Dexamethasone Induces Cross-Linked Actin Networks in Trabecular Meshwork Cells Through Noncanonical Wnt Signaling

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PURPOSE. Dexamethasone (DEX) regulates aqueous humor outflow by inducing a reorganization of the cytoskeleton to form cross-linked actin networks (CLANs) in trabecular meshwork (TM) cells. Rho-associated protein kinase (ROCK) has been demonstrated to have an important role in this process, but the upstream components leading to its activation remain elusive. The purpose of the study is to demonstrate that noncanonical Wnt signaling mediates the DEX-induced CLAN formation in TM cells.

METHODS. The TM cells were treated with 100 nM DEX in low serum medium for over 7 days. The medium was changed every 3 days. The cells were harvested and subjected to molecular analysis for the expression of Wnt ligands. Stress fiber structures were revealed by Phalloidin staining. Lentivirus-based shRNA against noncanonical Wnt receptor (*Ror2*) was used to determine the role of noncanonical Wnt signaling in DEX-induced CLAN formation.

RESULTS. The DEX induced stress fiber rearrangement in TM cells. A noncanonical Wnt ligand (*Wnt5a*) was upregulated by DEX as demonstrated by Wnt ligand degenerate PCR, real-time quantitative PCR (qRT-PCR), and Western blotting. Knocking-down *Ror2*, the receptor of noncanonical Wnt signaling, abolished the effects of DEX on the TM cells.

CONCLUSIONS. Our data suggest that DEX induces the upregulation of noncanonical Wnt ligand *Wnt5a*. Recombinant WNT5a protein induces CLAN formation through the noncanonical Wnt receptor ROR2/RhoA/ROCK signaling axis. Given the similarities between DEX-induced ocular hypertension and primary open-angle glaucoma, our results provide a mechanism of action for applying ROCK inhibitor to treat primary open-angle glaucoma.

Keywords: trabecular meshwork, steroid, glaucoma, noncanonical Wnt

Administration of steroids, such as dexamethasone (DEX), results in IOP elevation in a subset of the population. Primary open-angle glaucoma (POAG) patients and glaucoma suspects have a greater risk for elevated IOP after DEX treatment.¹ Prolonged DEX-induced ocular hypertension can lead to secondary open-angle glaucoma, which shares many aspects with POAG.² Therefore, a better understanding of the mechanisms underlying DEX-induced glaucoma will also shed light on the pathogenesis of POAG.

In 1994, Clark et al.³ first reported an unusual actin structure induced by DEX in cultured trabecular meshwork (TM) cells. Unlike normal TM cells, which displayed actin microfilament stress fibers running along the longitudinal axis of the cell, DEX-treated TM cells formed a polygonal network of actin microfilaments referred to as cross-linked actin networks (CLANs). They further demonstrated that similar structures also exist in human TM tissues.⁴ The CLAN structure may compromise the function of TM cells by changing the cell contractile property. The F-actin structure is associated with myosin light chain phosphorylation,⁵ but the upstream components are still not clearly defined in DEX-treated TM cells. In 2011, Filla et al.⁶ reported that the Rac1 inhibitor

NSC23766 decreased the percentage of CLAN-positive cells by 80% in DEX-treated TM cells. However, Rac1 is unlikely the only small GTPase transducing the signal from DEX to the microfilaments, because several other inhibitors can also relax the TM cells and increase aqueous humor outflow. Rosenthal et al.⁷ reported that the Rho-associated protein kinase (ROCK) inhibitor Y-27632 can reduce cell contraction induced by carbachol and endothelin-1. Khurana et al.⁸ reported that the protein kinase C (PKC) inhibitor GF109203X increased outflow by 46% in a porcine whole eye perfusion model. They suggested that PKC might have an important role in the modulation of aqueous outflow facility by regulating myosin light chain (MLC) phosphorylation, and, thereby, the morphologic and cytoskeletal characteristics of the TM cells.⁸ Recently, Fujimoto et al.⁹ reported that a ROCK inhibitor prevents the decrease in aqueous outflow induced by DEX. Taken together, RhoA, Rac1, and PKC all are potential transducers of the signaling cascade leading to MLC phosphorylation. Even though ROCK inhibitors are being investigated in several phase 2a clinical studies, the upstream agonists for the activation of small GTPase are not clearly defined. TGF- β ^{10,11} and endothelin-1¹² have been reported to be associated with glaucoma, but to

