approved by the animal ethics and the recombinant DNA committees of Tohoku and Osaka University. Nrf2$^{-/-}$ mice were generated previously in our laboratory [12]. Wild-type (WT) ICR mice were purchased from Japan SLC (Shizuoka, Japan). Mice used in the wound-healing experiments were 7–14 weeks of age. All mice were genotyped for Nrf2 status by PCR amplification of genomic DNA extracted from tail tips. PCR amplification was performed using three different primers: 5′-TGGACGGGACTATTGAAGGCTG-3′ (sense for both genotypes), 5′-GCCGCCTTTTCAGTAGATGGAGG-3′ (antisense for WT), and 5′-GCGGATTGACCGTAATGGGATAGG-3′ (antisense for LacZ). All mice were allowed water and rodent chow ad libitum and subjected to an alternating 12-h (0800 to 2000 hours, 2000 to 0800 hours) light–dark cycle.

Table 1
Sequences of primers for RT-PCR.

Gene	Primer (5′ to 3′)
Nrf2	Forward: TGCCCCTCATCAGGCCCAGT
Nrf2	Reverse: GCTCGGCTGGGACTCGTGTT
Keap1	Forward: GGTGGCGCGCTGTGCTTAGT
Keap1	Reverse: TGCTGGCTCAGGCGAAGCTC
GAPDH	Forward: TCTGACGTGCCGCCTGGAGA
GAPDH	Reverse: GGGGTGGGTGGTCCAGGGTT

RT-PCR

Total RNA was obtained from corneal epithelium of WT and Nrf2 KO mice by using the RNeasy total RNA kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed with the Super-Script First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's suggested protocol, and cDNA was used as a template for PCR. The sequences of the primer pairs for Nrf2, Keap1, and GAPDH are presented in Table 1. The thermocycle program consisted of an initial cycle at 94 °C for 5 min and 28 cycles, for Nrf2 and GAPDH, and 32 cycles, for Keap1, at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s (PCR thermal cycler MP; Takara Bio, Shiga, Japan).

Animal models for corneal epithelial wound healing

WT or Nrf2 KO mice were anesthetized by intramuscular injection of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). A circular piece of paper (2 mm in diameter) containing 0.2 μl *n*-heptanol was applied to the right eye of each of the Nrf2 KO ($n=12$) and WT mice ($n=10$) for 1 min [17]. The treated eyes were washed with 50 ml of saline, and Levofloxacin eye drops (Santen Pharmaceutical, Osaka, Japan) were administered to reduce the risk of bacterial contamination. The epithelial defect was stained with 1% fluorescein solution and photographed at 0, 6,

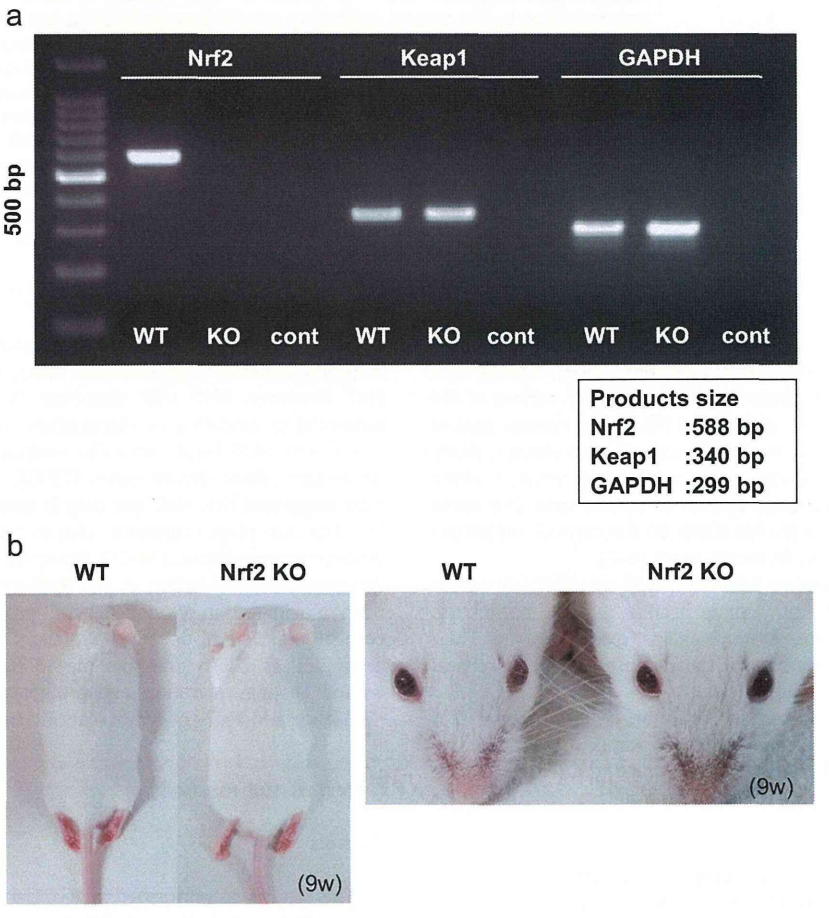

Fig. 1. Nrf2 and Keap1 mRNA expressions in corneal epithelium of WT and Nrf2 KO mice. (a) RT-PCR revealed that Nrf2 mRNA was expressed in the corneal epithelium of WT mice but not Nrf2 KO mice. Keap1, a binding partner of Nrf2, and GAPDH were expressed in the corneal epithelium of both WT and KO mice. The predicted product sizes for Nrf2, Keap1, and GAPDH were 588, 340, and 299 bp, respectively. (b) There was no apparent aberration in body size or cornea of Nrf2 KO mice compared to those of WT mice (9 weeks after birth).

