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Fgf receptor signaling plays a role in lens induction

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SUMMARY

We describe experiments showing that fibroblast growth factor receptor (Fgfr) signaling plays a role in lens induction. Three distinct experimental strategies were used: (1) using small-molecule inhibitors of Fgfr kinase activity, we showed that both the transcription level and protein expression of Pax6, a transcription factor critical for lens development, was diminished in the presumptive lens ectoderm; (2) transgenic mice (designated *Tfr7*) that expressed a dominant-negative Fgf receptor exclusively in the presumptive lens ectoderm showed defects in formation of the lens placode at E9.5 but in addition, showed reduced levels of expression for *Pax6, Sox2* and *Foxe3*, all markers of lens induction; (3) by performing crosses between *Tfr7* transgenic and *Bmp7*-null mice, we showed that there is a genetic interaction between Fgfr and Bmp7 signaling at the induction phases of lens development. This manifested as exacerbated lens development defects and lower levels of *Pax6* and *Foxe3* expression in *Tfr7/Tfr7, Bmp7*$^{+/-}$ mice when compared with *Tfr7/Tfr7* mice alone. As Bmp7 is an established lens induction signal, this provides further evidence that Fgfr activity is important for lens induction. This analysis establishes a role for Fgfr signaling in lens induction and defines a genetic pathway in which Fgfr and Bmp7 signaling converge on *Pax6* expression in the lens placode with the *Foxe3* and *Sox2* genes lying downstream.

Key words: Lens induction, Lens development, Dysgenetic lens, Pax6, Fgfr, Bmp7, Foxe3, Sox2, Mouse

INTRODUCTION

The lens is a relatively simple structure with which to study the signaling and gene regulation required for normal development. Key experiments have included those of Spemann (Spemann, 1901) that led to the idea of embryonic induction and raised the disputed (Mencl, 1903) possibility that the optic vesicle was required for lens development. In more recent times, molecular genetic approaches have identified a series of signaling molecules and transcription factors that are critical for the optic vesicle-presumptive lens interaction.

Of central interest is Pax6, a homeodomain transcription factor that is necessary for eye (Hogan et al., 1986b; Hill et al., 1991) and lens development (Ashery-Padan et al., 2000; Fujiwara et al., 1994) and, in the context of both invertebrate (Halder et al., 1995) and vertebrate (Altmann et al., 1997; Chow et al., 1999) embryos, is sufficient. The expression of *Pax6* in the lens lineage in vertebrates (Grindley et al., 1995) is controlled by at least one highly conserved transcriptional control element (Williams et al., 1998). This 341 bp enhancer has activity in directing gene expression to the lens placode, lens epithelium, immature primary lens fiber cells, corneal epithelium and the epithelium of the lacrimal gland (Kammandel et al., 1999; Makarenkova et al., 2000; Williams et al., 1998). In the mouse, *Pax6* expression in the lens lineage is maintained from the placode stage onwards through the action of bone morphogenetic protein (Bmp) 7 (Wawersik et al., 1999), a member of the transforming growth factor β superfamily (Massague, 1998). *Bmp7* is normally expressed in the lens placode and its deletion through gene targeting results in a failure of *Pax6* expression maintenance (Wawersik et al., 1999) and a variably penetrant phenotype that manifests as anophthalimia in its most severe form (Dudley et al., 1995).

Fibroblast growth factors (Fgfs) have been implicated in late lens development with the demonstration that Fgf-containing retina-conditioned medium and recombinant Fgf 1 and Fgf 2 could stimulate fiber cell differentiation in lens epithelial explants (Chamberlain and McAvoy, 1987; Chamberlain and McAvoy, 1989). In addition, a series of in vivo gain-of-function studies have shown that a number of Fgf ligands can modulate lens development. The placement of Fgf8-containing beads adjacent to the developing chick eye has suggested a role in early lens development (Vogel-Hopker et al., 2000), while overexpression of a variety of ligands in transgenic mice can stimulate premature differentiation of lens fiber cells (Lovicu and Overbeek, 1998; Robinson et al., 1995b). Some members of the Fgf receptor family are also expressed in the lens lineage (de Iongh et al., 1997; de Iongh et al., 1996). Fgfr1 is expressed in the presumptive ectoderm and lens pit. After lens vesicle separation, Fgfr1 and Fgf2IIIc are both expressed in the lens vesicle and presumptive corneal epithelium. As lens development proceeds, Fgfr2IIIc (bek) is expressed throughout

the lens epithelium and the transitional zone, but expression declines in maturing lens fiber cells. By contrast, Fgfr2IIIb (KGFR) shows strong expression in the early fibers of the transitional zone with weaker expression in the lens epithelium (de Iongh et al., 1997; de Iongh et al., 1996). Consistent with these observations, it has been shown that when dominant-negative or dimerized soluble forms of fibroblast growth factor receptors (Fgfrs) are expressed in the developing fiber cells, their differentiation is suppressed (Chow et al., 1995; Govindarajan and Overbeek, 2001; Robinson et al., 1995a), indicating that Fgfr activity is necessary. To date, the question of whether Fgfr signaling might be required for earlier phases of lens development, including lens induction, has not been addressed.

In this report, we describe such experiments. We use three distinct strategies to show that at both the morphological and molecular levels, lens induction does not proceed normally if Fgfr activity is perturbed. First, we demonstrate that reduced levels of *Pax6* reporter construct expression and Pax6 immunoreactivity result when presumptive lens ectoderm is cultured in the presence of small molecule inhibitors of the Fgfr kinases. Second, we show that when a dominant-negative Fgfr1IIIc is expressed in the presumptive lens ectoderm, early lens development is morphologically abnormal and, in addition, that molecular markers of lens induction, including *Pax6* and *Sox2*, are expressed at reduced levels. Finally, we perform analysis to show that there is a genetic interaction between Bmp7, an established lens inducer (Dudley et al., 1995; Wawersik et al., 1999), and Fgfr signaling at the stage of lens induction. This manifests as more severe lens defects and lower expression levels for *Pax6* and *Foxe3* (a lens lineage marker) in compound genotype animals. Combined, these analyses indicate that Fgfr signaling has an important role in lens induction and that this pathway converges with the activity of Bmp7 in upregulating the expression of *Pax6* (Ashery-Padan et al., 2000; Fujiwara et al., 1994) and *Foxe3* (Blixt et al., 2000; Brownell et al., 2000) – transcription factors necessary for lens development.

MATERIALS AND METHODS

Explant cultures

Explant cultures were prepared from embryonic day (E) 8.0-9.5 wild-type or *P6 5.0-lacz* reporter (Williams et al., 1998) mice. For E8.0-8.5 embryos, the anterior embryo from head-folds to just posterior to the developing heart was excised and placed within a 15 μl drop of collagen gel according to established procedures (Shannon et al., 1999; Wawersik et al., 1999). For E9.0-9.5 embryos, heads were bisected at the midline and half-heads placed in collagen gel. Explants were cultured in DMEM (Gibco BRL) supplemented with heat inactivated 10% fetal calf serum, glutamine, non-essential amino acids, cholera toxin and an antibiotic-antimycotic (Gibco BRL). For each explantation experiment, a minimum of five optic primordia were cultured for each condition tested and each experiment was repeated a minimum of three times. After culture for 8-48 hours, tissues were fixed for 5 minutes with 4% paraformaldehyde (for labeling with anti-Pax6 antibodies) or with 0.2% glutaradehyde, 1% formaldehyde [for X-gal staining according to established protocols (Song et al., 1996)].

Inhibitors of Fgfr signaling

SU9597 is a compound from a new family of inhibitors for the Fgf receptor tyrosine kinases (Mohammadi et al., 1997; Sun et al., 1999) and was used to block Fgf receptor signaling in vitro. The chemical name for SU9597 is 3-{2-[6-(3-methoxy-phenyl)-2-oxo-1,2-dihydro-indol-3-ylidenemethyl]-4-methyl-1H-pyrrol-3-yl}-propionic acid (Makarenkova et al., 2000). For explant cultures, SU9597 was dissolved in dimethylsulfoxide as a 100 mM stock solution, aliquoted and stored at –20°C.

Generation of transgenic mice

A restriction fragment encoding a C-terminally truncated mutant form of the Fgfr1IIIc (Reid et al., 1990) was subcloned into the P6 EE1.0 plasmid that consists of a PGEM4z backbone, the 341 bp *Pax6* ectoderm enhancer [designated EE (Williams et al., 1998)] and a 1 kb fragment containing a minimal *Pax6* P0 promoter. This minimal promoter fragment extends from an *Xho*I site located approximately 1 kb upstream of the P0 transcription start to an *Spe*I located at the transcription start. The SV40 small t splice and polyadenylation signals were downstream of the truncated Fgfr1 cDNA.

RT-PCR expression analysis

RT-PCR was carried out on separated presumptive lens ectoderm and optic vesicle from E9.0-E9.5 *Tfr7/Tfr7* transgenic mice. Separated tissues were obtained by harvesting mouse embryos at E9.0-9.5, removing and bisecting heads and then separating, using fine forceps, the head ectoderm from the epithelium of the neural tube. As the lens placode is very firmly adhered to the presumptive retina of the optic vesicle at E9.5, small volumes of 2 mg/ml dispase (Boehringer Mannheim, #1284908) were pipetted onto the lens placode-optic vesicle junction while the tissue was immersed in room temperature phosphate-buffered saline (PBS). After one or two dispase treatments, the lens placode could be separated from the optic vesicle with ease. The separated tissues were then trimmed to ensure that only presumptive lens ectoderm and optic vesicle was harvested. The Ultaspec reagent (Biotecx, TX) used to isolate total RNA and this reverse transcribed with the Reverse Transcription System (Promega) and oligo-dT primer. The resulting cDNA was then used as template in PCR amplifications with SV40 and GAPDH primers. The amplification conditions were 94°C for 45 seconds, 54°C for 30 seconds and 72°C for 30 seconds for 35 cycles. The SV40 sequence primers are specific for the transgene while the GAPDH primers were used as a positive control. Primer sequences are as follows: SV40-250, 5′TTTGCTCAGAAGAAATGCCA; SV40-450, 3′GCA-GTGCAGCTTTTTCCTTT; GAPDH, 5′CTACATGGTCTACATGT-TCCAGTA; and GAPDH 3′GTGATGGCATGGACTGTGGTCAT.

