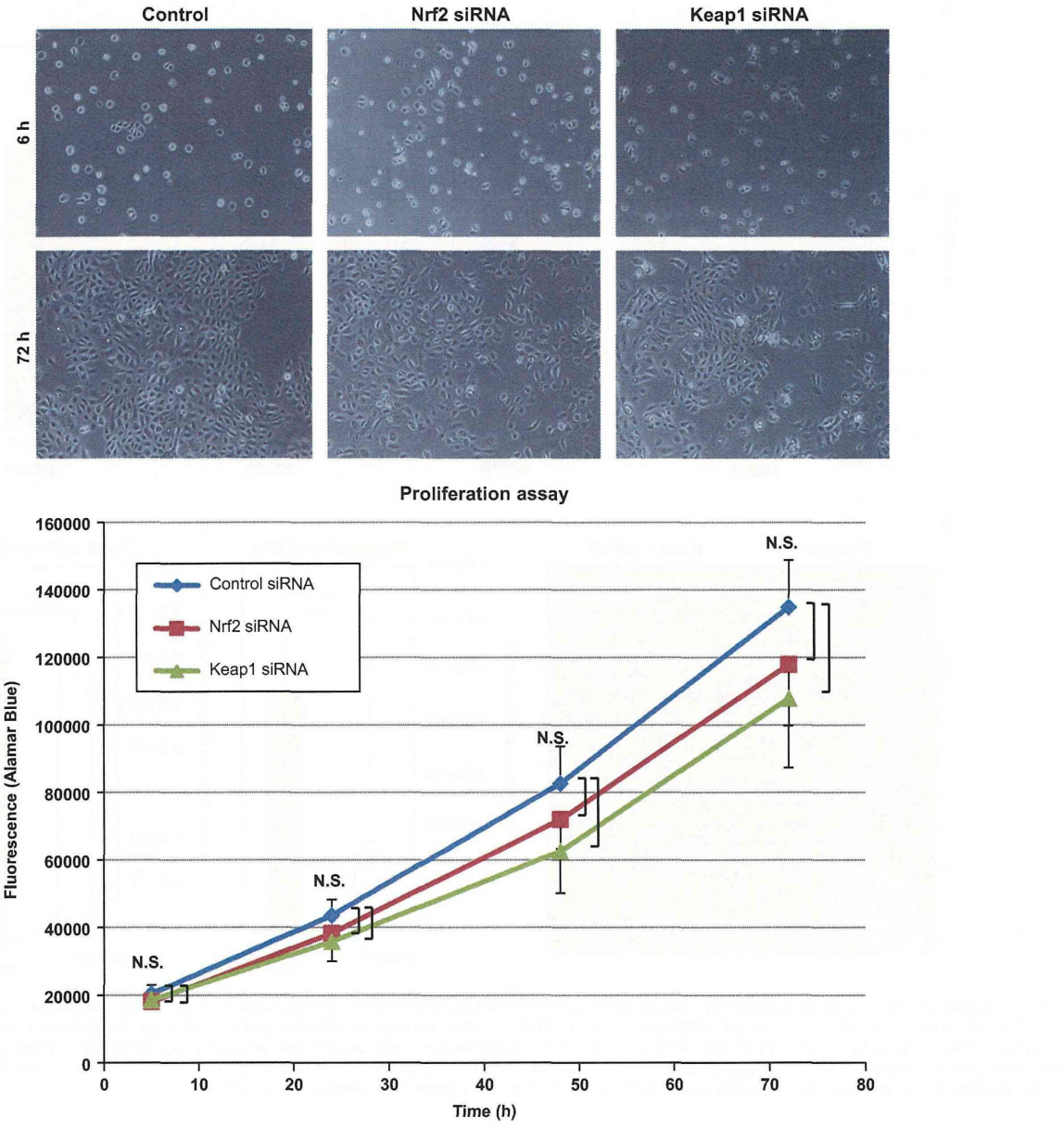


**Fig. 7.** The effect of Nrf2 suppression or activation on the proliferative capability of corneal epithelial cells in vitro. The Nrf2-specific and Keap1-specific C/TERT cells as well as control C/TERT cells into which siRNA was introduced were cultured in KSFM without growth factors. Their proliferative capability was then estimated by Alamar blue assay. The results showed that the proliferative capabilities of C/TERT cells were not significantly affected by either Nrf2 suppression or Nrf2 activation, even though proliferation appeared to be slightly reduced by Nrf2 activation caused by Keap1 knockdown. The graphs represent the means ± SE of seven to nine independent samples. N. S., not significant.

involves initial migration of cells from the limbus to cover the surface of the cornea, followed by intensive cell proliferation to form the stratified epithelial layers (Fig. 8) [20]. However, HE staining in this study revealed a delay in migration in the early processes of wound healing in the Nrf2 KO mice, suggesting that the delay of wound healing resulted from decreased migration capability rather than the cell-proliferation capability (Fig. 8).

These results provide motivation for further investigation of the effect of Nrf2 KO on cell migration activity in corneal epithelial cells in vitro. Initial efforts to isolate and culture the corneal epithelial cells in both WT and Nrf2 KO mice failed to produce stable cultures (data not shown), and to date, no stable method for the primary culturing of mouse corneal epithelial cells has been established. Therefore, we instead selected a stable human corneal epithelial cell line (C/TERT) for in vitro migration assays. siRNA-specific knockdown of Nrf2 mRNA in C/TERTs resulted in a significant decrease in the migration activities, but did not have a significant effect on the proliferative activities. Nrf2 knockdown by siRNA also significantly downregulated the target genes of Nrf2, including NQO1 and HO-1, indicating that Nrf2 was functionally suppressed by the siRNA. These results clearly indicate that the delay of corneal epithelial wound healing in the Nrf2 KO mice in vivo was caused by the reduction of their cell-migration capability rather than their cell-proliferation capability. Additionally, knockdown of Keap1, a suppressor of Nrf2, accelerated cell migration but exerted no apparent significant effect on the cell proliferation of C/TERTs. A previous study also indicated that the proliferative capability of esophageal epithelial cells did not differ remarkably between Keap1-knockout and WT mice [21].

