TGF β Signaling Induces Expression of Gadd45b in Retinal Ganglion Cells

Bin Liu,¹⁻³ Xiaoguang Sun,² Genn Suyeoka,³ Joe G. N. Garcia,² and Yannek I. Leiderman¹

PURPOSE. Growth arrest and DNA damage protein 45b (Gadd45b) functions as an intrinsic neuroprotective molecule protecting retinal ganglion cells (RGCs) from injury. This study was performed to elucidate further the induction pathway of Gadd45b expression in RGCs.

METHODS. The induction of Gadd45b expression in response to TGF β /NF κ B signaling was investigated in RGC5 cultures in vitro and murine retina in vivo. Gadd45b mRNA and protein expression were detected by quantitative real-time RT-PCR, immunoblot assay, immunohistochemistry, and immunocytochemistry. Activation of NF κ B and TGF β /Gadd45b signaling were assessed by measuring phosphorylation of NF κ B and using specific inhibitors. Gadd45b siRNA was transfected into RGC5 to silence Gadd45b mRNA expression.

RESULTS. Expression of TGF β receptors I and II was detected in RGC5 in vitro and RGCs in vivo. TGF β induced abundant Gadd45b mRNA and protein expression, exhibiting a dose-dependent response in vitro. Exogenous TGF β 1 induced upregulation of Gadd45b expression in RGCs in murine retina in vivo. TGF β stimulated phosphorylation of NF κ B, and inhibition of NF κ B phosphorylation blocked induction of Gadd45b by TGF β in RGC5 cells. Induction of Gadd45b by TGF β increased the resistance of RGC5 cells against TNF α cytotoxicity and paraquat oxidative stress.

CONCLUSIONS. TGF β signaling induced Gadd45b expression in RGCs. Modulation of the TGF β /NF κ B/Gadd45b signaling pathway may provide a means to enhance the neuroprotective effect of Gadd45b in RGCs. (*Invest Ophthalmol Vis Sci.* 2013; 54:1061-1069) DOI:10.1167/iovs.12-10142

Neurons possess intrinsic mechanisms to mediate metabolic stress, injury, and aging. We have previously shown that growth arrest and DNA damage-inducible protein 45b (Gadd45b) is intrinsically expressed in retinal ganglion cells

(RGCs) and functions as a neuroprotective molecule, protecting RGCs from various neuronal pathologies, including aging, glaucoma, and oxidative stress.¹ Gadd45b, a member of the Gadd45 family, likely plays a role in the cellular response to physiological and environmental stress, and promotes cell survival by activating antiapoptotic and DNA repair mechanisms.^{2,3} In adult neurons, Gadd45b promotes neurogenesis by epigenetic modulation of DNA demethylation.⁴

The induction pathway of Gadd45b expression in neurons is unclear. We sought to elucidate the extracellular and intracellular signals that induce Gadd45b expression in RGCs. In non-neuronal cells, TGF β signaling serves as an upstream regulator of Gadd45b expression.⁵ TGF β signaling is implicated in maintaining RGC survival,⁶ and is beneficial in neurodegenerative diseases⁷⁻⁹ by promoting neuronal survival.^{10,11} The endogenous expression of TGF β and its receptors has been detected in brain tissue under physiological and pathological conditions.¹²⁻¹⁴ We have investigated the effects of TGF β signaling on the induction of Gadd45b in RGCs.

This study demonstrates that TGF β signaling induces the expression of Gadd45b in RGCs. The TGF β signaling pathway is intrinsically present in RGCs, and exogenous TGF β significantly upregulates Gadd45b expression in RGCs in vivo. Induction of Gadd45b by TGF β appears to increase the resistance of RGC5 cells to injury in vitro. TGF β -induced activation of the nuclear factor-kappaB (NF κ B) pathway upregulates Gadd45b expression. Elucidation of the TGF β /NF κ B/Gadd45b signaling pathway may provide a means to enhance the neuroprotective effect of Gadd45b in RGCs.

