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Myocilin, a Glaucoma-Associated Protein, Promotes Cell Migration through Activation of Integrin-Focal Adhesion Kinase-Serine/Threonine Kinase Signaling Pathway

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Abstract

The *MYOCILIN* gene encodes a secreted glycoprotein which is highly expressed in eye drainage structures. Mutations in this gene may lead to juvenile open-angle glaucoma and adult onset primary open-angle glaucoma, one of the leading causes of irreversible blindness in the world. Functions of wild-type myocilin are still unclear. We have recently demonstrated that myocilin is a modulator of Wnt signaling and may affect actin cytoskeleton organization. Here we report that myocilin and its naturally occurring proteolytic fragments, similar to Wnt3a, are able to stimulate trabecular meshwork, NIH3T3 and FHL124 cell migration with the N-terminal proteolytic fragment of myocilin lacking the olfactomedin domain producing the highest stimulatory effect. Stimulation of cell migration occurs through activation of the integrin-focal adhesion kinase (FAK)-serine/threonine kinase (AKT) signaling pathway. Inhibition of FAK by siRNA reduced the stimulatory action of myocilin by 3 fold. Activation of several components of this signaling pathway was also demonstrated in the eyes of transgenic mice expressing elevated levels of myocilin in the eye drainage structures. These data extend the similarities between actions of myocilin and Wnt proteins acting through a β-catenin-independent mechanism. The modification of the migratory ability of cells by myocilin may play a role in normal functioning of the eye anterior segment and its pathology including glaucoma.

Keywords

Myocilin; Trabecular meshwork cell; focal adhesion kinase; integrins; serine/threonine kinase; Glaucoma

Introduction

Myocilin is a secreted glycoprotein with a length of 504 amino acids belonging to the family of proteins containing the olfactomedin domain (Kubota et al., 1997; Kwon et al., 2009b; Stone et al., 1997; Tamm, 2002; Tomarev and Nakaya, 2009). Myocilin is proteolytically cleaved at the C-terminus of Arg^{226} by calpain II, producing two stable protein fragments

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(Aroca-Aguilar et al., 2005; Sanchez-Sanchez et al., 2007). The N-terminal fragment has two coiled-coil domains containing a leucine zipper, while the C-terminal fragment contains the olfactomedin domain (Nguyen et al., 1998). The *MYOCILIN* (*MYOC*) gene is highly expressed in the trabecular meshwork (TM), iris, ciliary body, sclera, retinal pigmented epithelial cells and sciatic nerve, with lower levels of expression observed in skeletal muscle, mammary gland, thymus and testis (Adam et al., 1997; Ohlmann et al., 2003; Stone et al., 1997; Swiderski et al., 2000; Tamm, 2002; Tomarev et al., 1998; Tomarev et al., 2003; Torrado et al., 2002).

Mutations in the *MYOC* gene are found in more than 10% of juvenile open-angle glaucoma cases and in 3–4% of patients with adult onset primary open-angle glaucoma (Adam et al., 1997; Fingert et al., 1999; Fingert et al., 2002; Kwon et al., 2009b; Stone et al., 1997). Glaucoma is one of the leading causes of irreversible blindness in the world and primary open angle glaucoma is the most common form of glaucoma. It affects more than 60 million people and causes blindness in about 4.5 million people worldwide (Quigley and Broman, 2006). More than 70 glaucoma-causing mutations have been identified and greater than 90% of them are located in the region encoding the olfactomedin domain. Mutations causing severe glaucoma phenotypes, for example Tyr437His or Ile477Ser, lead to the retention of myocilin in the endoplasmic reticulum and prevent its secretion (Alward et al., 1998; Jacobson et al., 2001; Malyukova et al., 2006; Sohn et al., 2002). Moreover, secretion of wild-type myocilin is impeded in the presence of mutated myocilin protein (Caballero et al., 2000; Gobeil et al., 2004; Jacobson et al., 2001; Malyukova et al., 2006; Zhou et al., 2008). Expression of mutated myocilin sensitizes cells to oxidative stress-induced apoptosis (Joe and Tomarev, 2010) and accumulation of mutated myocilin in endoplasmic reticulum may lead to cell death (Joe et al., 2003; Liu and Vollrath, 2004).

The functions of wild-type myocilin are still unclear (Resch and Fautsch, 2008). The absence of open-angle glaucoma in an elderly woman homozygous for the Arg46Stop mutation (Lam et al., 2000) or in people hemizygous for *MYOC* (Wiggs and Vollrath, 2001) suggests that the loss of functional myocilin is not critical for the development of glaucoma or for normal eye functioning. Similarly, mice heterozygous and homozygous for a targeted null mutation in *Myoc* do not have a detectable eye phenotype (Kim et al., 2001). Some data suggest that myocilin may play a role in cell-matrix interaction (Goldwich et al., 2009) and may inhibit neurite outgrowth (Jurynec et al., 2003; Koga et al., 2009). Myocilin also modulates the organization of the actin cytoskeleton, stimulating the formation of stress fibers by interacting with components of the Wnt signaling pathway, and this may be essential for TM contractility and regulation of intraocular pressure (Kwon et al., 2009a).

In humans, the TM is made up of connective tissue beams covered by endothelial-like cells. The space between the beams is filled with extracellular material/matrix. Aqueous humor filters through the TM, passes through Schlemm's canal and eventually leaves the eye via the episcleral venous system. In the normal eye, the region of maximal resistance to aqueous humor outflow includes the peripheral juxtacanalicular TM, which is adjacent to Schelmm's canal, and the inner wall of Schlemm's canal. The juxtacanalicular TM has no collagen beams and consists of several layers of cells immersed in extracellular matrix that may act as a filter restricting aqueous humor movement through this region. The contractility of the TM

cells or changes in the composition of the extracellular matrix may modify their interaction leading to changes in aqueous humor outflow, and therefore intraocular pressure. It is well documented that TM cells have the capacity to migrate (Calthorpe and Grierson, 1990; Hogg et al., 1995; Wentz-Hunter et al., 2004). It has been suggested that modifications in the migratory ability of TM cells may play a role in glaucoma development (Hogg et al., 2000; Koga et al., 2006).

