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Overlapping effects of different members of the FGF family on lens fiber differentiation in transgenic mice

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SUMMARY

Fibroblast growth factors (FGFs), such as FGF-1, have been shown to induce differentiation of lens epithelial cells both in tissue culture and in transgenic mice. In the present study, using the αA-crystallin promoter, we generated transgenic mice that express different FGFs (FGF-4, FGF-7, FGF-8, FGF-9) specifically in the lens. All four FGFs induced changes in ocular development. Microphthalmic eyes were evident in transgenic mice expressing FGF-8, FGF-9 and some lines expressing FGF-4. A developmental study of the microphthalmic eyes revealed that, by embryonic day 15, expression of these FGFs induced lens epithelial cells to undergo premature fiber differentiation. In less severely affected lines expressing FGF-4 or FGF-7, the lens epithelial cells exhibited a premature exit from the cell cycle and underwent a fiber differentiation response later in development, leading to cataract formation. The responsiveness of lens cells to different FGFs indicates that these proteins stimulate the same or overlapping downstream signalling pathway(s). These overlapping effects of different FGFs on a common cell type indicate that the normal developmental roles for these genes are determined by the temporal and spatial regulation of their expression patterns. The fact that any of these FGFs can induce ocular defects and loss of lens transparency implies that it is essential for the normal eye to maintain very specific spatial control over FGF expression in order to prevent cataract induction.

Key words: FGF, FGF receptor, Lens, Fiber differentiation, Transgenic mice, Mouse

INTRODUCTION

Polypeptide growth factors play important roles in controlling cellular behaviour and differentiation decisions. Fibroblast growth factors (FGFs) constitute a large family of at least 17 distinct polypeptide growth factors (FGF-1 to FGF-17; Burgess and Maciag, 1989, Jaye et al., 1992, Tanaka et al., 1992; Miyamoto et al., 1993; Baird, 1994; Yamasaki et al., 1996; Coulier et al., 1997; McWhirter et al., 1997; Miyake et al., 1998; Hoshikawa et al., 1998) that play pivotal roles in a variety of developmental events, including embryogenesis, limb, ear, bone and skin differentiation, organogenesis, angiogenesis and growth (see Basilico and Moscatelli, 1992; Olwin et al., 1994). Although FGFs share structural similarities, they differ in their target specificities and spatial and temporal expression patterns. The prototype FGFs, FGF-1 and FGF-2, are widely expressed throughout development and continue to be expressed in adult tissues, implicating that they may be important for normal tissue homeostasis. The expression of some of the other FGFs, originally isolated as oncogenes (eg. FGF-3, FGF-4, FGF-5 and FGF-6), is predominantly restricted to embryonic tissues and certain types of cancers (see Basilico and Moscatelli, 1992).

The FGFs bind to and activate high-affinity cell surface receptors with intrinsic protein tyrosine kinase activity. In mammals, four closely related FGF receptor genes (encoding FGFR1 to FGFR4) have been identified and cloned (see Givol and Yayon, 1992; Johnson and Williams, 1993). Each of the receptor gene products is composed of an extracellular ligand-binding domain that contains up to three immunoglobulin (Ig)-like domains, a single transmembrane domain and a cytoplasmic tyrosine kinase domain that is activated upon ligand binding. The FGFRs are differentially expressed as various isoforms. Alternative exon usage in the C-terminal half of the third Ig-like domain for three of these receptors (FGFR1 to FGFR3) gives rise to transcript variants referred to as 'IIIb' and 'IIIc' isoforms. For example, at the FGFR2 locus, two variants are generated in this manner; FGFR2IIIb (also known as keratinocyte growth factor receptor, KGFR) and FGFR2IIIc (*bek*; Miki et al., 1992). The temporal and spatial patterns of expression of the FGFR isoforms suggest that they may play major roles in the determination of cellular responses to different FGFs in a variety of tissues (Ledoux et al., 1992; Givol and Yayon, 1992; Chellaiah et al., 1994). However, downstream pathways that are uniquely activated by each different FGFR isoform have yet to be identified.

The vertebrate ocular lens has been utilised as a model system to examine the molecular mechanisms by which growth factors regulate developmental processes such as cell proliferation and differentiation (McAvoy et al., 1991;

Robinson et al., 1995a; Reneker and Overbeek, 1996). The lens first develops as a thickening of the embryonic surface ectoderm adjacent to the optic vesicle (presumptive retina). This thickened lens placode invaginates and subsequently separates from the overlying surface ectoderm to form the lens vesicle. The distinct architecture of the lens is established early in development. Posterior lens vesicle cells elongate and make contact with a monolayer of anterior cuboidal epithelial cells, obliterating the lumen of the lens vesicle. Subsequent growth of the lens throughout life involves proliferation of the epithelial cells. At the lens equator, these epithelial cells are induced to differentiate into secondary fiber cells. The fiber cells undergo a distinctive elongation as they line up and encompass the previously differentiated fiber cells. As a result, the oldest fiber cells are located at the center of the lens, the youngest fiber cells are at the periphery and the lens maintains a cap of cuboidal epithelial cells. The lens fiber cells are postmitotic, express specific proteins such as β-crystallin and γ-crystallin and with continued maturation eventually lose their cytoplasmic organelles and nuclei (for review, see McAvoy, 1981). The precise spatial regulation of cell proliferation and differentiation in the lens is critical for the maintenance of its distinctive architecture and transparency.

Numerous studies have provided support for the hypothesis that FGF signalling plays an important role in development and growth of the lens (for review, see Chamberlain and McAvoy, 1997). In brief, in vitro studies have shown that members of the FGF family such as FGF-1 and FGF-2 can stimulate lens epithelial cell proliferation and differentiation in a dose-dependent manner (McAvoy and Chamberlain, 1989). This is supported by recent in vivo studies from our laboratory; a secreted form of FGF-1 expressed in the lens in transgenic mice induces the premature differentiation of lens epithelial cells (Robinson et al., 1995a). More recent in vivo studies suggest that FGF-2 also acts as a modulator of lens fiber cell differentiation and survival (Stolen et al., 1997). Several ocular tissues, including the lens, have been shown to express FGFs (FGF-1 and FGF-2) as well as several of their receptors, including FGFR1, FGFR2 (splice versions IIIb and IIIc) and FGFR3 (de Iongh and McAvoy, 1992, 1993; Lovicu and McAvoy, 1993, Orr-Urtreger et al., 1993; Peters et al., 1993; de Iongh et al., 1996, 1997; Lovicu et al., 1997). Further evidence to support the notion that normal lens fiber differentiation is regulated by FGF signalling has recently been provided by experiments using a signalling-defective FGF receptor. Lens fiber differentiation was impaired by expression of a truncated FGFR (no cytoplasmic domain) specifically in the fiber cells of transgenic mice (Robinson et al., 1995b; Chow et al., 1995). This dominant negative FGFR has been shown to inhibit nearly all FGF ligand and receptor interactions (Ueno et al., 1992). In a tissue like the lens that expresses at least four different FGFRs (see above), the use of this mutant FGFR does not provide information on which specific FGF or FGFR members are involved in lens fiber differentiation.

Over the last few years, other approaches have been adopted to examine the developmental roles of different FGFs and their receptors in vivo. One approach has been to use homologous recombination to generate null alleles in murine embryonic stem cells. The genes encoding FGF-3, FGF-4, FGF-5, FGF-7, FGFR1, FGFR2 and FGFR3 have been inactivated and mutant phenotypes have been demonstrated (Mansour et al., 1993; Hébert et al., 1994; Deng et al., 1994; Yamaguchi et al., 1994; Feldman et al., 1995; Guo et al., 1995; Deng et al., 1996; Colvin et al., 1996). None of these mutant mice exhibit ocular defects. For some of these genes, such as *Fgf-4* (Feldman et al., 1995), *Fgfr1* (Yamaguchi et al., 1994) and *Fgfr2* (Xu et al., 1998) mutations result in early embryonic lethality. As a result, it is still undetermined whether these genes play an essential role in normal ocular development. In addition, for multigene families, the loss of one member of the family may be compensated for by the overlapping expression of a closely related homologue (Schneider et al., 1994). In the present study, we have elected to alter the ocular pattern of FGF gene expression in order to investigate the signalling potential of different FGFs during ocular morphogenesis. We generated transgenic mice expressing different FGFs specifically in the lens to determine whether these FGFs have similar or distinctive effects on ocular development and differentiation. FGF-1 has been previously shown to induce lens fiber differentiation both in vitro (McAvoy et al., 1991) and in vivo (Robinson et al., 1995a); however, it activates all FGFR splice variants (Green et al., 1996; Ornitz et al., 1996). In contrast, some FGFs do not interact with certain FGF receptors. For example, FGF-7, FGF-8 and FGF-9 do not stimulate FGFR1 (Hecht et al., 1995; MacArthur et al., 1995a; Green et al., 1996) and FGF-3, FGF-5 and FGF-7 do not activate FGFR3 (Green et al., 1996; Ornitz et al., 1996). cDNAs encoding FGF-4, FGF-7, FGF-8 (variant 4) and FGF-9 were linked to the lens-specific αA-crystallin promoter and used to generate transgenic mice. Expression of any of these different FGFs in the lens induces epithelial cells to undergo premature differentiation into fiber cells. Our results suggest that stimulation of FGFR2 may be sufficient to induce this lens epithelial differentiation. Furthermore, normal ocular FGF distribution must be tightly regulated since inappropriate FGF accumulation results in cataract formation.

