Existence of the Canonical Wnt Signaling Pathway in the Human Trabecular Meshwork

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Purpose. We previously discovered elevated levels of *secreted frizzled-related protein 1 (sFRP1),* the Wnt signaling pathway inhibitor, in the glaucomatous trabecular meshwork (GTM), and found that key canonical Wnt signaling pathway genes are expressed in the trabecular meshwork (TM). The purpose of our study was to determine whether a functional canonical Wnt signaling pathway exists in the human TM (HTM).

Methods. Western immunoblotting and/or immunofluorescent microscopy were used to study β-catenin translocation as well as the actin cytoskeleton in transformed and primary HTM cells. A TCF/LEF luciferase assay was used to study functional canonical Wnt signaling, which was confirmed further by WNT3a-induced expression of a pathway target gene, *AXIN2*, via quantitative PCR. Intravitreal injection of an Ad5 adenovirus expressing Dickkopf-related protein-1 (DKK1) was used to study the in vivo effect of canonical Wnt signaling on IOP in mice.

Results. WNT3a induced β-catenin translocation in the HTM, which was blocked by co-treatment with sFRP1. Similarly, WNT3a enhanced luciferase levels in TCF/LEF luciferase assays, which also were blocked by sFRP1. Furthermore, *AXIN2* expression was elevated significantly by WNT3a. However, neither WNT3a nor sFRP1 affected actin cytoskeleton organization, which theoretically could be regulated by noncanonical Wnt signaling in HTM cells. Exogenous DKK1, a specific inhibitor for the canonical Wnt signaling pathway, or sFRP1 elevated mouse IOP to equivalent levels.

Conclusions. There is a canonical Wnt signaling pathway in the TM, and this canonical Wnt pathway, but not the noncanonical Wnt signaling pathway, regulates IOP. (*Invest Ophthalmol Vis Sci.* 2012;53:7043–7051) DOI:10.1167/iovs.12-9664

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Glaucoma is a leading cause of blindness worldwide.[1] It is characterized by optic neuropathy and subsequent visual loss. The exact disease mechanism(s) of primary open angle glaucoma (POAG), the most common form of glaucoma, is not clear. However, elevated IOP is the most important risk factor for the development and progression of this disease.[2–5] An intriguing question in glaucoma research is, at a basic mechanistic level, why and how IOP is elevated in POAG patients.

The trabecular meshwork (TM) is the key component of the aqueous humor outflow pathway, which regulates IOP because it contributes to the majority of outflow resistance.[6] In POAG, the TM undergoes a series of pathologic changes, causing increased outflow resistance and elevated IOP.[6] Many studies have shown that abnormalities in cell signaling pathways in the TM contribute to this pathogenesis,[7–12] and Wnt signaling appears to be important in this regard. Previously, we found that the Wnt signaling inhibitor *secreted frizzled-related protein 1 (sFRP1)* was elevated significantly in the glaucomatous TM (GTM).[13] sFRP1 binds and sequesters pathway activators (e.g., WNTs), thus blocking pathway activation.[14,15] Alternatively, sFRP1 inhibition might involve interaction with the Wnt receptors in the Frizzled (Fzd) family.[16] Exogenous sFRP1 caused ocular hypertension (OHT) in perfusion-cultured human anterior segments as well as in mouse eyes. Further analysis showed a significant decrease of β–catenin, the key mediator of the canonical Wnt signaling pathway, in sFRP1-perfused human eyes. In addition, the OHT caused by sFRP1 in the mouse eye could be alleviated by co-treatment with a small molecule GSK3β inhibitor, which enhances the canonical Wnt signaling activity.[13]

Wnt signaling is involved in various physiologic and pathologic events, including proliferation, differentiation, homeostasis, and tumorigenesis.[17] There are several different Wnt signaling pathways. β–catenin is the major mediator of the canonical pathway, while Ca^{2+}, Rac, and Rho are among the mediators of the noncanonical pathway.[18,19]

The canonical Wnt signaling pathway switches between on- and off-states.[20] In the absence of Wnt activators, the off-state forms a β–catenin protein destruction complex comprised of AXIN, APC, GSK3β, and CKI in the cytoplasm. This complex mediates the phosphorylation of β–catenin, leading to its proteasomal degradation. In the on-state, Wnt binds to Fzd and co-receptor low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), which activates Dishevelled (Dvl) and leads to the disassembly of the destruction complex. Consequently, β–catenin accumulates in the cytoplasm and translocates into the nucleus, where it alters gene expression by association with TCF/LEF transcription factors binding to TCF/LEF cis-elements.

In contrast to the canonical Wnt signaling pathway, the noncanonical Wnt signaling pathways do not require β–catenin or LRP5/6 for signal transduction. One of the well-characterized noncanonical Wnt pathways is the planar cell polarity (PCP) pathway.[18,19] In this pathway, Wnt-Fzd binding

activates Dvl, which then complexes with Dvl-associated activator of morphogenesis (DAAM). The Dvl-DAAM complex activates Rho, which subsequently activates Rho-associated kinase (ROCK). Activated ROCK induces actin fiber contraction by phosphorylation of myosin light chain (MLC) and MLC phosphatase.

We previously reported that several canonical Wnt signaling pathway genes are expressed in the TM.[13] The purpose of our study was to determine whether there is a functional canonical Wnt signaling pathway in the human TM (HTM).

Methods

TM Cell Culture

All primary HTM cells, including normal TM (NTM) and GTM cells, as well as transformed cells (GTM-3) were a kind gift from Alcon Research, Ltd. (Fort Worth, TX). NTM cells were generated from normal donors and GTM cells were generated from glaucoma donors. HTM cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-low glucose medium (Thermoscientific, Worcester, MA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) with 1% penicillin + streptomycin and 2 mM glutamine (Thermoscientific). Our previous studies showed that cell confluence affects sFRP1 expression[13] and we also wanted to mimic in vivo conditions under which TM cells are confluent. Therefore, primary HTM cells were cultured for an additional 7 days after becoming confluent before they were subjected to subsequent manipulation. The only exception was the luciferase assay, for which nonconfluent primary HTM cells were transduced with the lentiviral luciferase reporter vector to increase transduction efficiency.

