations in phenotype severity, dependent upon levels of transgene expression. In one line that expressed high levels of ΔkTβRI, a lens phenotype was clearly apparent at 6-8 weeks, whereas in another line that expressed only low levels of ΔkTβRI only minor morphological changes in the lens were detectable. However, both lines expressing ΔkTβRII showed more severe phenotypes with the most severe occurring in the line (OVE550) expressing the higher levels of the transgene. The phenotype was evident from late embryonic stages for OVE550, whereas it was not apparent until P3 for OVE591. It is important to note that the levels of expression in OVE591 (ΔkTβRII) and R20 (ΔkTβRI) were similar yet the phenotype in OVE591 was markedly more severe and occurred earlier during lens development.

Previous studies assaying the efficacy of dominant-negative forms of type I and type II receptors in myocytes and mink lung cells have indicated that dominant-negative type I receptors are less effective at blocking TGFβ signaling events than type II receptors (Brand and Schneider, 1995). This difference in effectiveness may be due, in part, to the different roles that type I and type II receptors play during ligand binding and subsequent receptor oligomerization and signaling. The type II receptor can bind TGFβ, but the type I receptor can only bind TGFβ once it has bound the type II receptor. Hence, in addition to blocking signal transduction, the dominant-negative type II receptor may also act by binding and sequestering active TGFβ from endogenous, functional type II receptors. By contrast, as the type I dominant-negative receptor cannot bind TGFβ directly, it can only act at the level of receptor signaling after formation of a heteromeric complex with the ligand-bound type II receptor. Type I dominant-negative receptors therefore cannot inhibit binding of TGFβ to endogenous type II receptors, which may subsequently still be able to recruit endogenous type I receptors to form signaling oligomeric complexes. Such 'leaky inhibition' may explain the extended timecourse for the appearance of the phenotype in the ΔkTβRI line (R20), compared with the ΔkTβRII lines (OVE591 and OVE550).

In the most severely affected line (OVE550), the eyes and lenses were markedly reduced in size, yet ocular tissues, other than lens, appeared to differentiate normally. It has been shown that development and growth of other ocular tissues, particularly the anterior segment, are modulated by the lens or by factors that it releases (Beebe and Coats, 2000). The reduction in size of the transgenic eyes may be mediated by the reduced size of the lens as a result of the transgene or alternatively may be mediated indirectly. As TβRII can bind TGFβ, it is possible that high level expression of ΔkTβRII in the lens leads to the sequestration of active TGFβ from the ocular milieu. The lens itself has been shown to express (Gordon-Thomson et al., 1998) and to secrete TGFβ2 into the ocular media (Allen et al., 1998). Moreover, studies of form deprivation myopia in chick lenses have suggested that TGFβ is involved in the regulation of ocular growth (Honda et al., 1996; Rohrer and Stell, 1994).

Previous studies have shown that TGFβ receptors are predominantly expressed in the transitional zone and outer cortex of the lens (de Iongh et al., 2001). Similarly, the dominant-negative TβR transgenes were most strongly expressed in these regions. The attenuation of fiber elongation in the transitional zone and outer cortex of transgenic lenses (see Fig. 5B) is consistent with TGFβ signaling being important for early fiber elongation and differentiation in these regions. It has been shown by targeted disruption, that the homeobox gene, Prox1, is a key factor that regulates lens fiber elongation (Wigle et al., 1999). However, it remains to be determined whether the attenuation of fiber elongation in the ΔkTβR mice involves altered expression of Prox1. The most marked effects of the dominant-negative inhibition did not become apparent until the later stages of fiber differentiation in the inner cortex and lens nucleus. This suggests that, while activation of TβR signaling pathways occurs during early fiber differentiation, the effects of inhibition in the transgenic lenses are cumulative, and degenerative effects are not manifested until much later during fiber differentiation. Another possible explanation for the degenerative effects of the transgenes is that they inhibit a TGFβ-dependent event(s) that occurs later during fiber differentiation, which is crucial for fiber survival.

