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Sef is a negative regulator of fiber cell differentiation in the ocular lens

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Abstract

Growth factor signaling, mediated via receptor tyrosine kinases (RTKs), needs to be tightly regulated in many developmental systems to ensure a physiologically appropriate biological outcome. At one level this regulation may involve spatially and temporally ordered patterns of expression of specific RTK signaling antagonists, such as Sef (similar expression to *fgfs*). Growth factors, notably FGFs, play important roles in development of the vertebrate ocular lens. FGF induces lens cell proliferation and differentiation at progressively higher concentrations and there is compelling evidence that a gradient of FGF signaling in the eye determines lens polarity and growth patterns. We have recently identified the presence of Sef in the lens, with strongest expression in the epithelial cells. Given the important role for FGFs in lens developmental biology, we employed transgenic mouse strategies to determine if Sef could be involved in regulating lens cell behaviour. Over-expressing Sef specifically in the lens of transgenic mice led to impaired lens and eye development that resulted in microphthalmia. Sef inhibited primary lens fiber cell elongation and differentiation, as well as increased apoptosis, consistent with a block in FGFR-mediated signaling during lens morphogenesis. These results are consistent with growth factor antagonists, such as Sef, being important negative regulators of growth factor signaling. Moreover, the lens provides a useful paradigm as to how opposing gradients of a growth factor and its antagonist could work together to determine and stabilise tissue patterning during development and growth.

Keywords

Sef; FGF; RTK signaling; Antagonists; Lens development; Cell differentiation

1. Introduction

Because of its relative cellular simplicity, the ocular lens provides a useful dynamic model to study many molecular, cellular and developmental processes, ranging from tissue induction, specification, morphogenesis and cell proliferation and differentiation, as well as

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receptor tyrosine kinase (RTK) activation and intracellular signaling. The defined spatial arrangement of lens cells provides the lens with distinct polarity; an anterior sheet of cuboidal epithelial cells overlying a mass of elongated fiber cells, enclosed by a thick basement membrane, the lens capsule. Lens cells are established early in development with the initiation of ocular morphogenesis, induced by the interaction of the evaginating neuroectoderm-derived optic vesicles with the overlying surface head ectoderm (see Robinson and Lovicu, 2004). A thickened placode of ectodermal cells invaginate to form the lens pit and subsequent lens vesicle; a hollow ball of cells that pinches off from the overlying embryonic surface ectoderm that will go on to form the prospective corneal epithelium. It is at this lens vesicle stage that the lens begins to visibly attain its distinct polarity. Posteriorly, the lens vesicle cells elongate to form the primary lens fibers that then make contact with the anterior cells that concomitantly differentiate into the lens epithelium. With the differentiation of the primary fibers comes the addition of new secondary fibers at the lens equator, where progeny of lens epithelial cell divisions exit the cell cycle, elongate and add cellular layers to the fetal fiber mass. This process continues throughout life as new secondary fibers form and mature, losing their intracellular organelles and nuclei as they do so (see Robinson and Lovicu, 2004). The transition from epithelial to fiber cells is also accompanied by molecular changes such as the successive accumulation of fiber-specific β- and γ-crystallins (McAvoy, 1978), as well as intermediate filaments such as filensin (Gounari et al., 1993).

The process of lens morphogenesis is very well established and over the last two decades many of the key molecules required for this process have been identified (Lovicu and McAvoy, 2005). One of the most extensively reported and studied families of growth factors involved in this process are the fibroblast growth factors (FGFs). With the initial discovery of their effects on lens cells in vitro, came the finding of their pleiotropic effects, with lens cell proliferation, migration and fiber cell differentiation induced by a progressive increase in dose of FGF (McAvoy and Chamberlain, 1989). More recent studies attributed these differential effects to differences in the levels and duration of MAPK/ERK1/2 phosphorylation (Lovicu and McAvoy, 2001; Le and Musil, 2001; Iyengar et al., 2006, 2007; Wang et al., 2009). Blocking studies further demonstrated that ERK1/2 signaling was required for the morphological changes (cell elongation) associated with the lens fiber differentiation process, supported by the loss of filensin expression (Lovicu and McAvoy, 2001). Interestingly, the accumulation of β- and γ-crystallins, also associated with fiber cell differentiation, was shown to be independent of ERK1/2 signaling (Lovicu and McAvoy, 2001; Wang et al., 2009).

It is well established that the distinct patterns of cell behaviour in the lens are primarily regulated by the ocular environment, in particular the ocular fluids that envelop the lens; the aqueous and vitreous humour (Coulombre and Coulombre, 1963; Schulz et al., 1993; Lovicu et al., 1995). The vitreous, as opposed to aqueous, has been shown to promote fiber differentiation and much of the fiber-differentiating activity of vitreous has been attributed to the presence of FGFs (Schulz et al., 1993). Consistent with a requirement for FGF signaling in lens fiber differentiation, recent in vivo studies using conditional gene targeting strategies have reported that FGF receptor signaling is essential for lens differentiation, as the additive loss of FGF receptors 1–3 led to impaired primary fiber cell differentiation and a block in lens morphogenesis (Zhao et al., 2008).

As noted earlier, FGF signaling in the lens is further complicated by the fact that differential doses of FGF can induce different cellular responses (McAvoy and Chamberlain, 1989). In many developmental systems, growth factor signaling must be regulated both temporally and spatially to ensure a precise and reproducible physiological cellular response. Clearly, generating a concentration gradient of growth factor is one way of achieving this; however,

another level of regulation may be achieved by specific growth factor signaling antagonists that provide a concurrent set of inhibitory signals. These may contribute to developmental patterning by restricting the range of a corresponding inducer (see Tsang and Dawid, 2004).

One of the first identified bona fide feedback regulators of the FGF pathway was Sprouty (Spry), which was initially discovered through a genetic screen in *Drosophila* and later shown to be important for FGF-induced tracheal branching in this species (Hacohen et al., 1998). Further screening for genes with restricted expression patterns during early development (Kudoh et al., 2001) revealed other modulators of the FGF pathway, including *Sef* (similar expression to *fgfs*). Sef, unlike Spry, is a transmembrane protein that is restricted to vertebrates; however, like Spry, it has been shown to function as an antagonist of FGF signaling during development (Tsang et al., 2002; Furthauer et al., 2002). As for Spry, over-expression of Sef can inhibit FGF signaling, and impaired Sef expression leads to overstimulation of FGF signaling (Tsang and Dawid, 2004). The mode of Sef inhibitory activity on FGF signaling is still contentious, as it has been shown to act at the level of the FGF receptors (FGFR1 & FGFR2; Tsang et al., 2002) as well as on downstream components of the Ras/ERK1/2 pathway (Furthauer et al., 2002; Torii et al., 2004). The co-expression of Sef with known sites of FGF signaling during embryogenesis lends credence to the important role it may have in cell fate specification; a close spatial and temporal interdependence between FGF signaling and Sef gene expression has been reported in different mammalian tissues (see Lin et al., 2002) as well as in the lens (Boros et al., 2006). As FGF plays an important role in lens biology, we investigated the role of Sef in this tissue by generating transgenic lines of mice over-expressing *Sef* specifically in the lens. We provide one of the first studies to demonstrate the inhibitory effects of Sef on lens development, an effect similar to that of conditionally deleting specific FGF receptors in the lens (Zhao et al., 2008).

2. Materials and methods

2.1. Animals

All animal procedures were carried out in accordance with the Animal Care Ethics Committee, at the University of Sydney (NSW, Australia) and conformed to the Association for Research in Vision and Ophthalmology Incorporated Resolution on the use of animals in Ophthalmic Research.

Different transgenic lines of mice were generated or obtained for this study. Murine (mSef; Tsang et al., 2002) or human (hSef-b; Preger et al., 2004) forms of Sef were over-expressed in transgenic mice, specifically in the lens using a modified αA-crystallin promoter, fused to a chick δ1-crystallin enhancer element, a rabbit β-globin intron and a human growth hormone polyA signal (see Reneker et al., 2004). Mice deficient for *Fgfr1* & *Fgfr2*, or *Fgfr1*, *Fgfr2* & *Fgfr3*, specifically in the lens (previously described; Zhao et al., 2008), were mated to our transgenic Sef lines in order to over-express Sef in this specific background.

2.2. Tissue collection

Embryonic murine tissues at different gestation days (E10.5–E15.5), as well as postnatal eyes, were fixed in 10% neutral buffered formalin for histological processing and subsequent immunolabeling. One hour prior to sacrificing, animals were injected with 5′2-bromodeoxyuridine (BrdU; 0.1 mg/g). Genotyping of mice was carried out using PCR of tail-derived genomic DNA with primers coding for the chick δ-crystallin enhancer element, as previously described (Reneker et al., 2004).

2.3. Histology and immunolabeling

Six micron thick paraffin wax sections of embedded tissues were used for all analysis, from haematoxylin and eosin staining to labeling studies. For BrdU-labeling, sections were successively treated with 3% hydrogen peroxide (H_2O_2), 0.02% pepsin, 0.01 N HCl and 3% normal goat serum, prior to incubation with a mouse anti-BrdU antibody (diluted 1:100, DakoCytomation, Denmark) as previously described (Lovicu and Overbeek, 1998). Following application of the secondary antibody (rabbit anti-mouse HRP antibody, diluted at 1:50, Zymed, CA, USA), BrdU was detected in the presence of 0.02% H_2O_2 (v/v) and 0.05% diaminobenzidine (w/v) in 50 mM Tris, pH 7.2. Sections were counterstained with haematoxylin and permanently mounted for microscopy.

For immunofluorescent labeling of Sef, $p57^{Kip2}$, filensin, and β- and γ-crystallins, tissue sections were hydrated to PBS/BSA (PBS supplemented with BSA), and incubated with 3% normal goat serum (rabbit serum for $p57^{Kip2}$) before application of primary antibody. Sections were treated overnight at 4 °C with polyclonal antibodies for Sef (rabbit anti-Sef antibody diluted 1:200; Phoenix Pharmaceuticals, Inc., California, USA), filensin (diluted 1:50), β-crystallin (diluted 5 μg/ml) or γ-crystallin (diluted 80 μg/ml), or $p57^{Kip2}$ (goat anti-$p57^{Kip2}$ antibody; Santa Cruz Biotechnology, CA, USA, diluted at 1:100). Following application of secondary antibodies; sheep-anti-rabbit Cy-3 antibody (Sigma, diluted 1:200 for Sef, filensin and γ-crystallin), goat anti-rabbit Alexa-fluor 488 (Invitrogen/Molecular Probes, Oregon, USA; diluted 1:1000 for β-crystallin) or rabbit anti-goat Cy-3 (Zymed, diluted 1:100 for $p57^{Kip2}$), sections were counterstained with bisbenzimide (Hoechst dye, Sigma 33342) at 2 μg/ml and mounted with 10% PBS/glycerol.

For immunofluorescent labeling of phosphorylated forms of ERK1/2 (pERK), Akt (pAkt) or FRS2α pFRS2), hydrated tissue sections were first subjected to heat-induced (105 °C) antigen-retrieval in 10 mM sodium citrate (pH 6) for 10 (pERK) to 30 min (pFRS2 and pAkt), before cooling for 20 min at room temperature and application of 10% normal goat serum (see Zhao et al., 2008). The anti-phospho-ERK1/2 (1:50), Akt (A11010, 1:100; Cell Signaling) and FRS2α 436 (AF5126, 1:100; R&D systems) antibodies were subsequently applied overnight at 4 °C and their binding was detected using either a goat anti-rabbit Cy3-conjugated secondary antibody (pERK; diluted 1:200; Sigma) or Alexafluor 546 goat anti-rabbit IgG (pAkt, pFRS2α, diluted 1:200; Molecular Probes). Sections were counterstained with Hoechst dye or in Vectastain mounting medium (containing DAPI, Vector Laboratories).

