LEDGF: Survival of Embryonic Chick Retinal Photoreceptor Cells

Makoto Nakamura, Dhirendra P. Singh, Eri Kubo, Leo T. Chylack, Jr, and Toshimichi Shinohara

Purpose. Lens epithelium–derived growth factor (LEDGF) is a novel adhesive, survival, and growth factor for lens epithelial cells, keratinocytes, fibroblasts, and cos7 cells. In the presence of LEDGF, these cells acquire resistance to environmental stresses, and in the absence of LEDGF they die. The effects of LEDGF on survival of embryonic chick retinal photoreceptor cells under serum starvation and heat stress were studied.

Methods. The expression pattern of LEDGF in embryonic chick retinal photoreceptor cells was investigated with protein blot analysis and immunohistochemistry using antibodies (Abs) to LEDGF. Retinal cells were cultured in serum-free medium for up to 6 days in the presence of varying amounts of LEDGF at 37° or 41°C. The photoreceptor cells were immunostained with Abs to arrestin and counted to evaluate the photoreceptor cell viability. Heat shock proteins in the cultured cells were quantified by protein blot analysis with Ab probes and semiquantitative reverse transcription–polymerase chain reaction analysis.

Results. LEDGF was found predominantly in the nucleus of neuroretinal cells, including photoreceptor cells. In the presence of LEDGF, photoreceptor cells manifested increased resistance to serum starvation and thermal stress and survived for a longer period. The levels of heat shock protein 90 were elevated in those cells. Most retinal cells died in the absence of LEDGF.

Conclusions. LEDGF enhanced survival of retinal photoreceptor cells under serum starvation and heat stress. Thus, LEDGF has a potency to enhance survival of neuronal cell types against environmental stresses, and it may be applicable as a therapeutic agent for those cells. (*Invest Ophthalmol Vis Sci.* 2000;41:1168–1175)

We have isolated a clone encoding a novel adhesive, growth, and survival factor from a human lens epithelial cell (LEC) cDNA library and named it "lens epithelium–derived growth factor" (LEDGF).[1] LEDGF is a secreted protein found at low levels in most tissues. It is taken up by cells and transported through the cytoplasm into the nucleoplasm.[2] LEDGF enhances adhesion, stimulates growth, and prolongs survival of mouse and human LECs, mouse keratinocytes, monkey kidney cos7 cells, and human fibroblasts cultured in a serum-free environment.[1,2] In the absence of exogenous LEDGF, most of the above-mentioned cells die after 2 to 7 days in culture, despite the fact that LEDGF is synthesized endogenously.[2] Antibodies (Abs) to LEDGF are prevalent in human serum[1] and are able to kill LECs in whole lenses.[3]

LEDGF belongs to a family of homologous proteins including hepatoma-derived growth factor (HDGF)[4] and HDGF-related proteins-1 and -2 (HRP-1 and HPR-2).[5] In addition, LEDGF has turned out to be identical to p75, a coactivator of transcription.[6]

We have shown that LEDGF enhances the resistance and prolongs the survival of LECs in culture when exposed to oxidative and thermal stress. LEDGF also increased the synthesis of heat shock protein (Hsp)27 and αB-crystallin but not Hsp90, Hsp70, Hsp60, or Hsp32.[2] Hsps protect a variety of cell types and enable cells to survive and recover from various cellular stresses.[7,8] In addition, they are molecular chaperones.[9,10] We have shown also that heparin potentiates the growth-enhancing properties of LEDGF, protects it from proteolytic degradation, and facilitates its uptake into the cytoplasm and its transport into the nucleoplasm.[11]

Nothing was known about the functional role of LEDGF in neuronal cells. In this article, we describe the localization of LEDGF in embryonic chick retina and characterize the survival-enhancing effects of LEDGF on cultured embryonic chick retinal photoreceptor cells. Because there is a well-established system of embryonic chicks retinal cell cultures, we took advantage of this to investigate the survival potency of LEDGF in embryonic retinal photoreceptor rods, the most abundant cell type in retinas. We investigated further a mechanism by which LEDGF prolonged the survival of photoreceptor cells.

Methods

Immunostaining of Embryonic Chick Retinas

Eyes of embryonic 14-day-old (E14) chickens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24

From The Center for Ophthalmic Research, Brigham and Women's Hospital; and The Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts.

Supported in part by NIH-sponsored RO1 project Grants EY10958, EY10824, and EY12015 (Bethesda, Maryland); Massachusetts Lions Eye Research Fund (Northborough, Massachusetts); and Shojin Research Associates fund (Studio City, California).

Submitted for publication August 4, 1999; revised October 22, 1999; accepted November 16, 1999.

Commercial relationships policy: N.

Corresponding author: Toshimichi Shinohara, The Center for Ophthalmic Research, Brigham and Women's Hospital, 221 Longwood Avenue, Room 107, Boston, MA 02115. tshinohara@rics.bwh.harvard.edu

hours, embedded in paraffin, and sectioned at approximately 10 μm. The sections were deparaffinized and placed in 0.3% hydrogen peroxide in methanol solution to block endogenous peroxidase activity. After being washed in PBS, they were immersed in 0.01 M citrate buffer (pH 6.0) and boiled for 10 minutes. After being cooled down and washed in PBS, they were incubated for 30 minutes at room temperature with 1% normal goat serum, followed by overnight incubation with affinity-purified rabbit Abs to LEDGF (1:100 dilution in PBS containing 5% dry milk) or Abs to Hsp90 (1:500 dilution in PBS containing 5% dry milk) at 4°C. They were then incubated with secondary Abs (biotinylated anti-rabbit immunoglobulins in PBS, containing carrier protein, and avidin–peroxidase–conjugated streptavidin, DAKO) for 2 hours at room temperature, followed by a 2-hour exposure to DAKO. After washing in PBS, the color was developed with 0.02% 3,3-diamino-benzidine-tetra hydrochloride (DAB; Bio-Rad Laboratories, Hercules, CA) and 0.04% hydrogen peroxide in Tris-buffered saline containing 1 mM $CaCl_2$, pH 7.6. Negative controls were incubated with Abs to LEDGF absorbed with full-size glutathione-S–transferase (GST)–LEDGF fusion protein[1,2] (1:100 dilution in PBS containing 5% dry milk) or a prebled rabbit serum (for Hsp90) overnight at 4°C. The immunostained specimens were counterstained with hematoxylin.

