대한체질인류학회지 제 28 권 제 1 호
Korean J Phys Anthropol Vol. 28, No. 1 (2015) pp. 37~44
http://dx.doi.org/10.11637/kjpa.2015.28.1.37

Original Article

Expression of Heat Shock Protein 27 and Alpha B Crystallin in the Retina and Optic Nerve of the Chick Embryo

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(Received 26 February 2015, revised 17 March 2015, accepted 20 March 2015, Published Online 30 March 2015)

Abstract : Heat shock protein 27 (HSP27) and alpha B crystallin (aBC) belong to the small heat shock protein (sHSP) family and have similar amino acid sequences. However, no study has compared the distributional patterns of these two sHSPs in the retina and optic nerve. In this study, we compared the spatiotemporal distributions of the expressions of HSP27 and aBC in the developing chick retina and optic nerve. Both HSP27 and aBC were first expressed in the retina and optic nerve at embryonic day 16 (E16). At E20 the expressions of the two proteins were increased in the retina and optic nerve. Double immunofluorescence demonstrated that HSP27 and aBC were expressed in oligodendrocytes of the retina and optic nerve. In addition, HSP27 was also found to be expressed in ganglion cells in the retina. The findings of this study suggest that HSP27 and aBC act to protect ganglion cells and oligodendrocytes during late development of the chick retina and optic nerve.

Keywords : HSP27, Alpha B crystallin, Retina, Optic nerve

Introduction

Cells express heat shock proteins (HSPs) when exposed to heat or deleterious stimuli. At a molecular level, HSPs prevent the aggregation of intracellular proteins and are involved in maintenance of protein structure through protein folding [1]. Numerous reports indicate that neurological disorders like Parkinson's disease, Alzheimer's disease, and multiple sclerosis are closely involved in deletion or misexpression of HSPs [2-5]. Furthermore, it has been reported that some HSPs are temporally or constitutively expressed during development and suggested HSPs play a crucial role in the survival and differentiation of cells [6-8]. HSPs with molecular weights from 15 to 30 kDa are classified as small HSPs (sHSPs), examples include, HSP20, HSP22, HSP25, HSP27, and alpha B crystallin (aBC) [9]. Of these, HSP27 and aBC exhibit high homology (38%) in terms of their amino acid sequences. However, few studies have compared the distribution of these two sHSPs in neural tissues.

The retina and optic nerve are derived from the optic cup, which during early development is a part of the neural tube. Therefore, cytoarchitecture of these tissues is similar to that of the central nervous system. The structure and development of the avian visual system has been well established. Similar to that observed in mammals, the retinae of birds contain four types of neuronal cells (ganglion cells, bipolar cells, amacrine cells, and horizontal cells) and three types of glial cells (Müller cells, oligodendrocytes, and microglia) as well as photoreceptors. During development, eye pigmentation becomes distinct at em-

*This work was supported by the research grant of the Chungbuk National University in 2012.
The author(s) agree to abide by the good publication practice guideline for medical journals.
The author(s) declare that there are no conflicts of interest.
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ISSN 2287-626X (Online) · ISSN 1225-150X (Print)

bryonic day 3.5 (E3.5), the ganglion cell layer of the retina is separated from the neuroepithelial layer at E10, myelinated nerve fibers are formed in the nerve fiber layer at E16, and basic connections among neurons are completed at E18 [10,11]. Therefore, the chick retina at E18 exhibits the same structure as the adult retina. In this study, we compared the spatiotemporal distributions of HSP27 and aBC expression in the developing chick retina and optic nerve in order to deduce the role of sHSP during normal development of the retina and optic nerve.

Materials and Methods

1. Experimental animals and tissue preparation

Fertilized eggs (Pulmuone, Korea) were incubated at 38˚C in a humidified atmosphere until they reached the appropriate embryonic days as described by Hamburger and Hamilton [10]. Embryonic chicks were cryoanesthetized on ice, rapidly decapitated, and lenses and vitreous bodies were removed from eyeballs. Retinal tissues were fixed by immersion in 4% paraformaldehyde (PFA, Sigma-Aldrich, USA) in 0.05 M phosphate buffered saline (PBS, pH 7.2) for 3 hrs, cryoprotected by immersion in 30% sucrose solution, embedded in frozen section compound (FSC22, Leica, Germany), and frozen rapidly. Retinae were horizontally cut through the middle of the optic fissure into 10～14 μm sections using a cryostat (CM3050S, Leica, Germany). The study was approved by the Institutional Animal Care and Use Committee of Chungbuk National University (Approval No. CBNUA-092-0906-01).