TABLE. Oligonucleotide Sequences Used in the Study

Gene Name	Accession Number	Forward Primer	Reverse Primer	Start	End
<i>Wnt ligands</i>		NNNGTCGACGCTTGYAARTGYCAYGG	NNNGTTAACTACGTRRCARCACCARTG		
<i>Wnt2</i>	NM_003391.2	TGGTGGTACATGAGAGCTACA	AATACAACCTCCAGCTGAGGAG	379	664
<i>Wnt3</i>	NM_030753.3	GACTATCCTGGACCACATGC	GGACTCACGGTGCTTCTCTA	707	875
<i>Wnt5a</i>	NM_001256105	AGAAGAAACTGTGCCACTTGTATCAG	CCTTCGATGTCGGAATTGATACT	298	398
<i>Wnt5aPtA</i>	NM_003392.4	TCGGGTGGCGACTTCCT	CAACTCCTGGGCTTAATATTCCAAT	618	694
<i>Wnt5aPtB</i>	NM_001256105	CCTCTCGCCCATGGAATT	GGGCTTAATATTCCAATGGACTTC	21	92
<i>Bmp2</i>	NM_001200	GCTAGACCTGTATCGCAGGC	TTTTCCCACTCGTTTCTGGT	40	185
<i>Bmp4</i>	NM_001202.3	CTGACCACCTCAACTCAACC	CCCACATCCCTCTACTACCA	1456	1637
<i>Tgf-β1</i>	NM_000660	CCCTGGACACCAACTATTGC	AAGTTGGCATGGTAGCCCTT	125	251
<i>Tgf-β2</i>	NM_001135599.2	AACAAGAGCAGAAGGCGAAT	TGCCATCAATACCTGCAAT	2025	2218
<i>Tgf-β3</i>	NM_003239	AAATTCGACATGATCCAGGG	CTGCTCGGAATAGGTTGGTT	116	245
<i>Foxc1</i>	NM_001453.2	CTCCCCCTCTCTTGCCCTTCTT	CGTCAGGTTTTGGGAACACT	2022	2233

our knowledge, have not yet been defined as components in DEX-induced ocular hypertension.

Recently, we found the activation of noncanonical Wnt signaling and an increase in myosin light chain phosphorylation in our new mouse model of glaucoma.¹³ In this model, targeted overexpression of TGF- α in corneal epithelium causes secondary angle-closure glaucoma. Noncanonical Wnt signaling is well known for modulating the cytoskeleton through RhoA, Rac1, and PKC.¹⁴ We, therefore, hypothesize that the noncanonical Wnt signaling pathway is one of the potential mechanisms mediating DEX-induced CLAN formation in TM cells. Here, we report that noncanonical Wnt ligand (*Wnt5a*) was upregulated by DEX as demonstrated by Wnt ligand degenerate PCR, real-time quantitative PCR (qRT-PCR), and Western blotting. We also found that recombinant WNT5a protein induced CLAN formation in TM cells, while knocking-down receptor tyrosine kinase-like orphan receptor 2 (*Ror2*), the receptor of noncanonical Wnt signaling, abolished the CLAN formation in DEX-treated TM cells. These observations demonstrated that dexamethasone induces CLAN formation through noncanonical Wnt signaling in TM cells.

