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TGF- β signaling is required for maintenance of retinal ganglion cell differentiation and survival

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Abstract

Purpose—To determine the role of TGF- β 1 in the maintenance of retinal ganglion cell line (RGC-5) differentiation and integrity.

Methods—RGC-5 cells were differentiated in media conditioned by human non-pigmented ciliary epithelial cells (HNPE) for four days before treatment with TGF- β 1 for 24 hr. Cells were examined for morphological changes and harvested for western blot and real-time PCR analysis. For study of apoptosis, differentiated RGC-5 cells were grown in serum-free medium for 24 hr in the presence or absence of TGF- β 1 and collected for Annexin V/Propidium iodide FACs analysis. The role of MAPK pathways in TGF- β 1-dependent signaling was determined by treatment with specific inhibitors of ERK, JNK and p38.

Results—Differentiation of RGC-5 cells in HNPE-conditioned media (CM) increased the neural cell markers, Brn-3c, NF-160, Thy1.2, Tau and PGP9.5. Treatment with TGF- β 1 significantly increased the length of neurites extended by differentiated RGC-5s, concomitant with increased expression of NF-160 and PGP9.5, but not Brn-3c, Thy1.2 or Tau. TGF- β 1 also decreased RGC-5 cell apoptosis in serum-free medium. p38 phosphorylation, but not smad2/3, JNK or ERK phosphorylation, was increased in TGF- β 1 treated cells. Specific inhibition of p38 signaling reversed TGF- β 1 induced neurite growth.

Conclusions—These findings demonstrate the induction of RGC-5 cell differentiation by HNPE derived CM and illustrate a role for TGF- β 1 in maintaining RGC-5 cell survival and promoting neurite outgrowth through p38 MAPK.

Keywords

TGF- β 1; retina; neurite; differentiation; apoptosis; p38

RGCs are highly specialized cells of the central nervous system situated in the inner layer of the retina. RGCs receive visual information from the photoreceptors of the outer retina via amacrine and bipolar cells and relay it to the brain. Raised intraocular pressure in glaucomatous patients is thought to target RGCs and their axons, particularly at the optic

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nerve head where damage is evidenced by cupping and distension of the lamina cribrosa plates occurs (Downs et al., 2008).

We have previously demonstrated that systemic neutralization of TGF- β in the adult mouse via expression of soluble endoglin (sEng), a TGF- β inhibitor, results in impaired visual acuity as detected by electroretinogram (ERG) recordings. sEng-expressing mice had impaired vascular perfusion and increased retinal vascular permeability, as well as RGC apoptosis as detected by TEM and TUNEL staining (Walshe et al., 2009). These studies did not determine whether RGC cell death and dysfunction were a reflection of neuroprotective properties of TGF- β on RGCs or a secondary effect of vascular dysfunction.

Ganglion cells of the developing postnatal mouse and rat retina express both TGF- β 1 ligand and TGF β R2, and TGF β R2 is detected in the body and axons of adult ganglion cells, suggesting a non-redundant role for TGF- β signaling in RGCs (Gordon-Thomson et al., 1998, Guérin et al., 2001, Close et al., 2005). Smad transcription factors, classical members of a family of transcription factors that are phosphorylated and mediate the TGF- β 1 response in numerous cell types, are expressed by RGCs in vivo (Walshe et al., 2009). Another network of interacting proteins that regulates a large number of cellular processes, the MAPKs, also mediate communication of extracellular signals into intracellular targets. The MAPK cascade is comprised of three main signaling pathways, ERK, JNK and p38. Each of these signal in RGCs and are known to mediate TGF- β 1 signaling in other cell types (Watanabe et al., 2001, Goldberg et al., 2002, Dhandapani et al., 2003, Rodríguez-Barbero et al., 2006).

