**Early Gene Expression Profile in Retinal Ganglion Cell Layer After Optic Nerve Crush in Mice**

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**Purpose.** Optic nerve crush (ONC) induces retinal ganglion cell (RGC) death, which causes vision loss in glaucoma. To investigate early events leading to apoptosis of RGCs, we performed gene expression analysis of injured retinas in the period before RGC loss.

**Methods.** The temporal changes of gene profiles at 0, 1, and 4 days after ONC were determined by DNA microarray. To verify the gene expression changes in RGCs, we enriched RGCs by laser-captured microdissection and performed real-time RT-PCR of 14 selected genes. In situ localization study was performed by immunohistochemistry.

**Results.** At 1 day and 4 days after ONC, 1423 and 2010 retinal genes were changed compared with 0 day, respectively; these genes were mainly related to apoptotic process, immune process, regulation of cell cycle, and ion transport. RT-PCR analysis revealed that expression levels of Activating transcription factor 3 (Atf3), Lipocalin 2 (Lcn2), and tumor necrosis factor receptor superfamily member 12a (Tnfrsf12a) were remarkably changed in RGC-enriched fraction within 4 days postcrush. Immunohistochemical analysis confirmed that all of these genes expressed highly in the ganglion cell layer of crushed retinas.

**Conclusions.** In response to ONC, the expression of apoptotic genes was stimulated soon after crush. Atf3, Lcn2, and Tnfrsf12a might be key molecules responsible for RGC loss in glaucoma.

Keywords: optic nerve crush, microarray, laser-captured microdissection

In glaucoma, the progressive loss of the retinal ganglion cells (RGCs) is a leading cause of blindness worldwide.1,2 Studies in humans and experimental animal models of glaucoma have demonstrated that RGC death is typically attributed to apoptosis.3–5 In pathologic conditions, including glaucoma, ischemic retina,6,7 optic neuritis,8,9 the degeneration of optic nerves is considered to be related strongly to RGC apoptosis. Ocular hypertension is one of the major risk factors for glaucoma,12 and it has been revealed that ocular hypertension directly affects the optic nerve head and that axonal crush is a primary event in the progression of ocular hypertensive glaucoma.5,7,15

The optic nerve crush (ONC) model in rodents is a well-established experimental model not only for glaucoma but also for investigating neuronal apoptosis due to axonal degeneration.14,15 ONC affects RGCs rapidly and specifically. After ONC, RGCs survive for 5 days and then gradually decrease in number by apoptosis. By 7 days after ONC, approximately 50% of RGCs are lost and within 14 days postcrush, 90% of RGCs are dead.16,17 Recently, several studies focused on the first 5 days after ONC as a therapeutic target for glaucoma, because neuronal protective and degenerative pathways are activated during this period. Some neurotrophic factors such as brain-derived neurotrophic factor,18,19 ciliary neurotrophic factor,15,19–21 and glial cell–derived neurotrophic factor22,23 are found to be effective for promoting RGC survival and axonal regeneration against ONC. Meanwhile, inhibitions of apoptotic or inflammatory pathways are also effective; Caspase inhibitors,24–26 transcriptional downregulations of apoptotic genes,27,28 and modification of inflammatory responses29–31 delay RGC death after ONC.

In this study, to investigate early events leading to apoptosis of RGCs after ONC, we first used microarray analysis with the retinas for screening candidate genes. Microarray analysis has been applied to characterize global gene expression changes in pathologic conditions of glaucomatous retinas so far.32–35 Despite their advantages as a high-throughput approach, there is a limitation. When performed with the whole retina, RGC-specific changes are masked by other cell types, because RGCs are a minor component in the retina. Then, we enriched RGCs by laser-captured microdissection (LCM) and carried out RT-PCR analysis for further evaluations.

