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Ribonuclease 5 coordinates signals for the regulation of intraocular pressure and inhibits neural apoptosis as a novel multi-functional anti-glaucomatous strategy

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ABSTRACT

Glaucoma is a vision-threatening disorder characterized by progressive death of retinal ganglion cells (RGCs), although little is known about therapeutic milestones. Due to its complex and multifactorial pathogenesis, multipronged therapeutic approach is needed. Angiogenin (ANG), now called ribonuclease (RNase) 5, has been previously known as angiogenic factor and more recently its biologic activity is extended to promoting cell survival via its ribonucleolytic activity. Here, we revealed the defect of ANG in human glaucomatous trabecular meshwork (TM) cells and identified novel multiple functions of ANG as an anti-glaucomatous strategy. ANG was highly expressed in normal eyes and normal TM cells compared to glaucomatous TM cells. ANG induced intraocular pressure (IOP) lowering in rat models of both normal and elevated IOP, and as a possible mechanism, activated Akt-mediated signals for nitric oxide (NO) production, an important regulator of IOP in glaucomatous TM cell. Moreover, we demonstrated ANG-induced production of matrix metalloproteinase (MMP)-1 and -3 and rho-kinase inhibition for TM remodeling. For anti-glaucomatous defense optimization, ANG not only elicited immune-modulative pathways via indolamine 2,3-dioxygenase (IDO) activation in TM cells and suppression of Jurkat T cells, but also rescued neural stem cells (NSCs) from apoptosis induced by glaucomatous stress. These results demonstrate that novel multi-functional effects of ANG may have benefits against glaucoma in ocular tissues.

1. Introduction

Glaucoma is an ocular disorder characterized by progressive death of retinal ganglion cells (RGCs), eventually leading to vision loss if untreated, and is the world's leading cause of the irreversible blindness [1]. As the world population grows and ages, the total number of people affected by glaucoma will continue to increase and is expected to be 80 million including 11.2 million blind by 2020 [2,3].

Primary open-angle glaucoma (POAG), which comprises the majority of primary glaucoma, has three targets: the trabecular meshwork (TM) in the anterior chamber of the eye, RGCs and visual cortex, in an ascending order [4–6]. It has been reported that the visual field defects due to RGC degeneration are directly proportional to TM damage [7]. Although still not fully revealed, IOP increases by malfunctioning TM, as one of the main culprits of visual field defect in glaucoma, may create a mechanical stress that is transmitted posteriorly and damages the RGCs [8]. To date, accordingly, understanding TM cell protection against damage has become a key topic for the treatment and prevention of glaucoma [9]. The various molecular alterations in anterior chamber induce apoptotic TM damage and TM cellularity [10], furthermore increase intraocular pressure (IOP) [11].

Thus, it is needed that targeting diverse pathogenic causes for glaucoma in TM beyond only ameliorating the resultant IOP increase should be emphasized. In the similar context, for example, dorzolamide hydrochloride and timolol maleate, which are the currently famous anti-glaucomatous agents, are recently known to exert protective effect towards oxidative stress in TM as well as to demonstrate well-known IOP lowering effect [12]. Several discoveries indicate that common factors are involved in TM and retinal changes in glaucomatous cascades [13,14] and encourage the hope that targeting TM itself is very important and may also cover the visual field alteration, a main event in glaucoma, at the end.

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Ribonuclease (RNase) 5 which is the 5th member of RNases and is also called angiogenin (ANG), is a 14.4 kDa single-chain polypeptide [15] and it was originally isolated from conditioned medium of cultured human colon adenocarcinoma cells [16]. Now, RNase 5 is known to undergo nuclear translocation and stimulate rRNA transcription to perform its various activities [17]. Recent studies about loss-of-function of ANG in amyotrophic lateral sclerosis [18–22] and Parkinson disease [22,23] demonstrates that RNase 5 is important for normal homeostatic functions. RNase 5 is also known to reprogram protein translation for cell survival under adverse conditions [17,24]. In the field of ophthalmology, Sack et al. reported that ANG is highly concentrated in normal tear fluid pooled overnight with maintaining the corneal avascularity, indicating probable physiologic roles for ANG rather than angiogenesis in normal eyes [25].

ANG has been suggested to be responsible for generating nitric oxide (NO) by activating endothelial NO synthase (eNOS) [26]. eNOS is well-known to be involved in modulating the endothelial permeability as a key enzyme of vascular homeostasis. Interestingly, the vascular balance mechanism between vasoconstriction and vasodilation is also necessary for homeostatic IOP regulation [27]. In a previous study, diverse vascular biomarkers were identified in POAG aqueous humor compared to normal [28] and ubiquitin, as one of them, is known to regulate eNOS activity [29]. Recently, NO is highlighted to contribute to the homeostatic mechanism maintaining aqueous humor outflow and normal IOP as an emerging target for the treatment of glaucoma [30,31]. Furthermore, we have reported the survival effect of NO in corneal fibroblasts under a low range of concentrations, metaphorically called the “double-edged sword” [32]. In addition, ANG can be a candidate survival booster for trabecular meshwork (TM) cells in TM, which is a key area for maintaining IOP, based on their embryonic origin from the neural crest [33] and the recently revealed neuroprotective roles of RNase 5.

As ANG is identified pathologically deficient in several human diseases, we investigated ocular expressions of ANG and further revealed the novel effects of ANG. For anti-glaucomatous ocular optimization, ANG, the synonym of RNase 5, showed multi-functional effects including IOP regulation via activation of an array of signaling pathways, TM cell survival, immune-modulation in TM and anti-apoptosis of neuronal lineage stem cell against glaucomatous damages.

2. Materials and methods

2.1. Study approval

The study protocol and the consent form were approved by the institutional review board of the Chung-Ang University Hospital. All procedures were performed according to the tenets of the Declaration of Helsinki, and informed consent was obtained for the use of cadaveric ocular tissues.

2.2. Reagents and antibodies

The detailed information about reagents and antibodies used in this study are shown in Supplementary Materials and Methods.

2.3. Immunohistochemical staining of cadaveric ocular tissue

The eyes donated for research were pre-fixed in 4% paraformaldehyde solution for 6 h, and cut perpendicular to the cornea, including cornea, limbus, and conjunctiva. The tissue was fixed at room temperature for 6 h. The next serial steps are shown in Supplementary Materials and Methods.

2.4. Animals

All animal experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague–Dawley rats (8-weeks-of-age; weight, 250–270 g), raised at the Clinic Research Center, Chung-Ang University, College of Medicine, were used.

2.5. Preparation of ocular hypertensive rats as a glaucoma rat model

To test and establish the glaucoma rat model, eight eyes of eight white rats were used in each vortex venous ablation glaucoma model and steroid-induced glaucoma model and the contralateral eyes were served as sham operated control. The information about preparation of both models is shown in Supplementary Materials and Methods.

2.6. Instillation of eye drops in the test rat models

We instilled two drops of ANG (50 μg/mL; 12 eyes) or 50 μg/mL latanoprost, a representative $PGF_{2\alpha}$ analogue (Xalatan™, Pfizer; 12 eyes) or normal saline (12 eyes) in 4 μL/drop in the normal IOP rat and high IOP rat models to compare the ocular hypotensive effects of ANG and the $PGF_{2\alpha}$ analogue. The eye drop agents were administered at 8 am and 8 pm to compare day- and night-time effects, respectively.

2.7. ANG-Cy3 tagging

Purified ANG was labeled with a Cy3 mono-reactive dye (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's protocol. The Cy3-conjugated ANG (ANG-Cy3) was purified using PD-10 columns (GE Healthcare). Labeling efficiencies were assessed using the Cy3 extinction coefficient at 550 nm and 280 nm. The protein-to-dye ratio was 0.6.

