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Crosstalk between TGF β and Wnt Signaling Pathways in the Human Trabecular Meshwork

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Abstract

Primary Open Angle Glaucoma (POAG) is an irreversible, vision-threatening disease that affects millions worldwide. The principal risk factor of POAG is increased intraocular pressure (IOP) due to pathological changes in the trabecular meshwork (TM). The TGF β signaling pathway activator TGF β 2 and the Wnt signaling pathway inhibitor secreted frizzled-related protein 1 (SFRP1) are elevated in the POAG TM. In this study, we determined whether there is a crosstalk between the TGF β /Smad pathway and the canonical Wnt pathway using luciferase reporter assays. Lentiviral luciferase reporter vectors for studying the TGF β /Smad pathway or the canonical Wnt pathway were transduced into primary human non-glaucomatous TM (NTM) cells. Cells were treated with or without a combination of 5 μ g/ml TGF β 2 and/or 100ng/ml Wnt3a recombinant proteins, and luciferase levels were measured using a plate reader. We found that TGF β 2 inhibited Wnt3a-induced canonical Wnt pathway activation, while Wnt3a inhibited TGF β 2-induced TGF β /Smad pathway activation (n=6, p<0.05) in 3 NTM cell strains. We also found that knocking down of Smad4 or β -catenin using siRNA in HTM5 cells transfected with similar luciferase reporter plasmids abolished the inhibitory effect of TGF β 2 and/or Wnt3a on the other pathway (n=6). Our results suggest the existence of a cross-inhibition between the TGF β /Smad and canonical Wnt pathways in the TM, and this cross-inhibition may be mediated by Smad4 and β -catenin.

Keywords

Glaucoma; Trabecular meshwork; TGF β signaling; Smad4; Wnt signaling; β -catenin; Crosstalk

Primary open angle glaucoma (POAG) is a leading cause of blindness worldwide characterized by progressive loss of retinal ganglion cells. The primary causative risk factor for the development and progression of POAG is elevated intraocular pressure (IOP) due to increased aqueous humor (AH) outflow resistance at the trabecular meshwork (TM) and the adjacent Schlemm's canal, where the majority of AH drains out of the eye (2000; Heijl et al., 2002; Kass et al., 2002; Lichter et al., 2001). At molecular level, excessive extracellular

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matrix (ECM) deposition and formation of cross-linked actin networks (CLANs) in the TM are associated with POAG (Braunger et al., 2015; Clark et al., 1995; Hoare et al., 2009). Excessive ECM deposition “clogs” the TM outflow pathway while excessive CLAN formation in TM cells may increase the stiffness of TM cells, both of which have been recognized as contributing factors to increased outflow resistance and IOP (Braunger et al., 2015; Last et al., 2011).

Recently, studies have shown that abnormal levels of growth factors and associated cell signaling pathway activities can cause these glaucomatous changes in the TM (Fleenor et al., 2006; Wang et al., 2008). Two important POAG-associated growth factors are transforming growth factor beta-2 (TGF β 2), an activator of the TGF β pathway (Tripathi et al., 1994), and secreted frizzled-related protein 1 (sFRP1), an inhibitor of the Wnt signaling pathway (Wang et al., 2008).

TGF β 2 activates the Smad-dependent TGF β (Smad/TGF β) pathway by binding to the type II receptor (TGF β RII). This binding phosphorylates and activates the type I receptor (TGF β RI), which phosphorylates the intracellular receptor Smad (R-Smad) proteins Smad2 and/or Smad3. Phospho-Smad2/3 (p-Smad2/3) associates with the common mediator Smad (co-Smad) protein Smad4, and translocates into the nucleus. The complex binds to the Smad binding element (SBE) and changes gene expression. The TGF β superfamily has long been implicated in several types of glaucoma (Fleenor et al., 2006; Fuchshofer and Tamm, 2012; Lutjen-Drecoll, 2005; Picht et al., 2001). Many studies showed that TGF β 2 is increased in the TM and AH of POAG patients (Ozcan et al., 2004; Tovar-Vidales et al., 2011; Tripathi et al., 1994). TGF β 2 induces excessive ECM deposition of proteins such as fibronectin, inhibitors of extracellular matrix degradation such as PAI-1, cross-linking proteins such as lysyl oxidase (LOX), LOX-like enzymes, and transglutaminase-2 (Fuchshofer et al., 2007; Medina-Ortiz et al., 2013; Sethi et al., 2011; Tovar-Vidales et al., 2011). Besides ECM and related proteins, TGF β 2 also increases CLANs in bovine and human TM cells (Clark et al., 1995; Hoare et al., 2009; Wade et al., 2009) and elevates IOP in human, porcine, and mouse eyes (Bachmann et al., 2006; Fleenor et al., 2006; Gottanka et al., 2004; Shepard et al., 2010).