Here we report that myocilin and its proteolytic fragments are able to stimulate cell migration. The stimulation of cell migration occurs through the activation of the integrin-focal adhesion kinase (FAK)-serine/threonine kinase (AKT) signaling pathway. The myocilin effects were demonstrated both in cell culture and in the eyes of transgenic mice expressing elevated levels of myocilin in the drainage structures.

Materials and Methods

DNA Constructs and Transgenic Mice

Human FLAG-tagged myocilin, myocilin-ΔC and myocilin-ΔN constructs have been described (Kwon et al., 2009a). Transgenic C57BL/6 mice producing 3 times higher levels of myocilin in the eye drainage structures and their wild-type littermates were used to study *in vivo* effects of myocilin. These transgenic mice were generated using bacterial artificial chromosome DNA containing the full length human *MYOC* gene, as well as 89 and 51 kb of the 5'- and 3'-flanking sequences, respectively (Kwon et al., 2009a).

Antibodies

Rabbit polyclonal antibody raised against the N-terminal part of mouse myocilin (positions 100–187) was previously described (Kim et al., 2001; Malyukova et al., 2006). Other antibodies were purchased from following sources: monoclonal β1-integrin, α3-integrin, αV-integrin, fibronectin, FAK and pFAK antibodies from BD Bioscience (San Jose, CA), rabbit polyclonal α5-integrin, pAkt and Akt antibodies from Cell Signaling Technology (Danvers, MA).

Transfection and Preparation of Conditioned Media

HEK 293, NIH3T3, FHL124, L-, and Wnt3a-expressing L-cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml). TM primary cell lines were cultivated as described previously (Tumminia et al., 1998) and were kindly provided by Dr. Paul Russell (UC Davis School of Veterinary Medicine). Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, using 1 μg of DNA/well in a 6-well plate (2×10^5 cells/well). 48 hrs after transfection, serum-containing DMEM incubation medium was removed, cells were washed three times with PBS and incubated in serum-free medium for additional 24 hrs. Conditioned medium (CM) was collected and used directly or frozen at −80° C. Myocilin-depleted CM was prepared by incubation of CM from myocilin-transfected HEK293 cells or Wnt3a-expressing L-cells with monoclonal antibodies against human myocilin (10 μg /ml) (R&D, Minneapolis, MN) for 30–60 min at room temperature.

Myocilin Purification

HEK293 cells were transiently transfected with FLAG-tagged myocilin constructs and incubated as above. Serum-containing media was replaced by serum-free media 6 hrs after transfection and cells were incubated for 48 hrs. CM was collected and myocilin-FLAG proteins were purified using anti-FLAG M2 agarose beads following manufacturer's instructions (Sigma, St. Louis, MO). For some experiments, myocilin was further purified by ion-exchange chromatography using HiTrap-SP FF 1ml columns (GE Healthcare, Piscataway, NJ). Purified myocilin was dialyzed against PBS and its purity was analyzed by SDS-PAGE.

Immunofluorescence

NIH3T3 cells were seeded on 2-well glass chamber slides (Nalge Nunc, West Chester, PA) in complete DMEM medium. The medium was removed 4–6 hrs after plating and cells were washed 2–3 times with PBS. Cells were incubated with different CM for 4 hrs and then fixed with freshly made 3.7% formaldehyde for 10 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After blocking with 5% BSA in PBS at room temperature for 1 hr, cells were incubated with Rhodamine-phalloidin (Molecular Probes, Eugene, OR) at room temperature for 30 min. For immunostaining, permeabilized cells were incubated in a solution of 0.5% Triton X-100 and 1% normal goat serum in PBS with the primary antibody at the indicated dilutions at 4°C for 16 hrs and the immunoreactive signal was revealed with secondary TRITC-anti-mouse antibody (dilution 1:200) (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Freshly dissected mouse eyes were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4°C for 24 hrs, cryoprotected in 30% sucrose for 24 hrs at 4°C and embedded in optimal cutting temperature compound (OCT; Tissue-Tek, Torrance City, CA). Eyes were cryosectioned at 10 μm directly onto microscope slides (Superfrost Plus; Diagger, Vernon Hills, IL). Sections were then incubated with primary antibodies for 1–2 hrs at room temperature or overnight at 4°C. Signal visualization was performed by incubating sections for 1 hr at room temperature with an appropriate secondary antibody conjugated to CY3 or an Alexa 488 fluorophore (1:200, Molecular Probe) diluted in PBS containing 0.5% Triton-X100 and DAPI (Molecular Probe) for counterstaining. Axioplan 2 fluorescent microscope and Axio camera (Carl Zeiss MicroImaging, Inc. NY) were used to detect fluorescence. The images were processed with Adobe Photoshop Elements 2.0 (Adobe Inc., San Jose, CA).

Western Blotting

Cells were washed 3 times with PBS and lysed on the plate in a lysis buffer (RIPA: 1 x TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide, protease inhibitor cocktail, Santa Cruz, Santa Cruz, CA). Lysates were separated by 4–12% gradient NU-PAGE (Invitrogen, Carlsbad, CA). 10 μg of each extract was loaded in each well. After separation and transfer to a PVDF membrane, the blots were incubated with a rabbit polyclonal antibody against myocilin at a 1:2000 dilution or with monoclonal antibodies against FAK and pFAK at a 1: 2000 dilution. Detection was made using a SuperSignal WestDura (Pierce, Rockford, IL). The amounts of loaded protein were normalized using an immunoblot for internal control protein, HSC70 (Santa Cruz, Santa Cruz, CA). For digital

quantification, membranes were scanned using Typhoon 9410 Variable Mode Image (Amersham Pharmacia Biotech, Piscatway, NJ) and analyzed using Image Scion Alpha 4.0.3.2 (Scion Cor, Frederick, MD).