2.8. Administration of ANG-Cy3 eye drops in the rat model

ANG (50 μg/mL) tagged with Cy3 mono-reactive fluorescence was applied to rat eyes every 4 h for 2 days. After the enucleation, the eyeball was cryo-sectioned axially, and frozen biopsy was performed immediately without washing or staining. Then, we observed the distribution of ANG in the eye under fluorescence microscope compared with that of 4′,6-diamidino-2-phenylindole (DAPI) staining.

2.9. Tear collection and aqueous humor extraction in eyes

The detailed information is noted in Supplementary Materials and Methods.

2.10. Primary TM cell

The normal TM tissues were obtained from four post-mortem non-diseased human eyes at the time of corneal transplant and four trabeculectomy specimens from POAG patients were used as glaucomatous TM tissues to prepare the non-transformed primary human normal TM cells (pNTM) and glaucomatous TM cells (pGTM), respectively. The TM tissues were dissected and isolated from eyes as previously described by Wordinger et al. [34]. The detailed processes for culture are shown in Supplementary Materials and Methods. Primary TM cells used for experiments were restricted to 3 to 5 passages.

2.11. Transformed TM cell line and neural stem cell (NSC)

SV40-transformed human TM cell lines derived from normal subjects and patients with glaucoma (NTM5 and GTM3, respectively) were provided by Alcon Laboratories (Fort Worth, TX, USA). The immortalized human NSC clone HB1.F3 was obtained from Prof. S.U. Kim (Chung-Ang University College of Medicine). The detailed processes are shown in Supplementary Materials and Methods.

2.12. Preparation of conditioned medium derived from human TM cells

Conditioned media were harvested from NTM5 and GTM3 with or without ANG treatment (2 μg/mL, 1 h).

2.13. In vitro drug treatment

The detailed concentrations and times of treatment of the drugs in this study are shown in Supplementary Materials and Methods.

2.14. Other experiments

The information about conventional *in vitro* experiments used in this study including semi-quantitative RT-PCR, real-time RT-PCR, ELISA, immunodot blot, western blot analysis, cell viability (MTT) assay, immunocytochemistry, flow cytometry, and Hoechst 33342 and PI staining is shown in Supplementary Materials and Methods.

2.15. Statistical analysis

Analysis of variance (ANOVA) and repeated measures ANOVA with *post-hoc* analysis were used to analyze the differences among three or more groups. Two-tailed Student's *t*-test or paired *t*-test and Mann–Whitney *U* test or Wilcoxon signed rank test were used for the statistical analysis between two groups. *P* values <0.05 were considered significant.

3. Results

3.1. Human ANG is expressed in normal ocular tissue and its decreased expression is associated with glaucoma

We examined ANG expression in the eyes (Fig. 1A) of dead subjects without previous ocular disorders or cancers. ANG was immune-expressed throughout all ocular tissues, including the cornea, conjunctiva,

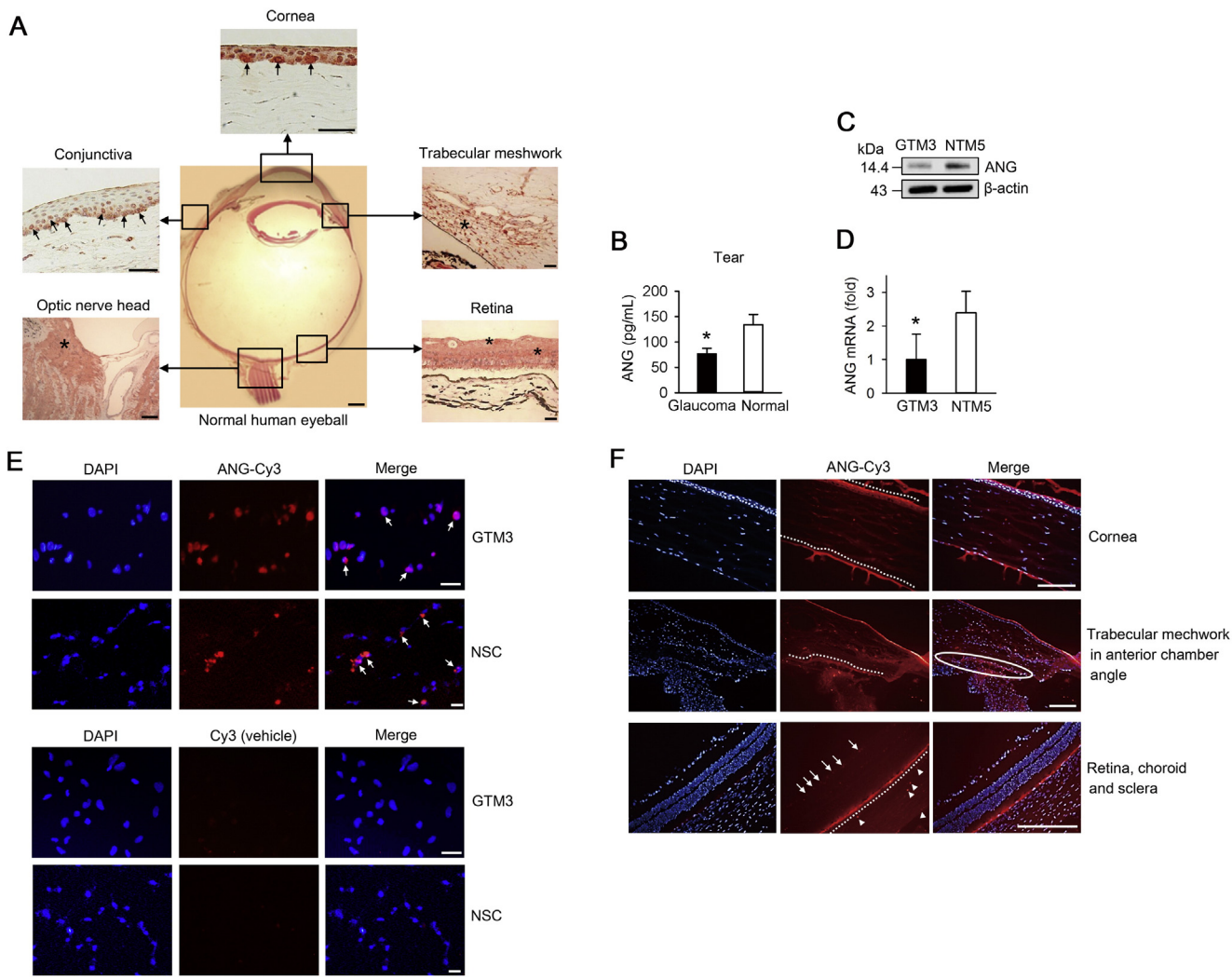

Fig. 1. Angiogenin (ANG) expression patterns in human ocular tissues and the delivery of treated ANG to target regions in rat eyes, human trabecular meshwork (TM) cells, and neural stem cells. (A) Immunostaining for ANG in a human normal eyeball. ANG-expressing cells and areas are indicated by arrows and asterisks. $n = 3$ eyes. Scale bars, 100 μm except for human eyeball, which is 2 mm. (B) Tear ANG levels by ELISA in normal controls and patients with open-angle glaucoma. $^*P < 0.05$ (compared to normal, *t*-test). $n = 12$ eyes per group. (C) Immunoblotting of ANG from transformed human normal and glaucomatous TM cell lines (NTM5 and GTM3, respectively). $n = 3$ independent experiments. (D) Quantification of ANG mRNA expression in NTM5 and GTM3. $^*P < 0.05$ (compared to NTM5, *t*-test). Data shown are the GAPDH-normalized fold changes. $n = 5$ independent experiments. Error bars represent SEM (B, D). (E) Representative intracellular fluorescence (red) images after treating cultured human GTM3 and the HB1.F3 immortalized human neural stem cell (NSC) clone with ANG-Cy3 (5 μg/mL, 3 h). Representative cells with nuclear internalized ANG are indicated by arrows and compared to control vehicle. (F) Representative ANG tissue fluorescence (red) images in ocular tissues of normal rats, including the cornea (up), anterior chamber angle (middle; oval, trabecular meshwork), retina, choroid, and sclera (bottom) after administration of Cy3-tagged ANG (50 μg/mL) eye drops every 4 h for 2 days. Prominent distribution of ANG are indicated by dotted lines, arrows, and arrow heads (up, corneal epithelium and endothelium by dotted lines; middle, trabecular meshwork by dotted line; bottom, retinal ganglion cells by arrows, circulation in suprachoroidal space by dotted line, and ANG flowed out to sclera from suprachoroid by arrow heads). $n = 3$ independent experiments (E, F). Scale bars, 100 μm (E, F).