METHODS

Cell Culture

RGC5, a retinal neuronal precursor cell line originally transformed via adenovirus carrying Ψ 2E1A, was obtained¹⁵ and maintained as described previously.¹⁶ The cellular origin of the RGC5 cell line is uncertain, but recent studies suggest the cell line to be of mouse origin expressing the cone photoreceptor-specific opsin protein.^{17,18} To induce neuronal differentiation, RGC5 cells were plated at 30% confluence in MEM media without pyruvate, glutamine, and serum, then treated with 5 μ M staurosporine for 5 minutes.^{1,16} Differentiated RGC5 cells exhibit dendritic morphology and undergo growth arrest. For immunocytochemistry, cells were grown on glass coverslips. Reagents were added to cell culture media to yield final concentrations as follows: TGF β 1 (R&D Systems, Minneapolis, MN) 5 to 40 ng/mL; TGF β 2 (R&D Systems), 5 to 40 ng/mL; SN50 (Calbiochem, San Diego, CA), 50 ng/ μ L; Bay 11-7085 (Calbiochem) 10 μ M; paraquat (Calbiochem), 600 μ M; and TNF α (R&D Systems) 20 ng/mL.

siRNA Transfection

For knockdown of Gadd45b expression, differentiated RGC5 cultures were transfected with Gadd45b small interfering RNA (siRNA) as

From the ¹Department of Ophthalmology and Visual Sciences, Illinois Eye and Ear Infirmary, Chicago, Illinois; ²Department of Medicine, University of Illinois Hospitals and Health Sciences System, Chicago, Illinois; and ³The Forsythe Laboratory for the Investigation of the Aging Retina, Department of Ophthalmology, Northwestern University School of Medicine, Chicago, Illinois.

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Corresponding author: Bin Liu, Department of Ophthalmology and Visual Sciences, Illinois Eye and Ear Infirmary, University of Illinois Hospitals and Health Sciences System, 1855 West Taylor Street, MC 648, Chicago, IL 60612; bliu925@uic.edu.

described previously.¹ Briefly, siRNA for rat Gadd45b transcript (NM 001008321) was designed and synthesized as follows: Sense: r(CGU UCU GCU GCG ACA AUG A)dTdT; Antisense: r(UCA UUG UCG CAG CAG AAC G)dAdT. Transfection of RNA interference (RNAi) duplex nucleic acid was facilitated by Lipofectamine RNAiMAX (Invitrogen). A nonspecific, red fluorescent-labeled, double-strand RNA (BLOCK-iT Alexa Fluor Red Fluorescent Oligo; Invitrogen, Carlsbad, CA), was transfected in parallel as an siRNA negative control, and to evaluate transfection efficiency. Gadd45b mRNA was assayed via quantitative RT-PCR. Cultures with transfection efficiency of greater than 70% were used for cytotoxicity assays. Cell viability was assessed by CellTiter AQueous One Solution Cell Proliferation Assay using tetrazolium compound, MTS (Promega, Madison, WI), as described previously.¹ Results are shown as a ratio using control cultures at the start point of cell transfection as a baseline. Each experiment was repeated at least three times. Student's *t*-test was used for statistical analyses.

Experimental Animals and Intravitreal Injection

Male 5-month-old C57BL/6 mice were used for studies in vivo. TGFβ1 (R&D Systems) was resuspended in sterile buffered solution (2 mM HCl-phosphate buffered saline, 0.5% insulin-free BSA) at a final concentration of 10 ng/μL according to the manufacturer's instructions. Each experimental animal received an intravitreal injection of TGFβ1 solution to one eye and injection of diluent to the fellow eye as a control. A peripheral anterior chamber paracentesis was performed to lower intraocular pressure. A sterile Hamilton microinjection needle (33 g) was directed through the sclera at the limbal region into the vitreous cavity without injury to the lens or retina, and 10 μL of TGFβ1 or diluent solution was slowly injected to replete intraocular volume. All mice were monitored for ocular injury and infection associated with the experimental procedure and maintained in a temperature-controlled environment on a 12 hours light/12 hours dark cycle. Mice were killed at 2, 6, 24, and 48 hours after injection, and retinas were immediately processed for isolation of RNA, immunoblot assay, or immunohistochemistry, accordingly. All animal studies were conducted in accordance with the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Visual Research. Each experiment was repeated at least three times and 48 mice were studied.

