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The function of FGF signaling in the lens placode

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ABSTRACT

Previous studies suggested that FGF signaling is important for lens formation. However, the times at which FGFs act to promote lens formation, the FGFs that are involved, the cells that secrete them and the mechanisms by which FGF signaling may promote lens formation are not known. We found that transcripts encoding several FGF ligands and the four classical FGF receptors are detectable in the lens-forming ectoderm at the time of lens induction. Conditional deletion of *Fgfr1* and *Fgfr2* from this tissue resulted in the formation of small lens rudiments that soon degenerated. Lens placodes lacking *Fgfr1* and *2* were thinner than in wild-type embryos. Deletion of *Fgfr2* increased cell death from the initiation of placode formation and concurrent deletion of *Fgfr1* enhanced this phenotype. *Fgfr1/2* conditional knockout placode cells expressed lower levels of proteins known to be regulated by FGF receptor signaling, but proteins known to be important for lens formation were present at normal levels in the remaining placode cells, including the transcription factors Pax6, Sox2 and FoxE3 and the lens-preferred protein αA-crystallin. Previous studies identified a genetic interaction between BMP and FGF signaling in lens formation and conditional deletion of *Bmpr1a* caused increased cell death in the lens placode, resulting in the formation of smaller lenses. In the present study, conditional deletion of both *Bmpr1a* and *Fgfr2* increased cell death beyond that seen in $Fgfr2^{CKO}$ placodes and prevented lens formation. These results suggest that the primary role of autocrine or paracrine FGF signaling is to provide essential survival signals to lens placode cells. Because apoptosis was already increased at the onset of placode formation in *Fgfr1/2* conditional knockout placode cells, FGF signaling was functionally absent during the period of lens induction by the optic vesicle. Since the expression of proteins required for lens formation was not altered in the knockout placode cells, we can conclude that FGF signaling from the optic vesicle is not required for lens induction.

Introduction

The tissue interactions that lead to lens formation begin at gastrulation. These create a "lens-forming bias" in the prospective lens-forming ectoderm, leading to specification of ectoderm cells to form a lens (Donner et al., 2006; Lang, 2004; Saha et al., 1989; Sullivan et al., 2004). In the final stage of lens formation, adhesion of the optic vesicle to the lens-forming ectoderm triggers "lens induction," leading to the formation of the lens placode and its subsequent invagination to form the lens vesicle. Contact between the optic vesicle and the ectoderm may also shield the lens from inhibitory signals from neural crest mesenchyme (Bailey et al., 2006; Sullivan et al., 2004). Previous studies implicated several factors in lens induction, including Bmp4 and 7 (Furuta and Hogan, 1998; Jena et al., 1997; Rajagopal et al., 2009; Wawersik et al., 1999), FGFs (Faber et al., 2001; Gotoh et al., 2004; Nakayama et al., 2008; Pan et al., 2006; Vogel-Hopker et al., 2000) and the Notch ligand, Delta2 (Ogino et al., 2008). Of these, only Bmp4 (mouse), Fgf19 (zebrafish) and Delta2 (frog) are known to be expressed in the optic vesicle and required for normal lens formation (Furuta and Hogan, 1998; Nakayama et al., 2008; Ogino et al., 2008) and only BMP receptors are known to be required in the responding ectoderm (Rajagopal et al., 2009). It is not yet clear whether FGFs from the optic vesicle fulfill the criteria to be considered classical "lens inducers" in mammals (one or more ligands produced by the optic vesicle, with receptors required in the lens-forming ectoderm; see Discussion).

The transcription factor, Pax6, is required in the surface ectoderm cells for lens formation (Ashery-Padan et al., 2000). *Pax6* heterozygous mice have smaller lenses that later develop cataracts (Grindley et al., 1997). Pax6 is expressed at low levels in the prospective lens ectoderm before placode formation ($Pax6^{pre\text{-}placode}$) and at higher levels during placode formation ($Pax6^{placode}$) (Lang, 2004). For these reasons, the amount of Pax6 protein in the nuclei of placode cells has

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been used as a measure of the extent of lens induction. If the inhibition of a signaling pathway decreases the accumulation Pax6, that pathway has been implicated in lens induction.

Several types of experiments have shown that FGF signaling participates in the establishment of lens competence, lens bias and lens specification [reviewed by Donner et al. (2006) and Lang (2004)]. Additional experiments suggest that FGFs may also be involved in lens induction. Expression in the lens placode of a kinase-deleted form of FGF receptor-1 was reported to reduce levels of Pax6 in the nucleus of placode cells and resulted in the formation of small lenses (Faber et al., 2001). Treatment of eye rudiments with an inhibitor of FGF receptor tyrosine kinase activity reduced lens cell proliferation and lens size (Faber et al., 2001). Germline deletion of *Ndst1*, which encodes an enzyme required for the synthesis of heparan sulfate, a co-factor for FGF receptor activation, disrupted the formation of the lens and optic vesicle and, in more severely affected eyes, decreased Pax6 levels in the lens placode (Pan et al., 2006). Mutation of critical amino acids in *Frs2α*, an adapter protein that participates in FGF receptor signaling, also disrupted optic vesicle and lens formation and reduced Pax6 levels in the placode (Gotoh et al., 2004). However, the identity and source of the FGF ligands involved in lens formation and the requirement for FGF receptors in the ectoderm have not been established (Smith et al., 2010). We determined the cell-autonomous function of FGF signaling during lens induction by conditionally deleting the two FGF receptors that are most abundantly expressed in the lens placode.

Materials and methods

Mice

Mice carrying floxed alleles of *Fgfr1* (Trokovic et al., 2003), *Fgfr2* (Yu et al., 2003) and *Bmpr1a* (Mishina et al., 2002) were mated to mice carrying the Le-Cre transgene, which is expressed in lens-forming ectoderm cells at E9 (Ashery-Padan et al., 2000). Animals were genotyped by PCR using primers described previously (Huang et al., 2009; Rajagopal et al., 2009). Matings were set up such that all animals were homozygous for the floxed allele(s), with the females also carrying a single copy of the Le-Cre transgene. This resulted in pregnancies in which approximately half of the embryos were Cre-positive. For timed matings, noon on the day on which a vaginal plug was detected was considered E0.5. The Le-Cre transgene has an internal ribosome entry site that drives the expression of green fluorescent protein (Ashery-Padan et al., 2000). Cre-positive embryos were identified using an Olympus SZX7 dissecting microscope with fluorescence detection.

Microarray analysis

Wild-type E9.5 or E10.0 embryos were frozen in OCT embedding compound and stored at −80 °C. Frozen sections were stained with hematoxylin and lens placode cells were isolated using a Leica LMD6000 laser microdissection system (North Central Instruments, Maryland Heights, MO). Tissue collected from both eyes of one embryo was lysed and RNA was isolated using a Qiagen RNeasy Micro kit (Qiagen, Valencia, CA). Total RNA (~50 ng) was amplified using the NuGEN WT-Ovation™ Pico RNA Amplification System (NuGEN, San Carlos, CA). The amplified DNA products were quantified and their size was determined using an Agilent 2100 Bioanalyzer and labeled using the NuGEN Encore™ Biotin Module. Labeled products were hybridized to Illumina Mouse6 v1.2 BeadArrays (Illumina, Inc., San Diego, CA), scanned on an Illumina® Beadstation 500, the images were decoded with Illumina Beadscan software and the results were analyzed using the Illumina BeadStation software, which reports the probability that transcripts were detected above background. Probe sets with detection p-values<0.05 were considered to represent transcripts that were expressed in the original samples. The results of 18 microarrays of wild-type tissues were used to identify the FGF and FGF receptor transcripts present in the E9.5 lens placode.