TUNEL assay was used to detect nicked DNA associated with apoptosis. Hydrated sections were treated with proteinase K (1 μg/ml) before incubation at 37 °C with terminal deoxynucleotidyl transferase (TdT) buffer (200 mM sodium-cacodylate, 30 mM Tris base, 10 mM cobalt chloride, pH 7.2). Sections were then incubated at 37 °C in TdT buffer containing 30 U/ml of terminal transferase and 4 M biotin-16 dUTP. Following a series of washes, sections were incubated at 37 °C with Cy3-conjugated streptavidin (diluted 1:1000). Sections were counterstained with Hoechst dye and mounted with PBS/glycerol for microscopy as above.

In situ hybridiasation of *Sef* mRNA transcripts was carried out as previously described (see Boros et al., 2006), using sense and antisense riboprobes labeled with digoxigenin. Immunolabeling with an anti-digoxigenin alkaline phosphatase conjugated antibody (Roche, Basel, Switzerland) was used to detect the distribution of the hybridized riboprobes.

2.4. Immunoblotting (hSefb)

Eyes from postnatal day 15 mice were homogenised with a micropestle in 1.5 mL tubes containing 2.5 mM EDTA, 25 mM Tris–HCl (pH 7.5), 0.375 M NaCl, 1% IGEPAL, 1.5 mM

sodium orthovanadate and a protease inhibitor cocktail (Roche). Homogenised samples were incubated for 2 h at 4 °C on a rotating wheel. Following protein estimation, for SDS-PAGE and Western blotting, 20 μg of each protein sample was combined with an equal volume of Laemmli sample buffer (BioRad, NSW, Australia) containing 5% (v/v) β-mercaptoethanol and loaded onto a 10% SDS-PAGE gel. Following electrophoresis, proteins were transferred onto a PVDF membrane that was subsequently incubated for 1 h at RT with 5% (w/v) skim milk powder in TBS-T (0.1% Tween 20 in tris-buffered saline, TBS). The blocked membrane was incubated overnight at 4 °C with an anti-Sef polyclonal antibody (Phoenix Pharmaceuticals, Inc.) diluted 1:1000 in blocking solution. Unbound primary antibody was removed with 3 × 5 min washes in TBS-T, prior to a 2 h incubation with goat anti-rabbit IgG-conjugated to HRP (diluted 1:5000; CST, USA). The membrane was rinsed several times with TBS-T, incubated for 3 min in an enhanced-chemiluminescence substrate (ECL, Amersham Biosciences, UK) and exposed onto film (Hyperfilm™, Amersham Biosciences) that was developed using standard autoradiography techniques.

2.5. Photography

Histological and BrdU-labeled sections were visualised under bright field illumination, while immunofluorescent sections were visualised with epifluorescence (Leica DMLB, Germany) or confocal microscopy. Photography was mostly carried out using a digital camera (Leica DCF-280, Germany).

3. Results

In the present study, three transgenic lines of mice (L22, L23, L24) were generated using the modified alpha-crystallin promoter (Reneker et al., 2004) to over-express *mSef* specifically in the cells of the ocular lens. All three lines of mice demonstrated a similar postnatal ocular phenotype, in the form of microphthalmia (see Fig. 1A–C), irrespective of whether they were genotypically hemizygous or homozygous for the transgene. Consistent with this ‘small’ eye phenotype, the lenses of the transgenic mice were proportionally smaller in size compared to lenses from wild-type littermates (Fig. 1D). As the ocular phenotypes observed were very similar between the different lines of mSef transgenic mice generated, representative data from L24 characterising this phenotype is presented.

3.1. Histology

At embryonic day 10.5 (E10.5), when the lens vesicle has formed, eyes from both transgenic and wild-type embryos look histologically similar and this persisted through to E11.5, when the posterior lens vesicle cells in both wild-type and transgenic mice started to elongate (data not shown). The elongation of the posterior lens vesicle cells indicates the initial stages of lens primary fiber cell differentiation as they approach the anterior lens vesicle cells, in the process obliterating the lumen of the lens vesicle by E12.5. It is at this developmental stage that there is a notable difference in the lenses of the transgenic mice compared to lenses from wild-type mice (Fig. 2). At E12.5, the lens vesicle of wild-type (Fig. 2A) and hemizygous transgenic mice (data not shown) have a similar structure, with the primary fibers extending to make contact with the anterior epithelial cells, eliminating the lumen. In contrast, the lens vesicle of homozygous transgenic mice (Fig. 2B) appears developmentally retarded as the primary fiber cells have failed to completely elongate anteriorly. The lens vesicle of these homozygous transgenic mice at E12.5, in fact, more closely resembles the lens vesicle of a wild-type mouse at an earlier stage of development (i.e. E11.5).

At E13.5 the lens of hemizygous lines of mice also start to show a phenotype that is distinctly different from that of wild-type littermates. The epithelia of the respective lenses appear similar; however, the fiber cells in the centre of the lens are less organised (data not

shown) in the transgenic mice. By E15.5, this is more pronounced, with pyknotic nuclei featured in many of the centrally situated primary fibers of lenses from mice hemizygous for the *mSef* transgene (Fig. 2D). The lens phenotype of the homozygous lines of transgenic mice is also more distinct, retaining a prominent lumen due to the failure of the posterior lens vesicle cells to elongate (Fig. 2E). Similar to the hemizygous lens, pyknotic nuclei are also evident in these lenses, as are loosely ordered rounded cells, typical of dying cells, in the lumen of the lens. By this embryonic stage, the abnormal morphogenesis of the lens in hemizygous and homozygous mice has clearly impacted on lens size, with transgenic lenses appearing much smaller than that of wild-type littermates (Fig. 2C). The characteristic smaller lens size and retention of the lumen in the lenses of the homozygous mice persists during fetal development and is still present postnatally (Fig. 2H, K). Moreover, pyknotic cell nuclei are also evident in the fiber cell mass of lenses of transgenic mice as they progress through to postnatal stages (see Fig. 2J). Note that throughout early development or postnatal growth, the retention of the lumen in the lens is only observed in lines of mice homozygous for the transgene. Although a lumen persists postnatally, there is evidence of some posterior cell elongation that may correlate with the reduced levels of *mSef* transgene expression with age (data not shown).

3.2. Expression of Sef

The expression of *Sef* was examined to determine the onset and extent of transgene expression. Consistent with previous studies (Boros et al., 2006), strongest expression of Sef in the developing lens (E15.5) is confined to the lens epithelium and earliest elongating secondary fiber cells (Fig. 3A). In transgenic littermates, expression extends to all lens cells, with stronger labeling in the posterior cells of the lens vesicle (incipient primary fibers; Fig. 3B, asterisk). The first notable difference in expression of Sef in transgenic lenses was at E12.5 (data not shown), with elevated Sef protein immunoreactivity in lenses at E13.5 (Fig. 3C, D). Due to the markedly elevated levels of Sef immunofluorescent labeling in the lens fiber cells of the transgenic lens, Sef labeling in the epithelium is not as apparent (Fig. 3D, arrows). Moreover, it should be noted that stronger labeling for Sef was evident in the lenses of homozygous mice compared to that of hemizygous mice (data not shown). This labeling pattern for Sef continued through to postnatal stages although, the overall levels of Sef appeared to drop with age (data not shown).

3.3. TUNEL analysis

Given the frequency of pyknotic nuclei in our histological analysis of the lens of transgenic mice, we used the TUNEL assay to determine whether the reduced size of the lens was potentially due to increased cell loss by apoptotic cell death. At E15.5, in contrast to wild-type lenses that had no appreciable TUNEL labeling (Fig. 4A, B), lenses of hemizygous (Fig. 4C, D) and homozygous lines of mice (data not shown) demonstrated strong TUNEL labeling of many of the pyknotic cell nuclei. Also observed was diffuse TUNEL-labeling, that overlapped with the diffuse Hoechst-labeled nucleic acids, associated with dying cells. This diffuse label may reflect a more accurate extent of the levels of fragmented DNA in these tissues.

3.4. pAkt labeling

Given that PI3-K signaling is associated with cell survival, we labeled for phosphorylated Akt. pAkt labeling in the lens of wild-type mice was present in the lens epithelium and particularly strong in early differentiating fiber cells (Fig. 5A, B). In contrast, pAkt labeling in the Sef transgenic mice (Fig. 5C–F) demonstrated a notable reduction in labeling, in both the lens epithelium and early differentiating lens fiber cells, in mice either hemizygous (Fig. 5C, D) or homozygous (Fig. 5E, F) for the transgene.

3.5. BrdU-labeling

Due to the reduced epithelium observed in lenses of transgenic mice, we also examined for changes in cell proliferation using the incorporation of BrdU as a marker. At E15.5 there were more positively labeled cells for BrdU in wild-type lenses (Fig. 6A) compared to transgenic lenses (Fig. 6B, C). Although the wild-type lenses had a greater number of epithelial cells compared to lenses from transgenic mice, when we calculated the percentage of BrdU-labeled epithelial cells, there was no significant difference between wild-type versus transgenic lenses (Kruskal–Wallis with post-hoc tests; T=0.96; P=0.62; N=15), with each demonstrating approximately 23% of cells labeled for BrdU. Occasionally, posteriorly situated cells of the homozygous lens that showed limited elongation were BrdU-labeled (Fig. 6C). Lenses from postnatal transgenic mice also presented the occasional BrdU-labeled cell in the fiber-like cell mass and also demonstrated no marked difference in the levels of epithelial cell proliferation (data not shown).

3.6. $p57^{Kip2}$ labeling

Given that some cells in the fiber cell mass of transgenic mice retained the ability to synthesise DNA (BrdU-positive), we examined the distribution of $p57^{Kip2}$, to determine whether their ability to exit the cell cycle was disrupted. $p57^{Kip2}$ labeling at E15.5 in wild-type lenses was localised to a defined group of cells at the lens equator, in the region just below where the epithelial cells incorporate BrdU, extending through to a select number of early differentiating fiber cells (Fig. 6D). There was no labeling of $p57^{Kip2}$ in the maturing fiber cells. This pattern was somewhat similar in lenses of mice hemizygous for the *mSef* transgene, with the notable exception that $p57^{Kip2}$-positive cells extended further into the fiber differentiation zone (Fig. 6E). Lenses of homozygous mice were different, mainly through the persistence of $p57^{Kip2}$ labeling in many, if not all, of the posterior lens vesicle cells that had failed to elongate and differentiate (Fig. 6F).

3.7. β- and γ-crystallin, and filensin expression

The expression of molecular markers for lens fiber differentiation, β- and γ-crystallin and filensin, were used to further characterise the differentiated state of the lens cells in the *mSef* transgenic mice. From embryonic day 12.5 through to E15.5 and postnatal stages, the pattern of expression of β-crystallin was similar in all lenses examined (see Fig. 7A, C, E). In the wild-type mouse lens, β-crystallin was highly expressed in the early fiber cells just posterior to the lens equator, where the lens cells begin to elongate into secondary fibers (Fig. 7A). This pattern was similar in lenses from both hemizygous (Fig. 7C) and homozygous (Fig. 7E) transgenic mice. The lumen of the homozygous lens was also strongly reactive for β-crystallin (Fig. 7E); which may reflect the released protein from dying cells in these lenses.