TM, retina and optic nerve head which collects retinal nerve fiber bundles. The representative photos of ocular tissues comparing ANG expression and the controls are showed in Supplementary Fig. 1. In particular, the TM cell network, which is a key site for aqueous outflow, thus IOP regulation, and the RGCs in the retina revealed prominent ANG expression.

Tear composition is affected by conjunctival secretions. Based on the presence of ANG in normal conjunctiva (Fig. 1A), we further evaluated ANG concentration in tears sampled from patients with open-angle glaucoma before starting anti-glaucomatous eye drops. Tear ANG levels determined by ELISA were significantly lower in patients with glaucoma than those in normal subjects (Fig. 1B). Furthermore, we investigated the ANG expression difference in transformed human normal TM cell line (NTM5) and glaucomatous TM cell line (GTM3) as well as primary human normal TM cells (pNTM) and glaucomatous TM cells (pGTM). ANG expression was defective in GTM3 at both the mRNA and protein levels compared to that in NTM5 (Fig. 1C and D). Moreover, pGTM also revealed down-regulated ANG expression in both mRNA and protein levels compared to pNTM (Supplementary Fig. 2), suggesting that primary TM cells (pNTM and pGTM) may reflect ANG-related phenotypes and responses of transformed TM cell lines (NTM5 and GTM3).

3.2. The ANG protein reaches target tissues and target cells following administration

We treated GTM3 and the immortalized human neural stem cell (NSC) clone HB1.F3 isolated from embryonic human brains as described previously [35] with ANG-Cy3 to confirm intracellular delivery of ANG to TM cells with glaucoma and nerve cells which are major therapeutic target in glaucoma. The red fluorescence was internalized, mainly to the nucleus, in both GTM3 and NSCs after the ANG-Cy3 treatment compared to the control vehicle (Fig. 1E).

In general, therapeutic use of proteins is still hindered by their large molecular size and by being recognized as foreign to recipient organs. Thus, we assessed whether ANG could be delivered to target tissues including TM and the retina after administration as an eye drop formula. After eye dropping Cy3-tagged ANG (ANG-Cy3), the Cy3 red fluorescence was observed in the corneal surface epithelial and endothelial layer, TM, RGCs, and suprachoroidal circulation, and sclera (Fig. 1F). This finding indicates that ANG protein penetrates the corneal tissue to reach the neuro-retina, especially the RGCs.

3.3. ANG induces the NO production pathway in human TM cells

NO that is produced by eNOS is known to have mechanoregulatory role in aqueous humor dynamics, and as a result, lead to IOP lowering [30,31]. The concentration of NO was elevated in the aqueous humor of normal rats after administering ANG (Supplementary Fig. 3). Thus, we investigated whether ANG elicits activation of intraocular NO production pathways and how such a pathway works in human TM cells. ANG induced time-dependent increase of both Akt and eNOS phosphorylation in GTM3 (Fig. 2A).

Because ANG is known as a ribonuclease and Cy3-tagged ANG was delivered mainly into the nucleus of GTM3 cells (Fig. 1E), we hypothesized that intranuclear translocation of ANG might be involved in NO production signal also. Pretreatment with neomycin, which suppresses nuclear translocation of ANG in vascular endothelial cells [36], inhibited ANG translocation into the nucleus and rather localized it in the cytoplasm in GTM3 cells (Fig. 2B).

Then, LY294002 (PI3k inhibitor) and neomycin were used to determine whether activation of eNOS was affected by the phosphatidylinositol-3-kinase (PI3k)-Akt pathway and by intranuclear localization of ANG, respectively in GTM3. Both phosphorylated eNOS (p-eNOS) and Akt (p-Akt) following ANG administration decreased after pretreatment with LY294002 or neomycin (Fig. 2C and D), indicating that there is a pathway inducing eNOS phosphorylation by activating Akt via PI3k and by intranuclear localization of ANG in glaucomatous TM cells. Although both LY294002 and neomycin inhibited phosphorylation of

