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Wnt inhibition induces persistent increases in intrinsic stiffness of human trabecular meshwork cells

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Abstract

Wnt antagonism has been linked to glaucoma and intraocular pressure regulation, as has increased stiffness of human trabecular meshwork (HTM) tissue. We have shown culturing HTM cells on substrates that mimic the elevated stiffness of glaucomatous tissue leads to elevated expression of the Wnt antagonist secreted frizzled related protein 1 (SFRP1), suggesting a linkage between SFRP1 and HTM mechanobiology. In this study, we document biomechanical consequences of Wnt antagonism on HTM cells. Cells were treated with the Wnt antagonists (SFRP1, KY02111, and LGK-974) for 8 days and allowed to recover for 4 days. After recovery, intrinsic cell stiffness and activation of the Wnt pathway via β-catenin staining and blotting were assayed. Basal cell stiffness values were 3.71±0.37, 4.33±3.07, and 3.07± kPa (median±S.D.) for cells derived from 3 donors. Cell stiffness increased after 0.25 μg/mL (4.32±5.12, 8.86±8.51, 4.84±3.15 kPa) and 0.5 μg/mL (16.75±5.59, 13.18±7.99, and 8.54±5.77 kPa) SFRP1 treatment. Stiffening was observed after 10μM KY02111 (10.72±5.63 and 6.57±5.53 kPa) as well as LGK-974 (9.60±7.41 and 11.40±9.24 kPa) treatment compared with controls (3.79±1.01 and 5.16±2.14 kPa). Additionally, Wnt inhibition resulted in decreased β-catenin staining and increased phosphorylation at threonine 41 after recovery. In conclusion, this work demonstrates a causal relationship between Wnt inhibition and cell stiffening. Additionally, these findings suggest transient Wnt inhibition resulted in durable modulation of the mechanical phenotype of HTM cells. When placed in context with previous results, these findings provide a causal link between Wnt antagonism and cell stiffness and suggest a feedback loop contributing to glaucoma progression.

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Keywords

human trabecular meshwork; Wnt; secreted frizzled related protein; cell stiffness; atomic force microscopy

Open angle glaucoma (OAG), a leading cause of blindness, is projected to afflict approximately 59 million individuals in 2020 (Quigley and Broman, 2006). Intraocular pressure (IOP) remains the only modifiable causative risk factor of the disease; however, the pathways underpinning IOP regulation remain incompletely understood. Elucidation of these pathways is therefore a primary objective in the prevention and treatment of OAG.

The human trabecular meshwork (HTM) is responsible for drainage of approximately 80% of the aqueous humor of the eye and dysfunction in the HTM is thought to be a critical risk factor in OAG (Gottanka et al., 1997; Johnson, 2006; Lutjen-Drecoll, 2005; Quigley, 1993; Rohen et al., 1993). Indeed, numerous structural changes in the HTM, both at the tissue and cellular levels, have been observed in OAG (Clark et al., 1995; Fuchshofer and Tamm, 2012; Gottanka et al., 1997; Hoare et al., 2009; Lutjen-Drecoll, 2005; Rohen et al., 1993; Saika, 2006). We have proposed that these structural changes increase the intrinsic stiffness of the HTM, increase outflow resistance, and contribute to elevated IOP in OAG. In support of this, we have shown the glaucomatous HTM to be ~20 fold stiffer than normal HTM (Last et al., 2011). We have also demonstrated *in vitro* that the intrinsic stiffness of HTM cells increases when cultured on substrates mimicking the stiffness of glaucomatous HTM (McKee et al., 2011), suggesting the biomechanical properties of the matrix modulate intrinsic cellular stiffness. Elucidating the molecular mechanisms underlying this stiffening at the cellular and tissue levels may point to novel therapeutic targets for the treatment of OAG. One key mechanism may be the inhibition of the Wnt signaling pathway, which is linked to both IOP elevation and HTM mechanobiology, as described below.

Secreted frizzled related protein 1 (SFRP1), a Wnt antagonist, is overexpressed in cultured glaucomatous TM cells and exogenous SFRP1 has been proven sufficient to increase IOP in *ex vivo* human organ culture and *in vivo* mouse models (Wang et al., 2008). Wnt can activate multiple signaling pathways, including a canonical β-catenin-mediated pathway and multiple non-canonical pathways (Clevers, 2006; Freese et al., 2010; Macdonald and He, 2012; Yu and Virshup, 2014). However, it appears that the non-canonical pathways are not involved in IOP regulation and that the effect is not specific to SFRP1, as another antagonist of canonical Wnt pathway (Dickkopf-related protein 1) had similar effects (Mao et al., 2012). These two studies provide firm support for a critical role of canonical Wnt antagonism in IOP regulation and OAG.