2. Immunohistochemical staining

Retinal sections were pre-blocked with 10% normal goat

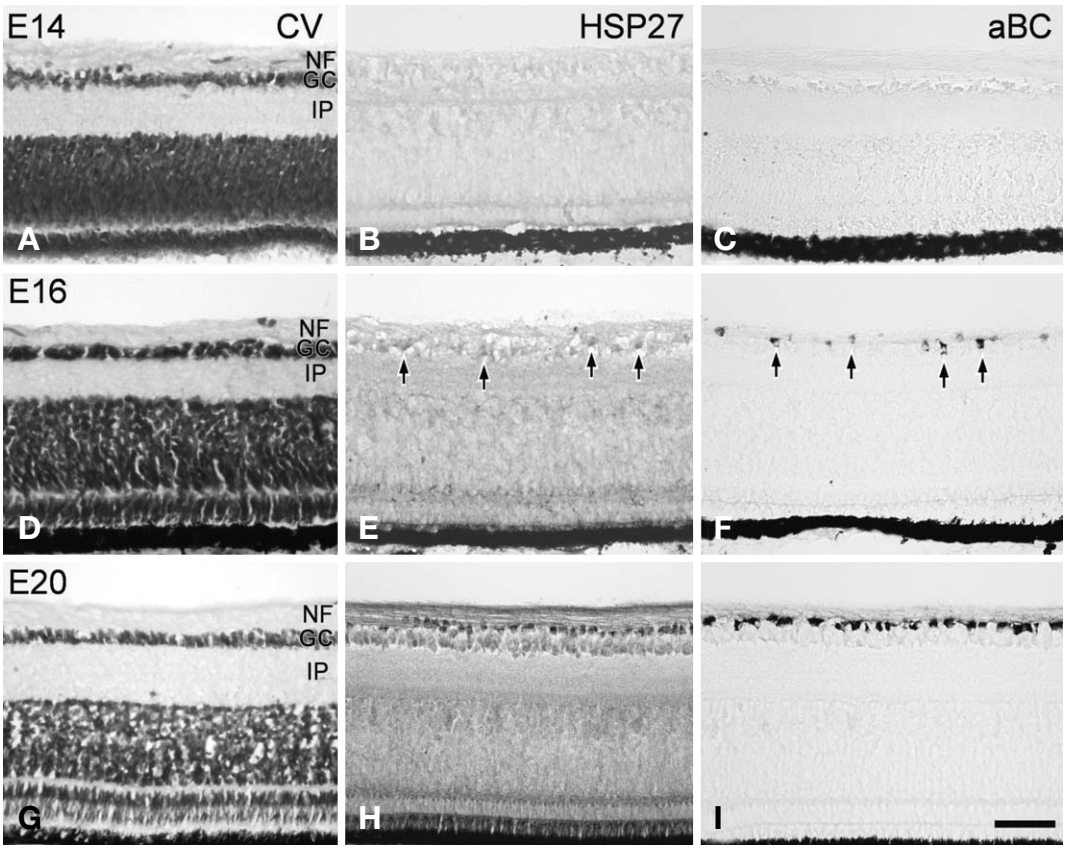

Fig. 1. HSP27 and aBC expressions in the developing chick retina. A-C: E14. HSP27 and aBC are not expressed in the E14 retina. D-F: E16. HSP27 and aBC are expressed in the ganglion cell layer and nerve fiber layer (arrows). G-I: E20. HSP27 and aBC are expressed in the nerve fiber layer, and HSP27 is also expressed in ganglion cell layer. CV, cresyl violet staining; NF, nerve fiber layer; GC, ganglion cell layer; IP, inner plexiform layer. Scale bar=50 μm.

serum for 30 min without the use of antigen retrieval techniques and incubated overnight at 4˚C with rabbit anti-HSP27 (1 : 200, ADI-SPA-803, Enzo Life Sciences, USA) and mouse anti-aBC (1 : 500, ADI-SPA-222, Enzo Life Sciences, USA) antibodies diluted in PBS. Subsequently, immunohistochemistry was performed using the avidin-biotin peroxidase complex method as described previously [12]. Briefly, retina tissues were incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, USA) and goat anti-rabbit IgG (Vector Laboratories, USA) diluted 1 : 400 in PBS, washed in PBS, incubated with avidin-biotin-peroxidase complex (ABC Elite kit, Vector Laboratories, USA), visualized with DAB (Sigma-Aldrich, USA), and mounted with Permount (Fisher Scientific, USA). For negative controls, primary or secondary antisera were omitted from the immunohistochemical staining procedure.

