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Expression Sites of Neural Stem Cell-Related Genes in the Monkey Retina

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Abstract

Objectives: Based on the hypothesis that undifferentiated retinal stem cell (RSC)-like cells exist in the fovea (the light-stressed, concave, avascular center of the retina where light is focused), we investigated the expression sites of neural stem cell (NSC)-related genes in the monkey retina.

Methods: Cynomolgus monkeys were euthanized, and both eyes were then enucleated. Each eye was hemisected near the limbus, and flat-mounted retina samples were then prepared. Using a stereomicroscope, 1-mm x 1-mm blocks of the retina at the fovea, mid-periphery, and extreme periphery were then excised. These samples were used for real-time polymerase chain reaction analysis of the NSC-related gene (nestin, PAX6, and SOX2) expression at each site.

Results: Nestin expression was high in the fovea, with a lower expression in the mid-periphery and extreme periphery. No differences in PAX6 gene expression were found in the fovea, mid-periphery, and extreme periphery. SOX2 expression was highest in the extreme periphery, with decreased expression in the mid-periphery and fovea.

Conclusions: Our finding that nestin expression was highest in the fovea suggests that foveal retinal cells may have more undifferentiated characteristics that are different from retinal cells at other sites.

Keywords: Retinal stem cells; Nestin; PAX6; SOX2; Real-time PCR; Fovea

Introduction

The existence of stem cells in the central nervous system (CNS) has been reported, and their use in regenerative medicine has recently attracted interest [1,2]. Retinal stem cells (RSCs), with the ability to differentiate into neurons, glia, and photoreceptors, have also been reported at the boundary between the retina and pars plana, which is the so-called ciliary marginal zone [3-6]. Stem cells have also been found to exist at sites other than tissue boundaries, such as in recessed areas like small intestinal crypts, hair follicles, and the dermis of the skin [7,8]. In addition, stem cells often exist at avascular sites such as the corneal limbus where corneal epithelial stem cells reside [9]. It is our belief that undifferentiated RSC-like cells may also exist in the fovea, which has a combination of these anatomical characteristics.

In a previous study using tissue sections obtained from monkey eyes, we found strong positive staining in the fovea for the neural stem cell (NSC) marker nestin, and discussed its possible involvement in the onset of idiopathic macular holes [10]. In the present study, we also used monkey eyes to analyze the gene expression of the neural stem cell (NSC) markers nestin, PAX6, and SOX2 at different sites in the retina using real-time polymerase chain reaction (PCR).

Methods

This study involved the use of two, healthy, male cynomolgus monkeys (body weight range: 11-14 kg). Both animals were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Excision of monkey retina and RNA extraction

For the excision of the retina and RNA extraction, the monkeys were first euthanized, and both eyes were then enucleated. Next, each eye was hemisected near the limbus and eye cups were prepared by removing the cornea, iris, lens, and vitreous. Flat-mounted retina samples were then prepared. Using a stereomicroscope, 1 mm ×1 mm blocks of the retina at the fovea, mid-periphery, and extreme periphery were excised. To prevent further RNase action and to stabilize the intracellular RNA, the samples were immersed in RNAlater® (QIAGEN, Valencia, CA, USA) stabilization reagent and the RNA was then extracted. The samples were homogenized in lysis buffer, and the RNA was extracted with the RNeasy Plus Mini Kit (QIAGEN). The RNA concentrations and purity were calculated from the absorbance at 260/280 nm.

Primer design and synthesis

Primers for the real-time PCR were designed using the algorithms of a primer design system [Perfect Real Time support system; Takara Bio Inc., Otsu, Shiga, Japan (http://www.takara-bio.co.jp/prt/intro.htm)] for the target genes nestin, PAX6, and SOX2, and for the reference gene GAPDH (Table 1).

Synthesis of cDNA

For each sample after pretreatment, cDNA was synthesized using a Prime Script® RT Reagent Kit (Perfect Real Time, Code: RR037A; Takara Bio).