sFRP1 inhibits the Wnt signaling pathway by binding and sequestering Wnt ligands in the extracellular space, prohibiting their binding with the receptor (Finch et al., 1997; Rattner et al., 1997). When Wnt proteins are uninhibited, they can bind to their transmembrane receptor, Frizzled and the co-receptor, lipoprotein receptor-related protein 5/6 (LRP5/6). With the assistance of Disheveled, the cytosolic β -catenin degradation complex that consists of Axin2, APC, CK1 and GSK3 β is disassembled via phosphorylation. Without this degradation complex, cytosolic β -catenin is no longer phosphorylated for proteasome degradation, and therefore can accumulate. Some cytosolic β -catenin will translocate into the nucleus, where they associate with the T-cell factors 1, 3, 4 (TCF1/3/4) or lymphoid enhancer-binding factor 1 (LEF-1), bind to the TCF/LEF binding element, and change gene expression. This is the β -catenin-dependent/canonical Wnt signaling pathway (Miller et al., 1999; Nusse and Varmus, 1992). Our previous studies showed that there is a functional canonical Wnt pathway in the human TM (HTM) (Mao et al., 2012a). In the human glaucomatous TM (GTM), we found increased mRNA and protein levels of sFRP1 as well

as decreased β -catenin, the latter of which is the key mediator of the canonical Wnt pathway as described previously (Mao et al., 2012b; Wang et al., 2008). We showed that the inhibition of the canonical Wnt signaling activity by SFRP1 or Dickkopf1 (Dkk1, an inhibitor that specifically inhibits the canonical Wnt pathway via the inhibition of LRP 5/6) elevates IOP in mouse eyes and perfusion cultured human eyes (Mao et al., 2012a; Wang et al., 2008). This IOP elevation can be blocked by co-treatment with a small molecule that activates the downstream canonical Wnt pathway (Wang et al., 2008). Exactly how canonical Wnt signaling maintains IOP, however, is still under investigation.

Since both the TGF β and Wnt pathways play important roles in the homeostasis of TM and regulation of IOP, it is very important to determine whether the two pathways crosstalk in the TM. Such crosstalk has been found in various non-TM cells, and more importantly, in fibrotic diseases. In renal fibrosis, the canonical Wnt pathway antagonizes the TGF β /Smad pathway and protects the tissues from fibrotic damage (Ho et al., 2012). Due to the fact that GTM alterations are very similar to those in fibrotic diseases (loss of resident functional cells and excessive ECM deposition), a similar crosstalk likely exists in the TM.

To determine whether a crosstalk exists in the TM, we used luciferase transcription reporter assays (also called luciferase assays) to measure whether the activation of one pathway by recombinant protein is able to affect the other pathway's activity. We transduced human primary non-glaucomatous TM (NTM) cells with lentiviral firefly luciferase reporter vectors containing the SBE for studying the TGF β /Smad pathway (SBE virus, Qiagen, Valencia, CA) or vectors containing the TCF/LEF binding element for studying the canonical Wnt pathway (TCF/LEF virus, Qiagen). TM cells were also co-transduced with the lentiviral renilla luciferase reporter vector containing a minimal CMV (mCMV) promoter as an internal control (renilla control virus, Qiagen). Because the transcriptional activity of the minimal CMV promoter is not affected by any signaling pathways, the amount of renilla luciferase can be used to normalize firefly luciferase for the difference in cell numbers and transduction efficiency. On day 1, 3×10^4 NTM cells were seeded into individual wells of 96 well white opaque plates in DMEM-low glucose medium supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin and streptomycin (ThermoFisher, Waltham, MA). On day 2, when cells reached 50% confluence, SBE or TCF/LEF lentivirus together with renilla control virus were mixed in serum-free and antibiotics-free medium containing a transduction reagent (SureEntry, 1:4000, Qiagen) and added to individual wells. The multiplicity of infection (MOI) was 75 for SBE or TCF/LEF lentivirus, and 50 for renilla control lentivirus. On day 3, medium was changed to serum-free medium. On day 4, cells were treated with the TGF β pathway activator TGF β 2 (5ng/ml, R&D Systems, Minneapolis, MN) and/or the Wnt pathway activator Wnt3a (100ng/ml, R&D Systems) in serum-free medium. On day 5, firefly and renilla luciferase levels were measured using the DualGlo kit (Promega, Madison, MI) and a plate reader (Infinite M200, Tecan, San Jose, CA). Experiments were performed in replicates (n=6) and luciferase luminescent signals were read three times per well and averaged. Statistical analysis was performed using Prism Graphpad (GraphPad Software, La Jolla, CA) using one-way ANOVA. Multiple-comparison post-hoc tests were applied. Three primary NTM strains were studied and representative data were shown. The TM cell strains were previously characterized by a combination of TM cell markers including collagen IV, laminin, α -smooth muscle actin, as well as