MATERIALS AND METHODS

DNA constructs

Transgenes for FGF-4, FGF-7 (KGF), FGF-8 and FGF-9 were generated by inserting the respective cDNA clones into the polylinker region of the αA-crystallin promoter vector 2 (CPV2; see Reneker et al., 1995) which also contains the small t intron and early region polyadenylation sequences from the SV40 virus (see Fig. 1). In brief, a 600 bp *Bam*HI-*Eco*RI fragment of murine FGF-4 cDNA (Hèbert et al., 1990) was inserted into a *Bam*HI-*Eco*RI digest of CPV2. Similarly, an 800 bp *Bam*HI-*Xho*I fragment of murine FGF-8 cDNA (variant 4; Crossley and Martin, 1995; Tanaka et al., 1992) was subcloned into *Bam*HI-*Sal*I-treated CPV2. For FGF-9, an 850 bp *Hin*dIII-*Eco*RI fragment of murine cDNA (Santos-Ocampo et al., 1996) was ligated into a *Hin*dIII-*Eco*RI digest of CPV2. The crystallin-FGF-4, crystallin-FGF-8 and crystallin-FGF-9 fragments for microinjection were isolated by *Sst*II digestion, agarose gel electrophoresis and recovery from agarose using the Qiaquick or QiaexII gel extraction kits (Qiagen, Hilden, Germany). For FGF-7, a 600 bp fragment was amplified by polymerase chain reaction (PCR; see below) from a cDNA clone encoding human KGF (Guo et al., 1993). The sense primer (5′ ACGCGTCGACACC**ATG**CACAAATGGATACTGAC 3′) for the 5′ end of KGF introduced a *Sal*I site as well as a Kozak ATG consensus sequence (Kozak, 1991). The antisense primer (5′ CGAGGTCGACGGTATCGATA 3′) was complimentary to sequences

in the 3′ untranslated region of the cDNA. The PCR product was agarose gel purified, digested with *Sal*I and *Hin*dIII and inserted into *Sal*I-*Hin*dIII digested CPV2. The DNA fragment for microinjection was isolated from vector sequences by *Not*I digestion and agarose gel electrophoresis as described above.

Generation of transgenic mice

The purified DNA fragments for CPV2-FGF-4 (1.8 kb), CPV2-FGF-7 (1.8 kb), CPV2-FGF-8 (2.0 kb) and CPV2-FGF-9 (2.0 kb) were diluted to a concentration of 2 ng/μl (in 10 mM Tris-HCl, pH 7.4 and 0.1 mM EDTA) and microinjected into individual pronuclei of 1-cell-stage FVB/N mouse embryos (Taketo et al., 1991). Injected embryos were transferred into pseudopregnant ICR female mice and allowed to develop to term or harvested at embryonic day 15 by Caesarean section. Potential transgenic mice were screened by PCR analysis (see below) of genomic DNA isolated from mouse tails (Saiki et al., 1988; Hogan et al., 1994). Founder mice were mated to non-transgenic FVB/N albino mice to establish individual transgenic lines.

Histology

To obtain embryonic tissues for histology, FVB/N female mice (6-8 weeks old) were superovulated by injection with 5 IU of pregnant mare serum gonadotrophin (Calbiochem, San Diego, CA) followed by injection with 5 IU of human chorionic gonadotrophin (HCG, Sigma, St. Louis, MO) 47.5 hours later. After administration of HCG, superovulated females were individually placed overnight with heterozygous transgenic males from different families. The following morning, females were examined for the presence of a copulation plug (embryonic day 0.5). Pregnant mice at various stages following copulation were killed and fetuses delivered by Caesarean section. Fetal heads or postnatal eyes were removed, fixed overnight in 10% phosphate-buffered formalin, dehydrated, embedded in paraffin and processed for routine histology. Transgenic fetuses were identified by PCR analysis of tail DNA (see below). For histochemical analysis, 5 μm thick sections were stained with haematoxylin and eosin.

PCR analyses

To identify transgenic mice, PCR screening was carried out on genomic tail DNA using primers that hybridize to the SV40 sequence present in each transgene (see Fig. 1). Primers used include: 5′-GTGAAGGAACCTTACTTCTGTGGTG-3′, a sense primer (SV40A) that hybridizes to SV40 sequences 5′ to the intron splice site, and 5′-GTCCTTGGGGTCTTCTACCTTTCTC-3′ (SV40B), an antisense primer that hybridizes to SV40 sequences 3′ to the intron splice site. PCR amplifications were performed for 28 to 32 cycles using the following conditions; denaturation (94°C) for 30 seconds, annealing (58°C) for 30 seconds and extension (72°C) for 60 seconds. A final extension was carried out for 2 minutes.

In situ hybridizations

The expression patterns of transgenic and endogenous FGF-4, FGF-8 and FGF-9 transcripts in the eye were examined by in situ hybridization using [^{35}S]UTP-labelled riboprobes as previously described (Robinson et al., 1995a). To assay for transgene expression, a riboprobe containing just the SV40 sequences was used (see Fig. 1). Sense and antisense transcripts were synthesised using T3 and T7 RNA polymerases (Promega), respectively. Antisense riboprobes specific for FGF-4, FGF-8 and FGF-9 were generated from the following: a 600 nt cDNA containing the full-length coding region of murine FGF-4, a 400 nt mouse cDNA for FGF-8, described previously by Crossley and Martin (1995), and an 850 nt cDNA containing the entire murine FGF-9 coding sequence. Hybridizations were performed on sections of embryos collected from timed pregnancies and processed as described above. Hybridization slides coated with photographic emulsion were exposed for 7 to 10 days before developing and counterstaining with Harris haematoxylin.

Immunohistochemistry

For β-crystallin immunohistochemistry, 5 μm sections of embryonic heads or postnatal eyes were hydrated and incubated for 30 minutes in 3% normal goat serum to reduce non-specific staining. Sections were then incubated overnight at 4°C with a polyclonal rabbit antibody specific for β-crystallin (diluted 1:800 with phosphate-buffered saline (PBS) supplemented with 3% normal goat serum). Following a brief rinse in PBS, sections were incubated for 1 hour at room temperature with an anti-rabbit Ig antibody conjugated to fluorescein-isothiocyanate (Vector Laboratories, Inc, Burlingame, CA), rinsed again in PBS and examined using fluorescence microscopy. The polyclonal antibody to β-crystallin was kindly provided by Dr J. S. Zigler (National Eye Institute, Bethesda, MD).

To examine the patterns of DNA replication (cells in S-phase of the cell cycle), incorporation of 5-bromo-2′-deoxyuridine (BrdU) was analysed using immunohistochemistry. Postnatal mice and pregnant female mice at 15.5 days postcopulation were injected intraperitoneally with 100 μg/g BrdU (Sigma) supplemented with one tenth the concentration of 5-fluoro-2′-deoxyuridine (Sigma) diluted with PBS. 1 hour following the injection, postnatal eyes or fetal heads were collected, fixed and processed as previously described (Robinson et al., 1995a). To detect BrdU incorporation, 5 μm thick sections of tissues were hydrated, quenched with 3% H_2O_2 in 10% methanol to block endogenous peroxidase activity and incubated with 0.02% pepsin (20 minutes at 37°C). Following pepsin digestion, sections were treated with 2 N HCl to denature the nucleic acids to facilitate