Morphologically, the swelling and sudden disintegration of the fiber cells is suggestive of a necrotic process. However, the pattern of nuclear pyknosis and TUNEL reaction indicates that an apoptotic process is involved. Disruption of fiber differentiation and induction of apoptosis have also been shown to occur when fibers are induced to inappropriately enter the cell cycle (Chen et al., 2000; Fromm et al., 1994; Pan and Griep, 1994). As TGFβ has been shown to regulate the cell cycle, it is possible that inhibition of TGFβ signaling altered cell cycle exit in lens fibers. However, the BrdU-labeling experiments indicate that expression of ΔkTβRII did not significantly affect cell cycle progression in epithelial or fiber cells.

Transgene expression also resulted in distinct changes in the lens epithelium (multilayering, apoptosis, filensin expression). However, these are likely to be secondary as the transgenes were not expressed in the epithelium. Additionally, the changes in the epithelium occurred only in regions overlying degenerate fibers, suggesting that the effects were due to disruption of the fibers. Previous studies have demonstrated epithelial multilayering after fiber cell disruption in transgenic mice (Stolen and Griep, 2000) or in mice that carry a mutation in the MIP gene (*Cat*^{FR}) (Shiels and Bassnett, 1996; Shiels et al., 2000; Zwaan and Williams, 1969). Additionally, there is evidence for direct communication between epithelial and fiber cells (Bassnett et al., 1994; Rae et al., 1996). Together these

studies suggest that the integrity of the epithelium is dependent, at least to some extent, upon the correct differentiation and formation of the fiber cells. Hence, the effects observed in the epithelium are likely to be due directly to disruption of the fiber-epithelial interface. Alternatively, the changes may be due to factors released by the degenerate fibers.

Effects on gene expression

Expression of dominant-negative receptors in lens fibers resulted in the modulation of expression of several genes. In particular, there was prolonged or increased expression of α- and β-crystallins, and MIP, and also changes in localization of the intermediate filaments, CP49 and CP115.

Crystallins and intermediate filaments

Consistent with a previous study (Ueda et al., 2000), which showed that αA-crystallin promoter activity in lens explants was negatively regulated by TGFβ, this study has shown that inhibition of TGFβ signaling by expression of a dominant-negative TβR in lens fibers resulted in increased expression of αB-crystallin. Increased βB2-crystallin expression was also observed, but only in the most severely affected line (OVE550), suggesting that there may be different thresholds for regulation of the crystallin genes by TGFβ. Alternatively, the increased expression of β-crystallin may be a secondary effect, occurring only when fiber cell maturation is compromised in the transgenic lens. α-crystallins have long been used as a marker of lens differentiation in vivo and in vitro (reviewed by Chamberlain and McAvoy, 1998). However, there is increasing evidence that α-crystallins may actually play an important and active role during the differentiation process. It was recently demonstrated that loading bovine epithelial cells with α-crystallins (particularly αB crystallin), but not β- or γ-crystallin, resulted in morphological changes characteristic of differentiating epithelial cells (Boyle and Takemoto, 2000), directly implicating α-crystallin in differentiation of epithelial cells into fibers. α-crystallins may play a variety of roles in lens cells. They have been shown to act as molecular chaperones (Horwitz, 1992; Nicholl and Quinlan, 1994), associate with cell membranes (Ifeanyi and Takemoto, 1990), localize to the nucleus (Bhat et al., 1999), bind actin (Del Vecchio et al., 1984) and complex with the intermediate filament proteins filensin and phakinin (Carter et al., 1995; Nicholl and Quinlan, 1994) to form the beaded filaments. Of particular relevance to the present study is the interaction of α-crystallin with the intermediate filaments, filensin (CP115) and phakinin (CP49). Similar to the altered expression of αA-crystallin, there was markedly increased expression of these filament proteins in cortical fibers of transgenic lenses. In particular, there were concentrations of CP49 in cortical fibers that were subsequently destined to degenerate. This localization pattern suggests that inappropriate accumulation of CP49 is one of the molecular events that leads to fiber degeneration. It is known that filensin is extensively processed during fiber differentiation, being proteolytically cleaved into distinct peptide fragment sets that are distinctly localized within the fiber and appear to have different functions (Sandilands et al., 1995). As α-crystallin has been shown to be a chaperone that is capable of protecting proteins from such proteolytic cleavage, it is possible that the increased expression of α-crystallin may alter the balance of the filensin fragment sets in the differentiated transgenic fiber cell. However, it remains to be determined whether such altered proteolytic processing occurs in the transgenic lenses.