12, 18, 24, 30, 36, 48, 60, and 72 h after epithelial debridement. The area of the epithelial defect was measured on photographs with a computer-assisted image analyzer (SL-7F; Topcon, Tokyo, Japan). Changes in the defect area were calculated (AxioVision; Carl Zeiss) and plotted on a graph. The experimental mice were euthanized by intraperitoneal injection of sodium pentobarbital solution, and then, the eyes were enucleated. Paraffin sections or frozen sections were processed for immunohistochemistry as described below.

Fluorescent immunostaining for Ki-67

Injured corneas healed at various time points were subjected to immunohistochemistry with Ki-67 antibody (ab15580; Abcam, Cambridge, MA, USA). The frozen corneoscleral tissue was cut into 10-μm-thick sections. After the sections were dried for 15 min at room temperature, they were fixed with cold methanol at −30 °C for 15 min and washed with Tris-buffered saline (TBS; Takara Bio) three times. For Ki-67 staining, slides were incubated with TBS containing 5% donkey serum and 0.3% Triton X for 1 h to block nonspecific reactions. The slides were incubated at 4 °C overnight with a 1:100 dilution of anti-Ki-67 antibody. The slides were again washed three times with TBS and incubated with a 1:200 dilution of Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA). All tissue sections were counterstained with Hoechst 33342 and then examined under a microscope (Axiovert200M; Carl Zeiss). The total number of Ki-67-positive cells in each section was counted. The length of the basement membrane from limbus to limbus was measured for the cornea, and the number of Ki-67-positive cells per 100-μm basement membrane was calculated.

Hematoxylin and eosin staining and diaminobenzidine immunostaining for Nrf2

Each eye sample was fixed in formalin and embedded in paraffin. The slides were stained with hematoxylin and eosin (HE). For Nrf2 immunostaining, slides were treated with anti-Nrf2 antibody (produced in our laboratory), and positive reactivity was visualized through sequential incubation with biotinylated anti-rat IgG, streptavidin-conjugated horseradish peroxidase, and diaminobenzidine for staining. Hematoxylin was used for nuclear counterstaining.

siRNA-dependent specific knockdown of Nrf2 or Keap1 in TERT-immortalized human corneal epithelial cells (C/TERTs)

C/TERTs were obtained from the Harvard Skin Disease Research Center. C/TERTs were cultured in keratinocyte serum-free medium (KSFM) (Gibco Invitrogen). The siRNA (Nrf2, ID: s9491, and Keap, ID: s18981) and introducing reagent were purchased from Ambion (Austin, TX, USA) and Invitrogen, respectively. Nrf2- or Keap1-specific siRNA and control scramble siRNA (negative control) were introduced into C/TERTs with the introducing reagent for 18 h, after which the medium containing siRNA was removed and fresh medium was added. The total RNA of C/TERTs was collected each day to confirm Nrf2 or Keap1 knockdown by real-time PCR.

Cell migration assay

Migration assays were performed according to the manufacturer's protocol (Oris cell migration assay kit; Platypus Technologies, Madison, WI, USA) [18,19]. Briefly, each of the Nrf2-specific,

