

# Blocking LINGO-1 Function Promotes Retinal Ganglion Cell Survival Following Ocular Hypertension and Optic Nerve Transection

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**PURPOSE.** LINGO-1 is a functional member of the Nogo66 receptor (NgR1)/p75 and NgR1/TROY signaling complexes that prevent axonal regeneration through RhoA in the central nervous system. LINGO-1 also promotes cell death after neuronal injury and spinal cord injury. The authors sought to examine whether blocking LINGO-1 function with LINGO-1 antagonists promotes retinal ganglion cell (RGC) survival after ocular hypertension and optic nerve transection.

**METHODS.** An experimental ocular hypertension model was induced in adult rats using an argon laser to photocoagulate the episcleral and limbal veins. LINGO-1 expression in the retinas was investigated using immunohistochemistry and Western blotting. Soluble LINGO-1 protein (LINGO-1-Fc) and anti-LINGO-1 mAb 1A7 were injected into the vitreous body to examine their effects on RGC survival after ocular hypertension and optic nerve transection. Signal transduction pathways mediating neuroprotective LINGO-1-Fc effects were characterized using Western blotting and specific kinase inhibitors.

**RESULTS.** LINGO-1 was expressed in RGCs and up-regulated after intraocular pressure elevation. Blocking LINGO-1 function with LINGO-1 antagonists, LINGO-1-Fc and 1A7 significantly reduced RGC loss 2 and 4 weeks after ocular hypertension and also promoted RGC survival after optic nerve transection. LINGO-1-Fc treatment blocked the RhoA, JNK pathway and promoted Akt activation. LINGO-1-Fc induced Akt phosphorylation, and the survival effect of LINGO-1 antagonists was abolished by Akt phosphorylation inhibitor.

**CONCLUSIONS.** The authors demonstrated that blocking LINGO-1 function with LINGO-1 antagonists rescues RGCs from cell death after ocular hypertension and optic nerve transection. They also delineated the RhoA and PI-3K/Akt pathways as the predominant mediator of LINGO-1-Fc neuroprotection in this paradigm of RGC death. (*Invest Ophthalmol Vis Sci.* 2008;49:975-985) DOI:10.1167/iovs.07-1199

The inability of injured central nervous system (CNS) neurons to spontaneously regenerate has been ascribed to the presence of Nogo66,<sup>1,2</sup> myelin-associated glycoprotein (MAG),<sup>3,4</sup> and oligodendrocyte myelin glycoprotein (OMgp).<sup>5,6</sup> LINGO-1 (LRR and Ig domain-containing, Nogo receptor-interacting protein-1)<sup>7</sup> was recently identified as a new member of Nogo66 receptor (NgR1)/p75 neurotrophin receptor<sup>8</sup> and NgR1/TROY<sup>9,10</sup> signaling complexes, which prevent axonal regeneration in the presence of three myelin inhibitors. While NgR1 directly binds the inhibitors, LINGO-1 and p75/TROY transduce the myelin inhibitory signal through the Rho pathway.<sup>11,12</sup> LINGO-1 is expressed in CNS neurons and is up-regulated after injury or cell death, suggesting that LINGO-1 may be involved in the cell injury response.<sup>7,13,14</sup> In addition to inhibiting axonal regeneration, LINGO-1 participates in injury-induced neuronal death.<sup>14,15</sup> A soluble LINGO-1 protein (LINGO-1-Fc) significantly reduced the apoptotic death of neurons and oligodendrocytes after spinal cord transection.<sup>15</sup> Inhibiting LINGO-1 function with LINGO-1-Fc or an antibody against LINGO-1 or dominant-negative LINGO-1 promotes mid-brain dopamine neuron survival in an animal model of parkinsonism.<sup>14</sup> The presence of LINGO-1 in the adult retina<sup>9</sup> prompted us to investigate the effect of LINGO-1 antagonists on protecting the neurons after retinal injury.

Glaucoma, a leading cause of blindness, is a neurodegenerative disease characterized by slow, progressive degeneration of retinal ganglion cells (RGCs) and their axons.<sup>16</sup> Elevated intraocular pressure (IOP) is an important trigger for the progression of glaucomatous optic neuropathy. Current standard therapy for glaucoma is to lower the IOP by medication or surgery, which may delay disease progression but does not alter RGC loss and axon degeneration. Therefore, more attention is being focused on new therapeutic strategies with the aim of preserving, protecting, and rescuing RGCs and their axons. In this study, we hypothesized that LINGO-1 antagonists may protect RGCs in glaucoma. To test this hypothesis, we induced rat ocular hypertension by laser photocoagulation and measured RGC survival with the treatment of LINGO-1 antagonists. Optic nerve transection, as an acute model of secondary degeneration, leads to significant optic neuropathy. We further confirmed the effect of LINGO-1 antagonists on the RGC survival after optic nerve transection.

A common crucial signaling event for three myelin inhibitors after binding with the NgR1 complex is the activation of RhoA, a member of small guanosine triphosphatase (GTPases), which are known regulators of the actin cytoskeleton.<sup>11,17</sup> The inactivation of RhoA promotes axon regeneration after spinal cord injury in rats and mice.<sup>17,18</sup> Besides its effect on the regulation of neurite growth, RhoA plays an important role in regulating apoptosis in neurons<sup>19</sup> and nonneuronal cells.<sup>20</sup> A Rho-antagonist, C3-05, promoted cell survival after spinal cord injury.<sup>19</sup> Application of C3-07, another Rho antagonist, promoted the regeneration of RGC axons and completely prevented RGC cell death for 1 week after optic nerve crush.<sup>21</sup> LINGO-1-Fc effectively blocks Rho activation and reduces neu-

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ron death after spinal cord injury.<sup>15</sup> Here we tried to examine whether LINGO-1-Fc reduces the activation of Rho and a downstream signaling pathway of RhoA, Jun N-terminal kinase (JNK), after ocular hypertension.

Phosphoinositides 3-kinase (PI3K)/Akt mediates neuronal survival and differentiation, protects neurons from apoptosis, and promotes axon regeneration.<sup>22,23</sup> The PI3K/Akt signaling pathway plays an important role in RGC survival in the damaged retina. Akt is activated by an inherent compensatory mechanism to protect RGCs after injury.<sup>24–26</sup> Partial inhibition of this increase of pAkt induced the neurons in the ganglion cell layer to commit apoptosis.<sup>24,26</sup> Intravitreal administration of brain-derived neurotrophic factor (BDNF) or insulin-like growth factor (IGF)-1 promoted Akt activation and prevented RGC death after optic nerve axotomy in experimental glaucomatous eyes.<sup>25,27–29</sup> LINGO-1-Fc promoted dopamine neuron survival in response to parkinsonism-inducing agents, in part by the activation of an Akt signaling pathway.<sup>14</sup> Similarly, we hypothesized that blocking LINGO-1 function by LINGO-1-Fc may exert neuroprotective activity in RGCs by activating the PI3K/Akt survival signaling pathway after ocular hypertension.

In this study, we investigated the neuroprotective effect of blocking LINGO-1 function with soluble LINGO-1 protein (LINGO-1-Fc) and a neutralizing anti-LINGO-1 mAb (1A7) and the signal transduction involved in their neuroprotection in a rat ocular hypertension model and in an optic nerve transection model.

## METHODS

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Generation of Recombinant LINGO-1-Fc

LINGO-1-Fc was prepared as described previously.<sup>7</sup> Residues 1–532 of human LINGO-1 were fused to the hinge and Fc region of human IgG1 and expressed in CHO cells. Human IgG1 (control protein) was purchased from Protos Immunoresearch (San Francisco, CA).