In this study, we examined the potential neuroprotective role of TGF- β 1 for RGC-5 cells and its effects on neurite outgrowth in vitro. We also characterized the role of the smad and MAPK pathways in mediating the effects of TGF- β 1 on RGC-5 cell differentiation. Identifying and understanding the role of neuroprotective factors for RGC-5 cells is of particular relevance for conditions characterized by RGC degeneration, such as glaucoma (Bode et al., 2011) or multiple sclerosis (Green et al., 2010).

Experimental Procedures

RGC-5 cell culture and differentiation

The RGC-5 cell line was developed as a retinal ganglion cell line by transformation of retinal cells; these cells have the characteristics of RGC based on Thy-1 and Brn-3C expression (Krishnamoorthy et al., 2001). RGC-5 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-GIBCO, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Inc., Lawrenceville, GA), 100 U/ml penicillin, and 100 μ g/ml streptomycin. RGC-5 cells were differentiated by adding media conditioned by HNPE cells (Tchedre et al., 2008b, Tchedre and Yorio, 2008). For preparation of CM, HNPE cells were grown to ~90% confluence and incubated for 24 hr with serum-free DMEM. RGC-5 cells were seeded sparsely at 100 cell/cm², media was removed 24 hr later and replaced with HNPE-conditioned medium supplemented with 0.2% heat-inactivated fetal bovine serum. The cells were then incubated at 37°C in 5% CO₂ for four days to allow the cells to differentiate.

To assess the possible role of TGF- β 1 in RGC-5 differentiation, 0.1, 1.0 and 10 ng/ml TGF- β 1 (R&D Systems, Minneapolis, MN) was added to cells that had been differentiated in HNPE CM. Cells morphology was examined and protein and mRNA analyzed after 24 hr treatment. A neurite was defined as a process that was longer than one cell body (typically >35–40 μ m) (Laketa et al., 2007, Endo et al., 2008). For each treatment, neurite length was

measured on 10–12 pictures taken at 10X magnification using ImageJ software and data were analyzed using Prism.

Apoptosis assay

RGC-5 cells were plated at a density of ~100 cells/cm² on 0.1% collagen coated flasks and differentiated for four days as described above. To assess the effect of TGF- β 1 on RGC-5 apoptosis, cells were incubated in 0.1 ng/ml TGF- β 1 in serum-free DMEM for 24 hr and apoptotic cells were detected using the Vybrant® Apoptosis Assay Kit (Invitrogen, Carlsbad, CA), followed by FACS analysis using a FACSCAN flow cytometer. Cellular ATP levels were assayed as a surrogate for viable cells using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) (Du et al., 2009).

SDS-PAGE and immunoblot analysis

Cell pellets were treated with lysis buffer (Cell Signaling, Danvers, MA) and equal protein was fractionated by 10% (wt/vol) polyacrylamide resolving gels (Bio-Rad, Hercules, CA). After transfer to nitrocellulose membranes, non-specific protein binding was blocked by a 60-min incubation in TBS-T (Tris-buffered saline, 0.1% Tween-20) containing 5% (wt/vol) nonfat skim milk and 2.5% BSA. Membranes were then incubated at 4°C overnight with one of the following antisera: pp-smad2/3 (1:1000; Abcam, Cambridge, MA), smad2/3 (1:1000; Abcam, Cambridge, MA), pp-smad1/5/8 (1:1000; Millipore, Temecula, CA), smad1/5/8 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), pp-p38 (1:1000; Cell Signaling, Danvers, MA), p38 (1:1000; Cell Signaling, Danvers, MA), pp-SAPK/JNK (1:1000; Cell Signaling, Danvers, MA), SAPK/JNK (1:1000; Cell Signaling, Danvers, MA), pp-Erk1/2 (1:1000; Cell Signaling, Danvers, MA), Erk1/2 (1:1000; Cell Signaling, Danvers, MA), Brn-3c (1:1000; Abcam, Cambridge, MA), neurofilament-M (NF-160) (1:500; Sigma, St. Louis, MO) or Thy1.2 (1:2000; Abcam, Cambridge, MA) diluted in TBS-T with 2.5% BSA. After four 6-min washes with TBS-T, membranes were incubated for 60 min at room temperature with horseradish peroxidase-conjugated rabbit polyclonal or mouse monoclonal IgG antibody (1:5000; GE Healthcare, Fairfield, CT). After four additional washes with TBS-T, immunoreactive proteins were identified by enhanced chemiluminescence. Scanning densitometry was performed with image-analysis software (ImageJ), with values normalized for loading using Ponceau staining for total protein.

