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Elevated amounts of myocilin in the aqueous humor of transgenic mice cause significant changes in ocular gene expression

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ABSTRACT

Myocilin is a 55–57 kDa secreted glycoprotein and member of the olfactomedin family, which is mutated in some forms of primary open-angle glaucoma. To assess the effects of elevated amounts of myocilin on aqueous humor outflow dynamics in an *in vivo* system, transgenic βB1-crystallin-MYOC mice have been developed that strongly overexpress myocilin in their eyes. The transgenic overexpression of myocilin results in an almost five-fold increase of secreted normal myocilin in the aqueous humor of βB1-crystallin-MYOC mice. In the present study, we wanted to use βB1-crystallin-MYOC as a tool to identify the response of ocular tissues to the presence of higher than normal amounts of myocilin, and to identify changes in gene expression that could help to shed light on the functional *in vivo* properties of myocilin. RNA was isolated from ocular tissues of βB1-crystallin-MYOC mice and wild-type littermates. Changes in gene expression were determined by hybridization of gene microarrays and confirmed by real time RT-PCR and Western blotting. The expression of genes that had been found to be differentially regulated in βB1-crystallin-MYOC mice was further analyzed in cultured human trabecular meshwork (HTM) cells treated with recombinant myocilin. Although βB1-crystallin-MYOC mice do not have an obvious phenotype, a statistically significant up- and downregulation of several distinct genes was found when compared to gene expression in wild-type littermates. Among the genes that were found to be differentially regulated were *Wasl*, *Ceacam1*, and *Spon2*, which are involved in cell adhesion and cell–matrix interactions. Differences in expression were also found for *Six1* which encodes for a transcription factor, and for *Pftk1* whose gene product is a cdc2-related protein kinase. The expression of these genes was also found to be regulated *in vitro* in HTM cells treated with recombinant myocilin. Substantially higher amounts in ocular tissues of βB1-crystallin-MYOC mice were found for connexin 46 and αB-crystallin. In addition, several genes that encode for olfactomedin proteins showed distinct changes in expression. *Olfml3* was significantly downregulated, while *Lphn1*, *Lphn2*, and *Lphn3* were significantly upregulated. Our findings support a role for myocilin in modulating cellular adhesion, and suggest functional processes that involve other proteins of the olfactomedin family.

1. Introduction

Myocilin is a 55–57 kDa secreted glycoprotein and member of the olfactomedin family, which is characterized by a coiled-coil domain near the amino terminus and an olfactomedin domain near the carboxyl terminus (Tamm, 2002). Mutations in the *MYOC* gene that encodes for myocilin are causative for some forms of juvenile and adult-onset primary open-angle glaucoma (POAG) (Stone et al., 1997). The mechanisms that cause POAG in affected patients have not been finally clarified, but appear to involve dominant-negative effects that are associated with non-secretion of mutated myocilin (Jacobson et al., 2001; Liu and Vollrath, 2004; Shepard et al., 2007). Myocilin is highly expressed in several tissues of the anterior eye such as iris, ciliary body, cornea and sclera (Adam et al., 1997; Karali et al., 2000; Swiderski et al., 2000). An extremely high expression has been observed in the trabecular meshwork (Adam et al., 1997; Swiderski et al., 2000; Tamm et al., 1999; Tomarev et al., 2003). The available data on the functional properties of myocilin have been derived exclusively from *in vitro* studies and indicate a matricellular role of myocilin that modulates cellular adhesion (Peters et al., 2005; Shen et al., 2008;

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Wentz-Hunter et al., 2004). As mutant *Myoc*-deficient mice do not express an obvious phenotype (Kim et al., 2001), the relevance of a matricellular role for myocilin *in vivo* is unclear. Some experimental evidence supports the hypothesis that secreted myocilin plays a role *in vivo* in modulating the hydrodynamic outflow resistance in the trabecular meshwork, and that elevated amounts of myocilin could obstruct the outflow system. The expression of myocilin is induced by treatment with dexamethasone in cultured trabecular meshwork cells and in perfused anterior segment organ cultures in a time-dependant manner and comparable to the time course that is observed during the development of steroid-induced ocular hypertension and glaucoma (Nguyen et al., 1998). Furthermore, recombinant myocilin is very effective at blocking polycarbonate filters with a pore size similar to that of the trabecular meshwork (Goldwich et al., 2003), and myocilin in the aqueous humor (AH) is tightly bound to polycarbonate filters that become obstructed after perfusion with AH (Russell et al., 2001). Finally, the strongest support for an obstructive role of myocilin has been contributed by Fautsch and colleagues who could show that perfusion of anterior segment organ cultures with human recombinant myocilin from a eukaryotic expression system causes a significant reduction in outflow facility (Fautsch et al., 2006). The effect required preincubation of myocilin with porcine aqueous humor indicating that recombinant myocilin appears to form a complex with other proteins in porcine aqueous humor that enables it to bind specifically within the trabecular meshwork. In contrast, a reduced secretion of myocilin in the trabecular meshwork correlates with an increase in outflow facility (Caballero et al., 2000), while perfusion with a C-terminal fragment of myocilin containing the entire olfactomedin domain has no effects on facility (Goldwich et al., 2003). To assess the effects of elevated amounts of myocilin on aqueous humor outflow dynamics in an *in vivo* system, we developed transgenic mice that strongly express myocilin in their lenses under control of the βB1-crystallin promoter (Zillig et al., 2005). The transgenic overexpression of myocilin from the lens results in an almost five-fold increase of secreted normal myocilin in the aqueous humor of βB1-crystallin-MYOC mice. Despite the high amounts of myocilin in their AH, intraocular pressure of βB1-crystallin-MYOC mice does not differ from that of control mice, a finding that argues against an obstructive role of myocilin for the mouse trabecular outflow system *in vivo*. Comparable data have been reported with another set of transgenic mice that have been genetically modified to overexpress myocilin (Gould et al., 2004). In the present study, we wanted to use βB1-crystallin-MYOC as a tool to identify the response of ocular tissues to the presence of higher than normal amounts of myocilin. The purpose of this study was to identify changes in gene expression that could help to shed light on the functional *in vivo* properties of myocilin. Our findings support a role for myocilin in modulating cellular adhesion, and suggest functional processes that involve other proteins of the olfactomedin family.