Quantitative Real-Time RT-PCR

Total RNA was extracted from cells or retinal tissue using RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. DNA-free RNA was assessed for integrity and quantity by electrophoretic analysis using an Agilent 2100 Bioanalyzer (Agilent, Clara, CA). A total of 100 ng of total RNA per sample was subject to reverse transcription into cDNA using a one-step supertranscript kit (Bio-Rad Laboratories, Inc., Hercules, CA) and was subsequently used for quantitative PCR. Primers for the Gadd45b gene and 18S gene (internal control) were synthesized as described previously,¹ as follows: rat Gadd45b: 5'-GCTGGCCATAGACGAAGAAG-3'; 5'-AGCC TATGCATGCCTGATAC-3'; mice Gadd45b: 5'-CCTGGCCATAGACGAAGAAG-3'; 5'-AGCCTCTGCATGCCTGATAC-3'; 18S: 5'-GTAACCGTTGAACCCATT-3'; 5'-CCATCCAATCGGTAGTAGCG-3'. The quantity of Gadd45b mRNA was measured by detection of hyperfluorescent PCR reaction products in real time using an iCycler (Bio-Rad Laboratories, Inc.), and normalized to the internal control gene 18S using Bio-Rad analysis software. All quantitative real-time RT-PCR reactions were performed in triplicate. Student's *t*-test was used for statistical analysis.

Immunoblot Assay

Cells grown in culture and retinal tissue were lysed in immunoblot buffer (0.5% SDS, 0.05 M Tris-HCl, pH 6.8, proteinase inhibitor cocktail,

phosphatase inhibitor cocktail), homogenized, and protein concentration measured by Bradford colorimetric assay; 50 μg of lysate was resuspended in loading buffer (2% SDS, 1% dithiothreitol, and 0.05 M Tris-HCl, pH 6.8, 10% glycerol, 0.001% bromophenol blue), separated by 15% SDS-PAGE, and transferred to a nitrocellulose filter. The blots were blocked with PBS/5% nonfat dry milk for nonspecific binding and incubated with antibodies as follows: anti-Gadd45b (rabbit monoclonal, working dilution 1:10,000; Abcam, Cambridge, MA), anti-phospho-NFκB p65 (rabbit polyclonal, working dilution 1:200; Cell Signaling Technology, Danvers, MA), anti-NFκB (Cell Signaling Technology, rabbit polyclonal, working dilution 1:100), or anti-β-actin (mouse monoclonal, working dilution 1:10,000; Sigma-Aldrich, St. Louis, MO). Immunoblot assays were performed with peroxidase-conjugated goat anti-rabbit IgG_{2a} or goat anti-mouse IgG and the enhanced chemoluminescence detection system (Amersham Life Science, Inc., Arlington Heights, IL) with β-actin used as an internal control. Each experiment was repeated at least three times.

Immunohistochemistry

After experimental animals were killed, eyes were fixed in 4% paraformaldehyde and processed for immunohistochemistry as described previously.¹ The following reagents were used in the detection of protein expression: anti-TGFβs (rabbit polyclonal, working dilution 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-TGFβRI (rabbit polyclonal, working dilution 1:1000; Santa Cruz Biotechnology, Inc.), anti-TGFβRII (rabbit polyclonal, working dilution 1:50; Santa Cruz Biotechnology, Inc.), anti-phospho-NFκB p65 (rabbit polyclonal, working dilution 1:100; Cell Signaling Technology), and anti-Gadd45b (rabbit polyclonal, working dilution 1:200; Santa Cruz Biotechnology, Inc.). Samples were then incubated with peroxidase-conjugated appropriate secondary antibody and the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) using diaminobenzidine as substrate, or fluorescent conjugated secondary antibodies. Double immunofluorescent labeling for Gadd45b and Thy1.1 (mouse monoclonal, working dilution 1:50; Serotec, Oxford, UK) was performed sequentially with an appropriate Alexa green-conjugated goat anti-rabbit secondary antibody and Rhodamine red-conjugated goat anti-mouse secondary antibody (working dilution 1:1000; Molecular Probes, Eugene, OR). Negative control samples were processed in parallel using specific blocking peptides to neutralize antibodies.