Histological analysis

Tissues for histological analysis included staged mouse embryos or whole eyes from postnatal animals. Tissue samples were prepared and stained either with Hematoxylin and Eosin or only Hematoxylin using conventional methods (Culling et al., 1985). An assessment of cellular proliferation through S-phase labeling of cells with 5-bromo-2′-deoxyuridine (BrdU) was performed according to standard procedures (Takahashi et al., 1993).

Immunofluorescence

Staged embryos were fixed with 4% paraformaldehyde, cryoprotected in 30% sucrose and 20 μm frozen sections prepared. These were immunofluorescently labeled according to conventional methods (Harlow and Lane, 1988). The anti-Pax6 antibody is an affinity-purified rabbit polyclonal available from Covance (cat#PRB-278P) that was used at a 1:500 dilution in an overnight incubation at 4°C.

In situ hybridization

All embryos were washed in PBS and fixed in 4% paraformaldehyde at 4°C. Antisense RNA probes were labeled with digoxigenin during in vitro transcription and whole-mount in situ hybridization performed as described previously (Nieto et al., 1996). The probe regions

comprised, for murine *Foxe3*, the entire intronless genomic clone on a *Apa*I-*Kpn*I fragment of 1064 bp (Brownell et al., 2000) and for murine *Sox2*, a 1047 bp *Xho*-*Acc*I fragment that encompasses most of the coding region (Wood and Episkopou, 1999).

Determining the rate and pattern of cell proliferation in the lens

The pattern of cell proliferation in normal and transgenic lenses at E13.5 was assessed by mapping BrdU-labeled cells onto a lens coordinate system adapted from previous analyses (McAvoy, 1978; Mikulicich and Young, 1963). Data were expressed as the proportion of total cells that were BrdU positive (the BrdU labeling index) in a given sector. Pregnant female mice were injected with BrdU at the appropriate stage of pregnancy, the mother euthanized 1 hour later and the embryos removed from the uterus by dissection. Heads from E13.5 embryos were fixed overnight in PBS-formalin (4%) and then processed for paraffin embedding. This quantitation was performed using sections that were within three sections either side of the lens center (defined by identifying the largest section) and counts from a minimum of four mice of each genotype pooled. Analysis on E10.5 embryos was performed in a similar way except that fixation was in 4% paraformaldehyde, counting was counting was restricted the lens pit and we used a secondary antibody that was conjugated with the Alexa594 fluorochrome (Molecular Probes) and Hoechst 33258 as a nuclear counterstain. The Student's *t*-test was used to assess the significance of the data derived.

RESULTS

An Fgfr inhibitor suppresses expression of *Pax6* in the lens placode

Expression of *Pax6* in the presumptive lens ectoderm begins as early as embryonic day (E) 7.5 as it is expressed throughout the head ectoderm of the embryo at the head-fold stage (Grindley et al., 1995). However, at E8.75, *Pax6* expression was upregulated in the presumptive lens ectoderm that overlies the optic vesicle (Grindley et al., 1995). This second phase of *Pax6* expression has been defined by previous investigation as a marker of lens induction (Furuta and Hogan, 1998; Wawersik et al., 1999). As the first in a series of experiments designed to determine whether Fgfr signaling played a role in lens induction, we explanted primordial eye tissues under conditions where Fgfr kinase activity was inhibited and determined whether expression of *Pax6* in the presumptive lens region was affected. We used two different methods to assess *Pax6* expression. First, we took advantage of a reporter transgenic mouse line (designated *P6 5.0-lacZ*) that expresses *lacZ* from a *Pax6*-derived lens lineage enhancer (Williams et al., 1998). The *P6 5.0-lacZ* reporter reproduces *Pax6* expression in the presumptive lens ectoderm from E8.75 and thus is a marker of lens induction. As a second method, we used antibodies to Pax6 to identify the translation product.

In explant experiments, we used the inhibitor SU9597 to examine the requirement for Fgfr signaling. This is one member of a family of compounds that bind Fgfr kinases at the ATP-binding site and suppress their activity. SU9597 (Sun et al., 1999) is a more cell-permeant variant of the more commonly used SU5402 (Mohammadi et al., 1997). Others have previously shown that these compounds are effective in inhibiting Fgf receptor kinase activity in the 5-20 μM concentration range and that in this range, they have no effect on the epidermal growth factor receptor, insulin-like growth factor receptor and platelet-derived growth factor receptor kinases (Mohammadi et al., 1997). Both SU5402 and SU9597 have also been used previously to demonstrate Fgfr signaling requirements in developmental systems (Makarenkova et al., 2000; McCabe et al., 1999; Schneider et al., 1999).

In one set of experiments, E8.5 embryos were harvested from homozygous *P6 5.0-lacz* reporter mice, the heads bisected at the midline and explanted into collagen gel either with or without SU9597 at 10 μM. Explants were allowed to incubate for 8 hours until they reached the equivalent of approximately E9.0 and then they were fixed and stained with X-gal in whole mount so that *lacZ* reporter activity could be detected. Compared with control explants, SU9597-treated 8 hour explants had a consistently observed but only slightly reduced level of X-gal staining (compare Fig. 1A with 1B).

Explants allowed to incubate for a period of 24 hours showed a significant increase in size and this indicated at a general level that the culture conditions permitted development to proceed (compare Fig. 1A,B with 1C,E). Interestingly, after 24 hours of incubation, the Fgfr inhibitor had a dramatic effect on expression of the *Pax6* reporter. Control explants showed the anticipated reporter-positive surface ectoderm overlying the optic vesicle (Fig. 1C). SU9597-treated explants showed a greatly reduced level of reporter expression (Fig. 1E). Histological sections prepared from 24 hour explants emphasized the lens placode-specific expression of the reporter in the control (Fig. 1D) and the reduced level of expression with SU9597 treatment (Fig. 1F). This observation implies that Fgf receptors lie upstream of the ectoderm enhancer that normally drives *Pax6* expression in the lens placode.

As evidence is emerging that at least two transcriptional enhancers regulate *Pax6* expression in the lens placode (Dimanlig et al., 2001; Kleinjan et al., 2001) it was advantageous to assess Pax6 levels in the eye primordium using indirect immunofluorescence. So that we could objectively compare levels of immunoreactivity in control and inhibitor-treated explants, sections were processed and labeled in the same experiment, images acquired digitally under identical lighting conditions, and all figure panels adjusted *en masse* in the same digital image file. In the Figure panels shown (Fig. 1G-L) both green and yellow represent immunoreactivity for Pax6. The peak signal intensity was replaced with the color yellow and, as such, indicates the highest levels of Pax6.

While Pax6 immunoreactivity was readily detectable in the lens placode of control E8.5 explants cultured for 24 hours (Fig. 1G,H) explants treated with SU9597 showed a reduced level (Fig. 1I,J). Pax6 immunoreactivity was also consistently reduced in the optic vesicle (Fig. 1G-J, ov). In explants harvested at E9.5 and cultured for 24 hours, control explants generated a lens pit with thickened epithelium and strong Pax6 immunoreactivity (Fig. 1K). The presence of SU9597 consistently reduced the width of the lens pit, the thickness of the pit epithelium and suppressed the level of Pax6 immunoreactivity (Fig. 1L). Combined, these data provide evidence that Fgfr kinase activity is required for a normal level of *Pax6* expression in the presumptive lens ectoderm, lens placode and lens pit. As *Pax6* is known to be critical for lens development (Ashery-Padan et al., 2000; Fujiwara et al., 1994; Wawersik et al., 1999) we can suggest that Fgfr signaling activity is required for normal lens induction.