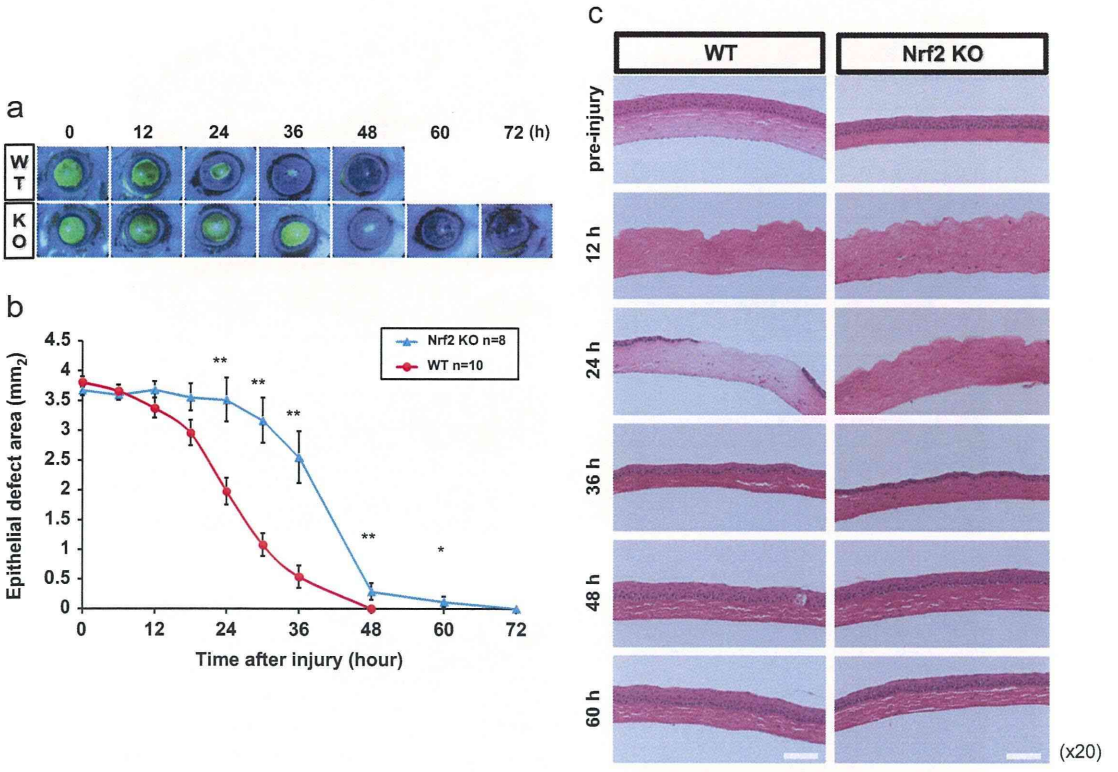

Fig. 2. Corneal epithelial wound healing in WT and Nrf2 KO mice. (a) The corneal epithelial layers of WT or Nrf2 KO mice were removed from the stroma by *n*-heptanol treatment. Epithelial defect regions were stained by fluorescein. The areas of the wounds were initially calculated and then (b) recalculated at various times after the injury. (c) HE staining was also performed at each time point. In WT mice, the closure of the corneal epithelial defect was completed within 48 h after the injury. Corneal epithelial wound healing was significantly delayed for 24 h or more in the Nrf2 KO mice compared with the WT mice. The graphs represent the means ± SE of 8–10 independent samples. $^{*}p < 0.05$, $^{**}p < 0.01$ (12, 24 h. *t* test; 35, 48, 60 h, Mann–Whitney *U*). Scale bars, 50 μm.

Keap1-specific, and control siRNA-introduced corneal epithelial cells was seeded outside of the partition in each well of a 96-well plate, and the partitions were subsequently removed. After 8 (Keap1 knockdown) or 24 h (Nrf2 knockdown), the migrated cells in the central parts of the wells were stained with calcein-AM; the fluorescence intensity was then measured by a fluorescence plate reader (ARVO MX; PerkinElmer, Norwalk, CT, USA). For the evaluation of the total cell number, all of the cells in the well were stained with Alamar blue (AbD Serotec, Oxford, UK) and the fluorescence signals were assessed by a fluorescence plate reader in each period.

Cell proliferation assay

Cell proliferation was evaluated by Alamar blue assay. The Nrf2-specific and Keap1-specific corneal epithelial cells as well as control corneal epithelial cells into which siRNA was introduced were seeded in each well of a 12-well plate and cultured in KSFM without growth factors. The Alamar blue assay was subsequently performed at 6, 24, 48, and 72 h.

Real-time RT-PCR

Total RNA was obtained from Nrf2-specific or control siRNA-introduced C/TERTs, and the cDNA was synthesized as a template for PCR. Quantitative real-time RT-PCR was performed using the ABI Prism 7500 fast sequence detection system (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's suggested protocol. Primer pairs and TaqMan MGB probes labeled with 6-carboxyfluorescein at the 5′-end and nonfluorescent quencher at the 3′-end were designed using Assay-by-Design (Applied Biosystems; GAPDH, Hs99999905_m1; Nrf2, Hs00232352_m1; NQO1, Hs0016-8547_m1; HO-1, Hs01110250_m1; and Keap1, Hs00202227_m1). Thermocycling programs consisted of an initial cycle at 50 °C for 2 min and 95 °C for 10 min and 45 cycles at 95 °C for 15 s and 60 °C for 1 min. All assays were run in duplicate for four or more individual samples.

Statistical analysis

Data were expressed as means ± SE. Statistical analysis was performed with Student's t test or the Mann–Whitney rank sum test. Differences were considered significant at $p < 0.05$. All statistics were calculated using SigmaPlot 11.0 (Systat Software, San Jose, CA, USA).

Results

Nrf2 and Keap1 expression in WT and Nrf2 KO mice

To examine the expression of Nrf2 mRNA and its binding partner Keap1 mRNA in the corneal epithelium of Nrf2 KO and WT mice, we performed RT-PCR for the genes encoding Nrf2 and Keap1. The results indicate that Nrf2 mRNA was expressed in the corneal epithelium of WT mice but not Nrf2 KO mice. In contrast, Keap1 mRNA was expressed in both WT and Nrf2 KO mice (Fig. 1a).

