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Original Article

ZO-1 associates with α3 integrin and connexin43 in trabecular meshwork and Schlemm's canal cells

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Abstract: Cellular structures that perform essential homeostatic functions include tight junctions, gap junctions, desmosomes and adherens junctions. The aqueous humor, produced by the ciliary body, passes into the anterior chamber of the eye and is filtered by the trabecular meshwork (TM), a tiny tissue found in the angle of the eye. This tissue, along with Schlemm's canal (SC) inner wall cells, is thought to control intraocular pressure (IOP) homeostasis for normal, optimal vision. The actin cytoskeleton of the tissue plays a regulatory role in maintaining IOP. One of the key risk factors for primary open angle glaucoma is persistent elevation of IOP, which compromises the optic nerve. The ZO-1 (Zonula Occludens-1), extracellular matrix protein integrins, and gap junction protein connexin43 (Cx43) are widely expressed in many different cell populations. Here, we investigated the localization and interactions of ZO-1, α3 integrin, β1 integrin, and Cx43 in cultured porcine TM and SC cells using RT-PCR, western immunoblotting and immunofluorescence labeling with confocal microscopy, along with co-immunoprecipitation. ZO-1 partially co-localized with α3 integrin, but not with β1 integrin, and co-immunoprecipitated with Cx43, as well as with α3 integrin. The association of ZO-1 with α3 integrin and Cx43 suggests that these proteins may form a multiple protein complex in porcine TM and SC cells. Since integrins interact with the actin cytoskeleton via scaffolding proteins, these results implicate junctional and scaffolding protein ZO-1 as a potential control point in regulation of IOP to normal levels for glaucoma therapy.

Keywords: Trabecular meshwork, extracellular matrix, integrins, connexin 43, ZO-1

Introduction

Primary open-angle glaucoma (POAG) is the major form of glaucoma worldwide [1], and is one of the leading causes of blindness, especially in the elderly, as well as those of African and Hispanic ancestry at younger ages [2, 3]. Approximately 70 million people are thought to be afflicted worldwide [4]. Intraocular pressure (IOP) elevation, common in POAG, results from increased resistance to aqueous humor (AH) outflow, and is one of the key risk factors for optic nerve damage [5]. Although progress has been made in understanding the pathogenesis of this disease, the regulation of IOP is complex and is not fully understood. Trabecular meshwork (TM) cells, along with the inner wall cells of Schlemm's canal (SC), are aqueous humor (AH) drainage structures, and play a pivotal role in regulating aqueous flow resistance and intraocular pressure (IOP) [6]. TM cells sense elevated IOP as mechanical stretch [6, 7]. TM cells are attached to multiple layers of beams; these beams contain extracellular matrix (ECM), composed of structural and functional connective tissue components that interact with TM cells via integrin proteins [8]. Integrins comprised of α and β subunits serve as the major receptors that connect the cytoskeleton to the ECM. To date, more than a dozen integrins have been detected in human TM cells, and these integrin receptors may play a functional role in TM-ECM interactions [9, 10].

ZO-1, also called tight junction protein 1 (TJP1), is widely expressed and associated with various cellular junctions, including tight junctions, gap junctions and adherens junctions in many cell types and tissues [11-17]. Former studies showed that ZO-1 is intimately associated with the actin cytoskeleton network [18], and integrins may be involved in regulating gap junction

expression and communication between cells [19]. Gap junction protein connexinx 43 (Cx43), a well known ZO-1 interacting protein, is associated with α5 integrin in bone cells [20], and associates with α5β1 integrin in lung cancer cells [21]. Recently, evidence showed that ZO-1 in TM cells [22, 23], and integrins interact with the actin cytoskeleton via ZO-1 associated molecules such as actinin, focal adhesion kinase (FAK), and junctional adhesion molecule (JAM) in other tissues [18]. Considering these disparate facts, we hypothesized that there may also be an association of ZO-1 with integrins and Cx43 in TM and SC cells. Since such an association could result in stimulatory or inhibitory control of the actin cytoskeleton and regulation of IOP in glaucomatous optic neuropathy, we investigated the expression and association of α3β1 integrin, Cx43, and ZO-1 in TM and SC cells using various molecular techniques.