PCR amplification

E9.5 lens placode cells were isolated by laser microdissection and total RNA was isolated as described above. Total RNA was also isolated from manually dissected adult mouse lens epithelia. Approximately 50 ng of total RNA was used to synthesize and amplify cDNA using the NuGEN WT-Ovation™ RNA Amplification System. PCR primers were selected using Primer 3 software. Transcripts were routinely amplified for 40 PCR cycles using standard procedures. To provide a semi-quantitative estimate of the abundance of transcripts encoding the four FGF receptors, cDNA was amplified for 33 or 35 PCR cycles.

Immunostaining

Embryos were fixed overnight in 10% neutral buffered formalin, washed, dehydrated, embedded in paraffin and sectioned at 5 μm using standard procedures or embedded in 4% agarose and sectioned at 150 μm in an oscillating tissue slicer (Electron Microscopy Sciences, Hatfield, PA). Antibody staining was performed on tissue sections using standard methods and detected with fluorescent-labeled secondary antibodies or with the Vectastain Elite Mouse IgG ABC kit. Antibodies used were mouse anti-chicken Pax6 (Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-Pax6 (sc-7750; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Pax6 (ab5790; ABCAM, Cambridge, MA), rabbit anti-phosphorylated Frs2α (AF5126; R and D systems, Minneapolis, MN), rabbit anti-Erm (*Etv5*; sc-22807; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Er81 (*Etv1*; ab36788, Abcam, Cambridge, MA), rabbit anti-FoxE3 (gift of Dr. Peter Carlsson) and mouse monoclonal anti-αA-crystallin (gift from Dr. Usha Andley). Fluorescent images for Pax6, Sox2, FoxE3 and αA-crystallin were acquired on a Zeiss LSM-510 Zeiss confocal microscope or an Olympus wide-field fluorescence microscope (Washington University). Images for Pax6, pFrs2α, ER81 and Erm were collected on a Zeiss LSM-710 confocal microscope (Miami University).

Quantifying immunofluorescence

Immunofluorescent images from sections stained for Pax6 or Er81 were analyzed using ImageJ (http://rsbweb.nih.gov/ij/). Fluorescent staining intensity in the optic vesicle cells was used as an internal standard to compare the immunofluorescence of wild-type and *Fgfr1/2*^{CKO} lens placode cells. A box was drawn around the distal optic vesicle and a separate box around the lens placode. The average pixel intensity within each box was recorded and the ratio of fluorescence in the two tissues was computed. Differences in pixel intensity between wild-type and conditional knockout eyes were evaluated using Student's t-test.

BrdU staining

Pregnant females were injected with 50 mg/kg of body weight of 10 mM BrdU (Roche, Indianapolis, IN) and 1 mM 5-fluoro-5-deoxyuridine (Sigma, St. Louis, MO) and sacrificed after 1 h. Staining was performed on sections of paraffin-embedded embryos using a monoclonal anti-BrdU antibody (1:250) (Dako, Carpinteria, CA) with a Vectastain Elite Mouse IgG ABC kit. Sections were counterstained with hematoxylin.

Measuring placode thickness

This was performed as described previously. Briefly, in frontal sections through the middle of the lens placode, the thickness of the

placode was measured at most dorsal and ventral points of contact between the optic vesicle and the lens-forming ectoderm and at three equally spaced locations between these extremes at mid-placode stage (E9.5).

TUNEL labeling

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end labeling (TUNEL) was done with an Apoptag kit (Chemicon, Temecula, CA). The deparaffinized slides were treated with 3% H_2O_2 in methanol for 30 min, followed by proteinase K treatment (20 μg/ml) for 15 min. Slides were incubated with TdT enzyme in equilibration buffer for 1 h at 37 ° C. The reaction was terminated with wash buffer provided by the manufacturer for 10 min at room temperature. Anti-digoxigenin-peroxidase conjugate was added for 30 min at room temperature, followed by DAB plus H_20_2 treatment. Slides were counterstained with hematoxylin.

Results

The FGFs and FGF receptors expressed in the ectoderm during lens induction

Twenty-two ligands and four receptors containing cytoplasmic tyrosine kinase domains mediate FGF signaling in mammals (Itoh and Ornitz, 2008; Ornitz and Itoh, 2001; Ornitz et al., 1996). Previous studies implicated FGF signaling in lens formation in the mouse, although the ligands and receptors required are not known (Faber et al., 2001; Gotoh et al., 2004; Pan et al., 2006; Smith et al., 2010).

To identify the FGF ligands and receptors expressed in the lens placode, placode cells were laser microdissected from E9.5 embryos, the RNA was reverse transcribed and amplified, and transcripts encoding FGF ligands and receptors were identified by microarray and RT-PCR analysis. These were compared to the transcripts detected by RT-PCR in adult lens epithelial cells. Table 1 lists the FGFs and FGF receptors and shows the number that were detected above background levels in 18 microarrays of RNA isolated from wild-type E9.5 or E10.0 lens placode cells. RT-PCR analysis was used to confirm the expression of FGF transcripts detected on the microarray or detected previously in mammalian lens placodes. Of the FGFs identified by microarray, transcripts encoding Fgf1, 9, 10 and 15 were readily detected by RT-PCR (Fig. 1A). A PCR product of the approximate size of the Fgf18 PCR product from the adult lens epithelial cells was present at trace levels. Although Fgf2 was detected on seven of the microarrays and in adult lens epithelial cells, Fgf2 transcripts were not detectable by RT-PCR in lens placode cDNA after 40 cycles of RT-PCR, suggesting that the transcripts detected by the microarray probes were due to off-target hybridization. Except for Fgf10, each of the PCR products tested was detected in adult lens epithelial cells. Fgf7 was included as a negative control for the PCR analyses, since it was not detected at E9.5 by microarray. Although Fgf7 transcripts were present in adult lens epithelial cells, only a trace was detected at E9.5 after 40 cycles of amplification. Although Fgf13 was detected on all microarrays, we did not test for this transcript by RT-PCR, since Fgf13 functions intracellularly and does not interact with FGF receptors (Goetz et al., 2009; Goldfarb et al., 2007; Itoh and Ornitz, 2008).

Table 1
Number of FGF and FGF receptor transcripts detected ($p<0.05$) in 18 microarray studies that used RNA extracted from E9.5 (15 arrays) or E10.0 (3 arrays) wild-type lens placode cells.

FGF ligands		FGF receptors	
Transcript	Number detected (of 18)	Transcript	Number detected (of 18)
Fgf1	4	Fgfr1	18
Fgf2	7	Fgfr2	18
Fgf3	1	Fgfr3	1
Fgf4	2	Fgfr4	0
Fgf5	4		
Fgf6	2		
Fgf7	0		
Fgf8	0		
Fgf9	2		
Fgf10	5		
Fgf11	1		
Fgf12	1		
Fgf13	18		
Fgf14	0		
Fgf15	15		
Fgf16	1		
Fgf17	4		
Fgf18	7		
Fgf20	0		
Fgf21	2		
Fgf22	0		
Fgf23	0		

Transcripts encoding Fgfr1 and 2 were readily detected by microarray and RT-PCR in the lens placode (Fig. 1B). At 35 PCR cycles, Fgfr3 and 4 were also detectable, but decreasing the PCR cycles to 33 reduced the Fgfr3 PCR product to trace level and made Fgfr4 undetectable (Fig. 1B).