### Generation of Anti-LINGO-1 mAb

Anti-LINGO-1 mAb 1A7 was generated in mice immunized with LINGO-1-Fc. The hybridoma cell line was maintained in Dulbecco modified Eagle medium, and the antibody was purified by Protein A Sepharose.<sup>14</sup>

### Animals

Experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23) revised in 1996 and approved by the University of Hong Kong Animal Ethics Committee. Adult female Sprague-Dawley rats weighing approximately 250 g each were used in the animal models. They were housed three rats per standard laboratory cage and were maintained on food and water ad libitum with a 12-hour light/12-hour dark cycle (7:00 AM–7:00 PM). All operations were carried out in animals anesthetized with intraperitoneal injections of ketamine (80 mg/kg) and xylazine (8 mg/kg). Before all operations, 0.5% alcaine (Alcon-Couvreur, Puurs, Belgium) was applied to the eyes, and anti-septic eye drops (Tobrex [Tobramycin 0.3%]; Alcon-Couvreur) were used to prevent infection after the treatment. Rimadyl (0.025 mg/mL) in drinking water was used to relieve the pain for 7 days after the surgeries. All animals were humanely killed with an overdose of anesthesia. Twelve animals were used for RGC counting for the phosphate-buffered saline (PBS) control group in the 4-week glaucoma model, and 10 were used for all other groups. Each group in the optic nerve transection model contained six animals. Each experimental group for Western blotting or immunohistochemistry contained four to five animals.

## Ocular Hypertension Model

To induce experimental ocular hypertension, Sprague-Dawley rats received argon laser photocoagulation of the limbal and episcleral veins in the right eye, as previously described.<sup>30,31</sup> A secondary laser surgery was delivered to block the reconnected vascular flow 7 days later. Approximately 90 spots were applied on the three episcleral veins, and 70 spots were applied around the limbal vein with the following settings: power, 1000 mW; spot size, 50  $\mu$ m in diameter; duration, 0.1 second. Each contralateral left eye was not operated on and was used as a control. After general anesthesia, the IOP of right and left eyes were measured using a tonometer (Tonopen XL; Reichert, Depew, NY) at different time points. Fluorogold (FG) labeling of RGCs was performed 7 days before kill. Both superior colliculi were exposed after removal of a small piece of skull and cortex, and a piece of absorbable gelatin (Gelfoam; Pharmacia & Upjohn, New York, NY) soaked with FG (6% in distilled H<sub>2</sub>O; Fluorochrome, Denver, CO) was placed on the surface of the superior colliculi. FG retrogradely labeled intact RGCs. Animals were killed 2 or 4 weeks after first laser exposure. In this article, the term *after laser* coagulation always means after the first laser coagulation.

## Optic Nerve Transection

After opening the dural sheath and exposing the superior extraocular muscles, the optic nerve was completely transected at 1.5 mm from the optic disc. Transection was performed on the right eye at day 0, and on the left eye at 2 days before kill as the control. Surviving RGCs were labeled in a retrograde fashion by placing a piece of absorbable gelatin (Gelfoam; Pharmacia & Upjohn) soaked with 6% FG at the ocular stump at 2 days before kill. Animals were killed 7 days after surgery. Care was taken to maintain the blood supply throughout the operation. Animals with compromised blood supply after the surgery, as determined by the fundus, were excluded.

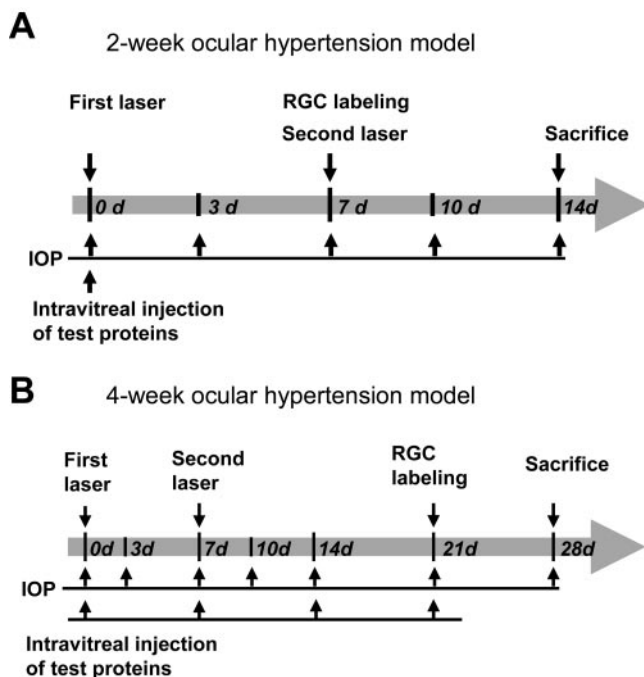
## Drug Administration

After the first laser or optic nerve transection of the right eyes, animals immediately received intravitreal injections of 2  $\mu$ g LINGO-1-Fc or 1A7 in 2  $\mu$ L PBS in the 2-week ocular hypertension model or the 1-week optic nerve transection model. Proteins were provided once a week in the 4-week ocular hypertension model. Control rats received intravitreal injections of either 2  $\mu$ g control protein in 2  $\mu$ L PBS or 2  $\mu$ L PBS. Before injection, 0.3% Tobramycin (Alcon-Couvreur) was applied to the eyes to prevent infection. The proteins were injected into the vitreous body using a 26-gauge sterile microsyringe (10  $\mu$ L, #80300; Hamilton). The site of injection was just below of the limbus of the cornea, which provided minimal possibility of injury to the retina. After injection, 0.3% tobramycin ophthalmic ointment (Alcon-Couvreur) was applied around the wound to prevent infection. Treatments were masked to avoid bias of investigators during counting of RGCs. Procedures of the glaucoma model are summarized in Figure 1.

A specific inhibitor of PI3K, LY294002 (LY; Calbiochem)—an enzyme required for the activation of Akt—was used to examine the functional significance of LINGO-1-Fc-induced Akt phosphorylation in vivo. LY294002 (10 mM) was dissolved in 100% dimethyl sulfoxide (DMSO; Sigma) and subsequently diluted to 1 mM using sterile PBS. Intravitreal injections of 2  $\mu$ L of 1 mM LY294002 or vehicle (10% DMSO) on days 0, 4, 7, and 10 after first laser photocoagulation were performed on the right eyes. Operated animals were killed on day 14, and FG-labeled RGCs were counted.

## Quantification of RGCs

At a predefined time, rats were killed with an overdose of anesthesia. After transcardial perfusion with 0.9% saline, both eyes of each animal were enucleated and fixed in 4% paraformaldehyde for 60 minutes. Retinas were prepared as flatmounts, and the FG-labeled RGCs were counted under a fluorescence microscope using an ultraviolet filter (excitation wave length, 330–380 nm), as described.<sup>24,30</sup> RGCs were



**FIGURE 1.** Schematic diagram showing procedure of ocular hypertension model. Rats received two treatments of laser coagulation with a 7-day interval. IOP was measured on days 0 (before the first laser), 4, 7 (before the second laser), 10, 14, 21, and 28. Seven days before kill, a piece of absorbable gelatin soaked with FG was placed on both sides of the superior colliculi to retrogradely label the RGCs. Animals were killed at 2 or 4 weeks. The proteins were provided once in the 2-week or once a week in the 4-week ocular hypertension model. (A) Procedure of 2-week ocular hypertension model. (B) Procedure of 4-week ocular hypertension model.

quantified under an eyepiece grid of  $200 \times 200 \mu\text{m}^2$  along the median line of each quadrant, starting from the optic disc to the border at  $500\text{-}\mu\text{m}$  intervals. Eight microscopic fields for each quadrant—a total of 32 per retina for four quadrants—were counted. The percentage loss of RGCs was used to examine the survival effects of different treatments. Data were expressed in terms of relative percentage of FG-labeled RGC loss in the right eye to that in the left eye (percentage left eye, mean  $\pm$  SEM).

