Short Communication

Activation of Master Autophagy Regulator TFEB During Systemic LPS Administration in the Cornea

Kyoko Uchida[1], Kana Unuma[1], Takeshi Funakoshi[1], Toshihiko Aki[1*], and Koichi Uemura[1]

[1] Section of Forensic Medicine, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

Abstract: The involvement of autophagy in the cornea during the systemic inflammatory response elicited by intravenous administration of lipopolysaccharide (LPS) was investigated. Eight-week-old male Sprague-Dawley rats were injected i.v. with 15 mg/kg body weight LPS. RC4 rabbit corneal keratocytes were also used and treated with 100 ng/mL of tumor necrosis factor α (TNFα) and/or cycloheximide (CHX). The nuclear translocation of transcription factor EB (TFEB), the master transcriptional regulator for autophagy, was observed after LPS administration in the corneal epithelium. Induction of autophagy-related proteins was observed in the cornea after LPS administration, as well as in RC4 cells after treatment with TNFα. Administration of trehalose, an inducer of TFEB, mitigated RC4 cell death caused by TNFα/CHX. These results demonstrate the importance of TFEB activation in cellular defense against the systemic inflammatory response in the cornea. (DOI: 10.1293/tox.2014-0004; J Toxicol Pathol 2014; 27: 153–158)

Key words: cornea, LPS, TFEB, autophagy, lysosome

Ocular tissue homeostasis is maintained in a highly regulated manner so as to retain transparency. The cornea, which is composed of five layers of tissue, the epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium, functions as a vital barrier against the outside environment to protect the eye[1]. Indeed, the corneal epithelium is separated from the environment only by tear fluids, suggesting that the corneal epithelium is always exposed to the risk of infection. Keratitis caused by viral, fungal and microbacterial infection can arise due to faulty use of contact lenses[2], contamination during corneal graft surgery[3], laser-assisted in situ keratomileusis (LASIK) surgery or trauma and is also associated with systemic inflammatory diseases such as sepsis[4]. Keratitis due to focal as well as systemic infection can ultimately lead to multiple sight-threatening defects such as ulceration, opacification and perforation.

Proteases and cellular protein degradation systems play essential roles in the maintenance of cellular homeostasis, especially in ocular tissues from which aberrant proteins should be eliminated to maintain transparency[5]. Autophagy, a cellular protein degradation pathway implicated in periods of starvation, is also known to play crucial roles in innate immune responses[6] as well as cellular defense against xenobiotics[7]. Recently, we have shown that autophagy is induced in the liver[8] and heart[9] in a model of systemic inflammatory disease developed by administering lipopolysaccharide (LPS) to rats. We have also shown that transcription factor EB (TFEB), which was first identified as a renal carcinoma-responsible gene[10], but later revealed to be a master regulator of lysosome biogenesis[11] and autophagy[12], is activated in the heart during LPS administration[9]. In this study, we examined whether the TFEB/autophagy pathway is also activated in the cornea of rats during the systemic inflammatory response.

The animal experiment protocols used in this study were approved by the Institutional Animal Care and Use Committee of the Tokyo Medical and Dental University. In brief, male Sprague-Dawley rats (eight weeks old) were intravenously injected with 15 mg/kg LPS (Sigma, St. Louis, MO, USA) (n = 3 or 4/group). Extraction of corneal tissues from the eyeball was performed under a stereomicroscope (Nikon, Tokyo, Japan), and the tissues were stored at –80°C until use. For immunohistochemical analysis, the tissues were fixed immediately with formalin.

Corneal tissues fixed with formalin were embedded in paraffin and subjected to immunohistochemical analysis as previously described[9]. In brief, tissue sections were incubated at 4 °C overnight with 1/100 diluted anti-TFEB (ab56330, Abcam, Cambridge, MA, USA) or 1/100 diluted anti-microtubule-associated protein light chain-3 (LC3) (#4445, Cell Signaling Technology, Beverly, MA, USA) antibodies and then incubated with peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA). Diaminobenzidine (DAB) was used as a substrate to visualize antigens. The

tissues were then analyzed under a light microscope (Olympus AX80).

