Myocilin from COS-1 cells binds specifically to NIH3T3 cells

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Abstract

Myocilin gene is directly linked to the most common form of glaucoma. The gene product is secreted into aqueous humor and is thought to be involved in the regulation of intraocular pressure. Most of the mutant myocilin proteins accumulate in cultured cells and are not secreted. In this study, through staining experiments of various cell lines, we demonstrated that secreted myocilin could bind to a particular cell surface to exert its function. Myocilin secreted by COS-1 cells was able to bind specifically to the surface of NIH3T3 cells. The binding of myocilin was dependent on the cell density. These results suggest that myocilin recognizes a specific cell and exerts its effect upon binding to the cell surface.

Introduction

Glaucoma is a progressive blinding disease that is characterized by the gradual loss of vision due to optic neuropathy and retinal ganglion cell death, and is the second largest cause of bilateral blindness in the world. It is speculated that there are an estimated 70 million glaucoma patients worldwide and thus, it is one of the most prevalent vision disorders that require immediate attention (Quigley, 1996). The relationship of the mechanism of onset with various factors, such as a mutation in the gene and the environment, has been extensively examined. However, little is known about the underlying pathological mechanism.

Myocilin was originally identified as trabecular meshwork inducible glucocorticoid response gene (*TIGR*) (Polansky et al., 1997). Mutations in the *myocilin* gene were found in 3-4% of patients with primary open angle glaucoma (POAG), the most common form of glaucoma (Stone et al., 1997), and in a subset of families with autosomal dominant juvenile-onset open angle glaucoma (OAG) and adult-onset OAG (Fingert et al., 1999, 2002). It has been shown that the *myocilin* knockout mouse and the transgenic mouse expressing a high level of wild-type myocilin in the angle tissue do not exhibit the glaucoma phenotype (Kim et al., 2001; Gould et al., 2004). Therefore, it is surmised that the change in myocilin expression level is not responsible for the onset of glaucoma. Senatorov et al. (2006) reported that the OAG-like phenotype was induced in mouse expressing a specific mutant myocilin. The above findings support the idea that specific mutant myocilins are responsible for the onset of various glaucoma.

Myocilin is ubiquitously distributed in various locations, including non-ocular tissues and organs, and strongly expressed in various components of the eye, namely, aqueous humor, sclera, iris, cornea, lens, ciliary body, optic nerve, retina, and trabecular meshwork (Kubota et al., 1997; Polansky et al., 1997; Nguyen et al., 1998; Karali et al., 2000; Noda et al., 2000; Rao et al., 2000; Clark et al., 2001; Fautsch and Johnson, 2001; Ricard et al., 2001; Russell et al., 2001; Tamm, 2002). Myocilin is a glycoprotein composed of 504 amino acids and has a molecular

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weight of 55-57 kDa. Myocilin has its own signal peptide sequence followed by a leucine zipper at the amino terminal region and an olfactomedin domain at the carboxyl terminal region (Kubota et al., 1997; Polansky et al., 1997; Nguyen et al., 1998; Tamm, 2002). Approximately 70 *myocilin* mutations have been identified in human glaucoma patients, and a majority of these glaucoma-associated mutations are localized in the olfactomedin domain (Fingert et al., 2002; Tamm, 2002; Gong et al., 2004). It is thought that most mutant myocilin proteins are misfolded, insoluble in detergent, and retained in the endoplasmic reticulum. Thus, the protein is poorly soluble in aqueous humor or culture medium (Jacobson et al., 2001; Sohn et al., 2002; Joe et al., 2003; Gobeil et al., 2004; Liu and Vollrath, 2004; Vollrath and Liu, 2006). Based on these results, some researchers predict that secreted myocilin may regulate intraocular pressure (IOP) and mutant myocilin proteins may impair eye function, leading to the onset of diseases. In fact, some neuronal conformational diseases are thought to be dominant neurodegenerative disorders in which the toxic effects of misfolded proteins lead directly to neuronal death, causing Parkinson's disease and neuroserpin-associated dementia (Davis et al., 2002; Lee and Trojanowski, 2006).

However, details of the molecular mechanisms underlying the pathogenesis of glaucoma caused by such mutations remain unclear. In this study, to gain an insight into the physiological functions of myocilin, we tried to determine the specific target of secreted-myocilin binding cell(s) using various cultured cell lines.

