Two-Dimensional Differential In-Gel Electrophoresis (2D-DIGE) Reveals Proteins Associated with Cross-Linked Actin Networks in Human Trabecular Meshwork Cells

Jaclyn Y Bermudez1*, Hannah C Webber1, Gaurang C Patel1, Liang-Jun Yan2, Abbot F Clark1 and Weiming Mao1

1North Texas Eye Research Institute, University of North Texas Health Science Center, 3500 Camp Bowie Blvd. Fort Worth, United States
2Department of Pharmaceutical Sciences, UNT System College of Pharmacy, University of North Texas Health Science Center, 3500 Camp Bowie Blvd, Fort Worth, United States

*Corresponding author: Weiming Mao, Ph.D, North Texas Eye Research Institute, University of North Texas Health Science Center, CBH449, 3500 Camp Bowie Blvd. Fort Worth, TX, 76107, Tel: 817-735-0564, E-mail: Weiming.mao@unthsc.edu

Received date: July 12, 2016; Accepted date: August 09, 2016; Published date: August 15, 2016

Abstract

Background: The primary risk factor for primary-open angle glaucoma (POAG) is increased intraocular pressure (IOP). In POAG patients, the outflow resistance through the trabecular meshwork (TM) is abnormally elevated. One of the important glaucoma-associated pathological changes in the TM is formation of excessive cross-linked actin networks (CLANs). CLANs are web-like polygonal structures found in confluent glaucomatous TM cells and tissues. Glaucoma-associated factors transforming growth factor beta 2 (TGFβ2) and glucocorticoids induce CLAN formation in non-glaucomatous TM cells (NTM). CLANs may increase cell stiffness and alter homeostasis, and thereby contribute to elevated IOP.

Methods: We used a proteomic approach to identify CLAN-associated proteins. We treated confluent primary NTM cells with 0.1% ethanol (EtOH; vehicle), 100 nM dexamethasone (DEX), or 5 ng/ml TGFβ2 plus 0.1% EtOH for 7 days to induce CLANs. The Triton insoluble fraction containing cytoskeleton was extracted for two-dimensional differential in-gel electrophoresis (2D-DIGE). Mass spectrometry (MS) was used to identify the differential expressed proteins in the 2D-gels. Co-localization of identified proteins with CLANs was confirmed using immunocytotfluorescence (ICF) microscopy.

Results: 2D-DIGE revealed 103 differentially expressed proteins in both treatment groups. MS identified 23 of the most enriched proteins. ICF showed that caldesmon, calponin, myosin light chain, and tropomyosin were co-localized with CLANs.

Conclusions: We identified a subset of proteins differentially expressed in NTM cells with DEX or TGFβ2-induced CLAN formation. The potential involvement of these proteins in CLAN formation and/or maintenance requires further investigation. These proteins may provide new insights into the pathogenesis of glaucoma.

Keywords: Glaucoma; Trabecular meshwork; Cross-linked actin networks; Proteomics

Introduction

The majority of the aqueous humor, which is produced by the ciliary body, is drained through the conventional outflow pathway [1]. In this pathway, the aqueous humor flows from the posterior chamber to anterior chamber and exits the eye through the trabecular meshwork (TM), a multilayered tissue in the iridocorneal angle of the eye.

In primary open angle glaucoma (POAG), glucocorticoid-induced ocular hypertension (GC-OHT), and glucocorticoid induced glaucoma (GIG), the outflow resistance at the TM is increased, which causes intraocular pressure (IOP) elevation. Elevated IOP is the major risk factor for the development and progression of glaucoma that leads to irreversible visual loss and blindness. Glaucomatous alterations in the TM have been extensively studied both morphologically and biochemically [2]. However, the mechanism of these alterations in the TM and how these changes lead to IOP elevation are not fully understood. One of the glaucomatous TM (GTM) alterations is the formation of excessive cross-linked actin networks (CLANs).