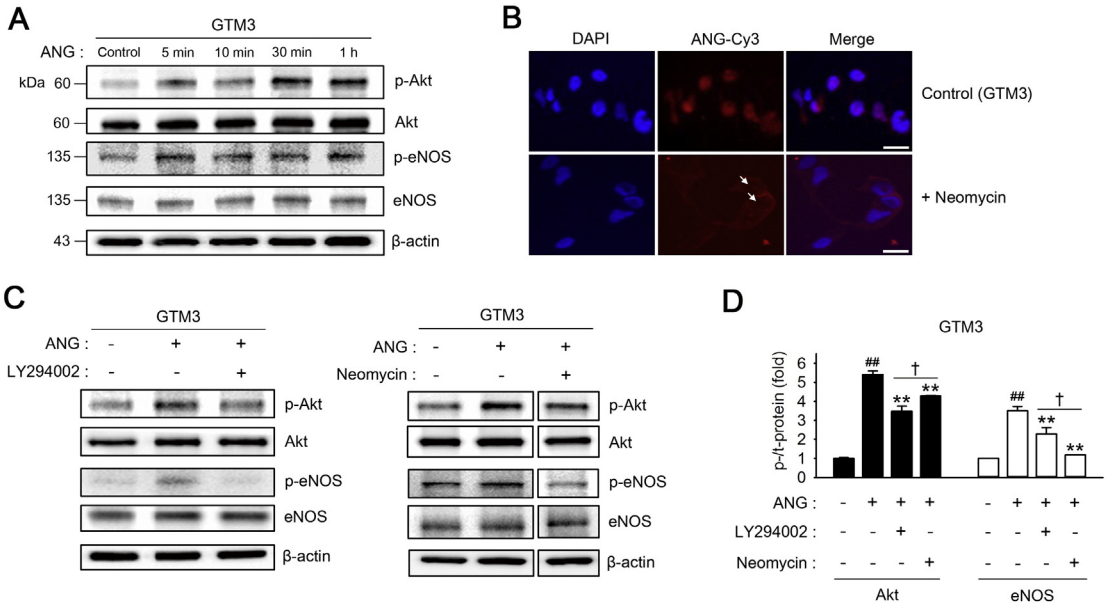

Fig. 2. Intraocular nitric oxide (NO) production pathways elicited by angiogenin (ANG) in GTM3 cells. (A) Immunoblotting of Akt and endothelial nitric oxide synthase (eNOS) in transformed human glaucomatous trabecular meshwork (TM) cells (GTM3) before (control) and after ANG treatment (5 μg/mL). (B) Representative intracellular ANG fluorescence (red) images after treating GTM3 cells with ANG-Cy3 (5 μg/mL, 3 h) in the absence or presence of neomycin (50 μM). Representative GTM3 cells with suppressed ANG-Cy3 nuclear translocation by neomycin are indicated by arrows. Scale bars, 50 μm. (C, D) Immunoblotting of Akt and eNOS (C) and quantitative analysis of the band density (D) from GTM3 cells with (5 μg/mL, 5 min) and without ANG and in the absence or presence of LY294002 (20 μM, 1 h), a PI3k inhibitor, and neomycin (1 mM, 1 h). Both p-Akt and p-eNOS induced by ANG were suppressed by LY294002 or neomycin. The small gaps indicate skipped lanes from the same membrane. (D) Both p-Akt and p-eNOS relative to total-Akt (t-Akt) and t-eNOS, respectively were induced by ANG and suppressed by LY294002 or neomycin. Akt phosphorylation was inhibited mainly by LY294002 compared to neomycin. On the contrary, p-eNOS was suppressed prominently by neomycin compared to LY294002. Akt: *vs* control ($^{\#\#}P < 0.01$), with *vs* without LY294002 ($^{**}P < 0.01$), with *vs* without neomycin ($^{**}P < 0.01$), with LY294002 *vs* with neomycin ($^{\dagger}P < 0.05$); eNOS: *vs* control ($^{\#\#}P < 0.01$), with *vs* without LY294002 ($^{**}P < 0.01$), with *vs* without neomycin ($^{**}P < 0.01$), with LY294002 *vs* with neomycin ($^{\dagger}P < 0.05$). ANOVA followed by Bonferroni's *post-hoc* analysis was performed for statistical analyses. Error bars represent SEM. $n = 3$ independent experiments (A–D).

Akt and eNOS, p-Akt was suppressed mainly by LY294002 and p-eNOS was suppressed mainly by neomycin in GTM3 (Fig. 2D). This suggests that the intranuclear ANG existence may activate eNOS through its independent pathway which is unrelated to Akt as well as via activating Akt in glaucomatous TM cells.

We intended to confirm that those signaling pathways elicited by ANG were not unique to transformed human TM cells. Through the parallel experiments, cultured primary human glaucomatous TM cells (pGTM) also revealed time-dependent Akt activation and upregulated eNOS by ANG in a similar fashion to GTM3 (Supplementary Fig. 4).

3.4. ANG activates MMP-1 and -3 and inhibits rho-kinase phosphorylation in human TM cells

To identify other signaling pathways of ANG for regulating IOP, we screened mRNA expressions of several candidates as defects in GTM3 rather than in NTM5 cells. Matrix metalloproteinase (MMP)-1 and -3, which are upregulated in human TM cells with enhanced trabecular aqueous outflow after $PGF_{2\alpha}$ analogue treatment [37], were expressed low in GTM3 although the difference of MMP-3 was not definite (Supplementary Fig. 5A). Additionally, rho-kinase signaling was uninhibited in GTM3 (Supplementary Fig. 5B). Rho-kinase mediates fibrogenic activity at the TM and eventually impacts resistance to aqueous outflow [38], and inhibiting rho-kinase induces cytoskeletal changes in TM cells and thus increases aqueous outflow [26]. Expression of MMP-1 mRNA increased in GTM3 (Supplementary Fig. 5C), and that of rho-kinase mRNA was prominently downregulated by ANG in both NTM5 and GTM3 (Supplementary Fig. 5D). Moreover, collagen I, a fibrogenic marker in TM [38], was also inhibited by ANG in prominently in GTM3 (Supplementary Fig. 5E).

MMP-1 and -3 protein expressions were lower in GTM3 than in NTM5 (Fig. 3A). MMP-1 and -3 expressions in GTM3 were augmented by ANG treatment over the course of time (Fig. 3B). In addition, phosphorylation of myosin phosphatase target subunit 1 (MYPT1), a downstream target of rho-kinase, was suppressed significantly after ANG treatment, particularly for 10 min in GTM3 (Fig. 3C). Phalloidin immunofluorescence staining indicated that 1 and 3 h ANG treatments retracted and rounded the cell bodies, suggesting cellular relaxation in GTM3 and NTM5 cells (Fig. 3D). It is thought that rho-kinase inhibition, which is represented as suppressed MYPT1 by ANG, may induce cytoskeletal change in TM cells, especially relaxation, to probably enhance the aqueous outflow for IOP regulation.

Primary glaucomatous TM cells (pGTM) revealed defective expression of MMP-1 and -3 compared to pNTM (Supplementary Figs. 6A and B) and pGTM showed time-dependent activation of MMP-1 and -3 proteins (Supplementary Fig. 6C) like GTM3 did. Moreover, MYPT1 was more robustly suppressed in pGTM compared in GTM3 (Supplementary Fig. 6D).

MMP-1 and -3 are representatives which are therapeutically upregulated by latanoprost, one of $PGF_{2\alpha}$ analogues, in human TM cells [37]. Interestingly, $PGF_{2\alpha}$ level was elevated by ELISA in aqueous humor of normal rats after applying ANG eye drops (Supplementary Fig. 7A). Conversely, tear ANG concentration in subjects with normal human eyes increased significantly by immunodot assay after applying $PGF_{2\alpha}$ analogue (latanoprost) eye drops (Supplementary Fig. 7B). It is very notable that these data indicate that there may be a probable reciprocal action mechanism between ANG and $PGF_{2\alpha}$ analogue in eye and ANG may be an important candidate target of action of latanoprost eye drops which mode of action are still veiled except its MMP activation pathway.

3.5. IOP-regulatory effect of ANG in rat models

Based on the ANG-elicited activation of diverse signals for IOP regulation in human TM cells, we applied ANG eye drops (50 μg/mL) in both of normal IOP- and high IOP-rat model approximating NTG and open-angle glaucoma, respectively to elucidate the IOP-regulatory effect of ANG in *in vivo*. We selected vortex vein cauterization method to prepare the high IOP model because of its superiority for lowering IOP rather than prolonged steroid administration (Supplementary Fig. 8).