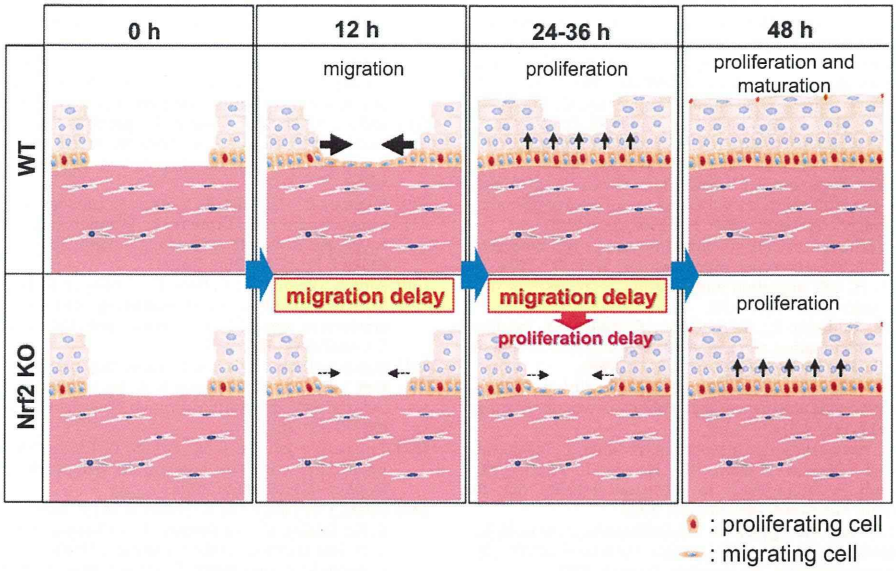


**Fig. 8.** The schema of corneal epithelial wound healing in WT and Nrf2 KO mice. In WT mice, corneal epithelial cells migrated into the injured region by 24 h after the injury. After the migrated cells covered the corneal stroma, cells began to proliferate at 24–36 h and finally reconstructed the corneal epithelial layers by 48 h. At the initial step of wound healing, cell migration was significantly delayed in Nrf2 KO mice compared to WT mice. At 36 h, the corneal surface was not covered with migrated cells; therefore, corneal epithelial cells were not actively proliferating. As a result, the initiation of proliferation was delayed in Nrf2 KO mice. At 48 h after injury, the proliferative activity recovered to levels equivalent to those observed in WT mice.

From these data, it is clear that the Nrf2-mediated defense system mainly affected cell migration rather than cell proliferation during corneal epithelial wound healing, although a detailed mechanism of Nrf2-mediated cell migration remains unclear. Previous studies have suggested that Nrf2 regulates Notch signaling [22], which has been identified as a key factor in corneal epithelial wound healing [23,24]. Thus, the reduction of corneal epithelial migration by Nrf2 KO might be caused by the inhibition of Notch signaling. Other studies have shown that NQO1 upregulates p63, the epithelial stem cell marker [25,26]. In the wound-healing processes of the corneal epithelium, the corneal epithelial stem cells play a central role in tissue generation by supplying daughter cells to the injured region [27]. In addition, several studies have suggested that HO-1 promotes wound healing in the cornea and other tissues [28–30]. The results of this study indicate that the downstream genes of Nrf2 were regulated by the Nrf2-mediated system in the corneal epithelium. It is therefore possible that Nrf2 regulates the migration of corneal epithelial cells via NQO1 or HO-1 expression. Further investigation will be required to clarify how the Nrf2-mediated signal transduction affects cell migration.

The results of this study are significant in that they indicate that Nrf2 activation by Keap1 knockdown increased the migration efficiency of corneal epithelial cells. This finding suggests that the Nrf2/Keap1 complex is a good target for therapies designed to improve corneal epithelium wound healing. Therefore, Nrf2-activating molecules, such as ebselen [31,32] or sulforaphane [33–36], could be excellent candidates in therapeutic medicine for the treatment of corneal epithelial diseases such as dry eye or chronic corneal epithelial defect.

In conclusion, this study provides evidence that the Nrf2-mediated defense system plays a crucial role in corneal epithelial wound healing, mainly by regulating the cell-migration activities of corneal epithelial cells.

## Acknowledgments

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# Therapeutic benefits of 9-amino acid peptide derived from prothymosin alpha against ischemic damages

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ABSTRACT

Prothymosin alpha (ProTα), a nuclear protein, plays multiple functions including cell survival. Most recently, we demonstrated that the active 30-amino acid peptide sequence/$P_{30}$ (amino acids 49–78) in ProTα retains its substantial activity in neuroprotection *in vitro* and *in vivo* as well as in the inhibition of cerebral blood vessel damages by the ischemic stress in retina and brain. But, it has remained to identify the minimum peptide sequence in ProTα that retains neuroprotective activity. The present study using the experiments of alanine scanning suggested that any amino acid in 9-amino acid peptide sequence/$P_9$ (amino acids 52–60) of $P_{30}$ peptide is necessary for its survival activity of cultured rat cortical neurons against the ischemic stress. In the retinal ischemia-perfusion model, intravitreous injection of $P_9$ 24 h after ischemia significantly inhibited the cellular and functional damages at day 7. On the other hand, 2,3,5-triphenyltetrazolium chloride (TTC) staining and electroretinogram assessment showed that systemic delivery with $P_9$ 1 h after the cerebral ischemia (1 h tMCAO) significantly blocks the ischemia-induced brain damages. In addition, systemic $P_9$ delivery markedly inhibited the cerebral ischemia (tMCAO)-induced disruption of blood vessels in brain. Taken together, the present study provides a therapeutic importance of 9-amino acid peptide sequence against ischemic damages.



## 1. Introduction

Ischemic stress in brain and retina causes common expression of cellular and functional damages, which include diverse injury-related cascades underlying necrosis and apoptosis, along with subsequent production and secretion of different cytotoxic mediators [7–10,21,31,34,40,44,51–53]. In addition to the release of cell-damaging mediators, some neuroprotective molecules, such as brain-derived neurotrophic factor, fibroblast growth factor and erythropoietin are simultaneously elevated after the onset of ischemia, and cause limited amelioration of ischemic injury through an inhibition of apoptosis, but not necrosis, a key mechanism of cell death [3,4,13,25,28,29,41,44,48]. Hence, it is essential to develop neuroprotective agents that target the mechanism of necrosis under ischemic condition.

