Light-Induced Lipocalin 2 Facilitates Cellular Apoptosis by Positively Regulating Reactive Oxygen Species/Bim Signaling in Retinal Degeneration

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Submitted: July 6, 2018
Accepted: November 16, 2018
Citation: Tang W, Ma J, Gu R, Lei B, Ding X, Xu G. Light-induced lipocalin 2 facilitates cellular apoptosis by positively regulating reactive oxygen species/Bim signaling in retinal degeneration. Invest Ophthalmol Vis Sci. 2018;59:6014–6025. https://doi.org/10.1167/iovs.18-25213

Purpose. Lipocalin 2 (LCN2) is reported to be one of the key regulators of cell survival and death; however, its effect on retinal degeneration is unclear. Therefore, we aimed to investigate the role of LCN2 and its underlying mechanisms in light-induced retinal degeneration.

Methods. A recombinant lentivirus expressing a short hairpin RNA targeting LCN2 mRNA and a recombinant lentivirus overexpressing LCN2 were used to downregulate and upregulate retinal LCN2, respectively. Seven days after intravitreal injection of the lentiviruses, rats were exposed to blue light (2500 lux) for 24 hours. Retinal function and morphology were evaluated with ERG and hematoxylin-eosin staining, respectively. TUNEL staining was used to detect apoptotic cells. The levels of reactive oxygen species (ROS) were evaluated with dihydroethidium labeling. Western blotting and real-time PCR were used to examine protein and mRNA expression levels, respectively.

Results. Retinal LCN2 expression was significantly upregulated after light exposure. Light exposure reduced the amplitudes of a- and b-waves on the ERG and the thickness of the outer nuclear layer and promoted photoreceptor apoptosis. These phenomena were clearly attenuated by LCN2 knockdown, whereas LCN2 overexpression had the opposite effects. The overexpression of LCN2 facilitated photoreceptor apoptosis by increasing ROS generation and Bim expression. On the opposite, LCN2 knockdown mitigated the generation of light-exposure-induced ROS and the activation of the Bim-mediated mitochondrial apoptotic pathway.

Conclusions. Light-induced LCN2 is a proapoptotic factor in the retina, and LCN2 knockdown protects photoreceptors from apoptosis by inhibiting ROS production and Bim expression. LCN2 is a potential therapeutic target for light-induced retinal degeneration.

Keywords: LCN2, oxidative stress, retinal degeneration, photoreceptor, apoptosis

Degenerative retinal diseases, featured by the irreversible retinal neuron damage, are a group of eye diseases that can cause permanent vision impairment and even vision loss.1 Nowadays, millions of people are affected by degenerative retinal diseases such as atrophic AMD and retinitis pigmentosa (RP) while there is still a lack of effective treatment for these diseases.2,3 Although the underlying molecular mechanisms of these retinal degenerative disorders vary, they share a common final pathway: irreversible photoreceptor loss.4,5 Therefore, it is critical to identify the factors that regulate photoreceptor death and develop strategies for the prevention of photoreceptor loss accordingly.

Lipocalin 2 (LCN2), also known as neutrophil gelatinase-associated lipocalin or 24p3, is a member of the lipocalin family, which binds and transports small lipophilic molecules, including iron, steroids, and fatty acids.6,7 LCN2 has been implicated in the regulation of cell apoptosis, although reports of the roles of LCN2 in cell apoptosis have been inconsistent.7 For example, Mishra et al.8 reported that LCN2 treatment reduced the number of apoptotic tubule cells after ischemic injury, and Borkham-Kamphorst et al.9 demonstrated that hepatocyte apoptosis was greater in LCN2−/− mice than in normal mice after acute liver injury, suggesting that LCN2 protects cells from apoptosis under pathological stimuli. In contrast, emerging studies have also demonstrated the proapoptotic effects of LCN2. LCN2 induced an increase in intracellular reactive oxygen species (ROS) and cell apoptosis in endometrial cancer cells.10 In cardiomyocytes, LCN2 activated the mitochondrial apoptotic pathway, leading to cell apoptosis.11 It has also been reported that LCN2 promoted cell apoptosis, which correlated with the induction of the proapoptotic protein Bim.12,13 Although increased LCN2 expression has been observed in the aqueous humor of AMD patients,14 the precise role of LCN2 in retinal degeneration is unclear.