Immunocytochemistry

Cells were cultured on glass coverslips. After treatment with TGFβ1 (10 ng/mL) or TGFβ2 (10 ng/mL) for 24 hours, cells were fixed in 4% paraformaldehyde and treated with 0.5% fetal bovine serum/0.2% Triton X-100/0.5% glycine in PBS. The coverslips were incubated with primary antibodies against Gadd45b (rabbit polyclonal, working dilution 1:50; Santa Cruz Biotechnology, Inc.), TGFβRI (rabbit polyclonal, working dilution 1:250; Santa Cruz Biotechnology, Inc.), TGFβRII (rabbit polyclonal, working dilution 1:50; Santa Cruz Biotechnology, Inc.) and with an appropriate secondary antibody; goat anti-rabbit Rhodamine red or goat anti-rabbit Alexa green conjugated IgG (working dilution 1:1000; Molecular Probes). The coverslips were mounted in Vectashield with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).

RESULTS

Induction of Gadd45b Expression by TGFβ in RGC5 In Vitro

We sought to demonstrate if the extracellular ligand, TGFβ is capable of upregulating Gadd45b expression in RGCs. RGC5, a transformed retinal neuronal precursor cell line, was initially used as a screening tool to test our hypothesis. We found that

FIGURE 1. Gadd45b protein expression is increased in RGC5 cells via TGF β . Immunocytochemical labeling for TGF β RI (A) and TGF β RII (B) was positive and localized to the cell membrane and cytoplasm. (C, D) Immunocytochemical labeling for Gadd45b was weakly positive in RGC5 control cultures (C). After treatment with TGF β 1 (10 ng/mL) for 24 hours (D), Gadd45b labeling increased in intensity in the cytoplasm (D, arrows) ($\times 400$). Immunoblot assays showed increased expression of Gadd45b in RGC5 cultures after treatment with TGF β 1 (10 ng/mL) for 12 to 48 hours (E).

TGF β signaling induced Gadd45b expression in RGC5 cells. TGF β RI (Fig. 1A) and TGF β RII (Fig. 1B) were present in abundance on the cell membrane and in the cytoplasm of RGC5.

We initially assessed TGF β 1-mediated induction of Gadd45b from the results of previous work.⁵ Baseline expression of Gadd45b protein was weakly positive in resting RGC5 cells (Fig. 1C). After treatment with TGF β 1 for 24 hours, Gadd45b expression was markedly increased and there was abundant labeling in the cytoplasm (Fig. 1D). By immunoblot analysis, increased Gadd45b protein concentration was observed in RGC5 treated with TGF β 1 for 12, 24, and 48 hours (Fig. 1E).

Gadd45b mRNA expression was detected by quantitative RT-PCR in RGC5 cells treated with TGF β 1. Gadd45b mRNA was significantly upregulated in RGC5 by treatment with TGF β 1 10 ng/mL at 2 hours and upregulation persisted for at least 24 hours (Fig. 2A). Upregulation of Gadd45b mRNA expression appeared to be dose dependent in response to TGF β 1 stimulation (Fig. 2B). Similarly, the expression of Gadd45b mRNA was significantly upregulated by treatment with TGF β 2 (Fig. 2C).

Induction of Gadd45b Expression by TGF β in RGCs In Vivo

Next we investigated the TGF β /Gadd45b signaling pathway in RGCs in vivo. We first assessed for the presence of TGF β receptors in retina in vivo. Figures 3A and 3B demonstrate that TGF β receptor I and TGF β receptor II were present predominantly in the retinal ganglion cell layer in adult mouse retina.

We further assessed whether exogenous TGF β 1 could induce Gadd45b expression in RGCs in vivo. Weak baseline expression of Gadd45b was present in the retinal ganglion cell layer of control eyes (Fig. 3C). In eyes treated with TGF β 1 for 24 hours (Figs. 3D–H), robust labeling of Gadd45b was detected in the ganglion cell layer. To confirm the localization of Gadd45b to RGCs, retinas were double labeled with Gadd45b (Fig. 3E) and the RGC cell marker Thy1.1 (Fig. 3F). As shown in Figure 3H, co-localization of Gadd45b and Thy1.1 indicated that RGCs expressed Gadd45b in response to TGF β 1 treatment in vivo. A marked increase in Gadd45b protein was detected by immunoblot analysis in retinas treated with TGF β 1 for 24 hours, and the effect persisted to 48 hours (Fig. 3J).