We previously identified prothymosin alpha (ProTα) as a necrosis-inhibitory factor in the conditioned medium of serum-free primary culture of cortical neurons [11,45]. It has been clarified that ProTα inhibits ischemia-induced damages in brain and retina through blockade of necrosis and apoptosis [12,13,46,48]. Several studies established a relationship between ProTα and cell survival [1,23,27,30,47,49,50], and distinct amino acid sequences in ProTα are separately involved in this survival phenomenon [6,24,43]. Among them, the peptide sequence (amino acids 32–52) of the central domain in ProTα participates in the cell defensive mechanisms against oxidative stress through an interaction with Nrf2–Keap1 inhibitory complex [18,24,33]. The N-terminal sequence in ProTα (amino acids 2–29), corresponding to thymosin alpha 1, shows anti-cancer activity and induces immunodefensive action against viral infections [5,14,15,36], whereas C-terminal sequence (amino acids 89–109, 99–109 and 100–109) of human ProTα is involved in the induction of pro-inflammatory activity through toll-like receptor signaling and dendritic cell maturation [42,43]. Recently, the cell survival action of mid part (amino acids 41–83) in human ProTα against mutant huntingtin-caused cytotoxicity has been discussed [6]. Most recently, we reported that active core peptide sequence comprised of 30 amino acids ($P_{30}$: amino acids 49–78) in ProTα exerts its full survival effect in cultured cortical neurons against the ischemic stress and potently blocks the ischemia-induced cellular and functional damages in brain and retina and reverses the damage of cerebral blood vessels in the *in vivo* studies using various ischemic models [17]. However, it is interesting to be investigated which peptide sequence with

*Abbreviations:* ERG, electroretinogram; GCL, ganglion cell layer; H&E, hematoxylin and eosin; INL, inner nuclear layer; IPL, inner plexiform layer; i.v., intravenously; i.vt., intravitreously; ONL, outer nuclear layer; OPL, outer plexiform layer; ProTα, prothymosin alpha; tMCAO, transient middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride.

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minimum amino acids of $P_{30}$ peptide in ProTα is responsible for neuroprotection. In the present study, we evaluated the neuroprotective effect of 9-amino acid peptide derived from ProTα against the ischemic stress.

## 2. Materials and methods

### 2.1. Animals

Male C57/BLJ mice weighing 20–25 g were purchased from Tagawa Experimental Animals (Nagasaki, Japan) and used for all the experiments. Mice were kept in a room maintained at constant temperature (21 ± 2 °C) and relative humidity (55 ± 5%) with an automatic 12 h light/dark cycle with free access to standard laboratory diet and tap water. Animal care and all experimental procedures were formally approved by Nagasaki University Animal Care and Use Committee (Animal Experiments Approval Number: 1104190914).

### 2.2. Determination of short peptide by alanine scanning

The procedure for the identification of neuroprotective peptide sequence $P_{30}$ ($P_{30}$: amino acids 49–78) in ProTα has been described previously [17]. To design ProTα-derived shorter neuroprotective peptide, alanine scanning of $P_{30}$ was performed to determine the contribution of specific amino acid residues that retain the original function of $P_{30}$ peptide. Neuroprotective activity of primary cultured cortical neurons was measured at 12 h after the start of serum-free culture in the presence or absence of various peptides. Cultures and methods of the measurement of survival activity were previously described [17,45].

### 2.3. Peptide administration

Peptide $P_9$ was dissolved in 0.05% dimethyl sulfoxide (DMSO), which was diluted with 0.1 M potassium ($K^+$)-free phosphate buffered saline (PBS). Following the protocol of injection in the eye as described previously [17], $P_9$ was administered intravitreously (i.vt.) with doses of 1, 3 and 10 pmol/μl at 24 h after retinal ischemia ($n = 5$, $n = 7$ and $n = 7$, respectively). On the other hand, $P_9$ was injected intravenously (i.v.) with doses of 0.1, 0.3 and 1 mg/kg ($n = 5$, $n = 6$, and $n = 7$, respectively) 1 h after the cerebral ischemia (tMCAO). Vehicles were treated with equal volume of 0.05% DMSO in similar manners.

### 2.4. Ischemic models

Two types of *in vivo* ischemic models were used throughout the experiments. Retinal ischemia was performed following the method as described previously [13,17]. Briefly, mice were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) and pupils were fully dilated with 1% atropine sulfate drops (Nitten, Nagoya, Japan). The anterior chamber of the eye was cannulated with a 33-gauge needle attached to an infusion container of sterile intraocular irrigating solution (BSS PLUS dilution buffer, Alcon, Fort Worth, TX, USA). Retinal ischemia was induced by elevating the IOP to generate a hydrostatic pressure of 130 mm Hg for 45 min by lifting the container. Following 45 min after retinal ischemic stress, the needle was withdrawn and 0.3% ofloxacin (Santen Pharmaceutical Co. Ltd., Osaka, Japan) was applied topically into the eye to avoid infection.

Another ischemic model is a transient middle cerebral artery occlusion (tMCAO) model, which was induced following the method as described previously [16]. Briefly, mice were anesthetized by 2% isoflurane (Mylan, Tokyo, Japan), and body temperature was monitored and maintained at 37 °C during surgery. After a midline neck incision, the middle cerebral artery was occluded transiently using 8-0 in size monofilament nylon surgical suture (Natsume Co. Ltd., Tokyo, Japan) coated with silicon (Xantopren, Bayer dental, Osaka, Japan) that was inserted through the left common carotid artery and advanced into the left internal carotid artery. Following 1 h tMCAO, the animals were briefly re-anesthetized with isoflurane and the monofilament was withdrawn for reperfusion studies. As the silicon-coated nylon suture also plugs the branch from middle cerebral artery to supply blood to hippocampus in mice, due to small brain size, the ischemia-induced brain damages are also observed in the hippocampus. Cerebral blood flow was monitored by laser Doppler flowmeter (ALF21, Advance Co., Tokyo, Japan) using a probe (diameter 0.5 mm) of a laser Doppler flowmeter (ALF2100, Advance Co., Tokyo, Japan) inserted into the left striatum (anterior: −0.5 mm, lateral: 1.8 mm from Bregma; depth: 4.2 mm from the skull surface) through a guide cannula.

