Journal of Clinical & Experimental Ophthalmology

Bermudez et al., J Clin Exp Ophthalmol 2016, 7:4
DOI: 10.4172/2155-9570.1000584

Research Article Open Access

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

Jaclyn Y Bermudez[1], Hannah C Webber[1], Gaurang C Patel[1], Liang-Jun Yan[2], Abbot F Clark[1] and Weiming Mao[1*]

[1]North Texas Eye Research Institute, University of North Texas Health Science Center, 3500 Camp Bowie Blvd. Fort Worth, United States

[2]Department 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 immunocytofluorescence (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 $MgCl_2$, 1 mM $CaCl_2$ 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 Ettan 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 1hr 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% Trition 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 Laemilli 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 Laemilli buffer. After protein estimation using the EZQ 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

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).

Antibody	Company	Monoclonal/ Polyclonal	Species	Dilution Factor
Caldesmon	Millipore	Monoclonal	Mouse	1:100
Calponin	Abcam	Monoclonal	Rabbit	1:50
Myosin light chain	Sigma-Aldrich	Monoclonal	Mouse	1:400
Tropomyosin	Sigma-Aldrich	Monoclonal	Mouse	1:800

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).

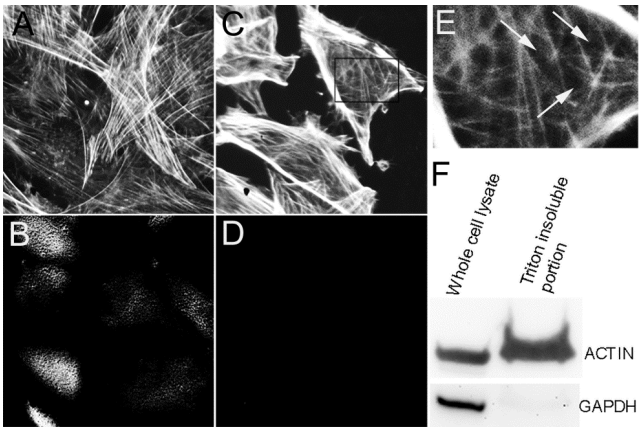

Figure 1: Justification of cytoskeletal protein extraction in BTM cells. **A and B)** ICF of F-actin and α-EGFR, a membrane protein, prior to lysis buffer treatment. **C and D)** After treatment with lysis buffer, F-actin was preserved but α- EGFR was removed. **E)** A magnified view of the boxed area in C shows that the CLAN structure was preserved. Arrows: hubs and spokes of CLANs. **F)** A comparison between whole cell lysate and the insoluble fraction using WB.