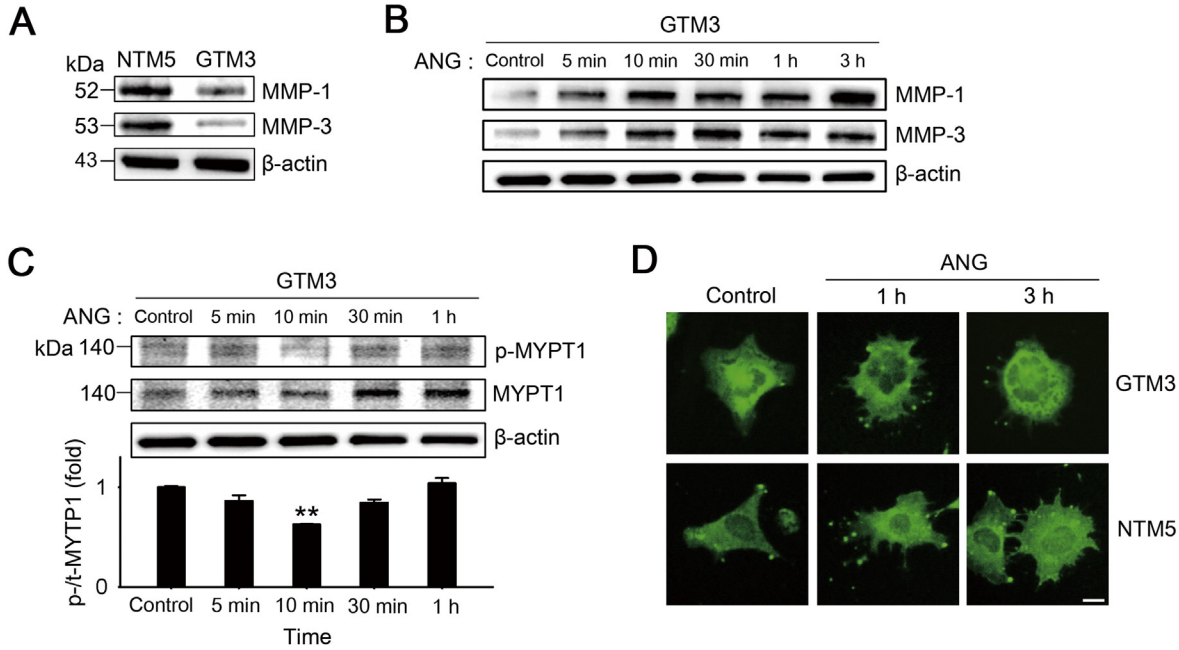

Fig. 3. Activation of matrix metalloproteinase (MMP)-1, MMP-3, and inhibition of rho-kinase phosphorylation by angiogenin (ANG) in GTM3 cells. (A) Immunoblotting of MMP-1 and -3 in transformed human normal trabecular meshwork (TM) cells (NTM5) and glaucomatous TM cells (GTM3). (B) Immunoblotting of MMP-1 and -3 in GTM3 before (control) and after ANG treatment (1 μg/mL, 5 min to 3 h). (C) Immunoblotting of myosin phosphatase target subunit 1 (MYPT1) in GTM3 before (control) and after ANG treatment (1 μg/mL, 5 min to 1 h). $^{**}P < 0.01$ (compared to the control, ANOVA followed by Bonferroni's *post-hoc* analysis). Error bars represent SEM. (D) Representative images of the changes in the actin cytoskeleton of GTM3 and NTM5 cells before (control) and after ANG treatment (5 μg/mL, 1–3 h). Scale bar, 20 μm. $n = 3$ independent experiments (A–D).

The ANG or prostaglandin (PG)$F_{2\alpha}$ analogue (latanoprost) eye drops significantly reduced IOP in both models within 24 h after application in both the 8 am and 8 pm application groups (Fig. 4A–D). The IOP lowering effect by ANG was maximal in the normal IOP model especially when administered at night (8 pm) (Fig. 4E). Notably, although not being analyzed quantitatively, it was found that the aqueous outflow channels of TM were dilated by tissue stain after applying ANG (Fig. 4F). It is suggested that ANG may enhance the aqueous outflow and thereafter lower IOP, although the IOP change in high-IOP glaucoma model was smaller compared to in normal IOP model (8 pm) probably due to the limited drainage capacity of remaining vortex venous outflow after cauterization.

Then, we administered ANG and latanoprost eye drops for 4 weeks to verify the mid-term maintenance of IOP lowering effect of ANG in rats with normal IOP. The daily application of ANG and latanoprost at night (8 pm), which had showed superior IOP lowering compared to application at 8 am, lowered IOP significantly over 4 weeks (Fig. 4G). There was no significant difference of IOP between ANG- and latanoprost-application groups at 4 weeks.

3.6. ANG promotes cellular protection of human TM cells via survival and immune-modulation signaling

Damage to the TM may contribute to loss TM tissue function with significance in cases of ocular hypertension and primary open angle glaucoma. mTOR and its downstream target P70s6k are well-known to reduce apoptosis and allow cellular proliferation dependent on PI3k and p-Akt in various cells. ANG treatment increased p-P70s6k expression in a time-dependent manner in NTM5 (Fig. 5A) and GTM3 (Fig. 5B). ANG treatment did not reduce the cell viability in NTM5 (Fig. 5C) and in GTM3 (Fig. 5D) up to 4 days. Interestingly, viability of GTM3 cells rather increased significantly 6 h after ANG treatment (Fig. 5D). It is proposed that rapid activation of mTOR and Erk pathways within 1 h after ANG treatment that is more prominent in GTM3 than in NTM5 (Fig. 5A and B) may reflect the transient enhancement of the viability of GTM3.

The cellular immune system is altered in glaucoma and, for example, the concentrations of T lymphocytes and IL-2 increases in the serum of patients with glaucoma [39]. The viability of T lymphocytes (Jurkat T cells) decreased when treated with TM cell (NTM5 or GTM3) protein extracts exposed to ANG (Fig. 6A) or when cultured in ANG-treated TM cell (NTM5 or GTM3)-conditioned medium (Fig. 6B). Specifically, ANG reduced the mRNA expression of phosphoinositide-dependent protein kinase 1 (PDK1) (Supplementary Fig. 9A) and IL-2 (Supplementary Fig. 9B) and decreased the subsets of $CD69^+$ (Supplementary Fig. 10A) or $CD25^+$ cells (Supplementary Fig. 10B) in the pool of activated Jurkat T cells induced by anti-CD3 and anti-CD28 antibodies.

Human ocular tissue is normally armed with local immunosuppressive factors to maintain its immune-privileged function. For example, the expression of indolamine 2,3-dioxygenase (IDO) is observed in ocular surface areas and indirectly inactivates T cells [40]. We investigated the IDO expression patterns as an immune-modulating factor in both NTM5 and GTM3 and evaluated the influences of ANG on IDO expression. IDO mRNA level was significantly lower in GTM3 than that in NTM5 (Fig. 6C) and ANG upregulated IDO expression in both types of cells (Fig. 6D and E).

To sum up, we speculate that the multiple functions of ANG elicit diverse anti-glaucomatous signals in TM cells as presumptively illustrated in Fig. 7.

3.7. ANG inhibits glutamate- or H_2O_2-induced apoptosis in NSCs

We prepared two apoptotic human NSC HB1.F3 lines against glutamate-induced excitotoxicity and oxidative stress-induced cell