3. Double immunofluorescence

Retinal sections were pre-blocked with 10% normal goat serum for 30 min and incubated with a couple of antibodies for double immunostaining. Mouse anti-aBC, anti-NeuN (mouse, 1 : 200, MAB377, Millipore, USA) and anti-nkx2.2 (mouse, 1 : 10, 74.5A5, Developmental Study of Hybridoma Bank, USA) antibodies were incubated with rabbit anti-HSP27 antibody. Anti-NeuN and nkx2.2 antibodies were used to label ganglion cells and oligodendrocytes, respectively [13,14]. Retina tissues were washed in PBS and incubated with Cy2- and Cy3-labeled secondary antibodies (1 : 500, The Jackson Laboratory, USA). Immu-

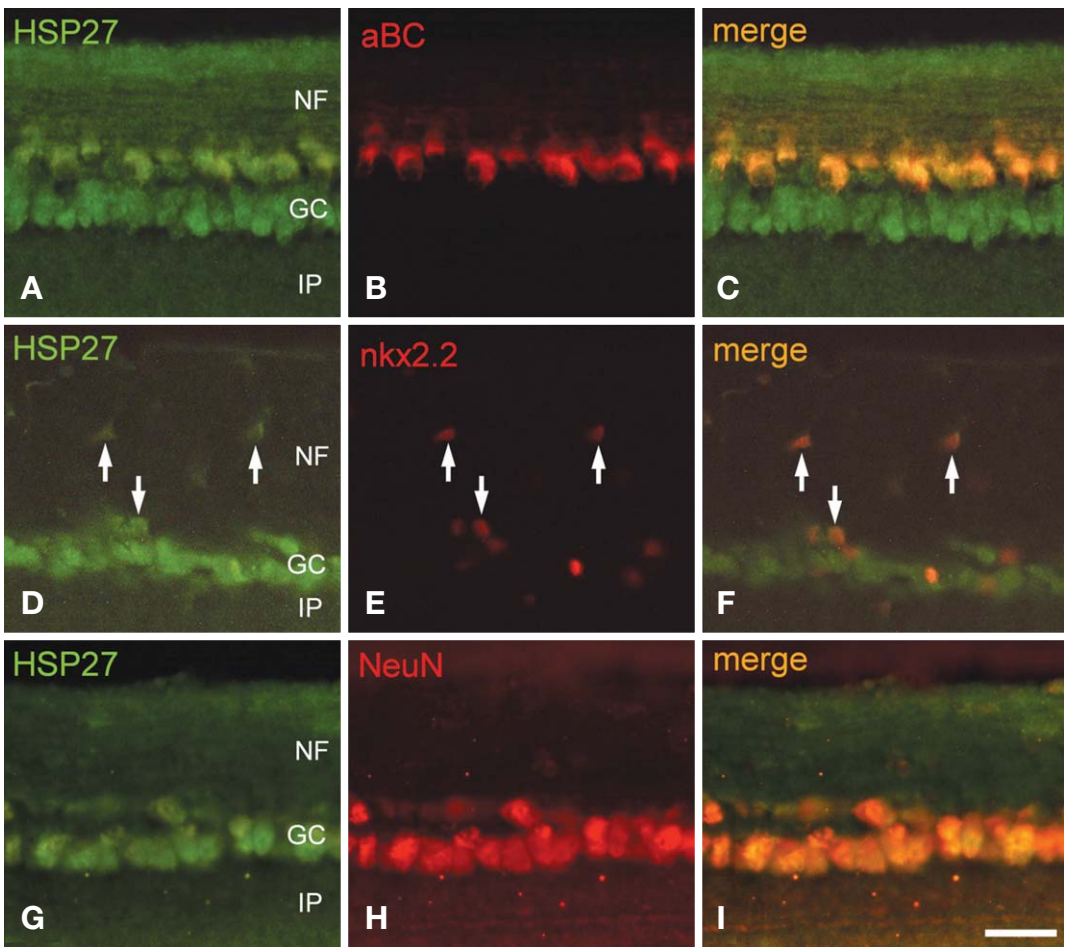

Fig. 2. Double immunolabeling in the E18 chick retina. A-C: HSP27/aBC. aBC immunolabeling oligodendrocytes almost completely overlap with HSP27 immunolabeling cells. However, ganglion cells in the ganglion cell layer exclusively express HSP27. D-F: HSP27/Nkx2.2. Nkx2.2 immunolabeling oligodendrocytes almost completely overlap with HSP27 immunolabeling cells (arrows). G-I: HSP27/NeuN. NeuN immunolabeling ganglion cells almost completely overlap with HSP27 immunolabeling cells. NF, nerve fiber layer; GC, ganglion cell layer; IP, inner plexiform layer. Scale bar=20 μm.

nostained samples were mounted with aqueous mountant and observed under a multipurpose microscope with an epifluorescence attachment (DMLB, Leica, Germany).

Results

1. Expression of HSP27 and aBC in retinae