Conditional deletion of FGF receptors lens placode cells

Conditional deletion of *Fgfr2* from lens placode cells did not prevent lens formation, although it resulted in the formation of smaller lenses that had high levels of cell death and defects in fiber cell differentiation (Garcia et al., 2005). In this earlier study, the effects of deleting *Fgfr2* were not examined at the lens placode stage. Since signaling through Fgfr1 could have moderated the effects of *Fgfr2* deletion, we deleted floxed alleles of *Fgfr1* and *2* from the lens placode using Le-Cre, which is expressed in the lens-forming ectoderm on E9 (Ashery-Padan et al., 2000). Littermate embryos homozygous for the floxed alleles, but not expressing the Cre transgene, served as controls. For simplicity, embryos that expressed the Cre transgene in the lens-forming ectoderm are referred to as $Fgfr1/2^{CKO}$, while those with no transgene expression are denoted as $Fgfr1/2^{WT}$ (Table 2).

Most $Fgfr1/2^{CKO}$ embryos did not have a lens at E12.5. In a few cases, rudimentary lens material was present soon after birth, but most eyes examined did not have detectable lens tissue. Fig. 2A–C shows a wild-type eye and two Cre-positive eyes at postnatal day 1 (P1). One of the Cre-positive eyes had a rudimentary lens. The other had no recognizable lens tissue. Fig. 2D and E shows sections through WT and $Fgfr1/2^{CKO}$ eyes at E12.5. In the $Fgfr1/2^{WT}$ eye, a normal-appearing lens is stained with antibody against the lens protein, αA-crystallin. In the $Fgfr1/2^{CKO}$ eye, the retina was folded and no morphologically identifiable lens was detected. A few αA-crystallin-positive cells, presumably remnants of the lens, were embedded in the mesenchyme that would normally form the corneal stroma.

Examination of embryos on E9.5 revealed that the lens placodes of $Fgfr1/2^{CKO}$ embryos appeared thinner than $Fgfr1/2^{WT}$ placodes. Measurement of thickness at five locations along the dorsal–ventral extent of the placode confirmed this impression (Fig. 3). Previous studies showed that interfering with FGF signaling reduced the BrdU labeling index in the prospective lens cells at E9.5 and E10.5 (Faber et al., 2001; Pan et al., 2006). We performed BrdU labeling at E9.5 to determine whether reduced cell proliferation could be responsible for the thinner placodes in $Fgfr1/2^{CKO}$ embryos. The BrdU labeling index was reduced in $Fgfr1/2^{CKO}$ lens placodes, but the difference between the knockout and $Fgfr1/2^{WT}$ placodes was not statistically significant (38.1 ± 5.6 and 33.3 ± 3.4 [SEM], respectively; $n = 6$–8 lenses, $p = 0.49$). Since deletion of *Fgfr2* increased cell death later in lens development (Garcia et al., 2005), we measured the TUNEL labeling index in early (20–24 somite [s]) and late (28–34 s) $Fgfr1/2^{WT}$ and

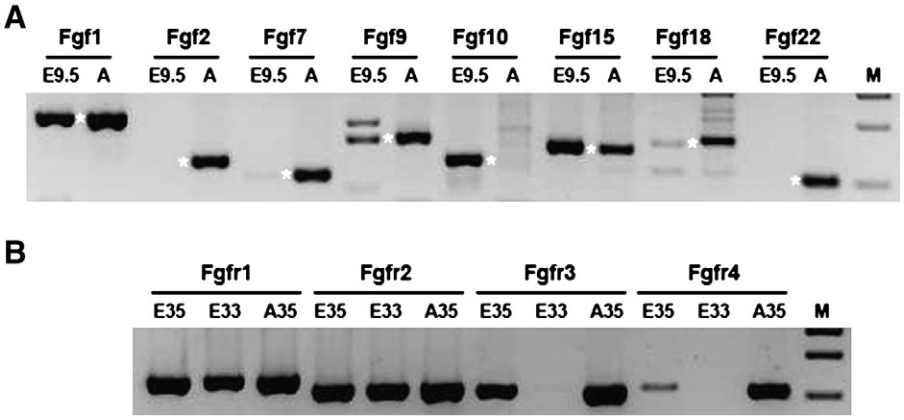

Fig. 1. Gel showing PCR products for FGF and FGF receptor transcripts. A. FGFs amplified using cDNA from E9.5 lens placodes (E9.5) and adult lens epithelial cells (A). Products of the expected size are denoted by asterisks. B. FGF receptor transcripts amplified using cDNA from E9.5 lens placodes for 35 (E35) or 33 (E33) PCR cycles or from adult lens epithelial cells amplified for 35 PCR cycles (A35). M, molecular weight markers.

Fgfr1/2^{CKO} lens placode cells. At both stages, the TUNEL labeling index was significantly increased in the *Fgfr1/2*^{CKO} placodes (Fig. 4). The increase in apoptosis was confirmed by labeling with an antibody against active caspase3 (Fig. 4G–I). Since deletion of *Fgfr2* alone did not prevent lens formation at E12.5 (Garcia et al., 2005), we compared the TUNEL labeling index in *Fgfr1/2*^{WT}, *Fgfr2*^{CKO} and *Fgfr1/2*^{CKO} placodes. Deletion of *Fgfr1* and *2* increased the level of cell death beyond that seen in *Fgfr2*^{CKO} placodes (Fig. 4J).

Effect of Fgfr1/2 deletion on known targets of FGF receptor signaling

FGF receptor activation leads to the recruitment of the adaptor protein, Frs2α. Frs2α is phosphorylated by the FGF receptor tyrosine kinase, permitting the assembly of a signaling complex that includes the phosphatase, Shp2, and the adapter protein, Grb2 (Ong et al., 2000). "Knock-in" constructs of *Frs2α* that lack the two phosphorylation sites required for binding Shp2 resulted in defects in retina and lens formation (Gotoh et al., 2004). We used antibodies to phosphorylated Frs2α (pFrs2α) to measure its activation in the lens placode and optic vesicle. Antibodies to pFrs2α stained *Fgfr1/2*^{WT} E9.5 lens placode cells at least as strongly as optic vesicle cells (Fig. 5). Staining for pFrs2α decreased markedly in *Fgfr1/2*^{CKO} placodes, compared to the optic vesicle (Fig. 5A and B). Staining for phosphorylated ERK (pERK), another downstream target of FGF receptor signaling, was also greatly reduced early in placode formation (Supplementary Fig. S1).FGF signaling often increases the expression of members of the Ets family of transcription factors. Staining for two of these, Erm and Er81, was markedly reduced in *Fgfr1/2*^{CKO} lens placode cells, when compared to *Fgfr1/2*^{WT} placode and adjacent optic vesicle cells (Fig. 5C–F).

Table 2
PCR primer sequences.