Materials and Methods

Cell lines and cell culture

COS-1, A549, and NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% fetal bovine serum. RGC-5 cells were grown in similar conditions except that DMEM with low glucose was used. HEK293 cells were maintained in minimal essential medium (MEM) supplemented with 2 mM L-glutamine, non-essential amino acids, and 10% fetal bovine serum. All cells were cultured under 5% CO_2 at 37˚C.

Plasmid construction

Wild-type full-length human *myocilin* cDNA was cloned into pcDNA3.1(-) (Invitrogen) as described previously (Izumi et al., 2003). This vector was digested with *Hind*III and *Xho*I, and the *myocilin* cDNA fragment was inserted into the *Hind*III-*Xho*I site of the mammalian expression vector pcDNA4/V5-HisA (Invitrogen) that produces C-terminus histidine-tagged fusion protein.

To construct the mutant myocilin (N57Q) expression vector, we inserted a point mutation using a QuickChange XL Site-Directed Mutagenesis Kit (Stratagene) with two oligonucleotides (5'-GTGTGGCCAGTCCCCAA GAATCCAGCTGCCC-3' and 5'-GGGCAGCTG GATTCTTGGGGACTGGCCACAC-3') according to the manufacturer's protocol.

Transfection and addition of myocilin

To perform indirect immunofluorescence experiments, COS-1 cells were grown to 80-90% confluence in a 24-well plate (Becton Dickinson) and cultured for 12-16 hours prior to transfection. For the transfection experiment, cells were washed with PBS(+) (phosphate-buffered saline containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and replaced with corresponding culture medium. Cells were transfected with pcDNA4/V5-HisA (negative control) or myocilin expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with a ratio of 2 μL of transfection reagent per 0.8 μg of plasmid. After transfection for six hours, cells were washed with PBS(+) three times to remove

transfection reagents and plasmids, and were replaced with 0.4 mL of fresh medium. After culture of forty-eight hours, the medium was collected and debris was removed by centrifugation. The supernatant was added to the pre-cultured cells as described below. In the experiments of Figure 1B, non-diluted myocilin-containing medium (1); the medium diluted 1:3 or 1:9 with control plasmid expression medium; and control medium (0) were added to NIH3T3 cells.

To the culture at 50-60% confluence, COS-1, A549, HEK293, and NIH3T3 cells were seeded at a density of 7.5 x 10^3 cells/cm^2, and RGC-5 cells were seeded at a density of 1.5 x 10^4 cells/cm^2 in an 8-well chamber (Nalge-Nunc). In the experiments presented in Figure 3, to test the relationship between cell density and binding capability of myocilin, NIH3T3 cells were seeded at a density of 1.5 x 10^3 cells/cm^2 (10-20% confluence) or 7.5 x 10^3 cells/cm^2 (50-60% confluence) in an 8-well chamber. The cells were cultured for 12-16 hours and myocilin-containing medium was added.

Anti-human myocilin antibody

Synthesized peptide (TRDTARAVPPGSREVST; corresponding to amino acid positions 188 to 204) was injected into rabbit, and anti-human myocilin antibody was affinity purified using a peptide-conjugated column. This antibody was diluted to 1:10000 for Western blot analysis and to 1:750 for indirect immunofluorescence experiment.

Western blot analysis

The medium was combined with Laemmli sample buffer (Laemmli 1970) and incubated at 100˚C for 5 min. Proteins were separated by SDS-PAGE under reducing conditions using a Mini-PROTEAN 3 Electrophoresis System (Bio-Rad), and transferred electrophoretically to PVDF membranes. Membranes were blocked with milk diluent/blocking solution (KPL) and incubated with anti-human myocilin antibody. Then, the membranes were washed with TBS-T (Tris-buffered saline containing 0.05% Tween20), incubated with horseradish-peroxidase-conjugated anti-rabbit IgG, and washed with TBS-T. Protein-antibody complexes were reacted with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and detected with Lumi-Imager F1 (Roche).