HSP27 and aBC immunoreactivities were not observed in E14 retinae (Fig. 1A-C). In E16 retinae, weak HSP27 immunoreactivities were observed in the ganglion cell layer and nerve fiber layer. However, aBC showed strong immunoreactivity only in the nerve fiber layer (Fig. 1D-F). In E20 retinae, both HSP27 and aBC showed strong immunoreactivity in the nerve fiber layer, and HSP27 showed weak immunoreactivity in the ganglion cell layer (Fig. 1G-I).

Double labeling analysis showed that almost all HSP27 immunolabeling cells in the nerve fiber layer were also aBC immunolabeling (Fig. 2A-C). In a previous study, we found aBC was exclusively expressed in oligodendrocytes in the chick retina [11], and in the present study we found that HSP27 was also expressed in oligodendrocytes. This result was confirmed by double labeling with HSP27 and nkx2.2 (oligodendrocyte marker). HSP27 immunolabeling cells in the nerve fiber layer were nkx2.2 immunolabeling oligodendrocytes (Fig. 2D-F), whereas most HSP27 immunolabeling cells in the ganglion cell layer were NeuN immunoreactive ganglion cells (Fig. 2G-I).

2. Expressions of HSP27 and aBC in the optic nerve

Cellular distributions of HSP27 and aBC showed similar patterns in the optic nerve. HSP27 and aBC immunoreactivities were not observed in optic nerves at E14 (Fig. 3A-C), but at E16, HSP27 and aBC immunoreactivities were observed in oligodendrocytes with typical small round or oval cell bodies (Fig. 3D-F). At E20 in optic nerves, the shapes of HSP27 and aBC immunoreactive cells