Cell Culture

Eyes of E15 chickens were dissected in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (CMF; GIBCO–BRL, Grand Island, NY). Neural retinas were then isolated from retinal pigment epithelium under a dissecting microscope. The retinas were incubated in CMF containing 0.25% trypsin at 37°C for 20 minutes, rinsed with Neurobasal (GIBCO–BRL), and dissociated by trituration through fire-polished Pasteur pipets. Twenty thousand to 28,000 neuroretinal cells in 0.5 ml of Neurobasal containing B27 supplement (GIBCO–BRL) were plated in 24-well tissue culture dishes (Falcon, Lincoln, NJ) that had been precoated with 0.1 mg/ml of poly-L-ornithine (Sigma Chemical, St. Louis, MO), for 1 hour at 37°C and incubated at 37°C in humidified 5% CO_2. We counted cell number under a microscope using the standard hemocytometer.

After culturing for 1 day, when approximately 50% to 70% of cells had attached to the bottom of the dishes, the cells were washed twice with Neurobasal and further cultured in 0.5 ml of Neurobasal without B27 supplement but with various concentrations of bacteria-expressed GST–LEDGF, GST, or purified LEDGF, which was prepared by cleaving GST–LEDGF with thrombin protease (Pharmacia Biotech, Piscataway, NJ).[1,2] In cytotoxicity experiments on Ab to LEDGF, the 24-well plates were washed and further cultured in 0.5 ml of Neurobasal with 10% fetal calf serum (FCS) and Abs to LEDGF (diluted 1/100). The Abs to LEDGF were to the C-terminal region of LEDGF.[1,2]

Immunostaining of Cultured Cells

The cultured cells were washed with PBS, fixed by cold methanol, dried, washed 3 times with PBS, permeabilized by 1% H_2O_2 in PBS for 10 minutes at room temperature, washed twice with PBS, and incubated with 10% bovine serum albumin (BSA) in PBS for 1 hour at room temperature to avoid nonspecific reactions. They were incubated with Ab to arrestin[12] (1:200 dilution) as the primary Ab in 1% BSA in PBS for 1 hour at room temperature and washed 3 times with PBS, followed by incubation with biotinylated affinity-purified anti-rabbit IgG (6.75 μg/ml) and blocked with normal horse serum (1:74 dilution) in PBS for 30 minutes. They were washed thrice with PBS, incubated with avidin and biotinylated peroxidase thrice at room temperature, and washed thrice with PBS. The biotinylated affinity-purified anti-rabbit IgG, avidin, and biotinylated peroxidase were from ImmunoPure Ultra-Sensitive ABC Peroxidase Staining Kit (Pierce, Rockford, IL) and were used according to its protocol. The color was developed with 0.04% hydrogen peroxide and 0.05% DAB.

Protein Extraction from Retinal Cells

Neuroretinal tissue from 6 eyes of E13 chickens were lysed in cold RIPA buffer (1% detergent NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS] in PBS). The sample was passed through a 22-gauge needle several times and then placed on ice for 1 hour. The lysate was centrifuged at 15,000*g* for 10 minutes, and the supernatant was stored at −20°C until used for protein blot analysis. To study Hsp expression induced by LEDGF, 8×10^5 embryonic chick neuroretinal cells were cultured for 24 hours in 6-well tissue culture dishes in Dulbecco's minimal essential medium (DMEM) with 10% FCS. Cells were washed with DMEM twice and further cultured at 37°C for 2 days in 2 ml of serum-free DMEM containing 100 ng/ml of GST–LEDGF fusion protein or GST protein. The wells were then washed thrice with PBS and scraped with a cell scraper (Costar, Cambridge, MA) under 0.3 ml of cold RIPA buffer. The protein concentration was determined with the Bradford method.[13]

Protein Blot Analysis

Proteins prepared from chick retinas were dissolved in SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer, separated on 10% SDS–PAGE, and blotted onto Immobilon-P (Millipore, Bedford, MA). The filters were preincubated with 7% (wt/vol) skim milk in PBS at 4°C overnight and then incubated for 2 hours at room temperature with rabbit Ab to LEDGF (C-terminal peptide) as primary Ab at 1:1000 dilution in PBS containing 0.05% Tween-20 (PBS–T) and 1% (wt/vol) skim milk. The filters were washed 3 times with PBS–T and incubated with anti-rabbit IgG labeled with horseradish peroxidase (1:1000 dilution, 200 ng/ml; Santa Cruz Biochemistry, Santa Cruz, CA) as a second Ab. Color was developed with 0.04% hydrogen peroxide and 0.05% DAB by following the company's protocol (Bio–Rad Laboratories, Hercules, CA). To confirm specificity of the Ab to LEDGF, the Ab was neutralized with the same volume of purified GST–LEDGF (1 g/l) overnight at 4°C. For Western blotting of Hsps, Abs to Hsp (goat polyclonal IgGs; 1:1000 dilution, 200 ng/ml; Santa Cruz Biochemistry) and anti-goat IgG labeled with horseradish peroxidase (1:2000 dilution, 200 ng/ml; Santa Cruz Biochemistry) were used. Optical densitometry (OD_{500nm}) of the immunostained filters with a optical densitometer (E.C. Apparatus Corp., St. Petersburg, FL) was used to quantitate Hsps. Protein size markers were purchased from Bio–Rad Laboratories.