From embryonic day 12.5 through to E15.5, the pattern of γ-crystallin expression was similar to that of β-crystallin, being more restricted to the central cells of the fiber mass. As expected, the expression of γ-crystallin in wild-type lenses commences slightly later in the fiber differentiation process to that observed for β-crystallin (compare Fig. 7A, B). This spatial and temporal trend remained in transgenic lenses, with γ-crystallin labeling appearing more restricted in the lens fiber cell mass of both hemizygous (Fig. 7D) and homozygous (Fig. 7F) transgenic mice. The lumen of the homozygous transgenic mouse lens is also weakly reactive for γ-crystallin at this developmental stage (Fig. 7F).

By comparing the distribution of the different lens molecular markers we begin to note some irregularities in their expression. Although the expression of the crystallins appeared relatively normal in all lenses examined, we did note the uncoupling of some lens cell markers in transgenic mice. For example, in wild-type lenses at E15.5, the expression of $p57^{Kip2}$ (Fig. 6D) and γ-crystallin (Fig. 7B) does not normally overlap; however, in

homozygous lenses of comparable age, these two proteins are co-expressed in the majority of posterior lens cells (see Figs. 6F and 7F).

Filensin is also normally strongly expressed in the maturing lens fiber cells of wild-type mice as shown at E15.5 (Fig. 8A–C). In the lenses of homozygous transgenic mice, we see a marked decrease in reactivity for filensin in the lens cells. More specifically in the majority of posterior lens cells that have failed to elongate, there is weak to no labeling for filensin (Fig. 8D–F).

3.8. Association of Sef with FGF receptor signaling

The characteristic lens phenotype reported in this study, resulting from the failure of the primary lens fiber cells in transgenic mice to elongate appropriately during lens morphogenesis, together with increased apoptosis, led to the retention of a prominent lens lumen. This is very reminiscent of the lens phenotype recently described where FGF receptor signaling was disturbed in the lens (Zhao et al., 2008). Deletion of three FGF receptors (*Fgfr1–3*) at approximately the lens vesicle stage resulted in a lens somewhat similar to that observed in this study (compare Figs. 2 and 9). These *fgfr*-deficient lenses were also markedly reduced in size compared to the wild-type lens, had fewer lens epithelial cells, increased apoptosis and the primary fibers failed to elongate appropriately resulting in the retention of a lens lumen (Fig. 9A). β- and γ-crystallins were also expressed in these *fgfr*-deficient lenses (Fig. 9B, C), with γ-crystallin reactivity being weaker and restricted to the apical region of the posterior ‘fiber’-like cells (Fig. 9C). Interestingly, in order to obtain this distinct lens phenotype, all three FGF receptors (Fgfr1–3) were required to be deleted. Conditional deletion of both Fgfr1 and Fgfr2 resulted in a normal lens (Fig. 10A, B). As mSef has only been reported to impair FGFR signaling by interacting with either FGFR1 or FGFR2 (Tsang et al., 2002; Xiong et al., 2003), we over-expressed *mSef* in the lens of lines of mice deficient in FGFR1 and FGFR2, but with a full complement of FGFR3 in the lens. Over-expression of mSef in these mice, resulted in the same phenotype we had observed in our primary transgenic lines over-expressing *mSef* (compare Figs. 2E and 10F). Lenses deficient for FGFR1 and FGFR2 from transgenic mice, now over-expressing mSef, were markedly smaller, had fewer lens epithelial cells and failed to promote the elongation of the primary fiber cells leading to the retention of a lens lumen (Fig. 10E, F). Interestingly, the retention of the lens lumen was no longer restricted to transgenic mice homozygous for the *mSef* transgene, as hemizygous lines of mice over-expressing lower levels of mSef, in lenses deficient for FGFR1 and FGFR2, also demonstrated an anterior lumen (Fig. 10C, D), although smaller than that of the homozygous mSef lines (Fig. 10F).

3.9. pFRS2 α and pERK1/2 labeling

With Sef potentially directly targeting FGFRs in our transgenic mice lenses, we were interested to determine whether downstream substrates distinctive of FGFR signaling, such as ERK1/2 (required for cell elongation, Lovicu and McAvoy, 2001) and FRS2α, were still phosphorylated. FRS2α phosphorylation in E15.5 wild-type lens tissue is strongly localised to the lens epithelium (Fig. 11A, B, arrow) and this appears to get stronger at the lens equator, in the transitional zone and inner cortex of the lens (Fig. 11A, B, asterisk) where the fibers undergo the earliest stages of differentiation. As the fiber cells mature and terminally differentiate, there is little to no phosphorylation of FRS2α. When we compared this to the lenses of transgenic mice, we observe an increase in FRS2α in the lens epithelium (Fig. 11C, D, arrow); however, labeling is markedly reduced in the poorly elongated equatorial and posterior lens cells (Fig. 11C, D, asterisk) and many of the more posterior lens cells of the mSef transgenic mice, that had not effectively elongated, had little to no reactivity for phosphorylated FRS2α.

As earlier studies have implicated ERK1/2 phosphorylation to be required for FGF-mediated lens fiber cell elongation, we looked for changes in the phosphorylation state of this MAPK. In lenses of wild-type postnatal day mice we see strongest phosphorylation of ERK1/2 in lens epithelial and early differentiating fiber cells, at the lens equator (Fig. 12A, B, arrowhead). As fiber cells elongate and mature, reactivity for pERK1/2 is markedly reduced. When comparing this labeling to that seen in lenses of transgenic littermates, we see that ERK1/2 is still phosphorylated in these cells with strong labeling in many but not all of the cells (Fig. 12C, D, arrows) that have that failed to elongate in the presence of elevated levels of Sef.

3.10. Sef-b transgenic mice

Further transgenic lines of mice (L25, L26, L27) were generated to over-express *hSef-b* specifically in the ocular lens using the same modified alpha-crystallin promoter adopted for over-expression of *mSef*. hSef-b, unlike mSef, is not a transmembrane protein and would not be expected to interact directly with the membrane bound FGF receptors. Although lenses from all lines of transgenic mice were shown to over-express higher levels of Sef-b protein compared to the wild-type lens (see Fig. 13B, D), the lenses from transgenic mice (bred to homozygosity) appeared to develop normally (Fig. 13A), and accumulated the normal spatial and temporal patterns of lens molecular markers, such as γ-crystallin (see Fig. 13C).

4. Discussion

Numerous studies have identified a key role for FGF in lens biology, with in vitro studies demonstrating that FGF elicits different responses from lens epithelial cells depending on its concentration (McAvoy and Chamberlain, 1989). This has led to the proposal that a gradient of FGF activity in the eye regulates the distinct patterns of cell proliferation and differentiation that determine lens polarity (see Lovicu and McAvoy, 2005). This finding was substantiated by different in vivo studies using transgenic mice (Robinson et al., 1995; Lovicu and Overbeek, 1998), with none more compelling than the recent studies demonstrating the necessity of FGF-mediated RTK signaling in governing lens development, differentiation and growth (Zhao et al., 2008). Given that tight regulation of such a potent growth factor in the eye is essential for the maintenance of the unique architecture of the lens, there is strong justification to identify the molecules and mechanisms important for the normal regulation of FGF signaling in vivo. A relatively new class of negative feedback antagonists, the Sef proteins, have recently been shown to be involved in a number of biological processes, including development, and much of this inhibitory activity has been through interaction with FGF-mediated ERK1/2 signaling (see Ron et al., 2008 for review).

Earlier in vitro studies by our laboratory and others have shown that ERK1/2 signaling in lens cells, induced by a number of growth factors (including FGF) and the ocular media, is important for inducing lens epithelial cell proliferation and their differentiation into fiber cells (Lovicu and McAvoy 2001; Le and Musil, 2001; Iyengar et al., 2006, 2007; Wang et al., 2009). Given our earlier finding that Sef is strongly expressed in the lens epithelium (Boros et al., 2006), it was important to investigate if this FGF antagonist could have a functional role in regulating lens polarity and growth patterns. In the present study, transgenic mice over-expressing murine *Sef* specifically in the lens were generated and characterized. The most striking effect of increased Sef levels in the lens was its ability to block the elongation of the posterior primary fibers of the early lens vesicle. This was observed primarily in the lens of homozygous mice where levels of Sef over-expression were considerably higher compared to lenses from hemizygote and wild-type littermates. This reduction in the ability of the lens fiber cells to elongate persisted through to postnatal stages, resulting in not only the inhibition of primary fiber cell differentiation but also

secondary fiber cell differentiation. As mentioned, the lens of hemizygous mice did not exhibit this same phenomenon; however, the lower levels of Sef over-expressed in these mice was still sufficient to disrupt the normal differentiation of the fiber cells, with lenses being markedly smaller compared to lenses from wild-type littermates. Although Sef over-expression and silencing in a number of cell lines can modulate cell behaviour, and over-expression in the chick impacts on the growth of the auditory nucleus (Abraira et al., 2007), to date this is the first in vivo report of Sef blocking cell differentiation in mammals. The inhibitory effect of Sef on lens fiber cell differentiation is consistent with its spatio-temporal expression during lens morphogenesis; expression of Sef is restricted to the lens epithelium and earliest fiber cells, with loss of Sef expression as cells differentiate into secondary fibers (see Boros et al., 2006).

Although we demonstrate the ability of Sef to block the elongation and differentiation of lens fiber cells, based on our BrdU-labeling studies, the rate of lens cell proliferation does not seem to be affected when comparing lenses from transgenic mice to those of wild-type mice. Interestingly, although there is no significant difference in the rate of cell proliferation, the smaller lens of our transgenic mice display a reduced epithelium, made up of fewer cells. Exactly why this is the case is uncertain but it may be related to our observations that mSef also induced the programmed death of many cells in these lenses. Both the human and mouse orthologues of Sef have previously been reported to induce apoptosis in different cell lines (Yang et al., 2004; Kovalenko et al., 2006; Ziv et al., 2006). We propose that Sef-induced cell death may also contribute to the distinct phenotype of our transgenic lenses. Our demonstration of reduced phosphorylation of Akt (compromised PI3-K signaling) in the transgenic lenses may also account for this reduced cell viability. Furthermore, as earlier studies in vitro have shown that impaired PI3-K signaling (using a selective inhibitor) can block FGF-induced fiber cell elongation (Wang et al., 2009), this may also contribute to the impaired fiber cell elongation phenotype observed in our transgenic lines. Given that FGF is not known to normally induce a strong phosphorylation of pAkt in rat lens epithelial cells in vitro (Wang et al., 2009; Iyengar et al., 2006), it will be interesting to elucidate exactly how and to what capacity Sef over-expression in these cells is influencing this signaling pathway.