dexamethasone-induced myocilin expression and formation of cross-linked actin networks at Alcon (Fort Worth, TX) and were a kind gift. Cell strain information: NTM340-07 male donor at age 80; NTM210-05 female donor with age unknown; NTM176-04 male donor at age 72.

The TGF β or Wnt signaling pathway activity was expressed as relative luciferase units (RLU), which represent firefly luciferase levels normalized by renilla luciferase levels. We found that TGF β 2 and Wnt3a were able to activate their respective pathways (n=6, p<0.05) (Figure 1). However, co-treatment with TGF β 2 and Wnt3a significantly inhibited TGF β 2-induced TGF β signaling activation as well as Wnt3a-induced Wnt signaling activation (n=6, p<0.05) (Figure 1). These data showed a cross-inhibition between these two pathways. In contrast, TGF β 2 or Wnt3a treatment alone had no effect on the basal activity of the other pathway except in NTM1022-05 cells in which Wnt3a inhibited basal TGF β signaling (Figure 1C), suggesting that a concurrent activation of both pathways is required for this cross-inhibition.

Since both TGF β and Wnt pathways have multiple subpathways (Smad-dependent and independent TGF β pathways; canonical and non-canonical Wnt pathways), we used siRNA to knock down Smad4 or β -catenin, the key mediators of TGF β /Smad pathway and canonical Wnt pathway, respectively, and performed similar luciferase assays. Smad4 is the common Smad required for the Smad signaling. Smad2 or Smad3 is able to activate the TGF β /Smad signaling but only in the presence of Smad4. The role of Smad4 in the TGF β pathway is somehow equivalent to β -Catenin in the Wnt pathway since there is no alternative for them, and therefore we call it the “key mediator”. Our rationale is that without Smad4 or β -catenin, the non-Smad pathway or non-canonical Wnt pathway remains functional, respectively. However, the TGF β /Smad or the canonical Wnt pathway will be disabled. This approach enabled us to dissect the subpathways involved in this cross-inhibition.

We performed luciferase assays using the transformed human NTM cell line HTM5 because it was technically challenging to conduct lentiviral transduction together with siRNA knockdown in primary TM cells. For these experiments, 3×10^4 HTM5 cells were plated in 96 well opaque plates in Opti-MEM medium (Invitrogen, Grand Island, NY) without antibiotics but with 5% FBS and 1% glutamine. Right after cells were seeded, they were transfected with 50 μ l transfection mixture containing 100ng TCF/LEF or SBE luciferase reporter plasmids (Signaling Reporter, Qiagen), 30 nM siRNA against β -catenin, Smad4 or non-targeting siRNA (OnTarget siRNA, GE Dharmacon, Lafayette, CO), and 0.6 μ l transfection reagent (Attractene, Qiagen). Different from lentiviral luciferase vectors, the luciferase plasmids are premixed with the TCF/LEF or SBE firefly luciferase reporter vector and mCMV renilla luciferase reporter vector according to manufacturer instructions. On day 2, medium was changed to serum-free Opti-MEM. On day 3, cells were treated with 100ng/ml Wnt3a, 5 μ g/ml TGF β 2, or both. On day 4, luciferase levels were measured as previously described. Experiments were performed in replicates (n=6), and data were analyzed as described previously.