Isolation of mRNA and Reverse Transcription–Polymerase Chain Reaction

For mRNA isolation, 2.5×10^6 of E15 chick retinal cells were cultured in a poly-L-ornithine–coated 10-cm culture dish in 13 ml of Neurobasal with B27 supplement. After 24 hours, the

cells were washed with Neurobasal and further cultured in Neurobasal with 100 ng/ml of GST–LEDGF or GST, without B27, for 2 days at 37°C. Messenger RNA was isolated from the cells with a MicroFastTrack 2.0 Kit (Invitrogen, Carlsbad, CA). The RNA concentration in diethylpyrocarbonate (DEPC; Sigma)-treated water at 100 ng/ml was measured spectrophotometrically at 260 nm. Reverse transcription (RT) was performed in 20 μl of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 1.0% Triton X-100, 1 mM of each dNTP, 100 pmol of each specific 3′ primer, 0.5 U/l of RNase inhibitor, 0.25 U/l reverse transcriptase and 100 ng of each mRNA, at 42°C for 1 hour and then 95°C for 5 minutes. After RT, the reaction mixture was supplemented with100 pmol of each specific 5′ primer and 5 units of *Taq* DNA polymerase (Promega, Madison WI) in a volume of 100 μl of the same buffer as for RT for use in PCR. After denaturation for 5 minutes at 94°C, 15, 20, 25, and 30 cycles of PCR amplification (denaturation at 94°C for 30 seconds, annealing at 55°C for 20 seconds, elongation at 72°C for 20 seconds) were carried out, followed by final extension for 5 minutes at 72°C. The PCR products were electrophoresed in a 1.3% agarose gel and visualized by ethidium bromide staining. The value of each band was measured with image-analysis (C-80 Epi-Illumination UV Darkroom, New England Scientific Associates, Salem, NH, and Scion Image 1.62, NIH). The primer sets were purchased from GIBCO BRL. The sequences of oligonucleotide probes were as follows: Hsp90, 5′ primer, 5′-ACTTTTGTCTGCATTCCCTC, bp 2310 to 2329, and 3′ primer, 5′-GAACACCCAGATGTCATACC, bp 2543 to 2562.

Results

LEDGF Present in Most Neuroretinal Cells

Protein blot analysis with Ab to LEDGF revealed a 60-kDa band in proteins from the E13 retina (Fig. 1, lane 1). The intensity of the band was greatly diminished when the blotting membrane containing the same protein samples was immunostained with the Ab neutralized with purified GST–LEDGF (Fig. 1, lane 2). This result indicated that LEDGF (60-kDa band) was expressed in the neuroretinal cells.

Immunohistochemistry of E14 chick retinas with Ab to LEDGF revealed strong immunostaining in the nucleus of most retinal cells including photoreceptors (Fig. 2A). All retinal cells in adult rats were positively immunostained (Eri Kubo, unpublished data, September 1999). Our limited observation suggests that cells having LEDGF live a long time but that cells without it die within short time. In contrast, we observed no staining in the retinal tissues with Ab to LEDGF neutralized with GST–LEDGF (Fig. 2B).

LEDGF-Promoted Survival of Photoreceptor Cells

We investigated whether LEDGF promotes survival of embryonic chick retinal photoreceptors in culture. Retinal cells were cultured for 1 day in Neurobasal with a B27 supplement. The cells were washed twice with Neurobasal, then further cultured up to 6 days in serum-free Neurobasal with or without 100 ng/ml of either LEDGF or GST–LEDGF. No additional LEDGF was added to the medium during the 6-day cultures. Live cells remained attached to the bottom of the dishes, and dead cells floated off into the culture medium. The number of photoreceptors among the live cells was quantified by immunostaining with Ab to arrestin,[12] as shown in Figure 3. Photoreceptor cells were the most abundant cell type found in the embryonic retinal cell culture[14] and they were labeled by Abs to arrestin. In the presence of LEDGF, nearly 50% of the cells survived by the end of the 1st day, 20% to 40% of cells survived through day 6 (Fig. 4A).

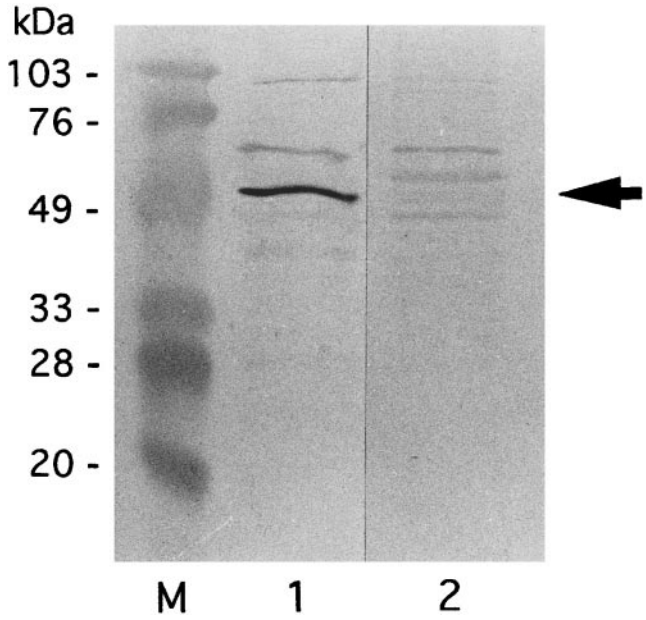

Figure 1. LEDGF is present in cells from E14 chick neuroretinas. The Immobilon-P membranes were immunostained with rabbit anti-LEDGF Abs (*lane 1*), or anti-LEDGF Abs neutralized with purified LEDGF (*lane 2*). M, molecular size markers. The *arrow* indicates LEDGF band.

Next we tried to determine the optimal concentration of LEDGF for survival of photoreceptors in serum-free medium. Similarly, retinal cells were cultured for 1 day, then further cultured for 6 days in serum-free Neurobasal with or without LEDGF or GST–LEDGF. All photoreceptors died in the medium with 0.01 ng/ml of LEDGF, in the GST (10^{-3}–10^{3} ng/ml), or in medium with no additive (Fig. 4B). More than 35% of cells survived in >0.1 ng/ml of LEDGF for 6 days' culture (Fig. 4B). Our results indicated that both GST–LEDGF and LEDGF at concentrations between 0.1 ng/ml and 100 ng/ml showed survival potency on the photoreceptor cells; an optimal concentration of LEDGF for photoreceptor survival was 100 ng/ml.

LEDGF-Promoted Survival of Retinal Photoreceptors from Serum Starvation and Thermal Stress

We investigated whether LEDGF promoted survival of photoreceptors under serum starvation and thermal stress. Twenty thousand to 28,000 cells were incubated at 41°C in triplicate in 24-well culture plates with 0.5 ml of Neurobasal with B27 supplement. The cells were washed twice with Neurobasal and cultured at 41°C for 5 more days in serum-free Neurobasal with or without LEDGF. In the absence of LEDGF, more than 95% of the cells died within 1 day, and no cell was alive after 2 days. In contrast, In the presence of LEDGF, more than 40% of the cells were alive at the end of the 1st day, and some cells survived through the 4th and 5th days in culture (Fig. 5). These data suggested that LEDGF enhanced cell survival under serum starvation and hyperthermic conditions.