FIGURE 2. TGFβ induced Gadd45b mRNA expression in RGC5. TGFβ1 (10 ng/mL) induced an increase in Gadd45b mRNA, detected by quantitative RT-PCR at 2 and 6 hours that persisted to at least 24 hours (A). TGFβ1 stimulation induced a dose-dependent increase in Gadd45b mRNA (B). TGFβ2 (10 ng/mL) treatment induced an increase in Gadd45b mRNA detected at 2 and 24 hours (C). Values are mean $\pm$ SEM of three independent experiments. * $P < 0.05$.

Gadd45b mRNA expression was assessed by quantitative RT-PCR following treatment with TGFβ1. An increase in Gadd45b mRNA was detected in treated retinas by 2 hours after intravitreal injection of TGFβ1, and the effect persisted to at least 6 hours (Fig. 3K). These data suggest that TGFβ signaling induces Gadd45b expression in RGCs in vivo.

Induction of Gadd45b Expression via NFκB

NFκB activation is an integral component of the TGFβ signaling pathway and regulates activity of the promoter modulating the Gadd45b gene in non-neuronal cells.¹⁹ We sought to determine whether TGFβ1 induces Gadd45b through activation of NFκB in RGCs. TGFβ1 stimulated phosphorylation of NFκB in RGC5 within 10 minutes of treatment and the activation of phosphorylation persisted to at least 30 minutes (Fig. 4A). Two specific NFκB inhibitors, SN50, which directly inhibits the nuclear translocation of NFκB, and Bay11-7085, which selectively inhibits phosphorylation of IκBα, were used to confirm the induction of Gadd45b expression via the activation of NFκB. Inhibition of the activation of NFκB by SN50 or Bay 11-7085, respectively, blocked the upregulation of Gadd45b mRNA transcription by TGFβ1 (Fig. 4B). These results suggest that TGFβ1 induces Gadd45b gene expression via NFκB activation.

We detected the presence of NFκB activation in response to TGFβ1 in RGCs in vivo. Detection of phosphorylated NFκB confirmed that NFκB was activated in cells within the ganglion cell layer after exposure to TGFβ1 for 30 minutes (Figs. 4C, 4D). Intense labeling of phosphorylated NFκB was detected in cell nuclei, indicating the activation and translocation of NFκB (Fig. 4D). The inner plexiform layer and a minority population of cells in the inner nuclear layer were also positive for phosphorylated NFκB. The activated form of NFκB, phosphorylated NFκB, was markedly increased after stimulation with TGFβ1 for 30 minutes (Fig. 4E).

TGFβ-Induced Expression of Gadd45b Protected RGC5 from Injury

We tested whether the upregulation of Gadd45b expression enhances cell resistance to neuronal injury, and whether Gadd45b is a significant mediator of the neuroprotective activity conferred by TGFβ in vitro. Differentiated RGC5 were exposed to cytotoxicity or oxidative stress mediated by TNF-α or paraquat, respectively. Gadd45b siRNA was used to knockdown the expression of Gadd45b mRNA in RGC5 cells. Gadd45b siRNA significantly attenuated Gadd45b mRNA expression in control and TGFβ-treated RGC5 cells (Fig. 5A).

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J

K

FIGURE 3. TGF β signaling induced Gadd45b expression in RGCs in vivo. Immunohistochemical labeling for TGF β receptor I (A) and II (B) (arrows) predominated in the retinal ganglion cell layer in adult murine retina. (C–E) Gadd45b protein was detected in the retinal ganglion cell layer in control eyes injected with saline (C) and markedly increased following treatment with TGF β 1 (D, arrows). (E–I) Double-labeling for Gadd45b (green) (E) and Thy1.1 (red) (F) demonstrated co-localization (yellow) of Gadd45b and Thy1.1 in RGCs (H) after treatment with TGF β 1. (G) Nuclear stain and (I) negative control. ([A–D, I], $\times 400$; [E–H], $\times 800$). (J) Immunoblot for Gadd45b showed increased Gadd45b following exposure to TGF β 1. (K) Quantitative RT-PCR demonstrated increased Gadd45b mRNA concentrations in retinas exposed to TGF β 1 for 2 and 6 hours. Values are mean $\pm$ SEM of three independent experiments. * $P < 0.05$.

