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Original Article

Neovibsanin B increases extracellular matrix proteins in optic nerve head cells via activation of Smad signalling pathway

Zhen Wang[1], Wei Xu[1], Ao Rong[1], Yan Lin[2], Xu-Ling Qiu[1], Shen Qu[1], Xian-Hai Lan[1]

[1]*Department of Ophthalmology, Tongji Hospital, Tongji University School of Medicine, Shanghai 200065, China;*
[2]*Department of Medical, Tongji Hospital, Tongji University School of Medicine, Shanghai 200065, China*

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Abstract: The present study demonstrates the effect of neovibsanin B on the synthesis and deposition of ECM proteins and the signalling pathways used in optic nerve head (ONH) astrocytes and lamina cribrosa (LC) cells. For investigation of the signalling pathway used by neovibsanin B, ONH cells were treated with neovibsanin B. Western blot and immunostaining analyses were used to examine the phosphorylation of proteins involved in Smad and non-Smad signalling pathway. The results revealed that ONH cells on treatment with neovibsanin B showed enhanced synthesis of extracellular matrix (ECM) proteins. Neovibsanin B induced phosphorylation of canonical signalling proteins, Smad2/3. However phosphorylation of non-canonical signalling proteins, extracellular signal-regulated kinases, p38, and c-Jun N-terminal kinases (JNK) 1/2 remained unaffected. There was also increase in co-localization of pSmad2/3 with Co-Smad4 in the nucleus of ONH astrocytes and LC cells indicating activation of the canonical Smad signalling pathway. Treatment of ONH cells with SIS3, inhibitor of Smad3 phosphorylation reversed the neovibsanin B stimulated ECM expression as well as activation of canonical pathway signalling molecules. In addition, inhibition of Smad2 or Smad3 using small interfering RNA (siRNA) also suppressed neovibsanin B stimulated ECM protein synthesis in ONH astrocytes and LC cells. Thus neovibsanin B utilizes the canonical Smad signalling pathway to stimulate ECM synthesis in human ONH cells. The neovibsanin B induced ECM synthesis and activation of the canonical Smad signalling pathway may be due to its effect on transforming growth factor-β2 (TGF-β2). However, further studies are under process to understand the mechanism.

Keywords: ECM expression, Smad signalling, glaucoma, phosphorylation, remodelling

Introduction

Glaucoma is accompanied by the presence of characteristic patterns of optic nerve head excavation and causes damage to the visual field. Identification of the elevated intraocular pressure (IOP) as a major causative risk factor attracted clinicians to target its lowering for the glaucoma treatment [1]. However, despite lowering of IOP, damage caused by the glaucoma in many cases could not be halted [2]. In human optic nerve head (ONH), two major cell types present are ONH astrocytes and lamina cribrosa (LC) cells [3, 4]. In LC proper orientation of the collagen and elastin fibers plays a vital role in preventing RGC axons from mechanical stress [5, 6]. However, altered deposition of elastin leads to a decrease in ONH elasticity. Both ONH astrocytes and LC cells are involved in the synthesis of growth factors and extracellular matrix (ECM) proteins in RGC axons [4, 7-9]. There are variations in the ECM constituting components like fibrillar collagens and elastin composition in the glaucomatous ONH [10-13]. It is reported that glaucomatous ONH shows characteristic cupping and excavation of the optic disc, remodelling of the LC, and activation of ONH astrocytes [14-16]. There are sieve like structures in LC region of the ONH through which RGC axons exit the eye [17, 18]. These sieve like structures are comprised of extracellular matrix proteins such as elastin and collagens [19].

Transforming growth factor type-β (TGF-β)/Smad pathway plays a key role in maintaining