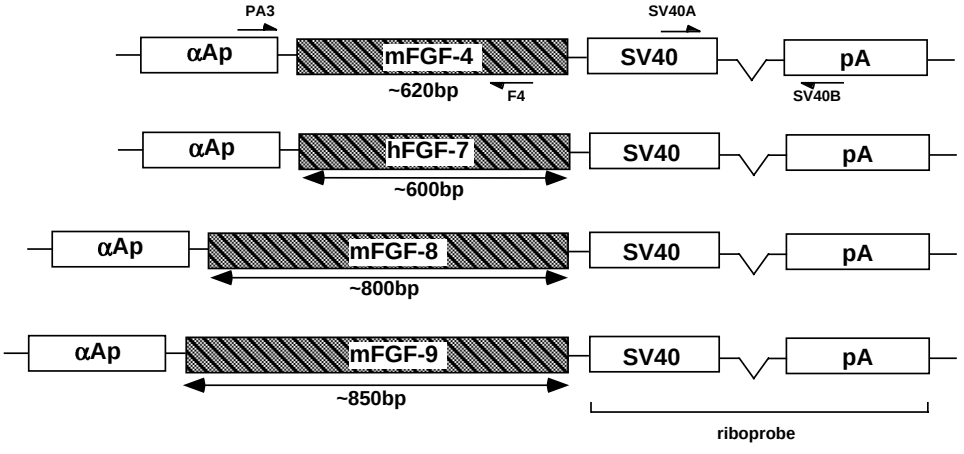

Fig. 1. Microinjection constructs used to generate transgenic mice. Murine cDNAs for FGF-4 (620 bp), FGF-8 (800 bp) and FGF-9 (850 bp) and a human cDNA for FGF-7 (600 bp) were subcloned into the polylinker region of the CPV2 plasmid between the αA-crystallin promoter (αAp) and the intron and polyadenylation sequences of the small t antigen from SV40 (SV40pA). Primers used for screening genomic tail DNA (SV40A/SV40B) and/or for RT-PCR (PA3/F4) are indicated as is the riboprobe that was used for in situ hybridization.

epitope recognition with the primary antibody. Sections were neutralised with 0.1 M sodium borate, pH 8.5, rinsed in PBS and incubated for 30 minutes in 3% normal horse serum to reduce non-specific staining. Sections were then incubated overnight at 4ºC with a monoclonal anti-BrdU antibody (Dako, Carpinteria, CA) diluted 1:50 with PBS. Binding of the primary antibody was visualised using a biotinylated secondary anti-mouse antibody (Vector Laboratories, Inc.), an ABC amplification kit (Vector Laboratories, Inc.) and a diaminobenzidine substrate kit (Vector Laboratories, Inc.) according to manufacturer's specifications. Sections were counterstained with Harris haematoxylin.

RESULTS

Transgenic mice

Transgenic mice were generated with constructs containing cDNAs encoding murine FGF-4, FGF-8, FGF-9 or human FGF-7, driven by the αA-crystallin promoter, which has been shown previously to direct transgene expression to the ocular lens (see Robinson et al., 1995a; Reneker et al., 1995). Potential transgenic founder mice, derived from microinjected FVB/N embryos, were screened by PCR using primers specific to the SV40 region (SV40A/SV40B) at the 3′ end of the constructs (see Fig. 1). Founder mice were mated to non-transgenic FVB/N mice and the following transgenic lines were established: FGF-4 (OVE 814, 815, 816, 817); FGF-7 (OVE 842, 843); FGF-8 (OVE 844, 846) and FGF-9 (OVE 1069, 1070). Mice from all transgenic families displayed ocular phenotypes (described in more detail below). In brief, by weaning, microphthalmic eyes were evident in transgenic mice expressing FGF-8 and some lines expressing FGF-4 (OVE 814, 816, see Fig. 2C) and FGF-9 (OVE 1070). The other lines, expressing FGF-4, FGF-7 or FGF-9, developed cataracts postnatally (see Fig. 2B). Transgenic mice expressing FGF-7 also demonstrated distinctive corneal changes (data not shown; F. J. L. and P. A. O., unpublished observations).

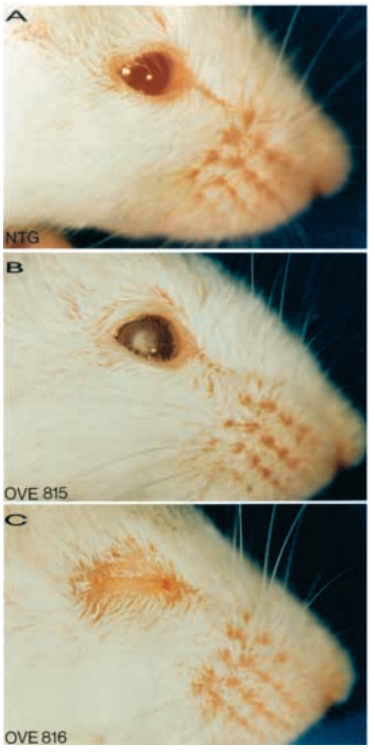

Fig. 2. Cataracts and microphthalmia induced by FGF-4. Representative eyes of postnatal day 21 mice are shown. (A) A non-transgenic (NTG) mouse. (B) A mouse from transgenic family OVE 815 exhibiting lens opacities. (C) A mouse from transgenic family OVE 816 exhibiting microphthalmia.

Lens-specific transgene expression

To assess the specificity of transgene expression, in situ hybridizations were carried out on embryonic heads from transgenic and non-transgenic mice. Using a transgene-specific ^{35}S-labelled riboprobe that hybridizes to the 3′ untranslated SV40 region of the constructs, transgene expression was detected exclusively in the elongating fiber cells of the ocular lens (Fig. 3). No specific hybridization was detected in non-transgenic tissues (see Fig. 3D). Sense riboprobes generated from the same SV40 sequence did not show any specific hybridization in transgenic or non-transgenic tissues (data not shown).

Developmental analyses of transgenic mice

Histological analyses were carried out on eyes from the different transgenic families and immunohistochemistry was used to examine changes in the patterns of cell cycle control (BrdU incorporation) and lens fiber differentiation (expression of β-crystallin).

FGF-4

Transgenic mice expressing FGF-4 specifically in the lens were represented by four families, which demonstrated two different ocular phenotypes, microphthalmia (OVE 814, 816) or postnatal cataractogenesis (OVE 815, 817; see Fig. 2). Transgenic family OVE 814 displayed distinctive changes in the architecture of the lens (Fig. 4). In these mice, as early as

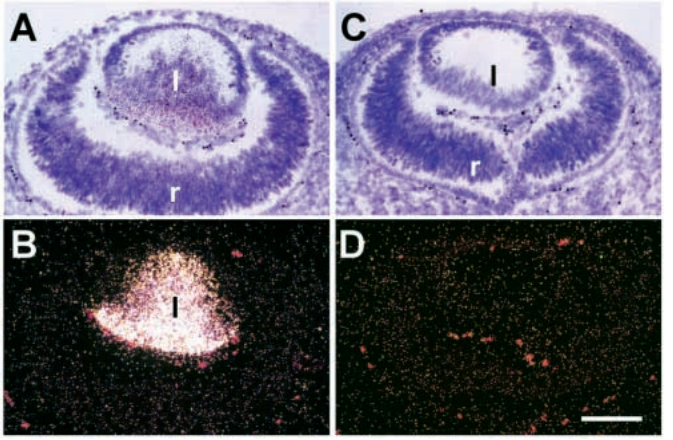

Fig. 3. Lens-specific transcription of CPV2-FGF-4 (family OVE 814). In situ hybridizations carried out with an antisense ^{35}S-labelled riboprobe specific to the SV40 sequence of CPV2-FGF-4. Bright-field (A,C) and dark-field microscopy (B,D) of embryonic day 12.5 transgenic (A,B) and non-transgenic (C,D) eyes are shown. High levels of transgene expression are detected specifically in the elongating fibers of the lens (l). No transgene expression was detected in non-transgenic littermates (C,D). Abbreviations: r, embryonic retina. Scale bar, 100 μm.