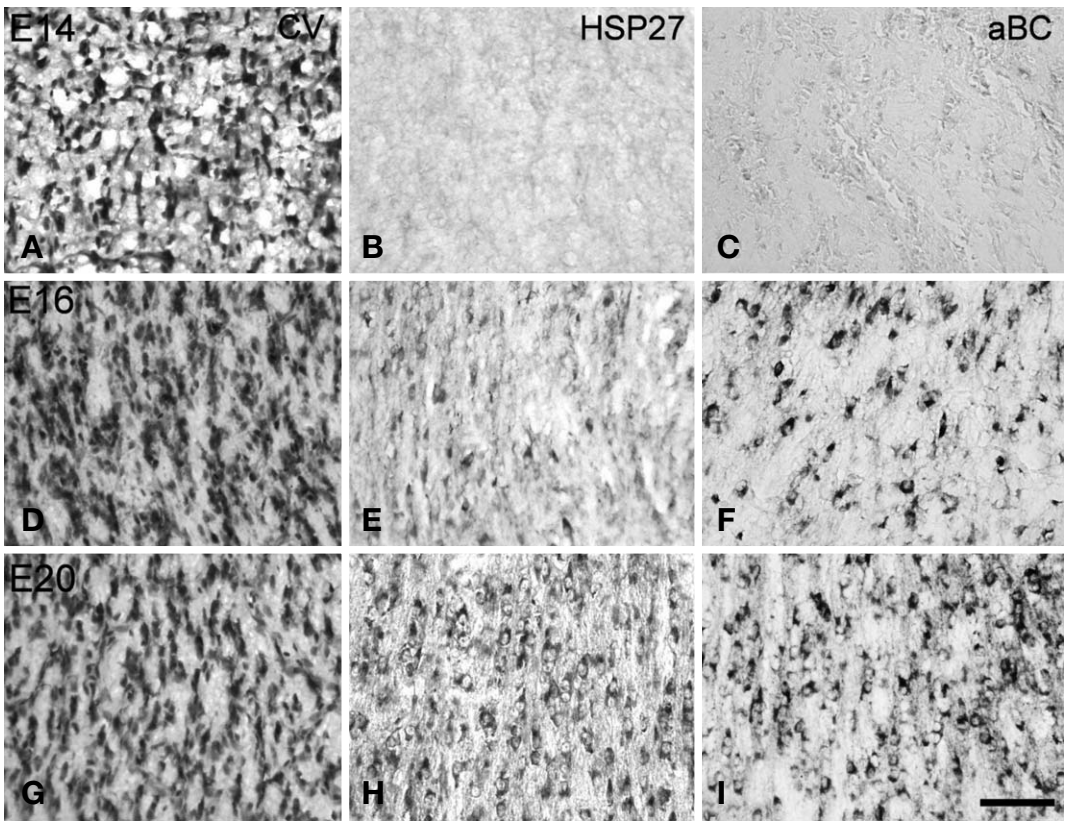

Fig. 3. HSP27 and aBC expressions in the developing chick optic nerve. A-C: E14. HSP27 and aBC are not expressed in E14 optic nerves. D-F: E16. HSP27 and aBC are expressed in oligodendrocytes of E16 optic nerves. G-I: E20. HSP27 and aBC are expressed in oligodendrocytes of E20 optic nerves. Scale bar=50 μm.

Table 1. Immunoreactivies of HSP27 and aBC in the developing chick retina and optic nerve

Embryonic days	Regions	HSP27	aBC
E14	Retina	−	−
	Optic nerve	−	−
E16	Retina		
	Ganglion cells	+	−
	Oligodendrocytes	+	++
	Optic nerve		
	Oligodendrocytes	++	+++
E20	Retina		
	Ganglion cells	+	−
	Oligodendrocytes	+++	+++
	Optic nerve		
	Oligodendrocytes	+++	+++

Staining intensities of HSP27 and aBC are classified as: −, negative; +, weakly positive; ++, moderately positive; or +++, strongly positive.

were similar to those of previous stages and numbers of immunoreactive cells were increased (Fig. 3G-I).

In summary, HSP27 and aBC were expressed in oligodendrocytes of the retina and optic nerve from E16, and HSP27 was also expressed in ganglion cells of the retina from E16 (Table 1).

Discussion

We reported in the previous study chicken (*Gallus gallus*) HSP27 and aBC showed amino acid sequence homology (44%) and that the distributions of these two proteins are similar in the developing chick cerebellum [14]. In the present study, we found that distributions of HSP27 and aBC in the retina and optic nerve also exhibit similar spatiotemporal patterns. Neither of the two proteins was expressed at E14, but both were expressed at E16 and these expressions were increased in the retina and optic nerve at E20. With regard to cellular distributions, HSP27 and aBC were commonly expressed in oligodendrocytes in the retina and optic nerve, although HSP27 was also expressed in ganglion cells. These observed similarities between the spatiotemporal expressions of HSP27 and aBC suggest that these two sHSPs play analogous roles during retina and optic nerve development.

Numerous studies demonstrated that many sHSPs act as a molecular chaperone which prevent irreversible aggregation of unfolded proteins and assist their folding under normal conditions as well as stress conditions [15]. They also function as anti-apoptotic agents by inactivation of caspase cascade [16-18]. Indeed, overexpressions of HSP 27 and aBC by gene delivery systems protect neurons from the apoptosis [19-21]. Furthermore, a few reports demonstrated that sHSPs are expressed in the retina under pathological conditions and suggested that sHSPs play a protective role on the retina. For example, both HSP27 and aBC are expressed in diabetic rat retina and they may protect the retinal neurons in chronic diabetes [22]. Expression of HSP27 in retinal ganglion cells after transection of the optic nerve may be involved in enhanced survival of ganglion cells [23]. In the same sense, it is plausible that our result of HSP27 and aBC expressions in the retina and optic nerve from E16 may be associated with protective role on ganglion cells and oligodendrocytes during late retinal development.