Furthermore, the developmental expression patterns of CP115 and CP49 (Blankenship et al., 2001) correlate with the developmental appearance of the phenotype in the transgenic (OVE550) lenses. By immunohistochemistry the beaded filament proteins first appear in the anterior cytoplasm of terminally differentiating embryonic fiber cells between E14.5 and E17.5 (Blankenship et al., 2001). The appearance of the fiber degeneration phenotype in the OVE550 line was first detectable in the anterior region of fiber cells at E18.5. Taken together, these findings indicate that disruption of beaded filament expression, assembly or complexing precedes fiber cell degeneration in the transgenic lens and reinforce the notion that the beaded filament network may be required for stabilization of the differentiated fiber cell phenotype (Blankenship et al., 2001).

MIP

MIP (MP26), the most abundant lens-specific membrane protein (Yancey et al., 1988), is known to function as a water channel (Varadaraj et al., 1999) and may also have cell adhesion functions (Fotiades et al., 2000). Distinct mutations of the *Mip* gene result in hereditary cataracts in mouse (*Lop* and Cat^{Fr}; Shiels and Bassnett, 1996) and human (Francis et al., 2000a; Francis et al., 2000b). The phenotype of the Cat^{Fr} lens, which involves degeneration of central lens fibers, nuclear pyknosis, multilayering of epithelium overlying degenerated fibers, but relatively normal cortical fibers (Zwaan and Williams, 1969), is very similar to the phenotype of the ΔkTβR lenses. The ΔkTβR lenses exhibited markedly increased MIP expression in those fibers that preceded the zone of degeneration, whereas MIP expression was lost abruptly before nuclear pyknosis and apoptosis. These results indicate that, in the normal lens, TGFβ signaling may play a role in modulating MIP expression in the fibers and that altered MIP expression precedes fiber degeneration in the ΔkTβR lenses. Although at present it is not clear by what mechanism MIP might be involved, if any, the similarity of the Cat^{Fr} and ΔkTβR phenotypes indicate that disturbance of MIP function or expression has serious consequences for lens fiber cell viability.

Inhibition of migratory response on laminin

The in vitro experiments showed that expression of a dominant-negative TGFβ receptor in lens cells results in a loss of ability to migrate on a laminin substratum. Moreover, the effects appear to be specific and a direct result of transgene expression. Transgenic epithelial cells, in which expression of the transgene had not been induced were still able to migrate on laminin and to assemble filamentous actin similar to wild-type cells. However, induction of α-crystallin promoter activity with FGF to induce expression of the transgene resulted in inhibition of cell migration and failure to assemble filamentous actin. As no exogenous TGFβ was added to these cultures, it would suggest that endogenous levels of TGFβ are sufficient and required to permit wild-type cells to migrate. The most likely source of TGFβ in the system is the lens epithelial cells, as they have been shown to express TGFβ2 mRNA and protein (Gordon-Thomson et al., 1998) and to release TGFβ2 into the

culture medium (Allen et al., 1998). The results also suggest that TGFβ signaling may be required for stabilization of the actin cytoskeleton during fiber cell migration/movement and differentiation of fiber cells. Indeed, the phenotype of the fiber cells in the transitional zone and outer cortex of transgenic lenses provides some support for this. In the transitional zone of transgenic lenses, particularly at embryonic stages, fiber cells appeared shorter than normal and there was an accumulation of cell nuclei. Taken together, this suggests impaired elongation and/or movement of these fiber cells into the fiber mass. Preliminary RT-PCR experiments (data not shown) indicate that the phenotype is not accompanied by changes in expression of α6β1 integrin, the putative laminin receptor for lens fiber cells (McAvoy et al., 2000; Menko and Philip, 1995; Walker and Menko, 1999). The mechanisms responsible for the altered actin cytoskeleton are as yet unclear, but may involve altered activation of integrins or α-crystallin. It has been shown, in colonic goblet cells, that expression of a dominant-negative TGFβ receptor greatly inhibited the conversion of β1 integrin precursor to its active form (Deng et al., 1999). A lack of active β1 integrin protein in the transgenic lens cells may explain the lack of migration by these cells on laminin. Alternatively, α-crystallin has been shown to interact with actin (Del Vecchio et al., 1984); therefore it is possible that the increased α-crystallin expression influenced the organization of the actin filaments in the differentiating fiber cells.