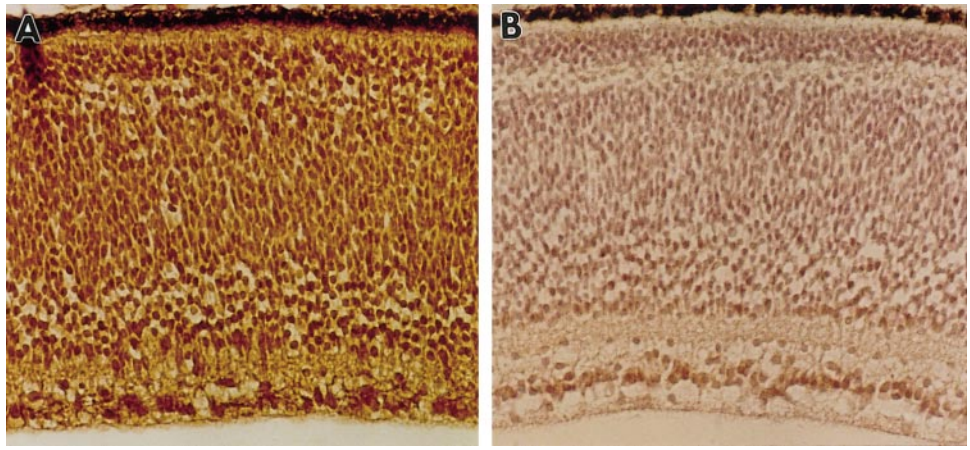

FIGURE 2. Immunohistochemistry of E14 chick retinas stained with Abs to LEDGF. The retinal sections were immunostained with Abs to LEDGF (**A**) or with Abs to LEDGF neutralized with GST–LEDGF (**B**). The retinal section was counterstained with hematoxylin. Photoreceptor cells (*middle* of photograph) were not fully differentiated, and retinal pigmented epithelial cells (*top* of photograph) and ganglion cells (*bottom* of photograph) were seen.

Effects of Ab to LEDGF on Photoreceptor Cells

To demonstrate the importance of LEDGF as a survival factor in a different way, we blocked the LEDGF effect with Ab to LEDGF. Primary retinal cells were cultured for 1 day and further cultured in Neurobasal containing 10% FCS and Ab to LEDGF (final concentration, 1/100 dilution). We speculate that FCS contains LEDGF, because most retinal cells, including photoreceptors, survived well in 10% FCS (data not shown). In the presence of the Ab to LEDGF, more than 70% of photoreceptors died after 1 day, and all cells died within 2 days (Fig. 6). In the absence of the Ab, more than 60% of the photoreceptors survived after 2 days. This toxic effect of the Ab was abolished by the addition of 1 ml LEDGF (100 μg/ml in PBS) to 1 ml of Ab to LEDGF (data not shown). These results suggest that LEDGF in the FCS is depleted by Ab to LEDGF and that the depletion of LEDGF kills photoreceptor cells.

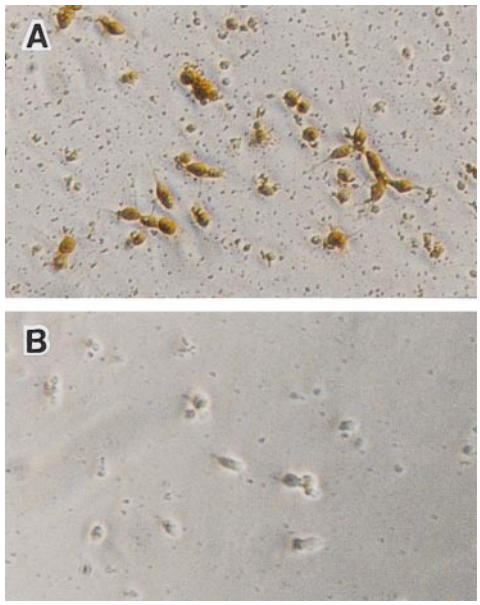

FIGURE 3. Immunostaining of cultured chick retinal cells with Abs to arrestin. Forty thousand of E15 chick retinal cells were cultured for 1 day in 0.5 ml of Neurobasal with a B27 supplement, then cultured in Neurobasal in the presence of 100 ng/ml of GST–LEDGF for 6 days, and stained with rabbit antiserum to arrestin.[12] Color was developed by DAB methods. (**A**) Immunostained with antiserum to arrestin (1/200 dilution). (**B**) Immunostained with normal rabbit serum (1/200 dilution) as a control.

LEDGF-Stimulated Expression of Hsp90 in Photoreceptors

We speculated that enhancement of survival of photoreceptors in the presence of LEDGF was related to expression of Hsps. Primary mixed neuroretinal cells were cultured at 37°C for 2 days with or without LEDGF. The amounts of each Hsp (90, 70, 60, 32, and 27) and of αB-crystallin were determined on the blot with each of the Ab probes. The level of Hsp90 was twice as high as in the control cells as determined by densitometric analysis in the cells cultured with LEDGF (Figs. 7A and 7B). Hsp60, expressed in the retinal cells at a higher level, was not stimulated by LEDGF (Figs. 7C and 7D). Other Hsps (70, 32, and 27) and αB-crystallin were undetectable by protein blotting analysis (data not shown).

To confirm that Hsp90 was stimulated in the neuroretinal cells, we conducted semiquantitative RT–polymerase chain reaction (RT–PCR) analysis with probes specific to chicken Hsp90. Similarly, chick retinal cells were cultured for 2 days with or without LEDGF. After 20 cycles of PCR, a DNA band on an agarose gel was detected in the cells treated with LEDGF, but no band was detected in nontreated cells. After 25 cycles, DNA bands were detected in both the treated and the nontreated cells, but the band from the treated cells was more intense (Fig. 8). Densitometric analysis showed the intensity of the bands from the LEDGF preparation to be 1.8 and 59.5 U by 20 and 25 cycles, respectively. In control experiments, intensity in GST-treated and untreated cells was 0 and 28.2 U and 0 and 13.6 U by 20 and 25 cycles, respectively. Thus, mRNA of the Hsp90 was indeed stimulated in the treated cells.