We first reported that actin microfilaments in the TM may rearrange to form CLANs, which are dome-like structures consisting of hubs and spokes [3]. CLANs are inducible by glucocorticoids and transforming growth factor beta 2 (TGFβ2) in TM cells and tissues [4-7]. The formation of CLANs has been found more frequently and abundantly in the GTM compared to the non-glaucomatous TM (NTM) [8,9]. Furthermore, CLAN formation is highly selective in confluent TM cells. The only non-TM ocular tissue that contains CLANs is the lamina cribrosa, another tissue that is involved in glaucoma pathology [10].

Although CLANs are inducible by glaucoma-associated factors such as glucocorticoids and TGFβ2, the exact mechanism of their formation is unclear. Gardel et al. constructed a mathematical model which suggested that CLAN formation increases cell stiffness [11]. Also, Last and colleagues showed increased stiffness in GTM tissues compared to NTM tissues using atomic force microscopy [12]. These studies led us to speculate that CLANs in the GTM may contribute to elevated cell
and tissue stiffness, increased outflow resistance, and ultimately ocular hypertension (OHT). In order to ascertain the mechanism of CLAN formation in the TM, we need to identify the proteins that are associated with the CLAN structure.

Several studies reported a set of CLAN-associated proteins using TM cells during their spreading phrase [13-15]. When cells are spreading after being seeded, virtually all adherent cell types form transient CLAN-like structures in order to organize their cytoskeleton. However, these structures disappear when these cells become confluent [3-5]. Only confluent TM cells retain CLANs and form DEX-induced CLANs, the latter of which is often used as a TM biomarker [5,16-18]. Due to the lack of evidence that CLAN-like structures share similar protein components and mechanisms of formation with CLANs in confluent TM cells, we studied the protein components of CLANs in confluent TM cells using a combination of CLAN protein enrichment and two-dimensional differential in-gel electrophoresis (2D-DIGE). We also confirmed the localization of these proteins with CLANs in cultured TM cells using confocal microscopy.

Materials and Methods

Cell culture
Primary NTM or bovine TM (BTM) cells were cultured in Dulbecco’s modified Eagle’s low glucose medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS; Atlas Biologicals, Collins, CO), 1% penicillin-streptomycin (Sigma-Aldrich), and 2 mM L-glutamine (GE Healthcare Life Sciences, Logan, UT) as previously described [3-5,16]. Once the NTM cells were confluent, they were treated with serum free medium containing either 0.1% EtOH as a vehicle control, 100 nM DEX (Sigma-Aldrich), or 5 ng/ml TGFβ2 (R&D System, Minneapolis, MN) plus 0.1% EtOH (subsequently referred to as “TGFβ2”) for 7 days to induce CLAN formation.

Protein extraction for 2D-DIGE
A Triton based approach was used to enrich CLAN-associated proteins [19]. NTM cells cultured in 100 mm dishes were treated with lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 4 mM MgCl₂, 1 mM CaCl₂ and proteinase inhibitor) containing 0.2% Triton-X-100 (Thermo Fisher scientific, Waltham, MA) to remove soluble proteins. After 2 minute (min) incubation at room temperature (RT), the buffer was carefully aspirated to remove the soluble fraction. The cells were rinsed with phosphate buffered saline (PBS) to remove remaining lysis buffer. After rinsing, PBS was added again to the dish and the insoluble fraction was scraped. The insoluble fraction was pelleted by centrifugation. The insoluble fraction was flash frozen in liquid nitrogen until analysis and sent to Applied Biomics (Hayward, CA) for 2D-DIGE, imaging, and mass spectrometry (MS) analysis. Please see supporting information for further details.

2D-DIGE
The 3 separate protein samples (DEX, TGFβ2, and vehicle control) were labeled with 3 different CyDye DIGE fluorophores, respectively. After protein estimation, equal amounts of proteins were separated using isoelectric focusing and SDS-PAGE.

Imaging
The SDS-PAGE was scanned using the Typhoon TRIO scanner (GE Healthcare, Pittsburgh, PA). The scanned images were then analyzed using the Image Quant software (GE Healthcare), and the ratio of differentially expressed proteins was obtained using the in-gel DeCyder software (GE Healthcare). Gel spots/proteins that showed differential expression in both DEX vs. Control and TGFβ2 vs. Control were picked and stored. Twenty-three of the most up-regulated/enriched proteins were selected for MS analysis.