Here we investigated the role of LCN2 in a light-induced rat model of retinal degeneration, which is widely used to study the molecular mechanisms of photoreceptor death.15,16 Our results show that LCN2 expression was significantly upregulated in the retina after light exposure, and this was accompanied by the increased expression of Bim and activated caspase-3. The knockdown of LCN2 protected the retina from cellular apoptosis.
and rescued the loss of function induced by light exposure by reducing ROS generation. LCN2 overexpression had the opposite effects. These results suggest that LCN2 is a potential therapeutic target for light-induced retinal degeneration.

**METHODS**

**Animals**

Adult male Sprague-Dawley rats purchased from SLAC Laboratories (Shanghai, China), weighing 180 to 200 g, were housed under normal cyclic light (200 lux, 12 hours on/off, 6 AM–6 PM) with free access to food and water. The rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) during examination and were killed with an overdose of pentobarbital at the end of the experiment. A total of 180 rats were used in the study and animal grouping is listed in Supplementary Table S1. For ERG and hematoxylin-eosin (H&E) staining analysis, both eyes of each rat were used. For other tests, only one eye of each rat was used. All procedures were approved by the Animal Ethics Committee of the Eye and Ear Nose Throat Hospital of Fudan University, Shanghai, China, and were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Lentivirus Construction**

To achieve LCN2 knockdown, three LCN2-mRNA-complementary short hairpin RNA (shRNA) oligonucleotides were cloned into a lentiviral vector (to produce LV-shLCN2) and synthesized (Genomeditech Co., Ltd., Shanghai, China). Three lentiviral shRNAs targeting three different sites in LCN2 mRNA and a negative control lentivirus that did not encode an LCN2 shRNA

**FIGURE 1.** LCN2, Bim, Bax, and cleaved caspase-3 expression increased in the rat retina after LE. (A) Real-time PCR of LCN2 mRNA expression in rat retina at 1, 3, and 7 days after LE. β-Actin was used as the internal control. (B, C) Western blotting analysis of LCN2 at 1, 3, and 7 days after LE. (D, E) Western blotting analysis of Bim at 1, 3, and 7 days. (F, G) Western blotting analysis of Bax at 1, 3, and 7 days. (H, I) Western blotting analysis of cleaved caspase-3 at 1, 3, and 7 days. β-Tubulin was used as the internal control for (B–I). Normal rats were used as controls. n = 3 for each group. *P < 0.05, **P < 0.01. Nor, normal.
(LV-shNC) was constructed (Supplementary Table S2). The lentivirus LCN2 shRNA with the best silencing efficiency was chosen for the subsequent experiments (Supplementary Fig. S1). To overexpress LCN2, the full-length rat LCN2 cDNA (NM_130741.1) was cloned into the lentiviral vector (LV-LCN2) and the empty lentiviral vector (LV-Vector) was used as the control (Genomeditech Co., Ltd.).

**Intravitreal Lentivirus Administration**

After anesthesia and pupil dilation with 1% atropine, an intravitreal injection of the relevant lentivirus (2 μL, 5 × 10⁸ transducing units/mL) was made into one eye of each rat 1 mm behind the limbus, using a 33-gauge needle (Hamilton, Reno, NV, USA) under a surgical microscope. After injection, the rats were maintained normally for 7 days to allow sufficient retinal transfection before the subsequent experiments.

**Model of Light-Induced Retinal Degeneration**

The light exposure procedures were performed as previously reported. Briefly, rats were randomly divided into light exposure (LE) and control (no LE) groups. The rats in the LE group were dark adapted for 24 hours and then the rats’ pupils were dilated with 1% atropine. Rats were then placed