Besides, to validate siRNA-mediated knockdown of target genes, we seeded 2×10^5 HTM5 cells into a 12-well plate. Right after seeding, cells were transfected with a mixture (200 μ l) with or without 30nM siRNA and/or 6.4 μ l Attractene (Qiagen). Three day post-transfection, whole cell lysate was extracted using the M-PER Mammalian Extraction Reagent (ThermoFisher) with 1:100 protease inhibitors. Proteins were separated using SDS-PAGE, transferred onto an Immobilon-P transfer membrane, blocked with 5% dry milk, and immunoblotted with anti-Smad4, anti- β -catenin (Cell Signaling, 1:500), and anti-GAPDH antibodies (Cell Signaling, 1:10,000) followed by HRP-linked anti-rabbit or anti-mouse IgG secondary antibody. Signal was developed using the Clarity Western ECL Blotting substrate (Bio-Rad, Hercules, CA). Images were taken using the FluroChem imaging system (Cell Biosciences, Santa Clara, CA) or the Bio-Rad ChemiDoc imaging system (Bio-Rad). Densitometry was performed and the level of the protein of interest was normalized to GAPDH. The level of non-targeting siRNA treated samples was set at 1. One-way ANOVA plus post-hoc tests were used to compare protein levels.

We found that HTM5 cells had similar responses to Wnt3a and/or TGF β 2 (Figure 2A and D) as in primary NTM cells (i.e. Wnt3a inhibited TGF β 2-induced TGF β signaling and TGF β 2 inhibited Wnt3a-induced Wnt signaling). Knocking down Smad4 or β -catenin resulted in complete inhibition of TGF β or Wnt pathway activation, respectively (Figures 2B and 2F), confirming the effectiveness of our siRNA in luciferase assays. β -catenin knockdown did not affect TGF β 2-induced TGF β pathway activation (Figure 2C) and Smad4 knockdown did not affect Wnt3a-induced Wnt pathway activation (Figure 2E), demonstrating the specificity of our siRNA. With the knockdown of either Smad4 or β -catenin, TGF β 2 and Wnt3a co-treatment were unable to inhibit TGF β 2-induced TGF β signaling (Figures 2B and 2C) or Wnt3a-induced Wnt signaling (Figures 2E and 2F), suggesting these pathway mediators are necessary for cross-inhibition to occur. Our Western immunoblotting and densitometry results showed that there was about 70% decrease in Smad4 and β -catenin proteins ($p < 0.01$, $n = 3$), confirming the effectiveness of our siRNAs (Figure 2G and H).

In summary, our studies showed that there exists a cross-inhibition between the TGF β /Smad pathway and the canonical Wnt pathway in the HTM. This inhibition requires both pathways' key mediators Smad4 and β -catenin.

Very few studies on such crosstalk in the TM have been reported. Villareal and colleagues showed that LiCl, a non-specific Wnt pathway activator, inhibits TGF β 2-induced expression of a subset of ECM and matricellular proteins (Villarreal et al., 2014). However, the same study suggested that this inhibition is mediated by canonical Wnt signaling as well as miR-29b, and the latter may be due to the non-specific effect of LiCl. Also, whether there is a direct inhibition of the TGF β signaling by the Wnt pathway or vice versa was not reported.

In non-TM cells and tissues, the crosstalk between the TGF β and Wnt pathways has been well studied. However, it has different mechanisms and also shows various effects in different cells and tissues. Jian and colleagues found that in bone marrow-derived adult human mesenchymal stem cells, Smad3 and β -catenin form a complex to facilitate their translocation (Jian et al., 2006). In chondrocytes, Zhang and colleagues reported that both Smad2 and Smad3 are required for the interaction with β -catenin, and they protect β -catenin

from being degraded (Zhang et al., 2010). Nawshad and colleagues found that the p-Smad2-Smad4-LEF1 complex inhibits TGF β 3-induced E-Cadherin expression in mouse palate medial edge epithelial cells (Nawshad et al., 2007). Nishita and colleagues showed that Smad4 is able to complex with β -catenin during *Xenopus* development (Nishita et al., 2000). In cancer cells, Smad4 and β -catenin can form a complex to regulate each other's activity (Hussein et al., 2003; Romero et al., 2008). The impaired crosstalk between the two pathways is also associated with cancer development and epithelial to mesenchymal transition in metastasis (Voorneveld et al., 2015; Xing et al., 2015). Because the interaction between the two pathways is highly cell/tissue specific, elucidating the exact mechanism that mediates the cross-inhibition between Smad/TGF β and canonical Wnt pathways in the TM is very important. Based on published studies and our findings that both Smad4 and β -catenin are required for this cross-inhibition, it is our hypothesis that the two transcription factors form a complex, and this complex represses both pathways' activity due to conformational changes and/or blockage of protein binding sites.