Wnt antagonism is also part of the HTM response to mechanical cueing. Our lab has identified SFRP1 as a gene that is upregulated in normal HTM cells grown on substrates that mimic the elevated stiffness of the glaucomatous HTM (Raghunathan et al., 2013). As noted above, we have previously reported HTM cells cultured on stiffer substrates exhibit increased intrinsic stiffness (McKee et al., 2011), suggesting a causal relationship between SFRP1 expression and intrinsic cell stiffness. In this study, we specifically examine whether a causal relationship between Wnt antagonism and HTM cell stiffness exists. We employed

atomic force microscopy (AFM) to determine the mechanical properties of HTM cells after culture in the presence of SFRP1 and other canonical Wnt antagonists.

All experiments were performed in compliance with the Declaration of Helsinki. Primary HTM cells were treated with recombinant SFRP1 (Sigma-Aldrich, St. Louis, MO), LGK-974 (SelleckChem, Houston, TX), a potent inhibitor of Wnt secretion (Liu et al., 2013), KY02111 (SelleckChem), a potent inhibitor of canonical Wnt signaling (Minami et al., 2012), or appropriate vehicle controls. Cells were isolated from donor corneoscleral rims (Saving Sight Eye Bank, St. Louis, MO) as described previously (Morgan et al., 2014). HTM cells were cultured in DMEM/F12 (Hyclone, Logan, UT) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 2 mM penicillin, streptomycin, amphotericin-B (Life Technologies, Carlsbad, CA). HTM cells were plated on glass coverslips for AFM and immunofluorescence and in 60 mm dishes for Western blotting at 25,000 cells/cm^2 and allowed to attach overnight. To remove confounding effects of serum, the cells were rinsed and incubated for four days in serum free DMEM/F12 before treatment. The cells were treated with the test compounds for two consecutive four day periods in serum free media. To isolate chronic effects of the treatment from transient effects, the cells were then allowed to recover for four days in serum free media before further analysis.

Cell mechanics were determined as described previously using the Asylum MFP-3D-Bio AFM (McKee et al., 2011; Murphy et al., 2014). Cells were rinsed in Hank's Buffered Saline (Hyclone), equilibrated on the AFM stage to minimize thermal drift, and indented in contact mode with silicon nitride cantilevers with square pyramidal tips (PNP-TR-50, Nano World, Switzerland). Prior to each experiment, thermal tuning and constant compliance methods were used to calibrate the spring constant and deflection sensitivity of each cantilever. The elastic modulus (E) of each sample was obtained by fitting indentation force versus indentation depth to the Hertz model as shown in Eq. 1, where F is the force applied by indenter, α is the tip half angle (35°), ν is Poisson's ratio (assumed to be 0.5 for incompressible biological materials with high water content (Ahearne et al., 2005; Anseth et al., 1996; Dimitriadis et al., 2002; Vinckier and Semenza, 1998)), and δ is indentation depth.

$$F=\frac{2}{\pi}\frac{E\tan(\alpha)}{1-\nu^2}\delta^2 \quad (1)$$

The Hertz model assumes that the samples were linearly elastic, homogenous, and infinitely thick. However, in the limit of small deformations, the Hertz model can be used for materials (such as cells) which are viscoelastic, heterogeneous, and finite (Mahaffy et al., 2000). For each sample, approximately 5–7 cells were indented 4–7 times at 2 μm/s with the contact point centered above the cell nucleus.