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**Figure 2.** LCN2 knockdown protected retinal function after LE. (A) Real-time PCR of LCN2 mRNA expression in rat retina exposed to LV-shNC or LV-shLCN2. β-Actin was used as the internal control. (B, C) Western blotting analysis of LCN2 in rat retina treated with LV-shNC or LV-shLCN2. β-Tubulin was used as the internal control. (D, E) Typical waveforms and statistical analysis of scotopic and photopic ERG in the normal control group receiving no LE or lentivirus, the LV-shNC group receiving no LE, the LV-shNC group receiving LE 3 days after LE, the LV-shLCN2 group receiving LE 3 days after LE, the LV-shNC group receiving LE 7 days after LE, and the LV-shLCN2 group receiving LE 7 days after LE. n = 3 for each group in (A–C); n = 6 for each group in (D, E). *P < 0.05, **P < 0.01.
individually in separate cages and exposed to 2500-lux blue fluorescent light for 24 hours. During exposure, the rats had free access to food and water and the room temperature was kept at 25±1°C. After LE, the rats were returned to the normal light/dark cycle for either 1, 3, or 7 days before further analysis. Age-matched normal control rats and rats that received LV-shNC or LV-Vector in the control group were dark adapted for 24 hours without exposure to the damaging 2500-lux and then returned to the normal light/dark cycle.

Electroretinography

ERG (Espion Electrophysiology System; Diagnosys LLC, Lowell, MA, USA) was performed 3 and 7 days after LE. The rats were dark adapted for 2 hours and then anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). After pupil dilation, platinum ring electrodes were placed centrally on their corneas, and two needles were inserted, through the nose and subcutaneously near the tail, as the reference electrode and ground electrode, respectively. The stimulation parameters were as previously reported.18 Scotopic ERG involved a single pulse flash of 0.01 cd s/m² intensity of 0.1 Hz from darkness, with 10 repetitions. There was then a 5-minute adaptation period (50 cd s/m²) before the photopic conditions were instated. Photopic ERG involved a single pulse flash of 20 cd s/m² intensity, at a frequency of 1 Hz with 20 repetitions on a 50 cd s/m² white background. The a-wave amplitude was measured from baseline to the trough of the a-wave, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave.

H&E Staining

After ERG, eyes were enucleated and fixed in 4% paraformaldehyde, immersed in 70% alcohol, and then embedded in paraffin. Serial sections (5-μm thick) were cut sagittally through the optic nerve head and stained with H&E. The retinal morphology was evaluated and imaged with a light microscope (Leica Microsystems, Bensheim, Germany). The thickness of the outer nuclear layer (ONL) in the inferior and superior hemispheres was measured at 500-μm intervals starting at the optic papilla, with CaseViewer 2.1 software (3DHistech Ltd, Budapest, Hungary). The ONL thickness was measured at 18 locations and the results were averaged.

TUNEL Staining

The enucleated eyes were fixed in 4% paraformaldehyde, dehydrated through a gradient of sucrose solutions, and embedded in optimal cutting temperature compound (Tissue-
Cryosections were cut sagittally through the optic papilla. The sections were rinsed with PBS and treated with 0.1% aqueous trisodium citrate containing 0.1% Triton X-100 for 10 minutes. The sections were then incubated in TUNEL reaction mixture (Roche, Mannheim, Germany) for 60 minutes. After they were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime, Jiangsu, China), the slides were examined and photographed with a confocal microscope (Leica Microsystems). The numbers of TUNEL-positive cells and DAPI-positive cells per field were counted, and the percentage of apoptotic cells (TUNEL/DAPI) was then calculated.

Measurement of ROS Production

ROS production in the retina was assessed with the fluorescent indicator dihydroethidium (DHE) (KeyGEN Biotech, Jiangsu, China), as previously described. Briefly, fresh retinas were harvested and processed for cryosectioning. The frozen sections were rinsed with PBS and incubated with 5 μM DHE for 30 minutes at 37°C. DHE specifically reacts with the superoxide anion and is oxidized to the red fluorescent compound ethidium, which binds to DNA in the nucleus. After counterstaining with DAPI (Beyotime), the sections were imaged under the same exposure conditions with a confocal microscope.
microscope (Leica Microsystems). The relative fluorescence intensities in the images were quantified with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Isolation of Cytosolic and Mitochondrial Fractions
The cytosolic and mitochondrial proteins in the retinas were isolated with a Mitochondrial Isolation Kit for Tissues (Beyotime). Briefly, fresh retinas were cut into small pieces, washed with ice-cold PBS, and placed in mitochondria isolation reagent A containing 1 mM phenylmethane sulfonyl fluoride (Beyotime). After homogenization, the homogenate was centrifuged at 600 g for 5 minutes at 4°C. The supernatant was then collected and centrifuged again at 11,000 g for 10 minutes at 4°C. The supernatant was collected as the cytosolic fraction. The pellet was resuspended in mitochondria isolation reagent B and centrifuged at 12,000 g for 10 minutes at 4°C. The pellet was then collected as the mitochondrial fraction.