**Methods**

**Optic Nerve Crush**

Eight C57BL/6 male mice at 9 weeks old were used for each group. Mice were deeply anesthetized under pentobarbital. The conjunctiva was incised, and the orbital muscles were deflected aside with care not to damage blood vessels. The optic nerves were exposed and were crushed by picking with forceps behind the eyeball.36 At 0, 1, 4 days after ONC, eyeballs were enucleated, and retinas were dissected from three mice for...
Microarray analysis, whereas another set of enucleated eyeballs was fixed by 4% paraformaldehyde to pool for immunohistochemical analysis. All animal studies were conducted in accordance with the Guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Committees for Animal Experiments of Suzuki University of Medical Science and Kindai University.

**Microarray Analysis**

Total RNAs were isolated from retinas using Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). After checking RNA quality by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), cDNA synthesis and RNA amplification were carried out using Amino Allyl aRNA kit (Thermo Fisher Scientific, Waltham, MA, USA). The resultant probes were labeled by Cy5 Mono-Reactive Dye (GE Healthcare, Little Chalfont, UK) and were hybridized on a DNA microarray, and the measurement of fluorescence was performed using a 3D-Gen Scanner 3000 (Toray, Kanagawa, Japan). Differentially expressed genes (fold changes ≥ 1.5) among three groups were selected, and we used the DAVID web tool (https://david.ncifcrf.gov/, provided in the public domain) to identify their functional annotation. The statistical significance of functional annotation analysis was determined by EASE score, and P ≤ 0.01 was considered to be significant. A PubMed search was conducted to select glaucomatous or apoptotic genes from the most changed 30 genes in microarray analysis.

**LCM and Real-Time RT-PCR**

At 0, 1, 4 days after ONC, eyeballs were collected from three mice and were divided in half at the sagittal plane. After removal of vitreous humor, the divided eyeballs were immediately embedded in frozen embedding medium (SCEM-L1; Leica, Wetzlar, Germany); 15-μm sections were mounted on membrane slides (2 μm PEN membrane; Leica) and were fixed with cold 5% acetic acid in ethanol. After staining with 0.025% toluidine blue solution, ganglion cell layer (GCL) and inner plexiform layer (IPL) were dissected from 72 frozen sections using the LCM system (LMD7000; Leica; Supplementary Fig. S1) and were directly captured into tissue lysis buffer. Total RNA was purified using RNeasy Plus Micro kit (Qiagen, Hilden, Germany) and cDNA was synthesized using Superscript IV Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturers’ instructions. RGC population in dissected RGC-enriched fraction was confirmed by relative expression level of Thy1 (Supplementary Fig. S1). Expression levels of selected genes in RGC-enriched fraction were determined by quantitative PCR using StepOnePlus real-time PCR system (StepOne software v2.3 and Power SYBR Green PCR Master Mix; Applied Biosystems, Carlsbad, CA, USA), using Gapdh as internal control. Gene-specific primers were designed with PrimerBank (https://pga.mgh.harvard.edu/primerbank/), provided in the public domain) and their sequences are listed in Supplementary Table S1. These experiments were carried out in duplicate and all data were presented as fold change compared with 0 day. Statistical analyses among three groups were evaluated with the Steel-Dwass test. A P ≤ 0.05 was considered to indicate statistical significance.

**Immunoblotting and Histologic Examination**

Polyclonal rabbit anti-Atf3 (HPA001562; Sigma-Aldrich Corp., St. Louis, MO, USA), polyclonal goat anti-Lcn2 (AF1857; R&D Systems, Minneapolis, MN, USA), and polyclonal goat anti-Tarfsf12a (ITEM-4 clone; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies were used to detect target molecules. Peroxidase-conjugated (GE Healthcare) or fluorescent-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used as secondary antibodies for immunoblotting or immunohistochemistry, respectively. Immunoblotting was carried out as described previously. In immunohistochemical analysis, rabbit antibodies to Atf3 or Tnfrf12a, the fixed eyeballs were paraffin-embedded and cut into 3-μm sections. After deparaffinization, the sections were heated at 95°C for 20 minutes in 10 mM citrate buffer (pH 6.0). For analysis of Lcn2, the fixed eyeballs were immersed in increasing concentrations of sucrose (10%–30%) and embedded in frozen medium, and cryosections (5-μm thickness) were further treated with cold methanol. Immunoreactivity of each target molecule and nuclear counterstaining were visualized with a BZ-X710 microscope (Keyence, Osaka, Japan). In situ apoptosis assay using cryoprotected retinas was carried out as in our previous study. In situ TUNEL assay kit using fluorescein-dUTP (11684795910; Roche, Mannheim, Germany) or alkyne-dUTP (C10245; Thermo Fisher Scientific) and polyclonal rabbit anti-cleaved caspase-3 antibody (9661; Cell Signaling Technology, Danvers, MA, USA) were used in this study.