Cell Migration Assays

Scratch or wound healing assays were performed with NIH3T3 and FHL124 cells seeded on glass chamber slides (Nalge Nunc). After cells were about 80% confluent, the monolayer was scratched with a pipette tip. The medium was replaced with serum free medium containing purified myocilin (3 μg/ml) or with CM from HEK293 cells transiently transfected with constructs encoding myocilin or its proteolytic fragments. In experiments with sFRP1, CM was preincubated with 10 μg/ml of sFRP1 at room temperature for 30 min before addition to cell cultures. Cultures were incubated 6–24 hrs in a CO_2 incubator at 37°C. Cell migration was evaluated using Axiovision Rel. 4.7 (Carl Zeiss MicroImaging, Inc. NY). Transwell assays were performed with 24 mm-diameter, uncoated cell culture inserts having an 8.0 μm pore size (Costar, St. Louis, MO) and 6 well culture plates. NIH3T3 cells were trypsinized, resuspended in serum free DMEM, and transferred to the upper chamber (1×10^4 cells in 250 μl). Purified myocilin (3 μg/ml) or Wnt3a (3 μg/ml) were added to the lower chamber. Cells were maintained for 6 hrs in a CO_2 incubator at 37°C. Cells migrating to the bottom surface of the filter were trypsinized and counted using a hematocytometer. In experiments with rabbit polyclonal anti-myocilin antibody, myocilin or Wnt3a were preincubated with the antibody (1: 1000 dilution) for 30 min at room temperature before addition to the lower chamber.

Transient Transfection of FAK siRNA

FAK and scrambled siRNAs were synthesized by Dharmacon (Chicago, IL). A mixture of equal amounts of four FAK siRNAs (GCGAUUAUAUGUUAGAGAU, GGGCAUCAUUCAGAAGAUA, UAGUACAGCUCUUGCAUAU and GGACAUUAUUGGCCACUGU) was used. FHL124 cells were plated at 60% confluence in a 24 well plate or glass chamber slide and transfected with different concentrations of FAK siRNAs mixure as above. Immunoblotting with anti-FAK antibody was performed to monitor the efficiency of FAK suppression.

AKT inhibition assay

Cells were treated with two phosphatidylinositol 3-kinase inhibitors, LY294002 and wortmannin, at a final concentration of 20 μM for 1 hr.

Statistics

Quantitative data are expressed as mean ± SEM. A two-tailed Students' *t*-test was used where comparisons between two groups were made. A one-way ANOVA (95% confidence interval) with Bonferroni multiple comparisons post-hoc test (only computed if overall $p<0.05$) was used when comparison of three or more treatment groups was made (IBM SPSS Statistics 19).

Results

Myocilin and Its Proteolytic Fragments Stimulate Cell Migration

Our previous results demonstrated that myocilin modulates organization of the actin cytoskeleton, stimulating the formation of stress fibers by interacting with components of Wnt signaling pathways (Kwon et al., 2009a). The organization of the actin cytoskeleton is critical for cell contractility and may affect cell motility (Pellegrin and Mellor, 2007). To test whether myocilin may regulate cell motility, we used cells derived from non-ocular (NIH3T3) and ocular (primary human TM cells and fetal human lens epithelial cells FHL124) tissues. First, we performed a transwell assay. In this assay, cells are placed in an upper chamber and migrate through a filter which is immersed in a lower chamber containing test proteins. NIH3T3 cells were treated with purified myocilin or Wnt3a as positive control, since Wnt3a is able to stimulate cell migration (Angers and Moon, 2009; Endo et al., 2005). In the presence of myocilin or Wnt3a, the amount of migrating cells was increased 3 ± 0.5 ($p = 0.003$) and 4 ± 0.6 ($p = 0.004$) fold, respectively (Fig. 1A). To confirm that cell migration was induced by myocilin and not by other contaminating protein(s), purified myocilin and Wnt3a were treated with antibodies raised against mouse myocilin before addition to the lower chamber. Such treatment did not reduce the stimulatory effect of Wnt3a but completely blocked the stimulatory effect of myocilin (Fig. 1A). Similar results were obtained when TM cells were used in another assay for cell migration, a scratch assay. Addition of myocilin induced cell migration and this induction was blocked by preincubation of myocilin with myocilin antibodies or sFRP1 (Fig. 1B).

CM from HEK293 cells transiently transfected with cDNAs encoding myocilin or its proteolytic fragments also stimulated NIH3T3 cell migration in a transwell assay by 3–7 fold, with CM from myocilin-ΔC expressing cells being the most active (Fig. 1C). Western blotting analysis showed that the CM contained similar molar amounts of myocilin and its proteolytic fragments (not shown). Analogous results were obtained in a scratch assay using NIH3T3 (Fig. 1D, E) or FHL124 (not shown) cells with CM from transiently transfected HEK293 cells. CM from cells expressing myocilin and its proteolytic fragments induced cell migration, with myocilin-ΔC producing the most dramatic effect (Fig. 1D, E). Previously we demonstrated that the N-terminal part of myocilin is essential for interaction with secreted frizzled-related proteins (Kwon et al., 2009a). Accordingly, preincubation of CM from cells expressing myocilin with sFRP1 reduced migration of NIH3T3 cells ($p = 0.005$), while no effects of sFRP1 on migration were observed when CM from cells expressing myocilin-ΔN was used ($p = 0.999$) (Fig. 1F).