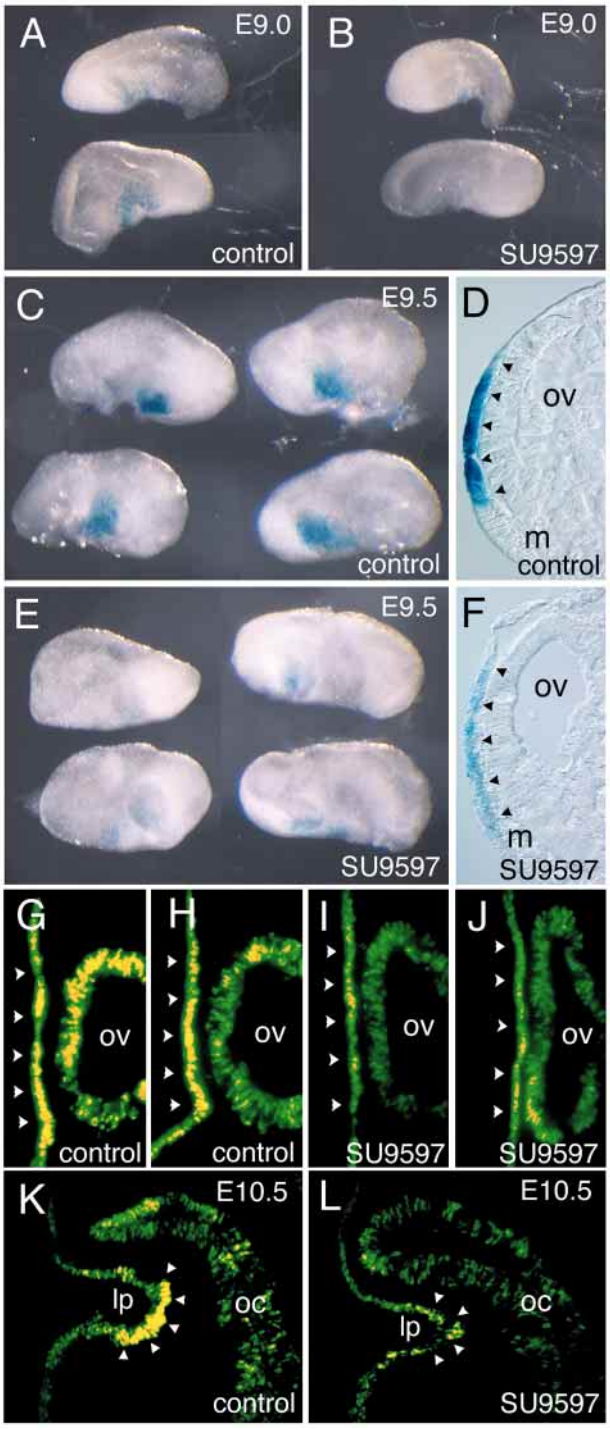

Fig. 1. Expression of the lens induction marker *Pax6* is dependent on Fgfr kinase activity. **(**A,B) Mouse half-heads from *P6 5.0-lacZ* embryos were explanted at E8.5 and cultured for 8 hours either in the absence (A) or presence (B) of 10 μM SU9597. At this stage, X-gal staining reveals only a modest reduction of reporter construct expression in SU9597-treated explants. (C-F) Mouse half-heads from P6 5.0-*lacZ* embryos were explanted at E8.5 and cultured for 24 hours either in the absence (C,D) or presence (E,F) of 10 μM SU9597. X-gal staining reveals that at this stage, there is a dramatic reduction in reporter construct expression levels in the lens placode in presence of SU9597 whether assessed in whole-mount (compare C with E) or in section (compare D with F, arrowheads). All explants shown in A-F were derived from the same litter of P6 5.0-*lacZ* embryos. (G-J) Mouse half-heads from wild-type embryos were explanted at E8.5 and cultured for 24 hours either in the absence (G,H) or presence (I,J) of 10 μM SU9597. Frozen sections of the explants were labeled with ant-Pax6 antibodies using indirect immunofluorescence. Both the green and yellow colors show Pax6 immunoreactivity, but the yellow shows the peak signal intensity. This indicates that Pax6 levels are reduced in explants treated with the Fgfr kinase inhibitor. This is true for the lens placode (arrowheads) but is also apparent in the optic vesicle (ov). (K,L) As in G-J, but explants were performed at E9.5 and allowed to proceed for 24 hours. In this case, it is apparent that SU9597 reduces the level of Pax6 immunoreactivity, particularly in the deepest epithelium of the lens pit (lp). The lens pit of the treated explant (L) is also narrower than in the control (K). Explants in A-C,E are shown at the same magnification. All panels are labeled with the approximate stage of development reached at the end of the explant period.

A truncated Fgf receptor expressed in the presumptive lens ectoderm perturbs early lens development

The described inhibitor experiments have the disadvantage that the inhibitor is active in all cells in the explant and as a result, we cannot determine whether Fgfr activity in the presumptive lens, presumptive retina or both, is required for *Pax6* expression. To overcome this limitation, we adopted a second experimental strategy and generated transgenic mice in which a truncated, dominant-negative Fgfr1IIIc was expressed only in cells of the lens placode and lens pit.

To this end, we took advantage of the *Pax6*-derived lens enhancer (used in the *P6 5.0-lacZ* reporter mouse described above) that directs transgene expression to the presumptive lens ectoderm. Thus, we generated a transgene construct (Fig. 2A, designated *Tfr*) that combines the lens enhancer (Williams et al., 1998), the P0 promoter of the *Pax6* gene (Xu et al., 1999) and the coding region of a truncated mouse Fgfr1IIIc (Bernard et al., 1991; Reid et al., 1990). SV40 sequences provide splicing and polyadenylation signals. Based on previous experimentation, this construct would be expected to be expressed in the presumptive lens ectoderm beginning at E8.75 (Williams et al., 1998) and to inhibit Fgfr-mediated signaling (Chow et al., 1995; Li et al., 1994; Peters et al., 1994; Robinson et al., 1995a).

The construct was used to generate transgenic mice on the FVB/N background (Taketo et al., 1991) using standard techniques of pronuclear injection (Hogan et al., 1986). Three potential founder animals were generated but only two produced transgenic offspring (Table 1). To confirm the pattern of expression, we performed both whole-mount in situ hybridization and RT-PCR analyses (Fig. 2B-F). An antisense probe to αA-crystallin gave the anticipated strong lens signal in a wild-type E10.5 embryo (Fig. 2B). An antisense probe to SV40 sequences present in the transgene mRNA gave a similar, albeit weaker, pattern of signal in a *Tfr7* embryo at E10.5 (Fig. 2C). Importantly, there was no indication of hybridization signal in the retina. A lens-specific hybridization signal was also observed in the *Tfr7* line at E11.5 (Fig. 2D) and in the *Tfr3* line at E12.0 (Fig. 2E).

Given the limited sensitivity of whole-mount in situ hybridization, we also performed an RT-PCR analysis of transgene expression in the *Tfr7* transgenic line. Thus, we harvested eye primordia from hemizygous *Tfr7* transgenic

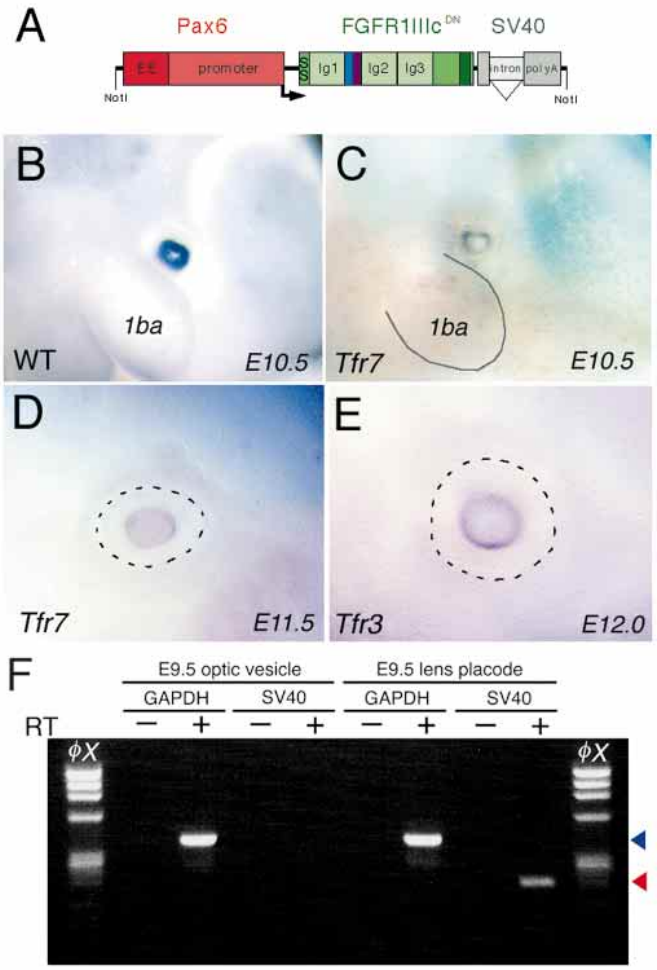

Fig. 2. *Tfr* transgene construct and expression. (A) Schematic showing the *Tfr* construct. Segments derived from the mouse *Pax6* gene are shown in reds and include the ectoderm enhancer (EE) and the P0 promoter. The promoter is encompassed in a fragment of approximately 1 kb 5′ to the start-point of transcription (right-facing arrow). The Fgfr1IIIc cDNA is positioned downstream of the *Pax6* gene elements. The various protein-coding domains within the cDNA are include the signal sequence (SS), three imunoglobulin-like domains (Ig1-3), the acidic domain (blue vertical bar), the CAM-homology domain (purple vertical bar) (Doherty and Walsh, 1996) and the transmembrane domain (dark green vertical bar). Included in the construct is the SV40 virus small t antigen intron and polyadenylation signals. (B-E) Whole-mount in situ hybridization on mouse embryos. (B) Control hybridization with an antisense probe to αA-crystallin showing signal in the lens pit in an E10.5, wild-type embryo (1ba – first branchial arch). (C,D) Hybridization of an antisense SV40 probe to *Tfr7* embryos at E10.5 (C) and E11.5 (D). Hybridization signal is apparent in the lens pit at E10.5 and the lens vesicle at E11.5. (E) Hybridization of an antisense SV40 probe to a *Tfr3* embryo at E12.0 showing signal in the lens epithelium. The broken line in D,E outlines the optic cup. (F) RT-PCR transgene expression analysis on lens placode and optic vesicle (E9.5) from *Tfr7* hemizygous mice. Bacteriophage ϕX174 DNA digested with *Hae*III is run as a size standard on both sides of the gel (ϕX). The GAPDH cDNA-derived product appears at 420 bp (blue arrowhead). The PCR product derived from the transgene appears at 200 bp (red arrowhead). This analysis shows that reverse-transcriptase (RT) must be included if either PCR product is to be amplified and that the transgene-specific product can be amplified only from lens placode cDNA.

mice at E9.5 and separated the presumptive lens ectoderm from the optic vesicle using dispase treatment and dissection. Total RNA was isolated and used to synthesize cDNA. The anticipated 420 bp GAPDH product (Stewart et al., 1997) was amplified from both lens placode and optic vesicle cDNA thus confirming quality of the template (Fig. 2F). PCR primers specific for transgene SV40 sequences amplified a product of the anticipated 200 bp size from lens placode but not optic vesicle (Fig. 2F). Finally, samples prepared without reverse transcriptase did not result in the amplification of any products and this indicated that there was no genomic DNA contamination of the cDNA (Fig. 2F). These data confirm that, as would be expected (Kammandel et al., 1999; Williams et al., 1998), transgene expression in the *Tfr7* mice is restricted to the presumptive lens ectoderm. Thus, any phenotypic changes in lens development in the *Tfr7* mice could be ascribed to inhibition of Fgfr signaling in lens lineage cells.