## Results

### Temporal Changes of Gene Profiles Within 4 Days Postcrush in Whole Retinas

At first, we conducted the two kinds of in situ TUNEL assay using fluorescein-dUTP or alkyne-dUTP and the immunohistochemical analysis of cleaved caspase-3 to detect apoptotic cells in the retinas at 0 to 7 days after ONC (Supplementary Fig. S2). In TUNEL assay with alkyne-dUTP and cleaved caspase-3 analysis, apoptotic RGCs had begun to be detected at 4 days after ONC, whereas there were no TUNEL- or cleaved caspase-3-positive RGCs at 1 day after ONC, in agreement with published studies. However, in TUNEL assay with fluorescein-dUTP, there were no positive RGCs at 4 days after ONC; this dissociation was thought to be caused by the difference between alkyne-dUTP and fluorescein-dUTP sensitivities. According to these results, we decided to examine gene expression profiles in the retinas before 4 days of ONC to elucidate proapoptotic events in RGCs.

To explore candidate genes related to proapoptotic events, microarray analysis was carried out to profile gene expression changes in the whole retinas at 0, 1, and 4 days after ONC. We examined the expression levels of total 23,474 mouse genes by one-color fluorescence method for hybridization and compared among three DNA microarrays. Overall, the amounts of upregulated genes were larger than that of downregulated gene as the days proceeded (Fig. 1). At 1 day after ONC, when compared with 0 day, the expression levels of 1423 genes were changed more than 1.5-fold; 980 genes were upregulated and 443 genes were downregulated (Fig. 1). At 4 days after ONC, the expression levels of 2010 genes were changed from 0 day; 1386 genes were upregulated and 624 genes were downregulated (Fig. 1). The range of expression change at 4 days was wider than that at 1 day, suggesting that a more drastic alteration in gene expression had occurred just before RGC loss (at 4 days after ONC) than just after crush (at 1 day after ONC).

Using those differentially expressed gene lists that showed more than 1.5-fold change in each comparison set among three DNA microarrays, we demonstrated gene ontology analysis to annotate their biological functions. Gene categories significantly altered (P ≤ 0.01) in each comparison are listed in Table 1. The genes associated with apoptotic process, immune system process, regulation of cell cycle, and response to...
mechanical stimulus were upregulated, whereas the genes associated with visual perception and ion transport were downregulated at both days (1 or 4 days after ONC). The gene categories for regulation of cell migration or cell shape, angiogenesis, and intracellular signal transduction were altered specifically at 1 day after ONC. The gene categories for DNA replication-dependent/independent nucleosome assembly, negative regulation of gene expression by DNA methylation or chromatin silencing, and response to metal ion were altered specifically at 4 days after ONC.

Gene Expression Changes of Glaucomatous/Apoptotic Genes in RGC-Enriched Fraction Within 4 Days Postcrush