In order to visualize β-catenin, cells were fixed for 20 minutes in 4% formaldehyde and 0.25% Triton-X 100 (Fisher Scientific, Waltham, MA) in PBS and blocked with 2% BSA (Fisher), 0.2% gelatin (cold-fish; Sigma-Aldrich), 0.1% Tween (Fisher) in PBS for 1 hr. Following blocking, cells were stained overnight using mouse anti-β-catenin (BD Biosciences, Franklin Lakes, NJ) with a secondary of DyLight 594 conjugated goat anti-

mouse (Fisher) and counterstained with DAPI. In order to quantify β-catenin phosphorylation, cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Pierce Protein Biology, Rockford, IL). 10 μg protein was loaded into a 10% Bis-Tris NOVEX gel (Life Technologies, Carlsbad, CA) and separation was carried out at 75 mA for 90 min in MOPS-SDS running buffer and proteins were transferred to nitrocellulose. The membrane was washed and blocked for 1 h blocking buffer (10% Superblock, 10% FBS, 3% Fish gelatin, 0.02% Na-Azide in TBS pH 7.4). Staining was performed overnight at 4°C using mouse anti-β-catenin pT41 (1:300; Abcam, Cambridge, England) and chicken anti-β-actin (1:1000; Abcam), washed, and incubated with HRP conjugated secondary antibodies (1:20000 anti-mouse IgG and anti-chicken IgG; KPL, Gaithersburg, MA). Bands were visualized using WesternBright ECL substrate (Advansta, Menlo Park, CA) and imaged (ChemiDoc-It^2; UVP, Upland, CA). Optical density was quantified using ImageJ (National Institutes of Health, Bethesda, MA). Band density was normalized to β-actin.

Experiments were performed on HTM cells isolated from multiple donors. For each experiment, significant differences from control were assessed after a one way ANOVA using Tukey's post-hoc test or a weaker level of significance using Student's t-test. Levels of significance are denoted throughout the manuscript by *** = $p<0.001$, ** = $p<0.01$, and * = $p<0.05$ (Tukey's) or # = $p<0.05$ (t-test). AFM data are shown for each donor as box plots with the boxes denoting the data range between the 1st and 3rd quartiles (bullseye indicates median), while whiskers indicate data distribution. Immunofluorescence quantification is displayed as mean ± SEM for three donors.

First, we determined the effect of SFRP1 on the intrinsic mechanics of HTM cells. HTM cells were cultured in the presence of different concentrations of SFRP1, 0, 0.25, 0.5, and 1 μg/mL. To mitigate the effect of serum and transient effects of SFRP1, HTM cells were incubated in serum free media for four days before and after the treatment. At the end of this period, cell stiffness was determined using AFM for cells derived from 3 donors (Figure 1A). The three donors have mean cell stiffness values of 3.71±0.37, 4.33±3.07, and 3.07± kPa (median±S.D.). These values are comparable to previously reported values for HTM cells grown on glass (2.7 and 2.4 kPa) (McKee et al., 2011; Murphy et al., 2014). Cell stiffness displayed a dose response to SFRP1. With 0.25 and 0.5 μg/mL SFRP1, there was a dramatic increase in cellular stiffness, while 1 μg/mL had no effect. The most potent increase in cell stiffness was observed after treatment with 0.5 μg/ml SFRP1 with median stiffness of 16.75±5.59, 13.18±7.99, and 8.54±5.77 kPa (~3 to 6 fold compared with control cells), while 0.25 μg/ml SFRP1 treatments had a lesser effect with median stiffness values 4.32±5.12, 8.86±8.51, 4.84±3.15 kPa (~2 fold increase). The lack of response at 1 μg/mL SFRP1 (3.15±2.09, 4.65±2.69, and 2.70±1.66 kPa) was initially surprising, but a similar biphasic response to SFRP1 has been previously reported (Elzi et al., 2012). In that study, exogenous SFRP1 was assayed for its ability to induce senescence in fibroblasts (IMR-90 cells), and while 0.25 and 0.5 μg/mL did, higher doses (1 and 2 μg/mL) did not. Combined, these results underscore the complexity of Wnt regulation.

To confirm the observed stiffening response was specific to Wnt inhibition and not an unrelated activity of SFRP1, we also employed small molecular inhibitors of Wnt: KY02111 and LGK-974. KY02111 intersects the Wnt pathway at a yet to be described point, but