### Immunohistochemistry

RGCs were retrogradely labeled with FG 7 days before kill. Eyes were enucleated 2 weeks after injury for LINGO-1 immunohistochemistry. To confirm the location of pAkt in the retina, eyes were removed 6 hours after laser coagulation. After transcardial perfusion with 0.9% saline, the eyes were postfixed in 4% paraformaldehyde for 4 hours and then transferred into 30% sucrose. Ten-micron-thick frozen retinal sections were washed with PBS, incubated in 0.5% Triton/PBS for 10 minutes, and blocked with 10% normal goat serum for 1 hour. Incubation with mouse anti-LINGO-1 (1:200; Biogen) or mouse anti-pAkt (1:100, Ser473, 587F11, 4051; Cell Signaling Technology, Beverly, MA) was performed at  $4^\circ\text{C}$  for 16 hours. Sections then were washed with PBS three times and incubated with fluorescent goat anti-mouse secondary antibody (AlexaFluor 568; Molecular Probes, Invitrogen, Carlsbad, CA) at room temperature for 2 hours. Tissue sections for LINGO-1 expression were also processed for immunoperoxidase staining. The difference between the immunofluorescent staining methods was that the sections were incubated with 1%  $\text{H}_2\text{O}_2$  instead of 0.5% Triton/PBS for 15 minutes at the beginning of immunoperoxidase staining. The sections were incubated with biotin-conjugated secondary antibody (goat anti-rabbit; Dako UK Ltd., Ely, Cambridgeshire, UK) and visualized with diaminobenzidine (DAB; 0.03% DAB, 0.003%  $\text{H}_2\text{O}_2$ , pH 7.2).

The nuclei were counterstained by hematoxylin. The sections were analyzed under a confocal microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany) or light microscope. Because this confocal microscope has no UV excitation laser, we used the multiphoton laser (720 nm) to excite the FG. We regarded blue as the color of FG. Five animals were used for each study.

### Western Blotting

To measure LINGO-1 in the retina, the animals were humanely killed 2 weeks after laser coagulation. To examine the temporal profile of Akt phosphorylation in retinas, animals with treatment of LINGO-1-Fc or PBS were humanely killed 6 hours, 1 day, 5 days, and 14 days after laser coagulation. For assessing the temporal profile of JNK phosphorylation, animals treated with LINGO-1-Fc or PBS were humanely killed at 5 days after laser coagulation. Treated retinas were dissected and homogenized in lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA) supplemented with 10% protease inhibitor cocktail and 1% phosphatase inhibitor cocktails from Sigma. After centrifugation at 13,000 rpm for 30 minutes to remove cell debris, the protein concentration of the supernatant was measured using a protein assay kit (DC; Bio-Rad Laboratories, Hercules, CA). A 40- to 80- $\mu\text{g}$  aliquot of proteins from each sample was subjected to 10% to 12.5% SDS-PAGE and transferred onto a PVDF membrane. The membranes were blocked with 5% nonfat dry milk and 2% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour in room temperature. Incubation with mouse anti-LINGO-1 (1:1000; Biogen), mouse anti-pAkt (1:1000, Ser473, 587F11, #4051; Cell Signaling Technology), rabbit anti-total Akt (1:1000, #9272; Cell Signaling Technology), rabbit anti-pJNK (1:1000, Thr183/Tyr185, #9251; Cell Signaling Technology), and rabbit total-JNK (1:1000, #9252; Cell Signaling Technology) antibodies were performed for 16 hours at  $4^\circ\text{C}$ . After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000; Dako) in 5% nonfat dry milk and 2% BSA in TBST for 1 hour at room temperature, and immunoreactive proteins were detected using the enhanced chemiluminescence method (ECL; Amersham). Protein loading was controlled using a monoclonal goat antibody against anti-actin (1:2000, C-11; Santa Cruz Biotechnology). The intensity of each band was quantified by densitometric scanning using gel documentation (Labworks; UVP, Inc, Upland, CA). All experiments for Western blotting were performed with four to five animals in each group and repeated two to three times. Protein levels were expressed as relative values compared with normal retinas.

### RhoA-GTP Pull-Down Assay

Retinas were collected 5 days after laser coagulation with the treatment of LINGO-1-Fc and then lysed. GTP-RhoA protein was pulled down using an assay kit (Rho Activation Assay Biochem Kit BK 036; Cytoskeleton, Denver, CO). GTP-RhoA protein and total RhoA protein were assayed by Western blotting using an antibody against RhoA (Cytoskeleton). Detailed Western blotting procedures were as described.

### Statistical Analysis

Statistical analysis was performed using Student's *t*-test for comparisons between two groups or by one-way analysis of variance (ANOVA) followed by post-hoc tests (Student-Neuman-Keuls) for comparisons of more than two groups. Data were analyzed statistically with SPSS software (SPSS 12.0; SPSS Inc., Chicago, IL). The mean difference was significant at the 0.05, 0.01, or 0.001 level.

## RESULTS

### LINGO-1 Expression in the Retina

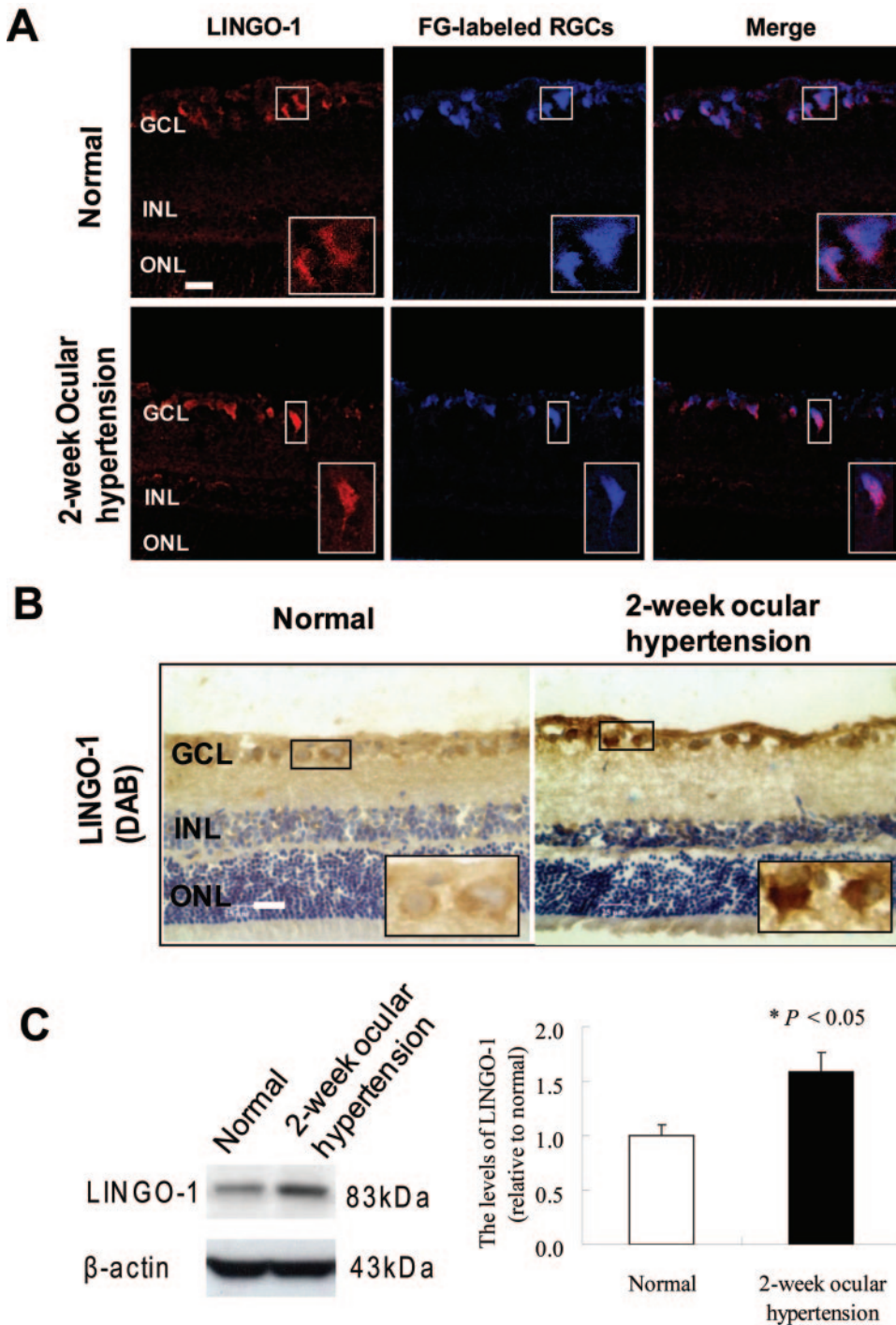
Previous studies have demonstrated that normal RGCs express LINGO-1.<sup>9</sup> Here we further examined LINGO-1 protein ex-