On the basis of microarray analysis with the whole retinas, the most changed 30 genes are listed in Table 2. We have selected 14 genes that are literally suggested to relate to glaucoma and/or apoptosis. To quantify gene expression changes specific to RGCs, we have dissected GCL and IPL by LCM (Supplementary Fig. S1) and have examined mRNA levels of 14 selected genes. Activation transcription factor 3 (Atf3), a member of the ATF/cyclic AMP response element-binding (ATF/CREB) family of transcription factors, increased by 56.4-fold ($P = 0.0284$) and 267-fold ($P = 0.0284$) at 1 and 4 days after ONC, respectively (Fig. 2). Lipocalin 2 (Lcn2), a multifunctional secreted protein, increased by 83.1 ($P = 0.0170$) at 1 day after ONC (Fig. 2). TNF receptor superfamily member 12a (Tnfrsf12a), also known as TNF-like weak inducer of apoptosis (TWEAK) receptor, increased by 16.8-fold ($P = 0.0110$) and 25.4-fold ($P = 0.0110$) at 1 and 4 days after ONC, respectively (Fig. 2). In our gene ontology analysis, Atf3 is a related gene to endoplasmic reticulum (ER) unfolded protein response; Lcn2 and Tnfrsf12a were included in the category of apoptotic process (Table 1). The expression levels of Metallothionein 2 (Mt2) and of Growth arrest and DNA damage-inducible 45 beta (Gadd45b) also increased significantly in RGC-enriched fraction at 4 days after ONC (Mt2, 7.16-fold, $P = 0.0110$; Gadd45b, 6.22-fold, $P = 0.0110$) (Fig. 2), Mt2 is an associated gene to the category of response to metal ion and Gadd45b is related to apoptotic process (Table 1). The expression levels of several genes, such as Protease serine 56 (Prss56), Tubulin beta 5 class I (Tubb5), Endothelin 2 (Edn2), and C1galt1-specific chaperone 1 (C1galt1c1), were not changed or detected in RGC-enriched fraction (Table 1). TNF receptor superfamily member 12a (Tnfrsf12a), also known as TNF-like weak inducer of apoptosis (TWEAK) receptor, increased by 16.8-fold ($P = 0.0110$) and 25.4-fold ($P = 0.0110$) at 1 and 4 days after ONC, respectively (Fig. 2). In our gene ontology analysis, Atf3 is a related gene to endoplasmic reticulum (ER) unfolded protein response; Lcn2 and Tnfrsf12a were included in the category of apoptotic process (Table 1). The expression levels of Metallothionein 2 (Mt2) and of Growth arrest and DNA damage-inducible 45 beta (Gadd45b) also increased significantly in RGC-enriched fraction at 4 days after ONC (Mt2, 7.16-fold, $P = 0.0110$; Gadd45b, 6.22-fold, $P = 0.0110$) (Fig. 2), Mt2 is an associated gene to the category of response to metal ion and Gadd45b is related to apoptotic process (Table 1). The expression levels of several genes, such as Protease serine 56 (Prss56), Tubulin beta 5 class I (Tubb5), Endothelin 2 (Edn2), and C1galt1-specific chaperone 1 (C1galt1c1), were not changed or detected in RGC-enriched fraction (Fig. 2), but those were changed in the rest of retinal layers outside of GCL and IPL (Fig. 5). In disagreement with the microarray data that showed that the expression levels of RNA polymerase I polypeptide C (Polr1c) and of mitogen-activated protein kinase kinase kinase 3 (Map4k3) decreased approximately 10-fold at 4 days after ONC (Table 2), RT-PCR analysis found that both genes tended to be upregulated (Figs. 2, 5). Furthermore, the results of RT-PCR analysis for DNA damage-inducible transcript 4 (Ddit4), Crystallin beta B2 (Crybb2), and Crystallin alpha A (Cryaa) were different from the microarray data, those expression levels were not changed in RT-PCR analysis (Table...
expression level at both 1 and 4 days after ONC (Fig. 4A). Finally, to investigate the in situ localization of Atf3, Lcn2, and Tnfrsf12a, we carried out immunohistochemistry. At 0 day of ONC, there was low Atf3-, Lcn2-, or Tnfrsf12a-immunoreactivity in the increased mRNA level of Lcn2 proteins translating in outer layers at 4 days after ONC (Fig. 3), most of Lcn2 proteins translating in outer layers at 4 days after ONC (Fig. 4B).

**DISCUSSION**

Using a combination of gene profiles of the retinas and RGC-enriched fraction, we determined that seven genes, Atf3, Lcn2, Tnfrsf12a, M12, Gadd45b, Polr1c, and Mapk4, were upregulated in response to crush in RGC-enriched fraction. Among them, we focused on the three molecules, Atf3, Lcn2, and Tnfrsf12a.