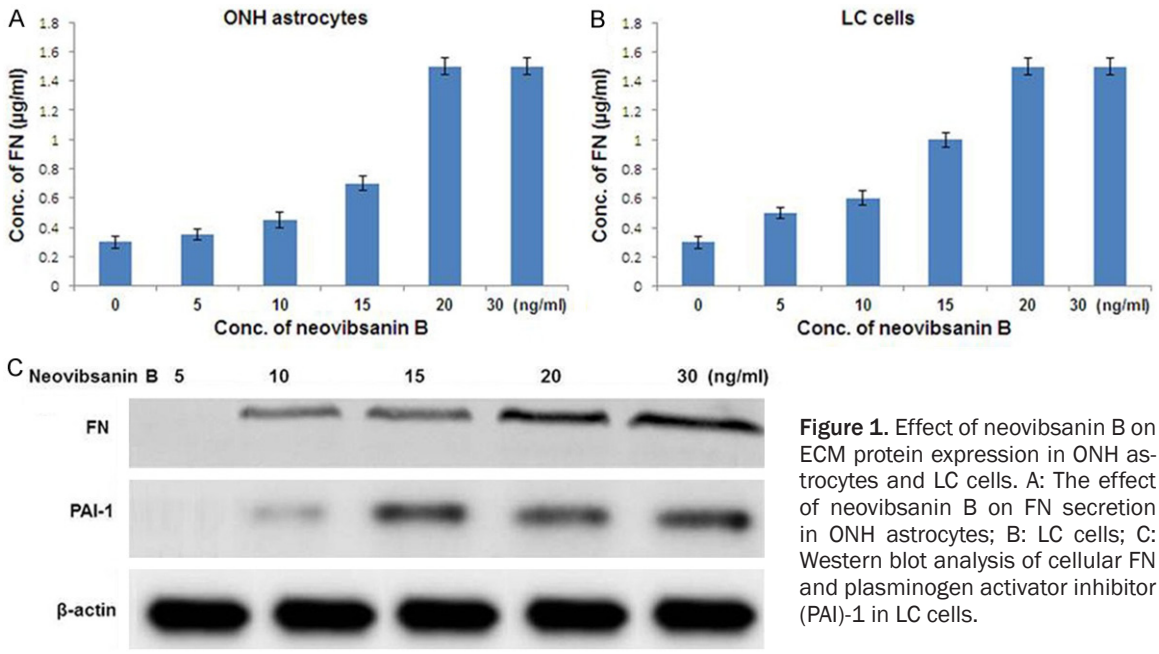

Figure 1. Effect of neovibsanin B on ECM protein expression in ONH astrocytes and LC cells. A: The effect of neovibsanin B on FN secretion in ONH astrocytes; B: LC cells; C: Western blot analysis of cellular FN and plasminogen activator inhibitor (PAI)-1 in LC cells.

normal vascular structure of cells [20-23] and its dysfunction may lead to vascular fibrosis [20-25]. In this pathway activated receptor-regulated Smads (RSmads), Smad2 and Smad3 mediate TGF-β signalling from cell membrane to the nucleus to regulate target gene transcription [26]. Additionally, reactive oxygen species (ROS) also mediate TGF-β signalling transduction and its inhibition prevents TGF-β1-induced over-production of collagens and tissue fibrosis [25, 27, 28]. Thus it seems that Smad and redox pathways are linked to each other in TGF-β signalling transduction. The neovibsanin B induced ECM synthesis and deposition in isolated human ONH astrocytes and LC cells along with the activation of Smad signalling pathways also suggests its effect on Transforming growth factor-β2 (TGF-β2).

Neovibsanins belong to the family of diterpenoids and were isolated from the plant, viburnum awabuki [29]. Neovibsanins are known to enhance neurite outgrowth activity in PC12 cells, therefore can be promising candidates for the treatment of neurological diseases [30]. Not only neovibsanins but their synthetic intermediates also promote neurite outgrowth activities. Taking into consideration the promising neurotrophic activity of neovibsanins, many strategies have been developed to achieve their total synthesis [31] and identify the scaffold responsible for the activity [32]. The present study demonstrates the effect of neovibsanin B on stimulation of ECM synthesis and deposition in human ONH astrocytes and LC cells and the signalling pathways used.

Material and methods

Dissection of optic nerve head and cell culture

Within 24 h of death, the human donor eyes were obtained and the LC region of the ONH was dissected. The explants from LC tissues were placed in culture plates containing Dulbecco's modified Eagle's medium supplemented with L-glutamine (0.3 mg/ml, Tarivid; Santen, Osaka, Japan), penicillin (100 units/ml)/streptomycin (0.1 mg/ml, Tarivid; Santen, Osaka, Japan), amphotericin B (3.8 μg/ml; Tarivid; Santen, Osaka, Japan), and 10% fetal bovine serum (Tarivid; Santen, Osaka, Japan).