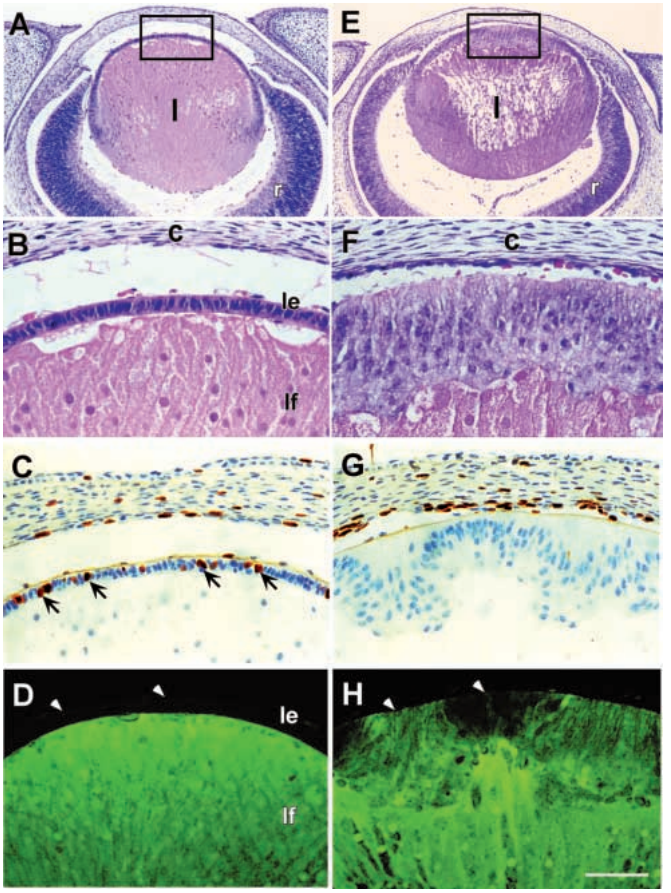

Fig. 4. Induction of cell cycle exit and fiber differentiation by FGF-4. Sections of embryonic day 15.5 non-transgenic (A-D) and OVE 814 transgenic (E-H) eyes either stained with haematoxylin and eosin (A,B,E,F), immunolabelled for BrdU incorporation, counterstained with haematoxylin (C,G) or immunolabelled for β-crystallin expression (D,H). In transgenic family OVE 814, anterior lens epithelial cells undergo extensive elongation (E, box, shown at higher magnification in F), accompanied by their exit from the cell cycle, as indicated by the absence of BrdU labelling (G), and the expression of β-crystallin (H). Small regions of epithelial cells did not express β-crystallin, unlike their more differentiated neighbouring cells (H, arrowheads). In non-transgenic mice, the lens maintains its distinct polarity with a monolayer of cuboidal epithelial cells (A, box, shown at higher magnification in B, le), overlying a full complement of fiber cells (lf). In these mice, numerous cells throughout the anterior lens epithelium show BrdU incorporation (C, arrows), with no detectable β-crystallin expression (D, le, arrowheads). The fiber cells of the transgenic mouse lens (E, l), in particular those situated at the lens nucleus, become vacuolated, do not incorporate BrdU (G) and are reactive for β-crystallin (H). In these mice, non-lenticular cells at the posterior edge of the cornea (c in F) show incorporation of BrdU as indicated by the brown-staining nuclei (G). The retina (r) of the transgenic mouse (E) is also significantly thinner than the retina of the non-transgenic littermate (A). Scale bar, (A,E) 240 μm; (B-D, F-H) 60 μm.

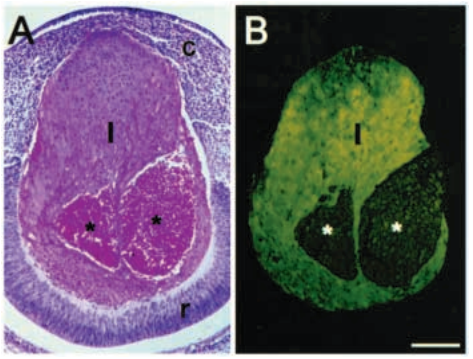

Fig. 5. Lenticular, corneal and retinal developmental changes induced by FGF-4. Representative sections of embryonic day 15.5 eyes from an OVE 816 transgenic mouse, stained with haematoxylin and eosin (A) or immunolabelled for β-crystallin (B). Lenses (l) are characterised by an ovoid shape (A,B) due to a rapid differentiation of the lens epithelial cells, the majority of which express β-crystallin (B). Epithelial-like cells, unreactive for β-crystallin, are still evident at the apical pole of the transgenic lens (B). The lens capsule has ruptured resulting in the infiltration of numerous eosinophilic blood cells within the lens (A, asterisks) which are not reactive for β-crystallin (B, asterisks). The embryonic cornea (c) demonstrates a normal epithelium, however, the underlying mesenchyme is disorganised and contains eosinophilic blood cells. Numerous mesenchymal cells also accumulate at the anterior retinal margin (A). The undifferentiated neuroblast layer of the retina (r) is abnormally thin, contributing to the thinner retina observed in these mice. Scale bar, 150 μm.

E13, lens epithelial cells had begun to change their morphology so that, by E15, the majority of the epithelial cells were no longer cuboidal but instead elongated, reminiscent of the elongation that occurs during normal fiber cell differentiation (Fig. 4E,F). The underlying lens fiber cells, in particular those situated at the lens nucleus became vacuolated (Fig. 4E). In non-transgenic mice (Fig. 4A,B), the lens has a distinct polarity with a monolayer of cuboidal epithelial cells overlying a full complement of elongated fiber cells. In the transgenic mice, lens epithelial cells have not only started to elongate by this stage of development but have also exited from the cell cycle as indicated by the absence of BrdU labelling (Fig. 4G). In comparison, non-transgenic littermates demonstrated numerous labelled cells throughout the lens epithelium (Fig. 4C). Immunohistochemistry also confirmed that these elongating lens epithelial cells are differentiating into fiber-like cells. Using a lens fiber-specific β-crystallin antibody, expression of this protein was observed in most of the elongated central epithelial cells in transgenic mice (Fig. 4D,H). In some regions, cells had exited from the cell cycle and started to elongate but did not express β-crystallin, unlike their more differentiated neighbouring cells (Fig. 4H). Due to the thickening of the lens epithelium observed at E15, the lenses of transgenic mice appeared larger when compared to non-transgenic littermates (Fig. 4A,E). However, by embryonic day 18 (E18), the transgenic lenses ceased growing because the lens epithelial cells had all exited from the cell cycle (data not shown). Interestingly, the retina also undergoes a dramatic reduction in rate of growth, leading to microphthalmia. A decrease in retinal cell numbers is already apparent at E15 (compare retinae in Fig. 4A,E).

Examination of the other microphthalmic family expressing FGF-4 (OVE 816) revealed a more severe phenotype, characterized by an ovoid shape of the lens (Fig. 5A). At E15, the cuboidal lens epithelium is nearly absent, due to the

premature differentiation of the majority of these cells (Fig. 5B). On close examination, epithelial-like cells are still present at the apical pole of the transgenic lens (Fig. 5A). A small population of these cells were positive for BrdU incorporation (data not shown), corresponding with the lack of β-crystallin reactivity (see Fig. 5B) and growth of the lens in this region. In most of the OVE 816 mice, the lens capsule appears to rupture resulting in the infiltration of numerous blood cells within the lens (Fig. 5A, asterisks). These cells do not express β-crystallin (Fig. 5B, asterisks).

Overexpression of FGF-4 in OVE 816 transgenic mice also induced developmental changes in the cornea and retina. Although the embryonic corneal epithelium appeared to be normal, the underlying mesenchyme (presumptive corneal stroma) was thickened, disorganised and appeared vascularized (Fig. 5A). A large number of mesenchymal cells also accumulated at the anterior retinal margins. Similar to OVE 814, the retinae in the OVE 816 transgenic mice were thinner due to the reduction in number of cells constituting the darker staining undifferentiated neuroblast layer. This correlated with a reduction in BrdU labelling in these cells (data not shown).

As mentioned earlier, transgenic mice expressing FGF-4 were represented by four families, two families characterised as microphthalmic (see above) and the other two families (OVE 815, OVE 817) developing cataracts postnatally. The latter two families developed similar phenotypes at similar stages of development, so they will be described collectively. A phenotype was not apparent in these less severely affected transgenic lines until postnatal day 7 when the lens epithelial cells acquired a columnar morphology (Fig. 6B). By postnatal day 14, the epithelial cells begin to multilayer (Fig. 6C) due to increased cell numbers associated with increased levels of BrdU incorporation (Fig. 6E). By postnatal day 17, many of the epithelial cells had exited from the cell cycle (data not shown) and elongated (Fig. 7B), prematurely differentiating into fiber-like cells expressing β-crystallin (Fig. 7D). Underlying fiber cells became vacuolated (Fig. 7B,D), similar to the fiber cells described earlier in the other FGF-4 transgenic families. In non-transgenic littermates, the lens epithelium remains as a monolayer of cuboidal cells (Fig. 7A), unreactive for β-crystallin (Fig. 7C).

As differences in phenotype were observed between the different FGF-4 families, we carried out in situ hybridization to examine transgene expression in all of the FGF-4 families. Representative sections of E15 heads from each transgenic family (OVE 814 to 817) were hybridized with the SV40 riboprobe and processed under identical conditions. Examination of these slides demonstrated a correlation between the levels of transgene expression and the severity of the ocular phenotypes (Fig. 8). The highest levels of transgene expression were detected in OVE 816, which demonstrated the most dramatic lenticular phenotype by E15 (Fig. 8A). The other microphthalmic FGF-4 family, OVE 814, demonstrated modestly lower levels of transgene expression (Fig. 8B). In both of these families, the transgene was transcribed in all of the fiber cells, including the prematurely elongated epithelial cells. Families OVE 815 (Fig. 8C) and OVE 817 (not shown), which do not display a phenotype at E15, had very low levels of expression, comparable to non-transgenic littermates (Fig. 8D); however, using RT-PCR analysis of total lens RNA, these families expressed the transgene postnatally (data not shown), consistent with the onset of the lens phenotype.