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Target Name	amplification site (mRNA)	5'-base sequence-3'
GAPDH		GAATGCCTTCATGGTGTGGTC
	306 429	GTCTGCGAGCTGGTCATGGA
Nestin		CCTGCTACCCTTGAGACACCTG
	886 1027	GGGCTCTGATCTCTGCATCTG
PAX6		AGATGAGGCTCAAATGCGACTTC
	398 479	GGCCTCAATTTGCTCTTGGGTA
SOX2		ACAGTCCGGACCGCGTTAAG
	113 226	GCTTGCTGATCTCCGAGTTGTG

Table 1: Primer information.

Real-Time PCR and selection of samples for the calibration curves

Using the synthesized cDNA as a template, real-time PCR reaction by the intercalator method for the target genes and reference gene was performed with SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (Code: RR820A; Takara Bio). The reactions were performed using a Thermal Cycler Dice® Real Time System II (Code: TP900; Takara Bio), with the Ct values determined by the second derivative maximum (SDM) method. Based on the obtained results, samples for the calibration curves were selected from the samples that exhibited high expression for each gene.

Real-Time PCR and preparation of calibration curves

After serially diluting the samples for the calibration curves in 8 steps and performing the real-time PCR reaction, the calibration curves for each analyzed gene were prepared from the Ct values and dilution series.

Measurement of Ct values for each sample

Reactions (n=2) were performed for each sample using 10 ng equivalents of the total RNA. The quantity (Qty(SDM)) for each gene was then calculated based on the Ct values and calibration curves.

Calculation of relative quantities

For each sample, the relative quantities [Rel. Qty(SDM)] of the target genes in each sample were calculated by normalizing the average Qty(SDM) [Qty Avg. (SDM)] of the target genes to the average Qty(SDM) of the reference gene. Multiplate RQ software (Takara Bio) was used to calculate the relative quantities.

Results

Graphs of the Rel.Qty(SDM) for the target genes in each sample, normalized for the Qty Avg. (SDM) of the reference gene in each sample, are shown in Figure 1 through 3. A high expression of nestin was found in the fovea, with a lower expression found in the mid-periphery and extreme periphery (Figure 1). No differences in PAX6 gene expression were found in the fovea, mid-periphery, and extreme periphery (Figure 2). SOX2 expression was highest in the extreme periphery, with decreased expression in the mid-periphery and fovea (Figure 3).

Discussion

It had long been believed that neurons in the CNS of adult mammals cannot regenerate. However, neuron regeneration in areas such as the hippocampus dentate gyrus has recently been reported [1,2]. It is now known that newly regenerated neurons in the hippocampus form synapses with existing neurons, and function as part of the neural network. The hippocampus is closely associated with learning and memory, and is an area that is easily injured by stress. Therefore, a continual regeneration of neurons in the adult hippocampus may be associated with the repair of the injured CNS. It has become clear that at the site of these newly regenerated neurons in the hippocampus, radial glia and astrocytes, with radial projections into the cortex, mainly function as neural progenitor cells (NPCs) [11]. This finding has also attracted attention in the field of regenerative medicine.

In the retina, which is also part of the CNS, RSCs (which have the ability to differentiate into neurons, glia, and photoreceptors) have additionally been reported at the boundary between the retina and pars plana; i.e., the so-called ciliary marginal zone [3-6]. Stem cells have reportedly also been found in the corneal limbus at the boundary of the conjunctiva and cornea [9]. Thus, stem cells are often present at tissue junctions. Furthermore, stem cells have also been found to exist at sites other than tissue boundaries, such as in recessed areas like small