To block MAPK signaling pathways, PD169316 (p38 inhibitor) (0.5 μ M; Sigma, St. Louis, MO), SP600125 (JNK inhibitor) (0.5 μ M; Sigma, St. Louis, MO), or 3-(2-aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione hydrochloride (ERK inhibitor) (0.5 μ M; Sigma, St. Louis, MO) were applied 1 hr prior to the addition of 0.1 ng/ml TGF- β 1.

RNA isolation and reverse transcription-PCR analysis

RT-PCR was carried out using total RNA isolated from cells using TRIzol™ (Invitrogen), according to the manufacturers' specifications. Total RNA (1–2 μ g) was reverse transcribed to cDNA using iSCRIPT as per the manufacturers' specifications (BioRad). The gene-specific oligonucleotide sequences used were: GAPDH: F-GGCACAGTCAAGGCTGAGAATG; R-ATGGTGGTGAAGACGCCAGTA; Thy1.2: F-AACTCTTGGCACCATGAACCC; R-GCTGGTCACCTTCTGCCCTC; Tau: F-TGAAGACGTGACTGCGCCCTA; R-CCTGGTTCGGGGTGTCTCCGA; PGP9.5: F-GCTTCGCGGACGTGCTAGGG; R-TTTTCATGCTGGGCCGTGAGGG. Reactions were performed on the LightCycler 480II (Roche) using 0.4 μ M primers and the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's instructions. Amplification of GAPDH was performed on each sample as a control for normalization.

Statistical analysis

Unless otherwise indicated, data are reported as mean $\pm$ SD of at least three independent experiments with three replicates per group. Data were analyzed by student's unpaired T-test using Prism software (and one-way ANOVA). P values <0.05 were taken to indicate statistical significance.

Results

TGF- β 1 induces neurite outgrowth

Undifferentiated RGC-5 cells grew rapidly in regular growth medium and appeared as flat cells, with few visible neurite extensions (Fig. 1A). Culture of the cells with HNPE-derived CM for four days led to differentiation, as evidenced by neurite extension (Fig. 1B). In order to assess the effect of TGF- β 1 on neurite maintenance or extension, RGC-5 cells grown in CM for four days were treated with 0.1, 1.0 or 10 ng/ml TGF- β 1 for 24 hr in CM. Addition of TGF- β 1 led to increased neurite length, with maximal effect at 0.1 ng/ml TGF- β 1 of ~25% (Fig. 1C and 1D).

TGF- β 1 modulates RGC-5 expression of neuronal markers

Since TGF- β 1 led to an increase in the extension of neurites by RGC-5 cells in culture, we sought to determine whether the expression of ganglion cell markers was influenced. Western blot analysis for the transcription factor Brn-3c revealed the absence of expression of Brn-3c in RGC-5 cells not exposed to CM (Fig. 2A). Differentiation of RGC-5 cells for four days led to a significant increase in the level of Brn-3c, which was not altered by the presence of TGF- β 1 for a further 24 hr (Fig 2A). NF-160, a structural component of the neuronal cytoskeleton, was also undetectable in undifferentiated RGC-5 cells (Fig 2B), but was expressed by differentiated RGC-5 cells and was more than doubled in cells treated with TGF- β 1 (Fig 2B). Thy1.2, a cell surface glycoprotein of unknown function found predominantly in ganglion cells of the retina was detectable in non-differentiated RGC-5s (Fig. 2C and 2D) and increased over 2-fold after differentiation, but not by the addition of TGF- β 1. Tau, an axonal microtubule associated protein, also increased upon differentiation (~7-fold) but not upon addition of TGF- β 1 (Fig. 2E). PGP9.5 (UCH-L1), a neuron-specific deubiquitinating enzyme, increased 2-fold after differentiation, and 3-fold upon incubation with TGF- β 1 for 24 hours (Fig. 2F).