MATERIALS AND METHODS

Cell Culture and DEX Treatment

A primary TM cell line was obtained from ScienCell Research Labs (Carlsbad, CA) and cultured to 100% confluence in Fibroblast Medium (catalog No. 2301; ScienCell Research Labs) before being placed in 1% charcoal-stripped serum medium containing 100 nM DEX or solvent (ethanol). Charcoal-stripped serum was used in the assay, because it can reduce background CLAN formation by removing the trace amount of steroids in the calf serum.³ The medium was changed every 3 days. After 7 to 8 days, the cells were subjected to immunostaining, Western blotting, and qRT-PCR assays. In some experiments, recombinant proteins were used to treat confluent TM cells. The source and dosage of the proteins are: WNT5a (catalog No. 645-WN-010, 50 ng/mL; R&D Systems, Minneapolis, MN), BMP4 (catalog No. 314-BP-010, 10 ng/mL; R&D Systems), and TGF- β 2 (catalog No. 302-B2-002, 2 ng/mL; R&D Systems).

Immunostaining

Cells were cultured in 12-well chamber slides (catalog No. 81201; Ibidi GmbH, Martinsreid, Germany) and fixed for 20 minutes in 1% glutaraldehyde. Cells were permeabilized with 0.2% Triton X-100 in PBS. Alexa Fluor 555 phalloidin

(Invitrogen, Carlsbad, CA) was used to visualize stress fiber structure. All incubations were performed at room temperature. Slides for immunofluorescence analysis were mounted (SlowFade Light Antifade Kit; Molecular Probes, Eugene, OR) in the presence of 4,6-diamidino-2-phenylindole (DAPI), and observed with the EVOS fluorescence microscope (Advanced Microscopy Group, Mill Creek, WA). The percentage of cells that developed CLANs was determined by examining four random fields using EVOS microscope's counting function. Only cells with at least five hubs and three triangulated arrangements of spokes were labeled as CLAN-formation cells.

Western Blotting

The WNT5a was enriched according to published methods¹⁵ using Blue Sepharose beads. Briefly, cells were lysed in RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1% Triton X-100) with protease inhibitors. The cleared lysate was incubated with Cibacron Blue 3GA Sepharose beads in the cold room for 2 hours. Beads were washed with RIPA buffer three times before loading onto SDS-PAGE gel. Anti-WNT5a antibody (ab72583; Abcam, Cambridge, UK) was used to reveal the protein band.

Degenerate PCR and qRT-PCR

Total RNA was isolated from cultured cells with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Then, 5 μ g total RNA were reverse transcribed with Maxima First Strand cDNA Synthesis Kit for qRT-PCR (Fermentas, Pittsburgh, PA). The Wnt ligand degenerate PCR was performed according to published methods,¹⁶ and the PCR products were cloned into the pCR4-TA sequencing plasmid (Invitrogen). Plasmids from a total of 18 colonies were sent for sequencing and the inserts were identified by BLAST database search. The qRT-PCR reactions were performed using the CFX96 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA) operated by CFX Manager software. Primer sequences used in the study are listed in the Table. A panel of 8 housekeeping genes (catalog No. HKK1; Real Time Primers, Elkins Park, PA) was used to normalize the expression levels.

Feline Immunodeficiency Virus (FIV)-Based shRNA Lentivirus

The FIV-based shRNA lentivirus expression system was obtained from System Biosciences (Mountain View, CA). The pSIF-H1 vectors are designed to express a single-stranded shRNA sequence with a fold-back stem-loop structure (also

FIGURE 1. Dexamethasone induces cross-linked actin networks formation in TM cells. Confluent TM cells were treated with 100 nM DEX or an equal volume of ethanol in 1% charcoal-stripped serum medium for 7 days. The medium was changed every 3 days. Stress fiber structure was visualized by phalloidin staining. (A) TM cells treated with solvent for 7 days, $\times 20$ objective lens revealed most cells had actin microfilament stress fibers running along the longitudinal axis of the cell. (B) TM cells treated with DEX for 7 days, $\times 20$ objective lens revealed most cells had polygonal networks of actin microfilaments referred to as CLANs. The dashed boxes indicate the region shown at higher magnification in (C, D). (C) Digital zoom revealed detailed actin structure in individual cells. (D) Digital zoom had a close-up view of the CLAN structure. The CLAN structures were indicated by the arrows. The region containing CLAN structure is encircled by a broken green line. The minimal CLAN structure (at least five hubs and three triangulated arrangements of spokes) is also labeled with yellow lines.