MS and database search
The protein spots were picked using an Etta Spot Picker (GE Healthcare). The spots were trypsin digested and analyzed by MALDI-TOF and TOF/TOF. Protein identification was performed using the GPS Explorer software equipped with the Mascot search engine (http://www.appliedbiomics.com/Services/mass-spectrometry.html). Eight or more peptides were used to positively identify the protein. Candidates with either protein score confidence interval (C.I.% ) or ion C.I.% greater than 95 were considered significant.

Immunocytofluorescence (ICF)
BTM and NTM cells were cultured on 12 mm diameter coverslips (NeuVitro, Vancouver, WA) in a 24-well culture plate. Confluent BTM cells were treated with lysis buffer as described previously, and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 30 min at 4°C. After a PBS wash, the remaining proteins were stained with Phalloidin-Alexa-488 (Life Technologies) diluted at 1:100 in Superblock (Thermofisher) for 1 hr at RT. They were further incubated with anti-epidermal growth factor receptor (EGFR) antibody (rabbit polyclonal antibody, 1:100 diluted, Santa Cruz, TX) for 2 h at RT, followed by a second incubation with donkey anti-rabbit-Alexa-594 secondary antibody (1:200, Life Technologies) for 2 h at RT. After another PBS wash, coverslips were mounted on slides using ProLong Gold Antifade Mountant (Life Technologies).

Confluent NTM cells were treated with DEX, TGFβ2 or vehicle control as described previously. After 7 days, cells were fixed, washed and treated with 0.2% Triton X-100 in PBS for 30 min at RT. After blocking, cells were incubated with a primary antibody (Table 1) overnight at 4°C followed by a second incubation with goat anti-rabbit-Alexa-488 or rabbit anti-mouse-Alexa-488 (1:500; Life Technologies) at RT for 2 hr and a third incubation with Phalloidin-Alexa-568 (1:100) at RT for 1 hr. Cover slips were mounted onto slides with ProLong Gold Antifade Mountant containing DAPI (Life Technologies). Images were taken using the Zeiss LSM 510 confocal scanning laser microscope (Zeiss, Oberkochen, Germany) or Nikon Ti Epifluorescent microscope (Nikon, Melville, NY) with the Nuance Imaging System (CRI, Hopkinton, MA).

Western immunoblotting (WB)
To collect whole cell lysate, confluent BTM cells cultured in 35 mm dishes were scraped in wash buffer (lysis buffer without Triton) and boiled in Laemmli buffer. To collect Triton insoluble fractions, confluent BTM cells were treated with lysis buffer, washed with wash buffer, and the insoluble fractions were scraped and boiled in Laemmli buffer. After protein estimation using the EQQ kit (Life Technologies), 30 µg of protein was used for SDS-PAGE. Proteins were transferred to a PVDF membrane, blocked with 5% dry milk, and probed with anti-β-actin (mouse monoclonal antibody, 1:1000 diluted, Millipore) and anti-GAPDH (rabbit monoclonal antibody, 1:500 diluted, Cell


Page 2 of 7
Signaling Technology) primary antibodies, as well as the corresponding secondary antibodies conjugated with HRP (1:10000, Santa Cruz). Signals were developed using the SuperSignal West Femto Kit (Thermofisher). Images were taken using the FluroChem imaging system (Cell Biosciences, Santa Clara, CA).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Monoclonal/ Polyclonal</th>
<th>Species</th>
<th>Dilution Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caldesmon</td>
<td>Millipore</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:100</td>
</tr>
<tr>
<td>Calponin</td>
<td>Abcam</td>
<td>Monoclonal</td>
<td>Rabbit</td>
<td>1:50</td>
</tr>
<tr>
<td>Myosin light chain</td>
<td>Sigma-Aldrich</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:400</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>Sigma-Aldrich</td>
<td>Monoclonal</td>
<td>Mouse</td>
<td>1:800</td>
</tr>
</tbody>
</table>

Table 1: Antibodies used for IFC.