Transcript	Primer sequences	Product size (bp)
Fgfr1	F 5′-CCCTCAGGAAACAGAAAACG-3′ R 5′-GAAGCAGCCCTAACCCCTAC-3′	238
Fgfr2	F 5′-CTCTCGAGGGATGGCAAAAG-3′ R 5′-AGCAAAGTGAGTGGGCGTAT-3′	216
Fgfr3	F 5′-CCCTGCAAGAAGGTTCAGAT-3′ R 5′-CCTAGGGCCCAGTGACAGTA-3′	209
Fgfr4	F 5′-CCCTTGGACTCATCCTCAGA-3′ R 5′-TCACCAAGATGCTGGAACAA-3′	214
Fgf1	F 5′-TGTGTACGAAGTCCCAAGACC3′ R 5-GTCTTCAATGGCAGCTGATGT3′	223
Fgf2	F 5′-CTTACCGGTCACGGAAATACTC-3′ R 5′-TCAGCTCTTAGCAGACATTGGA-3′	128
Fgf7	F 5′-CCAGTGAGAACTATAATCCGGAAA-3′ R 5′-CAGTACAGGCATGTTCCAAGC-3′	107
Fgf9	F 5′-CTGTGTATCACCCTGGGAAGT-3′ R 5′-GAGTGGTTTGGCTATCTGAGC-3′	165
Fgf10	F 5′-TTCTGCCTCCGTGGGAAGT-3′ R 5′-TGAGGATTAGGAGGAGGGAAG-3′	130
Fgf15	F 5′-CCAGGAGCTTGTCTCTGTCC-3′ R 5′-ACCAGAACTGAGAGCCAGGA-3′	154
Fgf18	F 5′ -CACAGTCACCAAGCGATCC-3′ R 5′-CCCCTCCTCCCAAGACTTTA-3′	184
Fgf22	F 5′-TTCTCCTCCACTCACTTTTTCC-3′ R 5′-GGACAGAACGGATCTCCACTAT-3′	103

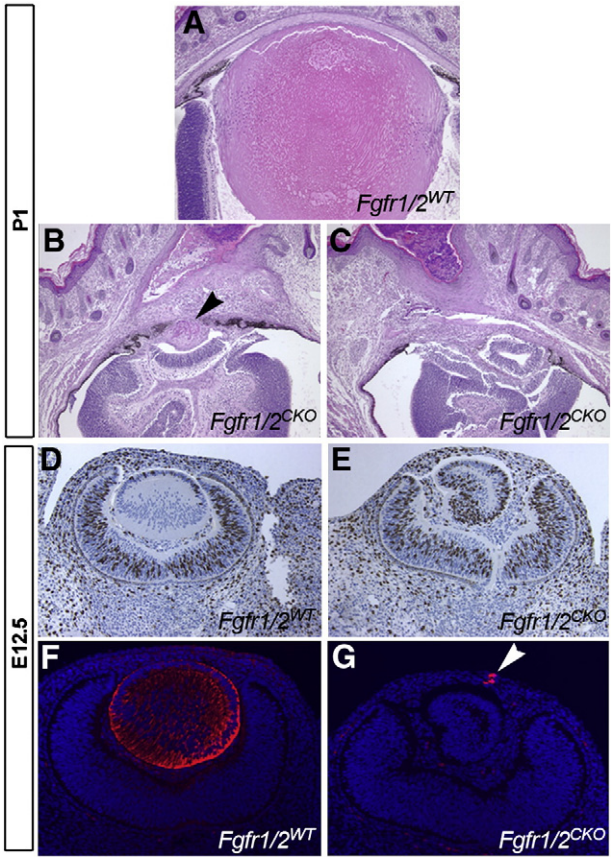

Fig. 2. The effect of deleting FGF receptors on lens morphology and αA-crystallin expression. Wild-type (A) and conditional knockouts (B and C) at P1. In rare cases, a lens rudiment was detectable (arrowhead in B). In most cases, no lens was detected. At E12.5, the wild-type lens had normal morphology (D) and stained for αA-crystallin (F). In conditional knockouts, the retina folded, sometimes appearing like an abnormal lens (E). However, only a few αA-crystallin-positive cells were present. These were embedded in the mesenchyme that will later form the corneal stroma (G).

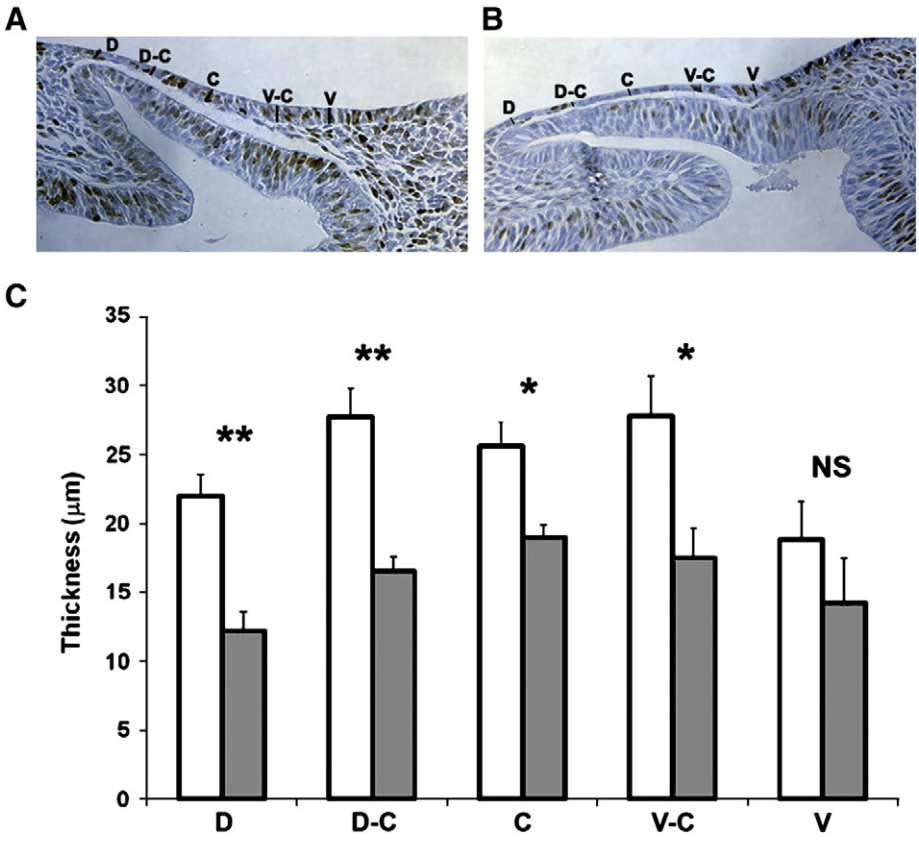

Fig. 3. Effect of deleting FGF receptors on the thickness of the lens placode. (A and B) Placode thickness was measured at five dorso-ventral locations at E9.5 (mid-placode stage) in wild-type (A) and Fgfr1/2^{CKO} (B) placodes. The brown nuclei in these sections are BrdU-labeled. (B) Fgfr1/2CKO placodes (shaded bars) were significantly thinner than wild-type placodes (open bars) at most locations. $^{*}p<0.05$; $^{**}p<0.01$; NS, not significantly different.