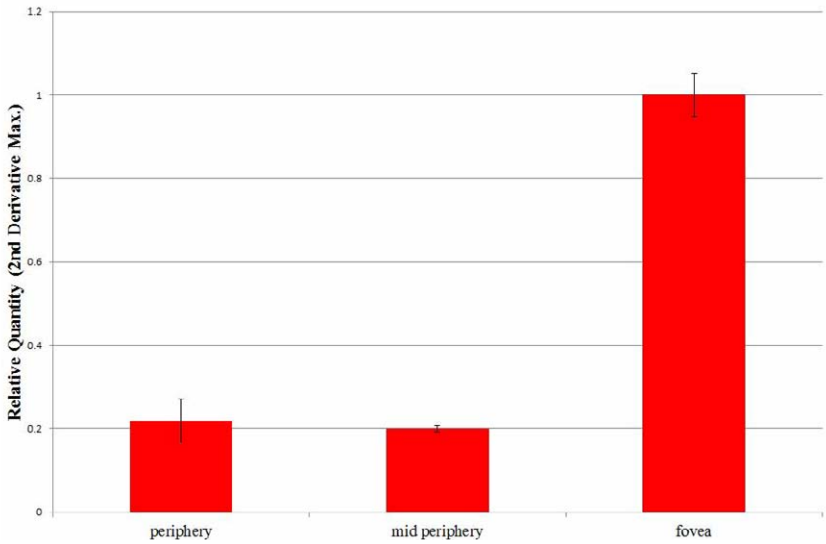

Figure 1: Graph of the relative quantity 2nd derivative max [Rel.Qty(SDM)] of the target gene nestin. Reference gene: GAPDH.

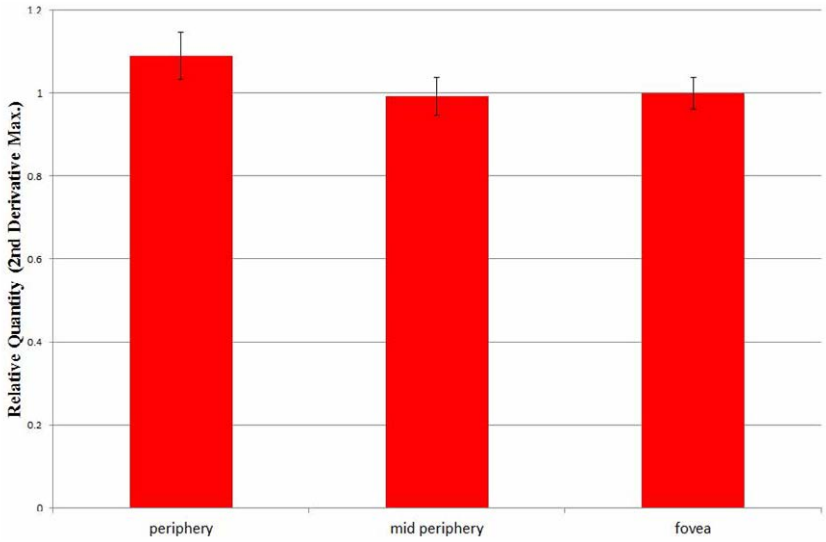

Figure 2: Graph of the Rel. Qty(SDM) of the target gene PAX6. Reference gene: GAPDH.