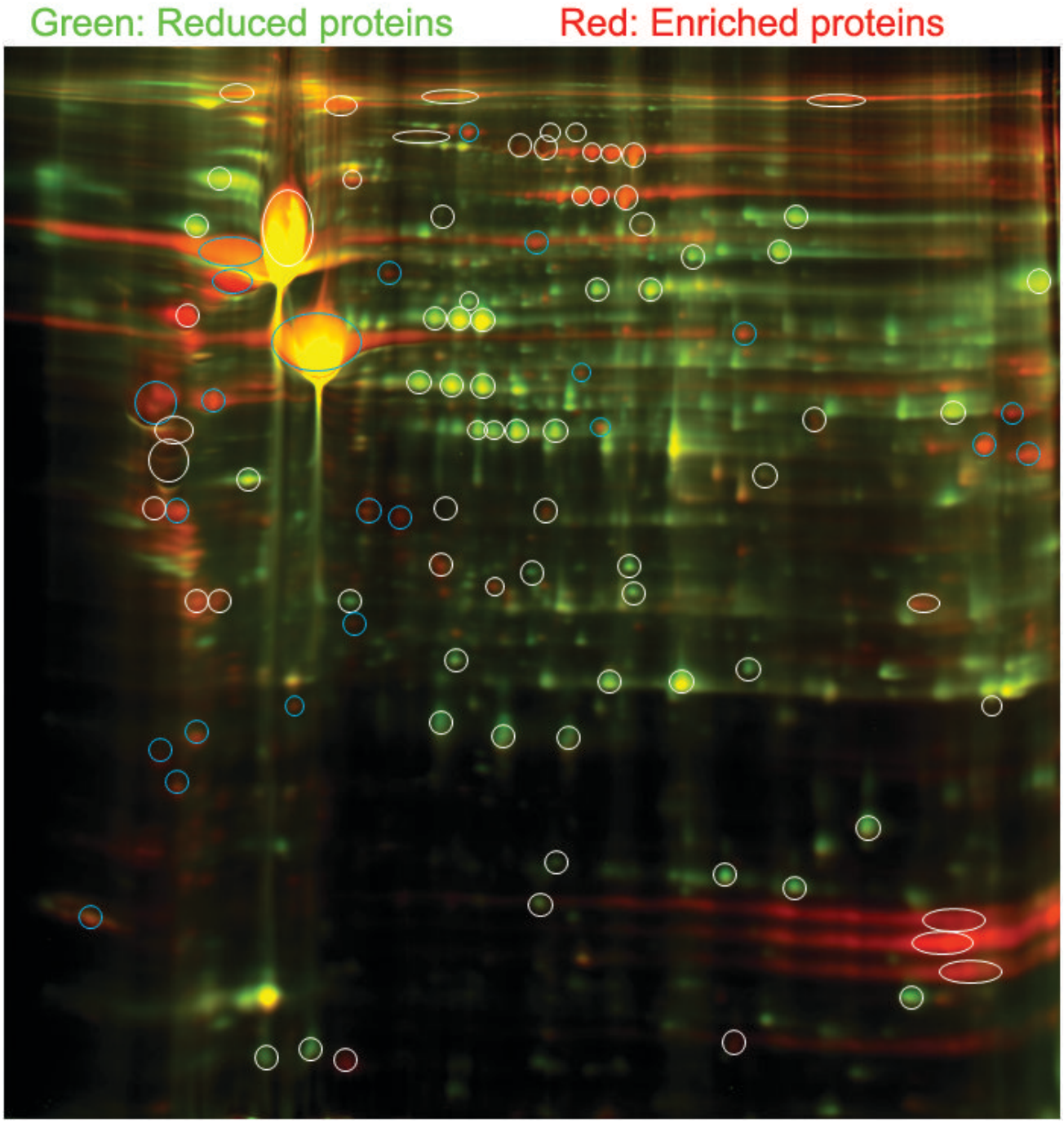

Figure 2: Enriched and reduced proteins associated with cytoskeleton and CLANs. Comparisons were made between DEX *vs.* Control and TGFβ2 *vs.* Control protein samples on the same DIGE gel. A representative image after 2D-DIGE overlay analysis is shown. Enriched proteins (red spots, pseudocolor) and reduced proteins (green spots, pseudocolor) found in both comparison groups are circled. Protein spots delineated by blue circles were selected for MS analysis.

Citation: Bermudez JY, Webber HC, Patel GC, Yan LY, Clark AF, et al. (2016) Two-Dimensional Differential In-Gel Electrophoresis (2D-DIGE) Reveals Proteins Associated with Cross-Linked Actin Networks in Human Trabecular Meshwork Cells. J Clin Exp Ophthalmol 7: 584. doi:10.4172/2155-9570.1000584