Deletion of Fgfr1/2 did not prevent the increased expression of Pax6 that occurs during placode formation, or reduce the expression of Pax6 downstream targets

Germline deletion of *Ndst1* or mutation of phosphorylation sites in *Frs2α* disrupted lens and retina differentiation and decreased Pax6 protein levels in the lens placode, at least in the most severely affected embryos (Gotoh et al., 2004; Pan et al., 2006). Antibody staining detected no obvious difference in Pax6 levels between wild-type and *Fgfr1/2*^{CKO} lens placodes (Fig. 6A–D). Since the results for Pax6 expression were different from those obtained in previous studies of FGF signaling in lens induction, they were repeated in two labs, using different litters of embryos, different antibodies to Pax6 and different methods of antibody detection (immunofluorescence or immunohistochemistry). Quantification of immunofluorescence intensity confirmed that Pax6 staining was not different in wild-type and *Fgfr1/2*^{CKO} lens placodes at E9.5. Quantification was performed by using staining in the adjacent, wild-type optic vesicle cells as an internal standard (placode/optic vesicle [P/O] ratio: 1.27 (WT) and 1.21 (CKO); $p=0.37$). Similar analysis showed that staining for Er81 was significantly reduced in the *Fgfr1/2*^{CKO} lens placodes (P/O ratio: 1.37 (WT) and 0.42 (CKO); $p=3.8\times10^{-7}$). Pax6 levels did decrease in the *Fgfr1/2*^{CKO} ectoderm cells remaining at E10.5.

Deletion of Pax6 in the lens-forming ectoderm prevents lens formation and the expression of several genes that are required for later stages of lens cell differentiation (Ashery-Padan et al., 2000). The transcription factor, Sox2, is downstream of and genetically interacts with Pax6 in the lens placode to promote lens development (Smith et al., 2009). As with Pax6, germline mutation or deletion of *Frs2α* or *Ndst1* reduced Sox2 expression in the lens placode of the most severely affected embryos (Gotoh et al., 2004; Pan et al., 2006). In the present study, antibodies to Sox2 stained nuclei in the retina and lens placode to a similar degree in *Fgfr1/2*^{WT} and *Fgfr1/2*^{CKO} eyes at E9.5 (Fig. 7A and B).

Pax6 is required for expression of the transcription factor, FoxE3, and the lens-preferred protein, αA-crystallin (Blixt et al., 2007; Brownell et al., 2000; Yang and Cvekl, 2005; Yang et al., 2006). These proteins are first detectable at the late placode/lens pit stage. Due to the extensive cell death in *Fgfr1/2*^{CKO} eyes, few cells remained at the onset of lens invagination. However, cells located deep in the small lens pits stained intensely with antibodies to FoxE3 and αA-crystallin (Fig. 7C–F), showing that Pax6 function was preserved in these cells.

Interactions between BMP and FGF receptors in the lens placode

The morphogens Bmp4 and 7 are required for lens formation (Furuta and Hogan, 1998; Jena et al., 1997; Wawersik et al., 1999). Conditional deletion of the type I BMP receptors, *Bmpr1a* and *Acvr1* in the lens placode prevented lens formation, demonstrating that BMP signaling is required in the ectoderm cells for lens induction (Rajagopal et al., 2009). Removal of one allele of *Bmp7* enhanced the phenotype of a dominant-negative *Fgfr1* transgene in the lens placode, suggesting that BMP and FGF signaling pathways interact in lens development (Faber et al., 2001). Deletion of either *Fgfr2* or *Bmpr1a* significantly increased cell death, resulting in the formation of smaller lenses (Garcia et al., 2005; Rajagopal et al., 2009). To explore the nature of the interaction between BMP and FGF signaling during placode formation, we conditionally deleted *Fgfr2* and *Bmpr1a* using Le-Cre. Cre-positive mice were born with small eyes resulting from absence of the lens (Fig. 8A and B). At E10.5, *Fgfr2/Bmpr1a*^{CKO} lens placodes were thin, with numerous TUNEL-positive cells (Fig. 8C and D). The TUNEL labeling index in the *Fgfr2/Bmpr1a*^{CKO} placodes increased 2.5-fold, compared to *Fgfr2/Bmpr1a*^{WT} (Fig. 8E). These results confirm that inhibition of FGF and BMP signaling can have