### 2.5. Electroretinogram

Electroretinogram (ERG) study was performed following the protocol as previously described [13,17]. Briefly, mice were dark-adapted for 3–4 h, then anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg) and pupils were dilated with 1% atropine. A contact electrode (KE-S, Kyoto contact lenses, Kyoto, Japan) was placed topically on the corneal apex and reference electrode was placed near the ipsilateral eye. The ground was a subdermal platinum needle electrode near the abdominal area. ERGs were produced by 20 J flash intensities. The flash stimulus source (SLS-3100, Nihon Kohden, Tokyo, Japan) illuminated the eye by diffuse reflection off the interior surface of the ganzfeld. Maximum flash luminance was measured with detector (MEB-9104, Nihon Kohden, Tokyo, Japan). After the intensity series, an incandescent background light sufficient to desensitize the rod system was turned on, and ERGs produced by the standard stimulus were recorded every 2 min for 20 min. The background was then turned off, and ERGs were produced by the standard stimulus every 2 min for the first 30 min of dark adaptation. The a- and b-wave amplitudes were measured online (Neuropack m, QP-903B, Nihon Kohden, Tokyo, Japan). ERG was performed at day 7 after retinal ischemia.

### 2.6. Tissue processing

All *in vivo* experiments were performed using retinal and brain tissues. For retinal tissue preparation, mice were deeply anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Eye was quickly isolated, washed with saline and 4% paraformaldehyde (PFA). Eye was then nicked through pupil, post-fixed in 4% PFA for 24 h and finally transferred to 25% sucrose solution (in 0.1 M $K^+$-free PBS) overnight for cryoprotection. Following freeze in cryoembedding compound, retinal sections were prepared at 10 μm thickness for staining. For brain tissue preparation, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 0.1 M $K^+$-free PBS, followed by 4% PFA. Brain was then quickly removed, post-fixed in 4% PFA and transferred immediately to 25% sucrose solution overnight. Brain was frozen in cryoembedding compound and coronal sections were cut at 30 μm thickness for staining.

### 2.7. Morphological assessment of retinal damages

For hematoxylin and eosin (H&E) staining, on the other hand, frozen retinal sections were washed with 0.1 M $K^+$-free PBS, immerged in Mayer's hematoxylin solution (WAKO, Osaka, Japan) for 5 min at room temperature (25 °C) and then washed with tap

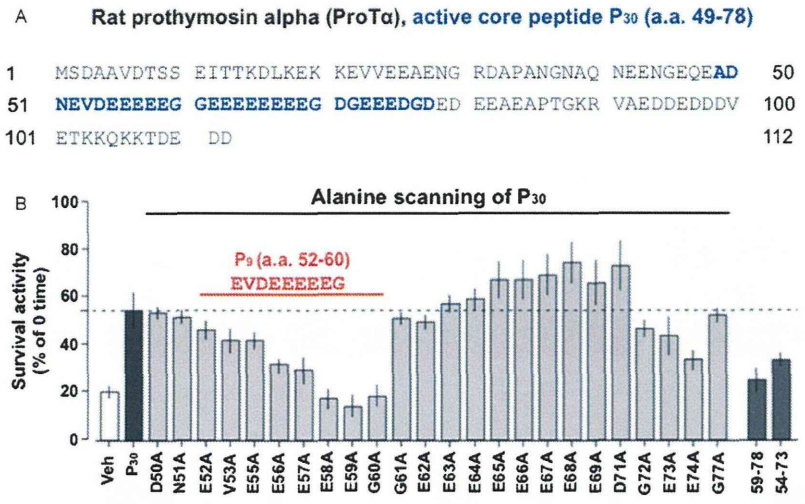


**Fig. 1.** Central 9-amino acid peptide in ProTα is an essential domain for its survival activity. (A) Amino acid (a.a.) sequence of rat ProTα and active core peptide $P_{30}$. Blue colored sequence indicates $P_{30}$ (a.a 49–78). (B) Screening of essential amino acid residue of $P_{30}$ in survival activity using alanine scanning of $P_{30}$ and deletion mutant analysis. Survival activity of primary cultured cortical neurons was measured at 12 h after serum-free ischemic stress. Survival activity of $P_{30}$ was significantly abolished by replacement of amino acids between E56 and G60 by alanine, whereas E52A, V53A, and E55A showed partial survival activity. However, the 54–73 amino acid peptide and the 59–78 amino acid peptide, C-terminus of $P_{30}$, have no significant survival activity. Red colored line and sequence indicate an essential 9-amino acid peptide $P_9$ and its survival activity. Dashed line indicates the level of the survival activity of $P_{30}$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

water for 20 min. Following brief treatment with 95% ethanol, sections were immerged in eosin–alcohol solution (WAKO) for 4 min at 25 °C. Sections were dehydrated through a series of ethanol solutions, xylene, and over-slipped with Permount (Fisher Scientific, Waltham, MA, USA). Sections were then analyzed using a BZ-8000 microscope with BZ Image Measurement Software (KEYENCE, Osaka, Japan).

### 2.8. TTC staining

For 2,3,5-triphenyltetrazolium chloride (TTC) staining, brain was quickly removed at 24 h after cerebral ischemia (1 h tMCAO) followed by $P_9$ administration ($n = 6$ for each group), sectioned coronally with 1-mm thickness and washed with $K^+$-free PBS. Brain slices were incubated in 2% TTC (Sigma–Aldrich, St. Louis, MO, USA) in 0.9% NaCl in dark place for 15–20 min at room temperature (25 °C) and transferred in 4% PFA overnight. Images of brain slices were then collected by scanner, and infarct volume was calculated by Image J software (NIH, Bethesda, MD, USA).