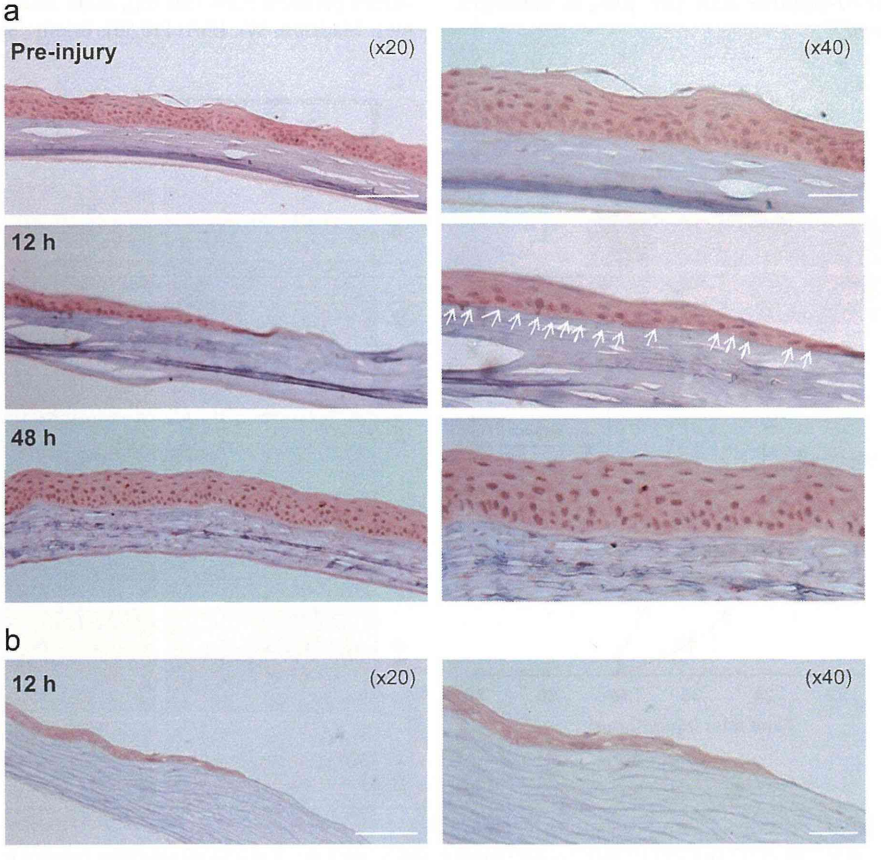

Fig. 3. Nrf2 activation in corneal epithelium during wound healing. Paraffin-embedded tissue sections were stained with anit-Nrf2 antibody to examine Nrf2 activation based on their translocation to the nuclei. (a) Nrf2 protein was expressed and located in the nuclei of preinjured corneal epithelia of WT mice (preinjury). During corneal epithelial wound healing, Nrf2 protein was also found in the corneal epithelium (12 and 48 h after injury, arrows indicate Nrf2-positive nuclei). (b) In Nrf2 KO mice, no signal for Nrf2 protein was detected in the corneal epithelium. Scale bars, 50 μm (20 × original magnification) or 20 μm (40 × original magnification).

There was no remarkable aberration in body size or eye of Nrf2 KO mice compared to those of WT mice (Fig. 1b).

Corneal epithelial wound healing in WT and Nrf2 KO mice

We compared the process of corneal epithelial wound healing in WT and Nrf2 KO mice to assess the involvement of Nrf2 in the wound-healing process. Immediately after corneal epithelial injury (0 h), no significant difference ($p=0.326$, t test) was observed in the area of the wound between the WT ($3.8 \pm 0.08\ mm^2$) and the Nrf2 KO ($3.7 \pm 0.10\ mm^2$) mice. In the WT mice, approximately 50% of the defect was resurfaced 24 h after injury, and no defect was observed 48 h after the injury (Figs. 2a and b). In contrast, the corneal epithelium of Nrf2 KO mice showed no sign of healing until 24 h after injury. The epithelial defect was gradually resurfaced between 24 and 48 h after injury in the Nrf2 KO mice, but the wound area was not completely healed until 48–60 h after the initial injury. The wound healing delay in the Nrf2 KO mice compared to the WT mice, after 24 h of wound healing, was statistically significant.

Histochemical analysis

HE staining showed that in the WT mice, corneal epithelial migration had already begun 24 h after injury in the central part of the cornea (Fig. 2c, WT). By 36 h, the corneal surface was covered by migrated corneal epithelial cells and epithelial stratification had commenced. By 48 h, four or five layers of corneal epithelium were fully reconstructed, in a state similar to the preinjured cornea. Conversely, in the Nrf2 KO mice, few corneal epithelial cells were observed in the central cornea even at 24 h after injury, suggesting a delay in cell migration in the Nrf2 KO mice (Fig. 2c, Nrf2 KO). At 36 h, the corneal surface was covered with one or two layers of migrated corneal epithelium (Fig. 3). By 48 h, the migrated corneal epithelium was stratified, and by 60–72 h, the corneal surface of Nrf2 KO mice appeared to be completely reconstructed, similar to the WT mice. These data demonstrate a delay in early cell migration 12–36 h after injury in the Nrf2 KO mice.

Activation of Nrf2 during corneal epithelial wound healing of WT mice

Nrf2 translocation to nuclei in the WT mice was evaluated by immunostaining to verify whether Nrf2 was activated during corneal epithelial wound healing. The results indicate that Nrf2 was present in the nuclei of preinjured corneal epithelial cells and also in the corneas of the WT mice at 12 and 48 h after injury (Fig. 3a), thus demonstrating that Nrf2 signaling was activated throughout the corneal epithelial wound-healing process.