below the β-catenin destruction complex as it is an effective Wnt inhibitor even in the absence of functional destruction complexes (Minami et al., 2012). LGK-974 inhibits porcupine, an O-acyltransferase required for the secretion of Wnt, and therefore inhibits endogenous Wnt signaling (Liu et al., 2013). Both were used at doses of 10 μM based on previously published work (Liu et al., 2013; Minami et al., 2012) and compared to 0.1% DMSO treated cells (vehicle control), with a similar treatment course to the SFRP1 experiments with cells isolated from 2 donors. Both inhibitors potently increased cellular stiffness (KY02111: 10.72±5.63 and 6.57±5.53 kPa; LGK-974: 9.60±7.41 and 11.40±9.24 kPa) compared with controls (3.79±1.01 and 5.16±2.14 kPa; Figure 1B). In conjunction with our SFRP1 results, these findings demonstrate an important link between HTM mechanobiology and Wnt antagonism known to influence outflow regulation (Mao et al., 2012; Wang et al., 2008). It is important to note that these studies were performed with HTM cells cultured on glass, known to profoundly increase cell stiffness compared to more biomimetic substrates (McKee et al., 2011). It is possible the effect of Wnt inhibition would be more dramatic if basal HTM stiffness was decreased by culture on softer substrates, or alternatively if Wnt inhibition has a synergistic effect with culture on glass. Future studies will be needed to determine which, if either, of these two possibilities is correct.

Though Wnt can activate several signaling pathways (Clevers, 2006; Freese et al., 2010; Macdonald and He, 2012; Yu and Virshup, 2014), relevant literature suggests these results are specifically due to antagonism of the canonical, β-catenin dependant pathway. While members of both canonical and non-canonical pathways are expressed in HTM cells (Shyam et al., 2010), available data suggest SFRP1 doesn't modulate noncanonical signaling in HTM cells (Mao et al., 2012). Further, while SFRP1 is known to inhibit both canonical and non-canonical action of Wnt by antagonizing receptor binding (Kawano and Kypta, 2003; Satoh et al., 2008), KY02111 intersects with the canonical pathway below the destruction complex, which has no known role in non-canonical signaling (Minami et al., 2012). Taken together, these data strongly suggest antagonism of the canonical pathway is responsible for the increased cellular stiffness. When taken in conjunction of our previous report demonstrating that elevated substratum stiffness increases SFRP1 expression (Raghunathan et al., 2013), these results suggest a potential feedback loop in the progression of glaucoma. If HTM cells contribute to overall tissue mechanics, stiffening induced by transient Wnt antagonism could induce SFRP1 expression and further stiffening. Unchecked, this loop could lead to progressive stiffening of HTM tissue and cells with resultant outflow restriction as we proposed previously (Last et al., 2011). Further studies are required to determine the impact of cellular stiffening, due to Wnt inhibition, on extracellular matrix dynamics (mechanics and composition) and their role in reduced outflow or elevation of IOP.

Having determined that transient exogenous Wnt inhibition results in long-term elevation of HTM cell stiffness, we wished to determine if there is also a lasting effect on canonical Wnt signaling by assaying β-catenin distribution in HTM cells. Using only the small molecule inhibitors (KY02111 and LGK-974) and 0.5 μg/mL SFRP1, we treated HTM cells as described above, and fixed and stained the cells for β-catenin. We noticed decreases in staining intensity after all three inhibitor treatments when compared to the vehicle control (Figure 2A–B). When quantified by integrating the background-subtracted intensity of β-

catenin staining, there was a small but significant decrease, suggesting durable inhibition of Wnt signaling (Figure 2C–D). Additionally, we performed a Western blot of β-catenin phosphorylated at threonine 41. T41 phosphorylation is a key regulatory post-translational modification which is not found in transcriptionally active β-catenin (Liu et al., 2002; Maher et al., 2009; Maher et al., 2010; Staal et al., 2002). When HTM cells were treated with SFRP1 (0.5 μg/mL) and a small molecular inhibitor (10 μM KY02111), there was a substantial increase in T41 phosphorylation, indicating Wnt inhibition (Figure 2E). Importantly, much like the stiffness results, β-catenin staining and phosphorylation was assayed 4 days after the exogenous inhibition was lifted. These results demonstrate a persistent downregulation of Wnt in HTM cells exposed to a transient inhibitory stimulus. Extrapolated to the tissue *in vivo*, these data indicate that temporary insults to the HTM could lead to long lasting changes possibly resulting in disease progression. Prolonged reduction in β-catenin levels can adversely impact cell proliferation and intercellular junction formation (Gess et al., 2008; Verma et al., 2003). Loss of cell-cell communication in the HTM may also lead to apoptosis and reduced cellularity. Indeed, sustained inhibition of Wnt can induce apoptosis (Bodine et al., 2005; Zhang et al., 2013) and can act as a mediator of senescence (Elzi et al., 2012). Both apoptosis and senescence have been hypothesized to play a role in progression of glaucoma. In fact, progressive loss of cellularity in the glaucomatous trabecular meshwork has been speculated to be the result of apoptosis and/or senescence (Alvarado et al., 1984; Baleriola et al., 2008; Liton et al., 2005).