**Atf3, Lcn2, and Tnfrsf12a Expressed Highly in GCL of Crushed Retinas**

To confirm the protein levels of Atf3, Lcn2, and Tnfrsf12a, we have demonstrated immunoblotting of retinas at 0, 1, 4 days after ONC. Protein expression changes were well correlated with the results of RTPCR: Atf3 and Tnfrsf12a were increased progressively during 4 days after ONC, Lcn2 showed high expression level at both 1 and 4 days after ONC (Fig. 4A). Finally, to investigate the in situ localization of Atf3, Lcn2, and Tnfrsf12a, we carried out immunohistochemistry. At 0 day of ONC, there was low Atf3-, Lcn2-, or Tnfrsf12a-immunoreactivity in the increased mRNA level of Lcn2 proteins translating in outer layers at 4 days after ONC (Fig. 3), most of Lcn2 proteins translating in outer layers at 4 days after ONC (Fig. 4B). Considering the increased mRNA level of Lcn2 in outer layers at 4 days after ONC (Fig. 3), most of Lcn2 proteins translating in outer layers might be secreted outside the tissue. Tnfrsf12a expression increased from GCL through the inner nuclear layer at 4 days after ONC (Fig. 4B).

**TABLE 2.** Top 30 Genes Most Significantly Altered Within 4 Days After ONC in Whole Retinas

<table>
<thead>
<tr>
<th>Gene Name (Symbol)</th>
<th>Fold Change, 0 vs. 1</th>
<th>Fold Change, 0 vs. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease, serine 56 (Prss56)</td>
<td>31.6</td>
<td>6.77</td>
</tr>
<tr>
<td>Cysteine-rich protein 61 (Cyr61)</td>
<td>26.9</td>
<td>3.45</td>
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<tr>
<td>Tabulin, beta 5 class I (Tubb5)</td>
<td>15.1</td>
<td>10.1</td>
</tr>
<tr>
<td>S100 calcium-binding protein A6 (S100a6)</td>
<td>8.50</td>
<td>9.31</td>
</tr>
<tr>
<td>Metallothionein 2 (M12)</td>
<td>7.79</td>
<td>2.57</td>
</tr>
<tr>
<td>Endothelin 2 (Edn2)</td>
<td>6.88</td>
<td>11.2</td>
</tr>
<tr>
<td>Lipocalin 2 (Lcn2)</td>
<td>6.86</td>
<td>2.03</td>
</tr>
<tr>
<td>Growth arrest and DNA damage-inducible 45 beta (Gadd45b)</td>
<td>6.65</td>
<td>2.77</td>
</tr>
<tr>
<td>TNF receptor superfamily, member 12a (Tnfrsf12a)</td>
<td>6.23</td>
<td>4.14</td>
</tr>
<tr>
<td>DNA damage-inducible transcript 4 (Ddit4)</td>
<td>1.08</td>
<td>4.29</td>
</tr>
<tr>
<td>Nuclear receptor subfamily 3, group E, member 3 (Nr3e3)</td>
<td>1.01</td>
<td>-4.33</td>
</tr>
<tr>
<td>RNA polymerase 1 poly peptide C (Polr1c)</td>
<td>-1.08</td>
<td>-11.7</td>
</tr>
<tr>
<td>Mitogen-activated protein kinase kinase 3 (Mapk3)</td>
<td>-1.19</td>
<td>9.09</td>
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<tr>
<td>NIM1 serine/threonine protein kinase (Nim1K)</td>
<td>-1.25</td>
<td>-7.84</td>
</tr>
<tr>
<td>CDK5 regulatory subunit associated protein 1 (Cdk5rap1)</td>
<td>-3.88</td>
<td>-1.26</td>
</tr>
<tr>
<td>Ribosomal L24 domain containing 1 (Rsl24d1)</td>
<td>-4.09</td>
<td>-1.12</td>
</tr>
<tr>
<td>Cigalt1-specific chaperone 1 (Cigalt1c1)</td>
<td>-5.21</td>
<td>-1.31</td>
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<tr>
<td>Crystallin, beta A2 (Cryba2)</td>
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<td>15.5</td>
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<tr>
<td>Crystallin, gamma S (Crygs)</td>
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<td>17.3</td>
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<tr>
<td>Crystallin, beta A1 (Cryba1)</td>
<td>-21.7</td>
<td>16.4</td>
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<tr>
<td>Crystallin, beta B2 (Crybb2)</td>
<td>-23.8</td>
<td>15.4</td>
</tr>
<tr>
<td>Crystallin, alpha A (Cryaa)</td>
<td>-44.5</td>
<td>19.1</td>
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</table>

Genes in bold were selected for analysis by real-time RTPCR.