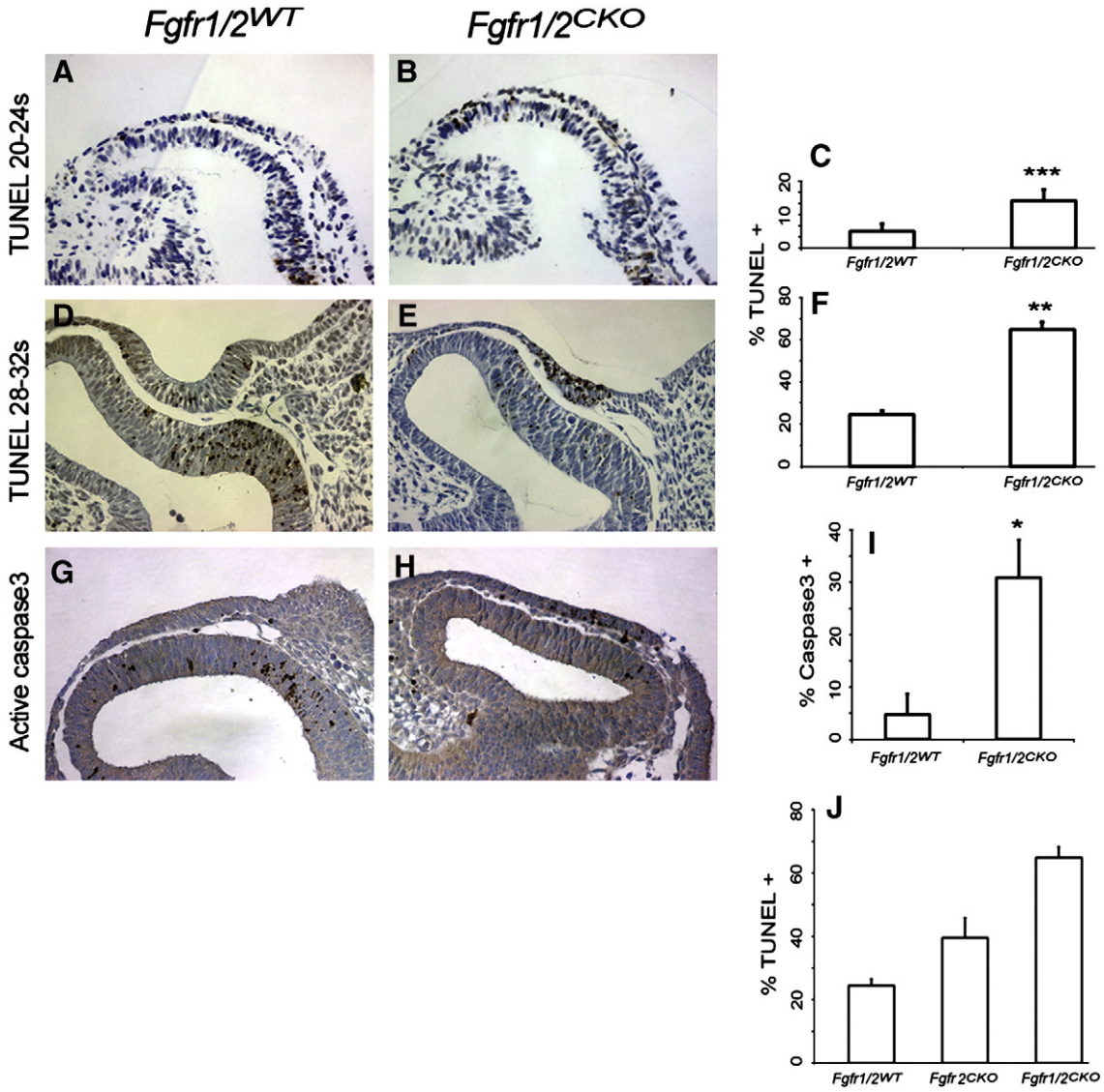

Fig. 4. Effect of deleting FGF receptors on apoptosis in the lens placode. TUNEL staining was greater in conditional knockout lens placodes from 20–24 somite and 28–32 somite embryos than in wild-type littermates (A, B, D, and E). The TUNEL labeling index is shown in (C) for 20–24 somite embryos and in (F) for 28–32 somite embryos. Antibody to activated caspase3 was also greater in conditional knockout than in wild-type embryos (G–I). Compared to wild-type, the TUNEL labeling index increased in a dose-dependent manner in *Fgfr2*^{CKO} and *Fgfr1/2*^{CKO} embryos (J).

additive effects on lens formation and show that these defects involve increased cell death.

Discussion

The tissue interactions required for lens formation have been studied for over a century (Grainger et al., 1988; Lang, 2004; Spemann, 1901; Swindell et al., 2008). The requirement for the optic vesicle to promote lens placode thickening and invagination is one of the most thoroughly studied examples of embryonic induction. Prior to lens placode formation, the prospective lens ectoderm is exposed to signals that permit it to respond to the final inductive stimulus from the optic vesicle and must be shielded from influences that inhibit lens formation (Bailey et al., 2006; Grainger et al., 1992; Sullivan et al., 2004). In the analysis that follows, we reserve the classical term "lens induction" for the effects of signals or other influences that originate from the optic vesicle.

The major phenotype observed after deleting the two most abundant FGF receptors that are expressed in the lens placode was extensive cell death, which, in most cases, resulted in loss of a morphologically identifiable lens. Although deletion of *Fgfr1* and *Fgfr2* decreased the levels of several proteins that depend on FGF signaling in the lens placode cells, the transcription factor Pax6 and its downstream targets, Sox2, FoxE3 and αA-crystallin were expressed in the remaining lens cells at normal levels. In the following sections, we reconcile these observations with the results obtained in previous studies of FGF signaling during lens induction. We then consider the implications of the experiments performed in this and other studies as they relate to the role of FGF signaling in lens induction.

Germline mutations and FGF receptor function

Two previous studies used germline genetic modifications to obtain information about the role of FGF signaling in lens formation. In one study, *Frs2α* was mutated to prevent Frs2α from recruiting the Shp2 phosphatase to FGF receptors (Gotoh et al., 2004). In the other, *Ndst1*, an enzyme involved in the production of heparan sulfate proteoglycans, was genetically inactivated (Pan et al., 2006). Heparan sulfate proteoglycans are important co-factors for the efficient binding of FGFs to their receptors (Ornitz, 2000). Because these

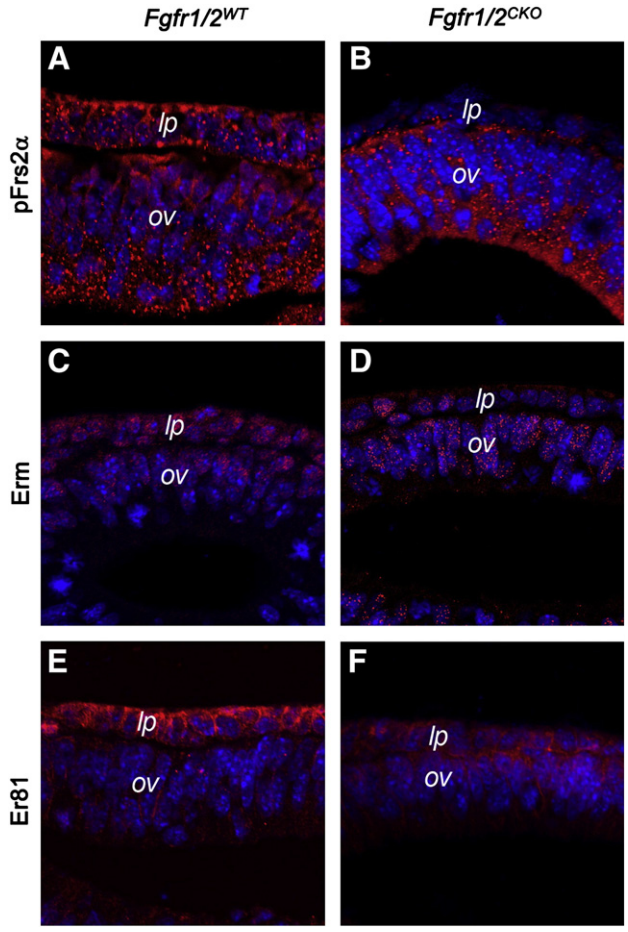

Fig. 5. The effect of FGF receptor deletion on the levels in the lens placode of the adapter protein, pFrs2α and the Ets family members, Erm and Er81. Staining for the phosphorylated (activated) form of Frs2α (A and B), Erm (C and D) and Er81 (E and F) decreased markedly in *Fgfr1/2*^{CKO} lens placode cells.

genes were inactivated in all cells, it was difficult to determine whether their deleterious effects on lens and eye formation occurred prior to or during lens induction and in which tissues FGF receptor function was most important.

Information relevant to these questions can be surmised from the phenotypes of the knockout embryos. In both studies, the authors classified the mutant phenotypes as "mild" or "severe" (Gotoh et al., 2004; Pan et al., 2006). In embryos with mild phenotypes, a smaller lens vesicle formed, with few or no abnormalities noted in the optic vesicle, the tissue responsible for lens induction. Some of these mildly affected embryos went on to form normal-appearing eyes with small lenses. Severe phenotypes were associated with disruption of the morphogenesis or differentiation of the optic vesicle and showed little or no lens formation. These embryos later became severely microphthalmic or anophthalmic. It seems possible that the severe phenotypes were due to defects in the differentiation or morphogenesis of the optic vesicle that occurred before lens induction.

In the *Ndst1* mutants, mildly affected eyes had normal levels of Pax6 in the lens placode, while more severely affected eyes had reduced Pax6 in the nuclei of placode cells (Pan et al., 2006). Since loss of *Ndst1* would be expected to impair FGF signaling in all cells of every embryo, it seems likely that the lower levels of Pax6 in severely affected embryos were due to defects in formation of the optic vesicle, rather than defects in the ability of placode cells to respond to FGFs. Pax6 expression was also reduced in the placode cells of severely affected *Frs2α* mutants (Gotoh et al., 2004). Pax6 levels were not reported for mildly affected eyes, making it unclear whether Pax6 levels were normal or reduced in the lens placodes of these less severely affected embryos. Mutation of *Frs2α* also had significant effects on the expression of several genes in the optic vesicle; levels of the transcription factors Six3 and Chx10 (Vsx2) and the morphogen Bmp4 were greatly reduced. Bmp4 from the optic vesicle is required for lens induction (Furuta and Hogan, 1998) and Six3 and Chx10 may regulate the expression of other genes needed for lens induction. Therefore, although the results of both studies are consistent with some role for FGF signaling in lens formation, they raise questions about when FGFs act and in what tissues.