Materials and methods

Antibodies

Monoclonal antibodies against α3 integrin (Cat. No. NBP1-97692), and β1 integrin (NBP2-22191) were obtained from Novus Biologicals Laboratories (Littleton, CO, USA). Rabbit anti-ZO-1 (Cat. No. 40-2200), and anti-Cx43 (Cat. No. 71-0700) antibodies were purchased from Life Technologies (Invitrogen, Carlsbad, CA, USA) and the specificity of ZO-1 and Cx43 antibodies was previously reported [24-26].

Cell culture

Porcine eyes were obtained from a local abattoir (Carlton Packing Co., Carlton, Oregon), and the eyes were dissected and cultured in Dulbecco's Modified Eagle's Medium (DMEM, containing 50/50% high and low glucose, Sigma-Aldriceh, St. Louis, MO). Supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, Utah) and 1% penicillin-streptomycin-fungizone (Sigma-Aldrich, St. Louis, MO), as described previously [27, 28].

Schlemm's Canal (SC) Cells Isolation. Since SC cells express endothelial cell marker PECAM-1 (CD31) [29, 30], we used Dynabeads® CD31 magnetic beads to isolate porcine SC (angular aqueous plexus cells) cells from porcine TM cells as described in detail by the manufacturer (Invitrogen, Carlsbad, CA, USA). These studies were conducted in accordance to the tenets of the Declaration of Helsinki and the postmortal porcine eyes are not considered animal research.

RT-PCR

Total RNA was isolated from PTM cells using Trizol reagent (Cat. No. 15596018) from Invitrogen. The Reverse Transcription (RT) reaction was conducted as we previously described [31, 32]. Briefly, 1 μg of total RNA was mixed in a solution containing 2 μL of 5 × RT buffer, pH 8.6, 0.1 M dithiothreitol, 2.5 mM dNTP, 0.01% bovine serum albumin, 0.5 μL dimethylsulfoxide, 10 units of RNAguard (Pharmacia Corporation, Peapack, NJ), 500 ng oligo-(dT) primer, and 20 units of RT in a total volume of 10 μL. The mixture was incubated for 1 hour at 37°C and then for 10 minutes at 95°C. The PCR was carried out in 20 μL of solution containing 2 μL of 10 × PCR buffer, 0.8 μL of 50 mM $MgCl_2$, 0.8 μL of 2.5 mM dNTP, 0.5 μL of 20 uM sense and antisense primers (some with 0.5 μL of dimethyl sulfoxide), one unit of TaqDNA polymerase and 1 μL of reverse transcript cDNA. The PCR conditions were 94°C for 3 min, then 40 cycles of amplification at 94°C for 45 s, 59°C for 30 s and 72°C for 45 s. This was followed by a final extension at 72°C for 10 min for T-A cloning. PCR products were separated by electrophoresis in a 2-3% agarose gel, stained with ethidium bromide. The following primers were used for RT-PCR. α3 integrin sense: 5'-TCC TCA ACC AGG CAC AGG CTC-3', α3 integrin antisense: 5'-GTC ACG TTG ATG CTC AGG AG-3' with predicted PCR product length at 227 base pars; β1 integrin sense: 5'-CTG TGA TGC CTT ACA TTA GC-3', β1 integrin antisense: 5'-TGG AAA ACA CCA GCA GCC GTG-3', the predicted PCR product length is 164 base pairs.