Immunofluorescence

To study β-catenin translocation, confluent GTM-3 cells cultured on coverslips in 24-well plates were treated with or without 100 ng/mL WNT3a (R&D Systems, Minneapolis, MN) for 4 hours. After washing in PBS, the cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS at 4°C for 30 minutes, washed with PBS, incubated with 0.5% Triton X-100 (Fisher Scientific, Pittsburgh, PA) at room temperature (RT) for 30 minutes, blocked with Superblock (Thermoscientific), and probed with a rabbit anti-β-catenin primary antibody (1:100; Cell Signaling, Beverly, MA) at 4°C overnight. After several PBS washes, cells were probed with a goat anti-rabbit-Alexa-488 secondary antibody (1:200; Invitrogen) at RT for 2 hours. Coverslips were transferred to glass slides and mounted with Prolong Gold mounting medium with or without DAPI (1:100; Invitrogen) for visualization of cell nuclei.

To study actin stress fibers, primary HTM cells were cultured on coverslips placed in 24-well plates. When primary HTM cells had been confluent for 1 week, they were treated with or without 100 or 500 ng/mL WNT3a, 2 or 10 μg/mL sFRP1 (R&D Systems), 10 μM Y27632[21] (Alcon), or 20 μM LPA[21] (Sigma-Aldrich, St. Louis, MO) for 1 or 24 hours. At the end of each treatment, cells were processed as described earlier except that they were stained with phalloidin-Alexa-488 or phalloidin-Alexa-568 (Invitrogen) at RT for 30 minutes.

Fluorescent images were taken using a Nikon Ti microscope (Nikon, Melville, NY) with the Nuance Imaging System (CRI, Hopkinton, MA).

Western Immunoblotting (WB)

Confluent GTM-3 cells or primary HTM cells cultured for an additional 7 days after they were confluent were treated with or without 100 ng/mL WNT3a and/or 2 μg/mL sFRP1 for 4 hours. Nuclear proteins and cytosolic proteins were extracted with the NE-PER kit (Thermoscientific). After protein concentrations were determined with the DC protein assay kit (Bio-Rad, Hercules, CA), equal amounts of proteins were boiled in Laemmli buffer, separated on 10% SDS-PAGE gels, transferred to PVDF membranes, blocked with 5% dry milk in tris-buffered saline/Tween (TBST), and probed with primary antibodies and corresponding secondary antibodies conjugated with horseradish peroxidase (HRP). The primary antibodies used in our study included mouse anti-β-catenin (1:500; R&D Systems), rabbit anti-laminA/C (1:1000; Cell Signaling), and rabbit antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000; Cell Signaling). The secondary antibodies included goat anti-mouse-HRP and goat anti-rabbit-HRP (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA). Signals were developed with the SuperSignal West Femto Substrate (Thermoscientific) and detected using the FluoroChem digital imaging system (Cell Biosciences, Santa Clara, CA). All experiments were performed in biologic replicates (cells from the same strain were used to repeat the experiments at least three times). Representative data are shown.

For the study of MLC phosphorylation, primary GTM cells were treated with WNT3a (100 or 500 ng/mL), sFRP1 (2 or 10 μg/mL), Y27632 (10 μM), or LPA (20 μM) for 1 hour. After a PBS wash, cells were lysed in 2D-buffer containing 7 M urea, 3.5 M thio-urea, CHAPs, and Tris. Protein concentration was measured with the EZQ kit (Invitrogen). For each batch of samples, 5 to 10 μg total protein were separated on two 18% SDS-PAGE gels. After protein transfer, the blots were probed with anti-double phospho-MLC (1:1000) or anti-total MLC (1:500; Cell Signaling), and then probed with goat-anti-rabbit-HRP secondary antibody (1:10,000; Cell Signaling). Images were developed as described above. Each blot was stripped with stripping buffer, and re-probed with anti-GAPDH antibody and secondary antibody for imaging.

Densitometry

The optical density (OD) of each protein band was measured by using the Image J software developed by the National Institutes of Health.

For β-catenin nuclear translocation study, the OD of β-catenin was normalized to laminA/C (for nuclear β-catenin) or GAPDH (for cytosolic β-catenin), that is β-catenin OD/laminA/C OD or β-catenin OD/GAPDH OD, respectively. The OD ratios of nuclear control and cytosolic control then were set at 1.0. The other ratios were normalized to their corresponding control.

Luciferase Assay

For GTM-3 cells, we used plasmid-based TCF/LEF luciferase reporter vectors (Cignal Report; Qiagen, Frederick, MD) according to the manufacturer's instructions. 2×10^4 GTM-3 cells were transfected with 100 ng TCF/LEF reporter plasmid and 0.6 μL SureFECT (Qiagen) in Opti-MEM (Invitrogen), and seeded into individual wells of 96-well opaque plates (BD Falcon, Franklin Lakes, NJ). At 16 hours after transfection, cells were incubated in assay medium (Opti-MEM with 0.5% FBS) for 8 hours. After incubation, cells were treated with or without indicated concentrations of WNT3a and/or sFRP1 for 16 hours. The Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to develop luciferase signals, which were measured using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Firefly luciferase levels were normalized to renilla luciferase. Experiments were performed in triplicate ($n = 3$).

For primary TM cells, we used lentivirus-based TCF/LEF luciferase reporter vectors (Cignal Lenti; Qiagen). We seeded 3000 TM cells into individual wells of 96-well opaque plates. The next day, cells were transduced with the lentivirus at a multiplicity of infection of 100:1 in a total volume of 50 μL medium containing 5 μg/mL SureEntry (Qiagen). After incubation for 24 hours, medium was changed. After another 24 hours of incubation, cells were treated with or without 100 ng/mL WNT3a and/or 2 μg/mL sFRP1 for 18 hours. The Bright-Glow Luciferase Substrate (Promega) was used to develop luciferase signals, which were measured using a M200 plate reader (Tecan, Durham, NC). Experiments were performed in pentaplicates.