The phenotype of the ΔkTβR lenses bears some similarities to lenses that overexpress a truncated FGFR1 (OVE498; Robinson et al., 1995a). However, the inhibition of fiber differentiation in ΔkFGFR1 lenses was more severe, particularly at later stages of development. At P14, almost all fibers in OVE498 lenses appeared markedly degenerate, whereas the ΔkTβR lines retained a relatively normal region of cortical lens fibers at postnatal stages. This suggests that TGFβ signaling functions at a later stage of fiber differentiation than FGF signaling. Interestingly, null mutations of the individual TGFβ genes have not resulted in any significant lens degeneration phenotypes (Kaartinen et al., 1995; Kulkarni et al., 1993; Proetzel., et al., 1995; Sanford et al., 1997) apart from a slightly smaller lens in the TGFβ2 null mouse at E18.5 (Sanford et al., 1997). This suggests that, with respect to lens differentiation, there is considerable overlap in the function of the TGFβs, detected in the eye (Granstein et al., 1990; Kurosaka and Nagamoto, 1994; Lutty et al., 1993).

In conclusion, these studies have shown that there is a requirement for TGFβ signaling during terminal lens fiber differentiation. Previous in vitro and in vivo studies (reviewed by Chamberlain and McAvoy, 1998) have indicated that FGF is required to initiate the fiber differentiation process. However, other studies indicate that once differentiation is initiated, signaling by other growth factors may be important for maintaining or sustaining fiber differentiation and maturation. For example, in vitro studies have shown that IGF can sustain the accumulation of fiber-specific crystallins (Leenders et al., 1997; Klok et al., 1998) in the absence of exogenous FGF. Other in vitro studies have shown that epidermal growth factor receptor signaling augments expression of cytoskeletal components of differentiating chick annular pad cells (Ireland and Mrock 2000). We present evidence that TGFβ signaling is also required during fiber differentiation. TGFβ signaling modulates expression of fiber-specific proteins and influences elements of the cytoskeleton during fiber differentiation. Its requirement at early stages of fiber differentiation is indicated by reduced fiber elongation in the transitional zone of ΔkTβR lenses. The importance of ongoing TGFβ signaling for terminal differentiation, maturation and survival fiber cells is demonstrated by the subsequent fiber degeneration in the ΔkTβR lenses. Thus, TGFβ signaling appears to be one of the important events initiated during lens fiber differentiation and may represent one of the key elements that is required for the normal completion of this process.

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REFERENCES

Allen, J. B., Davidson, M. G., Nasisse, M. P., Fleisher, L. N. and McGahan, M. C. (1998). The lens influences aqueous humor levels of transforming growth factor-beta 2. *Graefes Arch. Clin. Exp. Ophthalmol.* **236**, 305-311.

Bassnett, S., Kuszak, J. R., Reinisch, L., Brown, H. G. and Beebe, D. C. (1994). Intercellular communication between epithelial and fiber cells of the eye lens. *J.Cell Sci.* **107**, 799-811.

Beebe, D. C. and Coats, J. M. (2000). The lens organizes the anterior segment: specification of neural crest cell differentiation in the avian eye. *Dev. Biol.* **220**, 424-431.

Bhat, S. P., Hale, I. L., Matsumoto, B. and Elghanayan, D. (1999). Ectopic expression of alpha-B crystallin in Chinese hamster ovary cells suggests a nuclear role for this protein. *Eur. J. Cell Biol.* **78**, 143-150.

Blankenship, T. N., Hess, J. F. and FitzGerald, P. G. (2001). Development- and differentiation-dependent reorganization of intermediate filaments in fiber cells. *Invest. Ophthalmol. Vis. Sci.* **42**, 735-742.

Boyle, D. L. and Takemoto, L. (2000). A possible role for α-crystallins in lens epithelial cell differentiation. *Mol. Vis.* **6**, 63-71.

Brand, T. and Schneider, M. D. (1995). Inactive type II and type I receptors for TGFβ are dominant inhibitors of TGFβ-dependent transcription. *J. Biol. Chem.* **270**, 8274-8284.

Brand, T., MacLellan, W. R. and Schneider, M. D. (1993). A dominant-negative receptor for type β transforming growth factors created by deletion of the kinase domain. *J. Biol. Chem.* **268**, 11500-11503.

