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Perspective

FGFR and PTEN signaling interact during lens development to regulate cell survival

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ABSTRACT

Lens epithelial cells express many receptor tyrosine kinases (RTKs) that stimulate PI3K–AKT and RAS–RAF–MEK–ERK intracellular signaling pathways. These pathways ultimately activate the phosphorylation of key cellular transcription factors and other proteins that control proliferation, survival, metabolism, and differentiation in virtually all cells. Among RTKs in the lens, only stimulation of fibroblast growth factor receptors (FGFRs) elicits a lens epithelial cell to fiber cell differentiation response in mammals. Moreover, although the lens expresses three different *Fgfr* genes, the isolated removal of *Fgfr2* at the lens placode stage inhibits both lens cell survival and fiber cell differentiation. Phosphatase and tensin homolog (PTEN), commonly known as a tumor suppressor, inhibits ERK and AKT activation and initiates both apoptotic pathways, and cell cycle arrest. Here, we show that the combined deletion of *Fgfr2* and *Pten* rescues the cell death phenotype associated with *Fgfr2* loss alone. Additionally, *Pten* removal increased AKT and ERK activation, above the levels of controls, in the presence or absence of *Fgfr2*. However, isolated deletion of *Pten* failed to stimulate ectopic fiber cell differentiation, and the combined deletion of *Pten* and *Fgfr2* failed to restore differentiation-specific Aquaporin0 and DnaseIIβ expression in the lens fiber cells.

1. Introduction

The relative developmental simplicity of the ocular lens makes it an important model to study developmental mechanisms controlling cellular growth, survival, differentiation, and proliferation (Wormstone and Wride, 2011). Invaginations of surface ectoderm overlying the optic vesicles create bilateral lens vesicles during early mammalian development (reviewed in Robinson (2014)). Cells in the posterior hemisphere of the lens vesicle withdraw from the cell cycle, elongate, and turn on fiber cell specific genes as they differentiate into the primary fiber cells (reviewed in Bassnett and Beebe (2004)). The lens vesicle cells in the anterior hemisphere differentiate into the lens epithelium. Only lens epithelial cells proliferate, and as the lens matures, cell proliferation becomes increasingly restricted to the germinative zone, a narrow band of epithelial cells slightly anterior to the lens equator (Harding et al., 1971; McAvoy, 1978). Proliferation within the germinative zone displaces epithelial cells toward the equator where they differentiate into secondary fiber cells. Proliferation in the germinative zone and secondary fiber cell differentiation provide a constant source of new lens fibers throughout the mammalian lifespan.

Among the numerous receptor tyrosine kinases (RTKs) expressed in the developing lens, fibroblast growth factor receptors (FGFRs) play a unique and indispensable role in lens development (Garcia et al., 2011, 2005; Madakashira et al., 2012; Robinson, 2006; Zhao et al., 2008). As with most RTKs, ligand (FGF) binding by FGFRs leads to downstream activation of intracellular phosphorylation cascades culminating in the activation, by phosphorylation, of ERK1/2 and AKT kinases. Activation of these kinases leads to many of the cellular responses associated with growth factor stimulation (Lemmon and Schlessinger, 2010). In lens explants, or cultured lens epithelial cells, AKT and/or ERK1/2 phosphorylation results in enhanced cell survival, growth, proliferation, and differentiation (Chandrasekher and Sailaja, 2004a,b; Iyengar et al., 2006; Le and Musil, 2001a,b; Lovicu and McAvoy, 2001;

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Wang et al., 2009; Weber and Menko, 2006). However, eliminating or inhibiting FGFR signaling *in vivo* leads to decreased lens cell survival and differentiation without significantly altering cell proliferation (Chow et al., 1995; Garcia et al., 2011, 2005; Madakashira et al., 2012; Robinson et al., 1995; Stolen and Griep, 2000; Zhao et al., 2008).

The mouse lens specifically expresses three FGFR genes, *Fgfr1*, *Fgfr2* and *Fgfr3* (Hoang et al., 2014). Lenses lacking *Fgfr2* prior to the lens vesicle stage undergo degeneration marked by both apoptosis and differentiation defects, while simultaneously removing *Fgfr1* exacerbates this phenotype (Garcia et al., 2005, 2011). Conditional deletion of *Fgfr1, Fgfr2* and *Fgfr3* in the lens, subsequent to the lens vesicle stage, causes massive apoptosis and arrest of fiber cell differentiation (Zhao et al., 2008). Conversely, lenses that overexpress FGFs *in vivo* undergo ectopic fiber cell differentiation in the lens epithelium (Lovicu and Overbeek, 1998; Robinson et al., 1998, 1995). FGFR activation requires heparan sulfate in a ternary complex with FGF. The loss of heparan sulfate synthesizing enzymes *Ndst1* and *Ndst2* causes lens cell apoptosis, reduced proliferation, and defective fiber cell differentiation (Qu et al., 2011). However, expression of a constitutively active *Ras* allele in these lenses increased ERK1/2 phosphorylation and reversed the *Ndst1*/*Ndst2* deficient phenotypes.