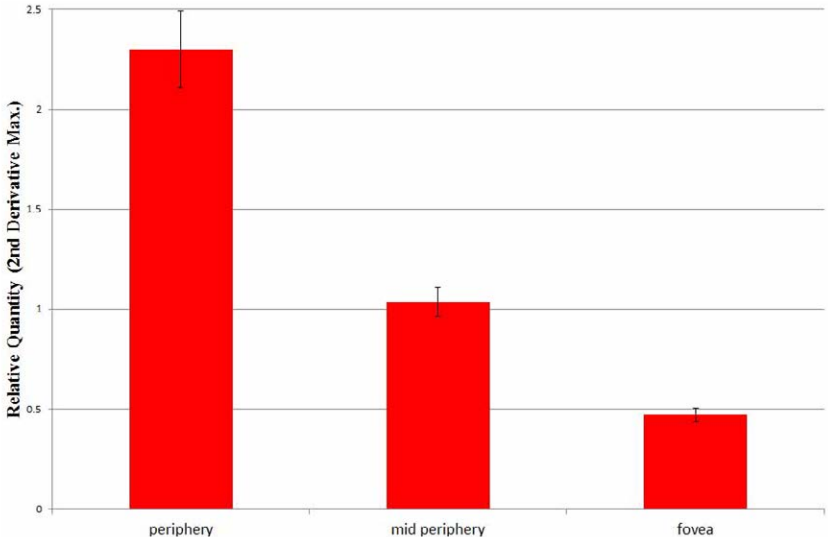

Figure 3: Graph of the Rel.Qty(SDM) of the target gene SOX2. Reference gene: GAPDH.

intestinal crypts, hair follicles, and the dermis of the skin [7,8]. They often tend to be found in avascular areas with low oxygen supply. These niches (recesses) are often hypoxic environments that are ideal for stem cells. The fovea shares many anatomical characteristics with these other tissues where stem cells reside. The fovea is a concave avascular area at the center of retina where light is focused, and it is continually light-stressed. Therefore, we theorize that the fovea may be a site of RSCs.

One clinical finding that supports our hypothesis is that there is a propensity for retinal diseases to involve the macula. Idiopathic epiretinal membrane is a disease with cell proliferation in the macula, and despite complete removal of the epiretinal membranes during a vitrectomy, recurrence of the membranes sometimes occurs postoperatively. This suggests that undifferentiated stem cells of the fovea differentiate and grow into glial cells. In fact, nestin-positive cells have been found in epiretinal membranes [12].

Moreover, during the postoperative course after vitrectomy for idiopathic macular holes, despite early improvement in the shape of the fovea as seen on funduscopy or optical coherence tomography (OCT) at the early postoperative stage, visual acuity continuously improves on a long-term basis. This suggests that undifferentiated RSCs of the fovea differentiate over time into neurons that form the sensory retina, with the visual function acquired later. Kishi et al. proposed that idiopathic macular holes are mainly caused by the vitreoretinal traction of vitreous gel that forms the posterior wall of a premacular vitreous cortex pocket [13]. However, much remains unknown about why macular holes concentrically enlarge, or why OCT shows findings of retinoschisis with macular holes. The same holds true for the question of why traumatic macular holes often spontaneously close.

In a previous study using vitreous samples obtained at vitrectomy, we reported increased activity of serine proteases such as chymase and

tryptase in cases of idiopathic macular holes and idiopathic epiretinal membrane [14]. Moreover, chymase activity was predominantly elevated in cases of idiopathic macular holes, and tryptase activity was predominantly elevated in cases of idiopathic epiretinal membrane. These serine proteases are produced in mast cells. In the eye, however, while mast cells are found in many areas including the ciliary body, choroid, sclera, and conjunctiva, among others, they are not found in the retina [15].

It remains unknown why serine protease activity is increased in the vitreous in cases of idiopathic macular holes and idiopathic epiretinal membrane. However, tryptase is involved in tissue fibrosis, and chymase induces apoptosis of cells with relatively low differentiation, including vascular smooth muscle cells, bronchial smooth muscle cells, and cardiomyocytes [16-20]. In other words, if we presume that there are undifferentiated cells in the fovea, then with regard to the action of these serine proteases, idiopathic epiretinal membranes may develop when tryptase activity is predominant, and idiopathic macular holes may develop when chymase activity is predominant. In fact, OCT often shows findings of retinoschisis near the fovea in cases of idiopathic macular holes. This suggests that there may be some type of dysfunction in Müller cells, which support the sensory retina.