Complementary DNA was synthesized from the corneal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and SuperScript II reverse transcriptase (Invitrogen). Quantitative RT-PCR (qPCR) was performed with a Real-Time PCR System (StepOnePlus, Applied Biosystems, Foster City, CA, USA) using SYBR green as a fluorescent dye. The primers used were as follows: 5'-CGGGTTGAGGAGACACACAA-3' and 5'-TCTTTGTTCGAAGCTCCGGC-3' for LC3; 5'-CGGGTTGAGGAGACACACAA -3' and 5'-TCTTTGTTCGAAGCTCCGGC-3' for p62; 5'-GCAAGGCGCTCGCCCTCAAT-3' and 5'-GCCCGCGTGACTCCTCTTCC-3' for LAMP1; 5'-AGCAGGTGGTTTCCGTGTCTCG-3' and 5'-AGGGCTGCTCCCACCGCTAT-3' for LAMP2; and 5'-CACCCGCGAGTACAACCTTCTTG-3' and 5'-CCTCTCTTGCTCTGGGCCTCGT-3' for actin. The conditions for PCR reaction were: 95 °C for 20 sec, followed by 40 cycle of 95 °C for 1 sec and 60 °C for 20 sec.

Total proteins were extracted from tissues or cells using a lysis buffer [10 mM Tris-HCl (pH 8.0), 320 mM sucrose, 1 mM EDTA, 50 mM Na_3VO_4, 2 mM NaF] supplemented with protease inhibitor cocktail (Complete, Roche, Mannheim, Germany). Equal amounts of protein per lane were subjected to SDS-PAGE, followed by immunoblot analysis. The following antibodies were used: anti-LC3 (#4445, Cell Signaling Technology, Beverly, MA, USA), anti-p62 (PM045, MBL, Nagoya, Japan), anti- lysosome-associated membrane protein-1 (LAMP1) (#3243, Cell Signaling Technology), anti-LAMP2 (ab37024, Abcam, Cambridge, UK), and anti-actin (Sigma-Aldrich, St. Louis, MO, USA). Peroxidase-conjugated secondary antibodies were obtained from Promega. A Western Lightning Chemiluminescence Reagent Plus Kit (Perkin Elmer Life Science, Boston, MA, USA) was used to visualize the antigens; signal intensities were quantified using an image analyzer (CS analyzer; ATTO, Tokyo, Japan).

RC4 rabbit corneal keratocyte-derived cells were obtained from RIKEN BioResource Center (Tsukuba, Japan), and cultured in DMEM supplemented with 10% FBS under a 5% CO_2 atmosphere at 37 °C. TNFα (mouse recombinant; T7539, Sigma) was added to the medium at a final concentration 100 ng/mL for the indicated time periods. In some experiments, cycloheximide (CHX; obtained from Sigma) was added along with TNFα at a final concentration 100 μg/mL. Trehalose (obtained from Sigma) was also used at a final concentration of 100 mM.

RC4 cells were transfected with vectors harboring fluorescence marker proteins using Lipofectamine2000 (Invitrogen). The following fluorescence marker proteins were used: TFEB fused with green fluorescence protein (GFP-TFEB, Addgene plasmid 38119)[13], LC3 fused to GFP (GFP-LC3)[14], and LAMP1 fused to monomeric GFP (LAMP1-mGFP)[15]. Fluorescence images were obtained under a BZ-8100 microscope (Keyence, Osaka, Japan).

Cell viabilities were measured using an LDH release assay kit (Wako, Osaka, Japan) according to the manufacturer's instructions. In brief, LDH activity in living cells (attached to the dish) as well as in the medium (including dead cells) was measured after lysing the cells with 0.2% Tween-20 in PBS. LDH activity in living cells was divided by that in total cells (living and dead cells) and expressed as cell viability (%).