TGF- β 1 is a survival factor for RGC-5 cells

In order to assess the potential role for TGF- β 1 as a neuroprotectant for RGC-5 cells, differentiated RGC-5 cells were incubated for 24 hr in serum-free media in the presence or absence of 0.1 ng/ml TGF- β 1. FACs analysis using an Annexin V/propidium iodide (PI)-based apoptotic assay revealed that the addition of 0.1 ng/ml TGF- β 1 reduced the level RGC-5 of apoptosis by ~50% (Fig. 3A). In addition to Annexin V/PI detection of apoptosis, we examined cellular ATP metabolism as a surrogate for cell health. When compared to control cells, addition of TGF- β 1 to RGC-5 cells significantly increased ATP levels 7% compared to control RGC-5 cells, demonstrating cellular metabolism was more active in the presence of TGF- β 1 (Fig. 3B).

Intracellular smad and MAPK signaling in RGC-5 cells

Intracellular smad and MAPK pathways have been reported to be involved in generating a TGF- β 1 signaling response either independently or simultaneously (Zavadil et al., 2001, Yu et al., 2002, Undevia et al., 2004, Valcourt et al., 2005, Yang et al., 2006). In order to determine the signaling pathway(s) that mediates TGF- β 1-induced differentiation of RGC-5, cells were treated for 24 hr with 0.1 ng/ml TGF- β 1 and harvested for protein analysis of

smad and MAPK signaling components. Western blot analysis revealed no change in the levels of smad2/3 or smad1/5/8 or in their phosphorylation (Fig. 4).

We then examined whether MAPK pathways might be involved in TGF- β 1-induced RGC-5 differentiation by measuring the phosphorylation and total protein levels of ERK, JNK and p38. TGF- β 1 did not alter the phosphorylation or total protein levels of ERK or JNK (Fig. 5A,B). However, treatment with TGF- β 1 led to a 2-fold increase in the phosphorylation of p38, while total p38 remained unchanged (Fig. 5C).

As MAPK, but not smad pathway components, were activated by TGF- β 1 treatment, we examined the role of MAPK signaling in TGF- β 1-induced RGC-5 differentiation. RGC-5 cells were treated with 0.5 μ M 3-(2-aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione, SP600125 or PD169316 for 24 hr to inhibit ERK, JNK and p38, respectively. There was no change in TGF- β 1-induced neurite growth with ERK or JNK inhibition (Compare Fig. 6A with Fig. 6C and Fig. 6D). Inhibition of p38, however, blocked the effects of TGF- β 1-induced RGC-5 neurite outgrowth (Fig. 6B), demonstrating a requirement for p38 MAPK signaling in TGF- β 1-induced RGC-5 differentiation. The basal level of neurite outgrowth was unchanged with ERK (Compare Fig. 6A with Fig. 6C) or p38 inhibition (Compare Fig. 6A with Fig. 6B); however, inhibition of the JNK pathway led to an increase in neurite length in control (untreated RGC-5 cells) (Fig. 6A and Fig. 6D control), pointing to a role for JNK signaling in the regulation of neurite outgrowth.

Discussion

Previous reports indicate the presence of multiple components of the TGF- β pathway in the developing retina, the adult retina and in cultured RGC (Yamanaka et al., 2002, Close et al., 2005, Ma et al., 2007). TGF- β signaling is complex, with autocrine and paracrine signaling reported for multiple cell types and widespread detection of both receptor and ligand expression (Gordon-Thomson et al., 1998, Guérin et al., 2001, Close et al., 2005). Recent observations from studies of neuronal morphogenesis in the brain demonstrated that TGF- β signaling is required for both axon formation and migration (Yi et al., 2010).