Finally, immunohistochemical study with Abs to Hsp90 indicated that Hsp90 is expressed in the normal photoreceptor cells. Cytoplasm of most retinal cells including photoreceptors showed strong immunostaining (Fig. 9A). Controls stained with prebled serum exhibited no immunostaining (Fig. 9B). We concluded that Hsp90 is present in the intact photoreceptor cells. We further speculate that LEDGF stimulates an expression of Hsp90 in the photoreceptor cells and protects them from stresses.

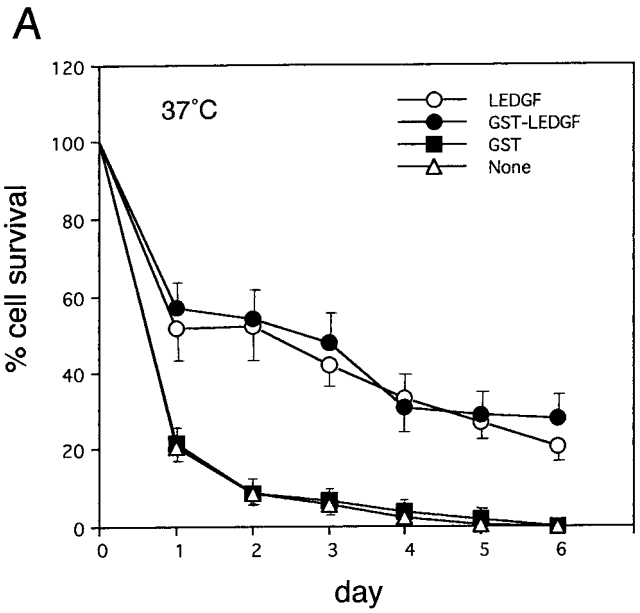

Figure 4. (**A**) Effects of LEDGF on the survival of the retinal photoreceptors. The cells (20,000–28,000 cells/well) were cultured in serum-free Neurobasal with or without LEDGF, GST–LEDGF, or GST (100 ng/ml) and then cultured for 1 to 6 days. The cells were stained each day with Ab to arrestin as in Figure 3. Photographs were taken of the cells in each well. The numbers of cells positively stained by Ab to arrestin were counted and divided by the initial photoreceptor cell numbers. *Vertical line* indicates % of cell survival, and *horizontal line* indicates incubation time. (**B**) Optimal concentration of LEDGF for survival of the retinal photoreceptor cells. Similarly, retinal cells (20,000–28,000 cells/well) were cultured in Neurobasal with 10^{-3} to 10^{3} ng/ml of LEDGF, GST–LEDGF, or GST for 6 days. *Vertical line* indicates % of cell survival, and *horizontal line* indicates amount of LEDGF (in nanograms per milliliter).

Discussion

Our results indicate that LEDGF is found predominantly in the nucleoplasm of most retinal cells, including photoreceptor cells. Cells cultured with LEDGF conferred resistance to hyperthermia and serum starvation. Our previous work established LEDGF as an adhesive, growth, and survival factor for lens epithelial cells, keratinocytes, fibroblasts, and cos7 cells.[1,2]

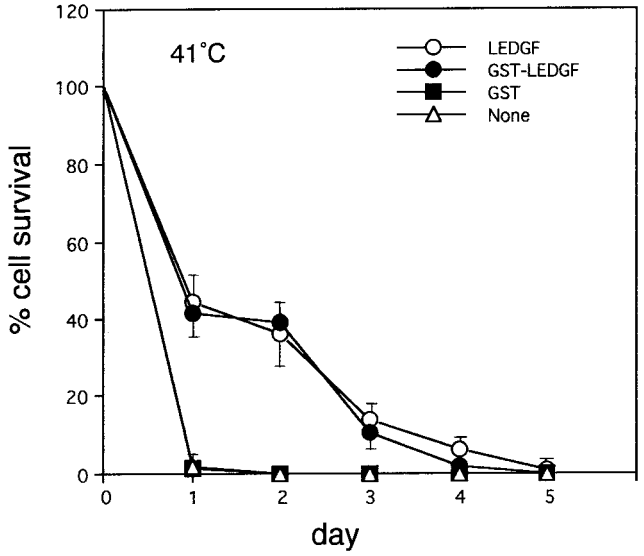

Figure 5. Effects of LEDGF on the survival of the photoreceptor cells under serum starvation and thermal stress. The retinal cells were cultured in serum-free Neurobasal at 41°C for up to 6 days. The percentage of live photoreceptor cells was determined by counting the positively immunostained cells and dividing this number by the initial number of photoreceptor cells. *Vertical line* indicates % of cell survival, and *horizontal line* indicates incubation time. T-bars indicate SD.

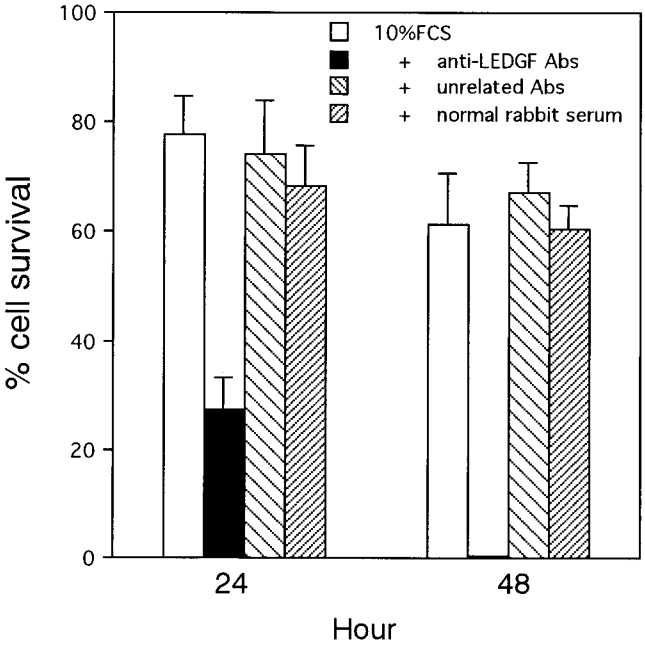

Figure 6. Effects of Ab to LEDGF on the retinal photoreceptor cells in culture. Cell survival was quantified by counting surviving cells and expressing that as a percentage of original cell number. *Vertical line* indicates % of cell survival, and *horizontal line* indicates incubation time. Normal rabbit serum indicates preimmune serum. T-bar indicates SD.