First, we examined whether TFEB is activated in the cornea during systemic LPS administration to rats. Representative images of corneas stained with an anti-TFEB antibody are shown in Fig. 1. Without LPS administration, TFEB localizes to the punctate structure in the cytoplasm of epithelial base layer cells (Fig. 1), corresponding to the lysosomal and/or cytoplasmic localization of TFEB in unstressed cells[13]. In contrast, TFEB localizes to the nuclei after LPS administration (Fig. 1), suggesting the activation of TFEB in the LPS-treated rat cornea. In correlation with the nuclear translocation of TFEB, an increased occurrence of autophagosomes in the LPS-treated cornea was demonstrated by staining the tissue with an anti-LC3 antibody (Fig. 1). These results indicate that in response to LPS administration, TFEB should be activated to induce autophagy in the cornea.

TFEB has been shown to transactivate the genes for proteins involved in autophagy to the lysosome pathway[11, 12]. To confirm TFEB activation during LPS administration in the cornea, immunoblot analysis was performed to evaluate the protein levels of LC3, p62, LAMP1, and LAMP2. Several hours (3−6 h) after LPS administration, LC3-II (activated form of LC3) and p62 increase in a time-dependent manner, whereas LAMP1 and LAMP2 levels increase transiently with a peak at 3 hours (Fig. 2A). We next performed qPCR analysis to determine whether or not LPS induces the expression of these genes. As shown in Fig. 2B, more than a 3-fold induction of the LC3 gene was observed 6 hours after LPS administration in the cornea. Although not statistically significant, the expression of p62 also showed a tendency to be induced by LPS (Fig. 2B). The LAMP1 and LAMP2 genes showed no induction after 6 hours of LPS administration (Fig. 2B), corresponding to the transient induction of these proteins during LPS administration (Fig. 2A).

To examine further the activation of TFEB and the induction of autophagy during the inflammatory response elicited by LPS in the cornea, we checked whether similar cellular responses could be observed in RC4 rabbit corneal keratocyte (fibroblast)-derived cells. As the LPS-stimulated inflammatory response in corneal tissues is associated with an elevation in pro-inflammatory cytokines such as IL-1, IL-6, and TNFα[16, 17], we investigated cellular responses during TNFα treatment in RC4 cells. Cells were transfected with expression vectors harboring GFP-tagged TFEB, LC3 and LAMP1, treated with TNFα for 6 hours, and observed under a fluorescence microscope. As shown in Fig. 3A, TNFα causes the translocation of TFEB from the cytoplasm to the nucleus, as well as LC3 punctuation, as observed in the LPS-treated rat cornea (Fig. 1). The structure and abundance of lysosomes do not seem to be affected by TNFα,
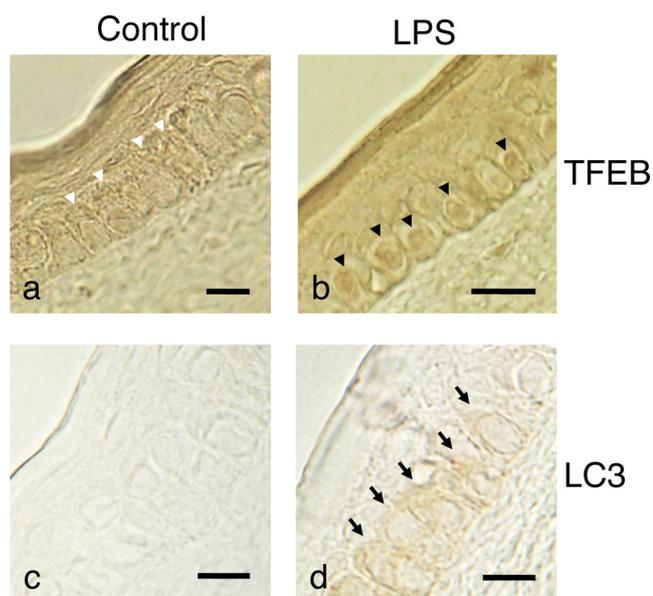
Fig. 1. Sections of corneal tissue from rats sacrificed 6 hours after administration of LPS (15 mg/kg body weight) or untreated control rats were stained with anti-TFEB and anti-LC3 antibodies. Representative images (original magnification, ×200) are shown: TFEB in the control cornea (a), TFEB in the LPS-treated cornea (b), LC3 in the control cornea (c), LC3 in the LPS-treated cornea (d). Bars, 10 μm. White arrowheads indicate cells which intracellular structures (putative lysosomes) were stained by the anti-TFEB antibody. Black arrowheads indicate cells which nuclei were stained by the anti-TFEB antibody. Black arrows indicate cells which autophagosomes were stained by the anti-LC3 antibody.