In a previous study, we performed immunostaining in cynomolgus monkey eyes for nestin, an NSC marker, in sections near the macula, mid-periphery, equator, and extreme periphery [10]. In that study, our comparison of nestin-positive cell density at each region clearly showed a higher density in the macula, but no significant differences among the other sites. Hematoxylin and eosin staining was also performed in monkey eyes intravitreally injected with chymase and in a sham control eye. No clear differences in the macula were seen between the sham control eye and the low-dose chymase-treated eye. However, findings in the high-dose chymase-treated eye suggested that there was thickening of the posterior hyaloid membrane in the macular region. TdT-mediated dUTP nick-end labeling (TUNEL) staining showed no positive cells in the sham control eye, but in the high-dose chymase-treated eye, scattered TUNEL-positive cells around the fovea were observed. Nestin is known as a marker for NSCs and RSCs. Thus, our findings suggested that undifferentiated cells with stem cell-like properties were present in the fovea.

Based on those previous results, we designed our present study to use real-time PCR to further analyze the expression of NSC-related genes at different regions of the monkey retina (i.e., the fovea, mid-periphery, and extreme periphery). Similar to our previous findings in the tissue sections of the monkey eye, the present results showed high nestin expression in the fovea. However, no differences in PAX6 gene expression were found among the different regions.

PAX6 is involved in foveal formation, and its expression in the retinal pigment epithelium (RPE) induces transdifferentiation into retinal cells [21]. PAX6 may be highly expressed in the fovea early in development, but in adult monkeys, this difference is probably lost. The results for SOX2 were opposite to those for nestin. Namely, SOX2 gene expression was lowest in the fovea and highest in the periphery. SOX2 is known to be a transcription factor that regulates self-replication of RSCs. In the adult rat brain, SOX2 is reportedly expressed not only by NPCs, but also by astrocytes [22]. SOX2 expression has also been reported in glial cells such as astrocytes in the retina, but since the fovea is avascular with few astrocytes, SOX2 expression in the fovea is probably low.

When retinal injury occurs in adult fish and birds, Müller cells in the retina reportedly become like NPCs, proliferate, and differentiate to retinal neurons, or again to Müller cells [23]. It has long been thought that this type of retinal regeneration does not occur in mammals, but recently, Müller-cell-derived regeneration in the retina of adult mammals has also been reported [24,25]. Since all retinal layers were excised in this present study, it is unclear which cells in the sensory retina expressed nestin. In our previous study that examined immunostaining for nestin in monkey eyes, many nestin-positive cells were observed in the inner nuclear layer and adjacent fovea [10]. This suggests that nestin may be expressed by Müller cells in the sensory retina or by Müller cell cones in the fovea.

Nestin expression is known to increase if the retina is exposed to mechanical injury or hypoxia [26,27]. In adult autopsy eyes, Bhatia et al. performed immunostaining for expression of NSC markers in Müller cells in different regions of the retina [28]. They reported cells positive for both vimentin and nestin mainly in the inner nuclear layer, thus suggesting the likelihood of Müller cells. Many cells in the extreme periphery area of the retina were also co-positive for vimentin and nestin. They also reported that although the number of co-positive cells decreased towards the posterior pole, there were still a substantial number of co-positive Müller cells at the posterior pole. However, it should be noted that the examination performed in that study was not limited to only the area around the fovea and not focused fovea.

Nestin-positive Müller cells are known to exist in fetuses [29], and nestin-positive Müller cells exist throughout most areas of the retina in adults. The results of this current study demonstrated that the number of nestin-positive Müller cells was relatively higher in the fovea than at other areas of the retina. The macular region is clinically considered to have a special physiological function not found in other parts of the retina. In other words, differences in the distribution of nestin-positive cells within the retina may be involved in disease onset. However, there is insufficient evidence at the present time to presume that this means that RSCs exist. If indeed there are a large number of nestin-positive undifferentiated Müller cells in the macula, then this may be related to the onset of many macular-specific diseases, including epiretinal membrane, macular holes, and macular edema. If further research studies can elucidate the pathogenesis of macular disease, then those findings could possibly lead to the development of novel treatments.

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