Western Blotting
The retinas were lysed in RIPA buffer (Beyotime) and the protein levels of the extracts were measured with a BCA Protein Assay Kit (Beyotime). Equal amounts of protein (40 μg) were loaded onto 12% SDS-PAGE gels, separated with electrophoresis, and then transferred onto polyvinyl difluoride membranes (Millipore, Billerica, MA, USA). After the membranes were blocked with 5% nonfat milk, they were incubated overnight at 4°C with the following primary antibodies: rabbit anti-LCN2 (1:1000, ab63929; Abcam, Cambridge, UK), rabbit anti-Bim (1:1000, ab32158; Abcam), rabbit anti-Bax (1:1000, 2772s; CST, San Antonio, TX, USA), rabbit anti-cytochrome c (1:1000, 10993-1-AP; Proteintech, Chicago, IL, USA), rabbit anti-cleaved caspase-3 (1:1000, ab65929; Abcam, Cambridge, UK), rabbit anti-voltage-dependent anion-selective channel 1 (VDAC1) (1:3000, ab154856; Abcam). The membranes were then incubated with a horseradish-peroxidase–conjugated goat anti-rabbit secondary antibody (1:2000, AB0101; Abways) for 1 hour at 37°C. The immunoblots were visualized with chemiluminescence (Pierce ECL, Thermo Scientific, Rockford, IL, USA). ImageJ software was used to quantify the density of the signals, and the levels were normalized against the internal controls.

Real-Time PCR
Total RNA was isolated from the retinal tissues with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Complementary
DNA was synthesized with the PrimeScript RT Reagent Kit with genomic DNA Eraser (Takara, Ohtsu, Shiga, Japan). Real-time PCR was performed with the StepOne Real-Time PCR System (Thermo Scientific) using the SYBR Premix Ex Taq Kit (Takara). The primer sequences were: LCN2 (forward) 5'-AACGTCACTTCCATCCTCGTC-3' and (reverse) 5'-AATCGCTCCTTCAGTTCATCG-3'; β-actin (forward) 5'-CGTTGACATCCGTAAAGACCTC-3' and (reverse) 5'-TAGGAGGCCAGGCGAATCT-3'. The thermal cycling conditions were 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds, 58°C for 20 seconds, and 72°C for 45 seconds. The melting curve profiles were generated at the end of each reaction. The expression levels of LCN2 mRNA were normalized to those of β-actin and calculated with the 2^-ΔΔCt method.

Statistical Analysis
All data are expressed as the means ± SEM. Unpaired Student’s t-test or 1-way ANOVA followed by Tukey’s post hoc multiple comparisons test was used to determine the statistical significance of differences between groups using the GraphPad Prism version 5 software (GraphPad, San Diego, CA, USA). P < 0.05 was considered statistically significant. Experiments were performed at least three times per condition.

RESULTS

Exposure to Light Induces the Expression of LCN2 and Promotes Cellular Apoptosis in the Retina
We first examined the expression of LCN2 in the retina after exposure to light. Our results showed that the expression of LCN2 mRNA was significantly and time-dependently increased by light injury compared with that in the normal retinas (Fig. 1A). Similarly, the expression of LCN2 protein was also notably elevated 3 and 7 days after LE, respectively (Figs. 1B, 1C). Moreover, the expression of Bim and Bax (two critical proapoptotic proteins) and the activation of caspase-3 in the retinas were increased by LE, in parallel with the changes in LCN2 expression (Figs. 1D–I). These results indicate that LCN2 is probably involved in the pathogenesis of light-induced retinal degeneration.