Fig. 2. (A) Immunoblot analysis of the levels of LC3-II, p62 and glycosylated mature forms of LAMP1 and LAMP2 in the cornea of LPS-treated (at 3 and 6 hours after administration of 15 mg/kg LPS) and control rats. Representative results obtained from four animals are shown. (B) qPCR analysis of the levels of LC3, p62, LAMP1 and LAMP2 transcripts in the cornea of LPS-treated (at 6 hours after administration of 15 mg/kg LPS) (black columns) and control (white columns) rats. Actin transcripts were also analyzed as an internal control. Each bar represents the mean ± SE of three animals. *, P<0.05 versus control by Student's *t*-test.

as estimated by GFP-LAMP1 staining (Fig. 3A). Immunoblot analysis also showed an increase in the LC3-II and p62 proteins, whereas LAMP1 and LAMP2 levels seemed to remain constant after TNFα treatment for 6 hours (Fig. 3B and C). These results correlate well with the results obtained in the LPS-treated rat cornea, further confirming activation of the TFEB/autophagy pathway during the inflammatory response in corneal cells.

Finally, we evaluated whether the activation of TFEB and subsequent induction of autophagy is involved in the homeostasis of corneal cells. Trehalose, an mTOR-independent but TFEB-dependent enhancer of autophagy to lysosome system[18, 19], was used for this purpose. Administration of 100 mM trehalose for 24 hours resulted in the nuclear translocation of TFEB in RC4 cells, confirming its effect on TFEB activation (Fig. 4A). Although several reports have indicated that corneal cells undergo apoptosis in response to TNFα treatment, we could not observe RC4 cell death during treatment with 100 ng/mL TNFα for 24 hours (data not shown). We thus exposed the cells to TNFα plus CHX (TNFα/CHX), another apoptosis-inducing stimulant of corneal cells[20, 21]. An approximate 50% loss of cell viability was observed following treatment of the cells with TNFα/CHX for 24 hours (Fig. 4B). Pretreatment with trehalose for

24 hours resulted in the suppression of cell death caused by the subsequent 24 hours of treatment with TNFα/CHX (Fig. 4B). Taken together, TFEB activation might represent an essential cellular reaction to maintain corneal homeostasis during the inflammatory response in corneal cells.