FIGURE 4. TGFβ1 induced Gadd45b through activation of NFκB in vitro and in vivo. (A) Immunoblot for phosphorylated NFκB (p-NFκB) showed increased p-NFκB in RGC5 treated with TGFβ1 for 10 and 30 minutes. (B) Quantitative RT-PCR demonstrated that upregulation of Gadd45b mRNA by TGFβ1 was blocked by a p-NFκB inhibitor, SN50, and by the p-IκB inhibitor, Bay11-7085. Values are mean $\pm$ SEM of three independent experiments. * $P < 0.05$. (C, D) In comparison with control retinas (C), immunohistochemical labeling for p-NFκB was increased in intensity and localized to the cytoplasm and nuclei of cells in the RGC layer following intravitreal injection of TGFβ1. The inner plexiform layer and a lesser population of cells in the inner nuclear layer were also positive for p-NFκB (D) ($\times 400$). (E) Immunoblot assays showed an increase in p-NFκB in retinas exposed to TGFβ1 for 30 minutes.

siRNA transfection or TGFβ1 treatment was not associated with significant changes in cell viability (Figs. 5B, 5C). Exposure to TNF-α 10 ng/mL for 16 hours caused moderate cell death in RGC5 and more extensive cell death in Gadd45b-attenuated

RGC5. As shown in our previous study,¹ the absence of Gadd45b did not affect the survival of RGCs under normal conditions, but significantly decreased the resistance of cells to neuronal injury. When RGC5 cells were pretreated with TGFβ1

FIGURE 5. Induction of Gadd45b by TGF β 1 protected RGC5 against cell death mediated by TNF- α and paraquat. (A) RGC5 cells were transfected with Gadd45b-specific siRNA (SiGadd45b) or nonspecific double-stranded RNA as a control (SiControl). Gadd45b mRNA expression was significantly increased in controls after treatment with TGF β 1 10 ng/mL for 2 hours, but upregulation of Gadd45b mRNA was significantly attenuated by Gadd45b siRNA transfection in the SiGadd45b group as assessed by quantitative RT-PCR. (B) Exposure to TNF- α 10 ng/mL for 16 hours decreased cell survival in Gadd45b-attenuated RGC5 (SiGadd45b) compared with control RGC5 (SiControl). Pretreatment with TGF β 1 10 ng/mL for 2 hours protected SiControl but not Gadd45b-attenuated RGC5 against TNF- α -mediated cell death. (C) Exposure to paraquat 600 μ M for 16 hours decreased cell survival in Gadd45b-attenuated RGC5 (SiGadd45b) compared with control RGC5 (SiControl). Pretreatment with TGF β 1 10 ng/mL for 2 hours protected SiControl but not Gadd45b-attenuated RGC5 against paraquat-mediated cell death. Values are mean $\pm$ SEM of three independent experiments. * P < 0.05.

for 2 hours prior to exposure to TNF- α , there was a significant increase in cell survival (Fig. 5B). In Gadd45b-attenuated RGC5 cells, there was a substantial loss of TGF β 1-mediated protection against cell death associated with TNF- α (Fig. 5B). Similar results were observed in RGC5 exposed to paraquat 600 μ M (Fig. 5C). Pretreatment with TGF β 1 protected RGC5 from paraquat-mediated oxidative stress, but the protective activity of TGF β 1 significantly diminished when Gadd45b expression was attenuated.

DISCUSSION

Previous work has identified Gadd45b as an intrinsic neuroprotective molecule in RGCs. Studies of non-neuronal cells have also shown that Gadd45b promotes cell survival against cytotoxicity, excitotoxicity, oxidative stress, and genotoxicity.^{2-4,20,21} Gadd45b exhibits neural activity-induced expression and promotes long-lasting neurogenesis in adult hippocampus.⁵ Activation of Gadd45b-mediated neuroprotection may represent a therapeutic strategy in glaucoma, and perhaps other neurodegenerative disorders.