As initial analysis of hemizygous *Tfr* transgenic mice indicated only a mild phenotype, both lines were bred to homozygosity to increase the level of transgene expression. This resulted in a more severe and informative range of phenotypes in both line 3 and line 7 (see summary, Table 1). Because *Tfr7* mice showed the most pronounced phenotype, this line has been emphasized. The observation that both lines showed similar changes (including reduced proliferation rates; see below) all but eliminated the possibility of transgene-mediated insertional mutagenesis. Clearly, lens defects can also be rationalized given the expression pattern of the transgene.

Transgenes driven by the *Pax6* ectoderm enhancer and P0 promoter are first expressed in the surface ectoderm overlying the optic vesicle at E8.75 but show higher levels at E9.5 as the lens placode forms (Williams et al., 1998). Histological examination of the lens placode in E9.75 *Tfr7*/*Tfr7* transgenic mice revealed a subtle but reproducible decrease in placode thickness and a delay in the initial stages of lens pit invagination (compare Fig. 3A, wild-type, with 3B, *Tfr7*/*Tfr7*). Similarly, at E10.5, *Tfr7*/*Tfr7* mice show a lens pit that is reproducibly narrower than in wild-type mice (compare Fig. 3C with 3D). The narrow lens pit observed is a very similar change to that observed in wild-type eye explants exposed to the Fgfr inhibitor (Fig. 1I and J) and is consistent with a role for Fgfr activity in progression of the lens placode to the lens pit.

Subsequent stages of lens development in the *Tfr7*/*Tfr7* mice all showed distinctive defects. At E11.5, when compared with wild-type mice (Fig. 3E) the *Tfr7*/*Tfr7* lens vesicle is small and remains attached to the surface ectoderm (Fig. 3F). At E12.5 in wild-type mice the lens vesicle has closed and the primary fiber cells have differentiated from the posterior wall of the lens vesicle (Fig. 3G, pfc). By contrast, in *Tfr7*/*Tfr7* mice, the lens vesicle has not separated; this is emphasized by a focal invagination of the surface ectoderm (Fig. 3H, red arrowhead) and a failure of mesenchymal cells to migrate between surface ectoderm and lens vesicle (Fig. 3H, black arrows). Normally, the lens vesicle interior is filled by primary lens fibers at this time, but in *Tfr7*/*Tfr7* mice, the vesicle remains small and empty (Fig. 3H). It is also apparent that at E11.5-E13.5, the entire eye in the homozygous transgenics is significantly smaller than in wild-type mice (compare Fig. 3E,F with 3G and H) although at the morphological level, retinal lamination

Table 1. Summary of phenotypic change in *Tfr* transgenic mice at E13.5

Locus/genotype	Line number	Germline transmission	Phenotype			
			Small lens	Epithelial proliferation	Fiber differentiation	Vesicle separation
Tfr3/homozygote	3	Yes	+	+ suppression	No change	Complete
Tfr5	5	No	NA	NA	NA	NA
Tfr7/homozygote	7	Yes	++	++ suppression	++ suppression	Incomplete

NA, not applicable.
Plus symbols indicate comparative severity of changes.

appears unaffected. At the day of birth, wild-type lenses (Fig. 3I) are larger than those in *Tfr7*/*Tfr7* mice and the persistent lens stalk is evident (Fig. 3J). In whole-mount preparations of adult wild-type eyes, the separated cornea and lens are clearly visible (Fig. 3K) while in *Tfr7*/*Tfr7* eyes, the connection between lens and cornea is observed as a column of translucent material. This is very similar to changes observed in Peters' anomaly in humans and in some heterozygous *small eye* mice (Hanson et al., 1994).

There was a degree of variability in the eye phenotypes observed both between and within the two transgenic lines. While the lens was clearly smaller in the *Tfr3*/*Tfr3* mice, we did not observe a persistent lens stalk (Table 1). *Tfr7*/*Tfr7* mice also showed phenotypic variability. Besides the typical changes described, in a few transgenic mice, the phenotype was more severe with an extremely small lens and ultimately, a degeneration of the lens and retina (data not shown). Despite this late degeneration response in a few mice, there was no indication of an increased level of programmed cell death in the early stages of lens development. An analysis of the expression pattern of the differentiation markers α-crystallin, β-crystallin (McAvoy, 1978) and MIP26 (Bok et al., 1982) showed that the development of lens fiber cells is delayed in the *Tfr7*/*Tfr7* mice (data not shown). In summary, the

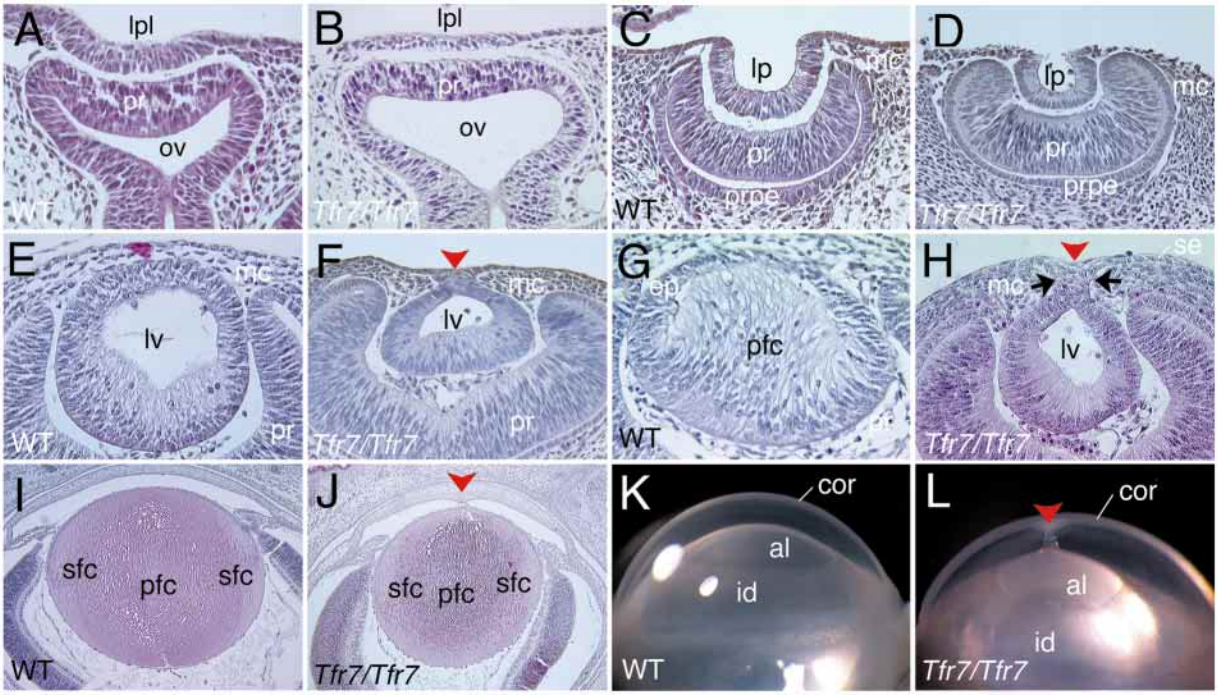

Fig. 3. Histological analysis of *Tfr7* mice. At E9.75 in wild-type embryos (A), the lens placode (lpl) has thickened adjacent to the presumptive retina (pr), and the optic vesicle (ov) has begun invaginating. By contrast, *Tfr7/Tfr7* embryos derived from the same litters show poorly developed lens placodes and a delay in the invagination of the optic vesicle. Wild-type (C) and *Tfr7/Tfr7* (D) eyes at E10.5 showing the arrangement of the lens pit (lp) presumptive retina, presumptive pigmented epithelium (prpe) and periorbital mesenchyme (mc). At this stage, the *Tfr7/Tfr7* lens pit is consistently smaller than that in the wild type. Wild-type (E) and *Tfr7/Tfr7* (F) mouse eyes at E11.5, showing that the lens vesicle (lv) in *Tfr7/Tfr7* embryos is smaller than in wild type and remains attached to the surface ectoderm (red arrowhead). Periorbital mesenchymal cells have been unable to migrate across the full width of the presumptive cornea. At E12.5 in wild-type mice (G) the primary fiber cells (pfc) have extended anteriorly to the lens epithelium (ep). By contrast, in *Tfr7/Tfr7* mice (H), no fiber cell differentiation has occurred as indicated by the small, hollow lens vesicle (lv). The lens vesicle remains attached (red arrowheads) to the surface ectoderm (se) and prevents the complete migration of periorbital mesenchyme (arrows). At the day of birth, both wild-type (I) and *Tfr7/Tfr7* (J) lenses have developed both primary and secondary fiber cells (pfc and sfc, respectively) but the *Tfr7/Tfr7* lens is significantly smaller. There is also a persistent lens stalk in the transgenic (red arrowhead). Whole-mount preparations of wild-type (K) and *Tfr7/Tfr7* (L) eyes also indicate the existence of the persistent lens stalk (red arrowhead). al, anterior lens; cor, cornea; id, iris diaphragm.

morphological analysis of *Tfr7/Tfr7* mice indicates that the dominant-negative Fgfr has an effect on all stages of lens development. Important for this analysis however, is the observation that there is an effect on the earliest discernible stage of lens morphogenesis.

The truncated Fgfr suppresses proliferation in the lens pit and lens epithelium

In seeking an explanation for the small size of lenses in *Tfr7*/*Tfr7* mice, we quantified the relative cell proliferation rate in controls and transgenics. We performed this analysis on the lens pit at E10.5 and on maturing lenses at E13.5 to determine whether an effect might be observed at multiple stages of lens development. For the assessment at E10.5 we labeled a series of sections (using indirect immunofluorescence; for example, Fig. 4A) from the central lens pit of wild-type and *Tfr7*/*Tfr7* mice and counted total cells and BrdU-positive cells within defined boundaries (Fig. 4A, white lines). The proportion of labeled cells was calculated, a statistical analysis performed and the results presented graphically (Fig. 4B). This indicated that there was a significant reduction of the proliferative index in *Tfr7*/*Tfr7* mice.

