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Myocilin modulates programmed cell death during retinal development

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ABSTRACT

Mutations in the myocilin gene (*MYOC*) are causative for 10% of cases with juvenile open-angle glaucoma and 3–4% of those with primary open-angle glaucoma. Myocilin is a secreted protein with relatively ill-defined matricellular properties. Despite its high expression in the eye, myocilin-deficient mice have originally been reported to have no obvious ocular phenotype. Here we revisited the ocular phenotype of myocilin-deficient mice and detected a higher number of neurons in their inner (INL) and outer (ONL) nuclear layers, as well as a higher number of retinal ganglion cells (RGC) and their axons. The increase in retinal neurons appears to be caused by a decrease in programmed developmental cell death, as apoptosis of retinal neurons between postnatal days 4 and 10 was found to be attenuated when compared to that of wildtype littermates. In contrast, when *Myoc*$^{-/-}$ mice were crossed with *βB1-crystallin-MYOC* mice with ectopic overexpression of myocilin in the eye, no differences in developmental apoptosis, RGC number and INL thickness were observed when compared to wildtype littermates. The amounts of the anti-apoptotic Bcl-2-like protein 1 (BCL2L1, Bcl-xL) and its mRNA were increased in retinae of *Myoc*$^{-/-}$ mice, while lower amounts of BCL2L1 and its mRNA were detected in mixed *Myoc*$^{-/-}$*/βB1-crystallin-MYOC* mice. The structural differences between *Myoc*$^{-/-}$ mice and wildtype littermates did not result in functional differences as measured by electroretinography. Noteworthy though mixed *Myoc*$^{-/-}$*/βB1-crystallin-MYOC* mice with ocular overexpression of myocilin had significant cone function deficits. Myocilin appears to modulate apoptotic death of retinal neurons likely by interacting with the intrinsic apoptotic pathway.

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1. Introduction

Mutations in *MYOC* encoding for myocilin have been reported in 10% of patients with juvenile open-angle glaucoma and 3–4% of cases with primary open-angle glaucoma (POAG) (Adam et al., 1997; Fingert et al., 1999, 2002; Kwon et al., 2009b; Stone et al., 1997). Glaucoma is the second leading cause of blindness worldwide and POAG is its most common form (Resnikoff et al., 2004). Myocilin is a secreted glycoprotein and member of the family of olfactomedin domain-containing proteins (Tomarev and Nakaya, 2009) with a predicted molecular weight of 55.3 kDa (Resch and Fautsch, 2009; Tamm, 2002). By Western blot analysis and SDS-PAGE, myocilin is detected as a doublet with an additional 57 kDa band which very likely results from N-glycosylation (Caballero and Borrás, 2001; Caballero et al., 2000; Clark et al., 2001). The olfactomedin-domain is localized in the C-terminal globular region of myocilin (Ricard and Tamm, 2005), while its N-terminus contains leucine zipper motifs within two coiled-coil domains that promote homodimerization of myocilin (Fautsch and Johnson, 2001) and most likely account for the presence of non-monomeric myocilin in the aqueous humor of the eye (Fautsch and Johnson, 2001; Russell et al., 2001). There is evidence that, in addition to the traditional secretory pathway, myocilin is released from the cell via exosomes, small vesicles with a membrane to which myocilin attaches via its coiled-coil domain (Perkumas et al., 2007; Stamer et al., 2006). Secreted myocilin associates with a variety of fibrillar extracellular matrix molecules including fibronectin, laminin and collagen types

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I, II, V, and VI (Filla et al., 2002; Peters et al., 2005; Tawara et al., 2000; Ueda et al., 2002, 2000).

Despite years of research efforts, no clear-cut physiological function of myocilin has been discovered (Resch and Fautsch, 2009). Based on cell biological experiments, a role of myocilin as a matricellular protein has been proposed. Matricellular proteins are non-structural proteins of the extracellular matrix that mainly serve a regulatory function (Bornstein et al., 2004; Murphy-Ullrich, 2001). Still, with regards to the nature of such a regulatory role, conflicting data have been reported. Overexpression of myocilin in trabecular meshwork cells has been shown to result in loss of actin stress fibers and focal contacts, and to result in reduced cellular adhesiveness (Shen et al., 2008). Recombinant myocilin added to a fibronectin matrix inhibited spreading and substrate adhesion of skin fibroblasts (Peters et al., 2005). On the contrary, other groups observed an increase in actin stress fibers (Goldwich et al., 2009; Kwon et al., 2009a) and enhanced substrate adhesion when recombinant myocilin was added to kidney podocytes or trabecular meshwork cells (Goldwich et al., 2009). The stimulating effects of myocilin on the formation of actin stress fibers appear to involve an interaction with components of the Wnt signaling pathway (Kwon et al., 2009a). In addition, recombinant myocilin stimulates cell migration through the activation of the integrin-focal adhesion kinase (FAK)-serine/threonine kinase (AKT) signaling pathway (Kwon and Tomarev, 2011).