### 2.9. Evaluation of damaged blood vessels

To perform fluorescence staining of blood vessels, biotinylated *Lycopersicon esculentum* (tomato) lectin (Vector Laboratories, Burlingame, CA, USA) was diluted with PBS and injected (1 mg/ml, 100 μl, i.v.) at 24 h after cerebral ischemia (1 h tMCAO). Mice were perfused 5 min after tomato lectin injection. Following tissue preparation as described in Section 2, coronal brain sections were washed with 0.1 M $K^+$-free PBS and blocked with 2% BSA in 0.1% PBST for 2 h at 25 °C. Sections were then incubated with Alexa Fluor 488 streptavidin conjugates (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature (25 °C). Sections were thoroughly washed with PBS and cover-slipped with Perma Fluor. Images were collected using an LSM 710 confocal microscope with ZEN Software (Carl Zeiss, Oberkochen, Germany).

### 2.10. Behavioral assessments

Following $P_9$ administration with doses of 0.1, 0.3 and 1 mg/kg (i.v., $n = 7$, $n = 6$ and $n = 7$, respectively) 1 h after cerebral ischemia (1 h tMCAO), clinical scores were assessed at 24 h after the ischemic stress. Clinical score was evaluated in the following way: 0, no observable deficits; 1, failure to extend the forepaw fully; 2, circling; 3, falling to one side; 4, no spontaneous movement; 5, death. In this study, 0.5 point was added to each score when the motor dysfunction was severe for scores between 1 and 4.

### 2.11. Statistical analysis

All results are shown as means ± S.E.M. Two independent groups were compared using the Student's *t*-test. Multiple groups were compared using Dunnett's multiple comparison tests after a one-factor ANOVA or a Repeated Measures ANOVA. Survival rate was compared using Logrank test after Kaplan–Meyer method. $P < 0.05$ was considered significant.

## 3. Results

### 3.1. Identification of functionally active short 9-amino acid peptide

More recently, we succeeded in identifying 30-amino acid peptide ($P_{30}$: amino acids 49–78) from the sequence of ProTα, bearing neuroprotective activity *in vitro* and *in vivo* [17]. To design shorter amino acid peptide from $P_{30}$ peptide in ProTα (Fig. 1A), we screened essential amino acid residue of $P_{30}$ by use of alanine scanning technique (Fig. 1B). Following replacement of amino acids D50 and N51 by alanine (D50A and N51A), no change in survival activity of $P_{30}$ in cultured cortical neurons against ischemic stress was observed at 12 h after the start of culture, whereas E52A, V53A, and E55A showed partial survival activity (Fig. 1B). Interestingly, the survival activity of $P_{30}$ was markedly abolished by replacement of amino acids between E56 and G60 by alanine (Fig. 1B). On the other hand, the survival activity was absent in peptide comprised of 54–73 amino acid residues as well as in C-terminus of $P_{30}$ (amino acids 59–78) against ischemic stress (Fig. 1B). These results indicated that 9-amino acid peptide (E52–G60) is core domain of $P_{30}$ for its full neuroprotective action.

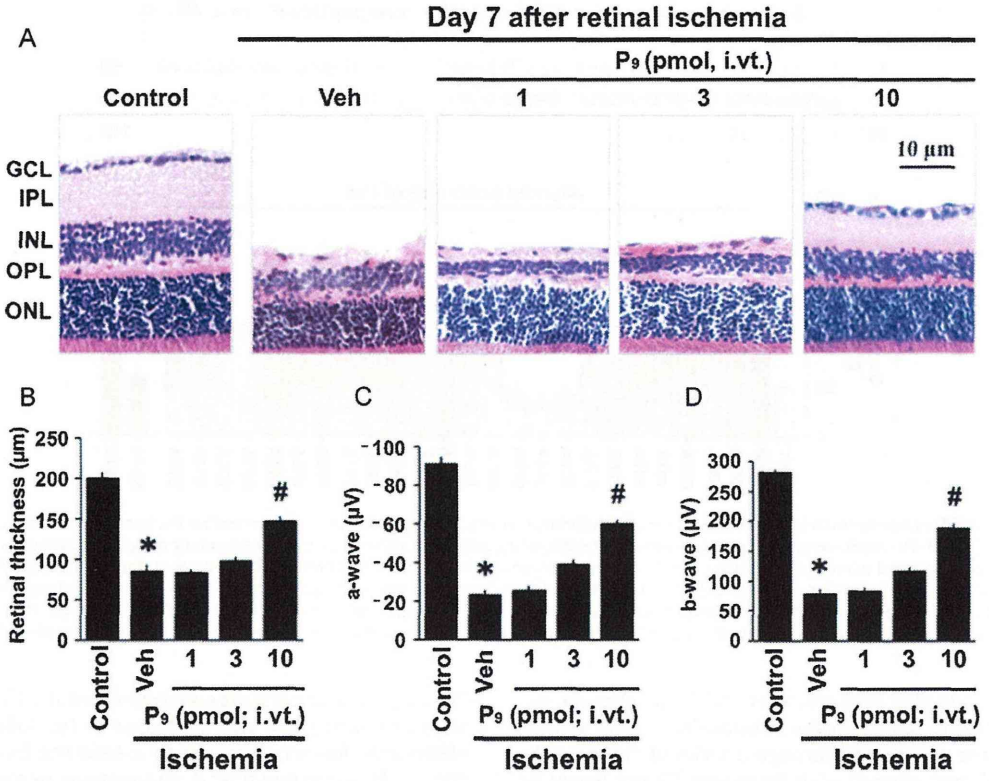


**Fig. 2.** $P_9$ peptide inhibits retinal ischemic damages. $P_9$ is injected intravitreously (i.vt.) with doses of 1, 3 and 10 pmol/μl in the ipsilateral eye at 24 h after retinal ischemia. Vehicle is treated with 0.05% DMSO in a similar manner. (A–D) Dose-dependent protective activity of $P_9$ against ischemic damages. (A) H&E staining of retinal section is performed in control (left panel), vehicle (middle panel) and $P_9$ post-treated mice (right panel) at day 7 after retinal ischemia. (B–D) Measurement of retinal thickness (B) as well as the a-wave (C) and b-wave (D) amplitudes of ERG analysis are performed at day 7 after retinal ischemia in control, vehicle and $P_9$ post-treated mice. The median effective dose of $P_9$ is 8 pmol/eye for a-wave ($n = 10$), and 4 pmol/eye for b-wave ($n = 10$) of ERG analysis. Data are mean ± S.E.M. (*$P < 0.05$, *vs.* control, #$P < 0.05$, *vs.* Veh) from experiments using 5–7 mice for each group.