2. Material and methods

2.1. Transgenic mice and cell cultures

βB1-crystallin-MYOC mice were obtained and kept as described previously (Zillig et al., 2005). This mouse strain was generated in a FVB/N background with hereditary retinal degeneration (Gimenez and Montoliu, 2001). Since the purpose of this study was to analyze gene expression in βB1-crystallin-MYOC animals with a phenotypically normal retina, animals were bred in a mixed FVB/N × CD1 background. Cell cultures of human trabecular meshwork cells and optic nerve astrocytes were established and grown as described previously (Fuchshofer et al., 2005, 2007).

2.2. cDNA microarray analysis

Total RNA was isolated from ocular tissues (whole eyes without lens) of 3-week-old βB1-crystallin-MYOC mice and wild-type littermates by using TRIzol (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. The RNA concentration was determined by absorbance at 260 nm (Eppendorf BioPhotometer; Eppendorf, Hamburg, Germany). Doubled-stranded cDNA was synthesized from 5 μg of purified total RNA with a kit (Superscript Double-Stranded cDNA Synthesis Kit, Invitrogen) and a T7-(dT)24 primer (Affymetrix, Santa Clara, CA). After the double-stranded cDNA was purified by phenol–chloroform extraction, *in vitro* transcription reactions were performed (Bioassay High Yield RNA Transcript Labeling Kit; Enzo Diagnostics, Farmingdale, NY), according to the manufacturer's protocol. Biotin-labeled cRNA was purified (Qiagen, Valencia, CA) and quantified using an ND-1000 Nano-drop spectrophotometer (Nano-Drop Technologies, Wilmington, DE), before being fragmented to 35–200 base fragments in an alkaline buffer. Five Affymetrix Mouse 430 2.0 Arrays (Affymetrix) containing 34,000 well-characterized mouse genes were used. Washing, staining, and scanning were performed by using the Genechip Instrument System (Affymetrix) as recommended in the manufacturer's technical manual. The arrays were scanned and data were analyzed on a computer (Microarray Suite algorithm, ver. 5; Affymetrix). The absolute analysis results of each chip were scaled to the same target intensity value of 150 and could then be directly compared with one another. The absolute analysis calculates a variety of metrics using the probe array's hybridization intensities measured by the scanner. The comparison analysis performs additional calculations on data from two separate probe array experiments to compare gene expression levels between two samples. The comparison analysis begins with the absolute analysis of one probe array experiment as the source of baseline data and a second probe array of the experimental conditions as the source of data to be compared to the baseline.

2.3. Real time RT-PCR

Total RNA was isolated from ocular tissues (without lens) of βB1-crystallin-MYOC mice and wild-type littermates as described above. First strand cDNA synthesis was performed by using 1.0 μg of total RNA and the iScript cDNA Synthesis Kit (BioRad Laboratories, Munich, Germany), as described by the manufacturer. Quantification by real time PCR was performed on a Rotor-Gene 3000 real time thermal analyzer (Corbett Life Science, Mortlake, Australia). The cDNA of *Lamin A* served as an endogenous control to normalize the differences in the amount of cDNA in each sample. PCR reaction was performed in a volume of 25 μl, consisting of 2.5 μl of 10× PCR buffer, 2.0–2.5 μl of $MgCl_2$ (25 mM), 0.5 μl of dNTPs (10 mM each; Promega, Madison, WI), 0.5 μl of Hot Star Taq (5 U/μl, Qiagen), 0.5 μl of primer mix (20 μM each) and 2.5 μl of 1× SYBR Green I solution (Sigma Aldrich, Seelze, Germany). For *Cryab* and *Gja3*, the real time RT-PCR was done on a Lightcycler real time analyzer (Roche, Basel, Switzerland) using TaqMan reagents and the One-Step RT-PCR kit according to the manufacturer's instructions (Applied BioSystems, Foster City, CA, USA). All samples that had to be compared for expression differences were run in the same assay as duplicates. Data were initially expressed as a threshold cycle and are expressed as fold increases in gene expression in βB1-crystallin-MYOC mice compared with the expression in wild-type littermates. For each experiment, the mean value in wild-type mice was set at 1. In experiments comparing gene expression between different ocular tissues, the mean value in the tissue with lowest expression was set at 1. After completion of PCR amplification, data were analyzed with Rotor-Gene Software version 6.0. For analyzing differentially expressed genes in individual tissues, the RNA from 3-week-old transgenic ($n = 3$) and wild-type littermates ($n = 4$) was pooled