Since lens cells rely on FGFR signaling for survival, decoupling the apoptotic phenotype from the differentiation phenotype in lenses with compromised FGFR signaling remains a challenge. During normal development, FGFR signaling in the lens may primarily promote cell survival with defective differentiation in FGFR-deficient lenses resulting as a secondary response to apoptosis. AKT enhances cell survival by a variety of mechanisms, including inhibiting FOXO transcription factors and destabilizing the pro-apoptotic BAD/Bcl-X_L complex (reviewed in Zhang et al. (2011)). FGFR stimulation activates phosphoinositide 3-kinase (PI3K) which converts the cell membrane lipid PtdIns (4,5)P2, hereafter referred to as PIP2, into PtdIns(3,4,5)P3, hereafter referred to as PIP3. PIP3 then recruits AKT to the cell membrane where phosphorylation by mTORC2 and PDK1 activates AKT (Sarbassov et al., 2005). The tumor suppressor protein, Phosphatase and tensin homolog (PTEN), counteracts PI3K by dephosphorylating PIP3 back to PIP2, leading to reduced AKT activation. In addition to inhibiting AKT activation, PTEN acts as a tumor suppressor by inhibiting cell proliferation and promoting apoptotic pathways (Chung and Eng, 2005; Franke et al., 2003; Weng et al., 2001a).

Given the antagonism between PI3K and PTEN, we hypothesized that PTEN acts as an important negative regulator of FGFR activity during lens development. In particular, PTEN activity may drive lens cells toward apoptosis by exacerbating the presumably decreased PIP3 levels in FGFR-deficient lens cells, which indirectly prevents the activation of AKT. Studies in both osteoprogenitor cells and keratinocytes reveal the importance of balancing FGFR and PTEN signaling. Deletion of *Fgfr2* rescues over-proliferation in osteoprogenitors caused by the loss of *Pten* (Guntur et al., 2011). Likewise, skin tumorgenesis resulting from *Pten* deletion requires *Fgfr2* (Hertzler-Schaefer et al., 2014). To specifically determine whether PTEN-signaling counter balances FGFR-signaling with respect to survival and/or differentiation in the lens, we used Cre-mediated recombination to facilitate the lens-specific removal of both *Pten* and *Fgfr2* during early lens development. We reasoned that the restoration of survival in FGFR-deficient lens cells would reveal survival-independent aspects of FGFR-mediated fiber cell differentiation.

Given the central importance of FGFR signaling in the development of many different tissues and organs (reviewed in Carter et al. (2015) and Teven et al. (2014)), it comes as no surprise that aberrant FGFR signaling causes numerous developmental disorders and drives the pathogenesis of many human cancers (reviewed in Ahmad et al. (2012), Katoh and Nakagama (2014) and Wesche et al. (2011)). Often, the same mutations that give rise to developmental disorders in the germline lead to specific cancers in somatic tissues. Likewise, *PTEN* mutations drive the genesis and malignancy of several human tumors (reviewed in Mester and Eng (2013)). Revealing how FGFR and PTEN signaling interact in the context of lens development may facilitate the discovery of new targets for therapeutic intervention to treat diseases or conditions caused by FGFR and/or PTEN dysfunction.

2. Materials and methods

2.1. Mice

Mice were used in accordance to the ARVO statement for the Use of Animals in Ophthalmic and Visual Research with approval from the Miami University Institutional Animal Care and Use Committee. Dr. Ashery-Padan at Tel Aviv University kindly provided the *Le-Cre* mice (Ashery-Padan et al., 2000). Mice engineered with loxP sites flanking exons 4 and 5 of Pten were previously described (Trimboli et al., 2009). Floxed *Fgfr2* mice (Yu et al., 2003) were obtained from Dr. Ornitz from the Department of Molecular Biology and Pharmacology, Washington University Medical School.

2.2. Histology and immunohistochemistry

The gestational age of experimental embryos was determined by vaginal plug detection, set at embryonic day 0.5 (E0.5). One hour prior to embryo collection, pregnant dams were injected intraperitoneally with (0.1 mg/g body weight) 5-bromo-2-deoxyuridine (BrdU) dissolved in phosphate-buffered saline (PBS). For paraffin wax-embedded sections, embryos were collected and fixed in 10% neutral buffered formalin (NBF). Standard protocols were used to process and embed tissues in paraffin wax before sectioning at 5 μm. Immunohistochemistry staining identified protein localization on the paraffin lens sections. Briefly, the sections were subjected standard xylene washes to remove excess paraffin followed by tissue dehydration. The sections were antigen retrieved as described in Zhao et al. (2008). Tissue sections were blocked using 10% normal horse or normal goat serum, dependent upon the antibody used. Primary antibodies for BrdU and Aquaporin0 (ab6326, ab15077 respectively) were obtained from Abcam, Cambridge, MA, USA. The primary antibody for p27KIP1 (BD610241) was obtained from BD Biosciences, San Jose, CA, USA. The primary antibodies for β and γ-crystallin were kind gifts from Samuel Zigler at Johns Hopkins University School of Medicine. All primary and secondary antibodies were used at a 1:100 dilution, with the exception of β- and γ-crystallin, which were used at a 1:250 dilution. Primary antibodies were detected using secondary antibodies attached to fluorescent probes (Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 546 goat anti-rat IgG, FITC for donkey antirabbit IgG, 711-095-152 and Cy3 for donkey anti-mouse IgG). Sections were counterstained with DAPI (H-1200, Vector Labs, Burlingame, CA, USA). Cells undergoing DNA degradation/apoptosis were detected using the In Situ Cell Death Detection Kit (TMR Red, Roche AppliedScience, 2156792). Photomicrographs were captured on a Zeiss 710 Laser Scanning Confocal System at the Center for Advanced Microscopy and Imaging at Miami University. Standard Hematoxylin and Eosin-stained sections were used to analyze the structure of the lens, and images were captured using a Nikon TI-80 microscope. The proliferation and apoptotic index represents a ratio of all the BrdU positive or TUNEL positive nuclei (respectively) over the total nuclei in the entire lens at E12.5. At