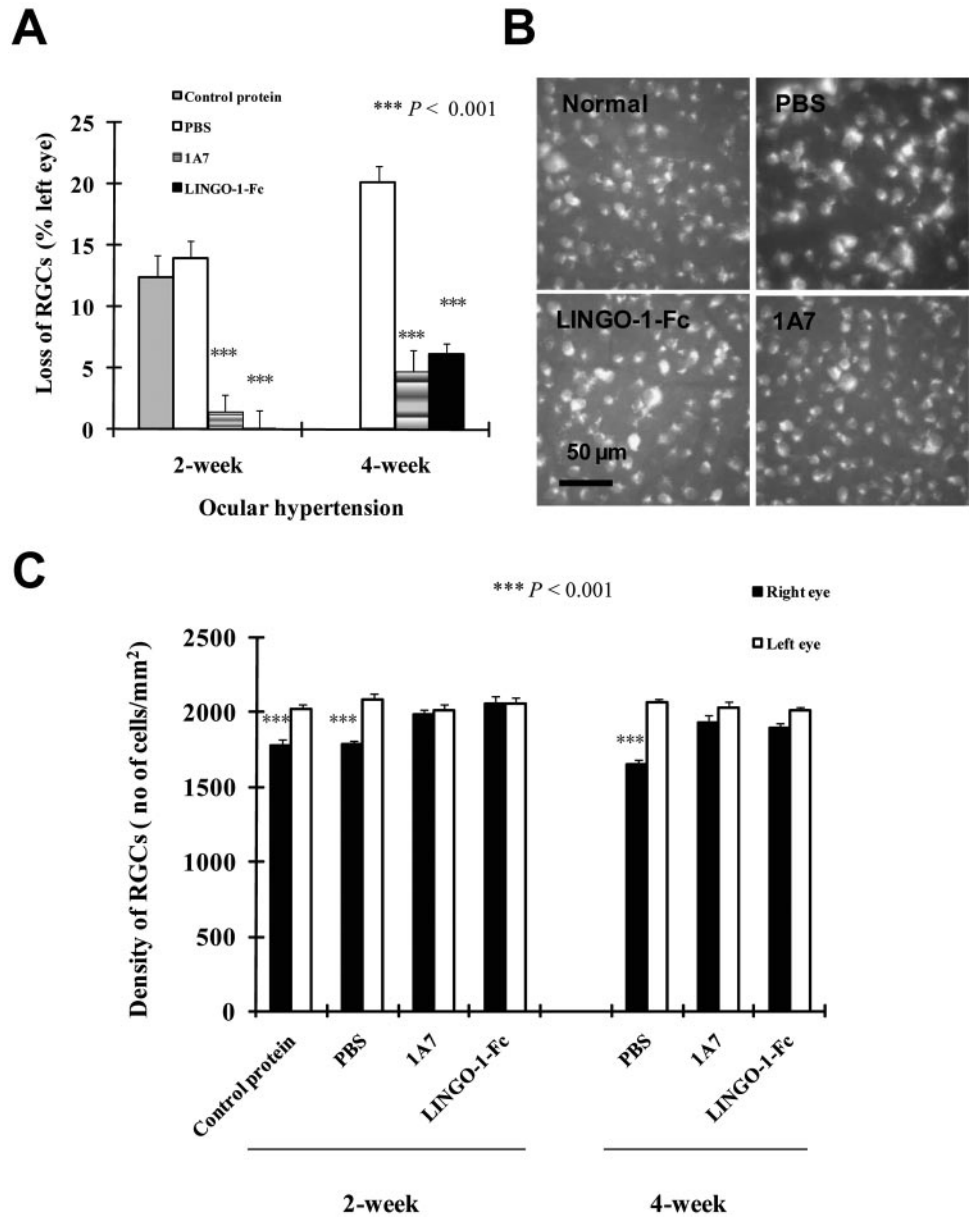
pression in normal and injured rat retinas using immunohistochemistry. As shown in Figure 2A and 2B, low LINGO-1 expression was observed at the cell surface of normal RGCs. In contrast, a much higher level of LINGO-1 was detected not only at the cell surface but also in the cytoplasm of RGCs 2 weeks after laser coagulation. No staining was detected in control experiments without the primary antibody (data not shown). We confirmed these findings using Western blotting (Fig. 2C). LINGO-1 expression was low in normal retina and increased to 1.6-fold 2 weeks after injury ( $P < 0.05$ ). This finding is consistent with the report that LINGO-1 expression increased in patients with Parkinson's disease and in animal models of parkinsonism.<sup>14</sup>

### Neuroprotective Effects of LINGO-1-Fc and 1A7 after Ocular Hypertension

To determine whether blocking LINGO-1 function protects RGCs in this model of ocular hypertension, we assessed surviving RGCs 2 weeks after laser injury by retrogradely counting FG-labeled RGCs in flatmounted retinas. As shown in Figure 3A,  $13.93\% \pm 1.44\%$  and  $12.37\% \pm 1.84\%$  RGCs were lost 2 weeks after laser coagulation in PBS and control protein treatment groups. In contrast, treatment with LINGO-1-Fc clearly prevented RGC death. LINGO-1-Fc-treated retinas had only  $0.09\% \pm 1.47\%$  RGC loss ( $P < 0.001$  compared with PBS and human IgG control groups). To further confirm the survival



**FIGURE 2.** Expression of LINGO-1 protein in the normal and ocular hypertensive retina. (A) Immunohistochemistry analysis of LINGO-1 expression in normal and injured retina after 2 weeks of ocular hypertension. LINGO-1 was expressed on the cell surface of normal RGCs. Strong LINGO-1 expression was found on the cell surface and in the cytoplasm of RGCs 2 weeks after the injury. LINGO-1 staining (red); FG-labeled RGCs (blue); merge of LINGO-1 and FG labeling (magenta). (B) LINGO-1 expression in normal and glaucomatous retinas shown with diaminobenzidine. Scale bar, 25  $\mu$ m. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; FG, fluorogold. (C) LINGO-1 expression in normal and injured eyes by Western blotting and densitometric analysis of Western blot analysis ( $P < 0.05$ , compared with normal retina).  $n = 5$  animals per group.



**FIGURE 3.** LINGO-1-Fc or 1A7 promotes the survival of injured RGCs after the induction of ocular hypertension. Each rat (10–12 per group) received an injection of PBS, control protein, LINGO-1, or 1A7 into the right eye immediately after the first laser coagulation for the 2-week model or once a week for the 4-week study. Each left eyes was untreated as the control. (A) Percentage of RGC loss in the right eye to the contralateral left eye 2 and 4 weeks after laser coagulation (mean ± SEM). LINGO-1-Fc or 1A7 rescued RGCs 2 and 4 weeks after ocular hypertension. *P* values compare PBS and control protein groups at the same time point. (B) Flatmount retinas showing surviving RGCs 4 weeks after laser coagulation with different treatments. Scale bar, 50 μm. (C) Density of RGCs was expressed as the number of RGCs/mm<sup>2</sup> (mean ± SEM) in each group. There was a loss of RGCs in right eyes of PBS or control protein groups 2 or 4 weeks after ocular hypertension. \*\*\**P* < 0.001 compared with the left eyes. Right eye, experimental eye; left eye, untreated control eye.

effect by blocking LINGO-1 function, we next examined the effect of the neutralizing anti-LINGO-1 antibody, mAb 1A7, on the survival of RGCs after injury. Similar to the effect of LINGO-1-Fc, 1A7 treatment reduced RGC loss at 2 weeks after laser coagulation and limited RGC loss to 1.46% ± 1.32% (*P* < 0.001 compared with the control groups). These results suggest that by blocking LINGO-1 function with LINGO-1-Fc or anti-LINGO-1 mAb, 1A7 exerted a significant neuroprotective effect on the retina after ocular hypertension.