The major disruption by Sef in the lens of our transgenic lines was on the fiber cell differentiation process, with the effective block in cell elongation. This was consistent with the marked reduction and loss of expression of the fiber cell-specific intermediate filament protein, filensin, in these aberrant cells. Interestingly, although the elongation of these cells was impaired, other molecular changes that normally accompany fiber cell elongation during the differentiation process, namely the accumulation of β- and γ-crystallins, did not seem to be affected. The normal successive temporal expression of these fiber-specific crystallins, with β-crystallins appearing prior to the γ-crystallins, was still evident in lenses of transgenic mice as is the case for the normal lens. Effectively, the over-expression of Sef has resulted in the uncoupling of the fiber differentiation process. This was also supported by the fact that we observed an extension of the normal expression of $p57^{Kip2}$ in our transgenic lens, with cells expressing γ-crystallins also expressing $p57^{Kip2}$. As can be seen from our findings in wild-type lenses, during the normal lens fiber differentiation process, the transient upregulation and expression of $p57^{Kip2}$ is lost before the cells accumulate γ-crystallin. This uncoupling of the lens fiber differentiation process has previously been reported in studies from our laboratory where we used a selective MEK inhibitor to block ERK1/2 signaling during FGF-induced fiber differentiation in vitro (Lovicu and McAvoy, 2001). These earlier studies clearly demonstrated that the elongation of fiber cells is dependent on ERK1/2 signaling. By blocking the increased levels of phosphorylated ERK1/2 during FGF-induced fiber differentiation, the elongation of lens epithelial cells was blocked, as was the expression of filensin (Lovicu and McAvoy, 2001), as we demonstrate

in the present study. Furthermore, consistent with our current in vivo findings, in these earlier studies the accumulation of β-crystallin (Lovicu and McAvoy, 2001) and γ-crystallin (Wang et al., 2009) was not impaired and hence did not appear to be dependent on ERK1/2 signaling. Our in vitro studies (Lovicu and McAvoy, 2001), together with previous reports that Sef can specifically inhibit ERK1/2 signaling (Furthauer et al., 2002; Kovalenko et al., 2003, 2006; Yang et al., 2003; Preger et al., 2004; Torii et al., 2004; Ziv et al., 2006), would suggest that the Sef-mediated block in cell elongation observed in our transgenic lenses is due to the disruption of ERK1/2 signaling. This is supported by the loss of filensin (which has been previously been shown to be a target of ERK1/2-signaling, Lovicu and McAvoy, 2001), in the lens of our transgenic mice. Our labeling studies; however, indicate that Sef over-expression is not necessarily sufficient to block the phosphorylation of ERK1/2. Mindful of this, if Sef was indeed negatively impacting on ERK1/2 activity in the transgenic tissues, it may be doing so by preventing the translocation of ERK1/2 to the cell nucleus as has been proposed earlier (see Torii et al., 2004). Although the exact mode of Sef action on ERK1/2-signaling is still contentious (see Torii et al., 2004; Ron et al., 2008), our ongoing studies will be aimed at identifying the exact mechanisms involved in this interaction.

There have only been a select small number of genetically modified mouse studies that present a lens phenotype similar to that demonstrated in the present study. Not surprisingly, one of the most similar has been the conditional deletion of three FGF receptors (*fgfr1–3*) specifically in the lens. This resulted in a block to lens development at the lens vesicle stage (see Zhao et al., 2008), with increased cell death and the block in elongation of the primary and secondary fiber cells, as we have reported. Although we propose there to be morphological similarities between the lenses of our mutants and those deficient for *fgfrs*, the two systems are essentially different. Not only is the timing of the onset of the *Sef* transgene expression different to the timing of the loss of all three *fgfrs* in the knockout lenses, but the latter has complete loss of FGFR activity. This would explain why lenses in these mutants have reduced pERK labeling and a more pronounced phenotype. In our mutants, we are at most blocking only part of the FGFR signaling pathway or at least reducing the overall level of this activity. It is not too unexpected; however, that there is such a similarity in phenotype between the two models, given that Sef has previously been reported to directly act on FGFRs by decreasing their tyrosine phosphorylation (Tsang et al., 2002; Xiong et al., 2003) and subsequent downstream signaling. More specifically, these earlier studies demonstrated that zebrafish Sef (zfSef, Tsang et al., 2002) and its human orthologue (hSef-a; Xiong et al., 2003) interacted with FGFR1 and FGFR2, with the latter study showing no interaction with FGFR3 (Xiong et al., 2003). Given that the loss of only *fgfr1* and *fgfr2* is not sufficient to impair lens development (Zhao et al., 2008), with all three FGFRs (*fgfr1, fgfr2* and *fgfr3*) required, we also over-expressed *mSef* in lenses deficient for *fgfr1* and *fgfr2*. The resultant progeny from these experiments demonstrated a similar phenotype to that seen with the conditional deletion of all three FGFRs (*fgfr1–3*) in the lens. Based on these findings and contrary to reports in the literature, we propose that mSef has the ability to also interact and effectively block the activity of FGFR3. Interestingly, over-expression of relatively lower levels of Sef (consistent with the lower levels in our hemizygote transgenic mSef lines), promotes a more severe phenotype on a *fgfr1/fgr2*-deficient lens background, compared to lenses with a full complement of *fgfrs*. The *fgfr1/fgr2*-deficient lens, hemizygous for the *mSef* transgene displayed a small anterior lumen, indicating a reduced ability of the primary fibers cell to elongate. In light of this, one possibility is that the lower levels of Sef are now more effective on lenses that had fewer FGFRs (*fgfr1/fgr2*-deficient), indicating that mSef may indeed be acting directly on the FGFRs. Furthermore, this supports our initial observations that the dosage of mSef (hemizygous versus homozygous phenotypes) influences the extent of inhibition on cell elongation. Although there is strong support for mSef directly acting on FGF-mediated RTK signaling, Sef has been reported to act on other growth factor-induced RTK-signaling

(Kovalenko et al., 2003; Xiong et al., 2003; Yang et al., 2003; Preger et al., 2004; Torii et al., 2004; Darby et al., 2006; Ziv et al., 2006; Zisman-Rozen et al., 2007). Based on this we cannot exclude the possibility that mSef may also have some effect on lens cells by antagonising an FGFR-independent signaling pathway(s).

A number of alternatively spliced isoforms of human *Sef*, including *hSef-b* (Preger et al., 2004) and *hSef-S* (Rong et al., 2007) have been reported. Although isoforms such as hSef-b retain the ability to associate with the FGFR and inhibit FGF activity by restricting the Ras/MAPK pathway (Preger et al., 2004; Ziv et al., 2006), their mode of activity is different to the prototypic Sef. The fact that hSef-b lacks a signal peptide and is localised to the cytosol (Preger et al., 2004), together with its restricted expression pattern (Preger et al., 2004; Zisman-Rozen et al., 2007), differentiates it from the full length Sef. To examine the effectiveness of this isoform on blocking lens fiber cell differentiation, we over-expressed it in the lens of transgenic mice. Although we demonstrated markedly high levels of Sef expression in the lens of transgenic mice, this did not impact on normal lens development, as demonstrated with mSef. Although hSef-b has been reported to block FGF activity, as well as the activity of other growth factors, such as EGF and PDGF (Kovalenko et al., 2003; Xiong et al., 2003; Yang et al., 2003; Preger et al., 2004; Torii et al., 2004; Darby et al., 2006; Ziv et al., 2006; Zisman-Rozen et al., 2007), it had no effect on the lens. This reinforces the fact that Sef antagonism of RTK signaling is dependent on isoform type and cellular context.

Based on our current findings, we propose an important role for Sef in regulating lens fiber differentiation. Its restricted higher expression in the normal lens epithelium (Boros et al., 2006) would suggest that it acts specifically in the lens epithelium to modulate growth factor activity and potentially maintain the lens epithelial phenotype. With the subsequent differentiation of the lens epithelial cells into secondary fiber cells, Sef is down-regulated (Boros et al., 2006). Whether this downregulation is required for fiber differentiation to proceed is unclear given that no impairment to lens or other ocular tissues has been reported in mice deficient for *Sef* (Lin et al., 2002). This can be attributed to the presence of other similar acting negative regulators of RTK-signaling, such as members of the Sprouty family, that have also be shown to be co-expressed with Sef in the developing lens (Boros et al., 2006). Clearly, the evidence is compelling that the distinct anterior–posterior polarity of the lens is maintained by an anterior–posterior gradient of FGF activity. Given our current findings that persistent over-expression of mSef in the lens fibers cells restricts the ability of the fiber cells to elongate, we provide evidence that one level of this regulation may involve a cell autonomous role for Sef (or like molecules, such as Sprouty) in orchestrating the regulation of RTK signaling, and in effect providing a double assurance mechanism for the maintenance of the normal lens polarity and growth patterns.

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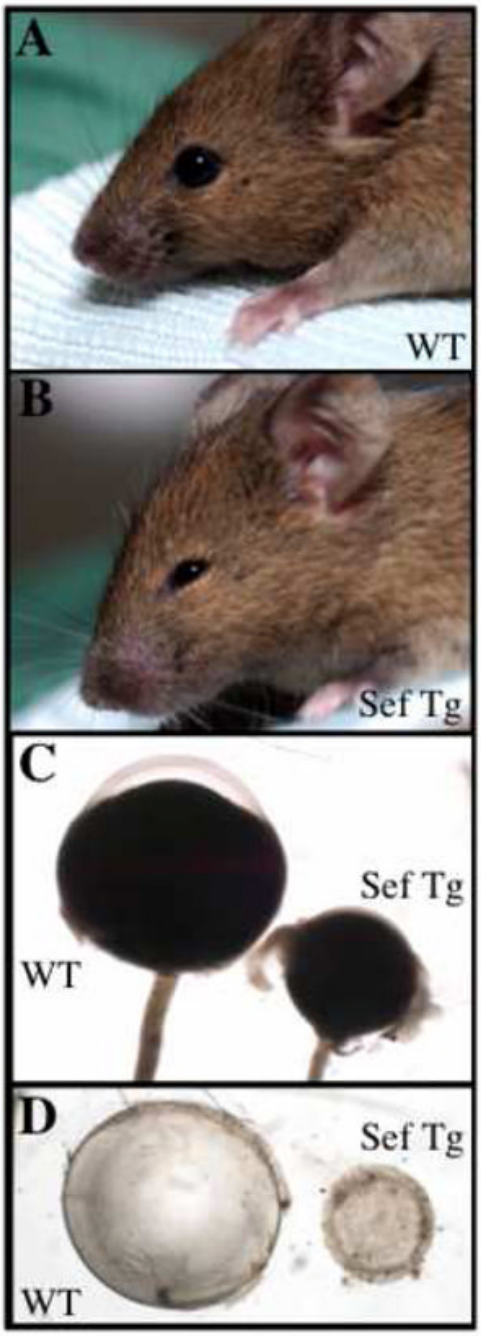

Fig. 1.

Transgenic lines of mice hemizygous for mSef display microphthalmia (B), compared to wild-type littermates (A). When comparing either whole eyes (C) or whole lenses (D), tissues from wild-type mice (WT, left) are proportionally larger in size compared to those from homozygous transgenic mice (Sef Tg, right).