Carter, J. M., Hutcheson, A. M. and Quinlan, R. A. (1995). In vitro studies on the assembly properties of the lens proteins CP49, CP115: coassembly with α-crystallin but not with vimentin. *Exp. Eye Res.* **60**, 181-192.

Chamberlain, C. G. and McAvoy, J. W. (1998). Fiber differentiation and polarity in the mammalian lens: A key role for FGF. *Prog. Ret. Eye Res.* **16**, 443-478.

Chen, Q., Hung, F-Q., Fromm, L. and Overbeek, P. A. (2000). Induction of cell cycle entry and cell death in postmitotic lens fiber cells by overexpression of E2F1 or E2F2. *Invest. Ophthalmol. Vis. Sci.* **41**, 4223-4231.

Chow, R. L., Roux, G. D., Roghani, M., Palmer, M. A., Rifkin, D. B., Moscatelli, D. A. and Lang, R. A. (1995). FGF suppresses apoptosis and induces differentiation of fiber cells in the mouse lens. *Development* **121**, 4383-4393.

de Iongh, R. U., Gordon-Thomson, C., Hales, A. M., Chamberlain, C. G. and McAvoy, J. W. (2001). TGFβ receptor expression in lens, implications for differentiation and cataractogenesis. *Exp. Eye Res.* **72**, 649-659.

Del Vecchio, P. J., MacElroy, K. S., Rosser, M. P. and Church, R. L. (1984). Association of α-crystallin with actin in cultured lens cells. *Curr. Eye Res.* **3**, 1213-1219.

Deng, X., Bellis, S., Yan, Z. and Friedman, E. (1999). Differential

responsiveness to autocrine and exogenous transforming growth factor (TGF) beta1 in cells with nonfunctional TGF-receptors type III. *Cell Growth Diff.* **10**, 11-18.

Dudley, A. T., Lyons, K. M. and Robertson, E. J. (1995). A requirement for bone morphogenetic protein-7 during development of the mammalian kidney and eye. *Genes Dev.* **9**, 2795-2807.

Filvarof, E. H., Ebner, R. and Derynck, R. (1994). Inhibition of myogenic differentiation in myoblasts expressing a truncated type II TGF-beta receptor. *Development* **120**, 1085-1095.

Fotiades, D., Hasler, L., Muller, D. J., Stahlberg, H., Kistler, J. and Engel, A. (2000). Surface tongue-and-groove contours on lens MIP facilitate cell-to-cell adherence. *J. Mol. Biol.* **300**, 779-789.

Francis, P., Berry, V., Bhattacharya, S. and Moore, A. (2000a). Congenital progressive polymorphic cataract caused by a mutation in the major intrinsic protein of the lens, MIP (AQP0). *Br. J. Ophthalmol.* **84**, 1376-1379.

Francis, P., Chung, J. J., Yasui, M., Berry Moore, A., Wyatt, M. K., Wistow, G., Bhattarchaya, S. S. and Agre, P. (2000b). Functional impairment of lens aquaporin in two families with dominantly inherited cataracts. *Hum. Mol. Genet.* **22**, 2329-2334.

Franzén, P., Heldin, C-H. and Miyazono K. (1995). The GS domain of the transforming growth factor-β type I receptor is important in signal transduction. *Biochem. Biophys. Res. Commun.* **207**, 682-689.

Fromm, L., Shawlot, W., Gunning, K., Butel, J. S. and Overbeek, P. A. (1994). The retinoblastoma protein-binding region of simian virus 40 large T antigen alters cell cycle regulation in lenses of transgenic mice. *Mol. Cell Biol.* **14**, 6743-6754.

Furuta, Y. and Hogan, B. L. M. (1998). BMP4 is essential for lens induction in the mouse embryo. *Genes Dev.* **12**, 3764-3775.

Go, C., He, W., Zhong, L., Li, P., Huang, J., Brinkley, B. R. and Wang, X. J. (2000). Aberrant cell cycle progression contributes to the early-stage accelerated carcinogenesis in transgenic epidermis expressing the dominant-negative TGFbetaRII. *Oncogene*, **19**, 3623-3631.

Gordon-Thomson, C., de Iongh, R. U., Hales, A. M., Chamberlain, C. G. and McAvoy, J. W. (1998). Differential cataractogenic potency of TGFβ1, -β2, and -β3 and their expression patterns in the postnatal rat eye. *Invest. Ophthalmol. Vis. Sci.* **39**, 1399-1409.