To investigate the effect of LINGO-1 antagonists on the long-term survival of RGCs, we injected LINGO-1-Fc and 1A7 intravitreally once a week; the animals were allowed to survive for 4 weeks. As shown in Figure 3A, similar to results in the 2-week injured study, blocking LINGO-1 function with LINGO-1-Fc or 1A7 significantly reduced RGC loss 4 weeks after laser coagulation (*P* < 0.001). Representative photomicrographs of retinal flatmounts 4 weeks after ocular hypertension are shown in Figure 3B. Similar data were obtained by evaluating the density of RGCs for each group (Fig. 3C). We have previously investigated the death of RGCs in our rat glaucoma model 2, 4, 8, and 12 weeks after laser coagulation. Results showed that

the loss of RGCs reached a maximal level after 4 weeks.<sup>32</sup> Hence, the findings of significant neuroprotection of LINGO-1-Fc and 1A7 4 weeks after laser coagulation provided sufficient evidence for the positive efficiency of LINGO-1-Fc and anti-LINGO-1 antibody in this glaucoma model. Unlike ciliary neurotrophic factor (CNTF) treatment (data not shown), neither LINGO-1-Fc nor anti-LINGO-1 antibody caused cataracts in the long-term survival experiment.

### IOP Profile

Experimental ocular hypertension can be monitored by measuring changes in pressure. IOP in the contralateral left eye of treated animals was approximately 13 mm Hg (Fig. 4) and remained at the same level through the experiment. The IOP of the laser-treated right eye in all four groups increased after the first laser surgery, reached approximately 22 mm Hg, and remained at this level until kill. Neither LINGO-1-Fc nor 1A7 treatments changed the IOP significantly in treated rats compared with control animals. Therefore, LINGO-1-Fc or 1A7 has a neuroprotective effect on RGCs without affecting IOP.

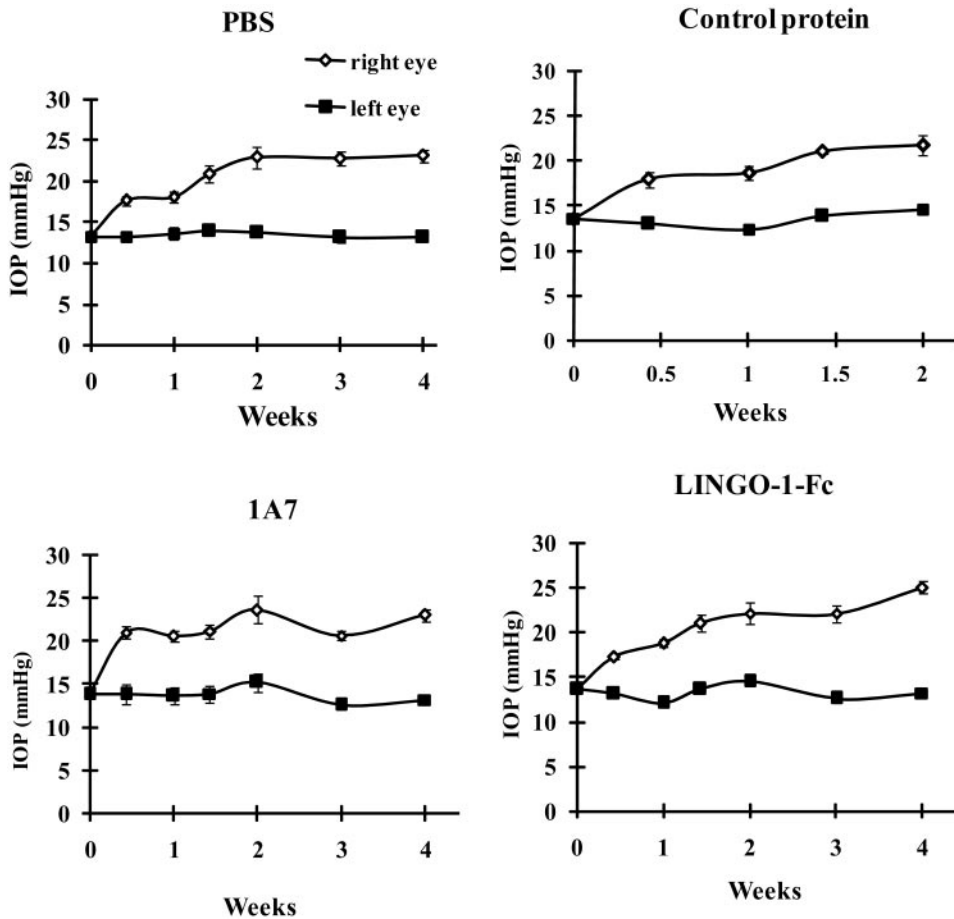


FIGURE 4. Graphs of IOP profile after ocular hypertension. The IOP of experimental right eyes was significantly elevated after laser coagulation compared with those of contralateral left eyes for all the groups. LINGO-1-Fc or 1A7 has no effect on lowering IOP. IOP values are expressed as the mean  $\pm$  SEM of the right or left eye for each time point.  $n = 10-12$  animals per group.

### Neuroprotective Effects of LINGO-1-Fc and 1A7 after Optic Nerve Transection

Transection of the optic nerve leads to acute optic neuropathy and massive RGC death. To further confirm the neuroprotective activity of LINGO-1 antagonists, we investigated the effect of LINGO-1-Fc and 1A7 on the RGC survival after optic nerve transection. Significantly more surviving RGCs were observed in the LINGO-1-Fc-treated rats (loss,  $17.3\% \pm 1.1\%$ ) than in the PBS ( $P < 0.001$ ) or control groups ( $P < 0.001$ ; loss,  $47.1\% \pm 0.9\%$  and  $43.5\% \pm 2.0\%$ , respectively) 1 week after injury (Fig. 5). Similar to LINGO-1-Fc, 1A7 also exerted neuroprotective activity on RGCs 1 week after optic nerve transection, with an RGC loss of  $17.2\% \pm 3.4\%$  ( $P < 0.001$ ) compared with PBS and control proteins (Fig. 5). These results suggest clear neuroprotection of LINGO-1 antagonists after chronic and acute retinal injury.

### Blocking of LINGO-1 Function Inhibits RhoA and JNK Activation and Promotes Akt Activation

To investigate the molecular mechanism of the neuroprotective effect of LINGO-1-Fc, we examined three related signaling pathways—RhoA, JNK, and Akt. As shown in the Figure 6, there was low level of GTP-RhoA in the normal retina. However, the GTP-RhoA level increased to almost twofold 5 days after laser coagulation ( $P < 0.05$  compared with normal group). In contrast, LINGO-1-Fc-treated retinas significantly reduced the high level of GTP-RhoA to the basal level ( $P < 0.05$  compared with the PBS group), suggesting that LINGO-1-Fc may exert neuroprotective activity by inhibiting RhoA activation.

JNK is phosphorylated as a downstream consequence of Rho activation.<sup>33</sup> The JNK pathway can be activated by many cellular stressors. The activation of JNK was temporally associated with the death of RGCs. Phosphorylated JNK locates in the RGCs after IOP elevation.<sup>34</sup> We next examined the level of