**TABLE 1.** Gene Categories Significantly Altered Within 4 Days After ONC in Whole Retinas

<table>
<thead>
<tr>
<th>Gene Category</th>
<th>Genes, ( n )</th>
<th>( P )</th>
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</thead>
<tbody>
<tr>
<td>0 day versus 1 day (Upregulated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptotic process</td>
<td>60</td>
<td>( 7.75E^{10} )</td>
</tr>
<tr>
<td>Immune system process</td>
<td>43</td>
<td>( 3.75E^{-8} )</td>
</tr>
<tr>
<td>Response to hypoxia</td>
<td>23</td>
<td>( 7.83E^{-5} )</td>
</tr>
<tr>
<td>Positive regulation of I-kappaB kinase/NF-kappaB signaling</td>
<td>19</td>
<td>( 1.19E^{-4} )</td>
</tr>
<tr>
<td>Aging</td>
<td>20</td>
<td>( 4.63E^{-4} )</td>
</tr>
<tr>
<td>Positive regulation of cell migration</td>
<td>21</td>
<td>( 7.80E^{-4} )</td>
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<tr>
<td>Positive regulation of transcription from RNA polymerase II promoter</td>
<td>66</td>
<td>( 9.21E^{-4} )</td>
</tr>
<tr>
<td>ER unfolded protein response</td>
<td>9</td>
<td>0.0019</td>
</tr>
<tr>
<td>Regulation of cell cycle</td>
<td>14</td>
<td>0.0043</td>
</tr>
<tr>
<td>Actin cytoskeleton organization</td>
<td>16</td>
<td>0.00162</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>22</td>
<td>0.00217</td>
</tr>
<tr>
<td>Response to mechanical stimulus</td>
<td>10</td>
<td>0.00247</td>
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<tr>
<td>Cell adhesion</td>
<td>36</td>
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<tr>
<td>Regulation of cell shape</td>
<td>15</td>
<td>0.00553</td>
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<td>Brain development</td>
<td>20</td>
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<tr>
<td>Intracellular signal transduction</td>
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<td>0.00683</td>
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<tr>
<td>Positive regulation of axon extension</td>
<td>7</td>
<td>0.00740</td>
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<tr>
<td>(Downregulated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visual perception</td>
<td>14</td>
<td>2.13E^{-7}</td>
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<tr>
<td>Eye development</td>
<td>5</td>
<td>0.00568</td>
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<tr>
<td>Ion transport</td>
<td>19</td>
<td>0.00564</td>
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<tr>
<td>0 day versus 4 day (Upregulated)</td>
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<td></td>
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<tr>
<td>Immune system process</td>
<td>58</td>
<td>8.87E^{-11}</td>
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<tr>
<td>DNA replication-dependent nucleosome assembly</td>
<td>11</td>
<td>1.80E^{-5}</td>
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<td>DNA replication-independent nucleosome assembly</td>
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<td>Protein heterotrimerization</td>
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<td>DNA methylation on cytosine</td>
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<td>Apoptotic process</td>
<td>56</td>
<td>2.54E^{-4}</td>
</tr>
<tr>
<td>Regulation of cell cycle</td>
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<td>3.81E^{-4}</td>
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<tr>
<td>Response to peptide hormone</td>
<td>14</td>
<td>5.44E^{-4}</td>
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<tr>
<td>Aging</td>
<td>23</td>
<td>0.00132</td>
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<tr>
<td>Chromatin silencing at rDNA</td>
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<td>0.00149</td>
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<td>Response to mechanical stimulus</td>
<td>11</td>
<td>0.00579</td>
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<tr>
<td>Response to hypoxia</td>
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<td>0.00866</td>
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<tr>
<td>Response to metal ion</td>
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<td>0.00985</td>
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<tr>
<td>Ion transport</td>
<td>37</td>
<td>5.51E^{-7}</td>
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<tr>
<td>Visual perception</td>
<td>16</td>
<td>1.20E^{-6}</td>
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<td>Chemical synaptic transmission</td>
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<td>3.25E^{-6}</td>
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<tr>
<td>Neuron differentiation</td>
<td>10</td>
<td>0.00567</td>
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</table>