Indirect immunofluorescence experiment

Cells were rinsed with PBS(+) three times and fixed with 4% paraformaldehyde solution at room temperature for 20 min. Cells were washed with PBS three times and permeabilized by treatment with 0.1% Triton X-100 solution for 10 min. Cells were washed with PBS three times and blocked with 5% goat serum in PBS at room temperature for 1 hour. Cells were washed with PBS three times and incubated with anti-human myocilin antibody diluted with 1% goat serum in PBS at 4˚C overnight. Cells were washed with PBS three times and incubated with Alexa488-conjugated anti-rabbit IgG (Invitrogen) and DAPI (4’,6-diamidino-2-phenylindole, dihydrochloride) (Sigma-Aldrich) solution at room temperature for 1 hour. Cells were washed with PBS three times and observed under a confocal microscope (Radiance 2100, Bio-Rad). To confirm that the interaction between myocilin and NIH3T3 cells occurs only on the cell surface, the results of which are presented in Figure 5, we carried out the above staining experiment without Triton X-100 treatment.

Results

Myocilin specifically binds to NIH3T3 cells

The extent of degradation of secreted myocilin depends on the cell species (Aroca-Aguilar et al., 2005). As most of the secreted myocilin was not degraded when COS-1 cells were used for expression, we selected this cell line for the

following experiments.

In order to verify the possibility that myocilin exerts its function upon binding to the cell surface, we examined whether myocilin is able to bind to various cells. Histidine-tagged recombinant myocilin was expressed in COS-1 cells and the culture medium containing secreted myocilin was collected. This medium was added to 50-60% confluent RGC-5, COS-1, A549, and HEK293 cells, and the cells were cultured for 96 hours. Cells were fixed and indirect immunofluorescence experiments were carried out with anti-human myocilin antibody. The results indicated that myocilin could not bind to these cells (Figure 1A). In contrast, myocilin bound specifically to NIH3T3 cells when non-diluted (1) or diluted myocilin-containing medium (1:3 or 1:9) was added (Figure 1B). Myocilin appeared as a dot-like pattern and did not localize at a definite site. We proposed two reasons why myocilin bound not to the other cells but to NIH3T3. One was that the amount of myocilin added was different in each medium because myocilin expression depends on the composition of the medium (Fautsch et al., 2005). The other was that myocilin was degraded during incubation.

To exclude the above possibilities, Western blot experiments were conducted to estimate myocilin expression. Myocilin expression was not significantly different among the media (Figure 2A). Signals derived from NIH3T3 cells were detected when myocilin-containing medium was diluted 1:3 or 1:9 with control plasmid expression medium. Under these experimental conditions, it was clear that myocilin expression in the diluted medium was apparently lower than that in the other media (Figure 2A). Also, myocilin was not completely degraded even after incubation for 96 hours (Figure 2B). Therefore, we concluded that undegraded wild-type myocilin could specifically bind to the surface of NIH3T3 cells.

Binding of myocilin to NIH3T3 cells depends on cell density

To analyze the binding pattern of myocilin to NIH3T3 cells in detail, we examined the relationship between the binding ability of myocilin and the density of NIH3T3 cells. After incubation for 48 hours, we could not detect the interaction of myocilin with NIH3T3 cells when the cell density was $1.5 \times 10^3/cm^2$ (Figure 3A). On the other hand, the interaction was observed when the cell density was $7.5 \times 10^3/cm^2$ (Figure 3B). Some detectable large dot-like signals could not be explained at present. After incubation for 96 hours, myocilin was able to bind to NIH3T3 cells regardless of the cell density.

Glycosylation of myocilin does not affect its binding ability to NIH3T3 cells

It is known that secreted myocilin is glycosylated and post-translational modification is involved in such molecular functions as cell-cell or receptor-ligand binding (Ohtsubo and Marth, 2004). Therefore, to investigate whether or not glycosylation is required for myocilin to exert its binding ability, we substituted the glycosylated asparagine residue at position 57 with glutamine (N57Q) and examined the binding ability of this mutant myocilin to NIH3T3 cells. The result indicated that the mutant myocilin was secreted into the medium and its binding ability was comparable to that of non-mutant myocilin (Figures 1B and 4).

Myocilin binds to cell surface

To confirm that the added myocilin is able to bind to the NIH3T3 cell surface, we performed indirect immunofluorescence experiments without Triton X-100 treatment (without permeabilization treatment). The result indicated that myocilin bound to NIH3T3 cells and the binding pattern was similar to that obtained with Triton X-100 treatment (Figures 1B and 5).