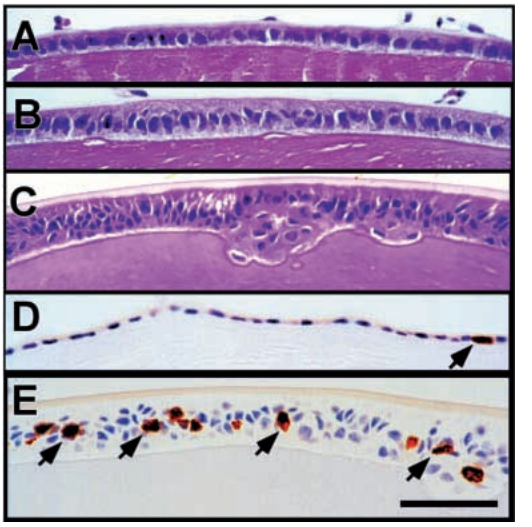

Fig. 6. Delayed lenticular changes induced by FGF-4 in transgenic family OVE 815. Sections of non-transgenic (A,D) and OVE 815 transgenic (B,C,E) lenses from 7-(A,B) and 14-(C-E) day-old mice were stained with haematoxylin and eosin (A-C) or assayed for BrdU incorporation and counterstained with haematoxylin (D,E). At postnatal day 7, lens epithelial cells from transgenic mice begin to elongate and acquire a columnar morphology (B) when compared to non-transgenic littermates (A). By postnatal day 14, the epithelial cells multilayer (C) due to increased cell numbers associated with increased levels of BrdU incorporation (E, arrows). BrdU incorporation in non-transgenic lenses is infrequent in anterior epithelial cells (see D, arrow). Scale bar, 200 μm.

FGF-7

The two transgenic families expressing FGF-7 specifically in the lens (OVE 842, OVE 843) demonstrated similar phenotypes. Histological analysis did not detect a phenotype in the lens of these mice until embryonic day 18 when elongation of the lens epithelial cells became apparent (Fig. 9). In addition, in some lenses, a small number of elongated displaced cells was observed between the epithelial and fiber cells (see Fig. 9B). By postnatal day 1 (P1), the thickening of the epithelium was more prominent (Fig. 9D). Using immunohistochemistry, the elongating epithelial cells were shown to be expressing β-crystallin (Fig. 9F) indicating that the epithelial cells were differentiating into fiber-like cells in response to FGF-7. The elongation of the epithelial cells was also accompanied by a marked reduction in the number of cells incorporating BrdU (Fig. 10). At P1, in the lenses of non-transgenic mice, there are numerous epithelial cells that incorporate BrdU (Fig. 10A, arrows); however, in the transgenic lenses, the majority of these cells have exited from the cell cycle with only a few cells showing immunoreactivity for BrdU (Fig. 10B, arrow). By postnatal day 7, we observed some variability in the lenticular phenotypes. For example, all the epithelial cells in some lenses underwent modest elongation, expressing β-crystallin (Fig. 11B,E). In other lenses, at a comparable age, the epithelial cells underwent a more dramatic response, with a greater thickening of the region of prematurely differentiated fiber cells. Interestingly, in these mice, a new epithelial layer was often established that was not immunoreactive for β-crystallin (see Fig. 11C,F). The

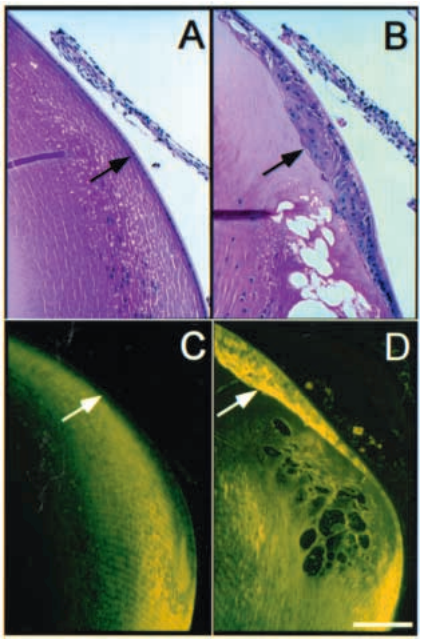

Fig. 7. Induction of β-crystallin expression by FGF-4. Sections of non-transgenic (A,C) and OVE 815 transgenic (B,D) lenses from 17-day-old mice stained with haematoxylin and eosin (A,B) or labelled for β-crystallin expression (C,D). The majority of epithelial cells in the transgenic lens are elongated (B, arrow) and express β-crystallin (D, arrow). Underlying fiber cells become vacuolated (B,D). In non-transgenic littermates, the lens epithelium (arrow) remains as a monolayer of cuboidal cells (A), unreactive for β-crystallin (C, arrow). Scale bar, 100 μm.

lenticular changes observed in both FGF-7 transgenic families are similar to those seen in low-expressing FGF-4 families described earlier (see Fig. 7), resulting in cataract formation by postnatal day 21.

A point worth noting is that, when transgene expression was examined using in situ hybridization, the FGF-7 transgene was expressed as early as E15, specifically in the lens (data not shown; Lovicu and Overbeek, unpublished observations), at comparable levels to those shown for OVE 814 (see Fig. 8B). Although there is no lens phenotype at E15 in transgenic mice expressing FGF-7, there is a dramatic change in the architecture of the differentiating cornea (data not shown; F. J. L. and P. A. O., unpublished observations).

FGF-8

Transgenic mice expressing FGF-8 (variant 4; see Crossley and Martin, 1995) specifically in the lens were represented by two families (OVE 844, OVE 846), both exhibiting microphthalmia. By E15, the lens epithelium except for the most central cells, had clearly elongated into fiber-like cells (Fig. 12B, asterisks). This premature elongation of the epithelial cells was accompanied by expression of the fiber-specific marker, β-crystallin (Fig. 12H) and by exit from the cell cycle (Fig. 12E). At E15, BrdU incorporation was restricted to a small central region of cells that had not yet elongated (Fig. 12E, arrowhead). This central region of cells was not immunoreactive for β-crystallin, similar to the lens epithelium of non-transgenic littermates (compare Fig. 12G with 12H). By E18, all the epithelial cells had exited from the cell cycle, elongated and differentiated into lens fiber cells resulting in a decline in growth of the lens and eye, subsequently leading to microphthalmia, as described above for the FGF-4 transgenic mice (data not shown).

FGF-9

Transgenic mice expressing FGF-9 specifically in the lens were represented by founder mice for two transgenic families (OVE 1069, OVE 1070) and by two mice that were microinjected as 1-cell-stage embryos and then delivered by Caesarean section at E15 for analysis. Similar to FGF-4, the FGF-9 transgenic mice were characterised by two different phenotypes: microphthalmia (OVE 1070) and cataracts (OVE 1069). The ocular phenotypes of the two founder fetuses at E15 were similar to the phenotypes observed for eyes from transgenic mice expressing FGF-8 or high levels of FGF-4 at this same age. From these observations, we would predict that the FGF-9 transgenic founder embryos would have developed microphthalmia if left to develop to full term.

In the lenses of the FGF-9 transgenic embryos, the majority of the peripheral epithelial cells had exited from the cell cycle, with only a small population of central epithelial cells incorporating BrdU (Fig. 12F, arrowhead). The peripheral cells that did not incorporate BrdU were elongated (Fig. 12C, asterisks) and had differentiated into fiber-like cells expressing β-crystallin (Fig. 12I). This premature differentiation of the peripheral epithelial cells altered the architecture of the lens, so that the transitional zones of the lens (normally situated at the lens equator; see Fig. 12G, arrows) became situated near the anterior pole (Fig. 12I, arrows), similar to the FGF-8 transgenic lenses (Fig. 12H, arrows).

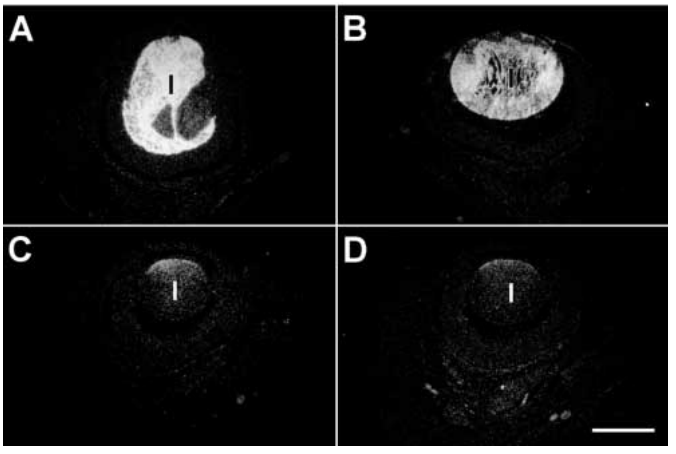

Fig. 8. Ocular phenotypes reflect levels of FGF transgene transcription. In situ hybridizations with an antisense ^{35}S-labelled riboprobe specific to SV40 sequences of the transgenic construct are shown as dark-field images of embryonic day 15.5 eyes from FGF-4 transgenic mice from families OVE 816 (A), OVE 814 (B), OVE 815 (C) and a non-transgenic FVB/N (D) mouse. Levels of transgene expression in the lens (l) were highest in OVE 816 embryos (A) with moderate expression in OVE 814 (B). Transgenic lenses from family OVE 815 (C) show barely detectable expression of transgene, comparable to non-transgenic embryos (D). Different levels of transgene expression clearly correlate with the severity of the lenticular changes. Scale bar, 400 μm.