Western immunoblotting

The procedures for western blotting were described previously [33, 34]. Briefly, cells were rinsed in cold PBS buffer (50 mM sodium phosphate buffer, pH 7.4, 0.9% saline) and were lysed in an IP buffer (20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml each of leupeptin, pepstatin A, and aprotinin). Homogenates were centrifuged at 20,000 × g for 20 min at 4°C, and the superna-

tants were taken for protein determination using the Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Proteins were boiled for 5 minutes and were separated by SDS-PAGE (50 µg of protein per lane) using 7.5% (for ZO-1) or 9% (for α3 and β1 integrins) gels according to predicted molecular weight of the targeted proteins followed by transblotting to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories) in standard Tris-glycine transfer buffer. Membranes were blocked for 2 h at room temperature in a blocking buffer, rinsed for 40 minutes four times in a TBSTw buffer (TBS +0.05% tween 20), and incubated overnight at 4°C with polyclonal or monoclonal primary antibodies diluted at 1:500 to 1:1000 in PBS buffer containing 0.05% Tween-20. The PVDF membranes were again washed four times in TBSTw for 40 min at RT, incubated with specific secondary antibodies (Li-Cor Bio-sciences, Lincoln, NE) for 1 h at RT. Membranes were once again washed four times using the same TBSTw buffer, then scanned on an imaging system (Odyssey Infrared; Li-Cor) utilizing companion software (Odyssey 2.0; Li-Cor).

Immunohistochemistry

Immunofluorescence procedures were described in detail previously [35-37]. Briefly, PTM cells or isolated SC cells grown on glass slides were fixed with 2% cold formaldehyde (PA) for 10 min, and washed with PBS. For immunolabeling, Cells on slides were incubated in 50 mM Tris-HCl, pH 7.4, containing 1.5% sodium chloride (TBS) and 0.3% Triton X-100 (TBSTr) and 5% normal goat serum (NGS) for 24 h at 4°C with primary antibody. For double immunofluorescence labelling, slides were incubated simultaneously with two primary antibodies. Slides were then washed for 1 h in TBSTr and incubated for 1.5 h at room temperature simultaneously with appropriate combinations of secondary antibodies, which included: Alexa Fluor 488-conjugated goat anti-rabbit IgG together with Alexa Fluor 594-conjugated goat anti-mouse IgG or Alexa Fluor 594-conjugated goat anti-rabbit IgG together with Alexa Fluor 488-conjugated goat anti-mouse IgG diluted at 1:1000 (Molecular Probes, Eugene, Oregon). All antibodies were diluted in TBSTr containing 5% normal goat serum. Following incubation with secondary antibodies, slides were sequentially washed in TBSTr for 40 min, in 50 mM Tris-HCl buffer, pH 7.4, for 30 min, and then coverslipped using antifade medium. To test for inappropriate cross-reactions between primary and secondary antibodies or between different secondary antibodies, control procedures included omission of one of the primary antibodies with inclusion of each of the secondary antibodies. Conventional immunofluorescence images were acquired on a fluorescence microscope. Confocal immunofluorescence images were gathered on a Fluoview confocal microscope with z-stacks of six to ten scans at 0.5 µm intervals.

Immunoprecipitation

Immunoprecipitations (IPs) were carried out as described previously [38, 39]. Briefly, porcine TM cell lysates in an IP buffer were sonicated and centrifuged at 20,000 × g for 20 min at 4°C. After protein determination of sample supernatants, volumes containing 500 µg of protein porcine TM cells were pre-cleared for 1 h at 4°C using 20 µl of protein-A-coated agarose beads (Santa Cruz Biotechnology Inc., Dallas, Texas), centrifuged at 20,000 × g for 10 min at 4°C, and incubated with 2 µl of polyclonal anti-alpha3 or anti-Cx43 antibodies for 2 h at 4°C. Beads with cell lysates and primary antibody omission served as negative controls. The mixture was incubated for 1 h at 4°C with 20 µl of protein-A-coated agarose beads and centrifuged at 20,000 × g for 10 min, and the pellet washed five times with 1 ml of wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.5% NP-40). Samples were mixed with an equal volume of SDS-PAGE loading buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 0.3 mM bromophenol blue, 0.14 M SDS, and 10% β-mercaptoethanol). Finally, samples were subjected to electrophoresis and transferred to be immunoblotted with the anti-ZO-1 antibody.