Discussion

A number of groups have examined the localization of myocilin and demonstrated that myocilin is distributed in both intra- and extracellular sites. For example, myocilin is localized in the mitochondria, Golgi complex, and endoplasmic reticulum of cells (Wentz-Hunter et al., 2003; Sakai et al., 2007). At the extracellular sites, myocilin is present at the site of type IV collagen, fibronectin, and laminin (Tawara et al., 2000; Lindsey et al., 2001; Filla et al., 2002; Ueda et al., 2002). In this study, we demonstrated by means of a staining experiment that recombinant myocilin secreted into the culture medium specifically bound to the surface of NIH3T3 cells, and added myocilin was not degraded during incubation under our experimental conditions (Figures 1 and 2). Therefore, we concluded that undegraded intact myocilin is able to bind to NIH3T3 cells. To our knowledge, this is the first finding that the binding ability of myocilin depends on the cell species.

Myocilin binding to NIH3T3 cells was dependent on cell density. Myocilin bound to NIH3T3 cells when NIH3T3 cell density was high. In contrast, myocilin did not bind to low-density pre-seeded cells (Figure 3). These results lead us to speculate that myocilin could directly bind to molecules that are involved in cell-cell adhesion (such as tight junction and/or adherens junction). However, the binding sites are not limited to the cell-cell adhesion sites. Therefore, we need further analysis to determine myocilin binding sites.

The physiological meaning of the interaction between myocilin and NIH3T3 cells remains unclear. From our examinations, we noted that there was neither drastic morphological change nor cell death following the addition of myocilin. However, from the fact that myocilin is ubiquitously expressed, it is plausible that myocilin acts on a cell that has characteristics similar to NIH3T3 cells, and regulates the flow rate of aqueous humor within the eye. Indeed, it has been reported that extracellular myocilin impairs focal adhesion formation, decreases cell motility, and increases outflow resistance (Caballero et al., 2000; Fautsch et al., 2000, 2006; Wentz-Hunter et al., 2004; Peters et al., 2005). Also, several data suggest that certain olfactomedin-domain-containing proteins could interact with proteins localized at the cell surface. For example, gliomedin, a glial ligand for neurofascin and NrCAM, interacts with these proteins at the nodes of Ranvier (Eshed et al., 2005, 2006). hCG1 may interact with cell surface lectins and cadherins (Liu et al., 2006). Amassin mediates the massive Ca^{2+}-dependent intracellular adhesion of sea urchin coelomocytes by binding to a cell surface protein (Hillier and Vacquier, 2003). We are currently trying to identify myocilin-associated proteins by mass spectrometry to further understand the function of myocilin.