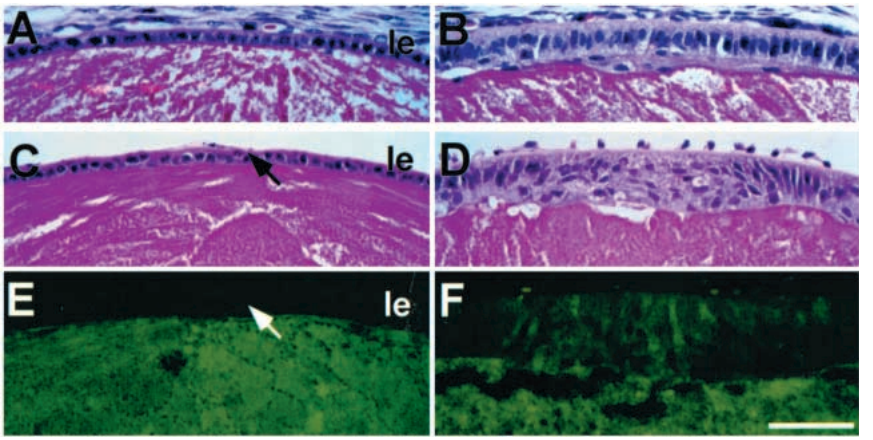

Fig. 9. Lenticular changes induced by FGF-7. Sections of non-transgenic (A,C,E) and OVE 842 transgenic (B,D,F) lenses from embryonic day 18 (A,B,E,F) and neonatal (C,D) mice stained with haematoxylin and eosin (A-D) or labelled for β-crystallin expression (E,F). At embryonic day 18, epithelial cells in transgenic lenses have begun to elongate (B) and express β-crystallin (F). In some lenses, a few elongated cells were observed between the epithelial and fiber cells (B). By postnatal day 1, the epithelium thickens (D) as cells continue to elongate and multilayer. In non-transgenic mice, the lens epithelium (le) remains as a monolayer of cuboidal epithelial cells (C, arrow), unreactive for β-crystallin (E, arrow). Scale bar, 50 μm.

The retinae of the FGF-9 transgenic mice were also clearly affected, appearing thinner at E15 when compared to retinae from non-transgenic littermates (compare Fig. 12A and C). The thinning of the retina, similar to the FGF-4 transgenic mice (OVE 814 and OVE 816), appeared to be due to a reduction in the number of cells constituting the neuroblast layer, consistent with a reduction in BrdU labelling in this cell layer (Fig. 12F) when compared to non-transgenic mice (Fig. 12D). Further characterisation of the retinal phenotype in these mice is currently under investigation.

Transgenic mice expressing FGF-9 that developed cataracts postnatally (OVE 1069) were not characterised in great detail in the present study. The cataracts observed in these mice were similar in severity to those previously described for FGF-4 transgenic families OVE 815 and OVE 817 (see Fig. 2B).

Expression of endogenous FGFs

In order to assay for endogenous ocular expression of FGF-4, FGF-8 and FGF-9, in situ hybridizations were carried out using histology sections from transgenic mice from each of the respective families, as well as from non-transgenic FVB/N mice at various developmental stages. For example, an antisense riboprobe for FGF-4 was used for hybridizations to sections from FGF-4 transgenic mice (OVE 814 and OVE 816), non-transgenic FVB/N mice and mice transgenic for some of the other FGFs (eg. FGF-8) to rule out cross-hybridization. In all cases, each of the specific FGF riboprobes only hybridized to the corresponding transgenic lenses (data not shown). For example, the FGF-4 riboprobe hybridized to transcripts in the lens of FGF-4 transgenic mice only, in a similar pattern to that shown previously using the riboprobe specific for the SV40 region (see Figs 3, 8). No endogenous FGF-4 transcripts were detected in ocular tissues of non-transgenic mice (data not shown). Similar to FGF-4, no endogenous transcripts for FGF-8 or FGF-9 were detected in ocular tissues during development (data not shown). Strong expression for FGF-8 was detected in the differentiating optic stalk, presumptive pituitary gland (Rathke's pouch) and apical ectodermal ridge of the developing limb bud as previously described (data not shown; see Crossley and Martin, 1995).

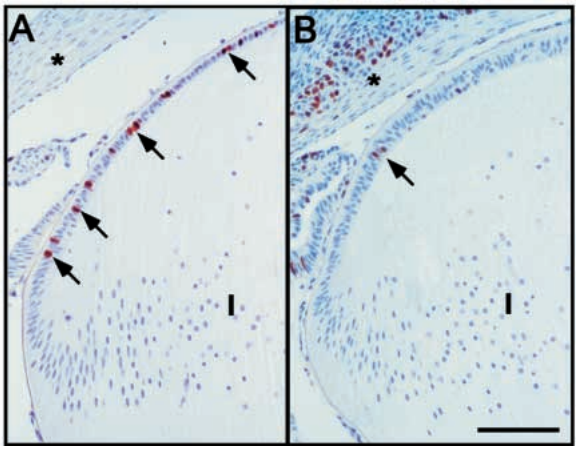

Fig. 10. Cell cycle changes induced by FGF-7. BrdU incorporation in lenses (l) of non-transgenic (A) and OVE 842 transgenic (B) neonatal mice was assayed by immunohistochemistry. In non-transgenic mice, numerous cells throughout the lens epithelium show BrdU incorporation (A, arrows). In the transgenic lens, epithelial cells have begun to elongate and exit from the cell cycle as indicated by the marked reduction of BrdU labelling (B, arrow). Incorporation of BrdU is elevated in the overlying cornea (B, asterisk). Scale bar, 100 μm.

DISCUSSION

In the present study, transgenic mice were generated that express different members of the FGF family specifically in the ocular lens. Using the αA-crystallin promoter, we were able to obtain expression of FGF-4, FGF-7, FGF-8 and FGF-9 in at least two lines of mice for each transgene. Using in situ hybridization, lens-specific transgene expression was observed as early as embryonic day 12 (Fig. 3), using riboprobes specific for either the SV40 region of the transgenic transcripts (see Figs 1 and 8) or the respective FGFs (data not shown). In all cases, transgene expression was restricted to the lens fiber cells, consistent with previous reports using this same promoter (Robinson et al., 1995b; Reneker et al., 1995). Although transgene expression was lens-specific, several ocular tissues, including the lens, cornea, anterior chamber and retina exhibited morphological changes in response to the different FGFs. The lenticular changes fell into two general phenotypes. The most severe phenotype was microphakia (small lens), which was exhibited by transgenic families expressing FGF-4, FGF-8 or FGF-9. In all of the families with readily detectable

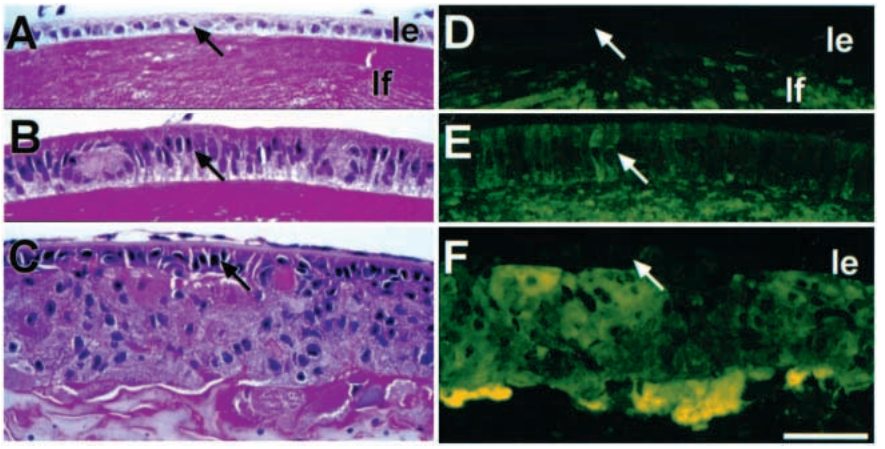

Fig. 11. Postnatal lenticular changes in FGF-7 transgenic mice. Sections of non-transgenic (A,D) and OVE 842 transgenic (B,C,E,F) lenses from 7-day-old mice stained with haematoxylin and eosin (A-C) or labelled for β-crystallin expression (D-F). The epithelial cells (arrows) in some transgenic lenses elongate (B) and express β-crystallin (E, arrow). In other lenses, by postnatal day 7, the epithelial cells undergo a more pronounced differentiation response (C) but also maintain an epithelial layer, unreactive for β-crystallin (F, arrow). Lens epithelial cells (le) of non-transgenic littermates remain as a monolayer of cuboidal cells (A), unreactive for β-crystallin (D). Abbreviations: lf, lens fibers. Scale bar, 50 μm.

embryonic expression of these FGFs, developmental examination revealed that these FGFs efficiently induced lens epithelial cells to exit from the cell cycle, lose their characteristic cuboidal morphology and elongate into fiber-like cells accumulating β-crystallin. In effect, lens epithelial cells prematurely differentiated into fiber cells in response to intralenticular synthesis of the different FGFs. Premature differentiation of the lens epithelium resulted in the loss of the lens stem cell population, and lead to a dramatic reduction in the growth rate of the lens and subsequent microphakia.