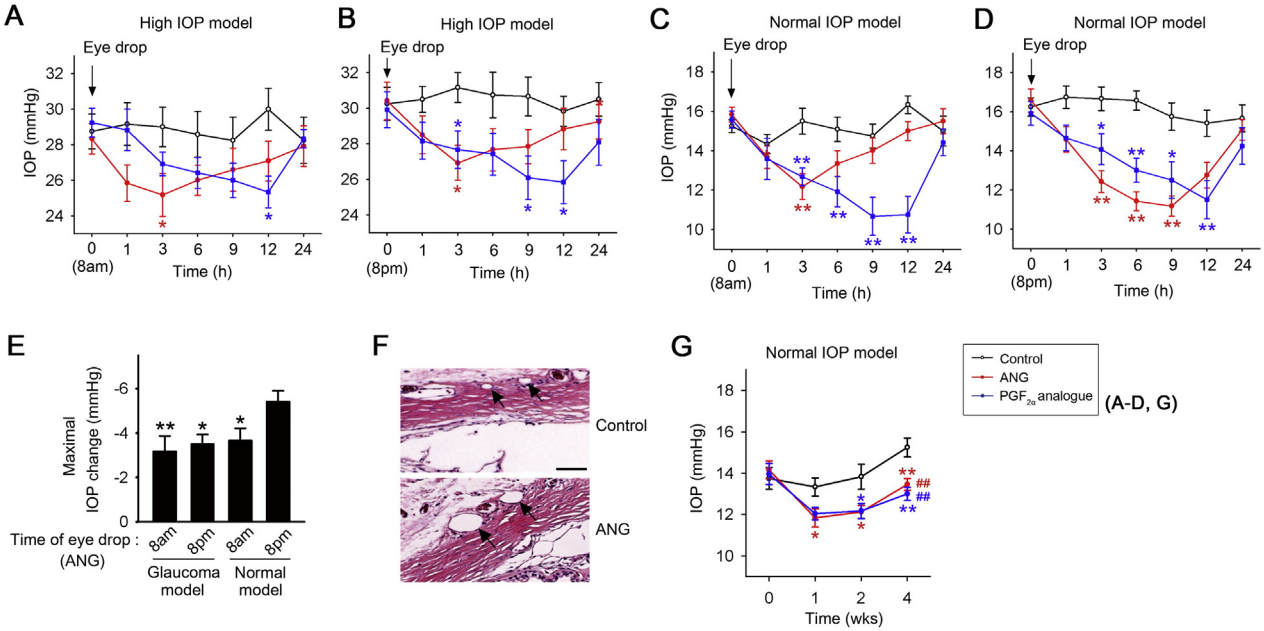

Fig. 4. Intraocular pressure (IOP)-lowering effect of angiogenin (ANG) eye drops in the normal and glaucoma rat models. (A–D) The estimated IOP over 24 h after application of ANG (50 μg/mL) or $PGF_{2\alpha}$ analogue (latanoprost, 50 μg/mL) eye drops compared to normal saline eye drops (control) in high-IOP glaucoma (A, B) and normal IOP rats (C, D). The eye drop application time occurred at 8 am (A, C) and 8 pm (B, D). $^*P < 0.05$ and $^{**}P < 0.01$ (compared to group control, ANOVA followed by Bonferroni's *post-hoc* analysis). Error bars represent SEM. $n = 12$ eyes of rats per group. (E) The maximum IOP changes by ANG according to eye drop application time and rat model. IOP decreased the most when applied to normal IOP rats at 8 pm and the maximal IOP change was higher compared to other model and times ($^*P < 0.05$ and $^{**}P < 0.01$ compared to 8 pm drop in normal model, ANOVA followed by LSD's *post-hoc* analysis). (F) Representative histological stains (hematoxylin and eosin) of the rat eye trabecular meshwork before (control) and 1 h after ANG eye drop administration. The aqueous outflow channels enlarged by ANG compared to the control are indicated by arrows. Scale bar, 50 μm. (G) Serial IOPs estimated at 1 pm over 4 weeks by ANG (50 μg/mL), $PGF_{2\alpha}$ analogue (latanoprost, 50 μg/mL) and normal saline (control) eye drops in normal IOP rats. $^*P < 0.05$ and $^{**}P < 0.01$ (compared to group control, ANOVA followed by Bonferroni's *post-hoc* analysis). The overall IOP lowering effects over 4 weeks among three groups were significantly different ($P < 0.01$, repeated measures ANOVA). Specifically, both group ANG and $PGF_{2\alpha}$ analogue showed difference of overall IOPs compared to group control, $^{\#\#}P < 0.01$ (*vs* control, repeated measures ANOVA followed by Bonferroni's *post-hoc* analysis). But, there was no difference of overall IOP lowering over 4 weeks between group ANG and $PGF_{2\alpha}$ analogue. Error bars represent SEM. $n = 8$ eyes of rats per group.

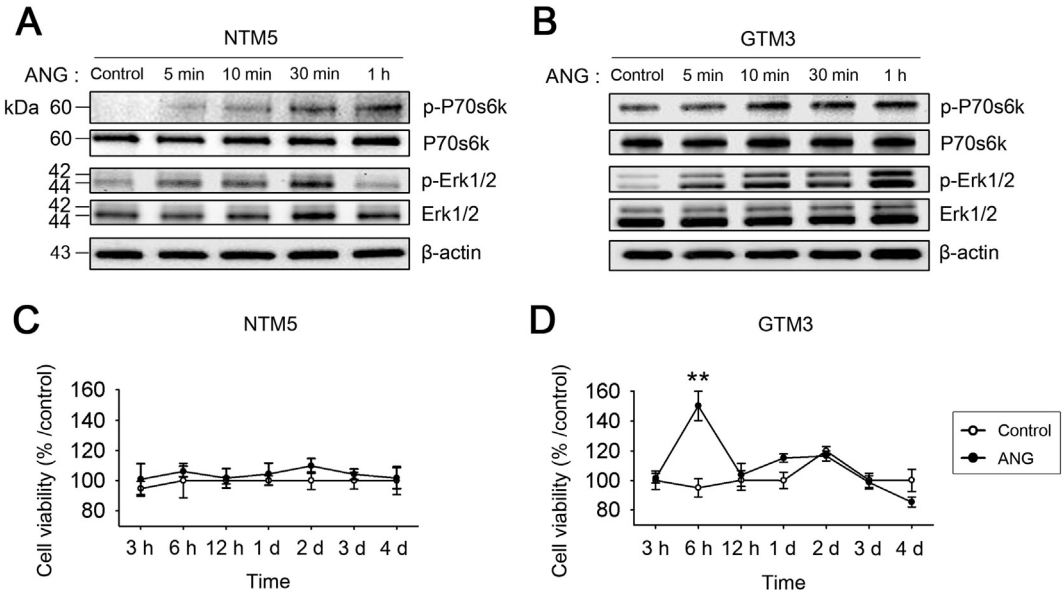

Fig. 5. Angiogenin (ANG) enhances survival pathways in NTM5 and GTM3 cells. (A, B) Immunoblotting of P70s6k and Erk1/2 phosphorylation from transformed human normal TM cells (NTM5, A) and glaucomatous TM cells (GTM3, B) before (control) and after ANG treatment (2 μg/mL in NTM5 and 5 μg/mL in GTM3, 5 min to 1 h). $n = 3$ independent experiments. (C, D) Cell viability analysis (MTT assay) of NTM5 (C) and GTM3 (D) exposed to ANG (5 μg/mL) compared to controls over 4 days. No statistical difference among times was noted when treated NTM5 cells with ANG (ANOVA). Error bars represent SEM. $n = 3$ independent experiments. (D) ANG enhanced GTM3 cell viability significantly after a 6 h treatment. $^{**}P < 0.01$ (compared to group control, ANOVA followed by Bonferroni's *post-hoc* analysis).

damage using H_2O_2, which both are known to be involved in glaucoma pathogenesis to evaluate the neuroprotective effect of ANG. Unfortunately, the RGC-5 cell line from rat RGCs, which had been widely used since 2001 [41], was shown to be a transformed photoreceptor cell line [42]. Although NSCs cannot directly reflect the features of RGCs, embryonic stem cell-derived neural progenitors may differentiate into RGC-like cells *in vitro* [43]. Therefore, we utilized immortalized human NSCs in place of RGCs.

Concentrations of 0.5 mM glutamate and 5 μM H_2O_2, representing approximately 50–70% cell viability, were selected to induce apoptosis