Results

Porcine TM cells express α3 and β1 integrin mRNA and protein

Expression of mRNA for α3 and β1 integrin in porcine TM cells was detected using RT-PCR. As shown in **Figure 1**, the two sequence-specific primer pairs used for PCR amplification of α3 and β1 integrin from porcine TM cDNA gave rise to products of 227 base pairs for α3 integrin and 164 base pairs for β1 integrin using 3% agarose gel electrophoresis. For each

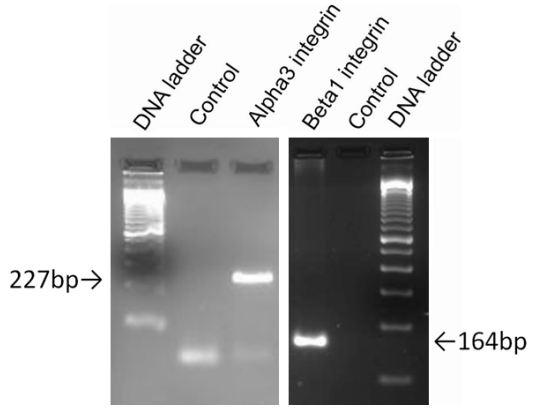

Figure 1. RT-PCR showing detection of α3 and β1 integrin mRNA transcripts in trabecular meshwork cells.

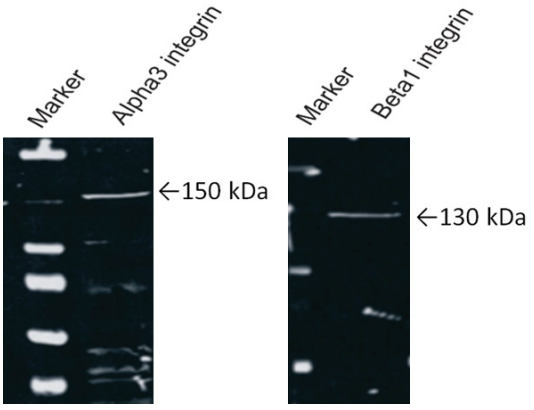

Figure 2. Western Blotting showing detection of α3 integrin and β1 integrin in trabecular meshwork cells. The weak lower molecular weight bands in α3 integrin immunoblotting may correspond the degradation products of α3 integrin protein.

primer pair, only a single PCR product with predicted molecular weight was generated. Using sterilized water instead of porcine TM cDNA as a negative control, no band other than the primer-dimer was detected.

Western immunoblotting

We examined α3 and β1 integrin protein expression using lysates of cultured porcine TM cells by western blotting. As shown in **Figure 2**, monoclonal anti-α3 integrin antibody detected a band migrating at 150 kDa, and monoclonal anti-β1 integrin antibody identified a band migrating at 130 kDa (**Figure 2**). The weak lower molecular weight bands in the α3 integrin immunoblot may correspond to the degradation products of α3 integrin protein. The results demonstrate the specificity of these antibodies.

Co-localization of ZO-1 with α3 integrin in cultured porcine TM cells

Double immunofluorescence labeling combined with confocal microscopy was used to determine the co-localization of ZO-1 with either α3 integrin or β1 integrin in cultured porcine TM cells. As shown in **Figure 3**, labeling of α3 integrin was mostly found at cell-cell contacts with a punctate appearance, and some weak labeling appeared intracellularly (**Figure 3A**). Labeling for ZO-1 typically appeared as fine puncta at cell-cell contacts (**Figure 3C** and **3D**). Double immunofluorescence labeling of α3 integrin and ZO-1 imaged by confocal microscopy showed that these two proteins were partially co-localized at cell-cell contacts of porcine TM cells (**Figure 3E**). Weak labeling of β1 integrin was found both at cell-cell contacts and intracellularly (**Figure 3B**). Double immunofluorescence labeling of β1 integrin and ZO-1 showed minimal co-localization of these two proteins at cell-cell contacts of porcine TM cells (**Figure 3F**).