In conclusion, this work demonstrates a causal relationship between Wnt inhibition and cell stiffening, both previously reported in association with OAG (Last et al., 2011; Mao et al., 2012; Wang et al., 2008). Importantly, our results were obtained after an extended recovery, indicating durable changes to HTM cells induced by Wnt antagonism. Further work will be needed to extend these findings to the tissue level and to elucidate the intracellular mechanisms connecting Wnt inhibition to cell and tissue stiffening. Modulation of the Wnt pathway may hold promise for altering the biomechanics of the outflow pathway to benefit treatment of patients with OAG.

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Highlights

- We transiently exposed to human trabecular meshwork (HTM) cells to Wnt antagonists.
- Atomic force microscopy (AFM) was used to measure HTM cell stiffness after recovery.
- Wnt antagonism resulted in long-term increases in HTM cell stiffness.
- This was accompanied by decreased β-catentin staining and increased phosphorylation.

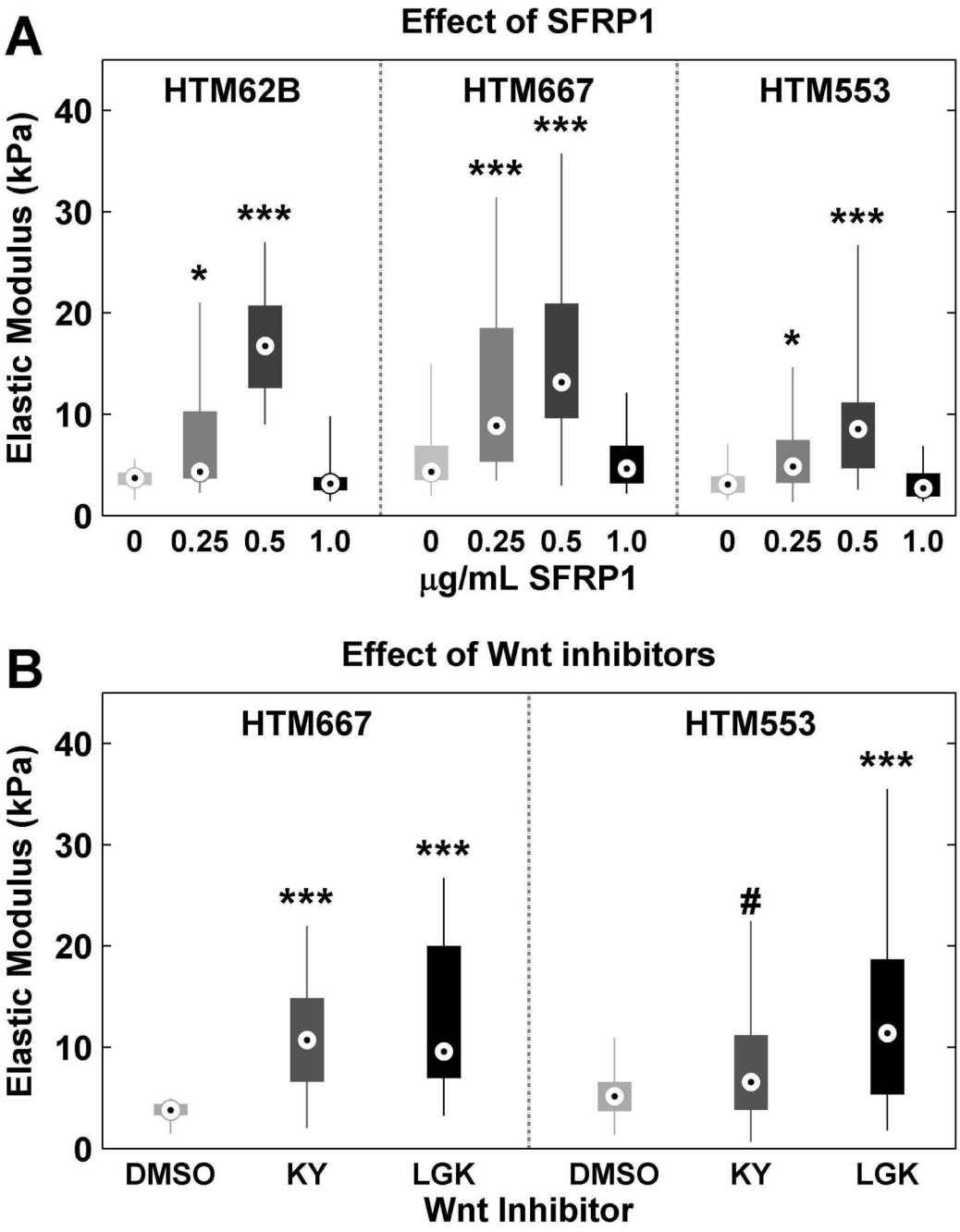

Figure 1. Wnt inhibition results in persistent increase in HTM cell stiffness

HTM cells were cultured for 4 days in serum-free media, treated with Wnt inhibitors for 8 days in serum free media, and recovered for 4 days in serum-free media before being measured using AFM. (A) HTM cells from 3 donors were treated with 0, 0.25, 0.5, 1 μg/mL SFRP1. At both 0.25 and 0.5 μg/mL the intrinsic stiffness of the cells increased significantly for all