2, Figs. 2, 3). One possibility for these dissociations between microarray and RTPCR is transfer of lens RNA to retinal sample in microarray analysis as described in a previous study, especially in those with crystallin genes. 40
FIGURE 2. mRNA levels of candidate genes for glaucomatous/apoptotic pathway in RGC-enriched fraction. mRNA level of each gene at 0, 1, and 4 days after ONC was analyzed by RT-PCR using dissected RGC-enriched fraction. Expression levels are represented as means ± SE of fold changes compared with 0 day. *P ≤ 0.05 in 0 vs. 1 day, †P ≤ 0.05 in 0 vs. 4 days, ‡P ≤ 0.05 in 1 vs. 4 days.
FIGURE 3. mRNA levels of candidate genes for glaucomatous/apoptotic pathway in residual retinal layers except GCL and IPL. mRNA levels of each gene at 0, 1, and 4 days after ONC were analyzed by RT-PCR using dissected retinal layers except GCL and IPL. Expression levels are represented as means ± SE of fold changes compared with 0 day. *P ≤ 0.05 in 0 vs. 1 day, †P ≤ 0.05 in 0 vs. 4 days, ‡P ≤ 0.05 in 1 vs. 4 days.
Tnfrsf12a, and confirmed their increasing protein levels and in situ localization. To date, multiple studies have revealed that Atf3, Lcn2, and Tnfrsf12a are upregulated in a rodent model of optic neuropathy, by using whole transcriptome analysis (Supplementary Table S2). In the ONC model, by using isolated RGCs with fluorescence-activated cell sorting (FACS), Fischer et al.41 showed Atf3, Lcn2, and Tnfrsf12a upregulation at 4 days after ONC. Yasuda et al.42–44 further confirmed this evidence in whole retinas at 2 days after ONC by using RNA sequencing or cap analysis of gene expression technologies. Sharma et al.45 investigated temporal gene expression changes in retinas of two models of optic neuropathy: ONC and ocular hypertension. Atf3 and Lcn2 were upregulated significantly

**Figure 4.** Protein levels and localizations of Atf3, Lcn2, and Tnfrsf12a in retinas at 0, 1, and 4 days after ONC. (A) Immunoblotting of Atf3, Lcn2, and Tnfrsf12a. Lamin B (Lmnb) and Gapdh were used as a loading control for nuclear and cytoplasmic fractions, respectively. Graphs represent fold changes of relative protein levels normalized with loading controls. (B) Immunohistochemical analysis of Atf3, Lcn2, and Tnfrsf12a in retinas at 0 (left) and 4 (right) days after ONC. Atf3-, Lcn2-, or Tnfrsf12a-immunoreactivity is shown by green, and nuclear staining is shown by blue. INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars: 20 µm
within 3 days after treatments in both models. Steele et al. and Panagis et al. reported increased Lcn2 expression level in the retinas and the focal loss area of GCL in DBA/2J mice, a model for chronic glaucoma with ocular hypertension. In the ocular hypertension model, LMD technique has been used to enable the whole transcriptome analysis. In these cases, GCL is disected by LCM in an experimental ocular hypertension rat. Overall, these previous studies of whole transcriptome analysis have provided important pictures for gene interaction networks. However, in most cases, the confirmatory analysis of protein level is lacking (Supplementary Table S2), even though mRNA levels do not always correlate with protein levels.1,2 Although our finding is not novel, it strongly supports previous studies and suggests Atf3, Lcn2, and Tnfrsf12a might be promising targets in future studies.