Partial co-localization of ZO-1/α3 integrin in Schlemm's Canal (SC) cells

We examined whether there was ZO-1/α3 integrin co-localization in cultured SC cells. As shown in **Figure 4**, immunofluorescence labelling of PECAM-1, which was used as a marker for SC cells [29, 40, 41], was found with a punctate appearance intracellularly in SC cells (**Figure 4B**), but was undetectable in porcine TM cells (**Figure 4D**). Immunofluorescence labeling of α3 integrin was found at cell-cell contacts, which it had a punctate appearance, and was also present intracellularly (**Figure 4A**). ZO-1 labeling typically appeared as fine puncta at cell-cell contacts (**Figure 4C**). Double immunofluorescence labeling of α3 integrin and ZO-1 showed partial co-localization of these two proteins at contacts between SC cells (**Figure 4E**), while Cx43 (red) and ZO-1 (green) showing partial co-localization in TM cells (**Figure 4F**).

Co-IP of α3 integrin with ZO-1

Since α3 integrin appeared to co-localize with ZO-1 in porcine TM and SC cells, we sought to determine whether these two proteins directly interacted in these cells. Cx43, a protein estab-

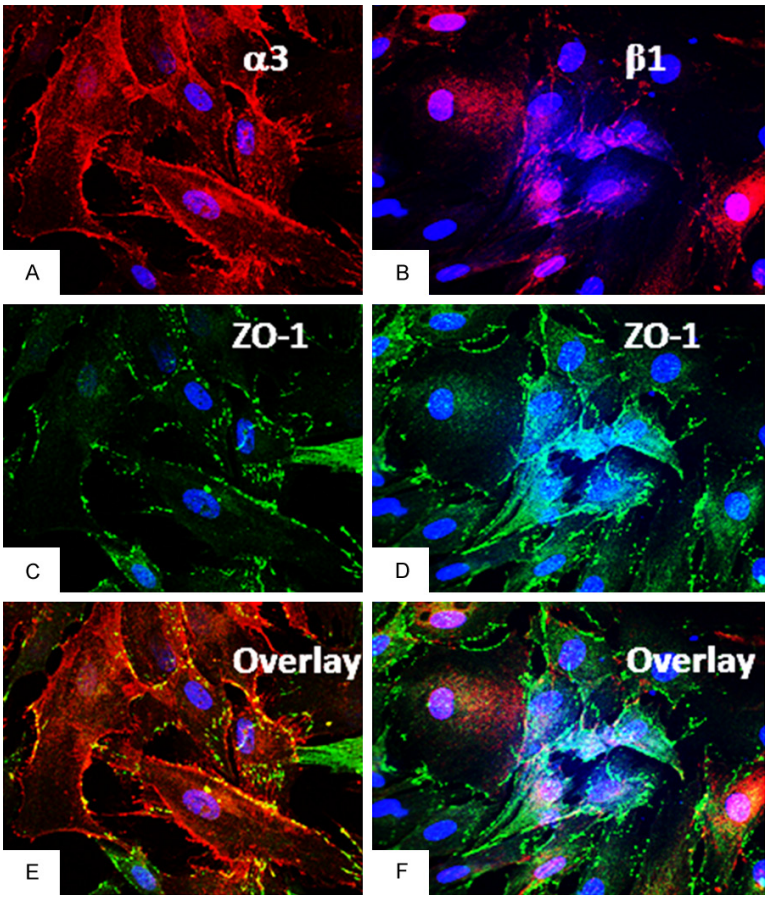

Figure 3. Partial co-localization of α3 integrin with ZO-1 in TM cells. Double immunofluorescence labeling showing α3 integrin (red, A) and ZO-1 (green, C) with partial co-localization in overlay (yellow, E) in TM cells, while β1 integrin (red, B) and ZO-1 (green, D) showing lack of co-localization in overlay (F).

lished to interact with ZO-1, was used as positive control. As shown in **Figure 5**, after IP of Cx43 and α3 integrin from lysates of porcine TM cells, immunoblots of IP material using anti-α3 integrin probed with anti-ZO-1 antibody revealed the presence of ZO-1 (lane 2), which co-migrated with ZO-1 in lysates of porcine TM cells (lane 1) and from IP materials using anti-Cx43 that were included as positive controls for ZO-1 detection (lane 3). ZO-1 was absent from IP material after omission of anti-α3 integrin during the IP procedure, which was included as a negative control (lane 4).