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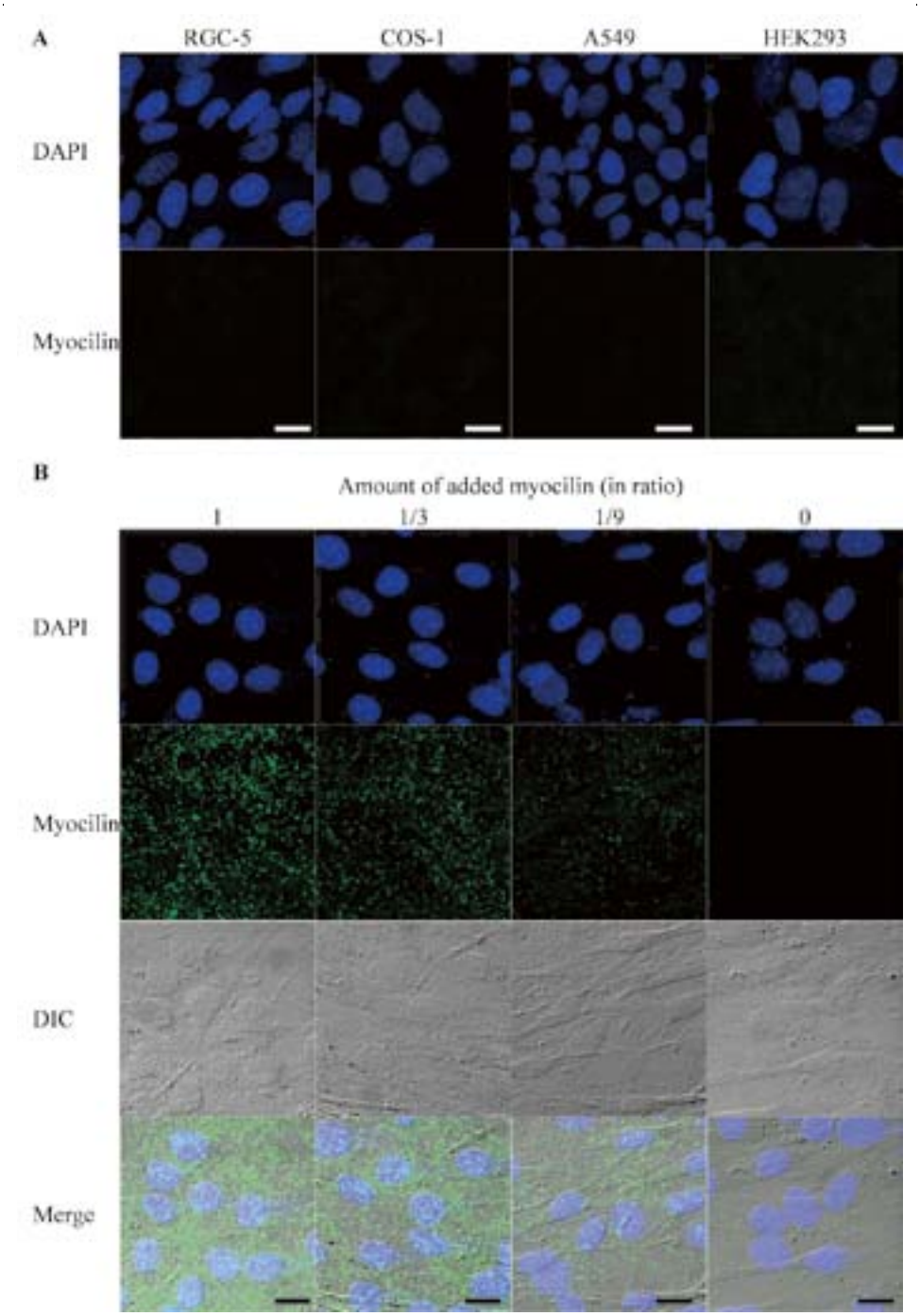

Figure 1. Myocilin binds specifically to NIH3T3 cells
(A) Myocilin-expression plasmid was transfected into COS-1 cells and medium containing secreted myocilin was added to pre-seeded RGC-5, COS-1, A549, and HEK293 cells. (B) Myocilin-containing medium was diluted 1:3 or 1:9 with control plasmid pcDNA4/V5-HisA expression medium. We added non-diluted myocilin-containing medium (1), diluted medium (1:3 and 1:9), and pcDNA4/V5-HisA expression medium only (0) into pre-seeded NIH3T3 cells. Scale bar, 20 μm.