LCN2 Knockdown Attenuated the Decrease of Retinal Function After LE
To determine the role of LCN2 in light-induced retinal degeneration, the expression of LCN2 was knocked down by the intravitreal injection of LV-shLCN2. Real-time PCR and Western blotting confirmed the efficiency of knockdown (Figs. 2A–C). Retinal function was then evaluated with ERG 3 and 7 days after exposure to light, as shown in Figures 2D and 2E. LE significantly reduced the amplitudes of the a- and b-waves on scotopic and photopic ERG compared with those in the controls 3 and 7 days after LE, respectively. However, the knockdown of LCN2 mitigated these reductions in the a- and b-wave amplitudes induced by LE 3 and 7 days after LE, respectively. These results suggest that the reduction in retinal function caused by light was mitigated by LCN2 knockdown.

LCN2 Knockdown Relieved Light-Induced Photoreceptor Loss
The thickness of the ONL was measured histologically 3 and 7 days after LE. As shown in Figures 3A and 3B, the average ONL thicknesses were markedly reduced by LE in the LV-shNC-treated groups 3 and 7 days after LE, respectively. In contrast, the knockdown of LCN2 with an intravitreal injection of LV-shLCN2 weakened the effect of LE on ONL thickness 3 and 7 days after LE, respectively. Because the expression of LCN2 and cell apoptosis were both significantly facilitated from 3 days...
after light injury, we chose this time point for further functional studies. A TUNEL assay was used to assess photoreceptor apoptosis 3 days after LE. The percentage of TUNEL-positive photoreceptor cells significantly increased in the ONL after exposure to light in the vehicle-treated eyes, whereas the proapoptotic effects of LE were attenuated when the expression of LCN2 was reduced (Figs. 3C, 3D). These results indicate that the knockdown of LCN2 protected the photoreceptor cells from light-induced apoptosis.

**LCN2 Overexpression Aggravated the Loss of Retinal Function and Photoreceptor Apoptosis After LE**

To identify the physiological functions of LCN2, LCN2 was overexpressed in the retina with the intravitreal injection of LV-LCN2. The overexpression efficiency was confirmed with real-time PCR and Western blotting (Figs. 4A–C). As shown in Figures 4D and 4E, the reductions in the amplitudes of the a- and b-waves on scotopic and photopic ERG caused by LE were exacerbated by the overexpression of LCN2 3 and 7 days after LE, respectively. Furthermore, the average ONL thickness was markedly reduced by LE in the LV-Vector-treated groups, whereas LCN2 overexpression significantly enhanced the effect of LE on ONL thickness 3 and 7 days after LE, respectively (Figs. 5A, 5B). Meanwhile, the percentage of TUNEL-positive photoreceptor cells significantly increased in the ONL after LE in the LV-Vector-treated groups, whereas the proapoptotic effects of LE were potentiated when LCN2 was overexpressed 3 days after LE (Figs. 5C, 5D). These results indicate that LE induced photoreceptor apoptosis and the loss of retinal function, at least in part, by upregulating the expression of LCN2.

**LCN2 Plays Its Proapoptotic Role by Positively Regulating ROS Generation and Bim Expression in the Retina**

Oxidative stress has been implicated as a major player in neuronal degeneration after LE. To determine whether LCN2 plays a role in the oxidative stress levels in light-exposed
retinas, in situ ROS generation was assessed with DHE staining in freshly prepared retinal sections. Compared with the controls, the intensity of oxidized DHE fluorescence increased markedly in the retinas after LE, particularly in the ONL, whereas LCN2 knockdown clearly mitigated ROS production, evident as reduced fluorescence intensity (Figs. 6A, 6B). Bim, a proapoptotic member of the intrinsic apoptosis pathway, plays an important role as a downstream target in oxidative-stress-mediated apoptosis.\(^{22,23}\) Therefore, we examined whether LCN2 has a regulatory effect on Bim in the retina. As shown in Figure 7A, the light-induced upregulation of Bim protein expression was markedly attenuated in the LV-shLCN2–treated group compared with that in the vehicle-treated group (Fig. 7A). Bax, another critical proapoptotic factor involved in the intrinsic mitochondria pathway,\(^{24}\) was also examined. LCN2 knockdown clearly attenuated the upregulation of Bax in response to light 3 days after LE (Fig. 7B). It is reported that under apoptotic stimuli, cytochrome c is released from the mitochondria into the cytosol when the permeability of mitochondrial membrane increases, and the released cytochrome c in the cytosol leads to the activation of caspase-3 and ultimately cell apoptosis.\(^{25,26}\) We then examined the release of cytochrome c and the activation of caspase-3. Our results show that the cytochrome c release from mitochondria to the cytoplasm and the subsequently increased cleavage of caspase-3 induced by LE were both alleviated by LCN2 mRNA interference (Figs. 7C–E).