known as a “hairpin”) from an RNA polymerase III H1 promoter. The hairpin-type siRNA (shRNA) template oligonucleotides against human *Ror2* (CGACAAGCTGAACGTGAAGAT) were cloned into the unique BamHI/EcoRI site of pSIF-H1 according to the manufacturer's protocol. The resulting plasmid (pSIF-H1-siRor2) and its control (pSIF-H1-siLuc) were packaged into viral particles. TM cells were transduced by the virus and positive cells were labeled by green fluorescent protein copGFP.

RESULTS

Noncanonical Wnt Ligand Is Upregulated by DEX in TM Cells

To delineate the signaling pathway leading to CLAN formation in DEX-treated TM cells, we adopted the protocol from the pioneer work of Clark et al.³ Confluent TM cells were treated with 100 nM DEX or an equal volume of ethanol in 1% charcoal-stripped serum medium for 7 days. Stress fiber structures were visualized by phalloidin staining. In control cells, most of the F-actin fibers were parallel to each other running along the axis of the cell (Figs. 1A, 1C). In DEX-treated cells, a dramatic increase in F-actin fiber density and the formation of CLANs (Figs. 1B, 1D) was observed. DEX-treated cells apparently were larger than control cells. All of these observations are in agreement with the initial report of Clark et al.³ on DEX-induced CLAN formation in TM cells. Approximately 34% of the cells from the DEX-treated culture had CLAN

structure, while no CLANs can be found in the control-treated culture.

We reported that several noncanonical Wnt ligands are upregulated in our mouse model of glaucoma.¹³ An unbiased approach was taken to profile the Wnt ligand expression in DEX-treated TM cells. Confluent TM cells were treated with 100 nM DEX or an equal volume of ethanol in 1% charcoal-stripped serum medium for 8 days, and total RNA was extracted and reverse transcribed into cDNA. Then, PCR was performed using a published degenerate primer pair that can amplify all known Wnt ligands.¹⁶ The DNA bands (Fig. 2A) were cut from the gel and purified before cloning into the pCR4 vector. A total of 18 individual colonies were picked and the plasmids were sent for sequencing. Sequencing data revealed that only three members of the *Wnt* gene family were found in both samples, but their relative abundance was very different. *Wnt2* was the most abundant ligand in the control sample (5 of 9 clones), while *Wnt5a* was the most abundant in the DEX-treated sample (6 of 9 clones). The dramatic shift in the expression of Wnt ligands from *Wnt2* to *Wnt5a* after DEX treatment was verified by qRT-PCR (Fig. 2B). *Wnt2* mRNA level was reduced approximately 3-fold, while *Wnt5a* mRNA level was increased more than 4-fold after DEX treatment. We also observed an increase in WNT5a protein level in DEX-treated TM cells by Western blotting (Fig. 2C).

Recombinant WNT5a Protein Induces the Formation of Cross-Linked Actin Networks in TM Cells

Next, we wanted to determine if recombinant WNT5a protein could also induce CLAN formation in TM cells. The TM cells were treated the same way as described previously (Figs. 3A, 3B), except 50 ng/mL recombinant WNT5a was added to the culture medium. After 7 days of culture, cells were stained with Alexa Fluor 555 phalloidin. As shown in Figure 3C, WNT5a induced widespread CLAN formation in TM cells. Cell size and CLAN structures were very similar to those of DEX-treated cells (Figs. 3E, 3F). Approximately 15% of cells in WNT5a-treated TM cells had CLAN structure. There were some subtle differences between DEX and WNT5a-treated cultures in terms of CLAN structure. CLANs in WNT5a-treated cells had a higher density of vertices and the size of the triangle was smaller comparing to those of DEX-treated cells.