Quantitative PCR (qPCR)

HTM cells cultured in 12-well plates were treated with or without 100 ng/mL WNT3a for 4 hours. Total RNA was isolated using the RNeasy Micro Kit (Qiagen). cDNA was synthesized with 0.5 or 1 μg total RNA using the High Capacity cDNA Reverse Transcription Kit (AB Applied Biosystems, Carlsbad, CA). qPCR was performed using one of two protocols. (1) The reaction mixture (25 μL) contained 2.5 μL of 10× PCR buffer (Sigma), 1.25 μL of 25 mM $MgCl_2$, 0.4 μL JumpStart Taq (Sigma), 0.5 μL of 10 mM dNTPs (Promega), 1.25 μL dimethyl sulfoxide (DMSO), 5 μL of 5M betaine (Fisher Scientific), 0.1 μL of 0.1 mM forward/reverse primer, 1.25 μL of 20× EvaGreen (Biotium, Hayward, CA), and 0.37 μL of the reference dye Rox in a MX3000P thermocycler (Agilent Technologies, Santa Clara, CA). The thermoprofile was 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds, consisting of 40 cycles followed by a dissociation curve. (2) An SSoAdvanced SYBR Green Supermix (Bio-Rad) in a total volume of 20 μL was used in a CFX96 thermocycler (Bio-Rad). The thermoprofile was 95°C for 15 seconds, 60°C for 30 seconds, consisting of 40 cycles followed by a dissociation curve. PCR primer (Sigma-Aldrich) sequences were:

AXIN2 forward: 5′CAGATCCGAGAGGATGAAGAGA3′
AXIN2 reverse: 5′AGTATCGTCTGCGGGTCTTC3′, giving an amplicon of 128 base pairs (bp).
GAPDH forward: 5′ GGTGAAGGTCGGAGTCAAC 3′
GAPDH reverse: 5′ CCATGGGTGGAATCATATTG 3′,[22] giving an amplicon of 153 bp.

The *GAPDH* gene was used as an internal control. Because the ΔΔCt method compares the relative expression level of one sample to the other, a "reference" sample is required for analysis. To compare *AXIN2* expression levels, one of the control samples was chosen as "reference" and its *AXIN2* level was set at 100%. Then, every other sample was compared to this one to obtain their relative *AXIN2* expression levels for statistical analysis. Therefore, the mean of the control group may not be exactly 100%. All experiments were performed in biologic replicates (cells from the same strain were plated onto multiple plates/wells).

Construction of the Recombinant Ad5 Adenoviral Vector

The Ad5-CMV-sFRP1 virus was constructed previously[13] and was a kind gift from Alcon Research, Ltd. For the Ad5-CMV-DKK1 virus, we first cloned the human *Dickkopf-related protein 1* (*DKK1*) cDNA (NCBI Gene ID: NM_012242) into the pGEMT vector (Promega). Then, the *DKK1* sequence was cut at KpnI (5′) and SpeI (3″) sites, and inserted at the same sites into the pacAd5-CMV shuttle vector (Gene Transfer Vector Core, University of Iowa, Iowa City, IA). After sequence confirmation, the pacAd5-CMV-DKK1 vector was sent to the Gene Transfer Vector Core at the University of Iowa for Ad5-CMV-DKK1 viral production.

Intravitreal Injection and Mouse IOP Measurement

All animal procedures performed in our study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all protocols and regulations established by the Animal Care and Use Committee of Alcon Research, Ltd. and the University of North Texas Health Science Center. Adult male BALB/c mice (20–25 g body weight, 2–6 months old; Jackson Laboratories, Bar Harbor, ME) were housed in transparent plastic rodent boxes under 12-hour light/dark cycle with lights on starting at 6 AM. Mouse chow and water were available ad libitum. For intravitreal injections, the animals were anesthetized with a mouse anesthesia cocktail (intraperitoneal injection of ketamine 73 mg/kg, acepromazine 1.8 mg/kg, and xylazine 1.8 mg/kg). Adenoviral vectors of Ad5-CMV-NULL (as a negative control), Ad5-CMV-sFRP1 or Ad5-CMV-DKK1 (3×10^7 PFU/eye) were injected intravitreally in a volume of 2 μL into a randomly-selected eye of each animal as described previously.[13] The contralateral eye was not injected. IOP measurements of conscious animals were performed in a masked fashion using the TonoLab rebound tonometer (Colonial Medical Supply, Franconia, NH) as described previously.[23]

Data Analysis

Data were analyzed using Student's *t*-test or one-way ANOVA and Bonferroni multiple comparison tests, with $P < 0.050$ considered statistically significant ($^*P < 0.050$, $^{**}P < 0.010$, and $^{***}P < 0.001$).

Results

Existence of the Canonical Wnt Signaling Pathway in Transformed HTM Cells

The transformed GTM-3 cell has been characterized to be similar to non-transformed, primary HTM cells in many biologic functions.[24] We used these cells in an initial evaluation of the Wnt signaling pathway due to their relatively rapid proliferation rate and susceptibility to transfection. After treatment with 100 ng/mL WNT3a for 4 hours, GTM-3 cells showed significant β-catenin accumulation in the cytoplasm and translocation to the nucleus as evidenced by immunofluorescence (Fig. 1) and WB analysis (Fig. 2A). The accumulation and translocation of β-catenin were inhibited by co-treatment with sFRP1 (Fig. 2A). These data suggested the presence of a functional canonical Wnt signaling pathway in GTM-3 cells.

To study whether this WNT3a-induced β-catenin translocation could alter gene expression via TCF/LEF cis-elements, a luciferase reporter vector, containing TCF/LEF cis-elements regulating luciferase expression, was transfected into GTM-3 cells. Treatment with WNT3a elevated Wnt signaling activity dose-dependently (Fig. 2B, $P < 0.001$, $n = 3$), and this elevation was blocked dose-dependently by sFRP1 (Fig. 2C, $P < 0.001$, $n = 3$). The luciferase assay showed that translocated β-catenin was able to induce gene expression through the TCF/LEF binding elements.