An alternative ocular phenotype was observed in FGF-4 transgenic families OVE 815 and OVE 817 as well as both FGF-7 families, OVE 842 and OVE 843. In these transgenic mice, a perinatal or postnatal cellular response was observed, with lens epithelial cells more gradually exiting from the cell cycle, elongating and differentiating into fiber-like cells, consequently leading to cataract formation. Interestingly, in transgenic families OVE 815 and OVE 817, at postnatal day 14, we observed a transient increase in BrdU incorporation in the lens epithelial cells prior to their differentiation into fiber-like cells by postnatal day 17. This transient increase in BrdU incorporation was not observed prior to differentiation, consistent with changes in responsiveness of lens epithelial cells to FGF with age (Lovicu and McAvoy, 1992). Using in situ hybridizations, we were able to correlate the different phenotypes observed for the FGF-4 families with the levels of transgene expression. At E15, the most severely affected FGF-4 family (OVE 816) expressed the highest levels of transgene (Fig. 8). The other FGF-4 microphthalmic family (OVE 814) appeared to have slightly lower levels of transgene expression (Fig. 8), consistent with its less pronounced fiber differentiation

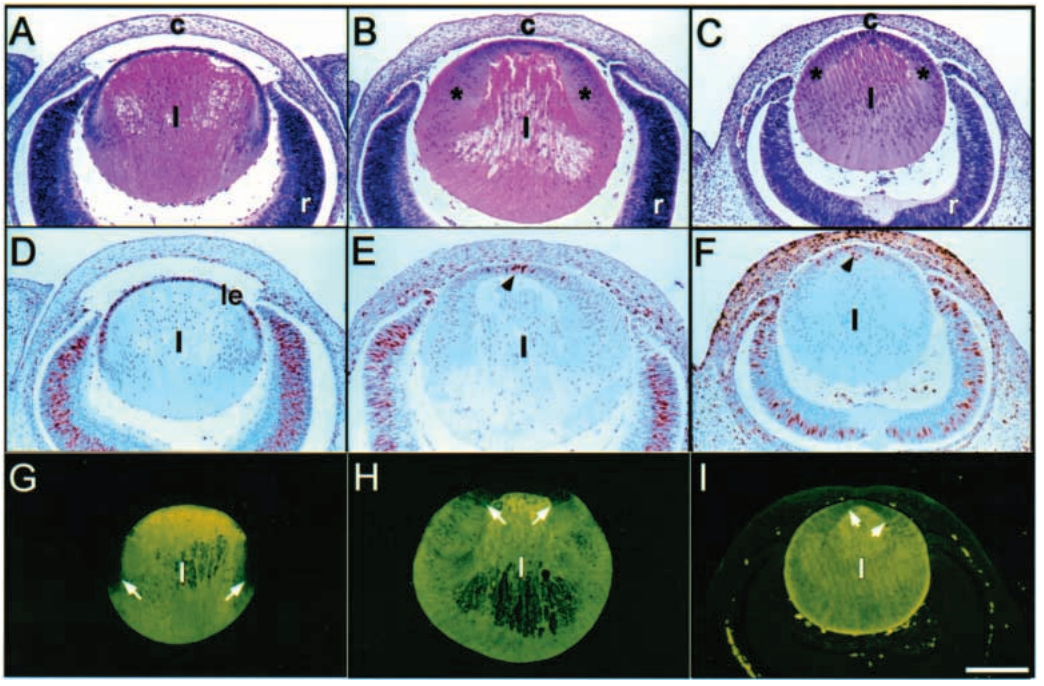

Fig. 12. Ocular changes induced by FGF-8 and FGF-9. Representative sections of eyes from embryonic day 15 non-transgenic (A,D,G), FGF-8 (B,E,H) and FGF-9 (C,F,I) transgenic mice either stained with haematoxylin and eosin (A-C), immunolabelled for BrdU incorporation, counterstained with haematoxylin (D-F) or immunolabelled for β-crystallin expression (G-I). Peripheral epithelial cells in lenses (l) of FGF-8 and FGF-9 transgenic mice elongate (B,C, asterisks), exit from the cell cycle as shown by the few remaining central cells incorporating BrdU (E,F, arrowhead) and differentiate into fiber cells expressing β-crystallin (H,I). This premature differentiation of the epithelial cells results in an anterior shift of the transitional zone of the lens, demarcated by arrows (H,I). In non-transgenic littermates, the lens epithelium (le) remains as a monolayer of cuboidal cells, incorporating BrdU (D, brown label) and unreactive for β-crystallin (G). The retina (r) in the FGF-9 transgenic mouse (C) is thinner compared to the retina of the non-transgenic mouse (A), possibly due to the reduction in number of proliferating, undifferentiated neuroblasts (compare BrdU incorporation in retinae of D and F). Scale bar, 200 μm.

response at this same age. In comparison, FGF-4 families OVE 815 and OVE 817, which do not develop a phenotype until postnatal day 7 (Fig. 6), had very low expression of the transgene at E15, comparable to non-transgenic littermates (see Fig. 8). Thus, the ocular phenotypes observed may be dependent on the different levels of epithelial cell stimulation by FGF, with high level stimulation resulting in microphakia and low level stimulation resulting in a retarded cell response leading to postnatal cataractogenesis.

The differences in phenotype between the different transgenic families may be also attributed to the differential responsiveness of the lens epithelium to the different FGFs. For example, although the FGF-7 transgene was transcribed at E15 at levels comparable to the strongly expressing FGF-4, FGF-8 and FGF-9 mice (data not shown), a phenotype in the lens epithelium of these mice was not observed until embryonic day 18. A possible explanation for this delayed responsiveness would be that the human cDNA used for FGF-7 is not as potent as the murine cDNAs used for the other FGFs. However, this is unlikely as the *Fgf-7* gene is highly conserved during mammalian evolution with human FGF-7 exhibiting a high degree of sequence identity to the murine cDNA (see Mason et al., 1994). The delayed response observed in FGF-7 transgenic mice may be the result of chronic, low level stimulation of the lens epithelial cells. A low level stimulation in the lens may be provided by either low level expression of FGF-4, as mentioned above, or by high-level expression of FGF-7. This response could reflect either a lower level of expression of the FGF-7 receptor (KGFR) in the lens epithelium (see Orr-Urtreger et al., 1993; de Iongh et al., 1997) or differences in the downstream signalling events following receptor activation. In addition, since all the FGFs examined in the present study are secreted proteins, the differential responsiveness of the lens cells is probably not due to the differences in protein secretion. Even though FGF-7 mice do not show a lens phenotype by E15, a prominent phenotype is observed in the cornea at this same age (data not shown; Lovicu and Overbeek, unpublished observations), implying that FGF-7 is efficiently secreted. Moreover, although FGF-9 has been reported to contain no identifiable signal sequence it has been shown to be secreted in vitro (Miyamoto et al., 1993). Since FGF-9 can induce the differentiation of the lens epithelial cells even when it is expressed specifically in the lens fiber cells, our results provide in vivo evidence that FGF-9 is efficiently secreted, presumably via an alternative secretory pathway (see Nauro et al., 1993; Song and Slack, 1996).

Ocular developmental changes were not restricted to the lenses. Other ocular tissues in our transgenic mice were constantly affected early in development by the lens-specific expression of these FGFs. As mentioned above, expression of FGF-7 had profound affects on the cornea while transgenic mice expressing FGF-9 and higher levels of FGF-4 exhibited retinal alterations. In transgenic mice expressing FGF-8, with the exception of the lens phenotype early in development, no other ocular tissues appeared to be affected. These mice still went on to develop microphthalmia. As dramatic lenticular changes were observed in all transgenic families, with most phenotypes progressing to microphthalmia, these findings are consistent with the hypothesis that the lens may be important for orchestrating later stages of ocular development.

Overall, results from the present study demonstrate that different FGFs have the potential to stimulate lens epithelial cells to undergo similar changes leading to fiber differentiation. The redundant effects of these different FGFs on lens cells suggests that their biological roles may be primarily specified by regulation of their expression patterns. Precise regulation of the levels and spatial expression of FGF(s) are therefore likely to be essential for normal ocular development. In fact, as low levels of FGF-4 expression can apparently induce changes in epithelial cell behaviour that result in cataract formation, inappropriate release of FGF into the ocular environment (eg. in response to cell injury) may contribute to cataractogenesis and visual impairment.