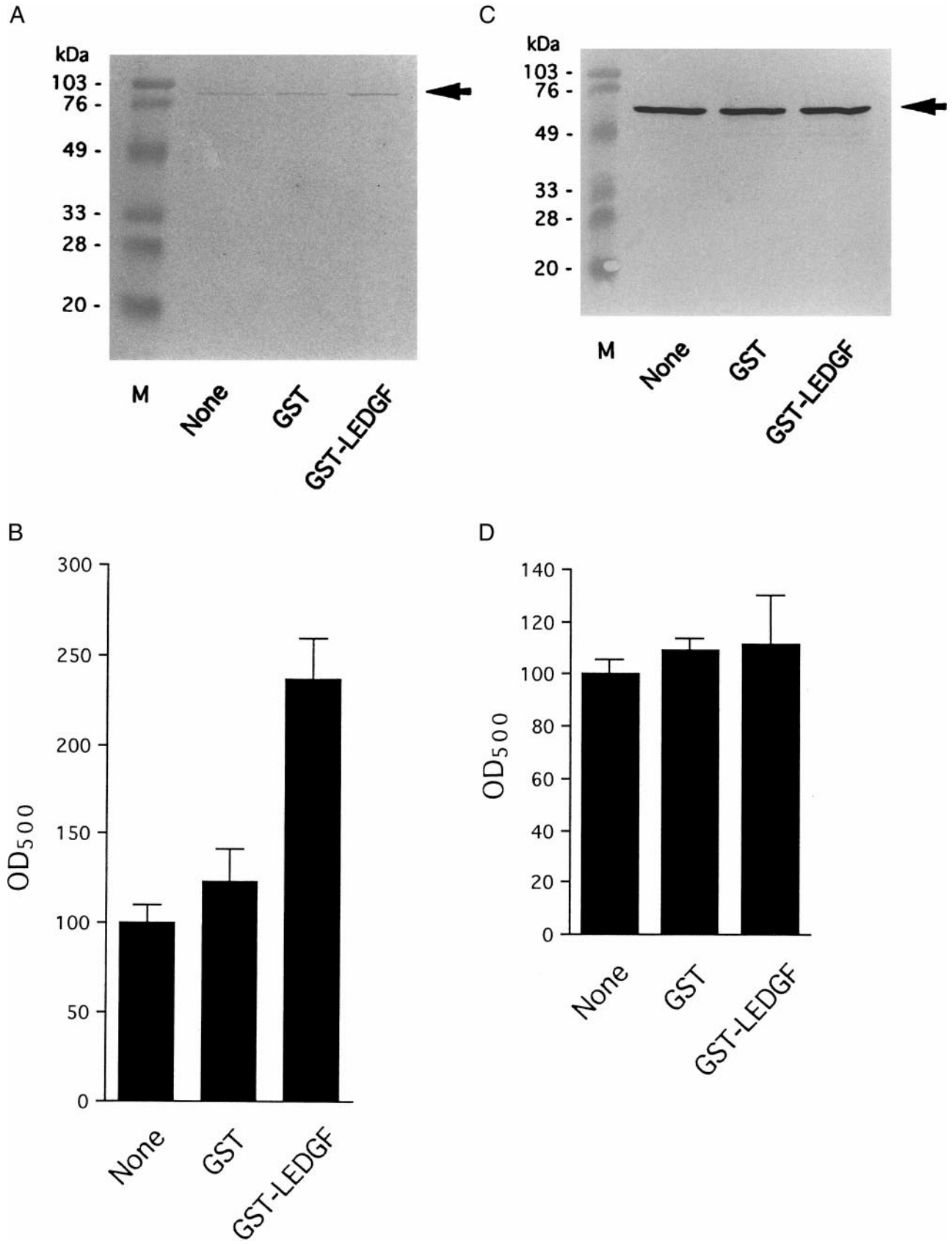

FIGURE 7. Detection of Hsp90 in the neuroretinal cells. Equal amounts (8 μg) of retinal cell proteins were applied to each lane of the SDS–PAGE gel, and proteins were transferred on the Immobilon-P membranes. The membranes were immunostained with the corresponding Abs to Hsp90 (**A** and **B**) and Hsp60 (**C** and **D**). M, molecular size. An *arrowhead* in the right margin indicates Hsp90 in (**A**) and Hsp60 in (**C**). The resulting values (OD_{500}) were obtained by densitometry and expressed for Hsp90 (**B**) and Hsp60 (**D**) as percentage change from baseline (without added LEDGF). At least three experiments were conducted to obtain statistically significant values. T-bar indicates SD.

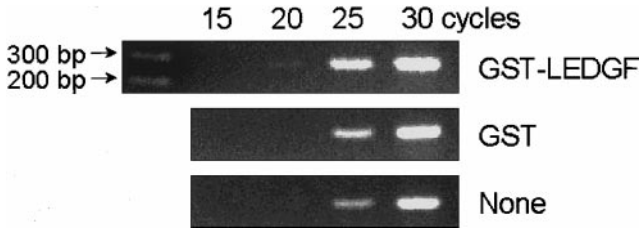

FIGURE 8. Semiquantitative RT–PCR analysis of Hsp90 expressed in the neuroretinal cell culture in the presence of LEDGF. RT–PCR was performed from the same amount of mRNA (100 ng/ml) of each sample with specific 5′ and 3′ primers to Hsp90. PCR amplifications were carried out 10, 15, 20, 25, and 30 cycles, and the products were electrophoresed in a 1.3% agarose gel and visualized by ethidium bromide staining. A band was detected after 20 cycles of PCR from mRNA of the cells treated with LEDGF, but not from the same amount of mRNA of nontreated cells. After 25 cycles of PCR, the band with LEDGF was more intense than the band without LEDGF.