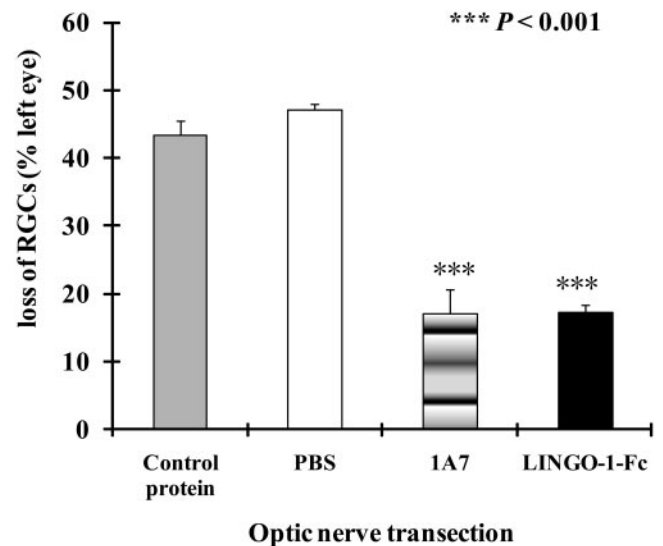
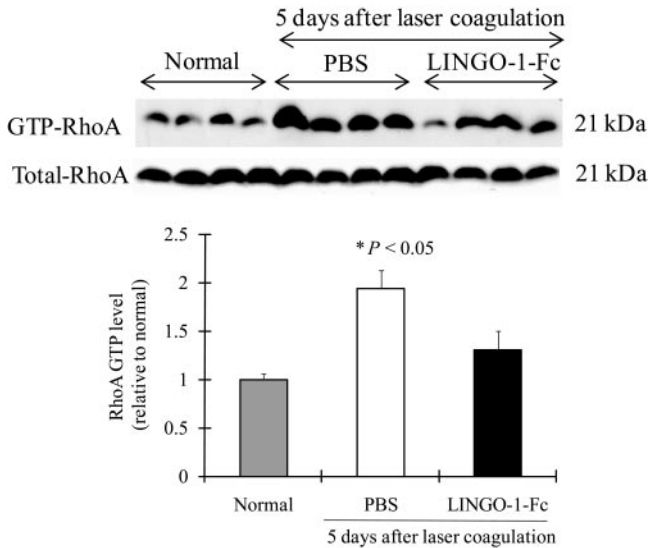


FIGURE 5. LINGO-1-Fc or 1A7 promotes the survival of injured RGCs after optic nerve transection. Percentage of RGC loss 1 week after optic nerve transection (mean  $\pm$  SEM).  $P$  values compared with PBS or control protein groups.  $n = 6$  animals per group.





**FIGURE 6.** LINGO-1-Fc reduces RhoA activation after ocular hypertension. RhoA-GTP isolated from retina homogenates 5 days after laser coagulation was detected by immunoblotting (relative to the normal group). RhoA was activated after laser coagulation and inactivated with the treatment of LINGO-1-Fc. Total RhoA remains at the same level.  $P$  value compared with the other groups.

p-JNK in the injured retina with LINGO-1 antagonist treatment after glaucoma. In our experiment, the total JNK-1 and JNK-2 remained unchanged up to 5 days after ocular hypertension (Fig. 7). The phosphorylated JNK-1 and JNK-2 were low in the normal retina, then increased to more than eightfold after 5 days. A similar response in the p-JNK levels after IOP elevation in a rat glaucoma model has been previously reported by the use of immunohistochemistry.<sup>34,35</sup> As shown in the Figure 7, LINGO-1-Fc or 1A7 treatment reduced activated JNK to almost the normal level ( $P < 0.01$ ).

PI-3K phosphorylation of Akt is an important survival signal for neurons<sup>36</sup> and for RGCs after injury. Even though there is no direct linkage to show the relationship between the suppression of RhoA and JNK with the activation of Akt, we speculated that LINGO-1-Fc promotes Akt activation because it promotes RGC survival.<sup>24</sup> We therefore examined the effects of LINGO-1-Fc on the PI3K/Akt signaling pathway by measuring total Akt and pAkt (the active form of Akt) at various times after laser coagulation surgery. Western blot analysis revealed that total Akt levels remained unchanged up to 14 days after laser coagulation (Fig. 8A). pAkt levels were very low in normal control retinas but increased sixfold by 6 hours after laser treatment and then declined from day 1 to day 14 after laser coagulation. A similar bell-shaped response in pAkt levels has been reported after optic nerve transection.<sup>24</sup> In LINGO-1-Fc-treated retinas, the level of pAkt followed a similar rise but slow decline pattern, peaking at 6 hours and then decreasing over 14 days after laser coagulation. The levels of pAkt on day 5 ( $P < 0.05$ ) and day 14 ( $P < 0.01$ ) were significantly higher than those in control retinas treated with PBS (Fig. 8A). We further confirmed that pAkt was expressed in RGCs. Figure 8B shows representative photomicrographs of pAkt immunolabeling of the retinas 6 hours after laser coagulation and treatment with LINGO-1-Fc. To confirm the role of pAkt in the neuroprotective activity by LINGO-1-Fc treatment after injury, we used a PI3K inhibitor, LY294002, to block Akt activation in the 2-week ocular hypertension model. Although LINGO-1-Fc promoted RGC survival 2 weeks after ocular hypertension (Fig. 3), combining LINGO-1-Fc with LY294002 abolished the neuroprotective effect (Fig. 9). Even though the combined treatment

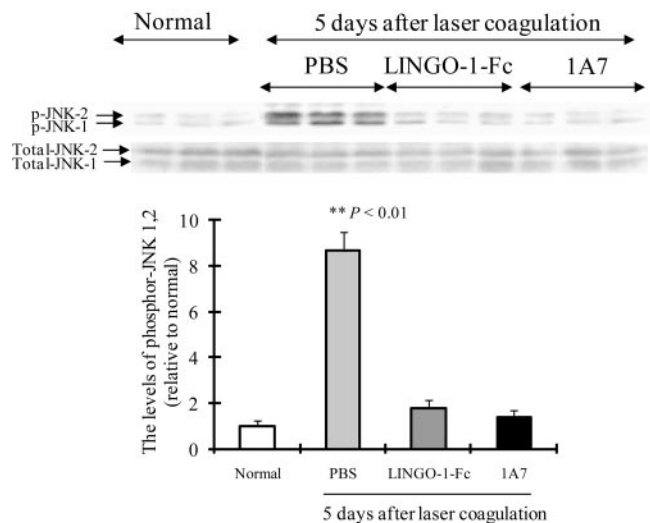
of LINGO-1-Fc with LY294002 rescued a portion of the RGCs compared with LY294002 used alone ( $P < 0.05$ ), LY294002 used alone did not augment RGC loss 2 weeks after laser photocoagulation, suggesting noninvolvement of Akt in this situation. Similar data were obtained by evaluating the density of RGCs. LY294002 alone or combined with LINGO-1-Fc did not lower IOP (data not shown). These results suggest that LINGO-1-Fc promotes RGC survival by blocking the RhoA, JNK signaling pathway and activating the Akt survival pathway after ocular hypertension.

## DISCUSSION

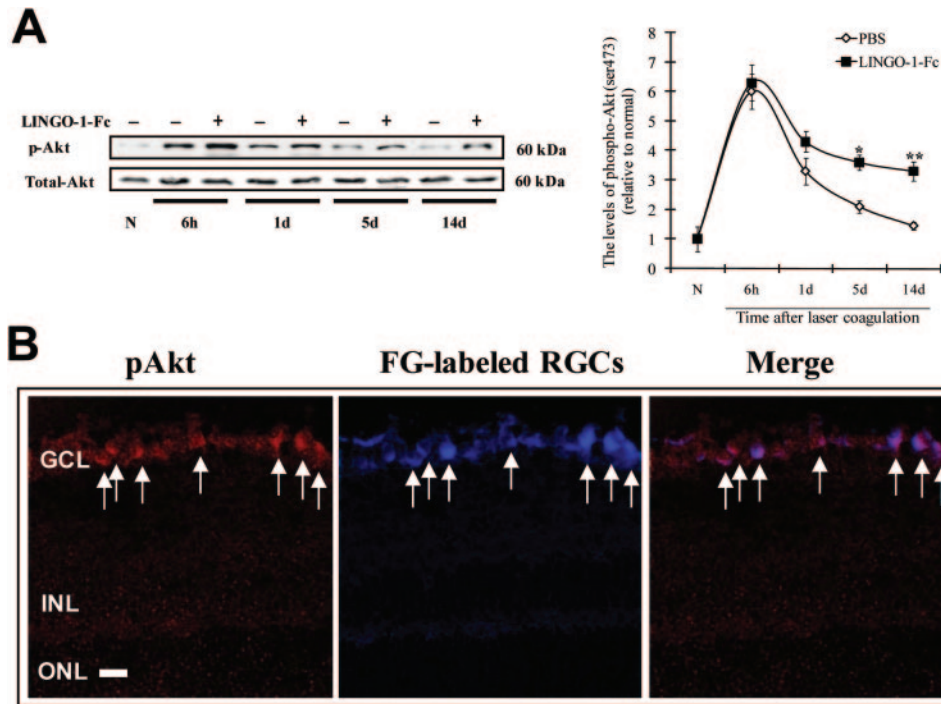
### Neuroprotective Effects of LINGO-1-Fc and 1A7 after Ocular Hypertension