The relevance of *in vitro* findings indicating a matricellular function of myocilin for the living organism have been less than clear, since myocilin-deficient mice were originally reported to express no obvious phenotype (Kim et al., 2001). Only fairly recently, some distinct physiological changes were detected in myocilin-deficient mice. The changes include a reduced cortical bone thickness and a diminished trabecular volume, findings that appear to be associated with a stimulating influence of myocilin on osteogenesis (Kwon et al., 2013b). Moreover, myocilin-deficient mice show a reduced thickness of the myelin sheath and a disorganization of the nodes of Ranvier in peripheral nerves (Kwon et al., 2013a) where myocilin is highly expressed (Ohlmann et al., 2003). The effects appear to involve myocilin signaling through ErbB2/3 receptors (Kwon et al., 2013a). The findings of a phenotype in bones and peripheral nerves of myocilin-deficient mice encouraged us to revisit the question, if myocilin-deficient mice show structural abnormalities in the eye, the organ where myocilin is most abundant (Tamm, 2002). Here we report on distinct structural changes in the retina of myocilin-deficient mice that involve an increase in neuronal number caused by a reduction in developmental programmed cell death. The effect is associated with increasing amounts of the anti-apoptotic Bcl-2-like protein 1 (BCL2L1, Bcl-xL) and its mRNA, and raises the possibility of a modulating role of myocilin in the adult eye that influences neuronal cell death upon injury.

2. Material and methods

2.1. Mice

All procedures conformed to the tenets of the National Institutes of Health Guidelines on the Care and Use of Animals in Research, the EU Directive 2010/63/E, and the Uniform Requirements for Manuscripts Submitted to Biomedical Journals. *Myoc*$^{-/-}$ mice (Kim et al., 2001) were generously supplied by Simon W.J. John (The Jackson Laboratory) and *βB1-crystallin-MYOC* mice were generated as described previously (Zillig et al., 2005). Both strains were bred in a BALB/c albino background for at least 10 generations and kept in cyclic light (12 h on/12 h off, lights on at 7 AM, light intensity approx. 400 lx). *Myoc*$^{-/-}$*/βB1-crystallin-MYOC* mice were generated by crossing *Myoc*$^{+/-}$ */βB1-crystallin-MYOC* mice with *Myoc*$^{+/-}$ animals. All experiments were performed in mice of either sex.

2.2. Western blot analysis

Neural retinae were treated with TRIzol (Invitrogen) following the manufacturer's instruction for protein isolation. For western blot analysis, the supernatant was separated by SDS-PAGE and transferred to a polyvinyl difluoride membrane (Millipore) which was incubated with PBS containing 0.1% Tween 20 (PBST; pH 7.2), and 5% bovine serum albumin or 5% non-fat dry milk (Table 1) overnight. Antibodies were used as described in Table 1. After washing with PBS-T, secondary antibodies (1:2000–1:5000) were added. Chemiluminescence was visualized by using Immobilon Western HRP Substrate (Millipore) or CDP-Star (Roche) and a LAS 3000 imaging workstation (Fujifilm). For normalization of signals seen in western blots, blotted membranes were stained for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or α-tubulin. The intensity of each band was evaluated by relative densitometry using appropriate software (AIDA Image Analyzer v.4.06 software; Raytest).

2.3. Light microscopy

Eyes of experimental and control pups were enucleated at postnatal (P) days P4, P9 and P14. Adult eyes, optic and sciatic nerves were taken from animals at 6–8 weeks of age. All

Table 1
Antibodies for Western blot analysis.

Primary antibody	Blocking	Secondary antibody
Akt (Cell Signaling) 1:1000	5% non-fat dry milk	Chicken-anti-mouse-HRP (Santa Cruz Biotechnology)
BAK (D4E4) (Cell Signaling) 1:1000	5% bovine serum albumin	Anti-rabbit-HRP (Cell Signaling)
BAX (Cell Signaling)	5% bovine serum albumin	Anti-rabbit-HRP (Cell Signaling)
BCL2L1 (Bcl-xL) (Cell Signaling) 1:1000	5% non-fat dry milk	Chicken-anti-rabbit-HRP (Santa Cruz Biotechnology)
β-Catenin (Cell Signaling) 1:1000	5% non-fat dry milk	Chicken-anti-rabbit-HRP (Santa Cruz Biotechnology)
ERK1/2 (Cell Signaling) 1:1000	5% bovine serum albumin	Chicken-anti-rabbit-HRP (Santa Cruz Biotechnology)
GAPDH (HRP-coupled) (Cell Signaling) 1:10,000	5% non-fat dry milk	
pAkt (Cell Signaling) 1:500	5% bovine serum albumin	Chicken-anti-mouse-HRP (Santa Cruz Biotechnology)
pERK1/2 (Cell Signaling) 1:500	5% bovine serum albumin	Chicken-anti-mouse-HRP (Santa Cruz Biotechnology)
pJNK (Santa Cruz) 1:1000	5% bovine serum albumin	Chicken-anti-mouse-HRP (Santa Cruz Biotechnology)
pSmad2 (Cell Signaling) 1:200	5% non-fat dry milk	Chicken-anti-rabbit-HRP (Santa Cruz Biotechnology)
pSmad3 (Cell Signaling) 1:200	5% non-fat dry milk	Chicken-anti-rabbit-HRP (Santa Cruz Biotechnology)
α-Tubulin (Rockland) 1:1000	5% non-fat dry milk	Chicken-anti-rabbit-HRP (Santa Cruz Biotechnology Anti-rabbit-AP (Cell Signaling)

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