Similar concerns apply to the use of the FGF receptor antagonist, SU9597, to inhibit lens formation (Faber et al., 2001). Embryo heads cultured in this inhibitor showed decreased Pax6 expression in the lens placode and formed smaller lens pits. However, the inhibitor also decreased Pax6 levels in the optic vesicle, such that Pax6 immunostaining was always greater in the lens placode cells. Therefore, its effects on lens differentiation could have been due to its effects on lens placode cells, optic vesicle cells, or both.

Targeted interference with FGF receptor signaling in the lens placode

Transgenic overexpression in the lens placode of a kinase-deficient form of *Fgfr1* delayed lens invagination, reduced lens size, decreased αA-crystallin mRNA and delayed the accumulation of α-crystallin, β-crystallin and MIP, markers of lens cell differentiation (Faber et al., 2001). In the transgenic embryos, the lens placode appeared thinner and the BrdU labeling index was decreased at the lens pit stage (E10.5) and in the lens epithelial cells at E13.5. Cell death was not measured. The strength of this study is that the transgenic construct was targeted to the lens placode, minimizing concerns that it might have effects before lens induction or on other tissues required for lens formation, like the optic vesicle. However, the transgene only modestly reduced the level of Pax6 in the lens placode, if at all. In the figures shown, levels of Pax6 were always greater in the lens placode than in the adjacent, non-transgenic optic vesicle and the extent of any decrease in Pax6 levels in the lens placode was not quantified (Faber et al., 2001). The persistence of Pax6 expression in the lens placode in the present study is consistent with continued Pax6 expression several days after deletion of *Fgfr1/2/3* at the lens

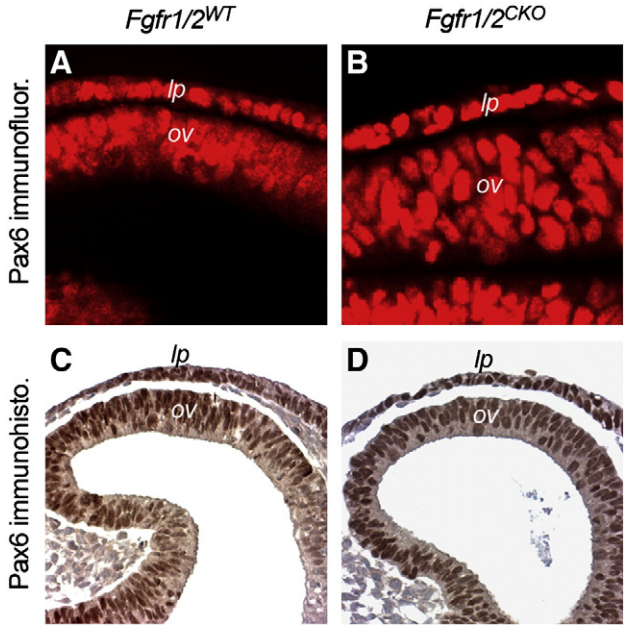

Fig. 6. Conditional deletion of Fgfr1/2 in the lens placode did not significantly decrease Pax6 levels. Sections of E9.5 lens placodes (*lp*) and optic vesicles (*ov*) were stained with antibodies to Pax6. Antibody staining was detected by immunofluorescence (A and B) or immunohistochemistry (C and D).

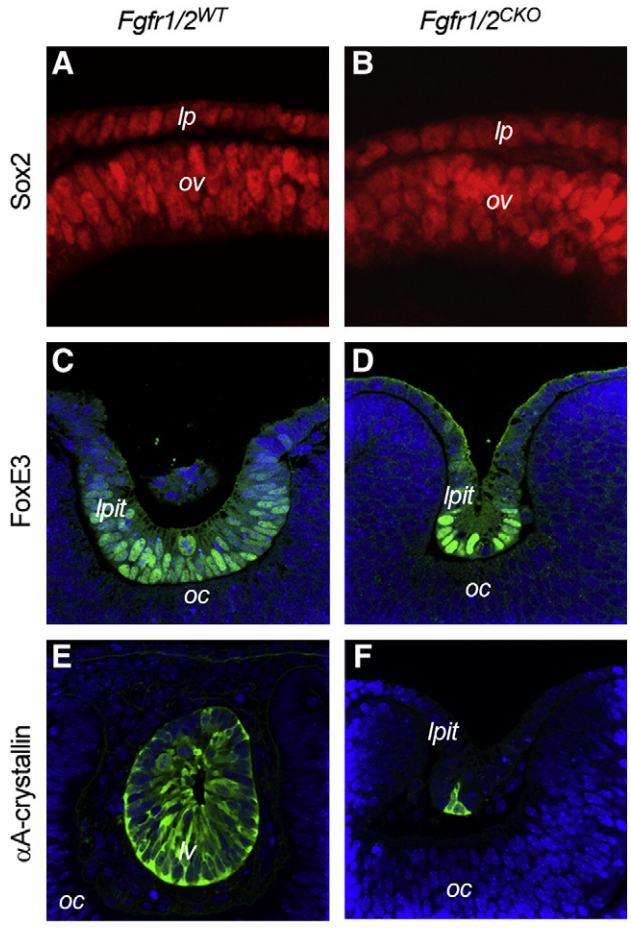

Fig. 7. The effect of deleting FGF receptors on the expression of the Pax6 targets, Sox3, FoxE3 and αA-crystallin. Conditional deletion of *Fgfr1/2* in the lens placode did not reduce the expression of Sox2 (A and B), FoxE3 (C and D), or αA-crystallin in lens placode (*lp*) or lens pit (*lpit*) cells. The lens vesicle shown in E is from an older embryo (36 somites) than the lens pit shown in F (33 somites).

vesicle stage (Zhao et al., 2008). An additional concern about this experimental design is that, to be effective, the "dominant-interfering" construct must be expressed at levels in excess of the endogenous receptors. This can result in "gain-of-function" phenotypes. For example, overexpression of a "dominant-interfering" construct encoding kinase-dead form of the TGFβ type 2 receptor (*Tgfbr2*) caused abnormal lens fiber cell differentiation and eventual degeneration, but deletion of *Tgfbr2* from the lens produced no lens phenotype . These results showed that TGFß signaling is not essential for lens development and that overexpression of a mutant receptor can interfere with normal development.

In the present study, FGF receptors were removed by conditional deletion using the Le-Cre transgene, which is targeted to the lens placode by the Pax6 P0 enhancer (Ashery-Padan et al., 2000). The lens placode does not form when Pax6 is deleted using Le-Cre (Smith et al., 2009). This indicates that Pax6 deletion is efficient and occurs before placode formation commences. Consistent with this observation, we found that cell death increased significantly in the lens-forming ectoderm of 20–24 somite *Fgfr1/2*^{CKO} embryos, which is before or at the onset of placode formation. This early increase in apoptosis and the decrease in FGF target genes, like Erm and Er81, in the lens placode show that FGF signaling was functionally absent throughout the time when lens induction was occurring.