We studied further whether activation of the Wnt pathway could upregulate the expression of an endogenous Wnt-regulated gene. AXIN2 can function as a component of the β-catenin destruction complex. There are TCF/LEF regulatory elements in the promoter region of *AXIN2,* and this gene often is upregulated by the Wnt signaling pathway, providing negative feedback regulation.[25–27] For this reason, *AXIN2* is used frequently as a marker for canonical Wnt/ β-catenin pathway activation. qPCR studies revealed that treatment with 100 ng/mL WNT3a for 4 hours significantly elevated *AXIN2* expression by 17-fold in GTM-3 cells (Fig. 2D, $P < 0.001$, $n = 6$).

The Existence of the Canonical Wnt Signaling Pathway in Primary HTM Cells

To confirm our findings in GTM-3 cells, we investigated further the Wnt pathway in two primary HTM cell strains. We cultured primary HTM cells, treated them with or without 100 ng/mL WNT3a and/or 2 μg/mL sFRP1 for 4 hours, and then extracted nuclear and cytosolic proteins for WB analysis. Similar to GTM-3 cells, WNT3a induced β-catenin translocation to the nucleus, which was inhibited by co-treatment with sFRP1 (Fig. 3A). These data suggested a functional canonical Wnt signaling pathway was present in primary HTM cells.

We also performed luciferase assays in primary HTM cells. In contrast to GTM-3 cells, primary HTM cells are very difficult to transfect by plasmid vectors using conventional methods,

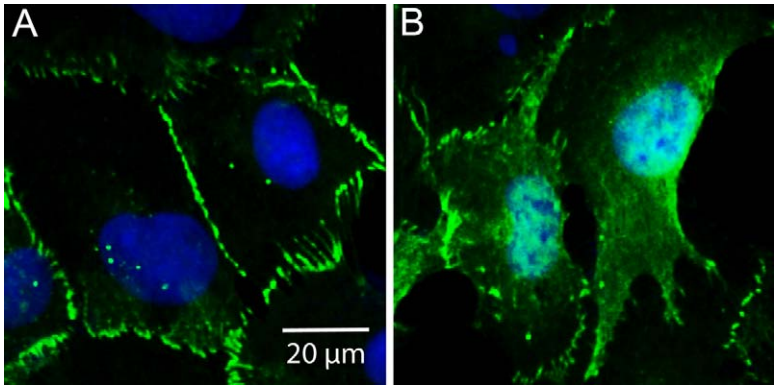

Figure 1. Immunofluorescent study of WNT3a-induced β-catenin translocation in transformed GTM-3 cells. GTM-3 cell cultures were treated without (**A**) or with (**B**) 100 ng/mL WNT3a for 4 hours. (**A**) β-catenin (*green*) was expressed at a low level in the control cells, and was located mainly at the submembranous region at the periphery of the cells. (**B**) WNT3a significantly elevated β-catenin levels in the cytoplasm and nucleus (*blue*: DAPI staining). Experiments were repeated three times using GTM-3 cells, and representative images are shown.