In addition to playing a role in neuronal structure and cell fate decisions, developmental studies reveal a potential role for TGF- β 1 as a neuroprotectant. Mice lacking TGF- β 1 exhibit a widespread increase in degeneration of neurons in the brain and prominent microgliosis prior to death (Brionne et al., 2003). Primary cultures of neurons from these mice display poor growth, possibly as a result of impaired TGF- β 1 regulation of apoptosis related genes (Brionne et al., 2003). Recent studies also demonstrate that Alzheimer's disease is associated with a decrease in circulating TGF- β 1 and an increase in sEng (Juraskova et al., 2010).

Motivated by these intriguing data and by the lack of studies in the retina, we sought to determine the effect of TGF- β 1 on differentiation and integrity of RGC-5 cells. RGC-5 cells have been widely used to examine RGC function in vitro (Das et al., 2006, Frassetto et al., 2006, Harvey and Chintala, 2007, Hong et al., 2007, Lieven et al., 2007, Krishnamoorthy et al., 2008, Tchedre et al., 2008a, Tchedre and Yorio, 2008) and a variety of treatments have been used to induce RGC-5 cell differentiation including the nonagglutinating lectin - succinyl concanavalin A (sConA), and the nonselective protein kinase inhibitor staurosporine (Krishnamoorthy et al., 2001, Frassetto et al., 2006, Lieven et al., 2007, Wood et al., 2010). More recently, a method of differentiation using media conditioned by cultured ciliary epithelial cells has been reported (Tchedre et al., 2008b, Tchedre and Yorio, 2008). We have shown that adapting this method by using more sparsely seeded RGC-5 cells and longer incubation times in CM, leads to reproducible and robust RGC-5 cell differentiation,

including growth arrest, neurite extension, and expression of the neuronal markers NF-160, Brn-3c, Thy1.2, Tau and PGP9.5.

Brn-3c, a transcription factor expressed predominantly by neuronal cells, is one of a number of markers that identifies the neuronal cell lineage. In vivo developmental studies have suggested a role for Brn-3c in promoting neurite outgrowth in differentiating RGCs in conjunction with Brn3b (Wang et al., 2002). Previous studies have failed to detect Brn-3c in cultured RGC-5 cells or in RGC-5 cells differentiated with either succinyl Concanavalin A, SS, or trichostatin A (Wood et al., 2010). Consistent with these observations, Brn-3c was not detectable in untreated RGC-5 cells but was expressed by cells differentiated in HNPE-derived CM. Brn-3c is an early transcription factor involved in the induction of RGC differentiation, thus, it was not unexpected that TGF- β 1 did not alter Brn-3c protein levels, as RGC-5 cells are differentiated before TGF- β 1 is applied.

NF-160, a structural component of neurites, was increased in cells treated with TGF- β 1. Although the original characterization of the RGC-5 line reported the presence of neurofilament (Krishnamoorthy et al., 2001), more recent studies have failed to detect its presence in RGC-5 cells (Van Bergen et al., 2009, Wood et al., 2010). Similarly, neurofilament was not seen in RGC-5 cells prior to differentiation in CM, but was expressed by cells following differentiation and was significantly increased with the addition of TGF- β 1. These findings suggest that although TGF- β 1 does not play a role in RGC-5 cell differentiation into a neuronal lineage, it does enhance the outgrowth of axonal structures of differentiated RGC-5 cells.

Thy1, a marker of RGC, and Tau another protein of predominantly of neuronal origin, were both detectable in RGC-5 cells pre-differentiation and were increased after four days in CM. TGF- β 1 did not alter levels of Thy1 or Tau in RGC-5 cells. Furthermore, analysis of PGP9.5, the ubiquitin-protein hydrolase present in neuronal cell bodies, revealed an increase in expression with both differentiation and as well as with the addition of TGF- β 1. These findings demonstrate differentiation of RGC-5 cells in HNPE-CM leads to expression of multiple markers of neuronal cells and addition of TGF- β 1 further increases some of these markers, namely NF-160 and PGP9.5 to increase neuronal characteristics of these cells.