Spot number	MALDI well number	Match Quality	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		Caldesmon OS=Homo sapiens GN=CALD1 PE=1 SV=3	CALD1_HUMAN	93,175	5.62	26	416	100	291	100
4	A2		Caldesmon OS=Homo sapiens GN=CALD1 PE=1 SV=3	CALD1_HUMAN	93,175	5.62	27	474	100	314	100
5	A3		Caldesmon OS=Homo sapiens GN=CALD1 PE=1 SV=3	CALD1_HUMAN	93,175	5.62	28	594	100	423	100
38	A4		Calponin-2 OS=Homo sapiens GN=CNN2 PE=1 SV=4	CNN2_HUMAN	33,675	6.95	9	117	100	76	100
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
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
3	A7		Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4	VIME_HUMAN	53,619	5.06	35	559	100	213	100
4	A8		Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4	VIME_HUMAN	53,619	5.06	33	1050	100	717	100
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
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
7	A11		Heterogeneous nuclear ribonucleoprotein D0 OS=Homo sapiens GN=HNRNPD PE=1 SV=1	HNRPD_HUMAN	38,410	7.62	12	306	100	233	100
8	A12		Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB PE=1 SV	ROAA_HUMAN	36,202	8.22	10	109	100	54	100
9	A13		Tropomyosin alpha-4 chain OS=Homo sapiens GN=TPM4 PE=1 SV=3	TPM4_HUMAN	28,505	4.67	16	466	100	358	100
10	A14		Nucleophosmin OS=Homo sapiens GN=NPM1 PE=1 SV=2	NPM_HUMAN	32,555	4.64	10	372	100	306	100
11	A15		Heterogeneous nuclear ribonucleoprotein D-like OS=Homo sapiens GN=HNRPDL PE=1 SV	HNRDL_HUMAN	46,409	9.59	10	109	100	61	100
12	A16		Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens GN=HNRNPA2B1 PE	ROA2_HUMAN	37,407	8.97	21	669	100	488	100
13	A17		Calponin-1 OS=Homo sapiens GN=CNN1 PE=1 SV=2	CNN1_HUMAN	33,150	9.14	16	273	100	144	100
14	A18		Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens GN=HNRNPA2B1 PE	ROA2_HUMAN	37,407	8.97	22	742	100	546	100
15	A19		Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4	VIME_HUMAN	53,619	5.06	18	269	100	154	100
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
17	A21		Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4	VIME_HUMAN	53,619	5.06	25	578	100	376	100
18	A22		Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1	ACTG_HUMAN	41,766	5.31	12	210	100	140	100
19	A23		RNA-binding protein 8A OS=Homo sapiens GN=RBM8A PE=1 SV=1	RBM8A_HUMAN	19,877	5.50	9	190	100	117	100
20	A24		Myosin regulatory light polypeptide 9 OS=Homo sapiens GN=MYL9 PE=1 SV=4	MYL9_HUMAN	19,814	4.80	10	371	100	299	100
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
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
23	B3		Myosin light polypeptide 6 OS=Homo sapiens GN=MYL6 PE=1 SV=2	MYL6_HUMAN	16,919	4.56	11	245	100	153	100
24	B4		Caldesmon OS=Homo sapiens GN=CALD1 PE=1 SV=3	CALD1_HUMAN	93,175	5.62	11	98	100	67	100
Control											
1 fmol	B5		Beta-galactosidase OS=Escherichia coli (strain K12) GN=lacZ PE=1 SV=2	BGAL_ECOLI	116,409	5.3	25	397	100	267	100
1 fmol	B6		Beta-galactosidase OS=Escherichia coli O127:H6 (strain E2348/69 / EPEC) GN=lacZ PE=3	BGAL_ECO27	116,343	5.2	25	427	100	297	100
1 fmol	B7		Beta-galactosidase OS=Escherichia coli O127:H6 (strain E2348/69 / EPEC) GN=lacZ PE=3	BGAL_ECO27	116,343	5.2	23	369	100	257	100
1 fmol	B8		Beta-galactosidase OS=Escherichia coli O127:H6 (strain E2348/69 / EPEC) GN=lacZ PE=3	BGAL_ECO27	116,343	5.2	24	287	100	166	100
high confidence											
low confidence											
no confidence											