Neovibsanin B treatment of ONH

Confluent ONH astrocytes and LC cells in 12 well plates were washed with PBS and kept in serum-free DMEM. After 24 h, the cells were grown in serum-free medium without (Control) or with a range of neovibsanin B concentrations from 5-30 ng/ml for 24 h. Cell lysates and the culture medium were then analyzed for ECM proteins. To analyze various signaling pathways, phosphorylation studies of Smads, ERK1/2, p38, or JNK1/2 were performed. The

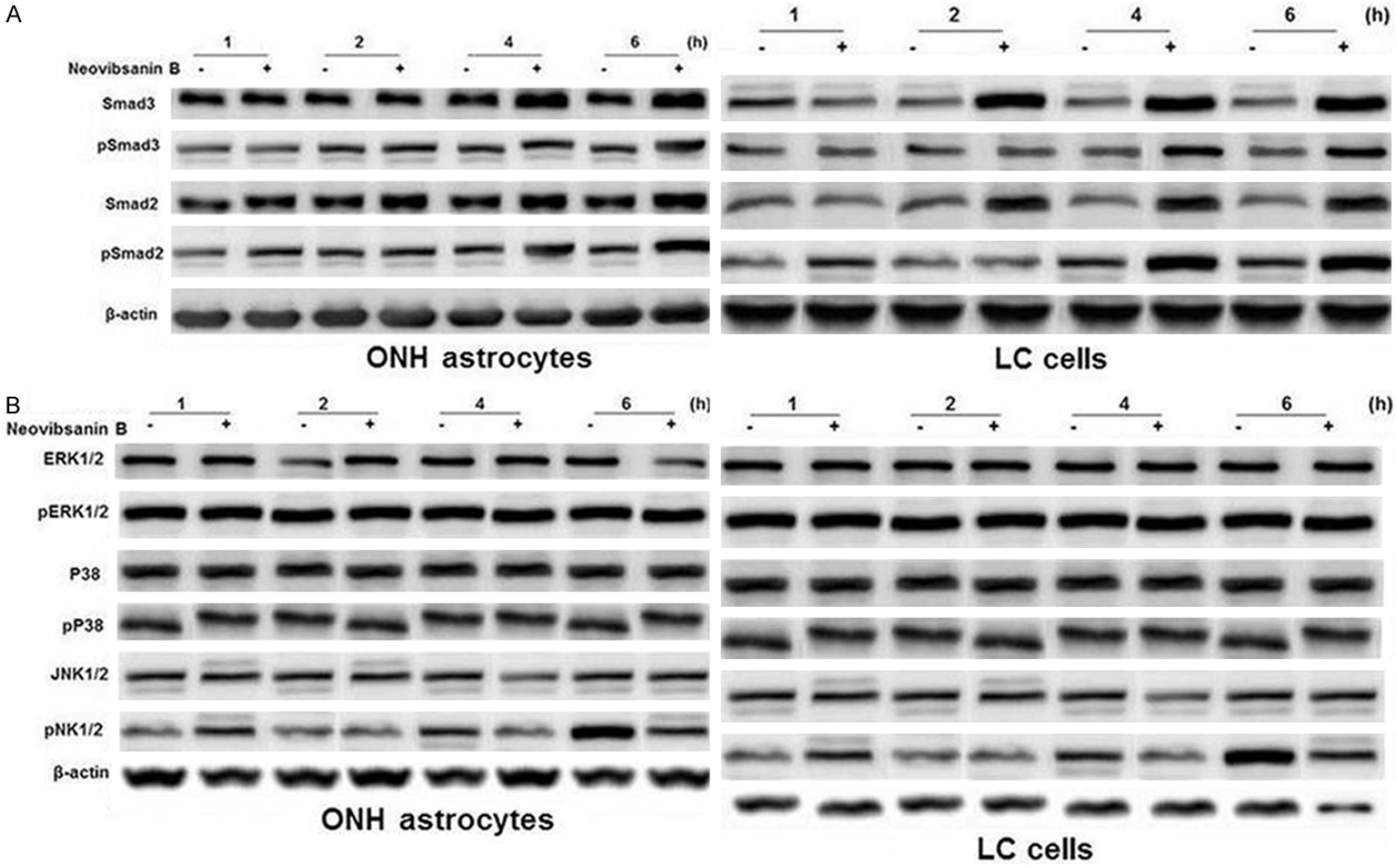

Figure 2. Effect of neovibsanin B on activation of Smad2/3 in ONH astrocytes and LC cells.