### 3.2. $P_9$-induced recovery of retinal ischemic damages

To examine whether $P_9$ has *in vivo* protective effect against ischemic damages, $P_9$ was injected intravitreously (i.vt.) in the ipsilateral eye with doses of 1, 3 and 10 pmol/μl at 24 h after retinal ischemia. The hematoxylin and eosin (H&E) staining data revealed that number of cells in different retinal layers and retinal thickness are significantly decreased in the vehicle-treated mice at day 7 after ischemia, whereas 10 pmol $P_9$, but not 1 and 3 pmol maximally and significantly blocked the cellular loss and decrease in retinal thickness at day 7, compared to vehicle (Fig. 2A and B). The a- and b-wave amplitudes in electroretinogram (ERG) study represent the functional activity of photoreceptor cells in the outer nuclear layer (ONL), and mixture of cells in different retinal layers including bipolar, amacrine, Muller and ganglion cells, respectively [13,17]. Experiments using ERG analysis showed that a- and b-wave amplitudes are significantly decreased in the vehicle-treated mice at day 7 after retinal ischemia, compared to control (Fig. 2C and D). Following $P_9$ injection, amplitudes of a- and b-waves were gradually increased in a dose-dependent manner and 10 pmol $P_9$ induced the maximum protective effect against ischemic damages at day 7 after ischemia, compared to vehicle (Fig. 2C and D). The a-wave value for naive (non-ischemia) was 94 ± 6 μV ($n = 7$), while 22 ± 3 μV ($n = 7$) for vehicle (ischemia alone). Thus, the median effective dose for $P_9$ to give 50 μV was 8 pmol/eye. On the other hand, b-wave values for naive (non-ischemia) and vehicle (ischemia alone) were 292 ± 8 μV ($n = 7$) and 90 ± 4 μV ($n = 7$), respectively. In the case of b-wave, the median effective dose for $P_9$ to give 150 μV was 4 pmol/eye.

### 3.3. $P_9$ ameliorates the cerebral ischemic brain

To evaluate the $P_9$-induced protection against the ischemic damages in brain, $P_9$ was intravenously (i.v.) administered with doses of 0.1, 0.3 and 1 mg/kg at 1 h after cerebral ischemia (1 h tMCAO), and subsequent 2,3,5-triphenyl tetrazolium chloride (TTC) staining and behavioral tests were performed at 24 h after the ischemic stress. The infarct volume was calculated as the percentage of damaged areas in the ischemic brain on the ipsilateral side to the contralateral side. TTC staining data showed that cerebral ischemia causes a significant increase in ipsilateral infarct volume in the vehicle-treated ischemic mice to 51 ± 3% ($n = 7$) of control. Following systemic injection of $P_9$ (1 mg/kg, i.v.), infarct volume was maximally and significantly decreased at 24 h in the ischemic brain treated with 0.3 or 1 mg/kg of $P_9$, which had been given at 1 h after the ischemic stress (33 ± 3%, $n = 7$, and 30 ± 2%, $n = 7$, respectively), compared to vehicle (Fig. 3A and B). On the other hand, the behavioral study showed that clinical scores are significantly increased at 24 h after ischemia in vehicle-treated mice (score: 3.5 ± 0.5, $n = 6$) (Fig. 3C). The systemic injection (i.v.) with 0.3 or 1 mg of $P_9$ at 1 h after tMCAO caused the significant decline in clinical scores (scores: 2.5 ± 0.5, $n = 6$, and 2.0 ± 0.5, $n = 7$, respectively), as shown in Fig. 3C.

### 3.4. Blockade of cerebral ischemia-induced blood vessel damages by $P_9$

To investigate whether $P_9$ inhibits the ischemia-induced blood vessel damages, $P_9$ was injected (1 mg/kg, i.v.) at 1 h after cerebral

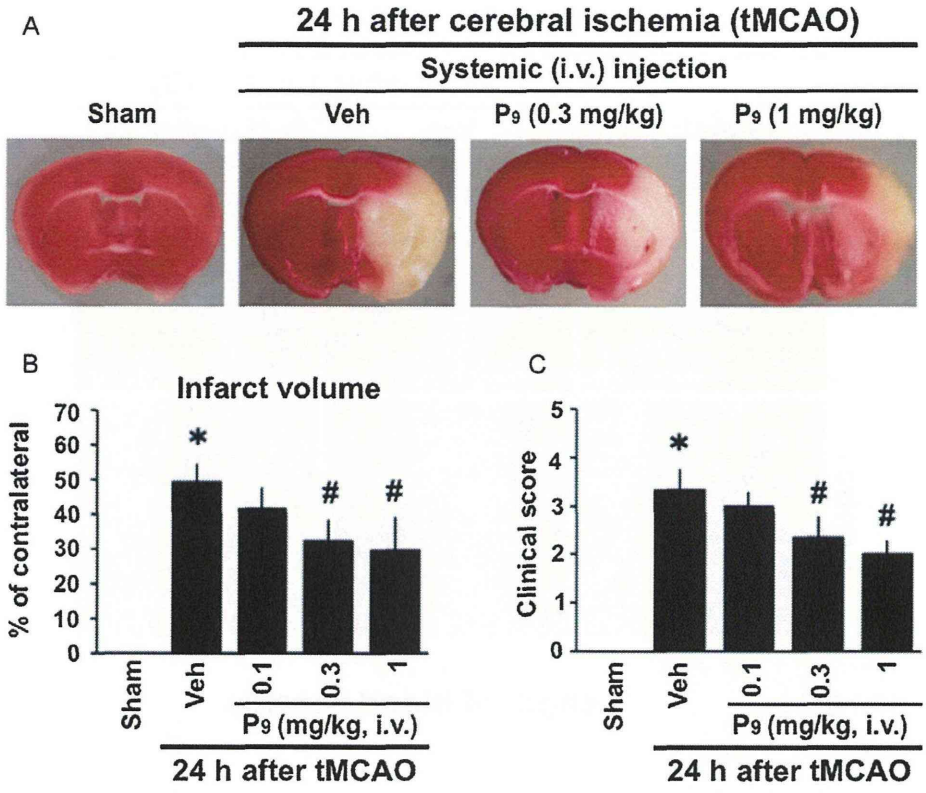


**Fig. 3.** $P_9$ improves cerebral ischemic brain. $P_9$ is injected intravenously (i.v.) with doses of 0.1, 0.3 and 1 mg/kg at 1 h after cerebral ischemia (1 h tMCAO). Vehicles are treated with equal volume of $K^+$-free PBS in a similar manner. (A–C) TTC staining (A), measurement of infarct volume (B) and clinical scores (C) are performed in sham-operated, vehicle-treated and $P_9$-treated mice at 24 h after tMCAO. Data represent the means ± S.E.M. (*$P < 0.05$, *vs.* Sham, #$P < 0.05$, *vs.* Veh). Experiments are performed using 5–7 mice for each group.