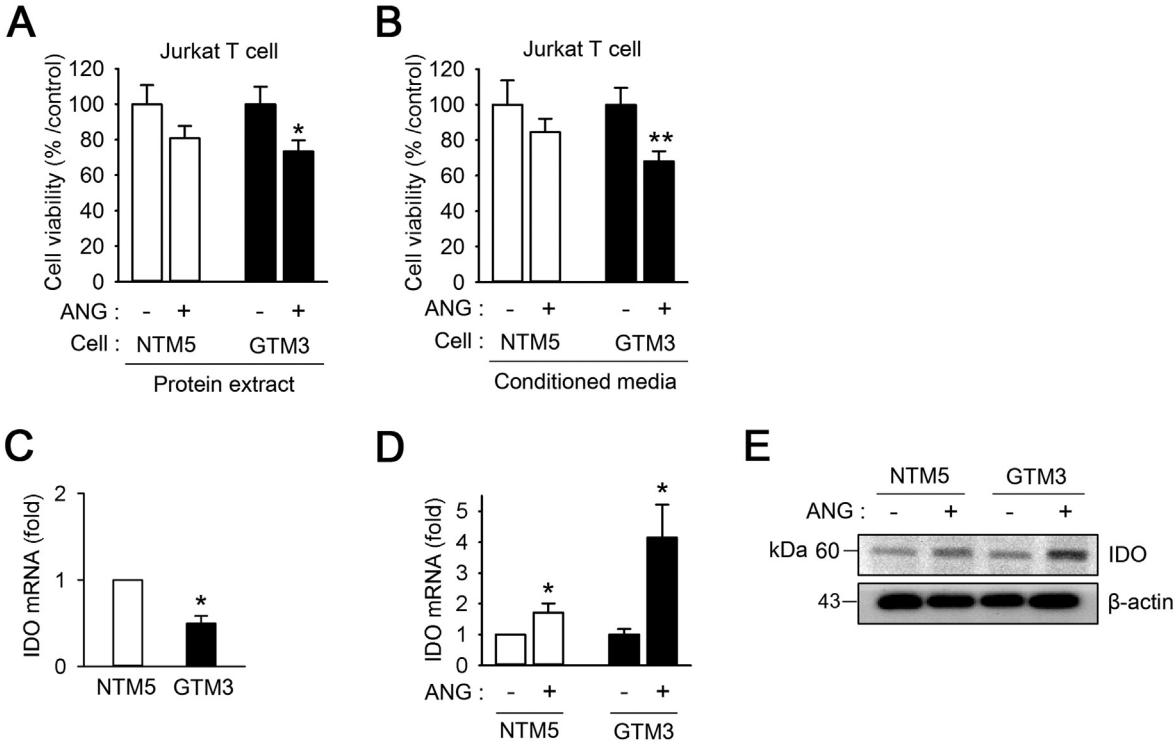

Fig. 6. Angiogenin (ANG) modulates the immune-inflammatory signal in human trabecular meshwork (TM) cells. (A, B) Cell viability analysis of Jurkat T cells (A) cultured with proteins from transformed human TM cells (NTM5 and GTM3) with or without ANG or (B) cultured in conditioned media from NTM5 and GTM3 with or without ANG (2 μg/mL, 1 h). $^{*}P < 0.05$ and $^{**}P < 0.01$ (compared to GTM3 without ANG, *t*-test). (C) Quantification of indolamine 2,3-dioxygenase (IDO) mRNA expression in NTM5 and GTM3 cells. $^{*}P < 0.05$ (compared to NTM5, *t*-test). (D, E) IDO expression in NTM5 and GTM3 cells before and after ANG treatment (2 μg/mL, 1 h) in mRNA (D) and in protein level (E). $^{*}P < 0.05$ (compared to NTM5 or GTM3 without ANG, *t*-test). Data shown are the GAPDH-normalized fold changes (C, D). Error bars represent SEM (A–D). $n = 3$ independent experiments (A–E).

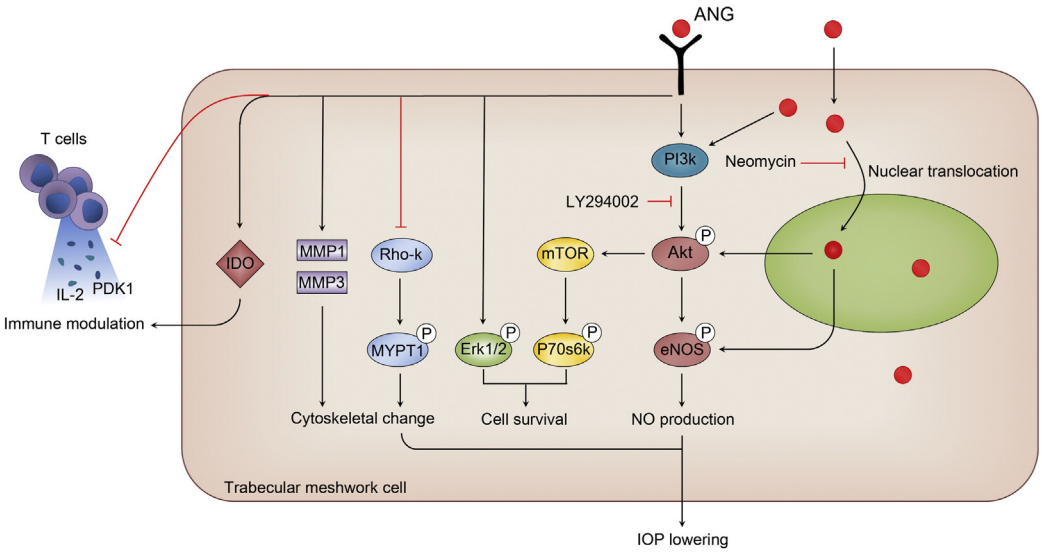

Fig. 7. Illustration showing proposed scenario of how angiogenin (ANG) elicits diverse anti-glaucomatous signals in human trabecular meshwork (TM) cells. eNOS, endothelial nitric oxide synthase. NO, nitric oxide. IOP, intraocular pressure. MMP, matrix metalloproteinase. IDO, indolamine 2,3-dioxygenase. PDK1, phosphoinositide-dependent protein kinase 1.

(Supplementary Fig. 11). Pretreatment with ANG prevented cellular damage from glutamate or H_2O_2-induced stress in NSCs (Fig. 8A). Then, we used Hoechst 33342 nuclear stain to determine whether ANG reverses apoptosis in NSCs (Fig. 8B1). ANG significantly rescued the NSCs from glutamate- and H_2O_2-induced apoptosis (Fig. 8B2). With confirming these results, pretreatment with ANG reversed glutamate- or H_2O_2-induced increase of cleaved caspase-9, -8, tBid, cleaved caspase-3, and cleaved poly ADP ribose polymerase (PARP) expressions in NSCs (Fig. 8C). This suggests that ANG rescues NSCs from both extrinsic and intrinsic apoptosis. We speculate that ANG alleviates pre-apoptotic signals under stressful conditions in NSCs.

4. Discussion

Cells must be equipped with protective mechanisms, as excess cellular stress accumulates due to aging and can evoke many biological