Results

Validation of triton-based cytoskeletal protein isolation

We adapted the approach developed by Sawada et al. [19]. Triton X-100 is a mild nonionic detergent that has been widely used for cell lysis and cytoskeletal protein extraction in the TM [20]. Treatment with 0.2% Triton X-100 is able to remove most of the membrane-associated proteins and cytosolic proteins while preserving the cytoskeleton system [20]. Since physical scraping of cells may disrupt CLANs and cause a release of CLAN-associated proteins into lysis buffer, we added Triton lysis buffer directly to BTM cell cultures to lyse cells in situ for 2 min.

We first used ICF to determine whether Triton lysis buffer is able to remove membrane proteins while preserving CLANs. After treatment with lysis buffer, BTM cells were fixed and stained with phalloidin-Alexa-488 for F-actin labeling, the backbone of CLANs, and an antibody against EGFR, a membrane protein that should be removed if cell lysis is complete. Cells subjected to conventional ICF protocols served as a positive control (Figures 1A and 1B). Our negative control was no primary antibody control (data not shown). We found this method did not disrupt actin stress fibers (Figures 1A and 1C) or endogenous CLANs (Figure 1E), but effectively removed membrane associated-proteins (Figures 1B and 1D).

We further confirmed our findings using WB. After treatment with lysis buffer, BTM cells were washed with wash buffer and the insoluble fraction was scraped in Laemmli buffer for WB. Whole cell lysate was also collected as a control. WB showed that there was significant enrichment of actin in the insoluble fraction. In contrast, the soluble cytosolic protein GAPDH was completely removed (Figure 1F).

2D-DIGE revealed proteins differentially expressed in CLAN forming TM cells

After validating our approach, we treated primary NTM cells with either 0.1% EtOH (Control), or DEX (containing 0.1% EtOH), or TGFβ2 (containing 0.1% EtOH) for 7 days, and collected insoluble cytoskeletal proteins for 2D-DIGE. The rationale is that both DEX and TGFβ2 change the expression of a large number of proteins. If we chose the overlap between DEX vs. Control and TGFβ2 vs. Control, we should be able to minimize non-CLAN related proteins.

Fluorescence-labeled protein samples were mixed, separated using isoelectric focusing as well as gel electrophoresis, and imaged. We found a total of 103 protein spots that were differentially expressed in both groups (DEX vs. Control and TGFβ2 vs. Control; Figure 2).