Formation of the tetrameric signaling complex of TGF- β 1 and TGF- β receptors type I and II (TGF- β RI, - β RII) typically results in phosphorylation of the downstream transcription factors of smad2 and smad3 (Feng and Derynck, 2005). However, TGF- β 1 did not lead to a change in smad2 or smad3 phosphorylation in RGC-5 cells. This finding led us to investigate the possible role of the MAPK signaling pathways, which have been shown to mediate TGF- β 1 signaling in some other cell types (Undevia et al., 2004, Horowitz et al., 2007, Yu et al., 2010) in the absence of smad signaling. Western blot analyses of differentiated RGC-5 cells treated with TGF- β 1 revealed a significant increase in phosphorylation of p38, but not ERK or JNK. Furthermore, inhibition of p38, but not ERK or JNK, suppressed TGF- β 1 induction of neurite outgrowth, demonstrating that TGF- β 1 signaling occurs via p38 MAPK, and is independent of smad signaling in RGC-5 cells. Smad-independent TGF- β 1 signaling similarly occurs via p38 signaling in epithelial and hepatocyte cell lines by inducing TRAF6 association with TGF β receptors (Yamashita et al., 2008). A mutant TGF- β receptor that retains p38 kinase activity, but is incapable of activating smads, was used to demonstrate smad-independent TGF- β 1 signaling via p38 in murine epithelial cells (Yu et al., 2002).

We have previously observed an increase in RGC apoptosis in mice in which TGF- β was systemically neutralized by adenoviral expression of sEng (Walshe et al., 2009). These mice also exhibited significant retinal vascular dysfunction, perfusion abnormalities and

permeability defects, but these experiments did not address if RGC apoptosis was due to the direct effect of TGF- β 1 on neural cells or if these effects were secondary to vascular dysfunction. To test whether TGF- β 1 is a survival factor for RGC-5s, the cells were exposed to TGF- β 1 under serum-free conditions for 24 hr. The administration of TGF- β 1 significantly reduced the level of apoptosis, demonstrating TGF- β 1 can indeed function as a survival factor for RGC-5 cells.

Consistent with these findings, mice lacking TGF- β 1 display extensive neural degeneration in the brain and are more susceptible to excitotoxic injury (Brionne et al., 2003). Similarly, overexpression of TGF- β 1 by astrocytes protects adult mice against neurodegeneration in an acute injury model (Brionne et al., 2003). In contrast to our observations, addition of TGF- β 1 to developing retinal explants has been shown to induce ganglion cell apoptosis (Franke et al., 2006). These effects were more pronounced at embryonic (E) day 5 as compared to E11, suggesting that RGCs respond differently to TGF- β 1 depending upon retinal maturity. This difference may also be the result of the higher concentration of TGF- β 1 used in the latter study (20-fold higher than the current study).

Previous studies demonstrated a role for the TGF- β family member bone morphogenetic proteins (BMPs) in neurite outgrowth and survival of primary cultures of RGCs; these studies also reported that TGF- β 1 did not influence RGC survival or differentiation (Kerrison et al., 2005). However, a very likely explanation for these discrepant results is the very high concentrations of TGF- β 1 (50 ng/ml) used in these studies whereas our own finding demonstrate maximal effects on RGC at 0.1 ng/ml. Interestingly, TGF- β 1 has been shown to display biphasic effects in other systems (Pepper et al., 1993). Moreover, observations in multiple cell types demonstrate that increasing growth factor concentration can lead to downregulation of the target receptor (Zhang et al., 2010).