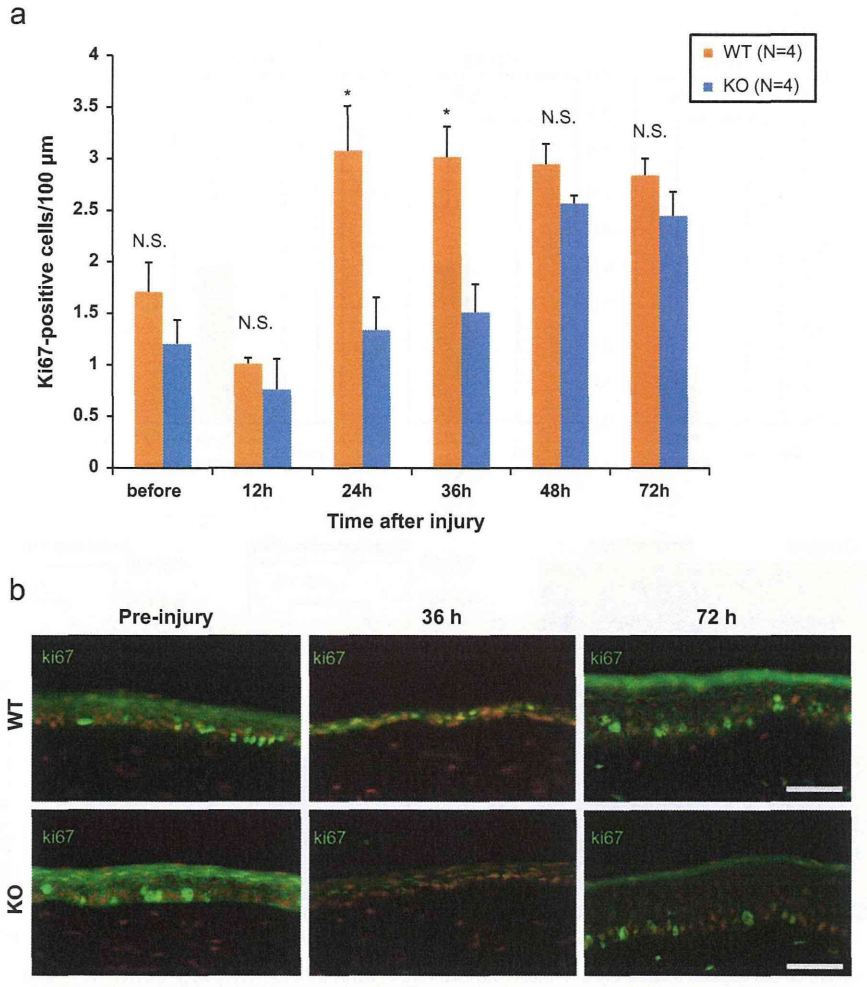

Fig. 4. Proliferative cells in WT and Nrf2 KO mice during corneal epithelial wound healing. (a and b) To assess cell proliferation in corneal epithelium in WT and Nrf2 KO mice, we performed Ki-67 staining at various times after the injury. Immunostaining revealed that at 24 and 36 h after injury, the number of Ki-67-positive cells in the Nrf2 KO mice was significantly reduced compared with that in the WT mice, whereas no significant difference was observed in the preinjured corneas and the injured corneas at 48–72 h after injury. The graphs represent the means ± SE of four independent samples. $^*p < 0.05$ (t test). Scale bars, 50 μm.

In contrast, no significant signal was observed in the nuclei of the corneal epithelium in Nrf2 KO mice (Fig. 3b).

Immunostaining for Ki-67 in corneal epithelium of WT and Nrf2 KO mice

To examine cell proliferation of the corneal epithelium during wound healing, we performed immunostaining for the proliferative marker Ki-67 in WT and Nrf2 KO mice. The results indicate that the numbers of Ki-67-positive proliferating cells in the Nrf2 KO mice transiently decreased at 24 and 36 h after injury. However, these values were nearly identical to those observed in the WT mice by 48 h after the injury (Figs. 4a and b), indicating that the proliferation was initiated later in the Nrf2 KO mice compared to the WT mice. Thus, Nrf2 probably affects the early migration process of corneal epithelial wound healing rather than cell proliferation.

The effects of Nrf2 knockdown on cell migration of corneal epithelial cells

To clarify the involvement of Nrf2 in cell migration in corneal epithelial wound healing, we performed Nrf2-specific knockdown by siRNA in a corneal epithelial cell line (C/TERT) in vitro. Over 70% of Nrf2 expression was suppressed by Nrf2-specific siRNA in C/TERT in vitro (76.6%, Fig. 5a). Furthermore, Nrf2-targeted siRNA significantly downregulated the Nfr2 downstream genes such as NQO1 (38.4%) and HO-1 (41.7%), indicating that Nrf2-mediated signal transduction was functionally suppressed by Nrf2 siRNA. The results of the migration assay revealed that Nrf2 suppression induced a significant reduction in the migration capability of C/TERTs (42.1%, Fig. 5b). Results of the Alamar blue assay indicated no significant difference in total cell number between the samples (Fig. 5c). These data indicate that Nrf2 knockdown in corneal epithelial cells affects cell migration capability.

The effects of Nrf2 activation throughout Keap1 knockdown on cell migration of corneal epithelial cells

To examine the effect of Nrf2 activation on cell migration, the Nrf2 suppressor Keap1 was knocked down by Keap1-targeted siRNA. The results of this experiment indicate that more than 70% of Keap1 expression was significantly suppressed by Keap1-specific siRNA in C/TERTs in vitro (Fig. 6a). In addition, Keap1-targeted siRNA significantly upregulated NQO1 (52.0%) and HO-1 (60.3%) but did not affect Nrf2 expression. This finding indicates that Nrf2 translocation to the nuclei was promoted and that Nrf2 was functionally activated by Keap1 siRNA. The results of the migration assay indicate that Keap1 suppression induces a significant increase in the migration capability of C/TERTs (214% increase, Fig. 6b). Similar to Nrf2 suppression, Keap1 suppression had no significant effect on the total number of C/TERT cells in this assay (Fig. 6c).