confluent ONH astrocytes and LC cells washed twice with PBS were kept in a serum-free medium for 24 h, followed by replacement of medium with neovibsanin B treated medium. The cell lysates were then subjected to immunoblotting with phospho-specific antibodies. The effect of Smad3 phosphorylation inhibition was investigated by treatment of ONH astrocytes and LC cells with or without SIS3 (50 μM; Tarivid; Santen, Osaka, Japan) with neovibsanin B for 24 h. The cell lysates and the conditioned medium were analyzed for their effects on ECM proteins. The effect of SIS3 on Smad and non-Smad signaling pathways was investigated by treatment of ONH astrocytes and LC cells with SIS3 for 1 h and followed by 1 h treatment with neovibsanin B. Western immunoblotting was used to examine phosphorylation of Smad and non-Smad signaling molecules.

Immunohistochemistry

Within 12 h of death 3 sets of normal and glaucomatous human eyes approximately of same age were obtained from the eye banks. The formalin fixed tissues after dehydration were paraffin embedded and sliced into 6 μm sections. The sections were treated with 10% normal serum after deparaffinization and rehydration. The sections after 12 h incubation with primary antibody were washed with PBS followed by 2 h incubation in appropriate Alexa Fluor™ secondary antibodies (1:200; Invitrogen Corporation, Carlsbad, CA). DAPI nuclear staining for 30 was followed by capturing of mages using Zeiss 410 confocal imaging system (Carl Zeiss, Thornwood, NY).

Co-localization of pSmad3 and Smad4

Confluent Lamina cribrosa cells after 24 h incubation in serum-free medium were incubated with 20 ng/ml of neovibsanin B for 1 h. After fixation with 3.5% formaldehyde, the cells were washed with PBS and treated with 0.05% triton for 20 min. Again cells were washed twice with PBS, blocked with 10% goat serum for 1 h and then incubated overnight with primary antibodies for pSmad3 or pSmad2 and Co-Smad4. Incubation with primary antibodies was followed by PBS washing and then incubation with secondary antibodies for 2 h. For nuclear staining incubation with DAPI for 30 min was performed. Zeiss 410 confocal imaging system (Carl Zeiss) was used to capture images.

Western blot analysis

Mammalian Protein Extraction Buffer (Tarivid; Santen, Osaka, Japan) and Bio-Rad Dc protein assay system (Bio-Rad Laboratories, Richmond, CA) were used for extraction and determination of protein concentration respectively. Equal protein amounts were loaded onto SDS-PAGE gels and after running gels, proteins were transferred onto nitrocellulose membranes. After blocking membranes in 5% nonfat milk incubation with primary antibody was performed with 3% BSA at 4°C. Antibodies used were anti-fibronectin (1:1000), anti-PAI-1 (R&D, 1:1000), anti-Pp38MAPK (1:500), anti-pJNK1/2 (1:500), anti-JNK1/2 (1:500), anti-pERK1/2 (1:500), anti-ERK1/2 (1:500), anti-β-actin (Santa Cruz, 1:1000), anti-Smad2, anti-Smad3, and anti-phosphorylated Smad3 (Cell Signaling Technology, 1:800). The membranes were then incubated with peroxidase-conjugated secondary antibody at room temperature for 2 hours. Specific band was detected with chemiluminescence assay (ECL detection reagents, Pierce) and recorded on x-ray film. Quantity one software was used to quantify the intensities of bands.

ELISA immunoassay

For quantification of soluble fibronectin the conditioned ONH astrocyte and LC cell line media were centrifuged at 12000 rpm. After removal cellular debris, 100 μl of dilution buffer was added to 50 μl of the conditioned medium. Immunosorbent assay (ELISA) kit was used for quantification of soluble fibronectin analysed using Graph Pad Prism 5.

Small interfering RNA transfection

Small interfering RNA (siRNAs) was synthesized using RiBoBio Co. Ltd (Guangzhou, China). The cultured cells were transfected in 6-well plates at 70% confluence. Transfection of siRNA was performed at a final concentration of 50 nmol/L using Lipofectamine 2000 (Invitrogen).