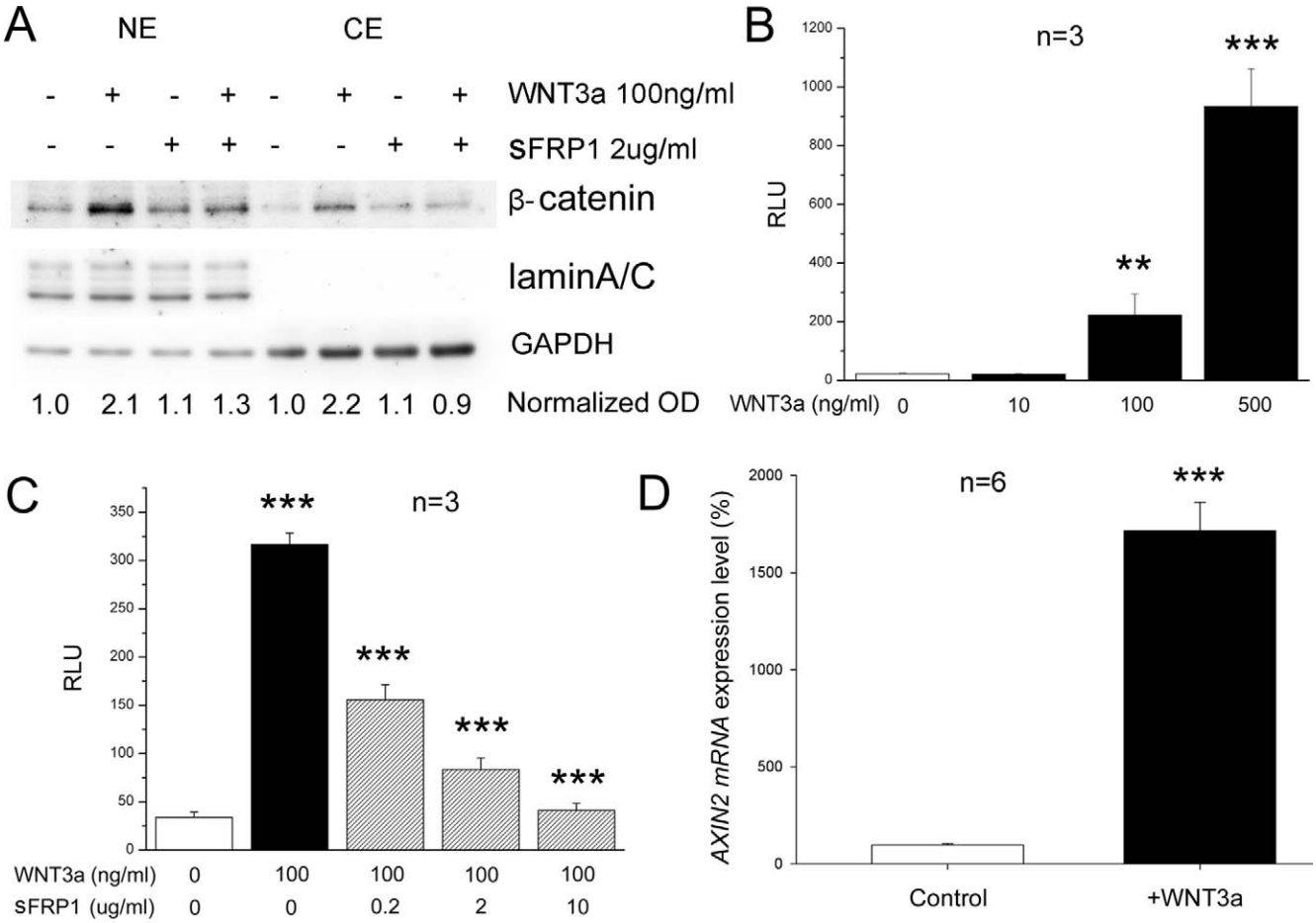

Figure 2. Existence of a functional canonical WNT signaling pathway in transformed GTM-3 cells. (**A**) Western blot of nuclear extract proteins (NE) and cytosolic extract proteins (CE) from GTM-3 cells treated with or without WNT3a and/or sFRP1 for 4 hours. LaminA/C served as a nuclear protein loading control, while GAPDH served as a cytosolic protein loading control. The OD of each β-catenin band was normalized and the values are shown on the bottom of the Western blot. (**B**, **C**) Plasmid-based luciferase assays of the canonical Wnt signaling pathway in GTM-3 cells. GTM-3 cells were transfected with TCF/LEF luciferase reporter vectors and treated with WNT3a in the absence (**B**) or presence (**C**) of sFRP1 at indicated concentrations. Columns represent relative luminescence units (RLU), as a measure of luciferase levels. *White columns*: control. *Black columns*: cells treated with WNT3a. *Hatched columns*: cells treated with WNT3a and sFRP1. Data were analyzed by ANOVA and Bonferroni multiple comparison tests. (**D**) Comparison of *AXIN2* mRNA expression levels of GTM-3 cells treated without (*left, white column*) or with (*right, black column*) 100 ng/mL WNT3a for 4 hours. Data were analyzed by Student's *t*-test. *Columns and error bars*: mean ± SD. $^{**}P < 0.010$. $^{***}P < 0.001$.

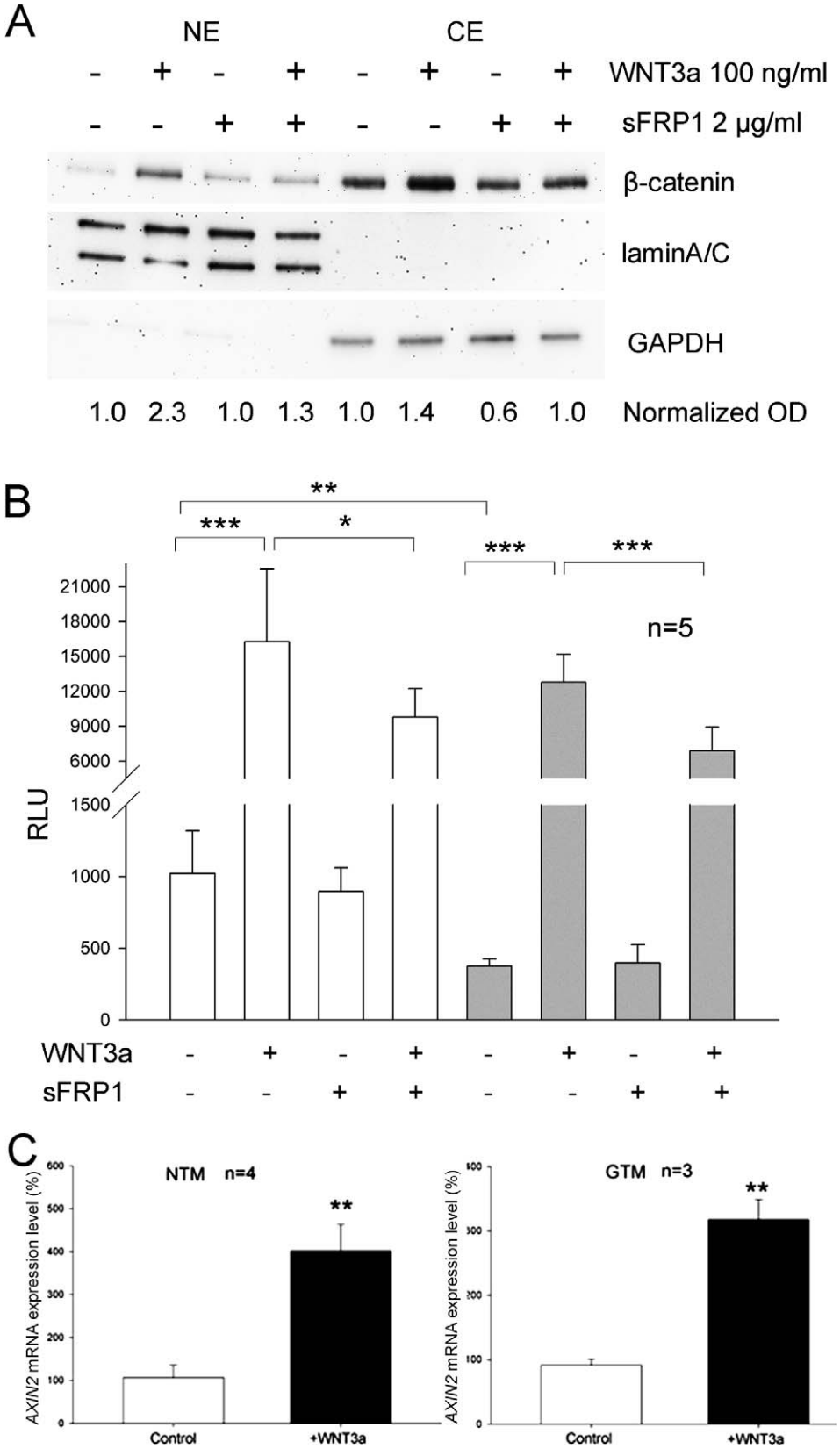

Figure 3. Existence of a functional canonical WNT signaling pathway in primary HTM cells. (**A**) Western blot of NE proteins and CE proteins from a primary NTM cell strain treated with or without WNT3a (100 ng/mL) and/or sFRP1 (2 μg/mL) for 4 hours. LaminA/C served as a nuclear protein loading control, while GAPDH served as a cytosolic protein loading control. Three different HTM strains were tested and representative data are shown. The OD of each β-catenin band was normalized first to laminA/C (for nuclear β-catenin) or GAPDH (for cytosolic β-catenin). They then were normalized to corresponding control (non-treated, which was set at 1.0), and the values are shown on the bottom of the Western blot. (**B**) Lentivirus-based luciferase assays of the canonical Wnt signaling pathway in primary NTM (*white columns, left panel*) and GTM (*grey columns,*