Govindarajan, V. and Overbeek, P. A. (2001). Secreted FGFR3, but not FGFR1, inhibits lens fiber differentiation. *Development* **128**, 1617-1627.

Granstein, R. D., Staszewski, R., Knisely, T. L., Zeira, E., Nazareno, R., Latina, M. and Albert, D. M. (1990). Aqueous humour contains transforming growth factor-β and a small (<3500 daltons) inhibitor of thymocyte proliferation. *J. Immunol.* **144**, 3021-3027.

Hales, A. M., Chamberlain, C. G. and McAvoy, J. W. (1992). Measurement of lens epithelial cell migration on a laminin substratum using image analysis. *J. Comput. Assist. Microsc.* **4**, 135-139.

Hales, A. M., Schulz, M. W., Chamberlain, C. G. and McAvoy, J. W. (1994). TGFβ1 induces lens cells to accumulate α-smooth muscle actin, a marker for subcapsular cataracts. *Curr. Eye Res.* **13**, 885-890.

Hales, **A. M.**, **Chamberlain**, **C. G. and McAvoy**, **J. W.** (1995). Cataract induction in lenses cultured with transforming growth factor-β. Invest. *Ophthalmol. Vis. Sci.* **36**, 1709-1713.

He, W. W., Gustafson, M. L., Hirobe, S. and Donahue, P. K. (1993). Developmental expression of four novel serine/threonine kinase receptors homologous to the activin/transforming growth factor-β type II family. *Dev. Dyn.* **196**, 133-142.

Hocevar, B. A. and Howe, P. H. (1998). Mechanisms of TGF-beta-induced cell cycle arrest. *Miner. Electrolyte Metab.* **24**, 131-135.

Honda, S., Fujii, S., Sekiya, Y. and Yamamoto, M. (1996). Retinal control on the axial length mediated by transforming growth factor-β in chick eye. *Invest. Ophthalmol. Vis Sci.* **37**, 2519-2526.

Horwitz, J. (1992). Alpha crystallin can function as a molecular chaperone. *Proc. Natl. Acad. Sci. USA* **89**, 10449-10453.

Ifeanyi, F. and Takemoto, L. (1990). Specificity of alpha crystallin binding to the lens membrane. *Curr. Eye Res.* **9**, 259-265.

Ireland, M. E. and Mrock, L. K. (2000). Differentiation of chick lens epithelial cells: involvement of the epidermal growth factor receptor and endogenous ligand. *Invest. Ophthalmol. Vis. Sci.* **41**, 183-190.

Joseph, H., Gorska, A.E., Sohn, P., Moses, H. L. and Serra, R. (1999). Overexpression of a kinase-deficient transforming growth factor–beta type II receptor in mouse mammary stroma results in increased epithelial branching. *Mol. Biol. Cell* **10**, 1221-1234.

Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N. and Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGFβ3 indicates defects of epithelial-mesenchymal interaction. *Nat. Genet.* **11,** 415-421.

Klok, E. J., Lubsen, N. H., Chamberlain, C. G. and McAvoy, J. W. (1998). Induction and maintenance of differentiation of rat lens epithelium by FGF-2, insulin and IGF-1. *Exp. Eye Res.* **67**, 425-431.

Kulkarni, A.B., Huh, C. G., Becker, D., Geiser, A., Lyght, M., Flanders, K. C., Roberts, A. B., Sporn, M. B., Ward, J. M. and Karlsson, S. (1993). Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc. Natl. Acad. Sci. USA* **90**, 770-774.

Kurosaka, D. and Nagamoto, T. (1994). Inhibitory effect of TGFβ2 in human aqueous humor on bovine epithelial cell proliferation. *Invest. Ophthalmol. Vis. Sci.* **35**, 3408-3412.

Leenders, W. P., van Genesen, S. T., Schoenmakers, J. G., van Zoelen, E.J. and Lubsen, N. H. (1997). Synergism between temporally distinct growth factors: bFGF, insulin and lens cell differentiation. *Mech. Dev.* **67**, 193-201.