Knocking-Down Wnt5a Receptor (*Ror2*) Prevents the Formation of DEX-Induced Cross-Linked Actin Networks in TM Cells

If DEX induces CLAN formation through Wnt5a, knocking-down its receptor (*Ror2*, receptor tyrosine kinase-like orphan receptor 2) should abolish the effects of DEX on TM cells. The TM cells were transduced with FIV lentivirus encoding shRNA against luciferase (control) or human *Ror2*. At 3 days after transduction, the media were switched to DEX-containing media as described previously. At 7 days after treatment, stress fibers were visualized by Alexa Fluor 555 phalloidin staining. Expression of green fluorescent protein revealed shRNA-expressing cells. As expected, after DEX treatment, control cells exhibited widespread CLAN formation whether or not they expressed copGFP (Figs. 4A, 4C). In *Ror2* shRNA-transduced culture, 6% of cells still had CLAN structure (Figs. 4B, 4D); however, these cells did not express *Ror2* shRNA (GFP-negative) and the GFP-positive cells, which constituted approximately 80% of the total culture, did not have any CLAN formation. The *Ror2* shRNA also seemed to affect normal stress

FIGURE 2. Noncanonical Wnt ligand is upregulated by DEX treatment in TM cells. (A) Wnt ligand degenerate PCR. The primers used to amplify Wnt ligands are shown in the *open arrows*. DNA electrophoresis gel revealed the 450 base pair (bp) bands of amplified PCR product from control and DEX-treated TM cells. The bands were gel purified and cloned into pCR4 plasmid, and nine positive clones were sequenced from each sample. The identities of the PCR inserts are shown in the *table*. *Wnt2* was the most abundant in the control sample (5 of 9 clones), while *Wnt5a* was the most abundant in the DEX-treated sample (6 of 9 clones). (B) qRT-PCR verified the finding by degenerate PCR. *Wnt2* was downregulated 3-fold after DEX treatment, while *Wnt5* was upregulated 4-fold. (C) WNT5a Western blotting demonstrated that there was more WNT5a protein in DEX-treated cells. TM cells were treated with control (ethanol) or 100 nM DEX for 7 days. Cells were lysed in RIPA buffer and incubated with Cibacron Blue 3GA Sepharose beads to enrich WNT proteins. The proteins were eluted from the beads by SDS-PAGE sample buffer. The results clearly showed there was an increase in WNT5a protein level after DEX treatment.

FIGURE 3. Recombinant WNT5a induces cross-linked actin networks formation in TM cells. Confluent TM cells were treated with 100 nM DEX, or an equal volume of ethanol or 50 ng/mL recombinant WNT5a in 1% charcoal-stripped serum medium for 7 days. The medium was changed every 3 days. Stress fiber structure was visualized by phalloidin staining. (A) The TM cells treated with solvent for 7 days, $\times 20$ objective lens revealed most cells had actin microfilament stress fibers running along the longitudinal axis of the cell. (B) The TM cells treated with DEX for 7 days, $\times 20$ objective lens revealed most cells had polygonal network of actin microfilaments referred to as CLANs. (C) The TM cells treated with 50 ng/mL WNT5a recombinant protein for 7 days, $\times 20$ objective lens revealed most cells had polygonal CLANs. The *dashed boxes* indicate the region shown at higher magnification in (D–F). (D) Digital zoom revealed detailed actin structure in individual cells. (E) Digital zoom had a close-up view of the CLAN structure. (F) Digital zoom had a close-up view of the CLAN structure. The CLAN structures were indicated by the *arrows*. The CLAN structures are very similar between DEX and WNT5a-treated cells. The CLAN structure in WNT5a-treated cells may have higher local vertex density.