Myocilin Induces Elevated Levels of Several Integrins *In Vitro* and *In Vivo*

Integrins, a family of cell-surface receptors that mediate cell attachment to the extracellular matrix, are critically involved in cell migration and in transmitting signals between extracellular matrix and the intracellular cytoskeleton (Huveneers and Danen, 2009; Streuli and Akhtar, 2009). Integrins are considered to be likely candidates for regulation of outflow facility in the eye (Faralli et al., 2009). Since primary TM cell cultures and lens epithelium-derived FHL124 cell reacted similarly to myocilin treatment and FHL124 cells can be transfected with high efficiency, most of the subsequent experiments were conducted with

FHL124 cells. To test whether the stimulatory effects of myocilin on cell migration involve signaling through integrins, FHL124 cells were treated with CM from HEK293 cells expressing myocilin or its proteolytic fragments and stained with antibodies that recognize activated β1-integrin in living cells (Galbraith et al., 2007). β1-integrin is the most ubiquitous integrin and is one of the major integrins in the human TM (Zhou et al., 1999). CM from cells expressing myocilin and its proteolytic fragments increased β1-integrin levels as judged by western blotting results (Fig. 2A, B). To test whether elevated levels of myocilin may affect β1-intergrin levels *in vivo*, we used transgenic mice that specifically overexpress wild type myocilin in the eye drainage structures (Kwon et al., 2009a; Zhou et al., 2008). Levels of β1-integrin were increased in the eye drainage structures of transgenic mice compared to wild-type mice with the most dramatic upregulation observed in the ciliary body (Fig. 2C).

Integrin receptors are assembled from different α- and β-subunits that combine to form 24 heterodimers having different compositions and ligand specificities (Humphries et al., 2006). β1 integrin may form complexes with at least 9 different α-integrins. It has been shown that α3- and αV-integrin subunits are expressed in the TM (Zhou et al., 1999) and that α3β1, α5β1, and αVβ1 integrin receptors are present in the TM (Diskin et al., 2009). The levels of α3-, α5 and αV-integrins in NIH3T3 cells were increased about 1.2 ± 0.4 ($p = 0.07$), 3 ± 0.3 ($p = 0.006$) and 1.5 ± 0.2 ($p = 0.005$) fold, respectively (Fig. 3A–C), while the level of αL-integrin was low and did not change ($p = 1.0$) after myocilin treatment (not shown). α5β1 integrin is the main receptor for fibronectin (Huveneers et al., 2008), one of the major extracellular matrix proteins in the human TM (Faralli et al., 2009; Hann et al., 2001). The levels of fibronectin was increased about 3 ± 0.3 fold ($p = 0.003$) (Fig. 3A–C). On the basis of these results we concluded that myocilin action may involve signaling through integrins.

Myocilin Stimulates Focal Adhesion Kinase (FAK) *In Vivo* and *In Vitro*

It is now well established that FAK is one of the key mediators of β1-integrin signal transduction. FAK may be activated by different stimuli and functions as a biosensor to control cell motility (Mitra et al., 2005; Sieg et al., 2000). To promote cell migration, FAK has to be activated via autophosphorylation at Tyr397 (Tamura et al., 1998), and this phosphorylation can be induced by a number of factors. We tested whether myocilin and its proteolytic fragments may also induce FAK phosphorylation. Treatment of FHL124 cells for 3 hrs with CM from HEK293 cells expressing different forms of myocilin indeed induced phosphorylation of FAK at Tyr397 as judged by western blotting with antibodies that recognize phosphorylated FAK (Fig. 4A, B).

Immunostaining of eye sections from wild-type and transgenic mice expressing elevated levels of wild-type myocilin with antibodies specific for the phosphorylated FAK protein revealed that levels of phosphorylated FAK were dramatically increased in the drainage structures of transgenic mice as compared with wild type mice (Fig. 4C).

Myocilin-Induced Cell Migration Is Mediated through FAK Signaling

To determine whether the effects of myocilin on cell migration are mediated through FAK signaling, we inhibited FAK using siRNAs. To validate efficiency of FAK silencing, HEK293 cells were transfected with increasing concentrations of FAK siRNAs, and the levels of FAK protein were determined by western blot. FAK expression was markedly inhibited in a dose-dependent manner (Fig. 5A), and the 100 μM concentration of FAK siRNAs that produced complete inhibition of FAK level was used in subsequent experiments. Treatment of FHL124 cells with this concentration of FAK siRNA or scrambled siRNA did not reduce migration of mock transfected control cells but reduced cell migration of myocilin-transfected cells by 3 ± 0.3 fold ($p = 0.003$) in wound healing assay (Fig. 5B).

It has been shown that wounding of cell monolayer *in vitro* may lead to a transient activation of FAK (Schlaepfer et al., 1999; Teranishi et al., 2009). To test whether myocilin treatment may potentiate FAK activation after wounding, FHL124 cell monolayers were scratched as described above, treated with CM from HEK293 cells expressing myocilin for 3 hrs, and stained with antibodies against phosphorylated FAK. Myocilin CM increased the levels of phosphorylated FAK, which was preferentially located at the wound margin (Fig. 5C). We conclude that myocilin stimulation of cell migration involves activation of FAK.

Myocilin Stimulation of Cell Migration Involves the PI3 Kinase Signaling Pathway

The downstream signaling pathways that mediate integrin-FAK signaling are diverse. One possible target is serine/threonine kinase Akt which is activated through phosphatidylinositide 3-kinases (PI3K). Treatment of FHL124 cells with myocilin (3 μg/ml) led to Akt activation as judged by its phosphorylation, although this activation was not as strong as after treatment of cells with PDGF (10 ng/ml), a known inducer of Akt (Fig. 6A). Inhibition of PI3K by two specific inhibitors, wortmannin (10 μM) and LY294002 (20 μM), completely blocked the Akt activation. The same concentration of LY294002 reduced migration of FHL124 cells and FHL124 cells treated with myocilin by 2.5 ± 0.5 ($p = 0.017$) and 4.7 ± 0.1 ($p = 1.19 \times 10^{-8}$) fold, respectively in scratch assay (Fig. 6B). IN summary, these results indicate that activation of Akt is critical for myocilin-induced cell migration.

Discussion

The functions of myocilin are still poorly understood despite significant efforts that have been made by many researchers since the seminal discovery of myocilin's role in glaucoma in 1997 (Adam et al., 1997; Stone et al., 1997). Our recent data suggested that myocilin may serve as a modulator of Wnt signaling by interacting with several components of the Wnt signaling pathway (Kwon et al., 2009a). Here we report that myocilin and its proteolytic fragments, similar to some Wnt proteins (Cohen et al., 2002; Kurayoshi et al., 2006), may increase cell migration by acting through the integrin-FAKPI3K-AKT signaling pathway.