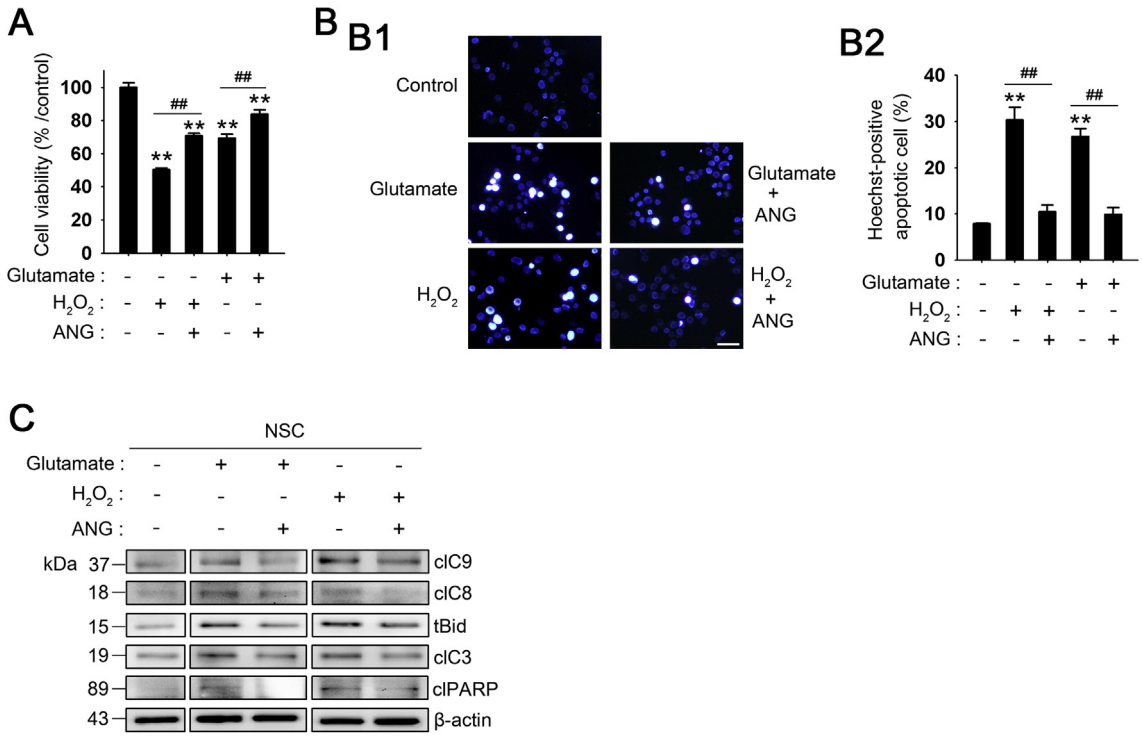

Fig. 8. Angiogenin (ANG) reduces glutamate- or H_2O_2-induced apoptosis in human neural stem cells (NSCs) HB1.F3. (A) Cell viability (MTT assay) of glutamate (0.5 mM)- or H_2O_2 (5 μM)-induced apoptotic NSCs with and without ANG (2 μg/mL, 30 min) and control. $^{**}P < 0.01$ (compared to control), $^{\#\#}P < 0.01$ (with ANG *vs* without ANG). (B) Concentrations of nuclear Hoechst 33342-stained NSCs (bright) with and without ANG (B1) and its quantification (B2). ANG significantly restored glutamate- or H_2O_2-induced apoptosis of NSCs. $^{**}P < 0.01$ (compared to control), $^{\#\#}P < 0.01$ (with ANG *vs* without ANG). (A, B) Scale bar, 100 μm. Error bars represent SEM. $n = 4$ independent experiments. ANOVA followed by Bonferroni's *post-hoc* analysis was performed for statistical analyses. (C) Immunoblotting of cleaved caspase-9 (clC9), cleaved caspase-8 (cCl8), tBid, cleaved caspase-3 (clC3), and cleaved poly ADP ribose polymerase (PARP) (clPARP) in glutamate- or H_2O_2-induced apoptotic NSCs with and without ANG and control. The small gaps indicate skipped lanes from the same membrane. $n = 3$ independent experiments.

responses. In this study, ANG (RNase 5) activated an array of pathways for IOP regulation and TM cell protection, and moreover showed anti-apoptotic roles in NSCs against glutamate- or H_2O_2-stimulated glaucomatous damage.

We assumed that the protective effects of ANG occurred under the notion of disrupted homeostasis. Decreased ANG expression in human glaucomatous TM cells, low ANG levels in tears of patients with glaucoma, and the pan-ocular existence of ANG in normal eyes, especially in the TM and neuro-retina, highlight that ANG may be an important key player in the maintenance of ocular homeostasis against glaucoma. Our results propose the association between ANG and glaucoma for the first time and strongly suggest a need to view glaucoma from a new perspective.

Although many investigators have proposed IOP-lowering effects of eNOS [30,44], MMP [37] or rho-kinase inhibition [38,45] separately, a substance that elicits multiple activation routes to regulate IOP is still being explored. Subsequently, we showed that ANG activated eNOS phosphorylation in a nuclear translocation-dependent and/or -independent (by PI3k-Akt-eNOS pathway) manner in both transformed and primary human glaucomatous TM cells (GTM3 and pGTM, respectively). Moreover, MMP-1 and -3 activities were upregulated and rho-kinase activities were inhibited by ANG to enhance trabecular aqueous outflow. The prominent IOP lowering effect of ANG, particularly when administered at night, parallels a high concentration of tear ANG at night, as reported by Sack et al. [25]. These results support the importance of timely supplementation of ANG to normalize levels under an ordinary rhythm.

TM cells have contractile property in nature being in apposition to the vascular smooth muscle cells, in which NO-cGMP signaling pathway is well known to be involved in cell relaxation [46]. NO, as one of promising future target for treatment of glaucoma, effects on the enhancement of aqueous outflow for IOP lowering and the major mechanism related is NO-induced TM and Schlemm's canal cell relaxation [31]. Although the key role of NO was initially focused on vasodilation in cardiovascular system in the past, the function of NO is actually varied according to the external cellular conditions or the concentrations of NO. Under normal conditions, eNOS and neuronal NOS (nNOS) are involved to generate relatively small amount of NO which regulates apoptosis, inflammatory responses and vascular tone [47]. On the other hand, in pathological condition such as infection or inflammation, inducible NOS (iNOS) is activated and overproduces large amounts of NO (micro to millimolar range) that is responsible for forming toxic products and thus potential metabolic damage to the TM [48]. As a critical limitation, we could not reveal the IOP regulation effect of ANG depending on the dose of NO in our study. But we suggest that NO would exhibit dual action on IOP regulation depending on the dose like as the biphasic nature of NO is broadly known in diverse cellular responses [49] including in the case of optic neuropathy [50,51]. Moreover, we think that treatment conditions of ANG applied in the present study may be for production of physiologic low concentrations of NO via eNOS activation in TM cells. Interestingly, mRNA expression of eNOS was increased after 1 h-treatment of ANG on GTM3, whereas iNOS was not affected by ANG (data not shown). Further studies are necessary to understand the actual NO levels for IOP lowering *in vivo*.

$PGF_{2\alpha}$ analogue eye drops are currently the most popular commercial agents for managing glaucoma. However, how they regulate IOP and treat glaucoma is poorly understood. We demonstrated that latanoprost, a typical $PGF_{2\alpha}$ analogue, increased tear protein levels of ANG in patients with glaucoma and that ANG elevated $PGF_{2\alpha}$ levels in the aqueous humor of rats. Thus, it is tempting to speculate that a reciprocal action mechanism may exist between ANG and $PGF_{2\alpha}$ analogue in eyes and that ANG is an important candidate target of latanoprost eye drops which still lack definite modes of action, veiled except its putative MMP activation pathway [37].

Endogenous molecules involved in normal physiology are generally quite safe and tolerable. Thus, it is notable that ANG maintains cell viability of NTM5 and GTM3. We demonstrated for the first time that the PI3k-Akt-mTOR and Erk pathways, master enhancers of cell proliferation and anti-apoptosis, are related to ANG action in NTM5 and GTM3 cells. Moreover, ANG alleviated possible immune-mediated damage in NTM5 and GTM3 cells by IDO production and suppressing T cells. These actions may contribute to maintain cellular homeostasis as a strategy to preserve normal TM function.

The final goal of glaucoma management is to prevent blindness due to death of RGCs. What was very encouraging in this study is that we found that ANG has a potential to reverse apoptosis in NSCs that can be differentiated into RGC-like cells. The importance of ANG enrichment in normal motor neurons is spotlighted in amyotrophic lateral sclerosis, a fatal neurodegenerative disease [52]. Similarly, we believe ANG-elicited protective mechanisms in NSCs may a key ocular homeostatic mechanism exploited by glaucoma, an ocular neurodegenerative disease.

The biologic activities of ANG have been extended to stimulating cell survival, anti-apoptotic effect [17,53], and modulation of inflammation in the eye [54]. As glaucoma is no longer a disease typified only by high IOP, multipronged therapeutic approach if required. Although detailed modes of action of ANG for IOP regulation should be confirmed in *in vivo* through the future studies, our findings provide novel insights into glaucoma as disrupted ocular homeostasis, which is an ANG defect, and propose newly unveiled anti-glaucomatous potential of ANG.

5. Conclusion

In conclusion, our findings inform new insights into glaucoma as a destroyed ocular homeostasis, that is ANG defect, and thus propose new therapy targets embracing the multifarious individual pathogenesis of glaucoma using the multifunctional effects of ANG (RNase 5).

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.11.005.

Author contributions

K.W.K., S.H.P., D.H.O., K.J., Y.S.C., and J.C.K. designed all the experiments. Among them, *in vitro* experiments were performed by S.H.P. and S.H.L., and animal experiments were performed by D.H.O. and K.S.L., S.O.C. and K.M.M. produced and provided Cy3-tagged angiogenin. K.W.K. and S.H.P. analyzed the data and wrote the manuscript. Y.S.C. and J.C.K. revised the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in online version.

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