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

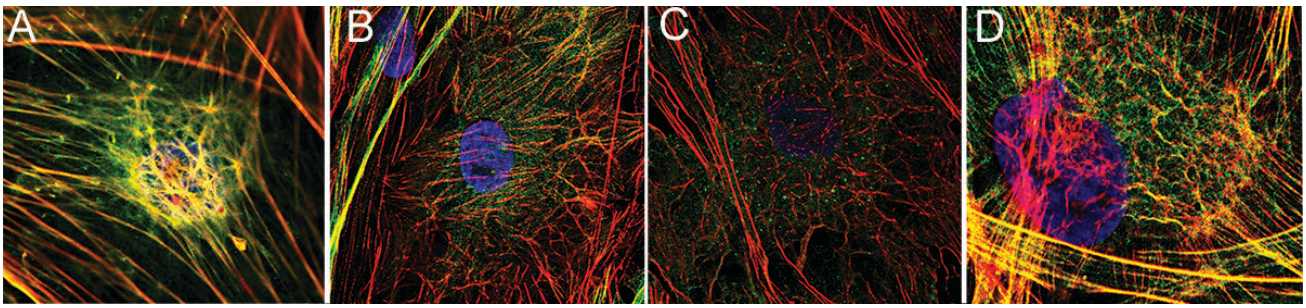

Figure 3: Localization of the 4 enriched proteins with CLANs. NTM cells were treated with DEX or TGFβ2 to induce CLAN formation. These cells were used for ICF and confocal microscopy. The 4 enriched insoluble fraction proteins were immunostained and are shown in green. F-actin and CLANs were labeled 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.

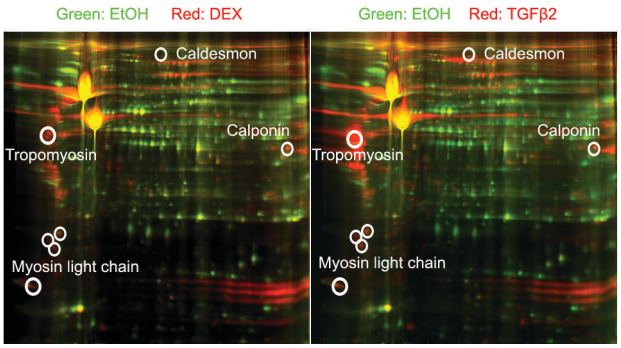

Figure 4: The 2D-DIGE image of the 4 proteins. A representative 2D-DIGE gel image was analyzed by assigning different pseudocolors to individual groups of protein samples. Left panel: DEX *vs.* Control. Right panel: TGFβ2 *vs.* Control. Red spots: enriched proteins. Green spots: reduced proteins.

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, Inoue 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.

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 dissembles 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 R21EY023048 (WM).