Discussion

The present results demonstrate that (1) ZO-1 co-localized with α3 integrin in cultured porcine TM and SC cells, but not with β1 integrin; (2) both α3 integrin and gap junction protein Cx43 interact with ZO-1, as indicated by their co-IP with ZO-1. These findings provide the molecular basis for considering scaffolding protein ZO-1 and its associated proteins to be involved in the regulation of extracellular cell matrix function in TM cells.

ZO-1 was originally cloned in 1986 [42]; it was considered a tight junction protein, but later was also found in some cell lines that are devoid of tight junctions [43, 44]. Subsequently, it was found that ZO-1 also associates with adherens and gap junctions [18]. To date, with more than 40 proteins have been found to interact with different domains of ZO-1 [18, 45-47]. ZO-1 is considered to be a key player in mammalian cell development, which was demonstrated by the lethal embryonic phenotype after knockdown of ZO-1 in mice [48]; and recently, ZO-1 gene mutations were found to be associated with arrhythmogenic cardiomyopathy [49]. In addition, it has been demonstrated that direct association of ZO-1 with α5 integrin occurs in other cell types and that this interaction plays an important role in the regulation of Cx43 hemichannel function [20].

Integrins are ECM cell surface receptors that are widely distributed in TM cells [9, 10, 50, 51]. Recently, changes in expression of some integrins in glaucoma has been reported [52]. In addition, manipulation of αvβ3 integrin expression in mouse anterior eye also changes IOP [53].

Cx43 is the first gap junction protein found to directly associate with ZO-1, and the interaction involves the second PDZ domain of ZO-1 and the C-terminal amino acid sequence of Cx43. This interaction is important for maintaining gap junction Cx43 size and organization. Recently, mutations in Cx43 were found to