FIGURE 4. Knocking-down Wnt5a receptor (*Ror2*) prevents DEX-induced cross-linked actin networks formation in TM cells. The TM cells were transduced with lentivirus encoding shRNA against luciferase (served as control) or human *Ror2*. At 3 days after transduction, the cells were treated with 100 nM DEX for 7 days. (A) In the control transduced culture, green fluorescence represents transduced cells expressing copGFP, $\times 20$ objective lens revealed CLAN structure in transduced and untransduced cells. (B) In *Ror2* shRNA transduced culture, few cells have CLAN structure and none of them has green fluorescence. The dashed boxes indicate the region shown at higher magnification in (C, D). (C) Digital zoom revealed detailed actin structure in individual cells. The CLAN structures were indicated by the arrow. (D) Digital zoom demonstrated unique stress fiber distribution pattern in transduced cells. Central stress fiber density significantly decreased in *Ror2* shRNA expressing cells.

fiber arrangement. In normal TM cells, actin microfilament stress fibers had an even distribution of parallel filaments running along the longitudinal axis of the cell (Fig. 3D). In *Ror2* shRNA-transduced cells, the density of stress fibers in the center of the cell decreased dramatically (Fig. 4D). Similar observations

were reported by Totsukawa et al.¹⁷ that ROCK inhibitor blocked the stress fiber formation in the center of the cells, while cortical actin bundles were not affected. Because ROR2 situates upstream of RhoA/ROCK in the signaling pathway, the two observations agree with each other.

FIGURE 5. Promoter B of *Wnt5a* is activated in TM cells and SMAD agonist BMP4 induces CLAN formation in TM cells. (A) The qRT-PCR analysis of DEX-treated TM cells. Promoter B specific transcript is upregulated by DEX treatment. *Bmp4* also is upregulated, while *Foxc1* is downregulated. (B) Recombinant BMP4 protein induces CLAN formation in TM cells. Confluent TM cells were treated with 10 ng/mL recombinant BMP4 or 2 ng/mL TGF- β in 1% charcoal-stripped serum medium for 7 days. The medium was changed every 3 days. Stress fiber structure was visualized by phalloidin staining, $\times 20$ objective lens revealed CLAN structure in BMP4-treated TM cells. (C) TGF- β -treated TM cells have a massive induction of large thickened parallel stress fiber bundles, but no visible CLAN structure. The dashed boxes indicate the region shown at higher magnification in (D, E). (D) Digital zoom revealed detailed CLAN structure in individual cells. The CLAN structures were indicated by the arrow. (E) Digital zoom revealed detailed stress fiber structure in individual cells.

Promoter B of *Wnt5a* Is Activated and the SMAD Agonist BMP4 Induces CLAN Formation in TM Cells

To figure out how DEX regulates *Wnt5a* expression, we focused on the transcription elements within the *Wnt5a* promoter. *Wnt5a* has two promoters: promoter A and promoter B. Neither has the glucocorticoid response element (GRE), suggesting an indirect effect of DEX on *Wnt5a*.¹⁸ We first determined which promoter was active in TM cells by qRT-PCR amplification of promoter-specific transcripts.¹⁹ Promoter A-specific primers did not amplify any PCR products from the samples, and only promoter B was active and responded to DEX stimulation in TM cells (Fig. 5A). Katoh and Katoh¹⁸ suggested there are several potential transcription binding sites in promoter B: CUX1 (cut-like homeobox 1), SBEs (Smad-binding elements), NFκB, and FOX. NFκB is repressed by glucocorticoid receptors,²⁰ and, therefore, is unlikely the mechanism for DEX-induced *Wnt5a* expression. Both CUX1 and SBEs are related to TGF-β signaling²¹ and *Foxc1* is a crucial transcription factor for anterior chamber development.²² Based on these findings, we analyzed the expression of several SMAD agonists and *Foxc1* in DEX-treated TM cells. As shown in Figure 5A, *Bmp4* was upregulated more than 2-fold, while *Foxc1* was downregulated by 2-fold, and none of the *Tgf-βs* was upregulated. Next, we examined if recombinant BMP4 protein could induce CLAN formation in TM cells. Low serum medium with BMP4 (10 ng/mL) or TGF-β2 (2 ng/mL) was added to confluent TM cells for 7 days. BMP4 induced CLAN formation (Figs. 5B, 5D), just as the DEX or WNT5a did, while TGF-β2 forced actin fibers to coalesce into large bundles (Figs. 5C, 5E). Approximately 10% of cells in BMP4-treated TM had CLAN structure and the overall CLAN structure was similar to that of WNT5a-treated cells.