In this study, we used luciferase assays, a well-established approach to study pathway crosstalk. Although the luciferase assay offers specific and quantitative measurement of pathway activities, this artificial promoter-based reporter assay does not reflect the complexity of the cis-element involved in transcription including silencers, enhancers, and epigenetic factors that may fine regulate the crosstalk between TGF β 2 and Wnt pathways. Therefore, more research including ex vivo and in vivo studies is needed to elucidate this cross-inhibition in the TM.

In the healthy TM, Wnt signaling may function as a negative regulatory mechanism by which the effects of TGF β 2/overactivated TGF β /Smad signaling on ECM, CLANs, and other cellular changes are alleviated. Since elevated TGF β 2 and SFRP1 are found in the AH and TM of many POAG patients, it is very likely that they co-exist in a number of POAG eyes. Under this situation, the inhibition of Wnt signaling by SFRP1 very likely disables the potential Wnt negative regulatory mechanism. Therefore, TGF β 2 and SFRP1 may contribute to POAG in a synergistic manner. Again, further studies are required to determine the molecular mechanism of this cross-inhibition and the exact molecules involved. This information may help us to identify novel therapeutic targets to provide new disease-modifying treatments for POAG.

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Abbreviations

AH	Aqueous humor
CLANs	Cross-linked actin networks
Co-Smad	common mediator Smad

Dkk1	Dickkopf1
ECM	Extracellular matrix
GTM	Glaucomatous trabecular meshwork
IOP	Intraocular pressure
LRP5/6	lipoprotein receptor-related protein 5/6
MOI	Multiplicity of infection
NTM	Non-glaucomatous trabecular meshwork
POAG	Primary open angle glaucoma
p-Smad	Phospho-Smad
RLU	Relative luciferase unit
R-Smad	receptor Smad
SBE	Smad binding element
SFRP1	Secreted frizzled related protein-1
TCF/LEF	T-cell factor/lymphoid enhancer factor
TGFβ2	Transforming growth factor beta-2
TGFβR	Transforming growth factor beta receptor
TM	Trabecular meshwork

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Highlights

- Overactivation of the TGF β /Smad and inhibition of the canonical Wnt signaling pathway have been implicated in the pathology of primary open angle glaucoma.
- TGF β /Smad signaling and canonical Wnt signaling cross-inhibit each other in human trabecular meshwork cells.
- The cross-inhibition of the TGF β /Smad and canonical Wnt signaling pathways is dependent on the transcription factors Smad4 and β -catenin.

Figure 1.

TGFβ and Wnt pathways inhibit each other's activity in primary NTM cells.

Primary NTM cells were transduced with the SBE (A, C and E) or TCF/LEF (B, D and F) lentiviral luciferase reporter vectors to study the TGF pathway (A, C, and E) and Wnt pathway (B, D, and F) activities, respectively. Cells were treated with or without 5ng/ml TGFβ2, 100ng/ml Wnt3a, or both (TGFβ2+Wnt3a) for 24 hours. Columns and bars: means and standard deviations of measured RLU, which represent individual pathway activity. *, ***, ****: $p < 0.05$, 0.001 , or 0.0001 , compared to Control (A–F); #, ###: $p < 0.05$ or 0.001 ,

compared to TGF β 2 (A, C and E); ##, ####: $p < 0.01$ or 0.0001 compared to Wnt3a (B, D, and F). N=6.

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Figure 2.

Smad4 and β -catenin are required for the cross-inhibition between TGF β and Wnt pathways. Smad4 (B and E) or β -catenin (C and F) was knocked down in HTM5 cells using siRNA. Non-targeting siRNA (NT) was used as a control (A). Cells were treated with or without 5ng/ml TGF β 2, 100ng/ml Wnt3a, or both (TGF β 2+Wnt3a) for 24 hours. TGF β (A–C) and Wnt (D–F) pathway activities were measured using SBE or TCF/LEF plasmid-based luciferase assays, respectively. Columns and bars: means and standard deviations of measured RLU, which represent individual pathway activity. *, **, or ****: $p < 0.05$, 0.01 or 0.0001, compared to Control; ##: $p < 0.001$, compared to TGF β 2 (A); ###: $p < 0.001$ compared to Wnt3a (D); NS: not significant ($p > 0.05$). $N = 6$. Also, Western immunoblotting was used to confirm protein knockdown in siRNA transfected HTM cells (G). Densitometry and one-way ANOVA plus post-hoc tests were used to compare relative protein levels (H); columns and bars: means and standard deviations; **: $p < 0.01$, compared to the non-targeting siRNA group; $n = 3$.