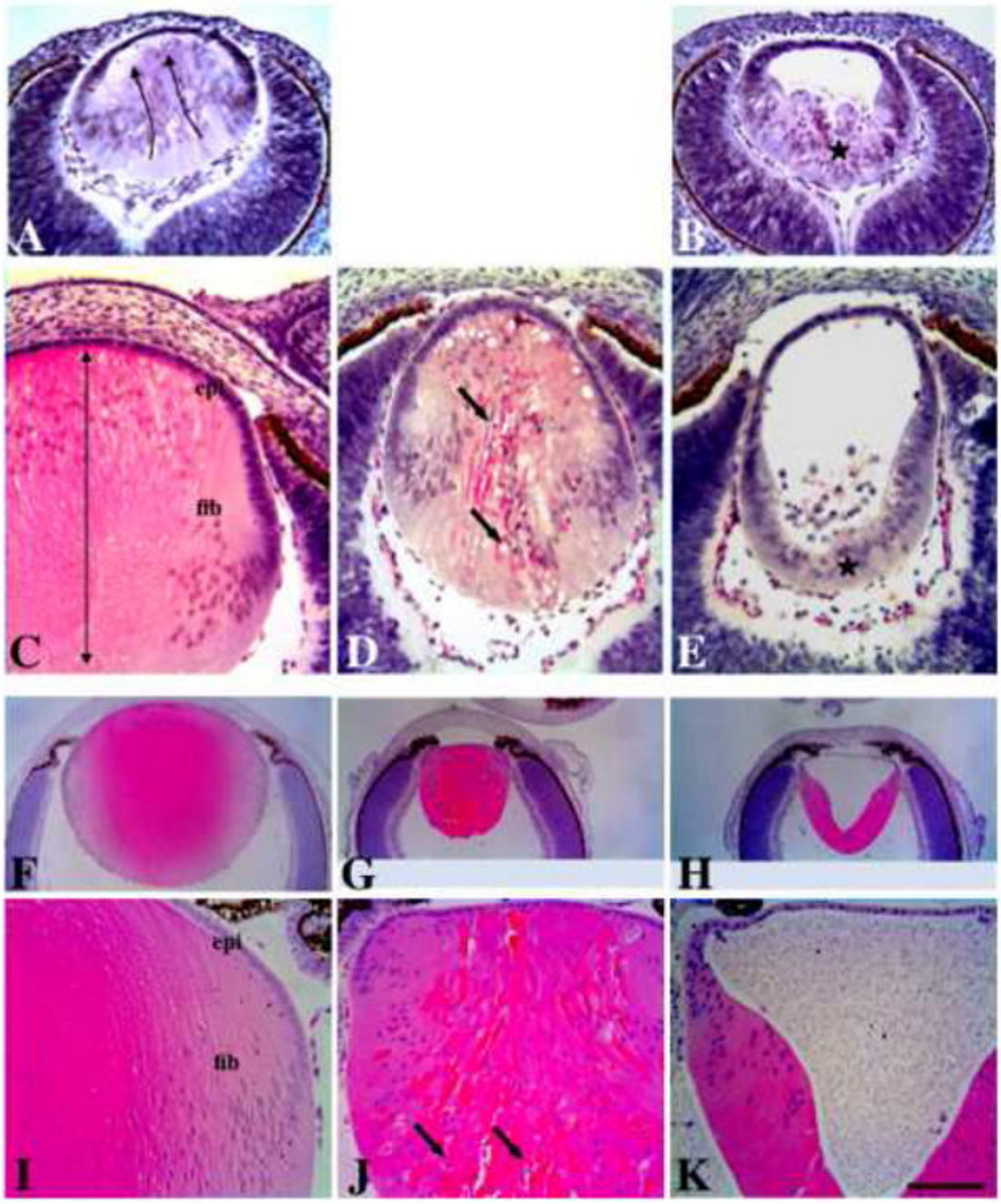

Fig. 2.

Comparative histology of ocular tissues from E12.5 (A, B), E15.5 (C, D, E) and postnatal day 1 (F–K) mice, stained with haematoxylin and eosin. The lens of wild-type mice (A, C, F, I) acquires its distinctive architecture from E12.5 (A, arrows indicate normal elongation of primary fiber cells) and maintains this throughout development (C, F, I), with a monolayer of lens epithelial cells (epi) overlying a mass of elongated lens fibers (fib). In eyes derived from mice hemizygous for the *mSef* transgene (D, G, J), the lens is relatively smaller than that of wild-type mice (C, F, I), and has a reduced and disorganised fiber cell mass, with prominent pyknotic nuclei (D, J, arrows), indicative of cell death. In mice homozygous for the *mSef* transgene (B, E, H, K), the lens is similar in size to that of

hemizygous tissue; however, the inability of the posterior cells to elongate (B, E, stars) results in the presence of a distinctive lumen that is maintained from E12.5 (B) through to postnatal stages (H, K). Although some elongation of posterior cells is apparent by postnatal day 1 (H, K), this differentiation process is grossly compromised in all homozygous lens tissues. Scale bar, A–E, 100 μm; F–H, 500 μm; I–K 125 μm.

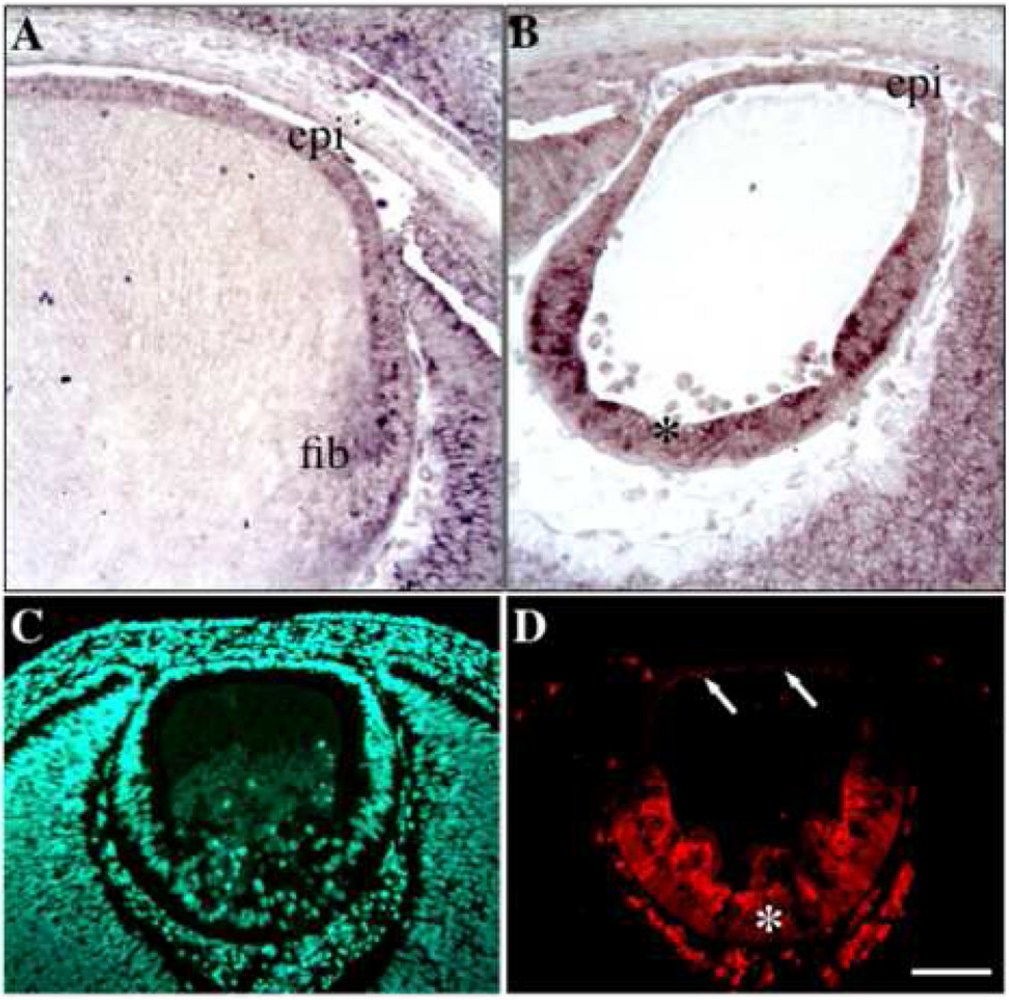

Fig. 3.

Representative micrographs of expression of *Sef* mRNA transcripts in lenses of E15.5 wild-type (A) and homozygous transgenic mice (B), as well as E13.5 transgenic lenses, homozygous for mSef (C, D), labeled with Hoechst dye (C) or immunofluorescently labeled for Sef protein (D). Lenses homozygous for the *mSef* transgene (B, D) had stronger labeling of Sef transcripts and protein in posterior cells (asterisk) than that seen in wild-type tissues (A), with immunolabeling also detected in the lens epithelium (D, arrows). Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, A–B, 50 μm; C–D, 100 μm.

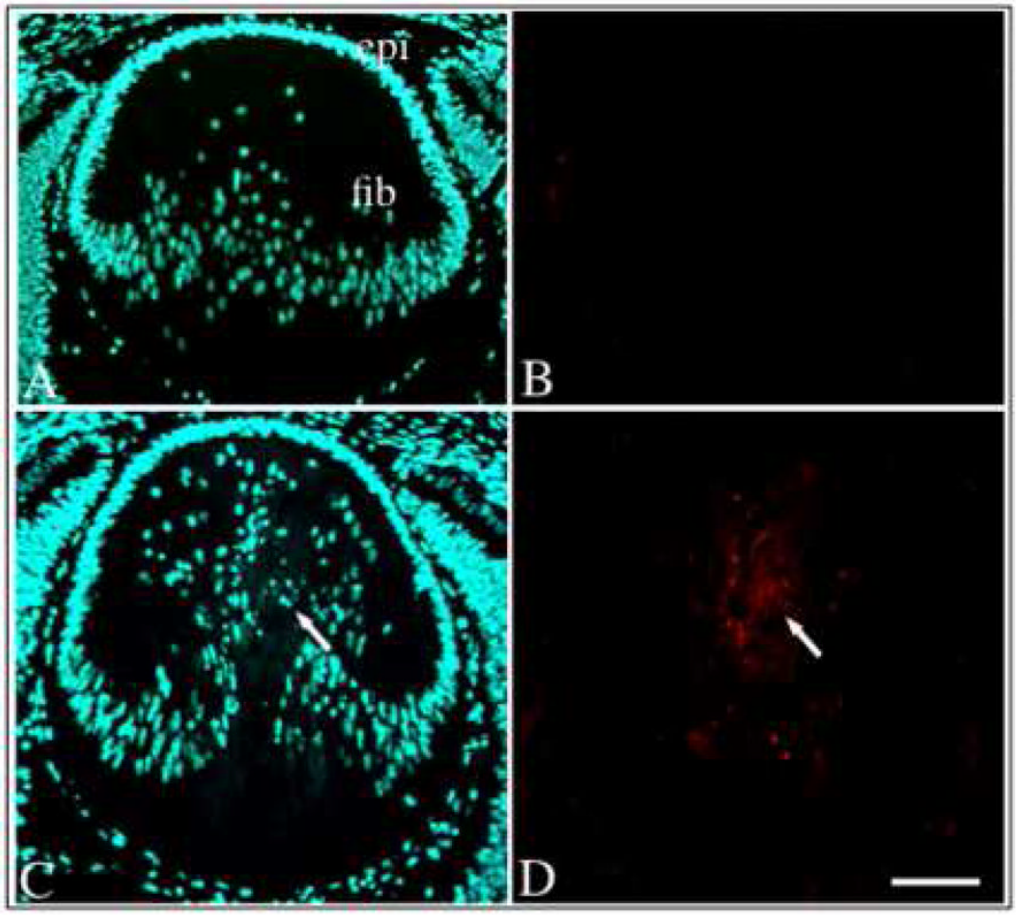

Fig. 4.

Immunofluorescent micrographs of ocular tissues from E15.5 wild-type (A, B) and transgenic mice hemizygous for mSef (C, D), labeled with either Hoechst dye (A, C) or for TUNEL (B, D) to identify apoptotic cell death. In contrast to wild-type lenses that had no cells labeled for TUNEL (B), lenses of hemizygous lines of mice (D) demonstrated distinct TUNEL labeling (arrow) of many pyknotic cell nuclei. A diffuse Hoechst-fluorescence (C), reflective of fragmented DNA, was also shown to overlap with the TUNEL-labeling (D). Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, 100 μm.