ischemia (1 h tMCAO). Following *in vivo* binding with biotinylated tomato lectin, the damages of blood vessels after fixation were evaluated by staining with Alexa Fluor 488 streptavidin at day 1 after tMCAO. When the damage was calculated as the ratio of averaged length of blood vessels in the region of somatosensory cortex on the ipsilateral side to the contralateral side, the cerebral ischemia caused a significant decrease to 45 ± 5% ($n = 6$) of control. The systemic injection of $P_9$ (1 mg/kg, i.v.) completely prevented the ischemia-induced damages of blood vessels (108 ± 6%, $n = 7$), as shown in Fig. 4A and B. Similar significant prevention by $P_9$ was observed in the striatum (control: 100%, $n = 6$; ischemia alone: 47 ± 4%, $n = 6$; ischemia + $P_9$: 104 ± 3%, $n = 7$) and hippocampus (control: 100%, $n = 6$; ischemia alone: 34 ± 5%, $n = 6$; ischemia + $P_9$: 102 ± 4%, $n = 7$).

## 4. Discussion

The present study includes the following important findings: (1) the 9-amino acid peptide $P_9$ (amino acids 52–60) derived from ProTα shows the full survival activity in cultured neuronal cells against ischemic stress, (2) $P_9$ significantly ameliorates the ischemic damages in retina and brain, and (3) $P_9$ prevents the cerebral ischemia-induced disruption of blood vessels.

The previous *in vitro* investigations suggested that the action of ProTα is related to cell survival [6,23,24,30,45,47], though the sequence-specific diverse functions of ProTα also have been reported [5,14,15,36,42,43]. Most recently, we demonstrated using various deletion mutants of GST-ProTα that the active core peptide $P_{30}$ (amino acids 49–78), but not N-terminal (amino acids 2–29) including the sequence (amino acids 1–48) or C-terminal sequence (amino acids 79–112 and 102–112) in ProTα induces original survival effect in cultured cortical neurons against the ischemic stress [17]. In the present study, we firstly applied alanine scanning technique for the determination of minimum peptide sequence of $P_{30}$ that exerts full neuroprotective effect in cultured cortical neurons under ischemic (serum-free) condition. The findings revealed that the replacement of amino acids D50 and N51 with alanine (D50A and N51A) retains the original survival activity of $P_{30}$ in cortical neurons against the ischemic stress, whereas alanine replacement of E52, V53, and E55 (E52A, V53A, and E55A) exerted partial survival action. Interestingly, the neuroprotective effect of $P_{30}$ was significantly abolished when replaced amino acids between E56 and G60 by alanine. However, the peptide comprised of 54–73 amino acid residues as well as the C-terminal sequence of $P_{30}$ (amino acids 59–78) induced no neuroprotective action against ischemic stress. Therefore, our *in vitro* study clarified that this 9-amino acid peptide $P_9$ (amino acids 52–60) represents the short active core sequence in $P_{30}$, which is required for full-length neuroprotection.

Next, we performed *in vivo* experiments to examine the neuroprotective effect of $P_9$ against ischemic stress in retina and brain. Retinal ischemia is associated with injury-related mechanism-induced destruction of cellular and functional response in the different cell layers of retina, leading to visual disorders and blindness [31,32]. Since retinal ischemic model provides higher reproducibility to comprehend the pathophysiological alternation and signaling cascades [37], this ischemic stress was used as a simple model for screening the neuroprotective action of $P_9$ by intravitreous administration. It is evident that ProTα improves the ischemic damages in retina [13,47,50]. Most recently, our *in vivo* study demonstrated that $P_{30}$ peptide in ProTα inhibits