References

1. Goel M, Lee RK, Bhattacharya SK (2010) Aqueous humor dynamics: a review. Open Ophthalmol J 4: 52-9.
2. Clark AF, Wordinger RJ (2009) The role of steroids in outflow resistance. Exp Eye Res 88: 752-759.
3. Wilson MM, Miggans ST, Clark AF (1993) Dexamethasone induced ultrastructural changes in cultured human trabecular meshwork cells. Current Eye Research 12: 783-793.
4. Clark AF, Brotchie D, Read AT, Hellberg P, English-Wright S, et al. (2005) Dexamethasone alters F-actin architecture and promotes cross-linked actin network formation in human trabecular meshwork tissue. Cell Motil Cytoskeleton 60: 83-95.
5. Clark AF, Wilson K, McCartney MD, Miggans ST, Kunkle M, et al. (1994) Glucocorticoid-induced formation of cross-linked actin networks in cultured human trabecular meshwork cells. Invest Ophthalmol Vis Sci 35: 281-94.
6. O'Reilly S, Pollock N, Currie L, Paraoan L, Clark AF, et al. (2011) Inducers of cross-linked actin networks in trabecular meshwork cells. Invest Ophthalmol Vis Sci 52: 7316-24.
7. Wordinger RJ, Clark AF (1999) Effects of glucocorticoids on the trabecular meshwork: towards a better understanding of glaucoma. Prog Retin Eye Res 18: 629-67.
8. Hoare MJ, Brotchie D, Pollock N, Cracknell K, Clark AF (2009) Cross-linked actin networks (CLANs) in the trabecular meshwork of the normal and glaucomatous human eye in situ. Invest Ophthalmol Vis Sci 50: 1255-63.
9. Clark AF, Wilson K, de Kater AW, Allingham RR, McCartney MD (1995) Dexamethasone-induced ocular hypertension in perfusion-cultured human eyes. Invest Ophthalmol Vis Sci 36: 478-89.
10. Job R RV, Grierson I, Currie L, O'Reilly S, Pollock N, et al. (2010) Cross-linked actin networks (CLANs) are present in lamina cribrosa cells. Br J Ophthalmol 94: 1388-92.
11. Gardel ML, Shin JH, MacKintosh FC, Mahadevan L, Matsudaira P, et al. (2004) Elastic behavior of cross-linked and bundled actin networks. Science 304: 1301-1305.
12. Last JA, Pan T, Ding Y, Reilly CM, Keller K, et al. (2011) Elastic modulus determination of normal and glaucomatous human trabecular meshwork. Invest Ophthalmol Vis Sci 52: 2147-2152.
13. Clark R, Nosie A, Walker T, Faralli JA, Filla MS, et al. (2013) Comparative genomic and proteomic analysis of cytoskeletal changes in dexamethasone-treated trabecular meshwork cells. Mol Cell Proteomics 12: 194-206.
14. Filla MS, Clark R, Peters DM A (2014) syndecan-4 binding peptide derived from laminin 5 uses a novel PKCepsilon pathway to induce cross-linked actin network (CLAN) formation in human trabecular meshwork (HTM) cells. Exp Cell Res 327: 171-182.
15. Filla MS, Schwinn MK, Nosie AK, Clark RW, Peters DM (2011) Dexamethasone-associated cross-linked actin network formation in human trabecular meshwork cells involves beta3 integrin signaling. Invest Ophthalmol Vis Sci 52: 2952-2959.
16. Mao W, Liu Y, Mody A, Montecchi-Palmer M, Wordinger RJ, et al. (2012) Characterization of a spontaneously immortalized bovine trabecular meshwork cell line. Experimental eye research 105: 53-59.
17. Mao W, Liu Y, Wordinger RJ, Clark AF (2013) A magnetic bead-based method for mouse trabecular meshwork cell isolation. Invest Ophthalmol Vis Sci 54: 3600-3606.
18. Pang IH, Shade DL, Clark AF, Steely HT, DeSantis L (1994) Preliminary characterization of a transformed cell strain derived from human trabecular meshwork. Curr Eye Res 13: 51-63.
19. Sawada Y, Sheetz MP (2002) Force transduction by Triton cytoskeletons. J Cell Biol 156: 609-615.
20. Inoue T, Pecen P, Maddala R, Skiba NP, Pattabiraman PP, et al. (2010) Characterization of cytoskeleton-enriched protein fraction of the trabecular meshwork and ciliary muscle cells. Invest Ophthalmol Vis Sci 51: 6461-6471.
21. Filla MS, Woods A, Kaufman PL, Peters DM (2006) Beta1 and beta3 integrins cooperate to induce syndecan-4-containing cross-linked actin networks in human trabecular meshwork cells. Invest Ophthalmol Vis Sci 47: 1956-1967.
22. Grosheva I, Vittitow JL, Goichberg P, Gabelt BT, Kaufman PL, et al. (2006) Caldesmon effects on the actin cytoskeleton and cell adhesion in cultured HTM cells. Exp Eye Res 82: 945-58.
23. Gabelt BT, Hu Y, Vittitow JL, Rasmussen CR, Grosheva I, et al. (2006) Caldesmon transgene expression disrupts focal adhesions in HTM cells and increases outflow facility in organ-cultured human and monkey anterior segments. Exp Eye Res 82: 935-944.
24. Jensen MH, Morris EJ, Gallant CM, Morgan KG, Weitz DA, et al. (2014) Mechanism of calponin stabilization of cross-linked actin networks. Biophys J 106: 793-800.
25. Rao PV, Deng PF, Kumar J, Epstein DL (2001) Modulation of aqueous humor outflow facility by the Rho kinase-specific inhibitor Y-27632. Invest Ophthalmol Vis Sci 42: 1029-1037.
26. Fatma N, Kubo E, Toris CB, Stamer WD, Camras CB, et al. (2009) PRDX6 attenuates oxidative stress- and TGFbeta-induced abnormalities of human trabecular meshwork cells. Free Radic Res 43: 783-795.
27. Zhao X, Ramsey KE, Stephan DA, Russell P (2004) Gene and protein expression changes in human trabecular meshwork cells treated with transforming growth factor-beta. Invest Ophthalmol Vis 45: 4023-4034.
28. Yu M, Sun J, Peng W, Chen Z, Lin X, et al. (2010) Protein expression in human trabecular meshwork: downregulation of RhoGDI by dexamethasone in vitro. Mol Vis 16: 213-23.