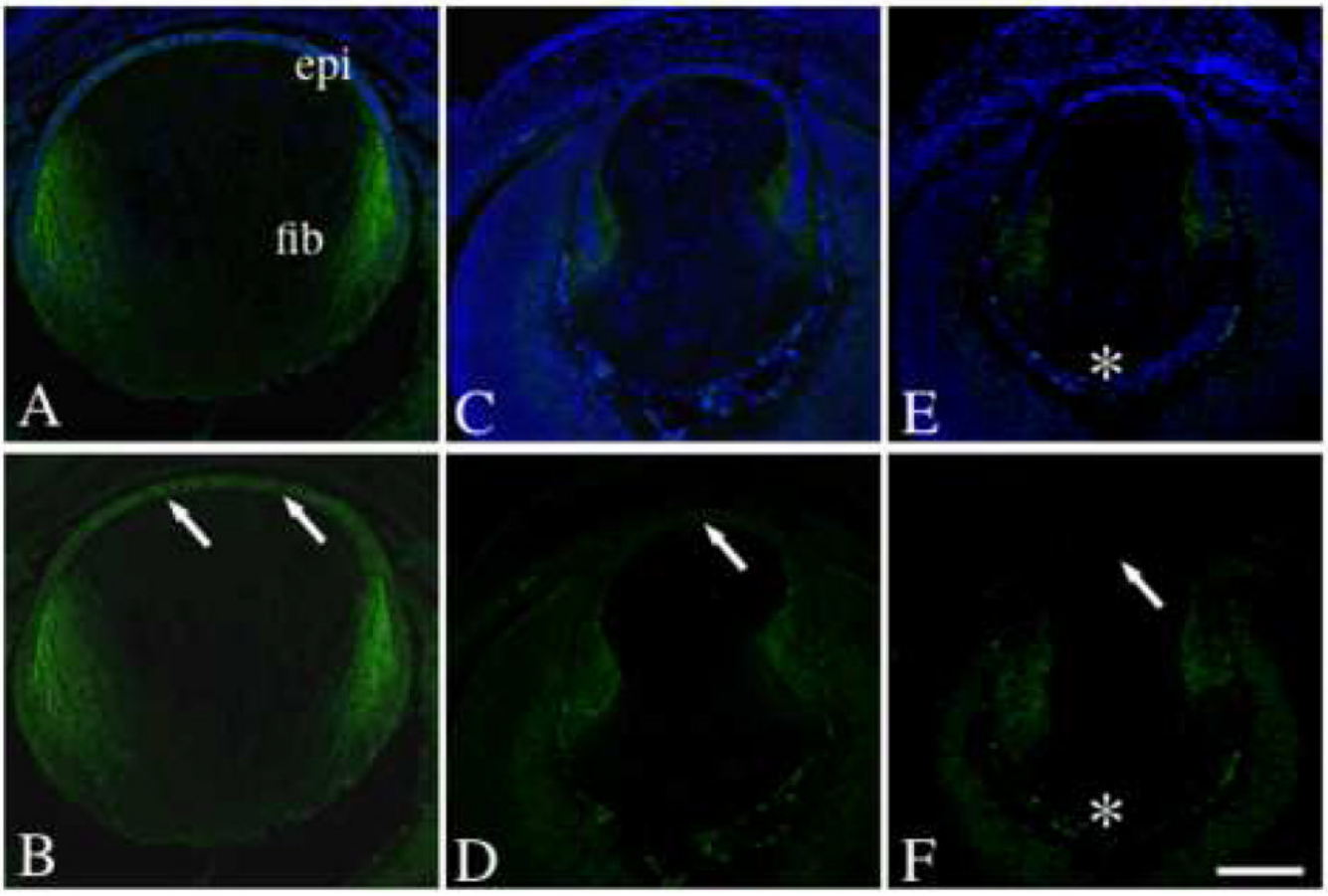

Fig. 5.

Immunofluorescent micrographs of ocular tissues from E15.5 wild-type (A, B) and transgenic mice hemizygous (C, D) or homozygous (E, F) for mSef, labeled for pAkt (green), counterstained with (A, C, E) or without DAPI (B, D, F). In wild-type lens tissues, pAkt labeling is strong in the lens epithelium (B, arrows) and persists as lens fiber cells begin to elongate and differentiate at the lens equator (A, B). In transgenic tissues, reactivity for pAkt is markedly reduced in both lens epithelial cells (D, F, arrows) and fiber cells, including those cells that failed to elongate in homozygous lines (F, asterisk). Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, 150 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

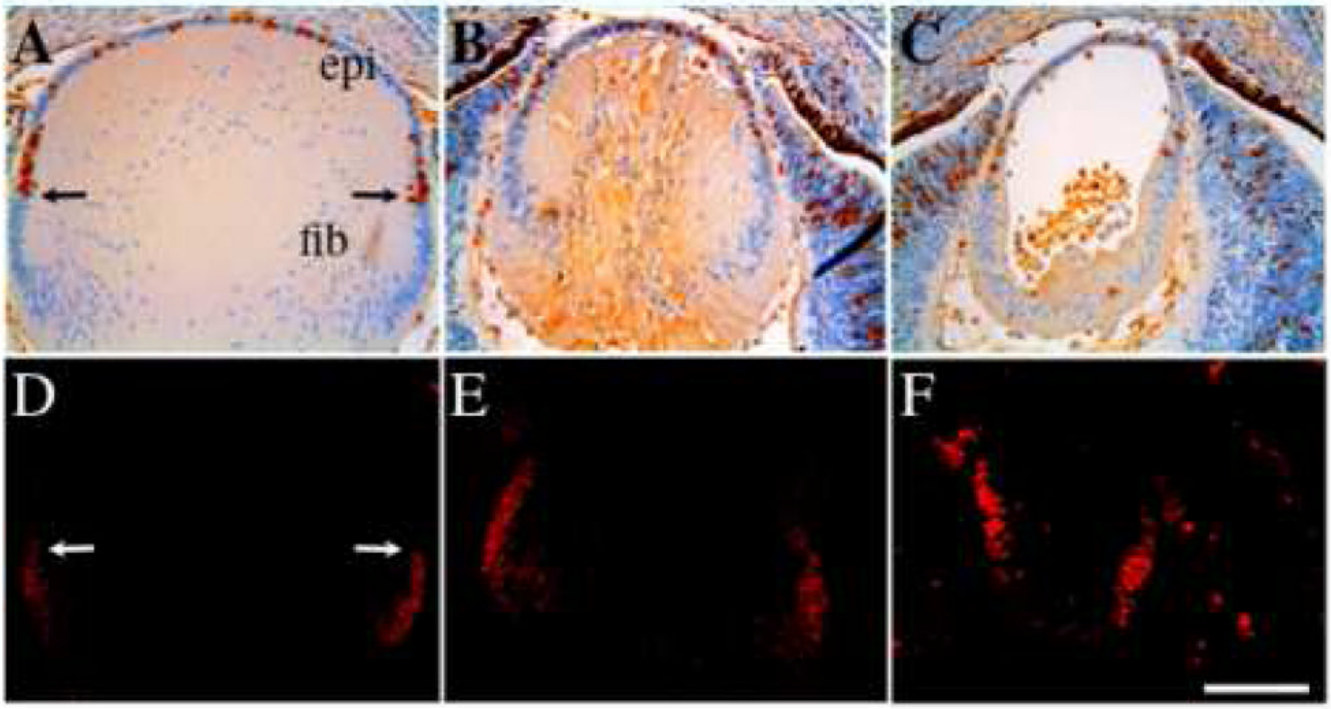

Fig. 6.

Representative micrographs of E15.5 lenses from wild-type mice (A, D), and mice hemizygous (B, E) or homozygous (C, F) for the *mSef* transgene, immunolabeled for either BrdU-incorporation (A–C) or $p57^{Kip2}$ (D–F). BrdU-labeled cells (labeled brown) in the wild-type lens (A) are highest in number just above the lens equator (arrows) and overall, higher in number than in lenses from mice hemizygous (B) or homozygous (C) for the mSef transgene. The percentage of BrdU-labeled cells, relative to total number of epithelial cells, however, is similar in all tissues. $p57^{Kip2}$-labeling in wild-type lenses is localised to cells just posterior to the lens equator (D, arrows), primarily in the early differentiating fiber cells (D) with no expression of $p57^{Kip2}$ in the more mature fiber cells. In lenses from mice hemizygous for mSef (E), $p57^{Kip2}$ is similar to that of the wild-type lens albeit extending a little further into the fiber differentiation zone. In lenses of homozygous mice (F), $p57^{Kip2}$ expression persists in many, if not all, of the posterior lens cells that had failed to appropriately elongate and differentiate. Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

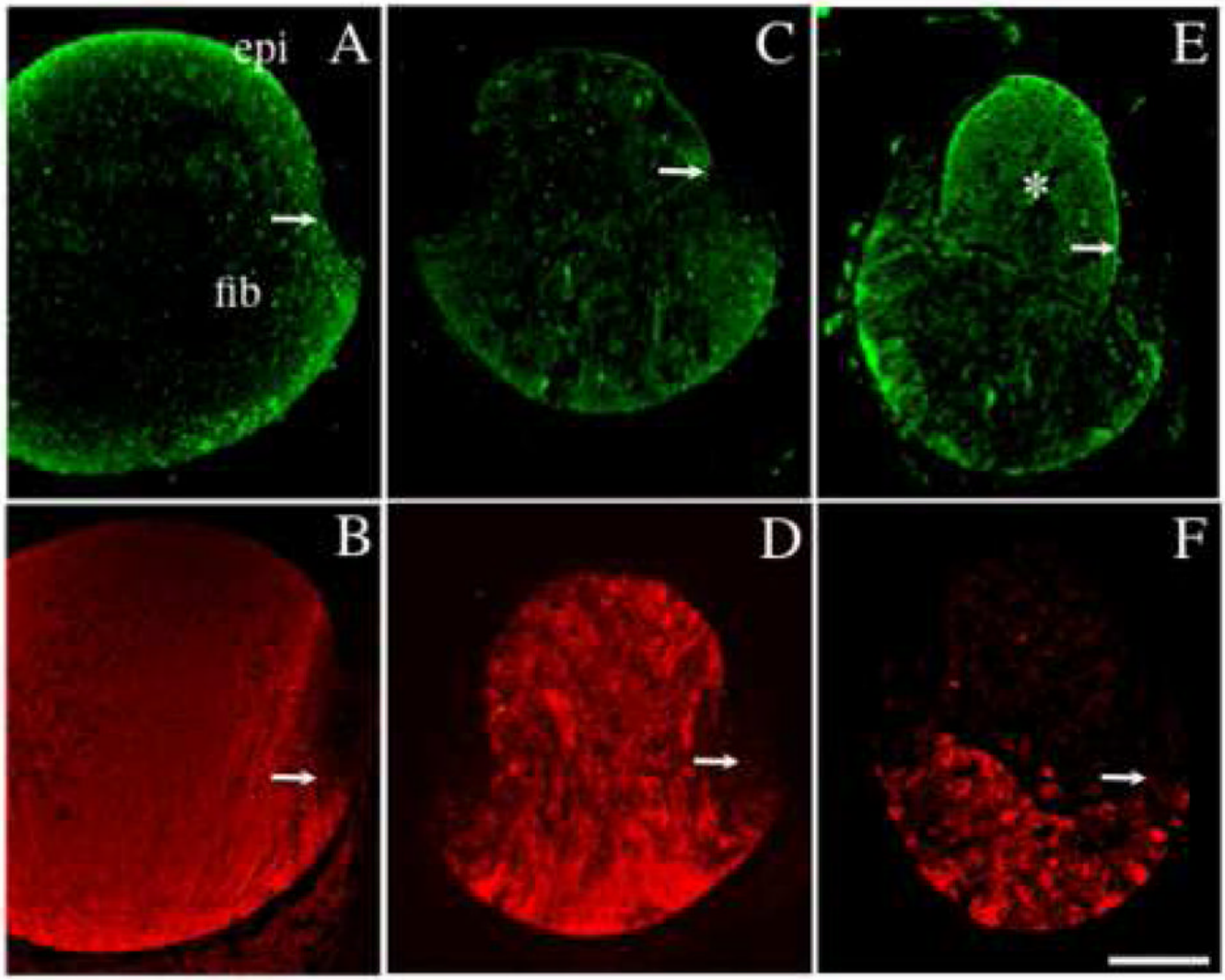

Fig. 7.