We also examined the effects of LCN2 overexpression in the retina. The oxidized DHE fluorescence intensity markedly increased after LE in the LV-Vector-treated group and LCN2 overexpression further enhanced the fluorescence intensity 5 days after LE (Figs. 8A, 8B). As shown in Figures 9A and 9B, the light-induced upregulation of Bim and Bax was further promoted by LCN2 overexpression. Meanwhile, light-exposure–induced cytochrome c release was significantly increased by the overexpression of LCN2 (Figs. 9C, 9D). These results indicate that light-injury–induced LCN2 facilitates the mitochondria-mediated apoptosis of photoreceptor cells by activating the ROS/Bim pathway.

**DISCUSSION**

Photoreceptor loss is a common final pathway in various vision-threatening degenerative retinal diseases, such as dry AMD and RP,\(^{4,27}\); however, there is no effective treatment for the prevention or mitigation of photoreceptor loss. In this study, we provide the direct evidence that the light-induced upregulation of LCN2 leads to retinal degeneration and photoreceptor apoptosis. Moreover, the knockdown of LCN2 protected the photoreceptors both histologically and functionally by reducing the levels of ROS and Bim expression. These results indicate that LCN2 acts as a proapoptotic mediator in light-induced retinal degeneration.

An important finding of our study is that exposure to light upregulates the expression of LCN2, which promotes the apoptosis of the photoreceptors. It is known that the apoptosis of photoreceptors is an important pathogenic factor in light-induced retinal degeneration.\(^{28,29}\) LCN2, a secreted protein, is reported to participate in a wide range of cell death events.\(^{30,31}\) Previous studies have shown that the upregulation of LCN2 is associated with the induction of apoptosis in lymphocytes,\(^{12}\) neurons,\(^{13}\) and fibroblasts.\(^{10}\) LCN2 also enhances the sensitivity of glial cells to apoptotic stimuli.\(^{32,33}\) The overexpression of LCN2 resulted in the apoptosis of a human chronic myelogenous leukemia cell line,\(^{34}\) whereas LCN2 knockdown with small interfering RNA inhibited the isotretinoin-induced apoptosis of human sebaceous gland cells. In contrast, there are also reports that LCN2 protects cells from the apoptosis induced by cellular stress.\(^{8,9,35}\) In retinal tissues, reports on the role of LCN2 are also controversial. Ghosh et al.\(^{36}\) reported that LCN-2, whose expression was significantly increased in early AMD, exerted a proinflammatory bias in the retina and led to retinal tissue injury in an AMD mouse model. However, Parmar et al.\(^{37}\) found that LCN2 deficiency led to exacerbated

![Figure 8](https://example.com/f8.png)
inflammation and upregulation of proinflammatory genes and microglial activation after LE in the retinas of Abca4−/− Rdh8−/− mice, and the overexpression of LCN2 protected human RPE cells from inflammation-associated cell death. Therefore, the roles of LCN2 are likely to be disease-, model-, and species-dependent. In this study, we found that light-induced LCN2 expression increased the proportion of TUNEL-positive cells among the photoreceptors. Moreover, the reduction in photoreceptor numbers and the consequent loss of retinal function induced by LE were aggravated by the overexpression of LCN2. Conversely, the knockdown of LCN2 had the opposite effects. The apoptosis of photoreceptors and the retinal dysfunction induced by LE was rescued by LCN2 knockdown. These results indicate that LCN2 is an important mediator of the light-exposure–induced apoptosis of photoreceptors.