Cell migration is a highly complex process that includes several coordinated steps and may be stimulated by many factors using different signaling pathways (Vicente-Manzanares et al., 2009). This study shows that myocilin-induced cell migration involves the activation of

the integrin-FAK-AKT signaling pathway. Integrins form one of the main classes of cell-matrix receptors that are essential for many aspects of cell biology including cell migration. There are 18 α-integrin and 8 β-integrin subunits in mammals that combine to produce at least 24 different heterodimeric receptors, each with their own ligand specificity (Streuli and Akhtar, 2009). Extracellular domains of integrins contain the binding sites for cell attachment to the extracellular matrix, while their cytoplasmic tails are critical for interactions with the actin cytoskeleton and signaling enzymes. Intracellular signals, acting through integrin cytoplasmic domains, induce conformational changes in integrin extracellular domains and increase their affinity for their extracellular ligands. One of the major extracellular ligands for integrins and one of the major extracellular matrix proteins in the TM is fibronectin (Faralli et al., 2009; Hann et al., 2001) which is synthesized by the human TM (Hogg et al., 1995). Eleven different integrin heterodimers can bind fibronectin (Leiss et al., 2008) and at least eight of them, α3β1, αVβ1, α5β1, αvβ3, αVβ5, αVβ6, α4β1, and α4β7, are present in the TM (Faralli et al., 2009; Zhou et al., 1999). Among integrins that we tested, the levels of β1-, α5, and αV-integrins as well as fibronectin were moderately elevated after treatment of cells with myocilin. Although it is not known whether myocilin binds integrins, available data suggest that myocilin binds specifically to the Heparin II domain of fibronectin (Filla et al., 2002) and such binding inhibits cell spreading (Peters et al., 2005). Myocilin alone also inhibited fibroblast spreading (Peters et al., 2005). The spreading of cells on a matrix is a major event that is necessary to engage cells in a process of movement and inhibition of cell spreading may inhibit cell migration. The discrepancy between these observations and our results may be explained by the differences in the source of myocilin. Peters and the coauthors (Peters et al., 2005) used myocilin expressed in insect sf9 cells. The major form of protein in their samples was the 66 kDa form. Low levels of 55 to 57 kDa forms and an 80 kDa form which appears to be unique for the sf9 cells, were also observed (Peters et al., 2005). In our case, myocilin was expressed in mammalian cells: 55 kDa and 57 kDa forms of myocilin were the major forms in our samples and the 66 kDa and 80 kDa forms were absent (see Fig. 1 in (Kwon et al., 2009a)). Our data and data of literature (Shepard et al., 2003) suggest that the 66 kDa form is not a glycosylated form of myocilin but an unrelated protein. In accordance with our results, another group also reported that the 55–57 kDa forms of myocilin promoted spreading of podocytes and mesangial cells (Goldwich et al., 2009).

Although it is well established that FAK may integrate growth factor and integrin signals to promote cell migration (Sieg et al., 2000), our data demonstrate for the first time that activation of FAK is necessary for myocilin-induced cell migration. Treatment of cultured cells with myocilin and its proteolytic fragments, or overexpression of myocilin in the eye angle tissues of transgenic mice led to FAK activation. Inhibition of FAK by siRNA significantly reduced myocilin-induced cell migration. We do not know at present whether FAK inhibition may inhibit myocilin-induced elevation of intergin levels. In a wound migration assay, activated FAK was preferentially localized at the wound margin and showed a pattern of staining consistent with its localization at focal adhesions. Downstream targets of activated FAK, including PI3K lipid kinase and Akt serine/threonine kinase were also activated after treatment of cells with myocilin and inhibition of these pathways reduced cell migration. It should be mentioned that in the presence of LY294002, an

inhibitor of PI3K, migration of myocilin-treated cells was 2.1 ± 0.4 ($p = 0.017$) higher than migration of control cells indicating that other downstream targets may be also involved.

Our previous results suggested that myocilin action in some respects resembles noncanonical Wnt signaling: myocilin interacts with several sFRPs and frizzled receptors, induces formation of stress fibers and activated Rac1 without nuclear accumulation of β-catenin (Kwon et al., 2009a). It has been shown that Wnt5a, similar to myocilin, has the ability to activate FAK, Rac1 and stimulate cell migration (Kurayoshi et al., 2006). Similarly, sFRP2 inhibited Wnt5a-induced migration of gastric cancer MKN-1 line (Kurayoshi et al., 2006), while we used sFRP1 to inhibit myocilin-induced migration of human trabecular meshwork cells. Recent data demonstrate the functional and physical interaction of Disheveled (Dvl) and adenomatous polyposis coli (APC) gene products is involved in Wnt5a/Fzd2-dependent focal adhesion dynamics during cell migration (Matsumoto et al., 2010). Dvl is a critical component of the Wnt signaling pathways. Our preliminary results indicate that treatment of cells with myocilin may induce Dvl (H.S.K and S.I.T, unpublished). We are currently investigating a possible role of Dvl in myocilin-induced cell migration. It is reasonable to suggest that, similar to Wnt5a, myocilin binds to frizzled receptors and integrins and cooperatively activate a signaling cascade that stimulates cell migration. A schematic diagram describing our proposed mechanism for myocilin signaling in cell migration is shown in Fig. 7.

The highest levels of myocilin expression in the body are observed in the TM and sclera (Adam et al., 1997; Swiderski et al., 2000; Tamm, 2002; Torrado et al., 2002). Although it is well demonstrated that both TM and scleral cells have the capacity to migrate, presumably, cells do not exhibit significant cell migration within the outflow pathway under normal conditions *in situ*. Specialized exceptions may be in response to extreme phagocytic challenge or putative stem cell repopulation after focal laser damage (Aga et al., 2008). Myocilin is also detected in several other ocular and non ocular tissues and aqueous humor (Jacobson et al., 2001; Russell et al., 2001) where it may affect the mobility of corneal endothelial cells and lens epithelial cells. The absence of clear phenotype in mice lacking myocilin as well in humans that do not have functional myocilin may be explained by compensatory action of other olfactomedin domain-containing and Wnt proteins that are expressed in the corresponding tissues and have a similar role in cell migration. Our data extends the similarities between actions of myocilin and Wnt proteins acting through β-catenin-independent mechanism.