In this study, we demonstrated the activation of the TFEB/autophagy cellular degradation pathway during the systemic inflammatory response in the rat cornea. The activation of this pathway seems to be involved in maintenance of corneal homeostasis, as activation of this pathway by trehalose significantly suppresses the death of corneal-derived
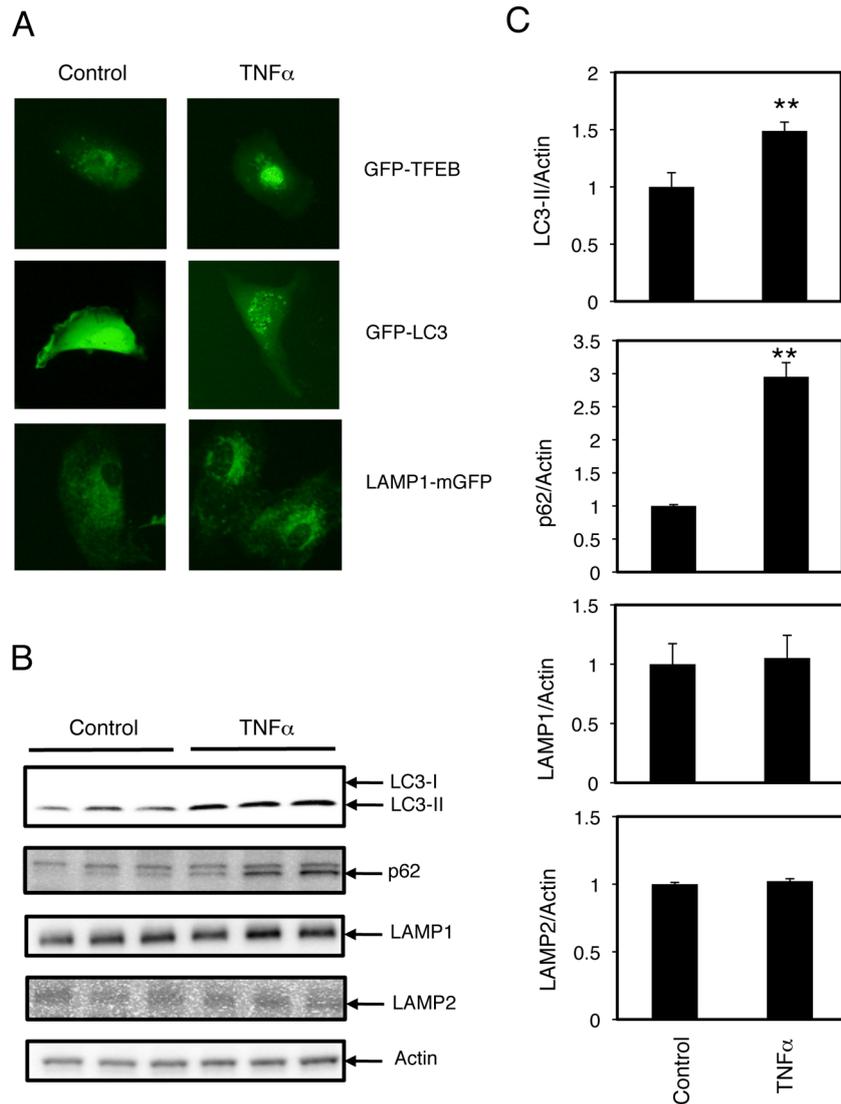
Fig. 3. (A) Nuclear translocation of TFEB and punctuation of LC3 during TNFα treatment of RC4 cells. Cells were transfected with GFP-TFEB, GFP-LC3 and LAMP1-mGFP expression vectors, treated with TNFα (100 ng/mL) for 6 hours and then observed under a fluorescence microscope. (B and C) Upregulation of autophagy-related proteins during TNFα treatment in RC4 cells. Immunoblot analysis of the levels of LC3-II, p62, LAMP1 and LAMP2 in RC4 cells treated with or without TNFα (100 ng/mL) for 6 hours. Each experimental group consisted of three independent samples. Protein levels are shown relative to actin (mean and SE, n=3). The control value was set to 1. **, $P < 0.01$ versus control by Student's t-test.

cells during an apoptosis-inducing inflammatory insult.