For analysis at E13.5, we examined mildly affected *Tfr7*/*Tfr7* eyes where lens-corneal separation was complete. In this instance, BrdU-labeling was performed using alkaline-phosphatase based immunohistochemistry (for example, Fig. 4C). The quantification (Fig. 4D) was performed using a method (McAvoy, 1978; Mikulicich and Young, 1963) in which the number of BrdU-positive cells is determined for a series of angular sectors (Fig. 4C). The data resulting gives a graphical comparison of the proliferation rate at any point along an axis that represents the lens epithelium. Wild-type mice (Fig. 4D, blue line) show the typical pattern of proliferation with peaks centered on 20° and 160° in domains adjacent to immature anterior chamber. Posteriorly, the labeling index decreases to zero at –20° and indicates the point within the lens that fiber cells differentiate. Anteriorly, proliferation becomes progressively lower from 40° to 60° and remains low through the anterior pole (approx. 60° to 120°). Both the *Tfr3*/*Tfr3* and *Tfr7*/*Tfr7* transgenics had reduced levels of proliferation. Line 7 was clearly more severely affected with the BrdU-labeling index lower in all sectors (Fig. 4D, red line). Line 3 showed significantly reduced levels of proliferation in the peak regions only but this was apparent on both the nasal and temporal sides of the lens (Fig. 4D, orange line). These results are consistent with a previous analysis (Robinson et al., 1995a) showing that expression of a truncated Fgf receptor reduced the proliferation rate in the lens lineage. These data are valuable for three reasons. First, they show that the activity of Fgfrs is required for proliferation in the lens lineage from an early stage of development. Second, they identify an additional phenotypic feature that the *Tfr3*/*Tfr3* and *Tfr7*/*Tfr7* transgenics share and emphasize that the changes observed are not a consequence of transgene insertional mutagenesis. Finally, these data provide a link to the phenotype of the *Foxe3* mutant mice (*dysgenetic lens*) where proliferation in the lens lineage is also reduced.

Fgf receptor and Bmp7 signaling cooperate in lens induction

Two models describing signaling events in early lens development suggest that Bmps participate (Dudley et al., 1995; Furuta and Hogan, 1998; Wawersik et al., 1999). In particular, analysis of null mice has indicated that the *Bmp7* gene is required for lens induction at the placode stage and that Bmp7 signaling lies upstream of *Pax6* expression in a genetic pathway (Wawersik et al., 1999). A role for *Bmp7* in lens induction is consistent with the observation that it is expressed

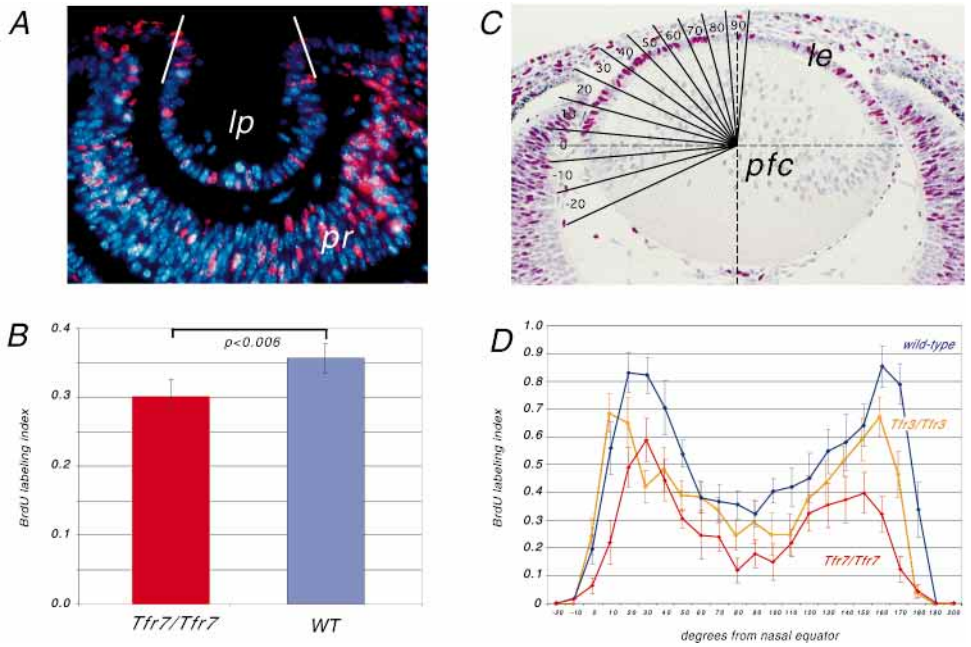

Fig. 4. Proliferation levels in the *Tfr3* and *Tfr7* lens lineage. (A) Histological section from an E10.5 mouse embryo showing typical BrdU labeling; the blue fluorescence identifies all nuclei, while the red identifies BrdU-positive cells. Both the total number of cells and the number of BrdU-positive cells in the lens pit were counted within the boundaries indicated (white lines) and the proportion of positive cells calculated. The results of this analysis for wild-type and *Tfr7/Tfr7* homozygous mice is shown in B. This indicates that there is a statistically significant reduction in proliferative index in the transgenics at E10.5. (C) An E13.5 wild-type lens section showing typical detection of BrdU incorporation. The section is overlaid with the radial grid used for counting. The graph (D) shows the BrdU labeling index at E13.5 in a series of radial sectors that represent different lens domains. In wild-type mice (blue trace) there are two peaks of proliferation at 20° and 160°; this represents a location adjacent to the immature anterior chamber and is a typical proliferation pattern for the mouse lens (McAvoy, 1978). The *Tfr3*/*Tfr3* (orange trace) and *Tfr7*/*Tfr7* (red trace) transgenic mice show reduced levels of proliferation; the *Tfr7*/*Tfr7* transgenic mice are more severely affected. Standard errors are represented by vertical bars.

in the presumptive lens ectoderm at E9.0 and persists in the lens placode and lens pit until it is downregulated at E11.0 (Wawersik et al., 1999). With this, we could use a third strategy for determining whether Fgfr activity was involved in lens induction by determining whether there was a genetic interaction between Fgfr signaling (as defined by the *Tfr7* transgene) and the established lens induction gene *Bmp7* [defined by the null allele (Dudley et al., 1995)].

To this end, we crossed *Bmp7* heterozygous null and *Tfr7/Tfr7* mice through two generations to produce mice with informative genotypes. Mice were initially assessed at the day of birth. A comparison of wild-type (Fig. 5A) and *Tfr7/Tfr7* lenses (Fig. 5B) indicated the expected small lens size in the transgenics. $Bmp7^{-/-}$ animals have a full range of phenotypes ranging from microophthalmia and associated small lenses (Fig. 5C) to anophthalmia (Fig. 5D). By contrast, *Bmp7* heterozygous mice have no discernible eye defects (Wawersik et al., 1999) (data not shown). Interestingly, mice that are *Tfr7/Tfr7* and *heterozygous* for the *Bmp7*-null allele show a more severe phenotype (the two examples represent the typical (Fig. 5E) and mild (Fig. 5F) forms of the phenotype). This was apparent from several features of the *Tfr7/Tfr7*, $Bmp7^{+/-}$ eye, including small lens size and the lack of lens vesicle separation and closure (compare Fig. 5B with 5E,F). The lack of lens vesicle closure is indicated by the extrusion of α-crystallin immunoreactive material (data not shown) into the conjunctival sac (Fig. 5E,F, black arrows). In some *Tfr7/Tfr7*, $Bmp7^{+/-}$ lenses, there were also extrusions of lens material into the vitreous (Fig. 5F, black arrowheads). Also interesting was the observation that in those *Tfr7/Tfr7*, homozygous *Bmp7* animals with eyes, lens formation was disproportionately affected when compared with other tissues. (Fig. 5G,H). This is illustrated by some lenses that were small, had ruptured capsules and were attached to the cornea (Fig. 5G), and others which were displaced and minute (Fig. 5G,H, arrowheads). By contrast, the retinae of *Tfr7/Tfr7*, $Bmp7^{-/-}$ animals were not dramatically reduced in size and showed pseudostratification similar to that observed in wild-type mice. To assess the frequency with which different features arose in wild-type, *Tfr7/Tfr7* and *Tfr7/Tfr7*, $Bmp7^{+/-}$ mice, we performed histological examination of groups of 10 mice of each genotype. This indicated that regardless of whether we examined lens size, lens vesicle separation, lens vesicle closure or capsule failure, the frequency of occurrence was higher in *Tfr7/Tfr7*, $Bmp7^{+/-}$ mice than in *Tfr7/Tfr7* or wild type (Table 2). Together with histological observations, this quantitation provides evidence for a genetic interaction between the *Bmp7* gene and the *Tfr7* transgene. In turn, this implies that Fgfr and Bmp7 signaling cooperate at some stage of lens development.

To extend this analysis and determine whether there might be a *Bmp7-Tfr7* genetic interaction during the induction stages of lens development, we examined the expression of lens lineage marker genes. Expression of Pax6 was assessed by immunofluorescence at E9.5 and E10.5. Objective comparisons of Pax6 levels were performed as described for Fig. 1. In this case, the panels included in any one experiment (see brackets, Fig. 6) can be directly compared as the sections shown were labeled at the same time, digital images combined and then adjusted together. As for Fig. 1, we have replaced the peak signal intensity with the color yellow to the same threshold level within an experiment.