DISCUSSION

Primary open-angle glaucoma (POAG), the most common form of glaucoma, is usually accompanied by elevated IOP due to failure of the TM to maintain normal levels of aqueous humor outflow. Steroid-induced ocular hypertension and glaucoma are clinically similar to POAG. Glucocorticoids, such as dexamethasone, induce CLAN formation in TM cells and tissues, making them excellent reagents to dissect the underlying mechanism. Rho-associated protein kinase (ROCK) and TM contractility have already been established as important regulators of IOP, and several ROCK inhibitors currently are being evaluated in phase 2a clinical studies in patients with glaucoma or ocular hypertension, but the upstream agonists of RhoA/ROCK in POAG still remain elusive. Here, we attempted to establish that noncanonical Wnt signaling has an important role in DEX-induced CLAN formation through ROR2/RhoA/ROCK pathway in TM cells with the implication of having a similar role in POAG.

Wnt ligand degenerate PCR is a powerful method that enabled us to determine Wnt ligand expression upon DEX treatment. Our result showed that two of the most abundant Wnt ligands in TM cells were *Wnt2* and *Wnt5a*. After DEX treatment, the *Wnt2* level decreased 3-fold, while the *Wnt5a* level increased 4-fold. This dramatic change in Wnt ligand expression indicated that the balance between canonical and noncanonical Wnt signaling is tipped toward the noncanonical Wnt pathway. Reduced expression of *Wnt2* in DEX-treated TM cells suggested a reduction in canonical Wnt signaling. The association between reduced canonical Wnt signaling and glaucoma has been well established. The Wnt inhibitor *Sfrp1* has been reported to be expressed preferentially in glaucomatous tissues, and has been found to reduce the aqueous outflow and elevate IOP in a perfusion model.²³ Others have

reported that myocilin, a DEX-induced glaucoma-associated protein,²⁴ represses canonical Wnt signaling and activates Rac1/JNK.²⁵ It still is unclear how reduced canonical Wnt signaling leads to glaucoma, because there is no direct link between canonical Wnt and CLAN formation. We hypothesized that the missing link is noncanonical Wnt signaling. Specifically, reduced canonical Wnt signaling may cause the activation of noncanonical Wnt, which, in turn, affects the stress fiber organization. We found the activation of noncanonical Wnt signaling in our new mouse model of glaucoma¹³ and applied that concept to DEX-induced CLAN formation. As expected, we verified the upregulation of noncanonical Wnt ligand (*Wnt5a*) in DEX-treated TM cells. We also demonstrated that DEX induces CLAN formation through noncanonical Wnt receptor (*Ror2*). We hypothesized that reduced canonical Wnt signaling is the initiator and increased noncanonical Wnt signaling is the executor in DEX-induced CLAN formation. It is known that noncanonical Wnt can suppress canonical Wnt signaling,²⁶ but it is not clear if canonical Wnt signaling can control the expression of noncanonical Wnt ligand (*Wnt5a*). Even though we observed downregulation of canonical Wnt ligand and upregulation of noncanonical Wnt ligand, we still do not know which occurs first.