right panel) cells. HTM cells were transfected with the lentiviral TCF/LEF reporter, and treated with or without 100 ng/mL WNT3a and/or 2 μg/mL sFRP1. Columns represent RLU. Data were analyzed by ANOVA and Bonferroni multiple comparison tests except for the comparison between columns 1 and 5 (*from left to right*), for which Student's *t*-test was used. Two different HTM strains were tested and representative data are shown. (**C**) Comparison of *AXIN2* mRNA expression levels of primary NTM (*white columns*) and GTM (*black columns*) cells treated without or with 100 ng/mL WNT3a for 4 hours. *Left panel*: NTM cells. *Right panel*: GTM cells. Data were analyzed by Student's *t*-test. *Columns and error bars*: mean ± SD. $^{**}P < 0.010$. $^{***}P < 0.001$.

which limits the application of plasmid-based luciferase assays in these cells. To circumvent this problem, we used a lentivirus-based luciferase assay system to study the Wnt/β-catenin signaling pathway. WNT3a (100 ng/mL) significantly induced luciferase expression in two HTM cell strains (one NTM and one GTM strain, $P < 0.050$, $n = 5$), and this induction could be blocked by co-treatment with 2 μg/mL sFRP1 ($P < 0.050$, $n = 5$, Fig. 3B). In addition, the basal Wnt signaling activity of the NTM cell strain was higher than that of the GTM cell strain (Fig. 3B, columns 1 vs. 5), supporting a previous study suggesting diminished Wnt signaling in GTM cells is due to higher endogenous levels of sFRP1.[13]

Besides luciferase assays, we also studied WNT3a-induced *AXIN2* expression in the same primary HTM cells. *AXIN2* expression levels were increased by approximately 3-fold in the two HTM cell strains treated with WNT3a (Fig. 3C, $n = 3$–4, $P < 0.010$).

The Wnt Pathway Ligands Did Not Affect Actin Cytoskeleton Organization in the TM

To determine whether IOP elevation in the human eye by exogenous sFRP1 was mediated by the noncanonical PCP-Rho-ROCK pathway, we treated primary HTM cells with WNT3a or SFRP1, and examined the organization of the F-actin cytoskeleton by immunofluorescence and the phosphorylation of MLC by WB.

We treated the primary HTM cells, including the NTM and GTM cell strains, with or without WNT3a or sFRP1 at two different concentrations. WNT3a had been shown to stimulate Rho activation and formation of stress fibers in other cells.[28] We included the ROCK inhibitor Y27632, which disrupts actin stress fibers, and the Rho activator LPA, which enhances the formation of actin stress fibers,[29,30] as negative and positive controls, respectively (Fig. 4, see Supplementary Fig. S1, available at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9664/-/DCSupplemental).[21] We found 1-hour (data not shown) or 24-hour (Fig. 4, see Supplementary Fig. S1, available at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9664/-/DCSupplemental) treatment with either WNT3a or sFRP1 did not alter actin stress fibers in GTM (Fig. 4, see Supplementary Fig. S1, available at http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9664/-/DCSupplemental) or NTM cells (data not shown).

We also studied the effects of WNT3a and sFRP1 on the phosphorylation of MLC, which is a key component that regulates the TM actin cytoskeleton. Similarly, we treated primary HTM cells with or without WNT3a or sFRP1 at two different concentrations, and isolated whole cell lysates for WB analysis. We did not find any significant changes in MLC

