Developmental Biology 351 (2011) 176–185

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

The function of FGF signaling in the lens placode

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ARTICLE INFO

Article history:
Received for publication 15 October 2010
Revised 30 December 2010
Accepted 4 January 2011
Available online 9 January 2011

Keywords:
Lens induction
Fibroblast growth factors
FGF receptors
Apoptosis
Pax6

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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doi:10.1016/j.ydbio.2011.01.001

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