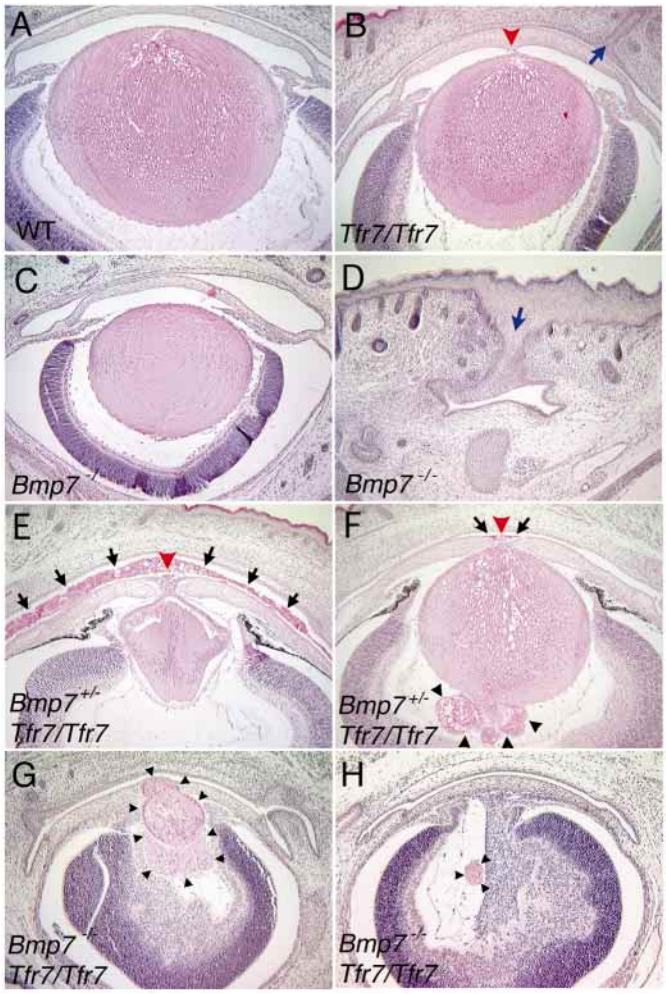

Fig. 5. Fgf receptor and Bmp7 signaling cooperate in lens development. All panels show Hematoxylin and Eosin stained sections of P0 mouse eyes. (A) Wild-type eye. (B) *Tfr7/Tfr7* mouse eye with persistent lens stalk (red arrowhead). The blue arrow indicates the eyelid suture. (C,D) $Bmp7^{-/-}$ eyes showing the range of possible phenotypes manifesting as microphthalmia (C) and anophthalmia (D). Only the eyelid suture (blue arrow) is recognizable in the case of anophthalmia (D is twice the magnification of other panels). This can be compared with the eyelid suture in B (blue arrow). (E) Eye section from *Tfr7/Tfr7*, $Bmp7^{+/-}$ mouse showing the typical phenotype featuring failure of lens vesicle closure and separation (red arrowhead), and extrusion of eosinophilic fiber cell material into the conjunctival sac (black arrows). (F) Eye section from a second *Tfr7/Tfr7*, $Bmp7^{+/-}$ mouse showing failure of lens vesicle closure and separation (red arrowhead) and extrusion of fiber cell material into the vitreous (arrowheads). (G,H) Two different examples of eye sections from *Tfr7/Tfr7*, $Bmp7^{-/-}$ mouse eyes showing the disproportionate effects on lens development compared with $Bmp7^{-/-}$ mouse eyes (C). In these cases, the lens (arrowheads) is disrupted (G) and smaller than observed in any other genotype (G,H).

In a first experiment, we detected the expected high level of Pax6 immunoreactivity (Fig. 6A, yellow signal) in the lens placode (Fig. 6A, arrowheads) of wild-type mice. By contrast, in *Tfr7/Tfr7* mice, the level of Pax6 immunoreactivity was greatly reduced (Fig. 6B, green signal) in the placode (Fig. 6B). Like small molecule inhibitor experiments, this suggested that

Table 2. Summary of phenotypic change in *Tfr7, Bmp7* compound mutants

Genotype	Phenotype at P0			
	Small lens	Failure of lens vesicle separation*	Failure of lens vesicle closure‡	Capsule failure§
Wild type	No	0/10¶	0/10	0/10
Tfr7/Tfr7	All	8/10	3/10	0/10
*Tfr7/Tfr7*** and *Bmp7*$^{+/-}$	Severe	10/10	9/10	3/10

*Failure of lens vesicle separation was indicated histologically by the presence of a persistent lens stalk (Figs 3, 5).
‡Failure of lens vesicle closure was defined by the presence of eosinophilic lens material in the conjunctival sac (Fig. 5).
§Capsule failure was indicated histologically by the presence of eosinophilic lens material in the vitreous (Fig. 5).
¶The fraction indicates the number of mice, out of 10 examined, that show a given phenotypic feature.
**Insufficient embryos of *Tfr7/Tfr7*, Bmp7$^{-/-}$ genotype for inclusion in this table; however, those with eyes showed the most severe lens defects (Fig. 5G,H).

Pax6 expression was in part dependent on Fgfr activity. In a second experiment (Fig. 6C-E), we compared Pax6 immunoreactivity levels in wild-type (Fig. 6C) *Tfr7/Tfr7* (Fig. 6D) and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ (Fig. 6E) mice. This clearly showed a progressive reduction in Pax6 levels in the lens placode (Fig. 6C-E, arrowheads). In a third experiment, we assessed Pax6 levels at E10.5 in wild-type (Fig. 6F) *Tfr7/Tfr7* (Fig. 6G) and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ (Fig. 6H) mice. As observed at E9.5, the level of Pax6 was progressively diminished as first Fgfr and then Bmp7 activity levels were reduced. Interestingly, it was also apparent that the level of Pax6 immunoreactivity in the presumptive retina was reduced in *Tfr7/Tfr7* and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ at both E9.5 and E10.5. This may reflect the existence of reciprocal induction signals from presumptive lens to presumptive retina that are perturbed when lens induction is abnormal. Regardless, the progressive reduction in Pax6 levels in the lens placode of compound genotype mice further supports the existence of genetic interaction between the *Bmp7*

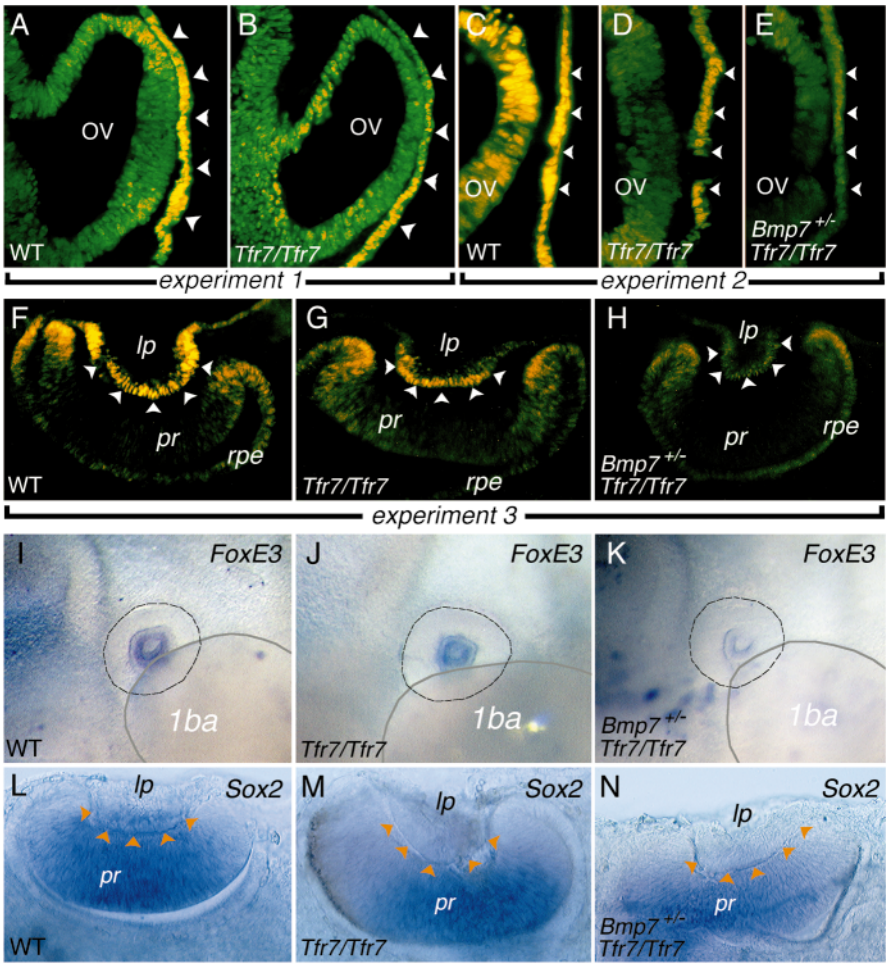

Fig. 6. *Pax6*, *Foxe3* and *Sox2* are downregulated in *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ mice. Immunohistochemical detection of Pax6 in the developing eyes of wild-type (A,C) *Tfr7/Tfr7* (B,D) and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ (E) embryos at E9.5. In all cases, the lens placode is indicated by the arrowheads. Both the green and yellow colors show Pax6 immunoreactivity, but the yellow shows the peak signal intensity. A,B represent one experiment and indicate that there is a reduction in the level of Pax6 immunoreactivity in the lens placode of *Tfr7/Tfr7* mice. The second experiment shows the lens placode at higher magnification and indicates that the level of Pax6 immunoreactivity is progressively reduced in *Tfr7/Tfr7* (D) and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ (E) embryos. (F-H) Immunohistochemical detection of Pax6 in wild-type (F) *Tfr7/Tfr7* (G) and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ (H) embryos at E10.5. The lens pit (*lp*) epithelium is demarcated by white arrowheads. pr, presumptive retina; rpe, presumptive retinal pigmented epithelium. These panels show that the lens pit is smaller and the per-cell Pax6 immunoreactivity lower in *Tfr7/Tfr7* and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ mice. For experiments 1-3, Pax6 immunoreactivity in the presumptive retina (pr) is also reduced in *Tfr7/Tfr7* (B,D,G) and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ (E,H) embryos compared with wild-type (A,C,F). (I-K) Whole-mount in situ hybridization with an antisense probe to *Foxe3* in wild-type (I) *Tfr7/Tfr7* (J) and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ (K) mice at E10.5. This shows that the level of *Foxe3* expression is reduced in *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ mice. The first branchial arch (1ba) is outlined in gray and the optic cup with a broken black line. (L-N) Thick sections of embryos hybridized with an antisense probe to *Sox2* in wild-type (L) *Tfr7/Tfr7* (M) and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ (N) mice at E10.5. This shows that *Sox2* expression in the lens pit (lp and red arrowheads) cannot be detected in *Tfr7/Tfr7* or *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ mice.

gene and the *Tfr7* transgene and implies that Fgfr and Bmp7 signaling pathways cooperate as early as E9.5.