donors. (B) HTM cells from 2 donors were treated with 10 μM KY02111 (KY) or LGK-974 (LGK). Both significantly increased the intrinsic stiffness of the cells. Data is presented as a box extending to the first and third quartiles with the median denoted as the

bullseye. Whiskers denote data range. Significant difference from control using Tukey's method denoted by * $p<0.05$; *** $p<0.001$; using Student's T-test denoted by # $p<0.05$. Each data set contains 26–47 force curves collected from 5–7 cells.

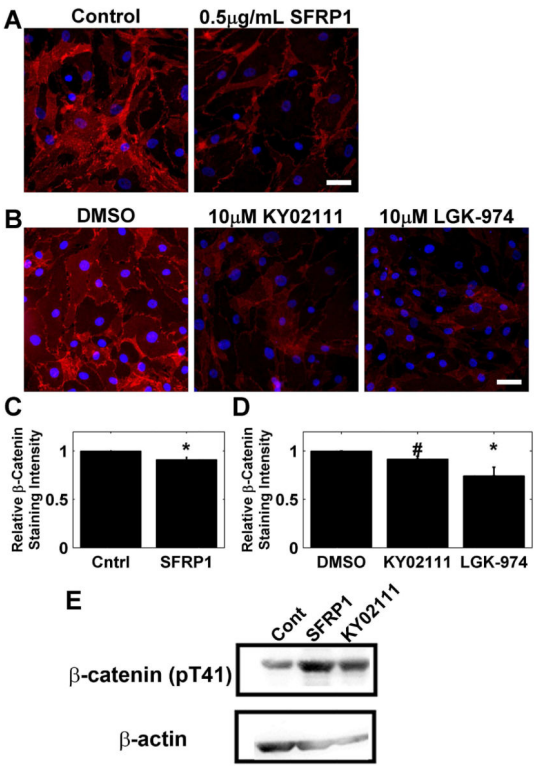

Figure 2. Transient Wnt inhibition results in persistent decrease of β-catenin

HTM cells were incubated for 4 days in serum-free media, treated with Wnt inhibitors for 8 days in serum free media, and recovered for 4 days in serum-free media before being fixed and stained for β-catenin. (A) HTM cells treated with 0.5 μg/mL SFRP1 show exhibit a decrease in the β-catenin staining intensity. (B) HTM cells treated with 10 μM KY02111 or LGK-974 likewise show a reduction of β-catenin staining intensity. (C–D) Quantification of β-catenin staining intensity in HTM cells from 3 donors indicates a statistically significant decrease in intensity after 0.5 μg/mL SFRP1 (SFRP1), 10 μM KY02111, and 10 μM LGK-974. (E) Western blot shows increased T41 phosphorylation of β-catenin after SFRP1 or KY02111 treatment. Data presented as mean ± SEM. Significant difference from control using Tukey's method denoted by * $p<0.05$; *** $p<0.001$; using Student's T-test denoted by # $p<0.05$. n = 3. Scale bar 50 μm.