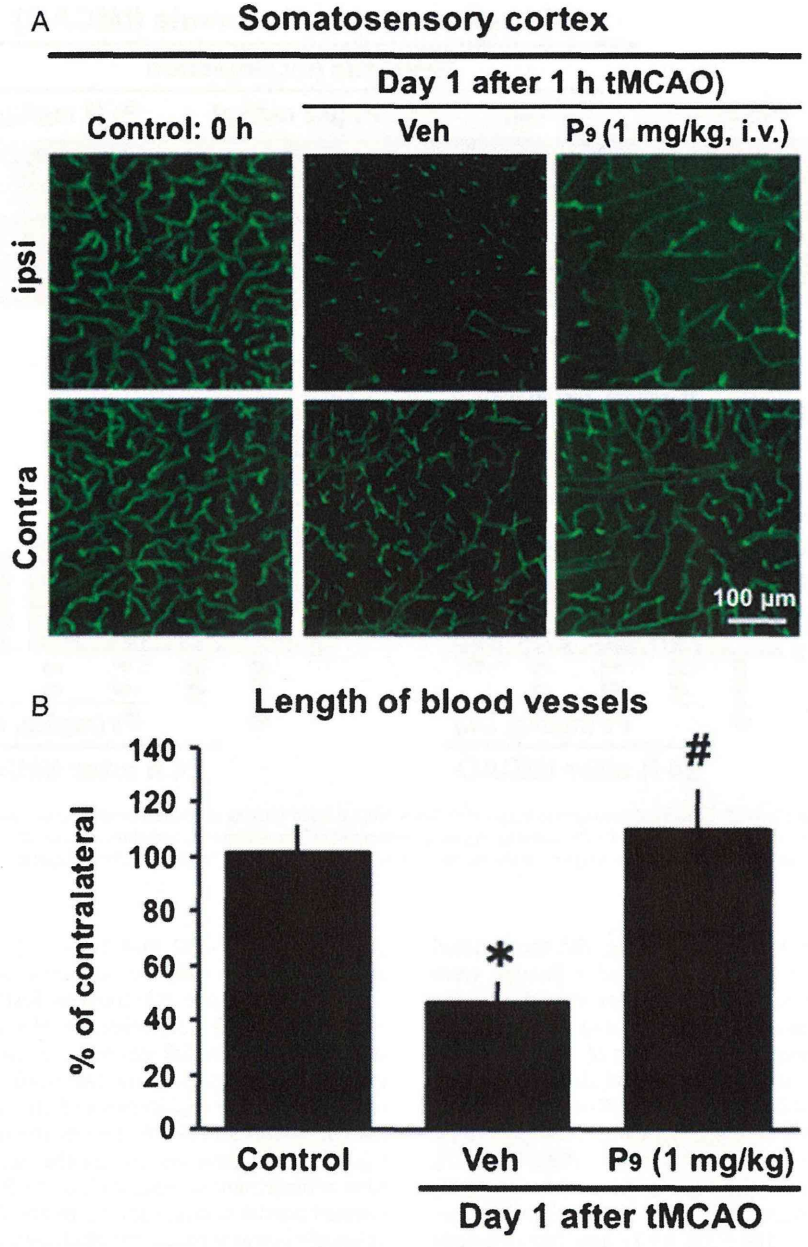


**Fig. 4.** $P_9$ prevents cerebral ischemia-induced blood vessel disruption. $P_9$ is administered (1 mg/kg, i.v.) at 1 h after cerebral ischemia (1 h tMCAO). (A and B) Following biotinylated tomato lectin injection (1 mg/ml, 100 μl, i.v.) at 24 h after tMCAO, and subsequent perfusion of mice 5 min after tomato lectin delivery, the staining of blood vessels using Alexa Fluor 488 streptavidin (A) as well as measurement of the length of blood vessels (B) is performed at day 1 after the ischemic stress. Data represent the means ± S.E.M. ($^*P < 0.05$, *vs.* control, $^\#P < 0.05$, *vs.* Veh) from experiments using 5–7 mice for each group.

ischemia-induced retinal damages [17]. The present findings using H&E staining and ERG analysis revealed that intravitreous treatment with 10 pmol of $P_9$ at 24 h after retinal ischemia markedly rescues the ischemia-induced decrease in retinal thickness and cell number in different layers in the retina.

Brain ischemia is one of the major clinical issue that is associated with irreversible neurological damages, along with dysfunction of motor, sensory and cognitive systems [10,20,39,40]. There are several reports about ProTα-induced blockade of ischemic brain damages [12,17,48]. Following cerebral ischemia (tMCAO), in the present study, the experiments using TTC staining and behavioral assessment in terms of clinical score suggested that systemic administration of $P_9$ (1 mg/kg, i.v.) at 1 h after tMCAO significantly improves the ischemic brain. Ischemic stress in the brain causes adequate breakdown of blood vessels [2,19], whereas this disruption of blood vessels is protected by $P_{30}$ peptide [17]. The experiment of fluorescence staining by *in vivo* tomato lectin administration showed the $P_9$-induced (1 mg/kg, i.v.) significant prevention of ischemia-induced cerebral blood vessel damages at day 1.

It is interesting to examine the potency ratio of $P_9$-induced protection to $P_{30}$, which has recently been reported. In the present retinal ischemia model, the median effective dose of $P_9$ to give 50 μV was approximately 8 pmol/eye for a-wave, and to give 150 μV was 4 pmol/eye for b-wave of ERG analysis, respectively, while these values for $P_{30}$ were 2 and 1 pmol/eye, respectively [17],

indicating that $P_9$ is 4 times less potent than $P_{30}$. In the cerebral ischemia model, on the other hand, systemic treatment (i.v.) with 0.3 or 1 mg/kg of $P_{30}$ causes markedly decrease in infarct volume at 24 h after the ischemic stress ($33 \pm 3\%$ and $30 \pm 2\%$, respectively), compared to ischemia alone ($52 \pm 3\%$), suggesting that the value with 0.3 mg/kg (approximately 0.3 μmol/kg) of $P_9$ is equivalent to that with 0.3 mg/kg (~0.1 μmol/kg) of $P_{30}$. Regarding the clinical score, the value (score: $2.0 \pm 0.5$) with 1 mg/kg (~1.0 μmol/kg) of $P_9$ is equivalent to that (score: $2.0 \pm 0.5$) with 1 mg/kg (~0.3 μmol/kg) of $P_{30}$ [17] indicating that $P_9$ is 3 times less potent than $P_{30}$ in cerebral ischemia-induced infarction and clinical score. All together, it is evident that the relative potencies of $P_9$ to $P_{30}$ in prevention of retinal and brain damages seem to be similar, though the administration routes are different (local/intravitreous and systemic/intravenous, respectively). Cerebral ischemic stress disrupts the blood–brain barrier (BBB) [26,35], but we previously found that ProTα through intraperitoneal (i.p.) route is penetrated into the damaged regions in cerebral and retinal ischemia models [12,13]. It is considered that smaller peptides are generally preferable in BBB-penetration, while at the same time they are more susceptible to enzymatic degradation [22,38]. Thus, similar relative potency of $P_9$ and $P_{30}$ in the present study may be a result of these opposite factors. The present study suggests that $P_9$ would be a promising prototype of small peptides to prevent the stroke. However, much more detailed studies should be performed prior to the future studies of peptide modification. They include (1) the quantitative beneficial actions in cultured neuronal cell death, and its mechanisms, (2) different administration schedules *in vivo*, (3) assessment studies of BBB-penetration and metabolic stability. All these studies are in progress in our group.

In conclusion, ProTα-derived 9-amino acid peptide $P_9$ induced potent actions against the cerebral and retinal ischemic damages. Thus, the present study would be a key demonstration to develop a new type of peptidic medicines against stroke.

## Conflict of interest

Authors have no conflict interest to report.

## Acknowledgments

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