Foxe3 and *Sox2* lie downstream of Fgfr and Bmp7 signaling in lens induction pathways

In order to understand further how Fgfr and Bmp7 signaling might function in a genetic pathway controlling lens induction, we chose to examine expression of additional lens lineage marker genes in the eyes of *Tfr7/Tfr7* and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ embryos.

The forkhead family transcription factor *Foxe3* has been implicated in lens development by its expression pattern and by the phenotype of the mouse mutant *dysgenetic lens* (*dyl*) (Blixt et al., 2000; Brownell et al., 2000). In this mouse, there is a failure of lens vesicle separation, inhibition and proliferation, and development of a dysplastic, cataractous lens (Blixt et al., 2000; Brahma and Sanyal, 1984; Brownell et al., 2000; Sanyal and Hawkins, 1979). As this phenotype was very similar to that observed in the *Tfr7/Tfr7* mice, we decided to determine whether the expression of *Foxe3* was affected. This analysis was performed on E10.5 embryos when the lens pit has just formed. As expected, *Foxe3* mRNA was detected in the lens pit of wild-type animals (Fig. 6I). In *Tfr7/Tfr7*, the *Foxe3* mRNA appeared at slightly reduced levels (Fig. 6J) while, in contrast, the *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ mice reproducibly showed a distinct down-regulation (Fig. 6K). This indicated that both Fgfr and Bmp7 signaling are required for a full level of *Foxe3* expression that *Foxe3* can be placed downstream of both in a genetic pathway.

The transcription factor Sox2 has also been implicated in lens development through regulation of crystallin genes (Kamachi et al., 1995; Kamachi et al., 2001). The *Sox2* gene has been used as a marker of lens induction in experiments examining the requirement for Bmp4 and Pax6 (Ashery-Padan et al., 2000; Fujiwara et al., 1994). To determine whether cooperative Bmp7 and Fgfr signaling might function upstream of *Sox2* expression, we performed whole-mount in situ hybridization with an antisense *Sox2* probe on E10.5 wild-type, *Tfr7/Tfr7*, and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ embryos; thick sections permitted the examination of *Sox2* expression in the lens pit. Wild-type mice showed the expected pattern of *Sox2* signal in the lens pit and the presumptive retina (Fig. 6L, arrowheads and pr, respectively). *Tfr7/Tfr7* mice did not show any detectable *Sox2* signal in the lens pit (Fig. 6M, arrowheads) and also appeared to have a modified pattern or level of *Sox2* expression in the presumptive retina (Fig. 6N, pr). Similarly, *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ mice showed no detectable *Sox2* expression in the lens pit and a modified level in the presumptive retina. These analyses indicate that combined Fgfr and Bmp7 signaling function upstream of Pax6, *Foxe3* and *Sox2* in a genetic pathway for lens development.

DISCUSSION

We have used three distinct experimental strategies to determine whether Fgf receptor signaling pathways play a role in lens induction. First, we have employed small molecule inhibitors of Fgfr kinases to show that expression of the crucial lens development gene *Pax6* is partially dependent on Fgfr activity. Second, we have generated transgenic mice that express a dominant-negative Fgf receptor in the presumptive lens ectoderm and, as a consequence, have abnormalities in lens induction and development. Finally, we have shown that there is a genetic interaction between the Fgfr and Bmp7 signaling pathways during the induction phases of lens development. Because Bmp7 is established as a lens inducer (Wawersik et al., 1999), this indicates that Fgfr signaling is also involved. This analysis shows for the first time that Fgfr signaling is involved in lens induction and defines a genetic pathway that regulates the process.

Fgf signaling plays a role in lens induction

Previous analysis has defined lens induction at multiple levels. Morphologically, the first indication of lens development (at E9.0-9.5 in the mouse) is the formation of the thickened lens placode in surface ectoderm that overlies the optic vesicle. At E9.5-10.5, the lens placode invaginates in coordination with the lens vesicle and forms the lens pit. At the molecular level, expression of a number of marker genes has defined lens induction. Among these, *Pax6* is probably best characterized; it is upregulated in the lens placode beginning at E8.75 and based on both tissue recombination (Fujiwara et al., 1994) and conditional mutation experiments (Ashery-Padan et al., 2000) is necessary for lens development. Misexpression of *Pax6* in *Xenopus laevis* embryos has shown that in this context, *Pax6* is also sufficient for lens development (Altmann et al., 1997; Chow et al., 1999). Finally, *Bmp7*-null mice show a variably penetrant absence of lens development that correlates with the loss of *Pax6* expression in the lens placode (Wawersik et al., 1999). When combined, these data provide a strong argument that *Pax6* expression in the lens placode defines lens induction. Similarly, it has been argued that increased expression of *Sox2* in the early eye is an excellent marker of lens induction; this is based on analysis of both the *Bmp4* (Furuta and Hogan, 1998) and *Bmp7* (Wawersik et al., 1999) null mice where lens development does not occur and *Sox2* expression is not upregulated.

With this, the analysis we have performed makes a strong case for involvement of Fgfr signaling in lens induction. Treatment of eye primordium explants with the Fgfr kinase inhibitor SU9597 downregulates *Pax6* expression in the lens placode according to both a *Pax6* gene-based reporter construct and immunofluorescent detection of the gene product. This response can be observed at both E9.5 and E10.5 and in the latter case, morphological changes to lens development (formation of a narrow lens pit) are also observed. While the use of small molecule Fgfr inhibitors has proven valuable for characterization of many developmental systems (Makarenkova et al., 2000; McCabe et al., 1999; Schneider et al., 1999) the strategy suffers from the limitation that all cells in an explant are subject to inhibitor effects and as a result, it cannot be determined whether the downregulation of *Pax6* expression in the lens placode is an autonomous response of placodal cells or an indirect consequence of the lack of Fgfr activity in the adjacent optic vesicle.

To overcome this limitation, we adopted a second experimental strategy and expressed a truncated, dominant-negative Fgfr1IIIc in the presumptive lens ectoderm. This technique has been employed widely to examine the function of Fgfr signaling in development and has the advantage that, depending upon the spectrum of Fgf ligands represented, a dominant negative mutant receptor may have inhibition activity

against all the Fgf receptor isoforms. Thus, with this approach, we can effectively answer a question about the requirement for Fgf receptor signaling even in a system that may involve multiple ligands and multiple receptors and as a consequence, may be refractory to analysis using induced mutations. In these experiments, a truncated mutant of Fgfr1IIIc (Li et al., 1994; Reid et al., 1990) was expressed in the lens lineage beginning at E8.75 using an enhancer from the *Pax6* gene (Williams et al., 1998). Two lines of transgenic mice were generated, both of which were used as homozygotes in order to increase transgene expression levels and enhance the phenotypic consequences. The demonstration that both lines of transgenic mice had the same defect in suppression of lens size and lens epithelial cell proliferation indicated that the phenotype observed was not a consequence of insertional mutagenesis. Finally, the confirmation that *Tfr7* transgene expression is restricted to lens lineage cells also indicates that cells of the lens placode and lens pit normally respond directly to Fgfr signaling during early lens development.

The morphological and molecular phenotype of the *Tfr7/Trf7* transgenic mice provides a strong argument for Fgfr signaling involvement in lens induction. First, we observe distinct morphological defects in lens development from the earliest stages. In particular, there is a delay in formation of the lens placode and its invagination to form the lens pit. At E9.75, The lens placode is thinner than normal, forms a narrow lens pit at E10.5, and throughout embryogenesis the lens of *Tfr7/Tfr7* transgenics is smaller than in wild-type mice. Second and more compelling is the observation that the *Tfr7/Tfr7* transgenic mice have reduced levels of expression of several genes that are either markers of, or are functionally involved in, lens development. *Pax6* expression is distinctly downregulated in the presumptive lens ectoderm of *Tfr7/Tfr7* transgenic mice at E9.5 and, importantly, this change is observed before any sign of a morphological defect. A downregulation of *Pax6* levels is also observed at E10.5 when the lens pit has formed. The observation that the *Tfr7/Tfr7* mice develop Peters' anomaly is consistent with downregulation of *Pax6* in the lens lineage as this phenotype is often associated with *Pax6* heterozygosity (Hanson et al., 1994). So too, expression of *Sox2* is diminished in the lens pit of *Tfr7/Tfr7* transgenic mice. As previous definitions of lens induction have used *Pax6* and *Sox2* expression in the lens lineage as key markers (Ashery-Padan et al., 2000; Furuta and Hogan, 1998; Wawersik et al., 1999) the current analysis, showing that *Sox2* and *Pax6* expression are reduced when Fgfr signaling is inhibited, indicates a role for Fgfr signaling in lens induction.