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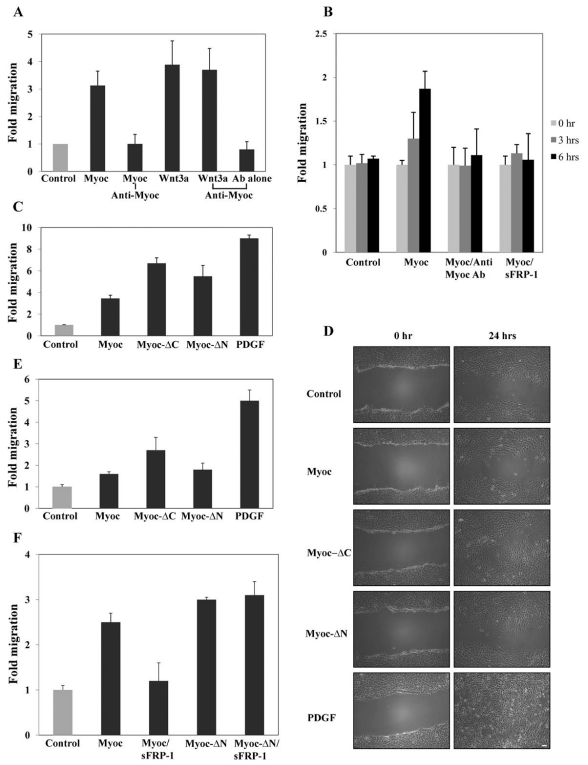

Figure 1.

Effects of myocilin on cell migration. (A) Transwell assay. NIH3T3 cells were plated in the upper chamber while myocilin (3 μg/ml) or Wnt3a (3 μg/ml) diluted in PBS were added to the lower chamber. PBS was used as control. The cells migrating to the bottom surface of the filter after 6 hrs were trypsinized and counted using a hematocytometer. In some cases, myocilin and Wnt3a were preincubated with antibodies against mouse myocilin (1:1000 dilution) for 30 min before addition to the lower chamber. Changes in the amounts of migrating cells after addition of indicated proteins of antibodies were calculated relative to PBS control. (B) Scratch assay. Subconfluent monolayers of human TM cells were plated on glass chamber slides. The monolayers were scratched with a pipette tip and incubated for 3 or 6 hrs with purified myocilin (3 μg/ml). Serum-free medium was used as control. In some cases, myocilin was preincubated with antibodies against mouse myocilin or purified sFRP1. Cell migration was evaluated using Axiovision Rel. 4.7 software. (C) Transwell assay as in A but CM from HEK293 cells expressing myocilin or its proteolytic fragments was added to the lower chamber instead of purified myocilin. Conditioned medium from HEK293 cells transfected with vector was used as control. PDGF (10 ng/ml) was used as a positive control for cell migration. (D) Scratch assay. Scale bar, 20 μm. Subconfluent monolayers of NIH3T3 cells were plated on non-coated glass chamber slides. The monolayers were scratched with a pipette tip and incubated for 24 hrs with CM from HEK293 transiently transfected with myocilin, its proteolytic fragments, or vector (control). PDGF (10 ng/ml) was used as positive control. Cell migration was evaluated as in (B). (E) Quantification of the results shown in (D). Comparison between all samples gave statistically significant differences ($p < 0.002$). (F) NIH3T3 cells were grown as in (D). CM from HEK292 cells transiently transfected with vector (control), myocilin or myocilin-ΔN was preincubated with 10 μg/ml of sFRP1 for 30 min before addition to cells. Cell migration was evaluated as above. Quantification of the results is shown.

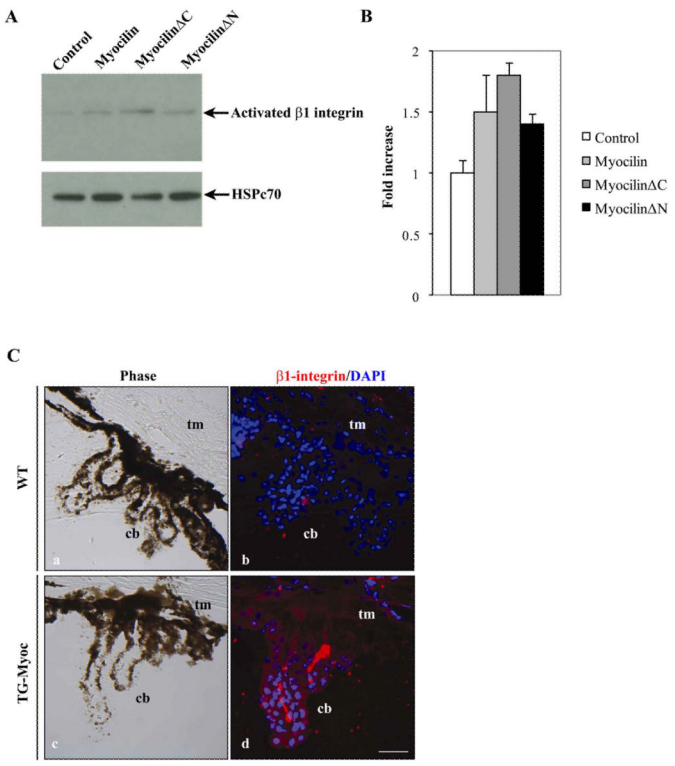

Figure 2.

Effects of myocilin on β1-integrin levels *in vitro* and *in vivo*. (A) FHL124 cells were treated with CM from cells expressing myocilin or its proteolytic fragments for 6 hrs. Conditioned medium from HEK293 cells transfected with vector was used as control. The levels of β1-integrin in cell lysates were estimated by western blotting. (B) Quantification of the results shown in (A). (C) Frozen eye sections from control (a, b) and transgenic mice expressing elevated levels of wild-type myocilin in the eye drainage structures (c, d) were stained with antibodies against β1-integrin. (a and c) represent phase contrast, b and d) show immunofluorescence. Scale bar, 20 μm; cb - ciliary body; tm – trabecular meshwork.