Representative micrographs of E15.5 lenses from wild-type mice (A, B) and mice hemizygous (C, D) or homozygous (E, F) for the *mSef* transgene, immunolabeled for either β-crystallin (A, C, E) or γ-crystallin (B, D, F). In all lenses, the pattern of expression of β- and γ-crystallin was similar, with β-crystallin highly expressed in the earliest fiber cells posterior to the lens equator (arrow), with γ-crystallin accumulation restricted to slightly more mature cells (arrow). Note that the strong reactivity for β-crystallin in the lumen of the homozygous lens (E, asterisk) most likely reflects the release of protein from dying cells. Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, 150 μm.

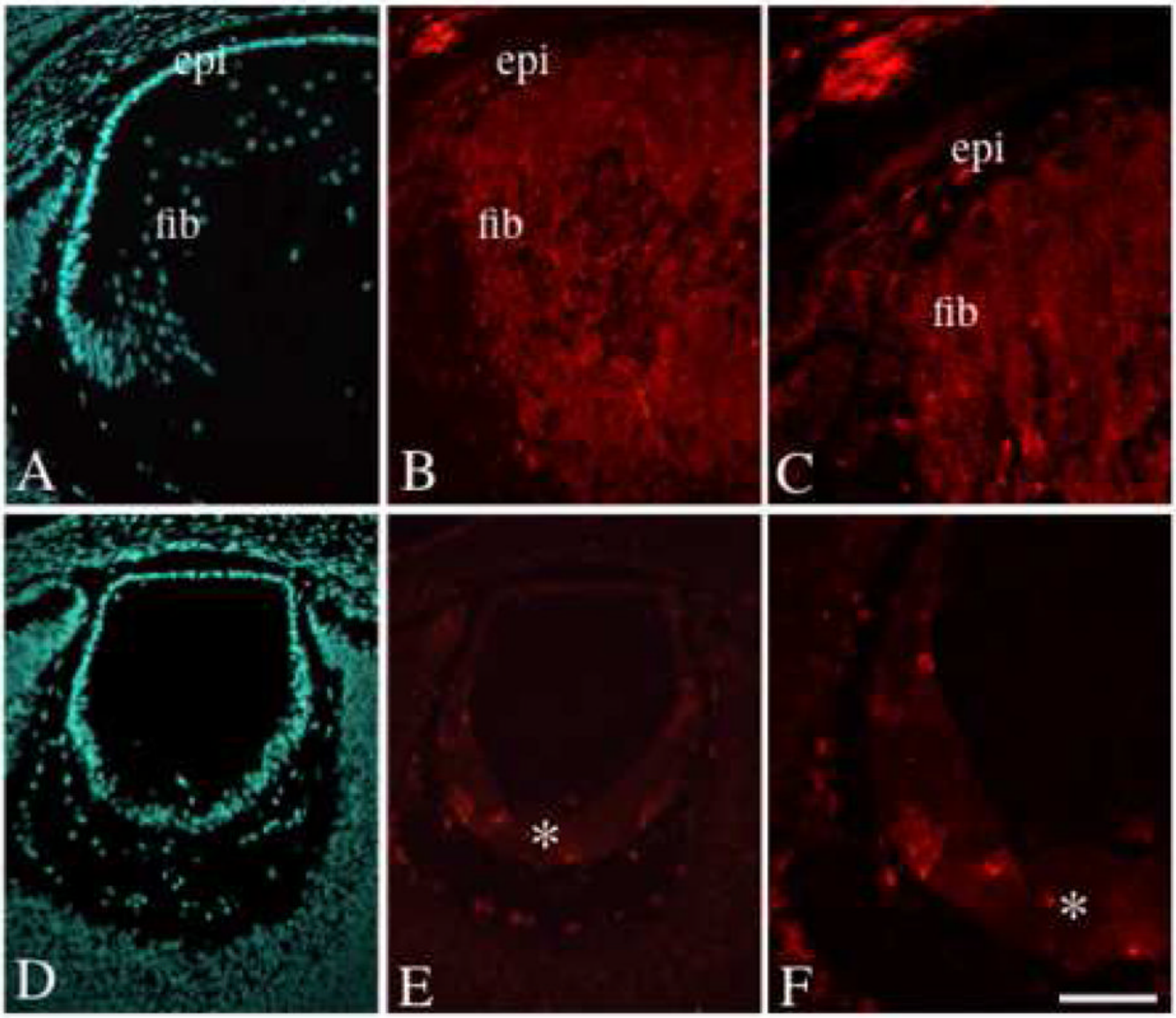

Fig. 8.

Representative micrographs of E15.5 lenses from wild-type mice (A–C), and mice homozygous (D–F) for the *mSef* transgene, either labeled with Hoechst dye (A, D) or immunolabeled for filensin (B, C, E, F). In the lens of wild-type mice, filensin was confined to the differentiating fiber cells with little to no reactivity throughout the lens epithelium (A–C). In lenses from transgenic mice, there was a markedly reduced label for filensin, with the majority of the aberrant posteriorly situated lens cells displaying little to no label (E, F, asterisk). Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, A, B, D, E, 150 μm; C, F, 75 μm.

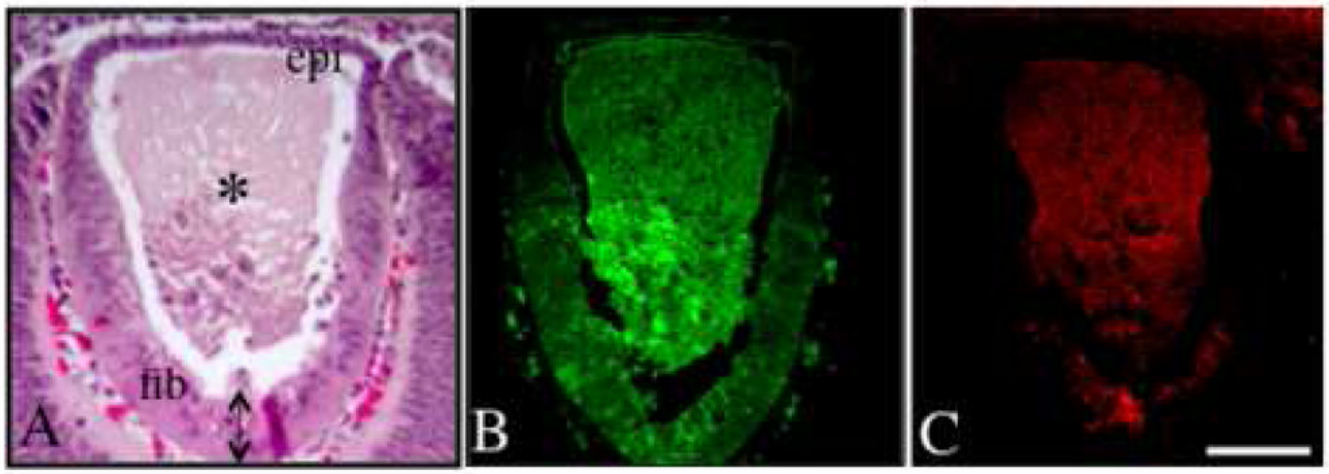

Fig. 9.

Defective lens fiber cell elongation in E15.5 mutant mice with lenses deficient for *fgfr1*, *fgfr2* and *fgfr3*, stained with haematoxylin and eosin (A), or immunolabeled for either β-crystallin (B) or γ-crystallin (C). The failure of the primary lens fiber cells to elongate appropriately (A, double arrow) led to the retention of a prominent lens lumen (asterisk), with a reduced lens epithelium (A). There was relatively normal expression of β-crystallin (B), with markedly lower expression of γ-crystallins (C), restricted to the apical pole of posterior 'fiber'-like cells. Reactivity in the lumen of the lens may reflect protein accumulation from dying cells or may be an artefact of immunolabeling. Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, 150 μm.

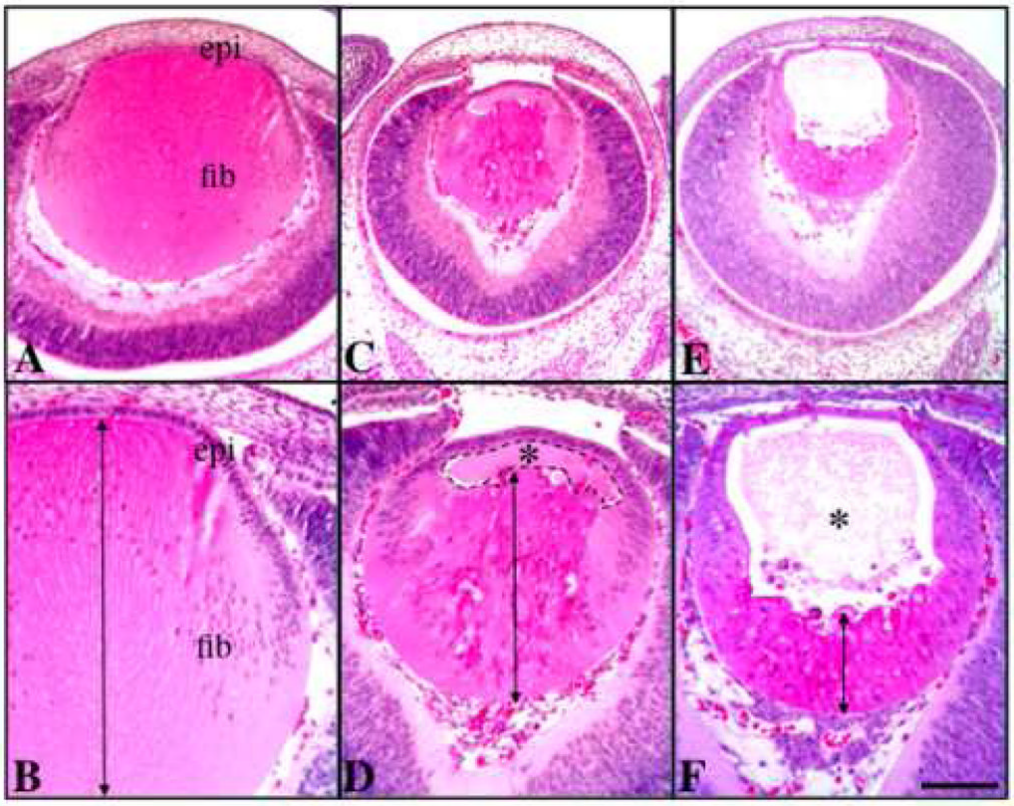

Fig. 10.

Haematoxylin and eosin staining of E15.5 eyes from mutant mice, with lenses deficient for *fgfr1* and *fgfr2*, either wild-type (A, B), hemizygous (C, D) or homozygous (E, F) for the *mSef* transgene. Conditional deletion of both *fgfr1* and *fgfr2*, results in a normal lens (A, B). Over-expression of mSef in lenses deficient in FGFR1 and FGFR2, but with a full complement of FGFR3 (C–F) are markedly smaller than wild-type lenses, with fewer lens epithelial cells and defective elongation (compare length of double-sided arrows) of the primary fiber cells resulting in a lens lumen (D, F, outlined with broken line). The lens lumen is larger in mice homozygous for the *mSef* transgene (E, F), where the inhibition of primary cell elongation is more pronounced. Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, A, C, E, 200 μm; B, D, F, 100 μm.