Statistical analysis

All the results are shown as mean ± SEM of at least three independent experiments. One-way ANOVA followed by Student-Newmann-Keuls multiple comparison tests was used for estimation of significance of differences. The differ-

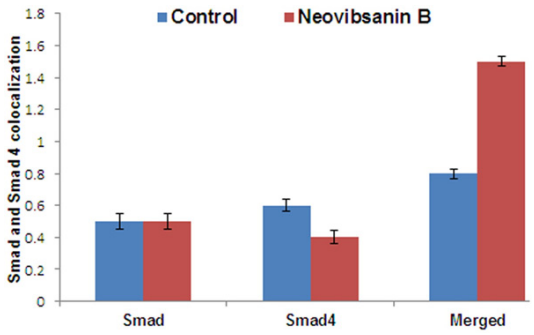

Figure 3. Effect of neovibsanin B on localization of Smad2/3 with Co-Smad4 LC cells.

ences were considered statistically significant if $P < 0.05$. The SPSS software (version 11.0, SPSS Inc) was used for statistical analyses.

Results

Neovibsanin B increases synthesis and deposition of ECM proteins in ONH astrocytes and LC cells

ONH astrocytes and LC cells were treated with 5, 10, 15, 20, and 30 ng/ml concentration of neovibsanin B for different time periods. The results from ELISA immunoassay revealed an increase in soluble FN in a dose and time dependent manner in both the cell types (**Figure 1A**, **1B**). The increase in soluble FN was maximum at 20 ng/ml after 48 h. The results from Western blot analysis also revealed induction of FN and PAI-1 in the cell lysates to reach a maximum at 20 ng/ml (**Figure 1C**).

Neovibsanin B activates the canonical Smad signaling pathway in ONH astrocytes and LC cells

For investigation of the signaling pathways the cells were treated with 20 ng/ml concentration of neovibsanin B for different time intervals and examined by western immunoblotting. The results showed an increase in phosphorylation of Smad2 and Smad3 in both ONH astrocytes and LC cells. However the total concentration of Smad2, Smad3 and actin remained unchanged (**Figure 2A**). We also studied the effect of neovibsanin B on phosphorylation of ERK1/2, p38 or JNK1/2 kinases in ONH astrocytes and LC cells using immunoblotting. The results showed that there was no effect of 20 mg/ml neovibsanin B on ERK1/2, p38 or JNK1/2 kinase phosphorylation (**Figure 2B**). These results confirm that neovibsanin B uses Smad signaling pathway for induction of ECM protein expression.

Neovibsanin B increases co-localization of phosphorylated Smad3 and co-Smad4 in LC cells

We also investigated the effect of neovibsanin B on co-localization of pSmad 2 or pSmad3 with Co-Smad4 in LC cells. In the nucleus of untreated LC cells some co-localization of pSmad3 with Co-Smad4 was observed (**Figure 3**) but immunostaining for pSmad2 was not observed. However, in neovibsanin B treated LC cells, the co-localization of phosphorylated Smad3 and Co-Smad4 was increased in both the cytoplasm and nucleus (**Figure 3**). In addition, p-Smad2 and Co-Smad4 levels were increased and these factors were co-localized. Similar results were observed in ONH astrocytes.

Inhibition of Smad3 phosphorylation blocks neovibsanin B stimulation of ECM proteins

To demonstrate that neovibsanin B induced stimulation of ECM proteins is mediated by activation of Smad3 a specific inhibitor of Smad3, SIS3 was used. For this purpose treatment of ONH astrocytes and LC cells with 25 and 50 μM of SIS3 respectively for 1 h was followed by neovibsanin B treatment for 24 h. The results showed a decrease in FN secretion in ONH astrocytes and LC cells by SIS3 treatment (**Figure 4**). This confirms that activation of Smad3 is required for neovibsanin B-induced ECM expression.

Inhibition of Smad3 or Smad2 suppresses neovibsanin B induced FN and PAI-1 expression

In ONH astrocytes and LC cells Smad3-siRNA caused a significant decrease of Smad3 protein expression compared to control siRNAs. Treatment of ONH astrocytes and LC cells with neovibsanin B in the presence or absence of Smad3-siRNA revealed a decrease in Smad3 via siRNA which significantly inhibited neovibsanin B-induced effects on FN and PAI-1 proteins (**Figure 5**). Transfection with Smad2 siRNA lead to significant reduction of Smad2 protein levels compared to control siRNAs in ONH