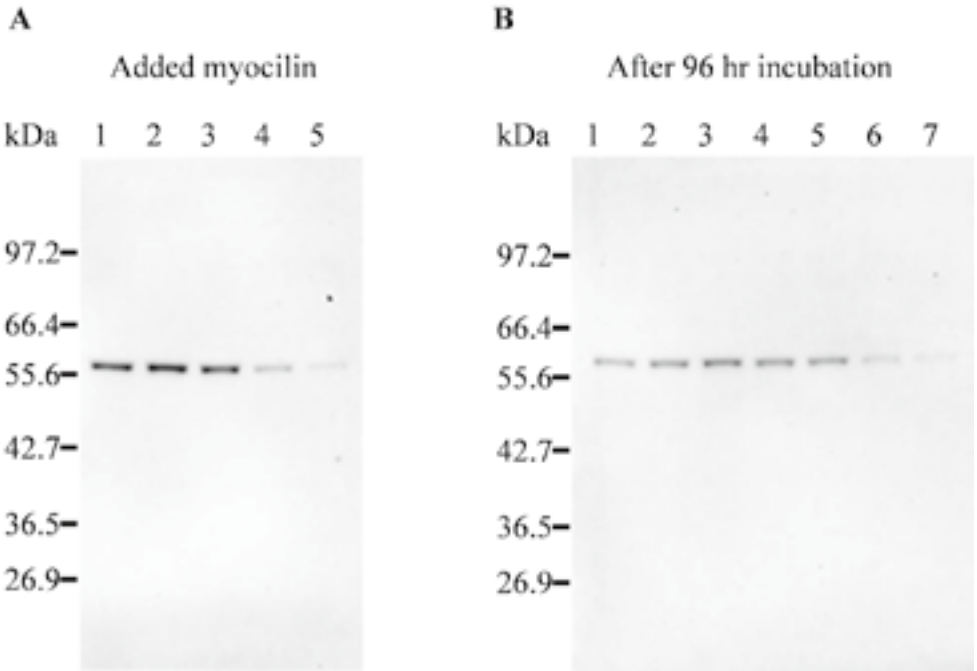

Figure 2. The amount of myocilin added is not responsible for the specific interaction
(A) Myocilin was expressed in different culture media. To estimate the amount of myocilin added, non-diluted or diluted myocilin-containing medium was mixed with Laemmli buffer, and Western blot experiment with anti-human myocilin antibody was carried out. Non-diluted myocilin-containing culture medium for HEK293 (lane 1), RGC-5 (lane 2), and NIH3T3, COS-1, and A549 (lane 3). Culture medium for NIH3T3, COS-1, and A549 was diluted 1:3 (lane 4) and 1:9 (lane 5). (B) To confirm that myocilin degradation did not occur during incubation, we examined myocilin expression by Western blot experiment using culture medium incubated for 96 hours. Non-diluted culture media were as follows: COS-1 (lane 1), HEK293 (lane 2), RGC-5 (lane 3), A549 (lane 4), and NIH3T3 (lane 5). Culture medium for NIH3T3 was diluted 1:3 (lane 6) and 1:9 (lane 7).