This study demonstrated that blocking LINGO-1 function with a soluble version of the extracellular domain of LINGO-1 (LINGO-1-Fc) or an antibody against LINGO-1 (1A7) promotes the survival of damaged RGCs in a chronic adult rat ocular hypertension model. Glaucoma is a common eye disease that can cause irreversible loss of vision if left undiagnosed and untreated. There are currently no therapies that prevent RGC death. Existing pharmacologic and surgical treatments, aimed at lowering IOP, do not fully prevent the loss of RGCs and their axons in glaucoma. Models of experimentally elevated IOP provide valuable opportunities for the discovery, study, and development of novel treatments for this disease. The model used in this study mimics the slow, progressive neuropathy produced by elevated IOP in humans by partially obstructing aqueous humor outflow, similar to elevated episcleral venous pressure glaucoma.<sup>37</sup> Of course, there are still some differences between this animal model and human glaucoma disease. Human chronic glaucoma is a progressive optic neuropathy characterized by slow visual field loss with a long-term—even several decades long—course, whereas the loss of RGCs in this rat ocular hypertension model reaches a maximal level after 4 weeks.<sup>32</sup>

LINGO-1 interacts with NgR1 and p75 or TROY and is an essential component for activation of the Rho signaling pathway, which inhibits axonal regeneration and induces cell death.<sup>7,9,10,14,15</sup> Blocking LINGO-1 function with LINGO-1-Fc



**FIGURE 7.** LINGO-1-Fc or 1A7 decreases JNK activation. The levels of total JNK-1 and -2 and p-JNK-1 and -2 at normal and 5 days after IOP elevation in control and LINGO-1-Fc- and 1A7-treated retinas.  $n = 4$  animals per group. Each band represents an individual animal.  $P$  values compared with the other groups.



**FIGURE 8.** LINGO-1-Fc increases Akt activation. **(A)** Levels of pAkt at different times after laser surgery in PBS- and LINGO-1-Fc-treated animals by Western blotting. LINGO-1-Fc significantly increased pAkt levels 5 and 14 days after laser coagulation. Four to five animals were tested in the each group. n, normal; h, hours after laser coagulation; d, day(s) after laser coagulation. *P* values compared with the PBS group at the same time point. \**P* < 0.05, \*\**P* < 0.01. **(B)** p-Akt staining (arrows) in retinal sections 6 hours after laser coagulation and treatment with LINGO-1-Fc. p-Akt is expressed in RGCs. Scale bar, 25  $\mu$ m. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. *Left*: stained with anti-pAkt. *Center*: FG-labeled RGCs. *Right*: merging of the pAkt and FG stains.

or 1A7 significantly promotes dopamine neuronal survival *in vitro* or in an animal model of parkinsonism.<sup>14</sup> Here we showed that LINGO-1 protein levels in the retina increase after ocular hypertension, suggesting that elevated endogenous LINGO-1 may play a role in cell death as well.<sup>7,14,15</sup> Using a model that mimicked the etiology and progression of glaucoma, we found that LINGO-1-Fc or 1A7 prevented RGC loss associated with increases of IOP. These agents prevented RGC loss even over a long-term, 4-week course of treatment. Because RGC loss stabilizes after 4 weeks in this ocular hypertension model,<sup>32</sup> the evident neuroprotection of LINGO-1-Fc or 1A7 in a 4-week course indicated the importance of preventing RGC damage in this ocular hypertension model. We also tested the efficacy of LINGO-1 antagonists in the extreme conditions of optic nerve transection. Even in that stringent environment, LINGO-1 antagonists provided a significant level of RGC protection for up to a week. Both LINGO-1-Fc and 1A7 are large molecules with molecular weights of 200 kDa and 140 kDa, respectively, and they exert local action if administered intravitreally. Even one injection of LINGO-1-Fc or 1A7 provided significant neuroprotection to the RGCs in our 2-week ocular hypertension model. The progress of chronic glaucoma in humans is much slower than in the animal model. The clinical implication of LINGO-1-Fc or 1A7 should be investigated through a series of clinical trials. Furthermore, LINGO-1-Fc and 1A7, reported here, are only the crude proteins. They can be improved to be more effective or longer acting for clinical use. Neurotrophic factors, such as BDNF<sup>38</sup> and CNTF, can delay the death of injured RGCs in a glaucoma model<sup>30,39,40</sup> and optic nerve injury.<sup>41,42</sup> However, subsequent studies have shown that BDNF can only slightly increase the survival rate of RGCs<sup>43</sup> and does not significantly rescue injured RGCs in hypertensive eyes after episcleral vein cauterization.<sup>44</sup> Similarly, other neuroprotectants, such as neurotrophin-3, neurotrophin-4, IGF, and glial cell-derived neurotrophic factor (GDNF), show only partial protective effects on RGCs after retinal injury.<sup>29,45,46</sup> Our data suggest that LINGO-1 antagonists can be a promising therapy for glaucoma because of their long-term effect.

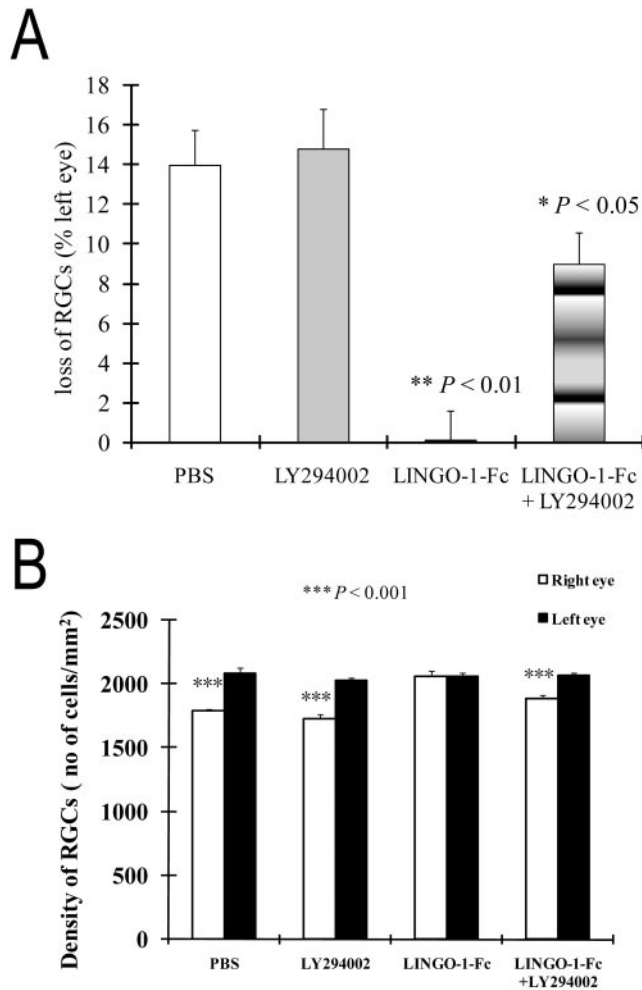
We confirmed that IOP in the right eyes was indeed elevated to a level significantly greater than in the left eyes. We observed no significant change in IOP in the right eyes that received LINGO-1-Fc or 1A7 compared with the left eyes. These results suggest that the protective effect of LINGO-1-Fc and 1A7 is independent of IOP in this model. It is known that increased IOP impairs axonal transport. However, there is no difference for IOP levels between LINGO-1-Fc- and 1A7-treated groups and the two control groups. The effect of increased IOP to the axonal transport can be counteracted between the groups. The neuroprotection of LINGO-1 antagonists was not an artifact from regulating the transport of the dye because of IOP change. Of course, the effect of LINGO-1 antagonists on the effect of axonal transport should be investigated in future studies.

A recent study has shown that LINGO-1-Fc blocked LINGO-1 binding to Ngr1 and reduced apoptotic death of neurons after spinal cord injury.<sup>15</sup> It is likely that the mode of action of the neutralizing 1A7 anti-LINGO-1 mAb is to bind directly to LINGO-1 or its coreceptors on the RGC cell membrane to disrupt their signal transduction pathway. Therefore, we sought to investigate the downstream signal pathway of LINGO-1 with the treatment of LINGO-1-Fc after ocular hypertension.