In an attempt to find the potential mechanisms for how canonical Wnt controls the expression of *Wnt5a*, we examined the transcription factor binding elements on promoter B of *Wnt5a*. Among them, SBEs (Smad-binding elements) and Fox-binding sites were chosen for their association with glaucoma. Gene expression assay revealed that *Bmp4* was upregulated, while *Foxc1* was downregulated by DEX treatment. *Foxc1* also is downregulated in our new mouse model of glaucoma,¹³ and *Bmp4* and *Foxc1* are well known for their association with congenital glaucoma.^{27–29} *Foxc1* is also related to canonical Wnt signaling³⁰ and potentially can bind to the FOX binding site on promoter B of *Wnt5a*.¹⁸ At this point, it still is not clear if knocking-down *Foxc1* expression in TM cells alone is enough to induce CLAN formation and we will explore the function of *Foxc1* in DEX-induced CLAN formation in the future. Two other observations led us to test the effect of *BMP4* on TM cells: first, *BMP4* activates SMADs, and *Wnt5a* promoter has several SBEs (Smad-binding elements). Secondly, corneal stroma-specific ablation of β-catenin or *Lrp5/Lrp6*, which blocks canonical Wnt signaling, leads to upregulation of *Bmp4* (Zhang Y, oral communication, 2012). We reported here that BMP4 protein induced CLAN formation just as the DEX or WNT5a did in TM cells. So, *BMP4* is a promising link between canonical and noncanonical Wnt signaling, but more studies are required to verify this hypothesis. Finally, it is worthwhile to test a model proposed by Grumolato et al.³¹ that canonical and noncanonical Wnt pathway reciprocally inhibit each other by competition of ligands for cell surface binding of Fzd. According to this model, reduced *Wnt2* expression will make more Fzd available to transducing WNT5a signaling, activation of ROR2 by WNT5a may trigger a positive feedback loop to stimulate the expression of *Wnt5a*. So, we proposed the following model: DEX suppresses the expression of *Wnt2*,³² which leads to reduced canonical Wnt signaling; reduced canonical Wnt signaling leads to the upregulation of *Wnt5a* through as yet undefined mechanisms; and WNT5a promotes CLAN formation through the ROR2/RhoA/ROCK pathway. Extensive loss-of-function and rescue studies in cell culture, organ culture, and experimental animals will be required to prove this model.

It is worthwhile to note that all of our observations were made in a cell culture system. We do not exclude the possibility that DEX may stimulate other RhoA/ROCK

agonists, such as endothelin³³ and TGF- β , in the surrounding tissues of TM in vivo. These soluble factors can then modulate stress fiber rearrangement in TM in a paracrine fashion. We also observed different effects of TGF- β 2 on TM cells. In our hands, TGF- β 2 failed to promote CLAN formation in TM cells; instead, it induced the formation of massive thickened, parallel stress fiber bundles. O'Reilly et al.³⁴ reported CLAN formation in TGF- β 2-treated bovine TM cells. One apparent difference between the two systems is the species of the cell. Bovine TM cells seem more sensitive to CLAN induction by various reagents.³⁴

Several companies are conducting phase 2 clinical studies to test the efficacy of ROCK inhibitors, including ATS907 (Altheos, Inc., San Francisco, CA), AR-12286 (Aerie Pharmaceuticals, Inc., Bedminster, NJ), and K-115 (Kowa Optimed, Inc., Torrance, CA), to treat POAG. The upstream agonists of ROCK still are elusive in POAG and DEX-induced ocular hypertension. Our study indicated that noncanonical Wnt signaling is the potential mechanism for ROCK activation in steroid-induced glaucoma. We demonstrated that DEX induces the upregulation of noncanonical Wnt ligand *Wnt5a*. WNT5a induces CLAN formation through the noncanonical Wnt receptor ROR2/RhoA/ROCK signaling axis. Based on our observations, the relationship between canonical and non-canonical Wnt signaling warrants more attention in searching for the pathogenic mechanism of POAG.

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