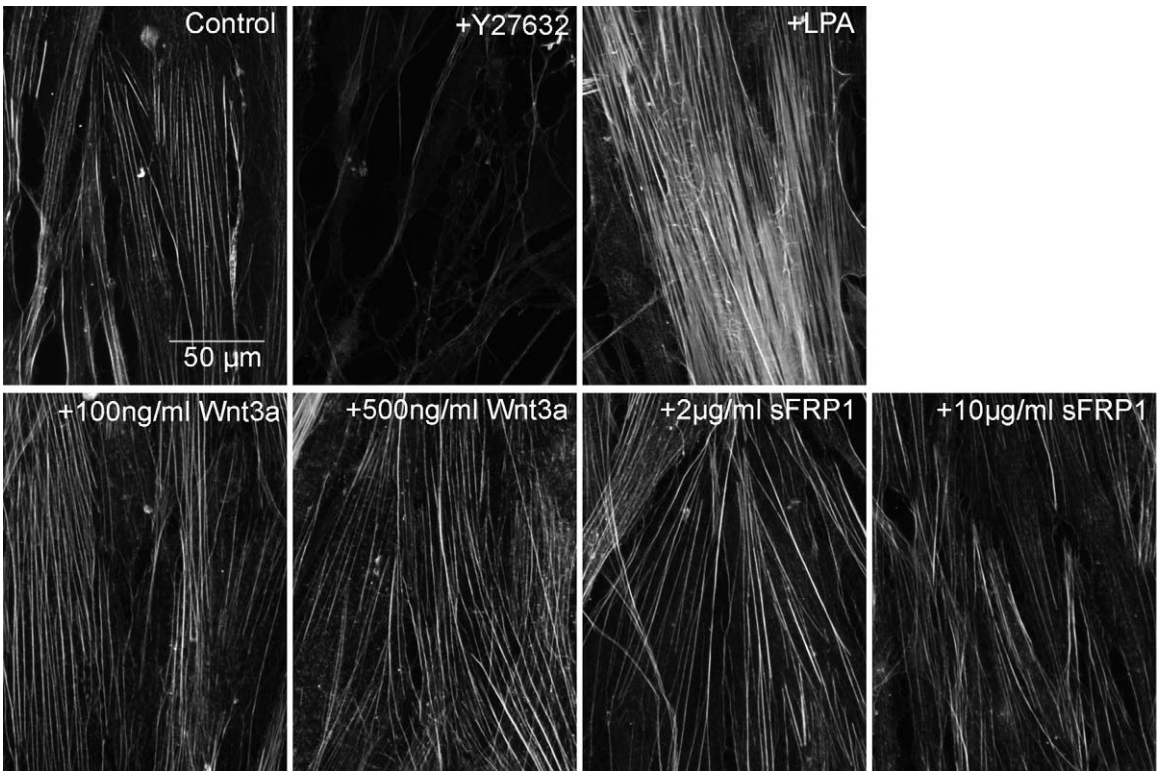

Figure 4. The organization of actin stress fibers was not affected by WNT3a or sFRP1. Primary GTM cells were treated with WNT3a (100 or 500 ng/mL), sFRP1 (2 or 10 μg/mL), Y27632 (10 μM), or LPA (20 μM) for 24 hours. Actin stress fibers were stained with phalloidin-Alexa-594. Neither WNT3a nor sFRP1 induced significant changes in actin stress fibers. For comparison, the ROCK inhibitor Y27632 disrupted actin stress fiber formation, while the Rho activator LPA enhanced actin stress fiber formation.

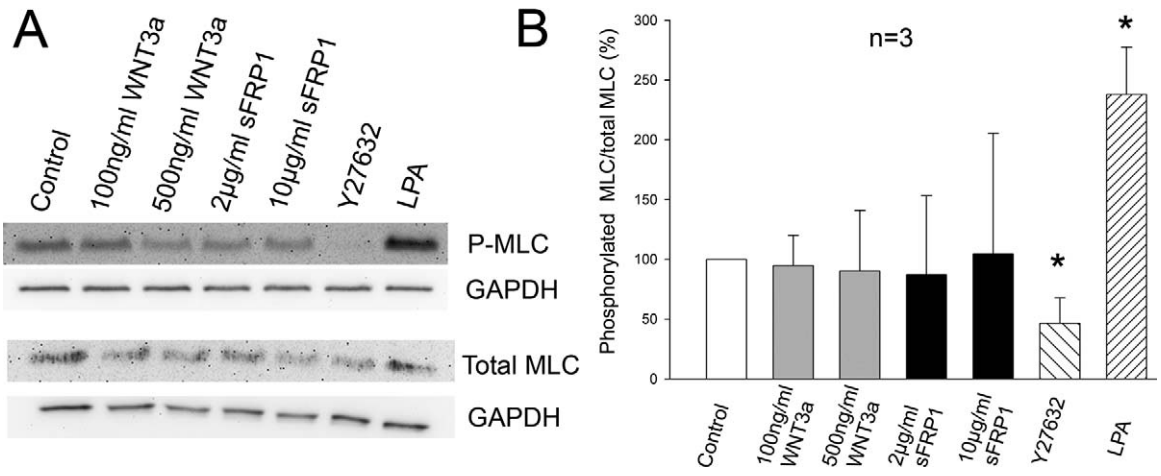

Figure 5. MLC phosphorylation was not affected by WNT3a or sFRP1. Primary GTM cells were treated with WNT3a (100 or 500 ng/mL), sFRP1 (2 or 10 μg/mL), Y27632 (10 μM), or LPA (20 μM) for 1 hour. WB of whole cell lysates was used for study MLC phosphorylation, as shown in (**A**). (**B**) Quantitative study of MLC phosphorylation. Double phospho-MLC (P-MLC) or total MLC was normalized first to GAPDH. Then, the amount of P-MLC was normalized to total MLC. Finally, the control was set at 100% and the other samples were normalized to the control. The paired Student's *t*-test was performed. *$P < 0.050$ compared to control.

phosphorylation caused by WNT3a or sFRP1 (Fig. 5). In contrast, Y27632 inhibited MLC phosphorylation while LPA enhanced it (Fig. 5).

These results suggested that PCP signaling is not stimulated by WNT3a or sFRP1 in the TM and that Wnt/β-catenin signaling is the major Wnt signaling pathway in these cells.

The Canonical Wnt Signaling Pathway Has a Major Role in IOP Regulation

To determine whether Wnt/β-catenin signaling or noncanonical Wnt pathways regulates IOP, we overexpressed the specific β-catenin pathway inhibitor DKK1 in mouse eyes using a recombinant Ad5-CMV-DKK1 vector. Ad5 vectors have selective trophism for the TM.[13,31] Whereas sFRP1 disrupts the binding of WNT3a to Fzds, DKK1 blocks signal transduction by inhibiting WNT binding to its co-receptor, LRP5/6.[32] Because activation of the noncanonical Wnt pathways does not require LRP5/6, DKK1 does not interfere with these signaling mechanisms.

We intravitreally injected the Ad5-CMV-NULL (as a negative control), Ad5-CMV-DKK1, or Ad5-CMV-sFRP1 (as a positive control) vector into one of the mouse eyes, leaving the uninjected contralateral eye as an additional negative control, and measured conscious IOP (Fig. 6). Significant IOP elevation was observed from the seventh day post-injection in the DKK1 and sFRP1 injected eyes ($P < 0.001$). In uninjected or Ad5-CMV-NULL virus injected eyes, the IOP was approximately 12 mm Hg throughout the study. Since DKK1 and sFRP1 had similar impact on IOP, it appears that the canonical, but not the noncanonical Wnt pathways, have a major role in IOP regulation in the mouse eye.