MS identified 23 enriched proteins

After getting the results from 2D-DIGE, we then picked 23 gel spots that were enriched in DEX and TGFβ2 treatment groups compared to Control. The gel spots that we selected were those showing an expression/enrichment level of 1.3 fold or greater compared to Control. The identity of the protein within these gel spots was identified using MS (Table 2).
Figure 2: Enriched and reduced proteins associated with cytoskeleton and CLANs. Comparisons were made between DEX vs. Control and TGFβ2 vs. Control protein samples on the same DIGE gel. A representative image after 2D-DIGE overlay analysis is shown. Enriched proteins (red spots, pseudocolor) and reduced proteins (green spots, pseudocolor) found in both comparison groups are circled. Protein spots delineated by blue circles were selected for MS analysis.
| Spot number | MALDI well number | Top Ranked Protein Name / Species | Accession No. | Protein MW | Protein PI | Pep.Count | Protein Score | Protein Score C.I.% | Total Ion Score | Total Ion C.I.% |
|-------------|-------------------|----------------------------------|--------------|------------|-----------|-----------|--------------|-------------------|----------------|----------------|----------------|
| 3 A1        | 3 A1              | Caldesmon OS=Homo sapiens GN=CALD1 PE=1 SV=3 CALD1_HUMAN | 93,175           | 5.62       | 26        | 416       | 100          | 291              | 100             | 100            | 100            |
| 4 A2        | 4 A2              | Caldesmon OS=Homo sapiens GN=CALD1 PE=1 SV=3 CALD1_HUMAN | 93,175           | 5.62       | 27        | 474       | 100          | 314              | 100             | 100            | 100            |
| 5 A3        | 5 A3              | Caldesmon OS=Homo sapiens GN=CALD1 PE=1 SV=3 CALD1_HUMAN | 93,175           | 5.62       | 28        | 594       | 100          | 423              | 100             | 100            | 100            |
| 38 A4       | 38 A4             | Calponin-2 OS=Homo sapiens GN=CNN2 PE=1 SV=4 CNN2_HUMAN | 33,675           | 6.95       | 9         | 117       | 100          | 76               | 100             | 100            | 100            |
| 1 A5        | 1 A5              | Mitochondrial inner membrane protein OS=Homo sapiens GN=IMMT PE=1 SV=1 IMMT_HUMAN | 83,626           | 6.08       | 26        | 368       | 100          | 192              | 100             | 100            | 100            |
| 2 A6        | 2 A6              | Pre-mRNA-processing factor 19 OS=Homo sapiens GN=PRPF19 PE=1 SV=1 | PRP19_HUMAN | 55,146 | 6.14 | 14 | 138 | 100 | 54 | 100 | 100 |
| 3 A7        | 3 A7              | Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4 | VIME_HUMAN | 53,619 | 5.06 | 35 | 559 | 100 | 213 | 100 | 100 |
| 4 A8        | 4 A8              | Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4 | VIME_HUMAN | 53,619 | 5.06 | 33 | 1050 | 100 | 717 | 100 | 100 |
| 5 A9        | 5 A9              | Spliceosome RNA helicase DDX39B OS=Homo sapiens GN=DDX39B PE=1 SV=1 | DX39B_HUMAN | 48,960 | 5.44 | 15 | 232 | 100 | 157 | 100 | 100 |
| 6 A10       | 6 A10             | Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1 | ACTG_HUMAN | 41,766 | 5.31 | 20 | 633 | 100 | 471 | 100 | 100 |
| 7 A11       | 7 A11             | Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB PE=1 SV=2 | ROAA_HUMAN | 36,202 | 8.22 | 10 | 109 | 100 | 54 | 100 | 100 |
| 8 A12       | 8 A12             | Heterogeneous nuclear ribonucleoprotein D0 OS=Homo sapiens GN=HNRNPD PE=1 SV=1 | HNRPD_HUMAN | 38,410 | 7.62 | 12 | 306 | 100 | 233 | 100 | 100 |
| 9 A13       | 9 A13             | Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB PE=1 SV=2 | ROAA_HUMAN | 37,407 | 8.97 | 21 | 669 | 100 | 488 | 100 | 100 |
| 10 A14      | 10 A14            | Heterogeneous nuclear ribonucleoprotein D-like OS=Homo sapiens GN=HNRPDL PE=1 SV=3 | HNRDL_HUMAN | 46,409 | 9.59 | 10 | 109 | 100 | 61 | 100 | 100 |
| 11 A15      | 11 A15            | Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens GN=HNRNPA2B1 PE=1 SV=2 | ROA2_HUMAN | 37,407 | 8.97 | 22 | 742 | 100 | 546 | 100 | 100 |
| 12 A16      | 12 A16            | Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB PE=1 SV=2 | ROA2_HUMAN | 37,407 | 8.97 | 21 | 669 | 100 | 488 | 100 | 100 |
| 13 A17      | 13 A17            | Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4 | VIME_HUMAN | 53,619 | 5.06 | 18 | 269 | 100 | 154 | 100 | 100 |
| 14 A18      | 14 A18            | Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 | K1C10_HUMAN | 58,792 | 5.13 | 15 | 100 | 100 | 27 | 72 | 100 |
| 15 A19      | 15 A19            | Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4 | VIME_HUMAN | 53,619 | 5.06 | 16 | 269 | 100 | 154 | 100 | 100 |
| 16 A20      | 16 A20            | Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 | K1C10_HUMAN | 58,792 | 5.13 | 15 | 100 | 100 | 27 | 72 | 100 |
| 17 A21      | 17 A21            | Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 | K1C10_HUMAN | 58,792 | 5.13 | 15 | 100 | 100 | 27 | 72 | 100 |
| 18 A22      | 18 A22            | Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1 | ACTG_HUMAN | 41,766 | 5.31 | 13 | 210 | 100 | 143 | 100 | 100 |
| 19 A23      | 19 A23            | Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1 | ACTG_HUMAN | 41,766 | 5.31 | 13 | 210 | 100 | 143 | 100 | 100 |
| 20 A24      | 20 A24            | Myosin regulatory light polypeptide 9 OS=Homo sapiens GN=MYL9 PE=1 SV=4 | MYL9_HUMAN | 19,877 | 4.80 | 10 | 371 | 100 | 299 | 100 | 100 |
| 21 B1       | 21 B1             | Myosin regulatory light chain 12A OS=Homo sapiens GN=MYL12A PE=1 SV=2 | ML12A_HUMAN | 19,782 | 4.67 | 8 | 210 | 100 | 158 | 100 | 100 |
| 22 B2       | 22 B2             | Myosin regulatory light chain 12B OS=Homo sapiens GN=MYL12B PE=1 SV=2 | ML12B_HUMAN | 19,767 | 4.71 | 10 | 406 | 100 | 333 | 100 | 100 |
| 23 B3       | 23 B3             | Myosin regulatory light chain 12B OS=Homo sapiens GN=MYL12B PE=1 SV=2 | ML12B_HUMAN | 19,767 | 4.71 | 10 | 406 | 100 | 333 | 100 | 100 |
| 24 B4       | 24 B4             | Caldesmon OS=Homo sapiens GN=CALD1 PE=1 SV=3 | CALD1_HUMAN | 93,175 | 5.62 | 11 | 98 | 100 | 67 | 100 | 100 |