Liu, J., Hales, A. M., Chamberlain, C. G. and McAvoy, J. W. (1994). Induction of cataract-like changes in rat lens epithelial explants by transforming growth factor-β. *Invest. Ophthalmol. Vis. Sci.* **5**, 388-401

Lovicu, F. J. and Overbeek, P. A. (1998). Overlapping effects of different members of the FGF family on lens fiber differentiation in transgenic mice. *Development* **125**, 3365-3377.

Luo, G., Hofmann, C., Bronckers, A. L., Sohocki, M., Bradley, A. and Karsenty, G. (1995). BMP-7 is an inducer of nephrogenesis and is also required for eye development and skeletal patterning. *Genes Dev.* **9**, 2808-2820.

Lutty, G. A., Merges, C., Threlkeld, A. B., Crone, S. and McLeod, D. S. (1993). Heterogeneity in localization of isoforms of TGFβ in human retina, vitreous, and choroid. *Invest. Ophthalmol. Vis. Sci.* **34**, 477-487.

Massagué, J. (1998). TGFβ signal transduction. *Annu. Rev. Biochem.* **67**, 753-791.

Massagué, J. (2000). How cells read TGFβ signals. *Nat. Rev. Mol. Cell Biol.* **1**, 169-178.

McAvoy, J. W. (1978). Cell division, cell elongation and distribution of α-β- and γ-crystallins in the rat lens. *J. Embryol. Exp. Morphol.* **44**, 149-165.

McAvoy, J. W., Chamberlain, C. G., de Iongh, R. U., Hales, A. M. and Lovicu, F. J. (1999). Lens development. *Eye* **13**, 425-437.

McAvoy, J. W., Wederell, E. D., Chamberlain, C. G. and de Iongh, R. U. (2000). Integrin expression during lens morphogenesis and differentiation. ARVO Abstract. *Invest. Ophthalmol. Vis. Sci.* **41**, S866.

Menko, A. S. and Philip, N. J. (1995). β1 integrins in epithelial tissues: a unique distribution in the lens. *Exp. Cell Res.* **218**, 516-521.

Millan, F. A., Denhez, F., Kondaiah, P. and Akhurst, R. J. (1991). Embryonic gene expression patterns of TGF β1, β2 and β3 suggest different developmental functions in vivo. *Development* **111**, 131-144.

Nicholl, I. D. and Quinlan, R. A. (1994). Chaperone activity of α-crystallins modulates intermediate filament assembly. *EMBO J.* **13**, 945-953.

Pan, H. and Griep, A. E. (1994). Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: implications for tumor suppressor gene function in development. *Genes Dev.* **8**, 1285-1299.

Pelton, R. W., Saxena, B., Jones, M., Moses, H. L. and Gold, L. I. (1991). Immunohistochemical localization of TGFβ1, TGFβ2, and TGFβ3 in the mouse embryo: Expression patterns suggest multiple roles during embryonic development. *J. Cell Biol.* **115**, 1091-1105.

Prescott, A. R., Sandilands, A., Hutcheson, A.M., Carter, J. M., Quinlan, R. A. (1996). The intermediate filament cytoskeleton of the lens: an ever-changing network through development and differentiation. *Ophthalmic Res.* **28 Suppl. 1**, 58-61.

Proetzel, G., Pawlowski, S. A., Wiles, M. V., Yin, M., Boivin, G. P., Howles, P. N., Ding, J., Ferguson, M.W. and Doetschman, T. (1995). Transforming growth factor–beta 3 is required for secondary palate fusion. *Nat. Genet.* **11,** 409-414.

Qi, Z., Atsuchi, N., Ooshima, A., Takeshita, A. and Ueno H. (1999). Blockade of type beta transforming growth factor signaling prevents liver fibrosis and dysfunction in the rat. *Proc. Natl. Acad. Sci. USA* **96**, 2345-2349.

Rae, J. L., Bartling, C., Rae, J. and Mathias, R. T. (1996). Dye transfer between cells of the lens. *J. Membr. Biol.* **150**, 89-103.

Reneker, L. W., Silversides, D. W., Patel, K. and Overbeek. P. A. (1995). TGF–α can act as a chemoattractant to perioptic mesenchymal cells in developing mouse eyes. *Development* **12**, 1669-1680.

Robinson, M. L., MacMillan-Crow, L. A., Thompson, J. A. and Overbeek, P. A. (1995a). Expression of a truncated FGF receptor results in defective lens development in transgenic mice. *Development* **121**, 3959-3967.