#### Effect of LINGO-1-Fc Treatment on RhoA Activation after Ocular Hypertension

Rho is the downstream pathway of Ngr1-LINGO-1-p75/TROY complex binding with myelin inhibitors. Small GTPases of the Rho family have been traditionally linked to the regulation of actin cytoskeleton.<sup>47</sup> Recent evidence has revealed that Rho-related GTPases, which include Rho, Rac, and Cdc42, also regulate cell death.<sup>48</sup> Paradoxical results suggest that Rho GTPases can trigger cell death or promote survival in nonneuronal cells.<sup>20,48-51</sup> However, most reports indicate that Rho promotes the cell death of neurons.<sup>15,19,21,52</sup> Rho antagonist not only reduced Rho activation but also promoted cell survival after spinal cord injury<sup>19</sup> and optic nerve crush.<sup>21</sup> LINGO-1-Fc effectively blocks Rho activation<sup>7,13</sup> and protects neurons after spinal cord injury.<sup>15</sup> We identified that RhoA was activated





**FIGURE 9.** LY294002 partially abolished the neuroprotective effect of LINGO-1-Fc. Specific inhibitor of Akt activation, LY294002, significantly blocked the neuroprotective effect of LINGO-1-Fc to RGCs 2 weeks after ocular hypertension. (A) Percentage loss of RGCs in the right eyes 2 weeks after laser coagulation treatment with LINGO-1-Fc, LY294002, or both.  $^{**}P < 0.01$  compared with the other groups.  $^{*}P < 0.05$  compared with the LY294002 group. (B) Density of RGCs was expressed as the number of RGCs/mm<sup>2</sup> (mean  $\pm$  SEM) in each group.  $^{***}P < 0.001$  compared with the left eyes.  $n = 10$  animals per group. *Right:* experimental eye. *Left:* untreated control eye.

after IOP elevation and LINGO-1-Fc inhibited the RhoA activation. Given that LINGO-1-Fc can block LINGO-1/NgR1 interaction,<sup>15</sup> LINGO-1-Fc treatment may rescue damaged RGCs through the inhibition of signaling by the NgR1-LINGO-1-TROY complex and, consequently, RhoA inactivation after ocular hypertension.

### Effect of LINGO-1-Fc Treatment on JNK Activation after Ocular Hypertension

Our studies also confirmed that the phosphorylation of JNK markedly increased after ocular hypertension.<sup>34,35</sup> The activation of JNK, an important proapoptotic factor, is involved in the death of RGCs in glaucoma. However, LINGO-1-Fc and IA7 administration strongly reduced p-JNK to the basal level. The activation of RhoA can induce apoptotic cell death pathways.<sup>53</sup> JNK activation can occur in response to Rho activation. LINGO-1-Fc can reduce the activation of RhoA *in vitro*<sup>7</sup> and after spinal cord injury.<sup>15</sup> LINGO-1-Fc also decreases the phosphorylation of JNK.<sup>15</sup> TROY and p75, additional components of the LINGO-

1/NgR1 signaling complex, can also mediate cell death through the activation of the JNK pathway.<sup>54</sup> The expression of TROY in RGCs<sup>9</sup> contributes to the activation of JNK after ocular hypertensive injury. This then provides a possibility that LINGO-1-Fc protects cells from inhibition of the activation of proapoptotic factors, which provides an alternative mechanism for LINGO-1 antagonist-mediated reduction in apoptotic cell death through the inhibition of TROY or p75 signaling.

### Effect of LINGO-1-Fc Treatment on Akt Activation after Ocular Hypertension

LINGO-1-Fc may exert neuroprotective activity through activation of the epidermal growth factor receptor (EGFR)/Akt intracellular signaling pathway, which is independent of RhoA.<sup>14</sup> Intracellular signaling of EGFR is mediated by the PI3K pathway, which activates Akt.<sup>55,56</sup> EGFR and its ligands are expressed in adult RGCs in the normal rat, mouse, and human retina.<sup>57</sup> Most RGC death in experimental animal models of ocular hypertension,<sup>58</sup> even in a monkey model,<sup>16</sup> occurs by apoptosis. Activated Akt exerts its antiapoptotic effect by inhibiting multiple downstream targets, including caspase activity and cytochrome *c* release.<sup>59,60</sup> An early and transient increase of pAkt levels occurs after episcleral vein cauterization<sup>25</sup> or optic nerve transection<sup>24</sup> and clamping,<sup>26</sup> leading to a delay in RGC death. Our data showed a rapid increase in pAkt levels after laser coagulation. The pAkt activation was stronger than in our previous findings in an acute injury model in hamster.<sup>24</sup> These differences in pAkt activation may be related to the severity of injury, to species differences, or to both. Strong activation of pAkt occurs in the ischemia reperfusion injury.<sup>61</sup> Injury from ischemia and reperfusion causes an inflammatory response due to oxidative damage, which triggers several signaling processes, such as Akt activation, that eventually result in cell apoptosis and death. In our model, IOP increased to approximately 32 mm Hg 6 hours after laser photocoagulation and remained at approximately 23 mm Hg thereafter.<sup>32</sup> This, in turn, led to unstable ocular perfusion and thereby to ischemia and reperfusion damage.<sup>62</sup> This may be why the p-Akt level increased highly after laser coagulation. Partial inhibition of this increase by specific pAkt inhibitors prompted the neurons in the ganglion cell layer to commit apoptosis.<sup>24,26</sup> It is a manifestation of the self-defense mechanism of RGCs against the injury insult, similar to the self-protection of increased erythropoietin (EPO) and EPO receptor to damaged RGCs after ocular hypertension.<sup>63</sup> Our previous study has shown that the rapid but transient increase of Akt phosphorylation after optic nerve transection may be implicated in delaying the onset of caspase-induced apoptosis in RGCs.<sup>24</sup> However, we found that the pAkt elevation after LINGO-1-Fc treatment continued for up to 14 days after the induction of ocular hypertension, consistent with our previous study that blocking LINGO-1 function increased Akt activation in a primary culture of mouse ventral midbrain.<sup>14</sup> p-Akt elevation was also observed *in vivo* in an animal model of Parkinson's disease in LINGO-1 knock-out mice.<sup>14</sup> The inhibition of Akt activation not only reduced pAkt but interfered with the neuroprotective effects of LINGO-1-Fc after elevated IOP. Thus, LINGO-1-Fc appears to operate through mechanisms similar to those of other neuroprotective factors, such as IGF,<sup>29</sup> insulin,<sup>64</sup> BDNF,<sup>28</sup> and EPO,<sup>65</sup> which rescue RGCs after axotomy by activating the PI3K/Akt signaling pathway. However, for the protective effect of LINGO-1-Fc, in addition to PI3K/Akt, other signaling pathways may be involved because blocking its action with LY294002 did not completely remove the neuroprotective effect on RGCs. We also identified that LINGO-1-Fc significantly reduced the level of phospho-JNK, a proapoptotic pathway in response to Rho activation, in rat

retinas after ocular hypertension. The reduced RhoA activation and increased Akt phosphorylation after LINGO-1-Fc treatment may be two independent signaling pathways for neuronal recovery after injury.<sup>14</sup> LINGO-1-Fc may protect cells from apoptotic death independently through the inhibition of RhoA, JNK activation or activation of the Akt pathway.

## CONCLUSIONS

LINGO-1 has attracted much interest as a regulator of axonal regeneration, and LINGO-1 antagonists may stimulate regeneration.<sup>7</sup> Our data indicate that blocking LINGO-1 function with LINGO-1 antagonists also has neuroprotective effects on damaged RGCs in well-established rat chronic glaucoma and acute optic nerve transection models. LINGO-1-Fc may exert neuroprotective activity by inhibiting RhoA activation or activating the Akt survival signaling pathway. Thus, LINGO-1 antagonists may provide an attractive therapeutic strategy to prevent neurodegeneration in glaucoma.

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