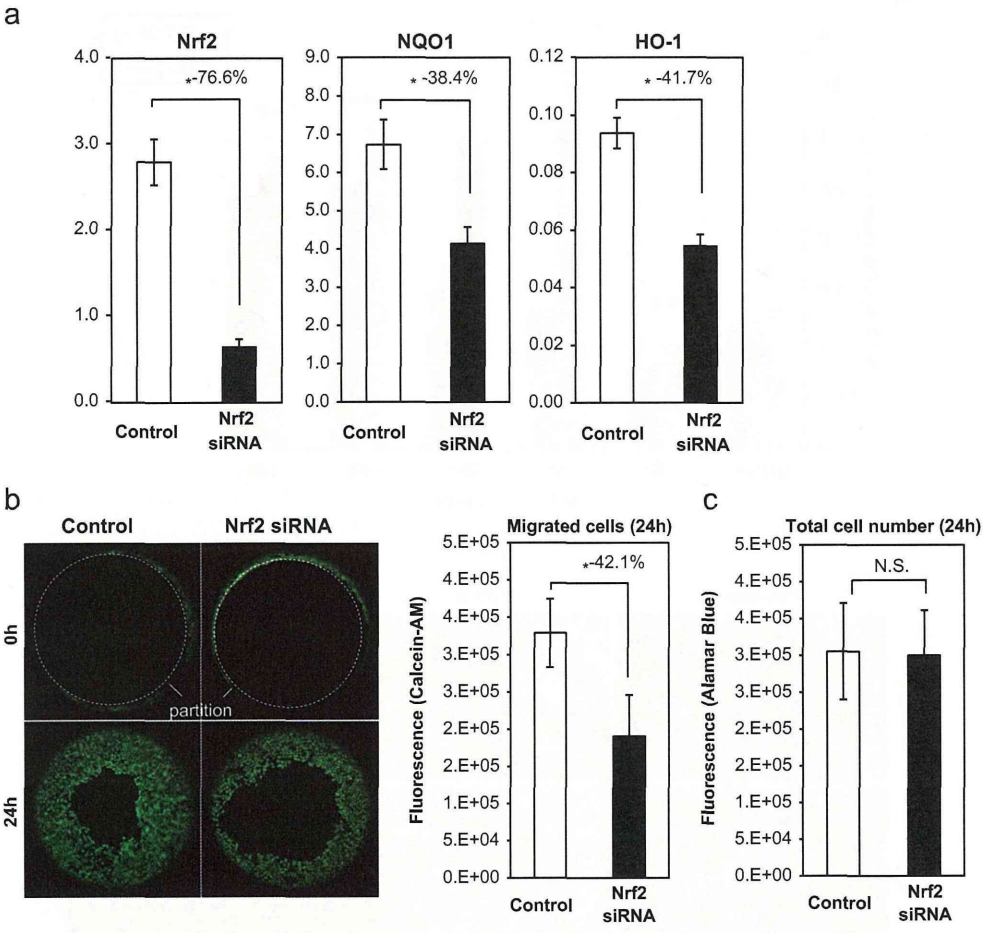

Fig. 5. The effect of Nrf2 knockdown on cell migration capability in corneal epithelial cells in vitro. (a) The effect of Nrf2-specific knockdown on the migration capability of C/TERT cells was examined; 76.6% of Nrf2 mRNA expression in C/TERT cells was suppressed by Nrf2-specific siRNA. Nrf2 knockdown also resulted in significant downregulation of its downstream genes, NQO1 and HO-1 (38.4 and 41.7%, respectively). (b) Nrf2- or control siRNA-introduced cells were allowed to migrate for 24 h and then stained with calcein-AM. The migration assay indicated that cell migration capability was significantly reduced by Nrf2 knockdown. (c) The Alamar Blue assay revealed no significant difference in the total cell number between the samples. The graphs represent the means ± SE of five independent samples. $^*p < 0.05$ (t test).