Here we found that LEDGF is also a survival factor for photoreceptor cells.

Omitting LEDGF from the culture medium induced photoreceptor cell death. Blocking LEDGF in the extracellular space with Abs to LEDGF also induced cell death.[1,2] LEDGF in the retinal photoreceptor cells in 10-day-old *rds/rds* mice was significantly lower than in normal mice (Jiro Usukura, personal communication, 1999, Nagoya University, Nagoya, Japan). These results suggest that lower levels of LEDGF or a lack of LEDGF in photoreceptor cells induces cell death and that higher levels of LEDGF promote cellular resistance to stresses.

Several factors have been reported to be survival factors for neuroretina (including nerve growth factor,[15] brain-derived neurotrophic factor, and ciliary neurotrophic factor),[16,17] activin,[18] and basic fibroblast growth factor.[19–22] Brain-derived neurotrophic factor and ciliary neurotrophic factor are effective in slowing retinal degeneration in mutant mice.[17,23,24] Nerve growth factor promotes survival of multiple neuronal cells and ganglion cells in culture[25–27] and basic fibroblast growth factor survival of photoreceptor cell in RCS rats[17,19,20] and in cell culture systems.[22] The survival mechanism of photoreceptor cells in the presence of LEDGF may differ from the above-mentioned factors.

Although the functional role of Hsp90 remains elusive, purified Hsp90 binds to denatured protein and displays antiaggregant properties.[28] In addition, ATP binding and hydrolysis are essential to the function of Hsp90 as a molecular chaperone in vivo.[29] Cytoplasmic Hsp90's function is complex; this protein is involved in many cell regulation and signaling pathways, including steroid hormone receptors,[30] helix–loop–helix transcription factors,[31] tyrosine and serine/threonine kinases,[32] and tumor suppressors.[33]

Hsp90 is an abundant cytoplasmic protein in the cerebellum, cerebral hemispheres, and the retina where it accounts for 1% to 2% of total protein.[34] It is constitutively expressed in the developing rat retina, and its levels are relatively constant during development, except for a short period during postnatal days 3 to 7, when it drops sharply[34] while developing retinal cells undergo apoptosis. LEDGF increases synthesis of Hsp27 and αB-crystallin in LECs[2] but not Hsp90, whereas in retinal cells the opposite was observed. Similarly, Hsp70 is induced in glial cells after hyperthermia, and Hps90 is not in rabbit cerebellum.[35,36] These results suggest that each Hsp is regulated independently in the ocular tissues. The basic mechanism of activation of Hsp90 by LEDGF is beyond the scope of the present study, but it is intriguing that LEDGF can activate expression of Hsp90, which at higher levels can protect retinal photoreceptor cells against multiple stresses.

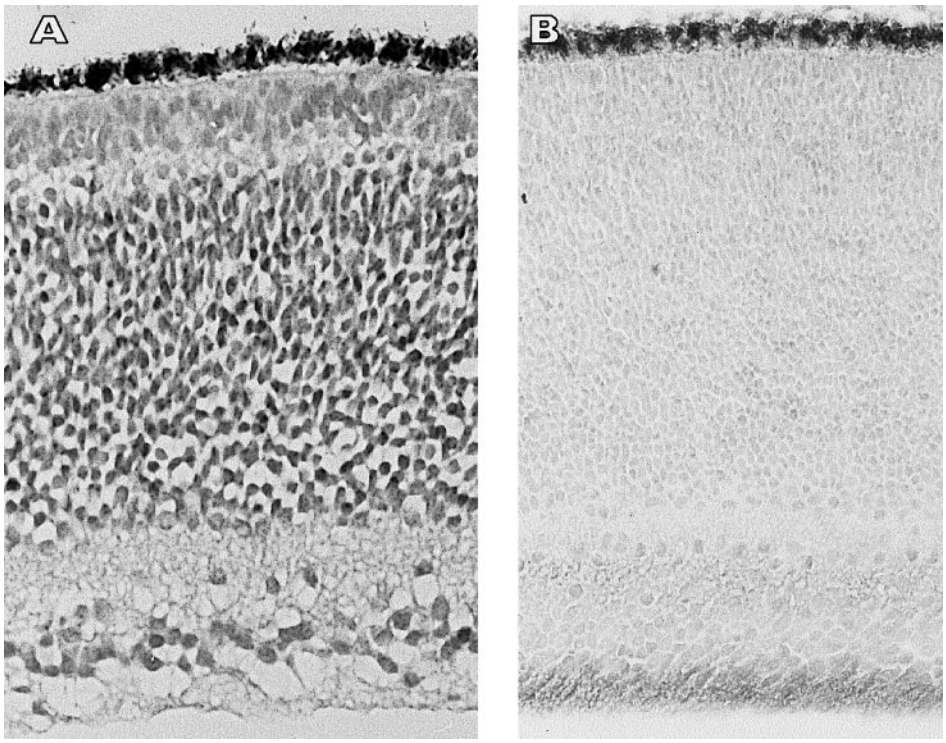

FIGURE 9. Immunohistochemistry of an embryonic chick retina stained with Abs to Hsp90. Embryonic chick retinas (E14) were fixed by cold methanol, and thin sections (10 μm) were prepared. The retinal sections were immunostained with Abs to Hsp90 (**A**) or prebled rabbit serum (**B**). The retinal section was counterstained with hematoxylin.

Acknowledgment

The authors thank Harold White for his help with the project.