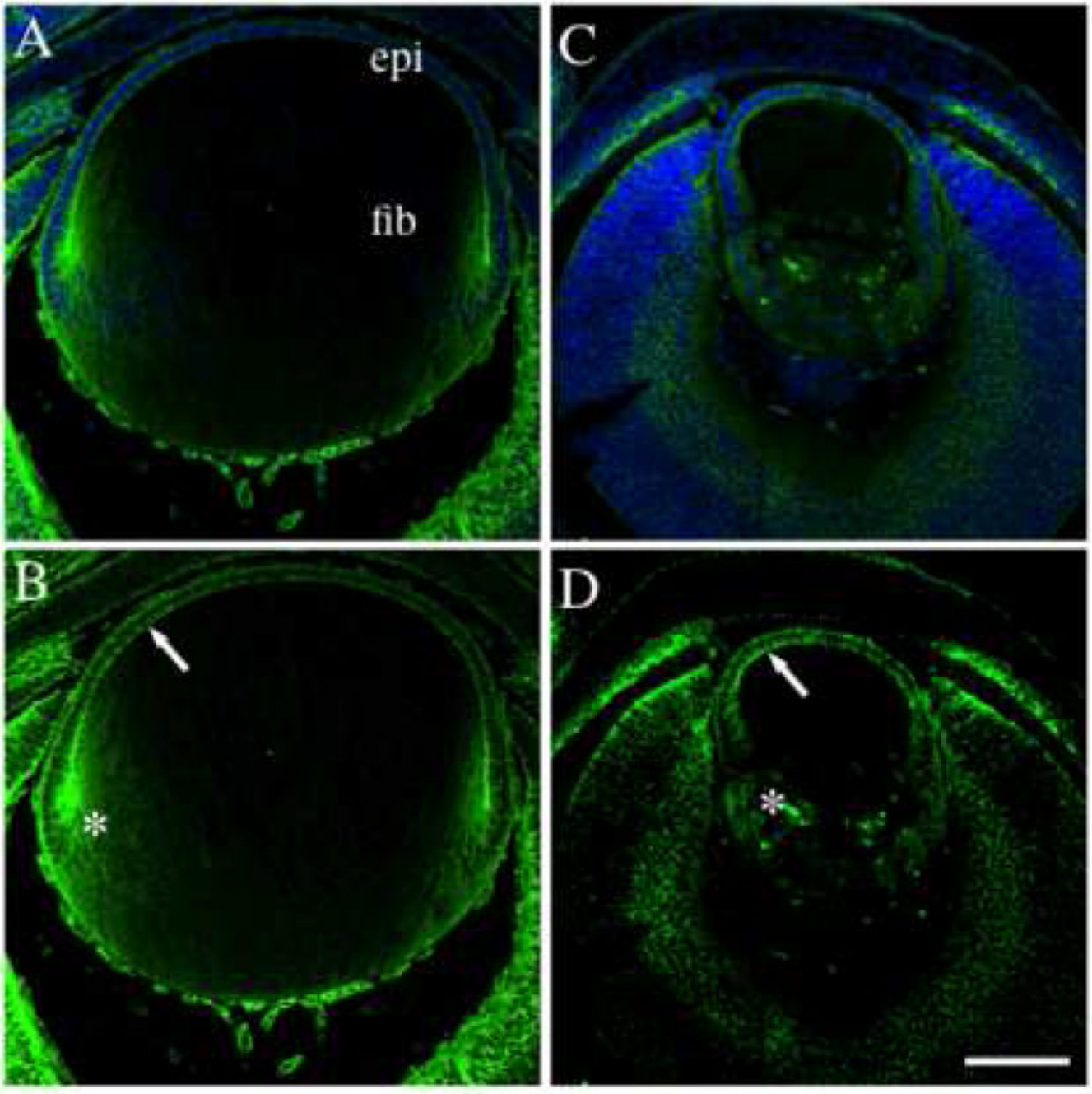

Fig. 11.

Immunofluorescent micrographs of ocular tissues from E15.5 wild-type (A, B) or transgenic mice homozygous for mSef (E, F), labeled for pFrs2α (green), counterstained with (A, C) or without (B, D) DAPI. In wild-type lens tissues, pFrs2α labeling is strong in the lens epithelium (B, arrow) and persists as lens fiber cells begin to elongate and differentiate at the lens equator (A, B, asterisk). In transgenic tissues, reactivity for pFrs2α is reduced in the posterior lens 'fiber' cells that have failed to elongate in homozygous lines, yet still persists, in the lens epithelium of these lenses (D, arrow). Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, 150 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

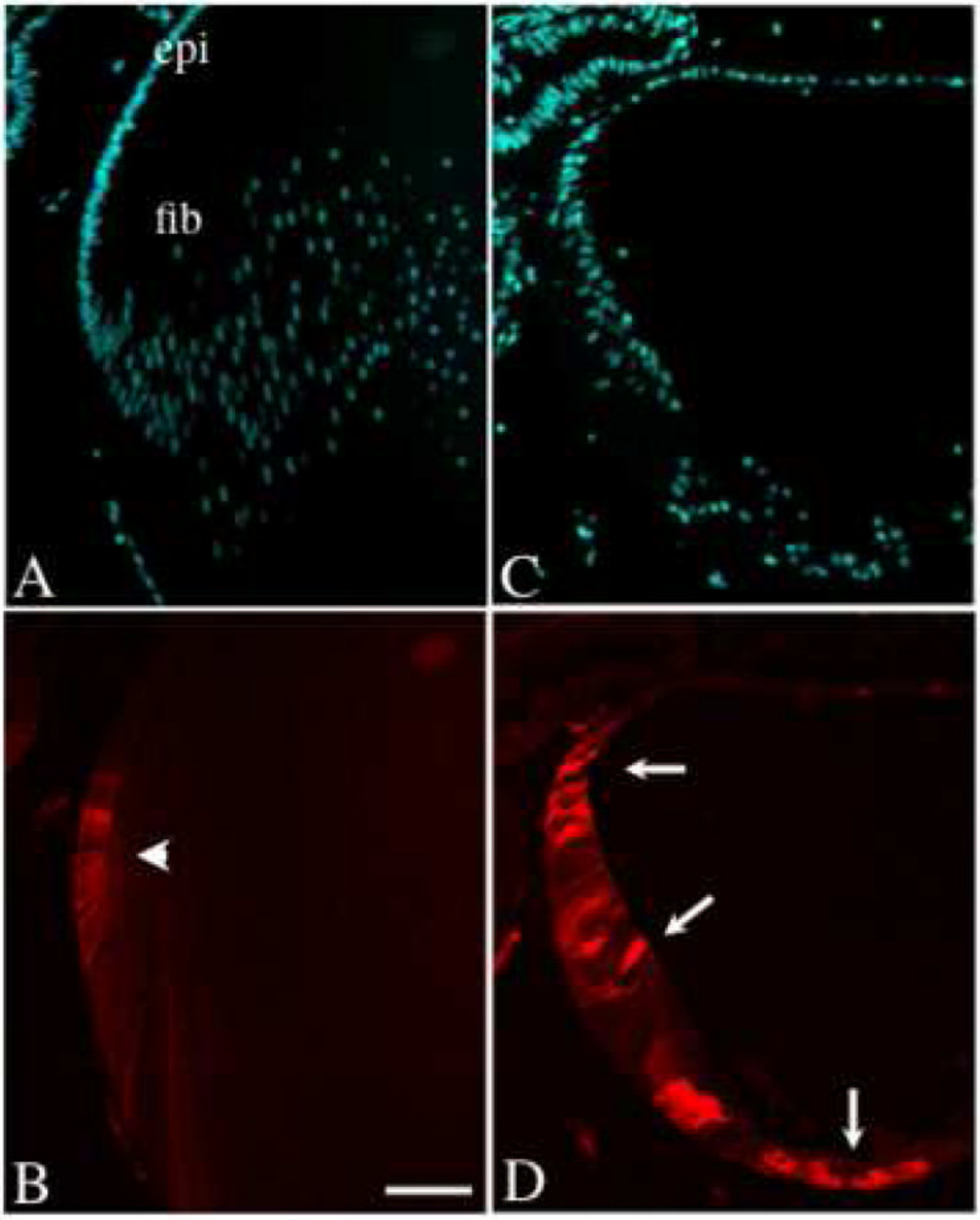

Fig. 12.

Immunofluorescent micrographs of ocular tissues from postnatal day 3 wild-type (A, B) or transgenic mice homozygous for mSef (C, D), labeled with either Hoechst dye (A, C) or pERK1/2 (B, D). In wild-type lens tissues, pERK1/2 labeling is strongest in the most posterior lens epithelial cells (B, arrowhead) and decreases in intensity as cells begin to elongate and differentiate into fibers (A, B). In transgenic tissues, reactivity for pERK1/2 is strong in some lens epithelial cells as well as many of the posterior lens 'fiber' cells that have failed to elongate in homozygous lines (D, arrows). Note that not all of these posterior lens cells are reactive for pERK1/2. Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, 50 μm.

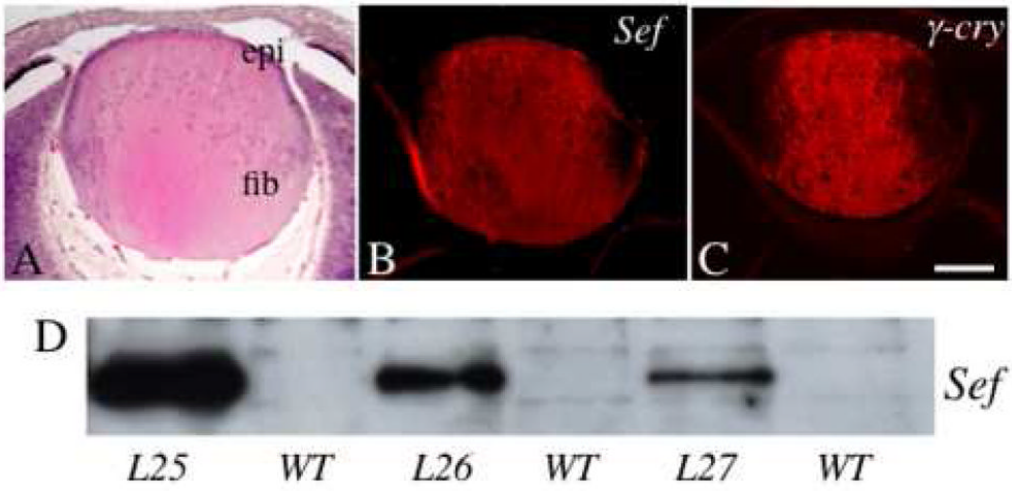

Fig. 13.

(A) Representative haematoxylin and eosin-stained section of an eye from an E15.5 mouse, homozygous for the over-expression of the *hSef-b* transgene, specifically in the lens. Although hSef-b protein is expressed at high levels in the lens of transgenic mice, as shown by immunofluorescence (B) and Western blotting (D) in different transgenic lines (L25, L26, L27), the lenses develop normally (A), expressing normal lens markers such as γ-crystallin (C). Abbreviations: epi, lens epithelium; fib, lens fibers. Scale bar, 200 μm.