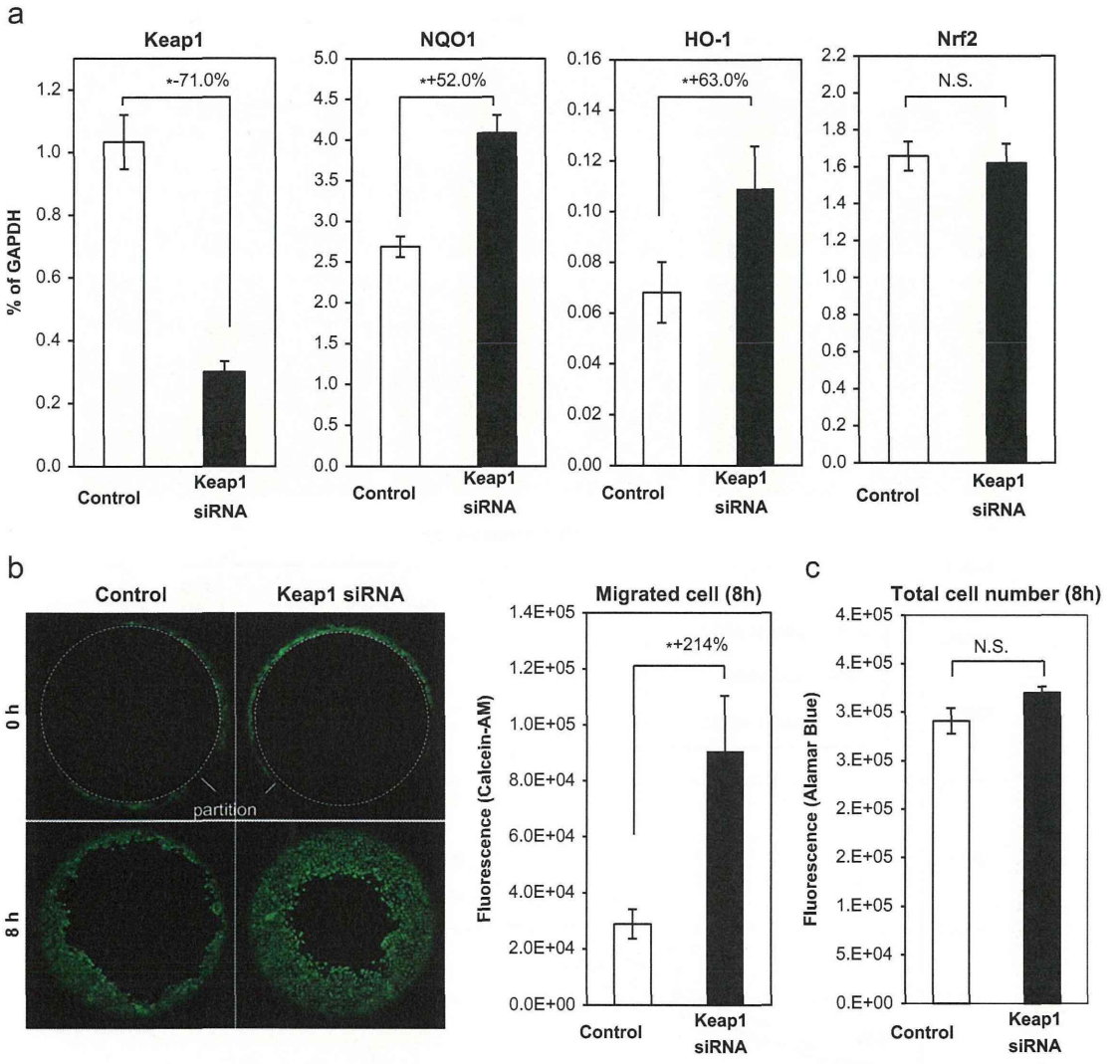

Fig. 6. The effect of Keap1 knockdown on the migration capability of corneal epithelial cells in vitro. (a) The effects of Keap1-specific knockdown on the migration capability of C/TERT cells were examined; 71.0% of Keap1 mRNA expression in C/TERT cells was suppressed by Keap1-specific siRNA. Keap1 knockdown significantly upregulated the expression of NQO1 (52.0%) and HO-1 (60.3%) but not Nrf2. (b) Keap1- or control siRNA-introduced cells were allowed to migrate for 8 h and then stained with calcein-AM. Migration assay indicated that the cell-migration capability was significantly promoted by Keap1 knockdown. (c) The Alamar blue assay revealed no significant effect of Keap1 knockdown on cell numbers. The graphs represent the means ± SE of four independent samples. $^*p < 0.05$ (t test).

Effect of Nrf2 suppression or activation by siRNA on proliferative capability of corneal epithelial cells

To evaluate whether the Nrf2/Keap1 system was involved in corneal epithelial proliferation, we investigated the proliferation profiles of Nrf2 and Keap1 knockdown C/TERT cells. Nrf2 suppression by siRNA had no significant effect on the proliferative capability of C/TERT (Fig. 7). The proliferation of C/TERT cells seemed to be slightly decreased by Nrf2 activation caused by Keap1 knockdown, but the decrease was not significant throughout the cell culture periods up to 72 h.

Discussion

The aim of this study was to clarify the role of the Nrf2-mediated defense system in corneal epithelial wound healing by using Nrf2 KO mice.

To first assess the involvement of Nrf2 in corneal epithelial wound healing in WT and Nrf2 KO mice, the corneal epithelia of mice from each group were injured by *n*-heptanol treatment. The HE and fluorescein staining showed that the *n*-heptanol treatment completely removed the corneal epithelial cell layer and that no inflammatory cells invaded the corneal tissue during wound healing. This finding suggests that the wound-healing process in this model is relatively simple, occurring without the involvement of inflammatory cells.

The results of the experiment using this model demonstrated that wound healing in the KO mice was significantly delayed for 24–48 h after injury. The results of HE staining during the process of corneal wound healing revealed that during the first 12–24 h after the injury, epithelial migration in Nrf2 KO mice was apparently delayed compared to that in the WT mice. Interestingly, 24–36 h after injury, the total number of Ki-67-positive corneal epithelial cells in the KO mice decreased significantly compared to that in the WT mice. These data suggest that the delay in wound healing in the KO mice resulted from decreased cell proliferation. However, the number of Ki-67-positive cells in the KO mice recovered to the same levels as in the WT mice by 48 h after injury. The process of corneal epithelial wound healing generally