Beneficial roles of TFEB in the pathogenesis of multiple diseases have been reported. Transfer of the TFEB gene improves autophagic flux and subsequently mitigates the progression of Pompe disease in skeletal muscle[22], α-synucleinopathy in the midbrain[23], and hepatic lesions due to the accumulation of a toxic mutant of alpha-1-antitrypsin[24]. A gene transfer strategy to defend the corneal epithelium has been successful in several experimental animal models including for graft rejection during cornea transplantation[20, 25]. Hence, TFEB should represent a possible candidate gene for the protection of the cornea against focal as well as systemic inflammatory diseases.

A

Trehalose

GFP-TFEB

B

Fig. 4. (A) Trehalose activates TFEB in RC4 cells. The cells were transfected with GFP-TFEB and then treated with trehalose (100 mM) for 24 hours. (B) Trehalose suppresses RC4 cell death caused by TNFα/CHX. Cells were treated with 100 mM trehalose for 24 hours, followed by treatment with 100 ng/mL TNFα plus 10 μg/mL CHX (TNFα/CHX) for another 24 hours. Cell viabilities were determined by the LDH release assay. Each bar represents the mean ± S.E. of three samples. **, $P < 0.05$ by Tukey's post hoc test.

References

1. Srinivas SP. Cell signaling in regulation of the barrier integrity of the corneal endothelium. Exp Eye Res. **95**: 8–15. 2012. [Medline] [CrossRef]

2. Leal SM Jr, and Pearlman E. The role of cytokines and pathogen recognition molecules in fungal keratitis - Insights from human disease and animal models. Cytokine. **58**: 107–111. 2012. [Medline] [CrossRef]

3. Hassan SS, Wilhelmus KR, Dahl P, Davis GC, Roberts RT, Ross KW, Varnum BH. Medical Review Subcommittee of the Eye Bank Association of America Infectious disease risk factors of corneal graft donors. Arch Ophthalmol. **126**: 235–239. 2008. [Medline] [CrossRef]

4. McCluskey P, and Powell RJ. The eye in systemic inflammatory diseases. Lancet. **364**: 2125–2133. 2004. [Medline] [CrossRef]

5. Im E, and Kazlauskas A. The role of cathepsins in ocular physiology and pathology. Exp Eye Res. **84**: 383–388. 2007. [Medline] [CrossRef]

6. Saitoh T, and Akira S. Regulation of innate immune responses by autophagy-related proteins. J Cell Biol. **189**: 925–935. 2010. [Medline] [CrossRef]

7. Aki T, Funakoshi T, Unuma K, and Uemura K. Impairment of autophagy: from hereditary disorder to drug intoxication. Toxicology. **311**: 205–215. 2013. [Medline] [CrossRef]

8. Unuma K, Aki T, Matsuda S, Funakoshi T, Yoshida K, and Uemura K. Inducer of heme oxygenase-1 cobalt protoporphyrin accelerates autophagy and suppresses oxidative damages during lipopolysaccharide treatment in rat liver. Hepatol Res. **43**: 91–96. 2013. [Medline] [CrossRef]

9. Unuma K, Aki T, Funakoshi T, Yoshida K, and Uemura K. Cobalt protoporphyrin accelerates TFEB activation and lysosome reformation during LPS-induced septic insults in the rat heart. PLoS ONE. **8**: e56526. 2013. [Medline] [CrossRef]

10. Steingrímsson E, Copeland NG, and Jenkins NA. Melanocytes and the microphthalmia transcription factor network. Annu Rev Genet. **38**: 365–411. 2004. [Medline] [CrossRef]

11. Sardiello M, Palmieri M, di Ronza A, Medina DL, Valenza M, Gennarino VA, Di Malta C, Donaudy F, Embrione V, Polishchuk RS, Banfi S, Parenti G, Cattaneo E, and Ballabio A. A gene network regulating lysosomal biogenesis and function. Science. **325**: 473–477. 2009. [Medline]

12. Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, Erdin SU, Huynh T, Medina D, Colella P, Sardiello M, Rubinsztein DC, and Ballabio A. TFEB links autophagy to lysosomal biogenesis. Science. **332**: 1429–1433. 2011. [Medline] [CrossRef]