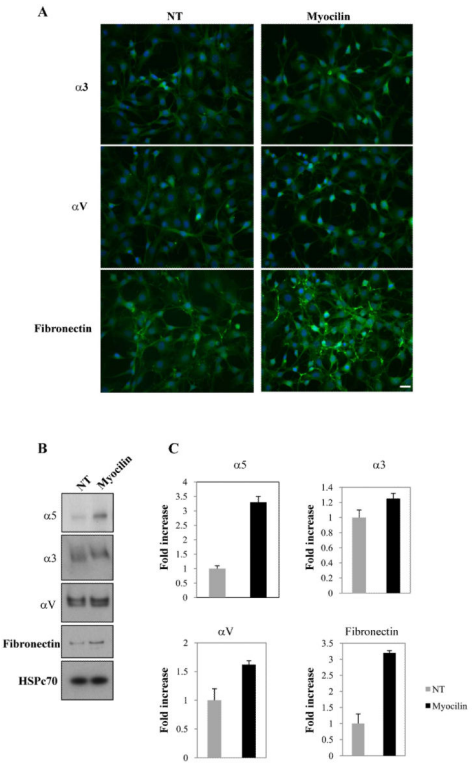

Figure 3.

Effects of myocilin on α-integrin and fibronectin levels *in vitro*. NIH3T3 cells were treated with CM from HEK293 cells expressing myocilin for 3 hrs. The levels of fibronectin and integrins were estimated after immunostaining of cells (A) or in cell lysates by western blotting (B). (C) Quantification of the results shown in (B). Scale bar in A, 10 μm.

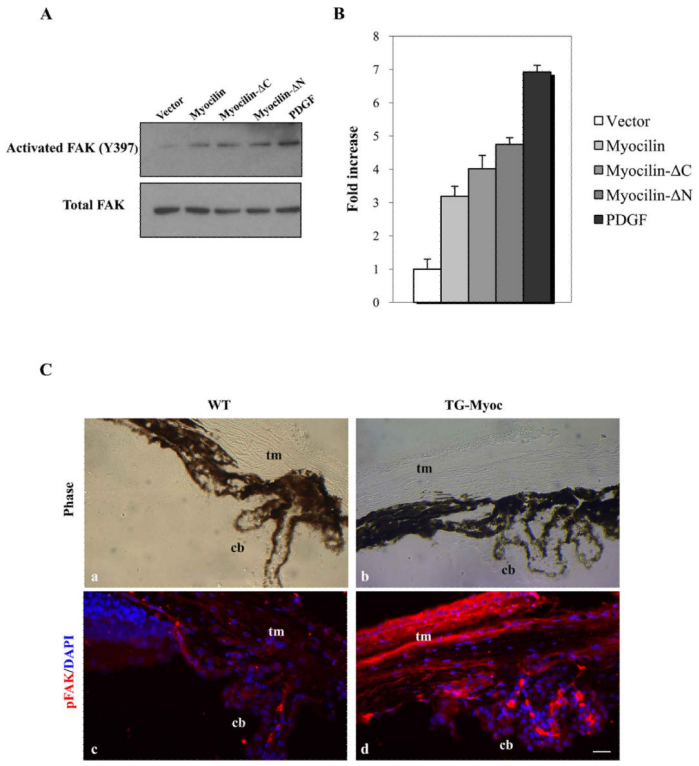

Figure 4.

Myocilin stimulates FAK both *in vitro* and *in vivo*. FHL124 cells were treated for 3 hrs with CM from HEK293 cells expressing myocilin or its proteolytic fragments. PDGF (10 ng/ml) was used as a positive control. Shown are western blots of cell lysates stained with antibodies against activated FAK (A). (B) Quantification of the results shown in (A). Comparison between different samples with one exception gave statistically significant differences ($p < 0.002$). Difference between the mean values obtained for Myocilin-ΔC and Myocilin-ΔN was statistically insignificant ($p = 0.37$). Upregulation of activated FAK in the eyes of 20-month-old wild-type and transgenic mice (C). Frozen sections of wild type (a, c) and transgenic (b, d) eyes were stained with antibodies to phosphor FAK (1:200 dilution) and DAPI. 3 pairs of animals were analyzed. A typical staining pattern is shown. cb, ciliary body; tm, trabecular meshwork. Scale bar, 20 μm.

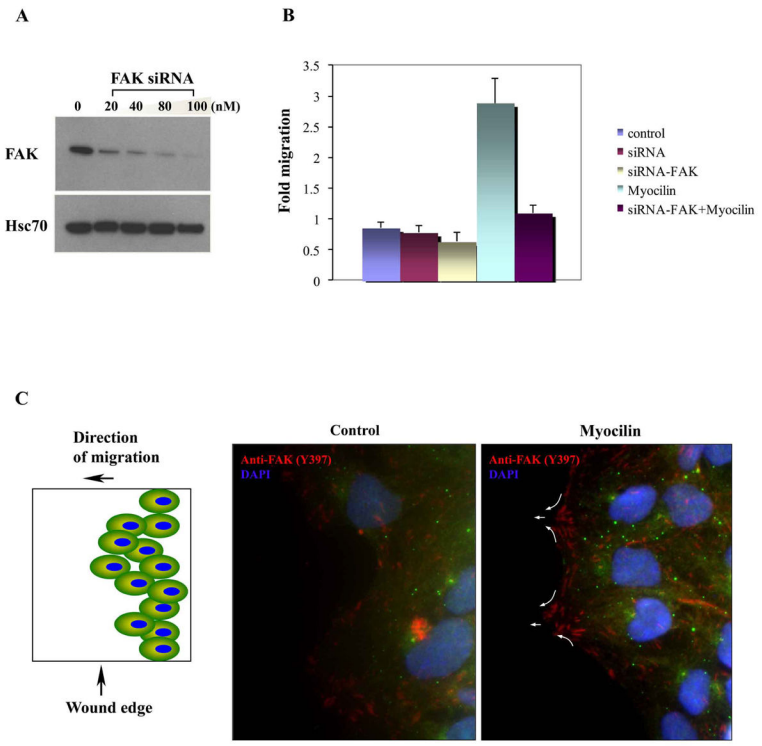

Figure 5.