Robinson, M. L., Overbeek, P.A., Verran, D. J., Grizzle, W. E., Stockard, C. R., Friesel, R., Maciag, T. and Thompson, J. A. (1995b). Extracellular FGF-1 acts as a lens differentiation factor in transgenic mice. *Development* **121**, 505-514.

Robinson, M. L., Ohtaka-Maruyama, C., Chan, C. C., Jamieson, S., Dickson, C., Overbeek, P. A. and Chepelinsky, A. B. (1998). Disregulation of ocular morphogenesis by lens-specific expression of FGF-3/int-2 in transgenic mice. *Dev. Biol.* **198**, 13-31.

Rohrer, B. and Stell, W. K. (1994). Basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) act as stop and go signals to modulate postnatal ocular growth. *Exp. Eye Res.* **58**, 553-561.

Sandilands, A., Prescott, A. R., Hutcheson, A. M., Quinlan, R. A., Casselman, J. T. and FitzGerald, P. G. (1995). Filensin is proteolytically processed during lens fiber cell differentiation by multiple independent pathways. *Eur. J. Cell Biol.* **67**, 238-253.

Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman,R., Boivin, G. P., Cardell, E. L. and Doetschman, T. (1997). TGFβ2 knockout mice have multiple developmental defects that are non-overlapping with other TGF beta knockout phenotypes. *Development* **124**, 2659-2670.

Shiels, A. and Bassnett, S. (1996). Mutations of the founder of the MIP gene family underlie cataract development in the mouse. *Nature Genet.* **12**, 212-215.

Shiels, A. Mackay, D., Al Ghoul, K. and Kuszak, J. (2000). Disruption of lens fiber cell architecture in mice expressing a chimeric AQP0-LTR. *FASEB J.* **14**, 2207-2212.

Srinivasan, Y., Lovicu, F. J. and Overbeek, P. A. (1998). Lens-specific expression of transforming growth factor β1 in transgenic mice causes anterior subcapsular cataracts. *J. Clin. Invest.* **101**, 625-634.

Stolen, C. M. and Griep, A. E. (2000). Disruption of lens fiber cell differentiation and survival at multiple stages by region-specific expression of truncated FGF receptors. *Dev. Biol.* **217**, 205-220.

Ueda, Y., Chamberlain, C. G., Satoh, K. and McAvoy. J. W. (2000). Inhibition of FGF-induced αA-crystallin promoter activity in lens epithelial explants by TGFβ. *Invest. Ophthalmol. Vis. Sci.* **41,** 1833-1839.

Varadaraj, K., Kushmerick, C., Baldo, G. J., Bassnett, S., Shiels, A. and Mathias, R. T. (1999). The role of MIP in lens fiber cell membrane transport. *J. Membr. Biol.* **170**, 191-203.

Walker, **J. L. and Menko**, **A. S.** (1999). α6 integrin is regulated with lens cell differentiation by linkage to the cytoskeleton and isoform switching. *Dev. Biol.* **210**, 497-511.

Wawersik, S., Purcell, P., Rauchman, M., Dudley, A. T., Robertson, E. J. and Maas, R. (1999). BMP7 acts in murine lens placode development. *Dev. Biol.* **207**, 176-188.

Wigle, J. T., Chowdhury, K., Gruss, P. and Oliver, O. (1999). Prox1 is crucial for mouse lens-fibre elongation. *Nat. Genet.* **21**, 318-322.

Yancey, S. B., Koh, K., Chung, J. and Revel, J. P. (1988). Expression of the gene for main intrinsic polyptide (MIP): separate spatial distributions of MIP and β-crystallin gene transcripts in rat lens development. *J.Cell Biol.* **106**, 705-714.

Zhao, J., Sime, P. J,. Bringas, P., Gauldie, J. and Warburton, D. (1998). Epithelium-specific adenoviral transfer of a dominant-negative mutant TGF-beta type II receptor stimulates embryonic lung branching morphogenesis in culture and potentiates EGF and PDGF-AA. *Mech. Dev.* **72**, 89-100.

Zwaan, J. and Williams, R. M. (1969). Morphogenesis of the eye lens in a mouse strain with hereditary cataracts. *J. Exp. Zool.* **169**, 407-421.