The LCM technique, which we used in this study, has advantages and disadvantages when compared with FAC5: unlike FAC5, LCM does not need labeling or enzymatic dissociation steps before collecting specific cells; therefore, cell morphology and cell-cell interactions are preserved in LCM; LCM allows comparison of the spatial expression pattern in the same tissue section. The purity of the separated population in LCM is lower than FAC5, because LCM separation relied on cell morphology and localization, whereas FACS separation relied on specific gene expression. In this study, GCL and IPL were dissected by LCM. Therefore, our dissected RGC-enriched fraction was contaminated by displaced amacrine cells mainly, but also by Müller cell endfoot, glial cells, axons of bipolar cells, and blood vessels. Although it remains ambiguous, unlike RGCs, displaced amacrine cells in GCL suggested being unaffected after ONC. Moreover, because Atf3, Lcn2, and Tnfrsf12a upregulation after ONC had been found in isolated retrograde labeling RGCs by FAC5, whose purity reaches up to 96%. mRNA levels do not always correlate with protein levels.1,2 Further evaluations using specific markers for each cell type are needed.

Signal transduction research has revealed signaling pathway in RGCs in response to axonal injury/axotomy. Among these pathways, phosphatidylinositol-3-kinase (PI3K)/Akt pathway, Bcl-2 family, caspase family are thought to be important for RGC death. ONC rapidly leads to elevation of dual leucine zipper kinase (Map3k12) expression, an initiator of c-Jun N-terminal kinase pathway that mediates proapoptotic gene expression in RGCs. In contrast, the PI3K/Akt pathway is known as a survival pathway. Several neuroprotective treatments activate PI3K/Akt signaling and prevent RGC death after ONC. Bcl-2 family members are well known to play a pivotal role in the apoptotic pathway. Proapoptotic Bcl-2 members, Bim, Bid, Bbc3 (p53-upregulated-modulator-of-apoptosis, PUMA) activate Bax/Bak and lead to mitochondrial-mediated apoptosis. Many studies have indicated the involvement of these Bcl-2 members in RGC apoptosis after ONC.

Atf3 has been known to be a stress response gene; its expression increases by several stresses, such as ischemia, wounding, toxicity, and injury. In response to ER stress, Atf3 expression is upregulated by the action of Atf4 or Atf6, other members of ATF/CREB family. These transcriptional factors induce the expressions of ER stress genes, C/EBP homologous protein (CHOP, also known as Ddit3 or Gadd153), and Gadd34 (also known as protein phosphatase 1 regulatory subunit 15A [Ppp1r15a]). In our microarray analysis, the expression level of Atf4 in crushed retinas was unchanged, but we found that the expressions of Atf6, CHOP, and Gadd34 coordinately increased (Atf6: 1.59-fold, CHOP: 1.55-fold, Gadd34: 5.18-fold, in comparison between 0 day and 1 day). Meanwhile, other studies investigating Atf3 functions in the nervous system have identified Atf3 involvements in axonal regeneration. Atf3 expression is activated following crush, and it conducts axonal regeneration as a transcriptional factor in various types of neurons. In zebrafish, whose optic nerves are known to be regenerative after ONC, Atf3 upregulation is also found in the GCL of retinas and optic nerves. Atf3 upregulates the genes of c-Jun, Heat shock protein 27, Small proliferine-rich protein 1a (Sprr1a), Galanin (Gald), Growth-associated protein 43 (Gap43), and Damage-induced neuronal endopeptidase (DINE) to promote neurite outgrowth. Some of these Atf3-targeted genes are involved in axonal regeneration by modifying cytoskeletal organization, which was identified as changed in our microarray analysis (Sprr1a: 1.53-fold, Gal: 4.49-fold, Gap43: 1.98-fold when compared between 0 day and 4 days).

In summary, our study provides early gene expression changes in GCL before RGC death. Some of them are potentially neuroprotective, whereas others are neurodestructive. The growing availability of molecularly targeted drugs that enhance survival processes or inhibit apoptotic processes may provide future avenues for preventing RGC loss in glaucoma.

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References