Another important finding of this study is that LCN2 plays its proapoptotic roles in the retina by increasing the accumulation of ROS and the expression of Bim. Oxidative stress, caused by the increased production of ROS and/or reduced oxidation resistance, is considered a critical pathogenic factor in retinal photic injury and AMD. LCN2 reportedly induces an intracellular oxidative environment that causes apoptosis in RL-95-2 cells and L929 cells. Kagoya et al. also found that LCN2 treatment induced a significant increase in intracellular ROS levels and oxidative DNA damage by elevating intracellular iron levels in hematopoietic cells. Therefore, we examined retinal ROS production to determine whether LCN2 exerts its proapoptotic effects by regulating oxidative stress in the retina. Our data show that the light-exposure–induced generation of ROS was enhanced by LCN2 overexpression, whereas the knockdown of LCN2 reduced the levels of ROS in the retina. These results indicate that ROS generation is positively regulated by LCN2 in the retina. However, the mechanism of LCN2-induced ROS accumulation in the retina remains unclear. It is likely that light-exposure–induced LCN2, with its iron-binding and transporting capacities, increases intracellular iron uptake, which elevates intracellular ROS through the Fenton reaction. However, further studies are required to test this hypothesis.

The B-cell lymphoma-2 (Bcl-2) family proteins are key mediators of the intracellular apoptotic signaling cascades. The Bcl-2 family is classified into antiapoptotic groups (Bcl-2, Bcl-XL, and so on) and proapoptotic groups, which are further divided into the multidomain proapoptotic group (Bax, Bak, and so on) and the BH3-only group (Bim, Puma, and so on).
Light-induced LCN2 Promotes Photoreceptor Apoptosis

Bim, as a potent inducer of apoptosis, can initiate the mitochondrial apoptotic pathway under pathophysiological stimuli.46–48 It favors apoptosis either by directly activating proapoptotic Bax or by neutralizing antiapoptotic Bcl-2.49 The directly or indirectly activated Bax integrates into the mitochondrial outer membrane and increases its permeability, facilitating the release of cytochrome c into the cytoplasm, the activation of caspase-3, and ultimately cell apoptosis.48

Previous studies have shown that Bim, as a downstream target, can be activated under the oxidative stress caused by ROS,22,23 and that the upregulation of Bim expression is linked to the apoptosis-inducing effects of LCN2.12,50,51 The knockdown of Bim expression abolished the cell-death–sensitizing effects of LCN2 in B35 rat neuroblastoma cells.52 In the present study, the enhanced expression of LCN2 in the retina after LE was accompanied by the upregulation of Bim. Moreover, light-induced Bim expression was mitigated by the knockdown of LCN2, whereas the expression of Bim was increased by LCN2 overexpression. This suggests that Bim acts as a downstream target in the LCN2-induced apoptosis of retinal photoreceptors. To demonstrate the regulatory roles of LCN2 in the intrinsic apoptosis pathway, we then examined the expression of Bax, the release of mitochondrial cytochrome c, and the activation of caspase-3. LE led to the upregulation of Bax expression, the release of cytochrome c into the cytoplasm, and promoted the activation of caspase-3, which were attenuated by the knockdown of LCN2. LCN2 overexpression had the opposite effects. These results indicate that LCN2 facilitates the Bim/Bax-mediated mitochondrial apoptosis (Fig. 9E).

This study had an important limitation. Intravitreal injection is routinely used to administer drugs into the eye. The lentiviral vectors might have been transmitted into retinal cells other than the photoreceptors after its intravitreal injection. However, our results show that they had an evident effect on photoreceptor apoptosis and on ROS generation in photoreceptors, as clearly shown in histological images. Nevertheless, more-efficient viral vehicles with higher tropism for photoreceptors should be developed to provide more direct evidence in future studies.

CONCLUSIONS

Our results demonstrate that LCN2 mediates light-induced photoreceptor apoptosis by upregulating ROS production and Bim and Bax expression, and that the knockdown of LCN2 protects the photoreceptors against light-induced retinal degeneration. Therefore, LCN2 can be considered a potential target for the treatment of photoreceptor apoptosis in degenerative retinal diseases, such as RP.

Acknowledgments

Supported by research grants from the National Key Basic Research Program of China (2013CB967503), the National Natural Science Foundation of China (81570854), the Youth Project of the National Natural Science Fund (81600739, 81700861, 81800846), Shanghai Sailing Program (16YF141003), and Science and Technology Commission of Shanghai Municipality (16411953700).

Disclosure: W. Tang, None; J. Ma, None; R. Gu, None; B. Lei, None; X. Ding, None; G. Xu, None

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