Discussion

In our study, we found that WNT3a treatment induced β-catenin nuclear translocation, increased expression of the TCF/LEF luciferase reporter gene, and upregulated the Wnt/β-catenin target gene *AXIN2* in cultured HTM cells. These findings demonstrated the existence of the canonical Wnt signaling pathway in the TM. The fact that neither WNT3a nor sFRP1 altered the actin cytoskeleton, and that exogenous DKK1 elevated IOP in the mouse further supported our hypothesis that β-catenin signaling, but not the noncanonical Wnt pathways, has a key role in IOP regulation and glaucoma pathogenesis.

We discovered previously elevated expression of the Wnt signaling inhibitor sFRP1 in the GTM.[13] Correspondingly, in our study, our lentivirus-based luciferase assays showed the basal canonical Wnt signaling activity was lower in the GTM, compared to the NTM, which most likely was due to higher basal sFRP1 levels in GTM cells. How inhibition of the canonical Wnt signaling affects IOP is not clear. Here, we speculated that the impact of the Wnt/β-catenin pathway on the TM occurs at two levels.

The first level is the change in gene expression due to the nuclear translocation of β-catenin. Many studies have shown that activation of the Wnt signaling pathway induces global alterations in the gene expression profile.[33] For example,

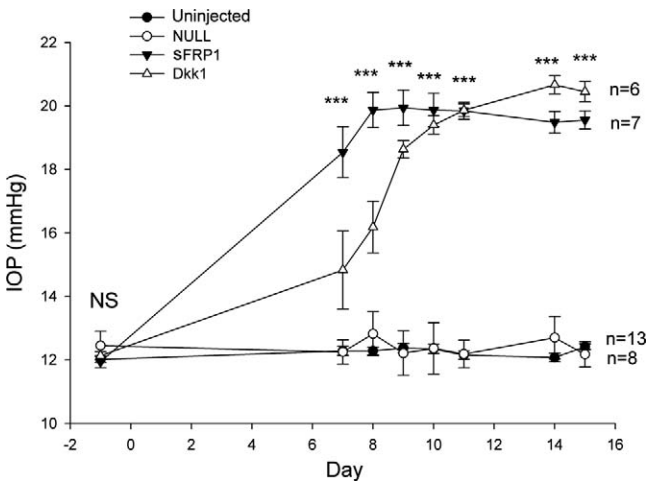

Figure 6. DKK1 elevated IOP in mouse eyes. One eye (randomly selected) of each mouse was injected with Ad5-CMV-NULL, Ad5-CMV-DKK1, or Ad5-CMV-sFRP1 vector on day zero. The Ad5-CMV-NULL as well as contralateral uninjected eyes were used as negative controls. Conscious mouse IOPs were measured, and the mean and SEM of each group were plotted over time. The IOP values of DKK1 and sFRP1 transduced eyes were elevated significantly on days 7 to 15. Data were analyzed by ANOVA. ***$P < 0.001$.

some well-characterized Wnt signaling pathway target genes (data available at http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes), such as *BMP4*, *GREMLIN*, and *ENDOTHELIN-1*,[34–36] are known to be involved in glaucoma pathogenesis.[11,37,38] It is very likely that disturbing the Wnt signaling pathway causes glaucomatous damage to the eye through these factors/pathways as well as other unknown mechanisms. The glaucoma gene *MYOCILIN(MYOC)* has been reported to be a modulator of Wnt signaling,[39] although it is unclear whether glaucomatous *MYOC* mutations, which inhibit MYOC secretion from the TM, have any effects on Wnt signaling in the TM.

The second potential effect of altered Wnt signaling on the TM could be changes in cell-cell junctions and junctional communication. The distribution of β-catenin, the key canonical Wnt signaling pathway mediator, can be divided into three pools.[40,41] The first pool consists of free cytosolic β-catenin, which typically undergoes rapid turnover in the absence of Wnt signaling. The second pool is nuclear β-catenin, which functions as a transcription factor. The third pool is the cadherin-associated β-catenin, which is located at the cell membrane and participates in the formation of adherens junctions. These three pools of β-catenin are interconnected.[42] Activation of Wnt signaling induces the stabilization of β-catenin in the cytosol and may be accompanied by translocation of cadherin-associated β-catenin into the nucleus,[43] which may weaken adherens junctions, cell-cell attachment, and junctional communication. In the presence of sFRP1, β-catenin is phosphorylated and degraded, which may affect all three pools of β-catenin. Although we currently know little about the role of these junctions in the TM, it is possible that the change of adherens junctions and/or cell-cell communication will influence aqueous humor outflow resistance and thereby regulate IOP.

In addition to the question on how the canonical Wnt signaling pathway regulates IOP, the mechanism(s) responsible for elevated sFRP1 expression in the GTM also remains unknown. We recently found that one of the epigenetic regulatory mechanisms responsible for elevated sFRP1 expression in certain types of cancers, DNA methylation, is not involved in the differential sFRP1 expression in NTM and GTM cells.[44] Other mechanisms, such as histone acetylation, microRNA, and cross-talk between the Wnt signaling and other signaling pathways, must be examined.

Finally, although many of the Wnt signaling responses were similar in transformed and primary HTM cells (e.g., β-catenin translocation, TCF/LEF luciferase assay, and *AXIN2* induction), there was some difference between them. First, it was difficult to show clearly significant β-catenin translocation in primary HTM cells by immunofluorescence analysis. Second, there was a 16-fold WNT3a induction of *AXIN2* expression in GTM-3 cells, while that in primary TM cells was only 3-fold. It is possible that due to the lack of major cell-cell contact in transformed cells, there are less adherens junctions in GTM-3 cells, which results in more free cytosolic β-catenin available for nuclear translocation. In addition, the difference in the Wnt signaling pathway components (e.g., Wnt receptors and co-receptors) also may account for the differential responsiveness.

In summary, we have shown that HTM cells contain a functional canonical Wnt signaling pathway, and this pathway regulates IOP. Our previous finding of elevated expression of the Wnt antagonist sFRP1 in the glaucomatous TM indicates that defects in canonical Wnt signaling are involved in glaucomatous IOP elevation. Further investigation is required to elucidate the downstream targets of this aberrant Wnt signaling and their roles in the pathogenesis of POAG.

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