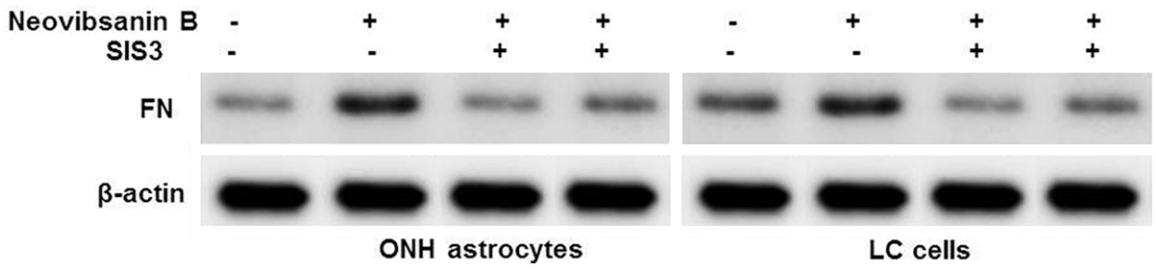

Figure 4. Effect of inhibition of Smad3 phosphorylation on neovibsanin B induced ECM stimulation.

astrocytes and LC cells (**Figure 5**). Western blot and densitometric analysis demonstrated that reduction of Smad2 via siRNA significantly reversed the stimulatory effects of neovibsanin B on FN and PAI-1 protein expression (**Figure 5**).

Discussion

In the whole ocular globe the LC region of the ONH being weakest and susceptible to injury leads to death of RGC axons [33]. It is reported that chronic IOP causes excavation of the optic disc, collapse and remodeling of the LC, and activation of ONH astrocytes [34-36]. Glaucomatous ONH are characterised by variations in the quality, synthesis and deposition of ECM proteins in the LC region which is fatal to RGC axons. Furthermore, in glaucomatous ONH extracellular matrix changes involve increase of collagens I, IV, and VI concentration [37] and degradation of elastin fiber [38]. The results from our study demonstrated that in ONH astrocytes and LC cells treatment with neovibsanin B induced synthesis of ECM protein. Neovibsanin B treatment leads to an increase in soluble FN and PAI-1 in a concentration dependant manner. There are reports that PAI-1 controls metalloproteases (MMPs) which are involved in fibrosis [39, 40].

We also observed that ONH astrocytes on neovibsanin B treatment enhanced secretion of FN, PAI-1, elastin, collagen I and VI. Similar results were observed in LC cells as well. Increase in expression of ECM proteins by LC cells on neovibsanin B treatment, clearly indicates the role of LC cells in changing the mechanical and elastic properties of LC. Thus neovibsanin B can be a promising candidate to improve the ECM changes in the glaucomatous ONH.

We also investigated the signaling pathway used by neovibsanin B to increase ECM protein expression in human ONH astrocytes and LC cells. Our results demonstrated the presence of endogenous pSmad2/3 and their co-localization with Co-Smad4, suggesting that ONH astrocytes and LC cells possess neovibsanin B mediated Smad signaling. Treatment of isolated ONH astrocytes and LC cells with neovibsanin B increased the levels pSmad2 and pSmad3 and their co-localization with Co-Smad4. Thus ONH astrocytes and LC cells respond to neovibsanin B via activation of canonical Smad signaling. However, neovibsanin B treatment did not activate phosphorylation of non-Smad signaling pathways such as ERK1/2, p38, or JNK1/2 in either ONH astrocytes or LC cells. Therefore, neovibsanin B does not appear to stimulate non-Smad pathways in ONH astrocytes and LC cells.

To investigate the involvement of canonical Smad signaling pathway in neovibsanin B-induced ECM protein regulation ONH astrocytes or LC cells were treated with SIS3 prior to neovibsanin B. The results showed inhibition of neovibsanin B-induced FN and PAI-1 expression. The suppression of Smad2 and Smad3 in ONH astrocytes and LC cells by siRNA decreased the total amount of Smad2 and Smad3. Suppression of Smad2 and Smad3 inhibited neovibsanin B-induced synthesis of FN and PAI-1 in ONH astrocytes and LC cells. Therefore, Smad2 as well as Smad3 is used for neovibsanin B stimulated ECM proteins. Since knockdown of either Smad2 or Smad3 completely reversed neovibsanin B stimulated ECM proteins to control levels, both signaling molecules may be required for neovibsanin B stimulation of ECM proteins.