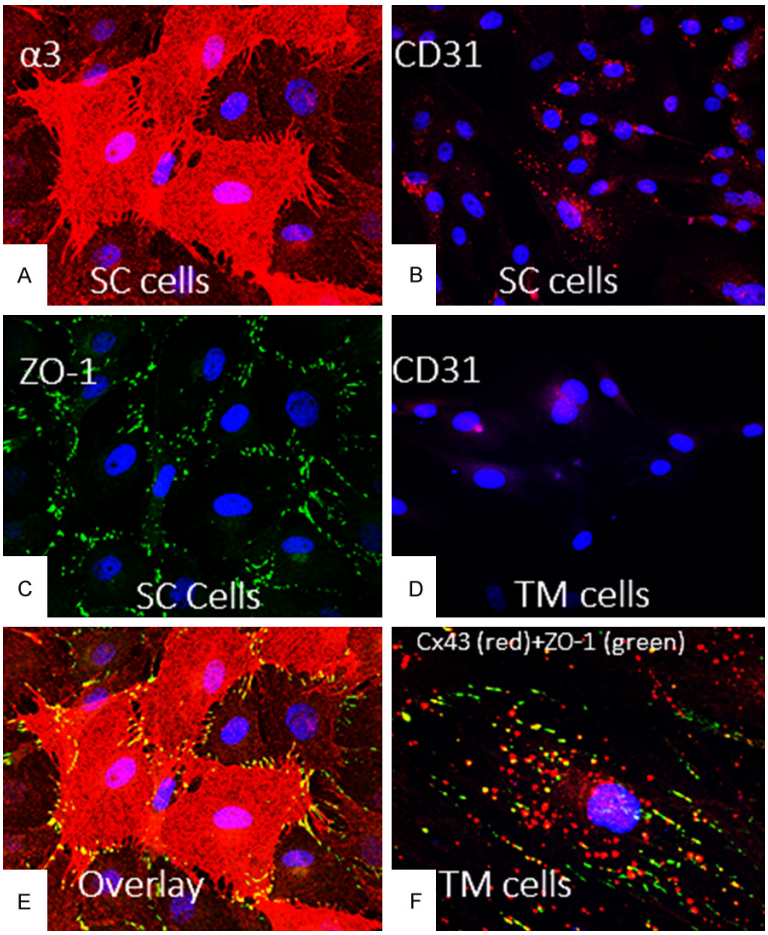

Figure 4. Partial co-localization of α3 integrin with ZO-1 in SC cells. Double immunofluorescence labeling showing α3 integrin (red, A) and ZO-1 (green, C) with partial co-localization in overlay (yellow, E) in SC cells. Immunofluorescence labeling of CD31 (red) in SC cells (B), but there is no CD31 labeling in TM cells (D), while Cx43 (red) and ZO-1 (green) showing co-localization in overlay in TM cells (F).

be associated with human oculodentodigital dysplasia (ODDD) disease and patients with ODDD are eight times more likely to develop glaucoma [54]. Therefore, it will be valuable to determine if there are changes in cellular localization of mutant Cx43, changes in Cx43 and ZO-1 association with these Cx43 mutants, and whether the integrin-ZO-1 association is also changed under conditions found in the Cx43 mutations. A recent investigation of glaucoma in the Chinese population showed that a Cx43 264 frame shift is linked to the development of primary open angle glaucoma.

In the present study, we found that ZO-1 is expressed in porcine TM cells , and the co-localization of α3 integrin with ZO-1 in these cells extends knowledge of ZO-1 co-localization with αvβ3 and α5β1 integrins in cultured rabbit corneal fibroblasts [55] and ZO-1 colocalization with α5 integrin in a non-small cell lung cancer cell line NCI-H460 cells [21]. In considering ZO-1 binding partners and gap junction protein Cx43 interaction with α5 integrin in osteocytic MLO-Y4 cells done by others [20], we demonstrated that both α3 integrin and Cx43 appear to interact with ZO-1, as demonstrated by co-IP. While Cx43 directly interacts with the second PDZ domain of ZO-1 [45, 57], ZO-1 can also bind to an internal PDZ-binding motif in the α5 cytoplasmic tail [58] or the second PDZ domain of ZO-1 can bind to α5 integrin [59]. Further, was also reported that integrins or α7 integrin signaling can regulate Cx43 expression [60, 61]. Whether ZO-1 can directly bind to α3 integrin or this interaction is mediated indirectly needs further investigation.

The link between Cx43 mutations and primary open angle glaucoma [56], together with the present finding of direct or indirect molecular associations between Cx43, ZO-1 and α3 integrin raises the possibility that these proteins may engage in a triple complex in TM cells and SC cells. The disassembly of the complex under Cx43 mutation conditions may disrupt the integrity of these cells and further contribute to disease progression.

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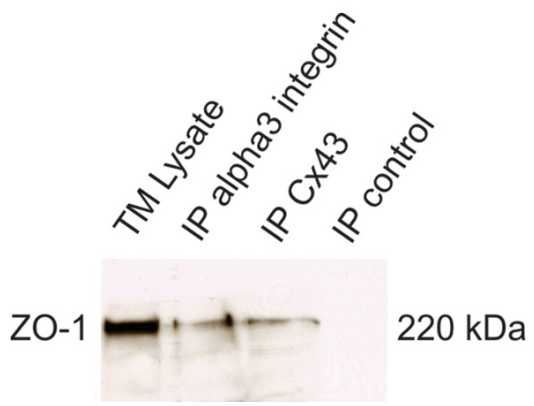

Figure 5. Co-IP of ZO-1 with α3 integrin and Cx43 in TM lysates. Immunoblots of IP material using anti-α3 integrin probed with anti-ZO-1 antibody revealed the presence of ZO-1 (lane 2), which co-migrated with ZO-1 in lysates of PTM cells (lane 1) and from IP materials using anti-Cx43 as positive controls for ZO-1 detection (lane 3). ZO-1 was absent from IP material after omission of anti-α3 integrin during the IP procedure, which was included as a negative control (lane 4).

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Disclosure of conflict of interest

None.

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