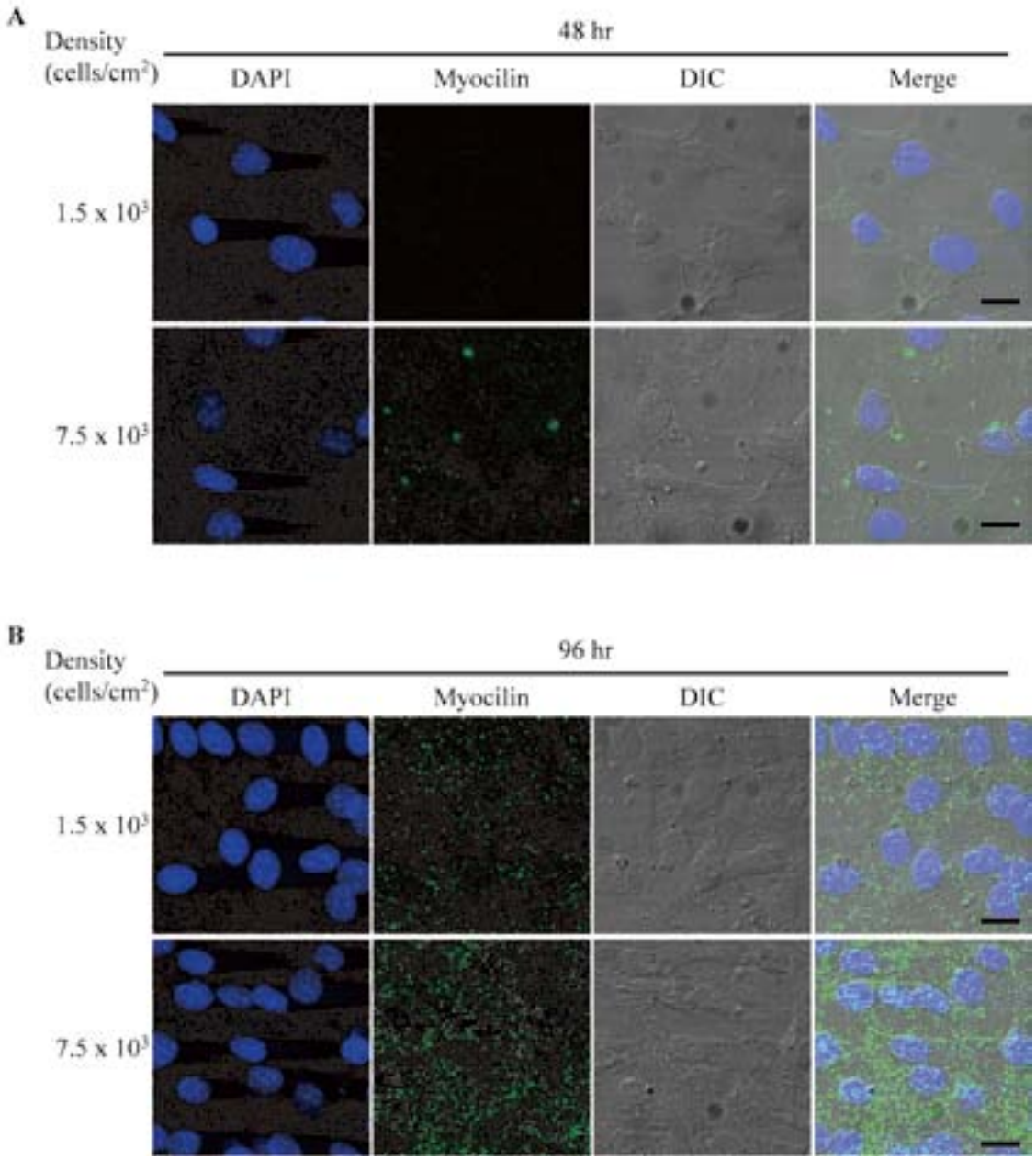

Figure 3. Binding of myocilin to NIH3T3 cells is dependent on cell density
NIH3T3 cells were pre-seeded at a density of 1.5 x 10^3/cm^2 or 7.5 x 10^3/cm^2. Myocilin-containing medium was added and indirect immunofluorescence experiment was carried out after incubation for 48 hours (A) or 96 hours (B). Scale bar, 20 μm.

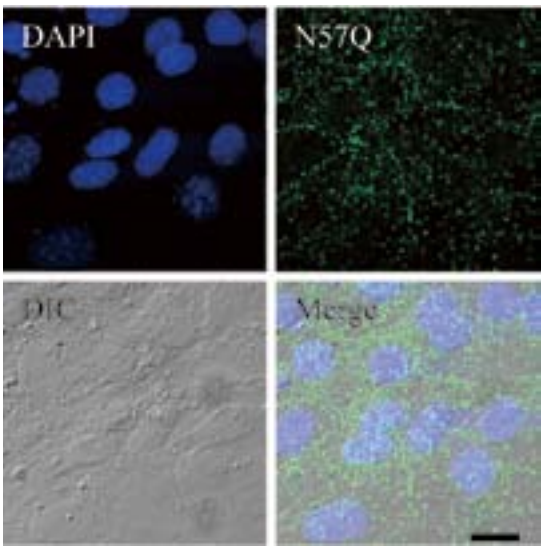

Figure 4. Glycosylation of myocilin does not affect binding ability
Glycosylation site of asparagine residue at position 57 was substituted with glutamine residue, and this mutant myocilin-expression plasmid was transfected into COS-1 cells. This mutant myocilin-containing medium was added to pre-seeded NIH3T3 cells and indirect immunofluorescence experiment was carried out after incubation for 96 hours. Scale bar, 20 μm.

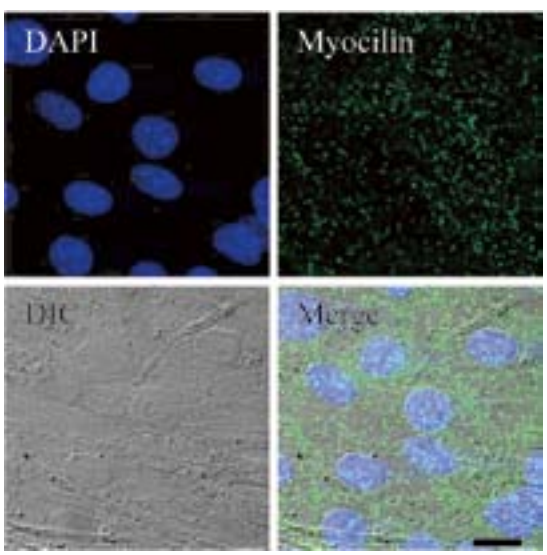

Figure 5. Myocilin binds to the surface of NIH3T3 cells
Myocilin-containing medium was added to pre-seeded NIH3T3 cells and indirect immunofluorescence experiment was carried out without Triton X-100 treatment after incubation for 96 hours. Scale bar, 20 μm.

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