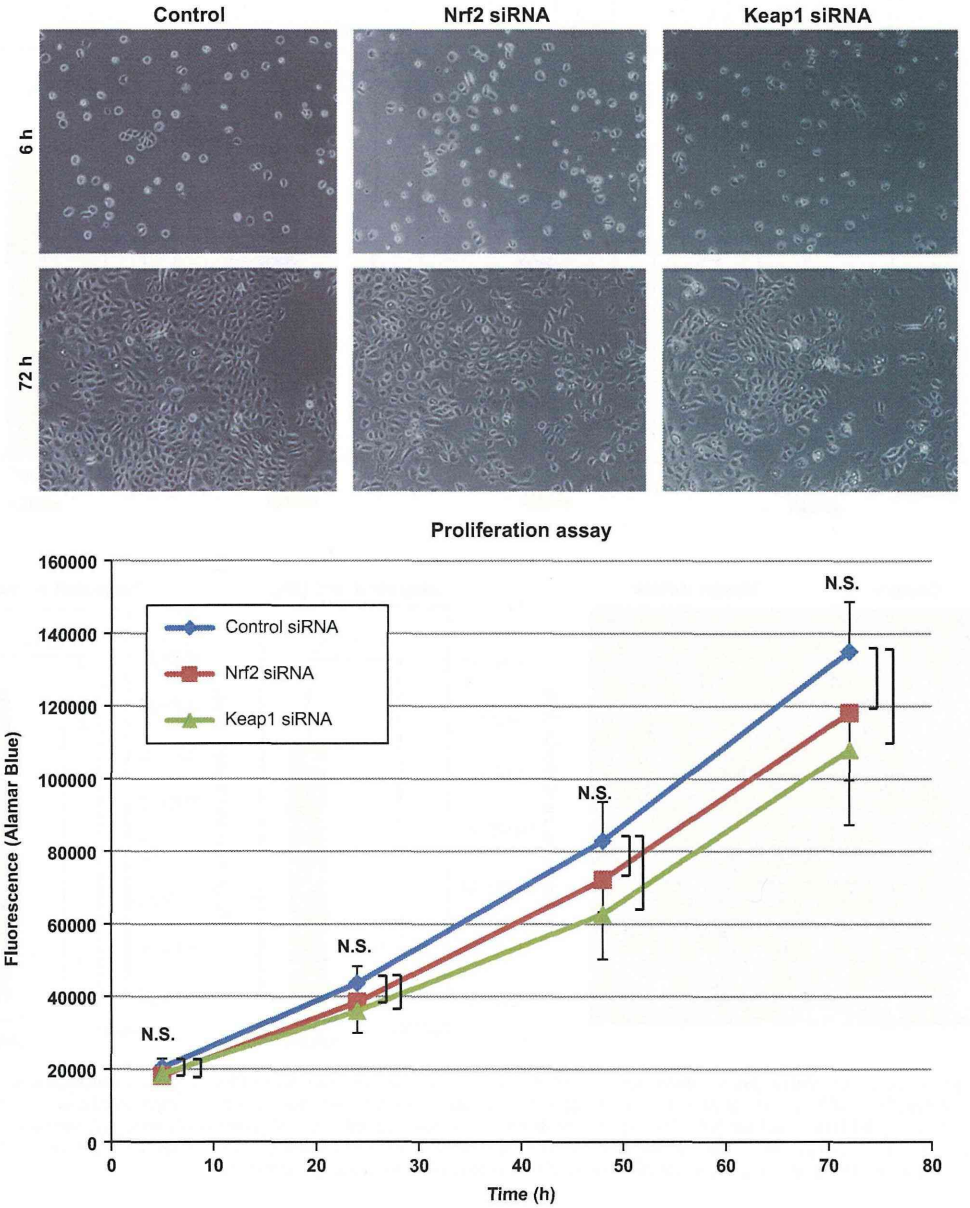

Fig. 7. The effect of Nrf2 suppression or activation on the proliferative capability of corneal epithelial cells in vitro. The Nrf2-specific and Keap1-specific C/TERT cells as well as control C/TERT cells into which siRNA was introduced were cultured in KSFM without growth factors. Their proliferative 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].

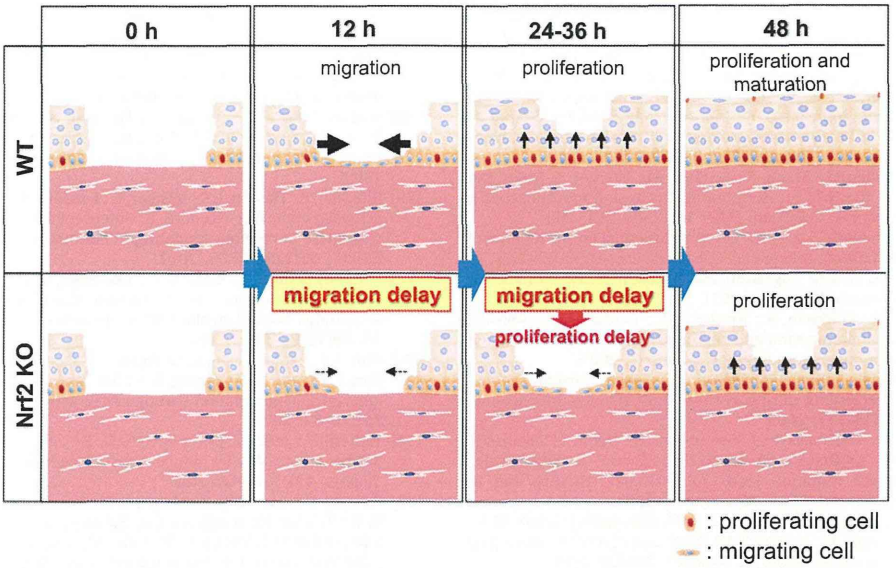

Fig. 8. The schema of corneal epithelial wound healing in WT and Nrf2 KO mice. In WT mice, corneal epithelial cells migrated into the injured region by 24 h after the injury. After the migrated cells covered the corneal stroma, cells began to proliferate at 24–36 h and finally reconstructed the corneal 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

Sebok Kumar Halder, Junya Sugimoto, Hayato Matsunaga, Hiroshi Ueda*

Department of Molecular Pharmacology and Neuroscience, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

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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.

* Corresponding author. Tel.: +81 95 819 2421; fax: +81 95 819 2420.
E-mail address: ueda@nagasaki-u.ac.jp (H. Ueda).

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