13. Roczniak-Ferguson A, Petit CS, Froehlich F, Qian S, Ky J, Angarola B, Walther TC, and Ferguson SM. The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. Sci Signal. **5**: ra42. 2012. [Medline] [CrossRef]

14. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, and Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. **19**: 5720–5728. 2000. [Medline] [CrossRef]

15. Falcón-Pérez JM, Nazarian R, Sabatti C, and Dell'Angelica EC. Distribution and dynamics of Lamp1-containing endocytic organelles in fibroblasts deficient in BLOC-3. J Cell Sci. **118**: 5243–5255. 2005. [Medline] [CrossRef]

16. Liang H, Brignole-Baudouin F, Labbé A, Pauly A, Warnet JM, and Baudouin C. LPS-stimulated inflammation and apoptosis in corneal injury models. Mol Vis. **13**: 1169–1180. 2007. [Medline]

17. Gupta D, Du Y, Piluek J, Jakub AM, Buela KA, Abbott A, Schuman JS, and SundarRaj N. Ethyl pyruvate ameliorates endotoxin-induced corneal inflammation. Invest Ophthalmol Vis Sci. **53**: 6589–6599. 2012. [Medline] [CrossRef]

18. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, and Rubinsztein DC. Trehalose, a novel mTOR-independent autophagy enhancer, accelerates the clearance of mutant huntingtin and alpha-synuclein. J Biol Chem. **282**: 5641–5652. 2007. [Medline] [CrossRef]

19. Dehay B, Bové J, Rodríguez-Muela N, Perier C, Recasens A, Boya P, and Vila M. Pathogenic lysosomal depletion in Parkinson's disease. J Neurosci. **30**: 12535–12544. 2010.

[Medline] [CrossRef]

20. Gong N, Ecke I, Mergler S, Yang J, Metzner S, Schu S, Volk HD, Pleyer U, and Ritter T. Gene transfer of cyto-protective molecules in corneal endothelial cells and cultured corneas: analysis of protective effects in vitro and in vivo. Biochem Biophys Res Commun. **357**: 302–307. 2007. [Medline] [CrossRef]

21. Goodkin ML, Epstein S, Asbell PA, and Blaho JA. Nuclear translocation of NF-kappaB precedes apoptotic poly(ADP-ribose) polymerase cleavage during productive HSV-1 replication in corneal epithelial cells. Invest Ophthalmol Vis Sci. **48**: 4980–4988. 2007. [Medline] [CrossRef]

22. Spampanato C, Feeney E, Li L, Cardone M, Lim JA, Annunziata F, Zare H, Polishchuk R, Puertollano R, Parenti G, Ballabio A, and Raben N. Transcription factor EB (TFEB) is a new therapeutic target for Pompe disease. EMBO Mol Med. **5**: 691–706. 2013. [Medline] [CrossRef]

23. Decressac M, Mattsson B, Weikop P, Lundblad M, Jakobsson J, and Björklund A. TFEB-mediated autophagy rescues midbrain dopamine neurons from α-synuclein toxicity. Proc Natl Acad Sci USA. **110**: E1817–E1826. 2013. [Medline] [CrossRef]

24. Pastore N, Blomenkamp K, Annunziata F, Piccolo P, Mithbaokar P, Maria Sepe R, Vetrini F, Palmer D, Ng P, Polishchuk E, Iacobacci S, Polishchuk R, Teckman J, Ballabio A, and Brunetti-Pierri N. Gene transfer of master autophagy regulator TFEB results in clearance of toxic protein and correction of hepatic disease in alpha-1-anti-trypsin deficiency. EMBO Mol Med. **5**: 397–412. 2013. [Medline] [CrossRef]

25. Kampik D, Ali RR, and Larkin DF. Experimental gene transfer to the corneal endothelium. Exp Eye Res. **95**: 54–59. 2012. [Medline] [CrossRef]