Increased cell death occurred throughout the placode stage, such that, by the time of invagination, few cells remained to form a lens. Deletion of FGF receptors had a dose-dependent effect on cell death; deletion of *Fgfr1* and *2* resulted in greater cell death than when only *Fgfr2* was deleted. Similarly, conditional deletion of *Bmpr1a* enhanced the cell death seen in *Fgfr2*^{CKO} placodes and prevented subsequent lens formation. In spite of the severe cell death phenotype and decreased expression of FGF targets in *Fgfr1/2*^{CKO} embryos, Pax6 and Sox2 were expressed at normal levels in the remaining placode cells and expression of Foxe3 and αA-crystallin was activated in the small number of lens pit cells remaining on E10.5. No morphological defects were observed in the optic vesicles in *Fgfr1/2*^{CKO} embryos at the lens placode or pit stages.

It is important to consider whether the results obtained in the present study can be explained by mechanisms other than the loss of FGF receptors. For example, the Le-Cre transgene might increase placode cell death, independent of the inhibition of FGF signaling. This is unlikely, since Le-Cre did not increase the TUNEL labeling index above that seen in Cre-negative placodes when used to delete the BMP receptor *Acvr1* or the mediator of BMP and TGFß signaling, *Smad4* (Rajagopal et al., 2009). Therefore, the presence of Cre recombinase in lens placode cells, independent of its effects on the floxed FGF receptors, is unlikely to be the cause of the cell death seen in this study.

Although smaller lens size was a common phenotype in all studies in which FGF signaling was impaired, only one other study of the role of FGFs in lens induction measured apoptosis in the lens placode or vesicle. In contrast to results obtained in the present study, *Ndst1* null lens vesicles showed no obvious increase in TUNEL labeling (Pan et al., 2006). Although heparan sulfate proteoglycans are important co-receptors for FGF signaling, deletion of *Ndst1* must not completely block FGF receptor activation, since germline deletion of *Fgfr1* causes developmental arrest at gastrulation, while *Ndst1* null embryos survive until after birth (Fan et al., 2000). Therefore, the cell death that occurs when FGF receptors are deleted in the lens placode may be avoided in *Ndst1* null embryos by the partial activation of FGF receptors.

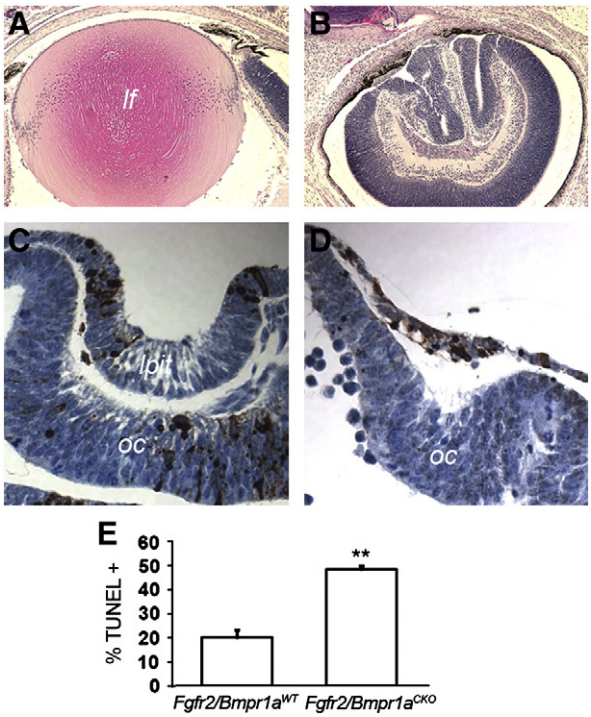

Fig. 8. Deleting *Fgfr2* and *Bmpr1a* increased apoptosis and prevented lens formation. Conditional deletion of *Fgfr2* and *Bmpr1a* resulted in eyes with no detectable lens at P1 (A, B). At E10.5, extensive apoptosis, decreased placode thickness and failure of lens pit formation was evident (C, D). At E10.5, the TUNEL labeling index increased greatly in double knockout lens placodes, compared to wild-type (E).

The role of FGF signaling in the lens placode

Previous investigations found that FGFs are expressed in the lens placode. These include Fgf1 and Fgf2 in rats (de Iongh and McAvoy, 1993) and Fgf19 in chickens and zebrafish (Fgf19 in humans and chickens is the ortholog of Fgf15 in the mouse, designated in this report as Fgf15/19) (Kurose et al., 2004; Nakayama et al., 2008; Wright et al., 2004). The present study extends this number to at least 5. Therefore, it is reasonable that FGF receptors in lens placode cells are activated in an autocrine or paracrine manner by FGFs produced within the ectoderm.

FGF signaling is required to establish the preplacodal region that gives rise to all cranial placodes (Bailey et al., 2006). However, later FGF signaling inhibited lens placode differentiation and promoted olfactory fate in the chicken embryo (Bailey et al., 2006). Fgf8 transcripts were detected in the optic vesicle of chicken embryos (Karabagli et al., 2002; Tian et al., 2002; Vogel-Hopker et al., 2000) and ectopic addition of Fgf8 prior to lens induction promoted the formation of cells that expressed L-maf, a marker of lens formation in chicken embryos (Vogel-Hopker et al., 2000). However, in situ hybridization analysis did not detect Fgf8 in the mouse optic vesicle at E9.0 (Crossley and Martin, 1995), a result consistent with our microarray studies of optic vesicle cells at E9.5 (data not shown). Fgf15/19 is important for lens formation in the zebrafish and may be involved in lens formation in chicken embryos (Kurose et al., 2005; Nakayama et al., 2008). Previous in situ hybridization studies showed that Fgf15/19 was abundant in the distal optic vesicle of the mouse embryo (McWhirter et al., 1997). However, germline deletion of Fgf15/19 in the mouse was not reported to affect eye development (Wright et al., 2004). Fgf15/19 binds selectively to Fgfr4 (Kurose et al., 2005), which was barely detectable in the mouse lens placode and germline deletion of *Fgfr4* did not result in an obvious eye or lens phenotype (Weinstein et al., 1998), a result that we have confirmed (data not shown). Therefore, a role for FGFs from the optic vesicle in lens induction remains unclear.

Conclusions

The data from the present study suggest that the primary role of FGF signaling in the mouse lens placode is to activate or maintain survival signaling pathways. Since other investigators did not detect increased cell death or extensive loss of placode cells when FGF signaling was impaired in the ectoderm, our results represent the most severe lens phenotype resulting from interference with FGF signaling in early lens development. Since no increase in TUNEL labeling was detected when the optic vesicle was severely disrupted in *Ndst1* knockout embryos, FGFs from the optic vesicle are unlikely to be required for cell survival in the lens placode (Pan et al., 2006). Endogenous FGFs may serve this function (Faber et al., 2001; Pan et al., 2006).

Apoptosis was 4- to 6-fold higher in $Fgfr1/2^{CKO}$ ectoderm at the beginning of placode formation, the time when lens induction is initiated. This indicates that Fgfr1 and 2 were functionally absent during the period of induction and could not have contributed to induction. In spite of the functional absence of FGF signaling, the increased expression of Pax6 that occurs during induction occurred normally and the expression levels of Pax6 downstream targets was unaffected. We conclude from these data that FGFs are not involved in lens induction by the optic vesicle.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.01.001.

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