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Crosstalk between TGFβ 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 TGFβ signaling pathway activator TGFβ2 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 TGFβ/Smad pathway and the canonical Wnt pathway using luciferase reporter assays. Lentiviral luciferase reporter vectors for studying the TGFβ/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 μg/ml TGFβ2 and/or 100 ng/ml Wnt3a recombinant proteins, and luciferase levels were measured using a plate reader. We found that TGFβ2 inhibited Wnt3a-induced canonical Wnt pathway activation, while Wnt3a inhibited TGFβ2-induced TGFβ/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 TGFβ2 and/or Wnt3a on the other pathway ($n = 6$). Our results suggest the existence of a cross-inhibition between the TGFβ/Smad and canonical Wnt pathways in the TM, and this cross-inhibition may be mediated by Smad4 and β-catenin.

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 (AGIS, 2000; Heijl et al., 2002; Kass et al., 2002; Lichter et al., 2001). At the molecular level, excessive extracellular 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 (TGFβ2), an activator of the TGFβ pathway (Tripathi et al., 1994), and secreted frizzled-related protein 1 (sFRP1), an inhibitor of the Wnt signaling pathway (Wang et al., 2008).

TGFβ2 activates the Smad-dependent TGFβ (Smad/TGFβ) pathway by binding to the type II receptor (TGFβRII). This binding phosphorylates and activates the type I receptor (TGFβRI), 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

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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translocates into the nucleus. The complex binds to the Smad binding element (SBE) and changes gene expression. The TGFβ 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 TGFβ2 is increased in the TM and AH of POAG patients (Ozcan et al., 2004; Tovar-Vidales et al., 2011; Tripathi et al., 1994). TGFβ2 induces excessive ECM deposition of proteins such as fibronectin, inhibitors of extracellular matrix degradation such as PAI-1, cross-linking proteins such as lysl 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, TGFβ2 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 GSK3β 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 TGFβ and Wnt pathways play important roles in the homeostasis of the 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 TGFβ/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 TGFβ/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 TGFβ pathway activator TGFβ2 (5 ng/ml, R&D Systems, Minneapolis, MN) and/or the Wnt pathway activator Wnt3a (100 ng/ml, R&D Systems) in serum-free medium. On day 5, firefly and renilla luciferase levels were measured using the Dual-Glo 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 TGFβ 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 TGFβ2 and Wnt3a were able to activate their respective pathways ($n = 6$, $p < 0.05$) (Fig. 1). However, co-treatment with TGFβ2 and Wnt3a significantly inhibited TGFβ2-induced TGFβ signaling activation as well as Wnt3a-induced Wnt signaling activation ($n = 6$, $p < 0.05$) (Fig. 1). These data showed a cross-inhibition between these two pathways. In contrast, TGFβ2 or Wnt3a treatment alone had no effect on the basal activity of the other pathway except in NTM210-05 cells in which Wnt3a inhibited basal TGFβ signaling (Fig. 1C), suggesting that a concurrent activation of both pathways is required for this cross-inhibition.

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