Our present work demonstrates that the induction of Gadd45b proceeds via the TGF β /NF κ B signaling pathway in RGCs. Given the neuroprotective function of Gadd45b in RGCs in response to various injurious stimuli,¹ activation of the

TGF β /NF κ B/Gadd45b pathway may be an effective means of protecting RGCs from death in neurodegenerative disorders.

Our findings relate Gadd45b-mediated neuroprotection with TGF β signaling in neurons. TGF β signaling is known to be beneficial for neuronal survival under pathological conditions. Deficiency of TGF β signaling has been implicated in the pathogenesis of Alzheimer's disease^{8,22-25} and Huntington's disease.²⁶ In brain tissue, TGF β signaling contributes to neuronal survival and development, and axonal growth.²⁷⁻³¹ TGF β 1 and TGF β 2 and their receptors are upregulated in brain tissue following injury,^{12,13,32} conferring a reduction in neuronal cell death and infarct size.⁹ Numerous in vitro studies have demonstrated the neuroprotective capability of TGF β 1 in neurons against a wide variety of death-inducing insults, including hypoxia/ischemia, glutamate excitotoxicity, β -amyloid, oxidative damage, and human immunodeficiency virus.⁷⁻¹¹

The neuroprotective features of Gadd45b¹ are consistent with the function of TGF β in neuroprotective processes. Our findings on the TGF β /Gadd45b signaling pathway indicate that Gadd45b is a major component of the neuroprotective activity of TGF β signaling. Although TGF β signaling and Gadd45b activity in neurons are considered neuroprotective, the mechanism by which TGF β induces Gadd45b expression is largely unclear. TGF β 1 mediates neuroprotection via upregulation of Bcl-2 proteins,³³ increasing Bad phosphorylation and

suppressing caspase-3 activation.³⁴ Gadd45b-deficient non-neuronal cells show enhanced activation of caspase-3 and PARP cleavage, and decreased expression of CIAP-1, Bcl-2, and Bcl-xL, which are anti-apoptotic molecules.²⁰ Gadd45b participates in epigenetic regulation by reversing specific DNA methylation and modulation of the dynamic transcription profile.³⁵ Gadd45b promotes DNA demethylation of regulatory regions of brain-derived neurotrophic factor and fibroblast growth factor 1, which function as potent neurotrophic molecules.⁴ Gadd45 proteins have been implicated in regulation of diverse cellular functions, including DNA repair, cell-cycle control, senescence, and genotoxic stress in which the proapoptotic and antiapoptotic activities of Gadd45 play essential roles in oncogenesis.³⁶

The TGF β receptor type I/ALK1 activates NF κ B³⁷ through the phosphatidylinositol-3-OH kinase/Akt and mitogen-activated protein kinase signaling pathways.³⁸ NF κ B has diverse functions in the nervous system, including neuroprotection.³⁹ Lack of NF κ B activation in RGCs after TNF- α stimulation has been implicated in TNF- α -mediated RGC death.⁴⁰ In non-neuronal cells, TNF- α -mediated activation of NF κ B opposes death signals via upregulation of antiapoptotic genes including Gadd45b.⁴¹ Our work has shown that TGF β signaling induces activation of NF κ B, which may subsequently stimulate survival signals via induction of Gadd45b to maintain RGC survival under stress.

The TGF β signaling is a multifunctional regulatory pathway with a broad spectrum of cellular activities ranging from the regulation of target gene activity to the control of cell development, growth, and apoptosis.^{22,23} During development, TGF β 2 plays important roles in the morphogenesis of the anterior segment of the eye.⁴² TGF β 2 is known to induce the expression of the extracellular matrix and contributes to the structural changes of the trabecular meshwork and optic nerve head in glaucoma.⁴³ Thus, additional investigations of the TGF β /NF κ B/Gadd45b signaling axis in RGCs are warranted to avoiding the adverse effects of TGF β signaling while protecting RGCs from injuries.

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