As a third strategy for determining whether Fgfr signaling was involved in lens induction, we determined whether there was a genetic interaction between the *Tfr7* transgene and the *Bmp7* null allele. This analysis showed that *Tfr7/Tfr7, Bmp7*$^{+/-}$ mice have an exacerbated lens phenotype compared with either *Bmp7*$^{+/-}$ mice or *Tfr7/Tfr7* mice. At the morphological level, *Tfr7/Tfr7, Bmp7*$^{+/-}$ mice have a smaller lens and more frequent lens vesicle closure and separation defects than either *Tfr7/Tfr7* or wild-type mice. This observation is particularly striking given previous analysis showing that *Bmp7* heterozygous mice have no detectable defects (Dudley et al., 1995; Wawersik et al., 1999). Furthermore, we show that the most severe lens defects arise in animals that are homozygous for both *Bmp7* and *Tfr7*. This assessment is complicated by the fact that some *Bmp7* homozygous null mice have no eyes, but in those *Bmp7*$^{-/-}$, *Tfr7/Tfr7* that do, the consequences for lens development is disproportionate; the retina is near normal in size and morphology while lenses are highly disrupted and very small. From a genetic standpoint, these observations argue that Bmp7 and Fgfr signaling cooperate in an early lens development pathway.

To enhance our understanding of how Fgfr and Bmp7 signaling cooperate, we examined the expression of lens lineage marker genes during induction phases of lens development. This was performed on embryos of wild-type, *Tfr7/Tfr7* and *Tfr7/Tfr7, Bmp7*$^{+/-}$ genotypes using the markers Pax6, *Foxe3* and *Sox2* (Furuta and Hogan, 1998; Wawersik et al., 1999). We examined Pax6 immunoreactivity in the lens placode at E9.5 and the lens pit at E10.5 and showed that the lowest levels could be observed in embryos with the *Bmp7*$^{-/-}$, *Tfr7/Tfr7* genotype. Similarly, at E10.5, *Foxe3* expression levels were reduced progressively in the lens pits of *Tfr7/Tfr7* and *Tfr7/Tfr7, Bmp7*$^{+/-}$ embryos. Combined, these data indicate that the placodal phases of *Pax6* and *Foxe3* expression require both Bmp7 and Fgfr signaling. Interestingly, *Sox2* expression was undetectable in *Tfr7/Tfr7* embryos and remained so in *Tfr7/Tfr7, Bmp7*$^{+/-}$ embryos. As it has also been shown that *Sox2* expression is lost in the *Bmp7* null lens pit (Wawersik et al., 1999) this might imply that either signaling pathway is necessary for *Sox2* expression. This interpretation must be applied cautiously, however, as in both this and previous analysis (Wawersik et al., 1999) there may be a limit on *Sox2* transcript detection sensitivity given the techniques employed. Regardless of this, this analysis indicates that *Sox2* expression in the lens lineage is dependent on both Bmp7 and Fgfr signaling. Because Bmp7 is an established lens inducer, the interaction between the *Bmp7* null allele and the *Tfr7* transgene indicates that Fgfr signaling is also involved in lens induction. A future challenge will be to identify the ligands for Fgfrs that regulate early lens development. Good candidates are Fgf8 (Lovicu and Overbeek, 1998; Vogel-Hopker et al., 2000) and Fgf15 (McWhirter et al., 1997) as they are both expressed with a timing that might imply an early role.

A genetic pathway defining lens induction

These experiments define the relationship between several elements of a genetic pathway for lens induction (Fig. 7). Two phases of *Pax6* expression in the lens lineage have been defined previously (Grindley et al., 1995). *Pax6* is expressed in the pre-placodal head ectoderm (Wawersik et al., 1999) (defined here as *Pax6*$^{pre-placode}$) but also later in the lens placode (defined as *Pax6*^{placode}). The placodal phase of *Pax6* expression is dependent on the activity of Pax6 in the earlier phase (Grindley et al., 1995). The first novel feature of the pathway described is the requirement for Fgfr signaling for *Pax6*^{placode} (this relationship could help explain why there is a mild phenotype in the *Tfr7/Tfr7* transgenic mice as expression of the dominant-negative Fgfr1 will impose a feedback suppression on the transgene). Furthermore, the model suggests that *Pax6*$^{pre-placode}$, Bmp7 and Fgfr signaling act in concert to permit the full level of expression of *Pax6*^{placode} (Fig. 7). This model is also consistent with previous analysis of the *Bmp7*-null mice that indicates *Bmp7* is upstream of *Pax6*^{placode} (Wawersik et al., 1999).

Similar phenotypes in the *dysgenetic lens* and *Tfr7/Tfr7, Bmp7*$^{+/-}$ mice suggested that *Foxe3*, the gene mutated in *dyl*,

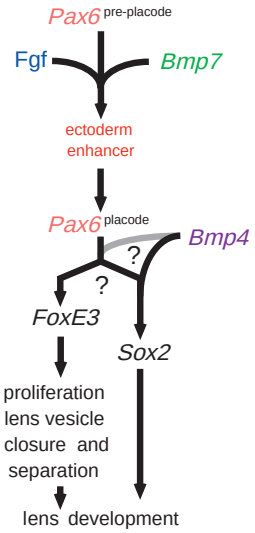

Fig. 7. A model for the genetic pathway describing lens induction and development. The arrows indicate genetic interactions determined by these and previous analyses. Gray arrows indicate a possible rather than a demonstrated pathway element. At the apex of the hierarchy is the pre-placodal phase of *Pax6* expression (*Pax6*$^{pre-placode}$). It is understood that the later phase of *Pax6* expression in the lens placode (*Pax6*^{placode}) is dependent upon earlier function of Pax6. The genetic pathway described reflects this interaction. As we observe that Fgfr and Bmp7 signaling cooperate to maintain the placodal phase of Pax6 expression, it follows that their input to the pathway must be upstream of *Pax6*^{placode}. Previous analysis has shown that the early phase of *Pax6* expression is unaffected in the *Bmp7*-null mice and, thus, Fgfr and Bmp7 signaling must converge on the pathway downstream of *Pax6*$^{pre-placode}$. Evidence that *Foxe3* is downstream of *Pax6*^{placode} and Fgfr and Bmp7 signaling includes (1) the reduced level of Foxe3 expression in *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ embryos, (2) similar phenotypes in *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ and *dyl* (*Foxe3*$^{dyl/dyl}$) embryos, (3) the absence of *Foxe3* expression in *small eye* embryos, and (4) the absence of *Foxe3* expression in embryos carrying a targeted deletion of the *Pax6* ectoderm enhancer (Dimanlig et al., 2001). Thus, *Foxe3* expression must lie upstream of events such as lens lineage proliferation, vesicle closure and separation. While we currently do not understand the genetic relationship between *Foxe3* and *Sox2*, it is clear from this and previous analyses performed that *Sox2* lies downstream of *Pax6*^{placode}. Because *Sox2* (but not *Pax6*) expression is diminished in the *Bmp4*-null mice, Bmp4 signaling must contribute to the pathway between *Pax6*^{placode} and *Sox2*.

might lie in the same developmental pathway as Fgfr and Bmp7 signaling. The observation that the level of *Foxe3* expression is lowest in *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ embryos and appears to be at an intermediate level in *Tfr7/Tfr7* mice argues that Fgfr and Bmp7 signaling also lie upstream of *Foxe3* and in combination stimulate a full level of expression (Fig. 7). Consistent with this proposal is the demonstration that *Foxe3* expression in the lens placode and pit is absent in the *small eye* mice (Brownell et al., 2000), thus indicating that its expression is dependent on one of the phases of *Pax6* expression. Our accompanying work analyzing a targeted deletion of the *Pax6* ectoderm enhancer indicates that *Foxe3* expression is lost (Dimanlig et al., 2001). As the ectoderm enhancer is responsible, at least in part, for the placodal phase of *Pax6* expression, the combined evidence indicates that *Foxe3* lies downstream of *Pax6*^{placode} (Fig. 7).

Sox2 is an sry-related HMG family transcription factor that has been implicated in the regulation of lens crystallins (Kamachi et al., 1995; Kamachi et al., 2001) and has been used as a marker to define the process of lens induction (Furuta and Hogan, 1998; Kamachi et al., 1998; Wawersik et al., 1999). It is normally upregulated in both the presumptive lens ectoderm and the optic vesicle at E9.5 when the lens placode thickens (Furuta and Hogan, 1998). *Sox2* expression is not upregulated in *small eye* or *Bmp7*-null mice and this has suggested that *Sox2* lies downstream of both *Pax6* and *Bmp7* (Furuta and Hogan, 1998; Wawersik et al., 1999). In the current analysis, we show that in both *Tfr7/Tfr7*, and *Tfr7/Tfr7*, *Bmp7*$^{+/-}$ embryos, *Sox2* expression in the lens pit is undectectable. This suggests that *Sox2* also lies downstream of Fgfr signaling in a lens induction pathway (Fig. 7). A weakness in the current model is our lack of understanding about the relationship between *Sox2* and remaining pathway elements. This uncertainty could be resolved by determining whether *Sox2* expression is perturbed in the *dyl* mouse and, as *Bmp4* lies between *Pax6* and *Sox2* in a lens development pathway (Furuta and Hogan, 1998), whether *Foxe3* expression is affected in the *Bmp4* null. Inclusion of the sFRP2 marker (Wawersik et al., 1999) in our analysis will also help define distinct stages of lens development.

A future challenge will be to understand which cell-cell interactions are mediated by Fgfr and Bmp7 signaling. It is possible, for example, that placodal Fgf or Bmp7 could initiate an exchange of signals between presumptive lens and retina that is required for placodal *Pax6* expression. Some evidence for this comes the observation that when Fgfr or Bmp7 activity is inhibited in the lens placode, *Pax6* and *Sox2* expression is reduced in the presumptive retina (Fig. 6) (Wawersik et al., 1999). Alternatively, Bmp7 may act in a paracrine or autocrine manner within the placode. The genetic pathway we have described here will form a basis for defining the process of lens induction in molecular detail.

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