Thus neovibsanin B increases ECM expression in the human ONH. Neovibsanin B induced ECM expression requires activation of the canonical Smad signaling pathway. Therefore,

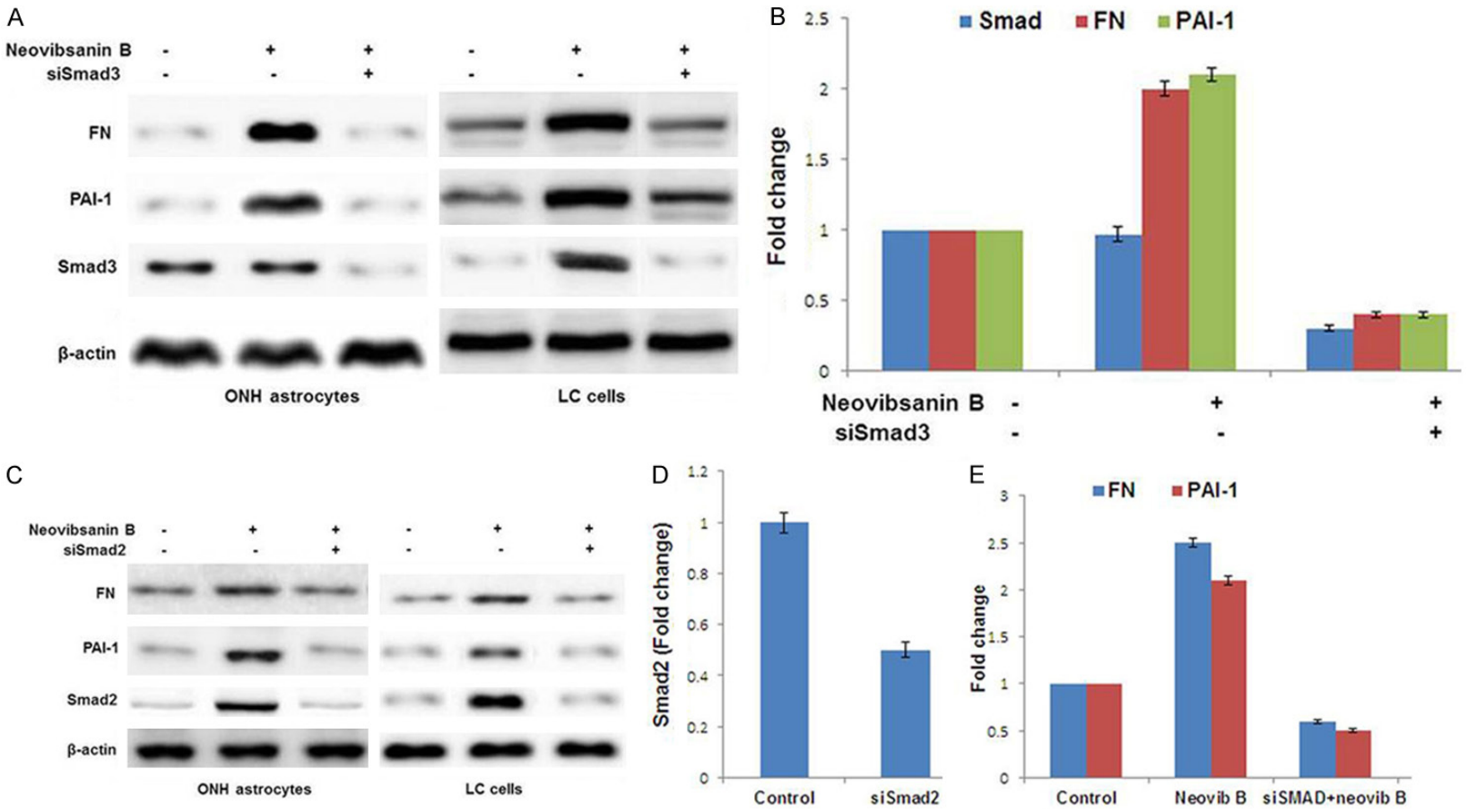

Figure 5. Effect of Smad2 or Smad3 siRNA on neovibsanin B induced FN and PAI-1 expression in ONH astrocytes and LC cells.

it appears that neovibsanin B may be interacting with transforming growth factor-β2 (TGF-β2). However further studies are needed to understand the mechanism.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wei Xu, Department of Ophthalmology, Tongji Hospital, Tongji University School of Medicine, 389 Xincun Road, Shanghai 200065, China. Tel: 0086-21-66111464; Fax: 0086-21-66111464; E-mail: xuwei2616@gmail.com

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