References

1. Singh DP, Ohguro N, Kikuchi T, et al. Lens epithelium-derived growth factor (LEDGF): effects on growth, and survival factor of lens epithelial cells, keratinocytes, and fibroblasts. *Biochem Biophys Res Commun.* 2000;267:371–381.
2. Singh DP, Ohguro N, Chylack LT Jr, Shinohara T. Lens epithelium-derived growth factor: increased resistance to thermal and oxidative stress. *Invest Ophthalmol Vis Sci.* 1999;40:1444–1451.
3. Ayaki M, Sueno T, Singh DP, Chylack LT Jr, Shinohara, T. Antibodies to lens epithelium-derived growth factor (LEDGF) kill epithelial cells of whole lenses in organ culture. *Exp Eye Res*. 1999;69:139–142.
4. Nakamura H, Izumoto Y, Kambe H, et al. Molecular cloning of complementary DNA for a novel human hepatoma- derived growth factor. *J Biol Chem.* 1994;269:25143–25149.
5. Ishimoto Y, Kuroda T, Harada H, Kishimoto T, Nakamura H. Hepatoma-derived growth factor belongs to a gene family in mice showing significant homology in the amino terminus. *Biochem Biophys Res Commun.* 1997;238:26–32.
6. Ge H, Si Y, Roeder RG. Isolation of cDNAs encoding novel transcription coactivators p52 and p75 reveals an alternate regulatory mechanism of transcriptional activation. *EMBO J.* 1998;17:6723–6729.
7. Lindquist S, Craig EA. The heat-shock proteins. *Annu Rev Genet.* 1988;22:631–677.
8. Hightower LE. Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell.* 1991;66:191–197.
9. Georgopoulos C, Welch WJ. Role of the major heat shock proteins as molecular chaperones. *Annu Rev Biochem.* 1993;62:601–634.
10. Hendrick JP, Hartl F–U. Molecular chaperone functions of heat-shock proteins. *Annu Rev Biochem.* 1993;62:349–384.
11. Fatma N, Singh DP, Shinohara T, Chylack LT Jr. Stabilization of recombinant adhesive and survival factor (ASF) through heparin binding [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 1999;40(4): **S**519. Abstract nr 2738.
12. Singh VJ, Usukura J, Shinohara T. Molecular mimicry: uveitis induced in *Macaca fasicularis* by microbial protein having sequence homology with retinal S-antigen. *Jpn J Ophthalmol.*1992; 36:108–116.
13. Bradford MM. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem.* 1976;72:248–254.
14. Saga T, Scheurer D, Adler R. Development and maintenance of outer segments by isolated chick embryo photoreceptor cells in culture. *Invest Ophthalmol Vis Sci.* 1996;37:561–573.
15. Cattaneo E, Mckay R. Nerve growth factor regulates proliferation and differentiation of neuronal stem cells. *Nature.* 1990;247:762–765.
16. La Vail MM, Yasumura D, Matthes MT, et al. Protection of mouse photoreceptors by survival factors in retinal degenerations. *Invest Ophthalmol Vis Sci.* 1998;39:592–602.
17. Faktrovich EG, Steinberg RH, Yasumura D, Matthes MT, La Vail MM. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature.* 1990;347:83–86.
18. Schubert D, Kimura H, LaCorbiere M, Vaughan J, Karr D, Fisher H. Activin is a nerve cell survival molecule. *Nature.* 1990;344:868–870.
19. Akimoto M, Miyatake S, Kogishi J, et al. Adenovirally expressed basic fibroblast growth factor rescues photoreceptor cells in RCS rats. *Invest Ophthalmol Vis Sci.* 1999;40:273–279.
20. Perry J, Du J, Kjeldbye H, Gouras P. The effects of bFGF in RCS rat eyes. *Curr Eye Res.* 1995;14:585–592.
21. Smith SB, Titelman R, Hamasaki DI. Effects of basic fibroblast growth factor on the retinal degeneration of the mi^{vit}/mi^{vit} (Vitiligo) mouse: a morphologic and electrophysiologic study. *Exp Eye Res.* 1996;63:565–577.
22. Fountaine V, Kinkl N, Sahel J, Dryfus H, Hicks D. Survival of purified rat photoreceptors in vitro is stimulated directly by fibroblast growth factor-2. *J Neurosci.* 1998;18:9662–9672.
23. Lin LF, Mismer D, Lile JD, et al. Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). *Science.* 1989;246: 1023–1025.
24. Leibrock J, Lottspeich F, Hohn A, et al. Molecular cloning and expression of brain-derived neurotrophic factor. *Nature.* 1989; 341:149–152.
25. Lindsey JD, Weinreb RN. Survival and differentiation of purified retinal ganglion cells in a chemically defined microenvironment. *Invest Ophthalmol Vis Sci.* 1994;35:3640–3648.
26. Meyer–Franke A, Kaplan MR, Pfrieger FW, Barres B. Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron.* 1995;15:805–819.
27. Johnson JE, Barde Y–A, Schwab M, Thoenen H. Brain-derived neurotrophic factor supports the survival of cultured rat retinal ganglion cells. *J Neurosci.* 1986;6:3031–3038.
28. Wiech H, Buchner J, Zimmermann R, Jakob U. Hsp90 chaperones protein folding in vitro. *Nature.* 1992;358:169–170.
29. Panaretou B, Prodromou C, Roe SM, et al. ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. *EMBO J.* 1998;17:4829–4836.
30. Joab I, Radanyi C, Renoir M, et al. Common non-hormone binding component in non-transformed chick oviduct receptors of four natural steroids. *Nature.* 1984;308:850–853.
31. Wilhelmsson A, Cuthill S, Denis M, Wikstrom AC, Gustafsson JA, Poellinger L. The specific DNA binding activity of the dioxin receptor is modulated by the 90 kDa heat-shock protein. *EMBO J.* 1990;9:69–76.
32. Dai K, Kobayashi R, Beach D. Physical interaction of mammalian CDC37 with CDK4. *J Biol Chem.* 1996;271:22030–22034.
33. Chen CF, Chen YM, Dai K, Chen PL, Rily DJ, Lee WH. A new member of the Hsp90 family of molecular chaperones interacts with the retinoblastoma protein during mitosis and after heat-shock. *Mol Cell Biol.* 1996;16:4691–4699.
34. Kojima M, Hoshimaru M, Aoki T, et al. Expression of heat shock proteins in the developing rat retina. *Neurosci Lett.* 1996;205:215–217.
35. Manzerra P, Rush S, Brown IR. Temporal and spatial distribution of heat shock mRNA and protein (hsp70) in the rabbit cerebellum in response to hyperthermia. *J Neurosci Res.* 1993;36:480–490.
36. Quraishi H, Brown IR. Expression of heat shock protein 90 (hsp90) in neural and nonneural tissues of the control and hyperthermic rabbit. *Exp Cell Res.* 1995;219:358–363.