Retinal oligodendrocytes in the chicken arise from the floor of the third ventricle at E5. Thereafter, they migrate ventrolaterally and enter into the retina at E10 through the optic nerve [24]. Unlike that observed in mammals, the avian optic nerve contains loose lamina cribrosa sclerae and permits oligodendrocyte passage [24,25]. Immature oligodendrocytes entering the retina finally become mature myelinating oligodendrocytes by repeated proliferation and differentiation. We suppose because myelin basic protein (MBP; a prominent myelin marker) is first expressed in the chick retina at ~E16 [11], the HSP27 and aBC immunoreactive cells observed in the retina and optic nerve in the present study were not immature oligodendrocytes but mature myelinating oligodendrocytes.

Oligodendrocytes are vulnerable to oxidative stress by free radicals [26], and mature oligodendrocytes may be vulnerable to free radicals, because the myelin of mature oligodendrocytes contains higher levels of lipids than any other cell membrane [26,27]. Nevertheless, mature oligodendrocytes are more resistant to oxidative stress than immature oligodendrocytes [28]. We previously found mature oligodendrocytes exclusively express aBC and suggested that aBC might protect cells [28]. The present study suggests that HSP27 and aBC act as molecular chaperones to maintain the biological activities of mature oligodendrocytes exposed to oxidative stress. Further studies are required to explore this suggestion and identify the mechanism responsible.

More than 50% of neurons undergo apoptosis during brain development [29], and subsequently, differentiated

neurons with neural connections must survive for a long time, because they cannot proliferate. In vitro and in vivo evidences indicate that HSP27 may enhance the survival of differentiated neurons. For example, several studies have demonstrated that HSP25 and HSP27 protect Purkinje cells in the developing rodent cerebellum [30-32], and in other studies, the expressional suppression of HSP27 provoked the apoptosis of PC12 cells [33] and HSP27 microinjection increased neuronal survival and decreased apoptosis under stressful conditions [34]. In the present study, HSP27 was expressed in ganglion cells, which suggests that it prevents the apoptosis and increase the survival of these cells.

In summary, we compared the spatiotemporal distributions of HSP27 and aBC in the developing chick retina and optic nerve. HSP27 and aBC were expressed in oligodendrocytes of the retina and optic nerve from E16, and HSP27 was expressed in ganglion cells of the retina. These results suggest that HSP27 and aBC act to protect ganglion cells and oligodendrocytes during late development of the chick retina and optic nerve.

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닭 배아 망막과 시각신경에서 열충격단백질27과 alpha B crystallin의 발현 비교

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간추림 : 저분자열충격단백질(small heat shock protein)에 속하는 열충격단백질27 (HSP27)과 alpha B crystalline (aBC)은 서로 유사한 아미노산 서열을 갖지만, 망막과 시각신경에서 두 열충격단백질 발현의 분포양상을 비교한 연구는 없다. 이 연구에서는 닭 배아 망막과 시각신경의 발생과정에서 HSP27과 aBC의 발현시기와 분포를 비교하였다. HSP27과 aBC의 발현은 망막과 시각신경에서 동일하게 발생 16일에 처음 관찰되었다. 발생 20일에는 두 열충격단백질의 발현이 망막과 시각신경에서 증가하는 양상을 보였다. 망막에서 HSP27은 신경절세포와 희소돌기아교세포에서 발현하였지만, aBC는 희소돌기아교세포에서만 발현됨을 이중면역형광염색을 통해 확인하였다. 한편 시각신경에서는 HSP27과 aBC가 모두 희소돌기아교세포에서 발현하여 동일한 발현양상을 보였다. 이 연구의 결과는 저분자열충격단백질인 HSP27과 aBC가 망막과 시각신경의 발생 후기단계에서 신경절세포와 희소돌기아교세포의 보호 작용에 관여할 가능성을 제시한다.

찾아보기 낱말 : 열충격단백질27, 알파비크리스탈린, 망막, 시각신경

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