Table 2: Mass spectrometry results of proteins selected from the 2D-DIGE gel (Figure 2).

Figure 3: Localization of the 4 enriched proteins with CLANs. NTM cells were treated with DEX or TGFβ2 to induce CLAN formation. These cells were used for ICF and confocal microscopy. The 4 enriched insoluble fraction proteins were immunostained and are shown in green. F-actin and CLANs are labeled with phalloidin and are shown in red. Cell nuclei were stained with DAPI and are shown in blue. A) caldesmon; B) calponin; C) myosin light chain; and D) tropomyosin.
ICF showed 4 proteins associated with CLANs

We performed ICF to determine which of the 23 identified proteins are associated with CLANs. Among these proteins, we found that caldesmon, calponin, and tropomyosin were co-localized with the spokes and/or hubs of CLANs (Figure 3). Although myosin light chain did not show co-localization, it was in proximity to CLANs, which appeared to be parallel to the spokes (Figure 3C). All 4 proteins are involved in binding and regulation of the actin cytoskeleton. The position and intensity of these 4 proteins in the 2D-DIGE are highlighted in Figure 4.

Discussion

In this study, we used a detergent-based approach to enrich CLAN-associated proteins from TM cells. Using 2D-DIGE and MS, we identified 23 proteins associated with CLANs induced by both DEX and TGFβ2. Among these proteins, caldesmon, calponin, myosin light chain, and tropomyosin co-localized or were in close proximity to CLANs.

Although CLANs in TM cells and tissues have been studied for many years, our knowledge of the proteins that are associated with CLANs is limited. α-Actinin was previously shown to be at the vertices (hubs) of CLANs in cultured TM cells [4,5]. Filla et al. reported Syndecan, Filamin, α-Actinin, and PDLM1 to co-localize with CLANs in the TM using ICF [21]. However, no further reports have shown their potential roles in CLAN formation. More importantly, the methods in those studies were different from ours because they used non-confluent TM cells. The formation of CLANs in confluent TM cells is unique to TM cells. Without further evidence showing that the components of transiently formed CLANs/CLAN-like structures are identical/similar to CLANs in confluent TM cells, it is our belief that using confluent TM cells for CLAN research is more physiologically and potentially pathologically relevant.