In earlier studies from our laboratory, using an analogous strategy to that adopted in the present study, it has been shown that other FGF family members such as a FGF-3, FGF-5 and a secreted form of FGF-1, can also induce the premature differentiation of lens epithelial cells (Robinson et al., 1998; Srinivasan and Overbeek, 1996; Robinson et al., 1995a). Taken together, using the transgenic mouse system, FGF-1, FGF-3, FGF-4, FGF-5, FGF-7, FGF-8 and FGF-9 have the ability to induce lens fiber differentiation. As lens epithelial cells express several FGFRs (see Introduction), cross reactivities may account for the similar effects generated by many FGFs on a common cell type. This suggests that downstream signalling events in lens epithelial cells leading to fiber differentiation are analogous in each case of FGF induction.

Examination of the FGFs and their cognate FGFRs can provide insights into which receptors are the major role players in lens fiber differentiation. FGF-7, FGF-8 and FGF-9 reportedly do not bind to FGFR1 (Hecht et al., 1995; Santos-Ocampo et al., 1996; Ornitz et al., 1996) but still have the ability to stimulate lens fiber differentiation. Similarly, FGF-3, FGF-5 and FGF-7 do not interact with FGFR3 (Green et al., 1996, Ornitz et al., 1996) but induce lens fiber differentiation (see Srinivasan and Overbeek, 1996; Robinson et al., 1998). Together, these data suggest that FGFR1 and FGFR3 are not essential for FGF-induced lens fiber differentiation. A similar conclusion can be made from the analysis of mouse embryos homozygous for null alleles of the different FGFR genes. Disruption of the *Fgfr3* gene by homologous recombination results in viable mice with no reported abnormalities in lens and eye development (Colvin et al., 1996; Deng et al., 1996). Similarly, although null mutations for FGFR1 result in embryonic lethality (Yamaguchi et al., 1994; Deng et al., 1994), chimeric mice made by combining wild-type embryos with FGFR1-null embryonic stem cells are viable. The FGFR1-null lens epithelial cells can apparently undergo normal fiber differentiation (Dr J. Rossant, personal communication). Since FGFR2 is expressed in the lens epithelium and different splice versions of FGFR2 can be stimulated by FGFs that have been shown to induce lens fiber differentiation, our studies in transgenic mice indicate that activation of FGFR2 may specify the changes in gene expression that result in fiber cell formation. Future studies using knockout mice generated by the targeted disruption of the *Fgfr2* gene can be used to test our proposal for a significant role for this receptor in normal lens fiber differentiation.

Fgf-8 was originally identified as a gene encoding a secreted, androgen-dependent growth factor that regulates growth of a mammary carcinoma cell line (Tanaka et al., 1992). Recent studies have shown that the *Fgf*-8 gene is structurally the most

complex member of the FGF family. Unlike the majority of FGFs whose amino terminal region is encoded by a single exon, the N-terminus of FGF-8 is encoded by 4 exons which are alternatively spliced to generate at least 7 secreted isoforms (FGF-8a to FGF-8g) that differ only in the short domain that lies between the signal sequence and the start of the conserved FGF core sequence (Crossley and Martin, 1995; MacArthur et al., 1995a). Using recombinant FGF-8 protein isoforms, MacArthur and co-workers (1995a) tested the ability of these isoforms to induce a mitogenic response in BaF3 cells transfected with different splice versions of FGFR1, FGFR2, FGFR3 and FGFR4. Unlike the other FGF-8 isoforms, recombinant FGF-8a (variant 4, Crossley and Martin, 1995), the FGF-8 isoform used in the present study, did not induce a mitogenic response in these cultured cells. On the contrary, NIH3T3 cells transfected with the cDNA for this same FGF-8 isoform or treated with recombinant FGF-8a could be morphologically transformed (MacArthur et al., 1995b). One potential model to explain these results is that mitogenic stimulation requires different downstream signal(s) than induction of transformation. An interesting alternative hypothesis is that the FGF-8a isoform does not activate any individual FGFR isoform (MacArthur et al., 1995a) but has the ability to stimulate cells containing more than one FGFR isoform, both in vitro (NIH3T3 cells) and in vivo (lens epithelial cells). Therefore, in lens epithelial cells, FGF-8a-induced differentiation may be mediated via FGF receptor heterodimerization. However, FGF-8a could alternatively be activating a novel FGFR in the lens.

A key event in transmembrane signalling by receptors with tyrosine kinase activity is ligand-induced dimerization. Receptor dimerization stimulates an increase in intracellular kinase activity resulting in autophosphorylation and the induction of a cellular response (see Schlessinger and Ullrich, 1992). As the different FGFR isoforms differ in their ligand-binding specificities, heterodimerization may serve to increase the repertoire of ligands that can bind, increasing the diversity of intracellular signalling and biological responses. There is indirect evidence that FGF receptors can undergo heterodimer formation and heterologous transphosphorylation in response to FGF. Numerous studies using dominant negative FGF receptor mutants, deleted of their cytoplasmic domain, have demonstrated inhibition of other FGF receptors, presumably through the formation of inactive heterodimers upon ligand binding (Amaya et al., 1991; Ueno et al., 1992; Werner et al., 1994). Other studies using a kinase-defective FGF receptor also provide strong support for both homologous and heterologous transphosphorylation of FGF receptors in response to FGF (Bellot et al., 1991; Shi et al., 1993). Furthermore, in vitro studies by Mohammadi et al. (1991) show that a recombinant fragment containing the C-terminal autophosphorylation sites of either FGFR1 (*flg*) or FGFR2 (*bek*) can be phosphorylated on tyrosine by a recombinant *flg* cytoplasmic kinase domain.

In vitro studies examining the effects of FGF on lens epithelial explants have shown that responsiveness of lens epithelial cells is dependent on the concentration of FGF (see McAvoy and Chamberlain, 1989). For example, a low concentration of FGF will only stimulate cell division whereas, at higher concentrations, FGF induces lens fiber differentiation. The mechanism regulating this differential responsiveness of lens cells to different levels of FGF is not clear at present. However, since higher doses of FGF may promote FGF receptor heterodimerization (Shi et al., 1993), FGF-induced signal transduction leading to the differentiation of lens epithelial cells may be dependent on transphosphorylation between heterologous FGF receptor tyrosine kinases. Stimulation of heterodimeric protein tyrosine kinase receptors may broaden the spectrum of signal transduction molecules activated through the transphosphorylation of novel sites not autophosphorylated in homodimeric receptor complexes (Rupp et al., 1994). As lens epithelial cells express more than one form of FGF receptor, we propose that heterologous transphosphorylation, perhaps between the different FGFR2 isoforms, may have significant biological implications for regulating lens cell behaviour. Future studies will be aimed at addressing this proposal.

To date, eight members of the FGF family (FGF-1, FGF-2, FGF-3, FGF-4, FGF-5, FGF-7, FGF-8 and FGF-9), including the four presented in this study, have been shown to induce lens fiber differentiation either in vitro or in vivo (Chamberlain and McAvoy, 1989; Robinson et al., 1995a; Srinivasan and Overbeek, 1996; Robinson et al., 1998). Of these, only FGF-1 and FGF-2 have been shown to be highly expressed in the lens and surrounding ocular tissues (de Iongh and McAvoy, 1992, 1993; Lovicu and McAvoy, 1993; Schulz et al., 1993; Lovicu et al., 1997). FGF-3 is expressed transiently in the developing murine retina (Wilkinson et al., 1989), FGF-5 in primate adult retina (Kitaoka et al., 1994) and FGF-7 in perioptic mesenchyme and cornea (Mason et al., 1994; Wilson et al., 1993). Using in situ hybridizations to assay for expression of FGF-4, FGF-8 and FGF-9, the present study did not detect endogenous transcripts for any of these FGFs in the eye, although previously reported expression patterns in other tissues were observed (data not shown). Although FGF-1 and FGF-2 have all the necessary activities of lens differentiation factors and are temporally and spatially expressed at the right time and place (Lovicu et al., 1997; de Iongh and McAvoy, 1993), they are not secreted in the 'classical' sense (for review, see Basilico and Moscatelli, 1992). The other FGFs examined to date, although efficiently secreted and shown to have the potential to induce lens fiber differentiation, do not appear to be present in the eye when required. Unless there is a novel mechanism that locally releases FGF-1 and/or FGF-2, it is unlikely that either is the endogenous lens differentiation factor. The identification and characterisation of additional FGF family members and homologues (Kinoshita et al., 1995; Yamasaki et al., 1996; Smallwood et al., 1996; Coulier et al., 1997), together with studies of the FGFR signalling pathway, should lead to a greater understanding of the role(s) of FGFs in lens differentiation and development.

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