FAK is essential for myocilin-induced cell migration. (A) Inhibition of FAK in HEK293 cells transfected by increasing concentrations (0–100 nM) of FAK siRNAs. Shown are western blots of cell lysates stained with anti FAK antibodies 48 hrs after transfection. (B) Inhibition of myocilin-induced FHL124 cell migration by FAK siRNAs as judged by wound healing assay. Cells were mock transfected (control), transfected with scrambled siRNA or transfected with 100 nM FAK siRNAs and myocilin. Changes in cell migration between control, scrambled siRNA, siRNA-FAK and siRNA-FAK + myocilin were statistically insignificant (p between 0.06 and 0.33). (C) Accumulation of activated FAK at the leading edge of FHL124 migrating cells in wound healing assay. FHL124 cell monolayers were scratched and treated with CM from HEK293 cells expressing myocilin or CM from mocked transfected HEK293 cells (control) for 3 hrs, and stained with antibodies against phosphorylated FAK.

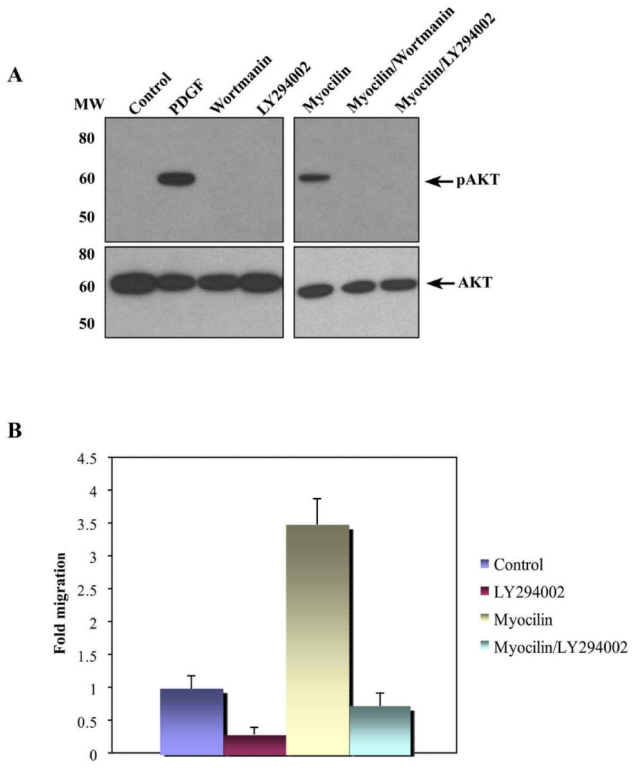

Figure 6.

Myocilin induces Akt activity. (A) Treatment of FHL124 cells with myocilin (3 μg/ml) or PDGF (10 ng/ml) for 1 h led to activation of Akt. PBS was added to control samples. Inhibitors of PI3K, wortmannin (10 μM) and Ly294002 (20 μM), completely blocked the Akt activation. (B) Inhibition of myocilin-induced migration of FHL124 cells in would healing assay by LY294002 (20 μM). The monolayer of FHL124 cells was scratched with a pipette tip and incubated for 24 hrs with purified myocilin (3μg/ml) and LY294002 (20 μM). Control was incubated with PBS. Comparison between different samples with one exception gave statistically significant differences ($p < 0.017$). Difference between the mean values obtained for control and myocilin/LY294002 was statistically insignificant ($p = 0.42$).

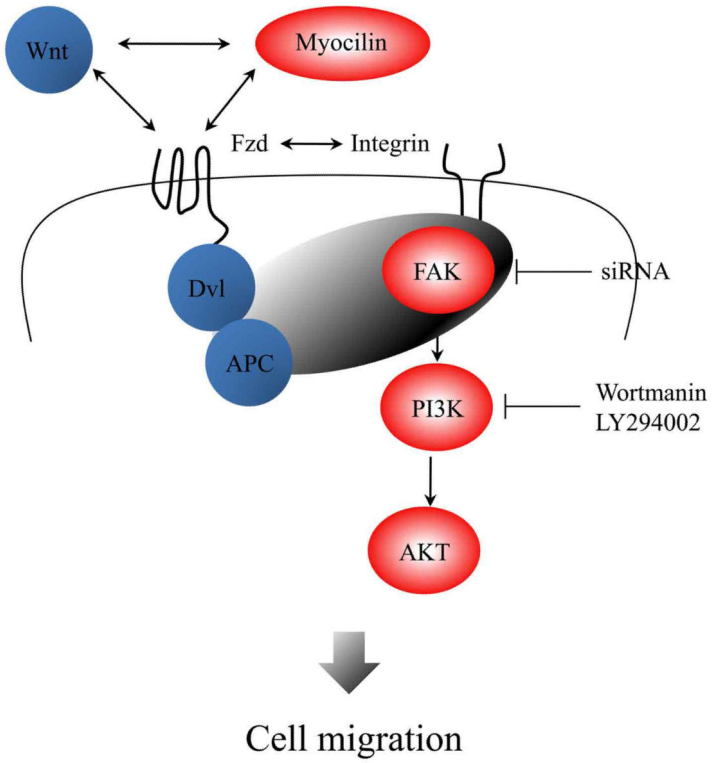

Figure 7.

Schematic diagram of myocilin signaling in cell migration. Binding of myocilin or Wnt to frizzled receptors may enhance the association of Dvl with APC leading to the activation of FAK and its accumulation at the leading edge. Downstream targets of activated FAK include PI3K lipid kinase and Akt serine/threonine kinase. Inhibition of these kinases reduced cell migration. Myocilin proteolytic fragments also stimulate cell migration through the same mechanism.