The source of TGF- β signaling in the retina is unclear. Müller cells in vitro release TGF- β 1 and thus may serve as a source of TGF- β 1 for RGC in vivo (Abukawa et al., 2009). Both vascular endothelial and mural cells, which comprise the inner vascular plexus within the ganglion cell layer, are known to synthesize TGF- β 1 (Hirschi et al., 1998, Ding et al., 2004). Astrocytes, which are abundant in the retina and in close proximity to the RGCs, also synthesize TGF- β 1 (Dhandapani et al., 2003). Lastly, RGCs themselves synthesize TGF- β 1 (Gordon-Thomson et al., 1998), raising the possibility of an autocrine role for TGF- β 1 in vivo. Taken together, these studies describe an important role for TGF- β 1 signaling in differentiated RGCs and point to a potential therapeutic role for TGF- β 1 in neuroprotection.

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Glossary of terms

RGC	retinal ganglion cell
HNPE	human non-pigmented ciliary epithelial cells
CM	conditioned media
sEng	soluble endoglin
ERG	electroretinogram

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Research Highlights

- We examine retinal ganglion cell neuroprotection with TGF- β 1
- TGF- β 1 maintains health of retinal ganglion cells
- TGF- β 1 enhances neurite length and neuronal differentiation
- TGF- β 1 effects these changes via phosphorylation of p38 MAPK

Figure 1. Effect of TGF-β1 on RGC-5 cell differentiation

(A) Undifferentiated RGC-5 cells; (B) RGC-5 cells differentiated in HNPE cell CM for four days; (C) Differentiated RGC-5 cells treated with 0.1 ng/ml TGF-β1 induces neurite extension. (D) Addition of TGF-β1 increases neurite length, with the maximal effect at 0.1 ng/ml TGF-β1. *** $p < 0.0001$

Figure 2. Effect of TGF- β 1 on expression of neuronal-specific markers by RGC-5 cells
 Western blot analysis for: (A) Brn-3c, (B) NF-160 and (C) Thy1.2 and real-time-PCR for: (D) Thy1.2, (E) Tau and (F) PGP9.5. Brn-3c and NF-160 were not detected in undifferentiated RGC-5 but were expressed by differentiated RGC-5. Thy1.2 was present in undifferentiated RGC-5s and increased after differentiation. Differentiation increased expression of Thy1.2, Tau and PGP9.5. Addition of TGF- β 1 to differentiated RGC-5 cells increased NF-160, but not Brn-3c or Thy1.2 protein and increased PGP9.5, but not (D) Thy1.2 or (E) Tau mRNA. N = 3; *p<0.05

Figure 3. Effect of TGF-β1 on RGC-5 cell apoptosis

(A) Annexin V/Propidium iodide analysis of apoptosis by of differentiated RGC-5 cells cultured in serum-free medium in the presence or absence of TGF-β1 for 24 hr led to a significant decrease in apoptosis with TGF-β1. (B) TGF-β1 treatment increased RGC-5 cellular metabolism as evidenced by levels of ATP. N = 3; *p<0.05

Figure 4. Effect of TGF- β 1 on smad signaling in RGC-5

(A) Western blot analysis of (A) phospho-smad2/3, total-smad2/3 and (B) phospho-smad1/5/8 and total smad1/5/8 revealed no significant changes. N = 3.

Figure 5. Role of ERK, JNK and p38 MAPK in TGF- β 1 signaling in differentiated RGC-5 cells
 (A) Western blot analysis of phospho-ERK, total-ERK, (B) phospho-JNK and total JNK revealed no significant changes. (C) Addition of TGF- β 1 increased phospho-p38, with no changes in total p38. N = 3; *p<0.05

Figure 6. Role of p38 MAPK, ERK and JNK MAPK in TGF-β1-induced differentiated RGC-5 cell neurite outgrowth

Cells were differentiated, and treated with TGF-β1 in the presence or absence of: (A) no inhibitors; (B) the p38 inhibitor (PD169316); (C) the ERK inhibitor (3-(2-aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione hydrochloride); or (D) the JNK inhibitor, SP600125. (E) Neurites were quantified as described in the methods. p38, but not ERK or JNK MAPK, mediates the effects of TGF-β1 on RGC-5 cell neurite outgrowth. N = 3; *p<0.01 compared to control; # p<0.01 compared to TGF-β1 control.