The challenge of a proteomic study of CLANs requires a proper method to enrich CLAN-associated proteins. Filla et al. reported a method using differential centrifugation to study the cytosolic and membrane-associated protein fractions collected from spreading TM cells pretreated with DEX [13]. CLANs are initially found in the perinuclear region, and therefore, without studying the nuclear fraction some potential components might have been missed (e.g. proteins that may anchor CLANs to the nuclear structure). In contrast, our detergent-based approach enabled us to preserve all proteins associated with CLANs as long as their binding to F-actin is resistant to Triton. Previously, Insue and colleague used a similar Triton-based approach to extract cytoskeletal proteins from the TM [20]. Our data showed that this approach is not only suitable for cytoskeletal proteins but also ideal for the preservation of CLANs. However, the disadvantage of our method is that the proteins with weak or more dynamic association with CLANs might have been excluded from analysis. In addition, CLAN formation may result from post-translational protein modifications (e.g. phosphorylation), which was not examined in our study. Therefore, a combination of different protein enrichment approaches would be helpful in future studies of CLAN components.

![Figure 4: The 2D-DIGE image of the 4 proteins. A representative 2D-DIGE gel image was analyzed by assigning different pseudocolors to individual groups of protein samples. Left panel: DEX vs. Control. Right panel: TGFβ2 vs. Control. Red spots: enriched proteins. Green spots: reduced proteins.](image-url)

Both DEX and TGFβ2 induce CLAN formation in the TM [5,6]. Although the signaling mechanism of the two pathways may be different, it is very likely the CLANs that they induce are structurally similar. We found that a number of enriched proteins were either up or down-regulated similarly with DEX and TGFβ2 treatment compared to Control. Our unique strategy not only minimizes background and non-specific proteins, but also suggests that DEX-induced CLANs and TGFβ2-induced CLANs are structurally similar.

The CLAN-associated proteins that we identified are all related to binding and contraction of actin microfilaments. Caldesmon and calponin are both thin-filament associated proteins that are involved in smooth muscle contraction. TM cells have certain smooth muscle characteristics, including expression of α-smooth muscle actin [5]. These proteins are also capable of binding actin and tropomyosin. The overexpression of caldesmon in NTM cells causes rearrangement of actin stress fibers and formation of triangular networks resembling CLANs [21,22]. This overexpression also results in the disruption of focal adhesions by decreasing the expression of β-catenin and the disruption of cadherin junctions [22,23]. Similar to caldesmon, an increase of calponin also results in actin filament rearrangement in the TM, suggesting its role in stabilizing CLANs [24].

In the presence of calmodulin, myosin light chain is phosphorylated by myosin light chain kinase, which activates myosin causing smooth muscle contraction. The whole process is regulated by the Rho/Rho kinase (ROCK) system. The activation of Rho/Rock enhances the formation of actin stress fibers, focal adhesion, and TM cell contraction [25]. In the present study, we found several isoforms of myosin light chain to be associated with CLANs. Although no direct co-localization was observed, myosin light chain was within the vicinity of the CLANs, and demonstrated a pattern similar to CLANs. We believe that myosin light chain may participate in CLAN formation or its maintenance, which is supported by our data that the ROCK inhibitor Y27632 blocks CLAN formation and disassembles established CLANs (Montecchi-Palmer et al. submitted for publication).

Tropomyosin regulates cell contraction by blocking the interaction between actin and myosin. It can be induced by TGFβ2 and DEX, and it is elevated in the GTM [13,26-28]. We found that tropomyosin co-
localized with CLANs, which further suggests its role in CLAN formation and glaucoma.

In summary, we identified and confirmed 4 proteins that are co-localized or associated with CLANs, suggesting their involvement in CLAN formation and/or stabilization. Further investigation is required to determine their roles in formation and/or stabilization of CLANs, which may provide novel therapeutic targets to inhibit CLANs and lower IOP.

Acknowledgments

This study was supported by a Seed Grant provided by University of North Texas Health Science Center (W.M.) and National Eye Institute (B21YE023048 (W.M.)).

References