The Cellular Expression of Antiangiogenic Factors in Fetal Primate Macula

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PURPOSE. To characterize the cellular expression patterns of antiangiogenic factors differentially regulated in the fetal human macula.

METHODS. RNA was extracted from macular, nasal, and surrounding biopsies of three human fetal retinas at midgestation. Relative levels of expression of pigment epithelium-derived factor (PEDF), brain natriuretic peptide (BNP), collagen type IVα2 (COL4A2), and natriuretic peptide receptors A and C (NPRA and NPRC) were determined with quantitative PCR. Cellular expression of PEDF and BNP was investigated by in situ hybridization on retinal sections from monkeys aged between fetal day 55 and 11 years. BNP, COL4A2, and NPRA proteins were localized by immunohistochemistry. Labeling was imaged and quantified by confocal microscopy and optical densitometry.

RESULTS. Quantitative PCR confirmed higher levels of PEDF and BNP and lower levels of COL4A2 in the macula at midgestation. PEDF mRNA was detected in ganglion cells (GCs) and the pigment epithelium (REPE). BNP mRNA was detected in GCs and macroglia, although BNP immunoreactivity (IR) was predominantly perivascular. COL4A2IR was detected in large blood vessels and NPRA-IR on the retinal vascular endothelium. GC axons in fetal retinas, and cone axons at all ages. Optical densitometry showed a graded expression of PEDF and BNP at all ages, with highest levels of expression in GCs in the developing fovea.

CONCLUSIONS. Because the retinal vessels initially form in the GC layer, it is likely that PEDF has a key role in defining and maintaining the foveal avascular area. The precise role of BNP is unclear, but it may include both antiangiogenic and natriuretic functions. (Invest Ophthalmol Vis Sci. 2010;51:4298–4306) DOI: 10.1167/iovs.09-4905

The retinal vasculature in primates is characterized by an arcuate pattern of blood vessel growth around the macula, a lack of large-diameter vessels in the macula, and an absence of retinal vessels from the foveal region.1–5 Finite element analysis modeling suggests that definition of the foveal avascular area is a prerequisite for development of the foveal depression.6,7 and several clinical studies confirm that the foveal depression does not form in the absence of a foveal avascular area. Absence of a foveal depression is often associated with vision loss,8–13 but may also occur as part of the normal spectrum.14 In mature eyes, neovascularization of the macula occurs in age-related macular degeneration and high myopia and is the cause of sudden vision loss. We have recently identified three antiangiogenic factors among the more than 2000 genes differentially expressed in the macula15,16: natriuretic peptide precursor B, also known as brain natriuretic peptide (BNP), pigment epithelium-derived factor (PEDF), and collagen type IVα2 (COL4A2). As part of the validation of our microarray study, we confirmed higher levels of PEDF expression in the fetal macula by quantitative PCR (QRT-PCR) and, by in situ hybridization (ISH), we showed PEDF mRNA expression by ganglion cells (GCs) in fetal macaque retinas.

We report a longitudinal study of the expression patterns and immunoreactivity of these three factors. First, we report QRT-PCR findings for collagen type IVα2, BNP, and its receptors, and PEDF, to verify the data from the microarray. Second, we show for the first time that PEDF mRNA is highly expressed by foveal GCs throughout fetal life and in the postnatal period and discuss the effect of this expression on retinal vascular development in the macula. Third, we show that BNP mRNA, like PEDF, is expressed by GCs in the macula and report quantitative findings that show for the first time that BNP is differentially expressed in the macula into adulthood in macaque retinas. We also report the immunolocalization of BNP and its receptor NPRA in fetal and adult macaque retinas. Finally, in view of these findings and a recent report on expression patterns of the guidance factor EphA6 and its receptors,17 we provide an update on development of the primate retinal vasculature, including the likely roles of PEDF and EphA6.

Understanding the genes that normally regulate vascular patterning in the macula and around the fovea is central to understanding the disease processes that lead to pathologic neovascularization and can contribute to the development of therapeutic strategies to inhibit pathogenic blood vessel growth in the macula.

METHODS

Specimens

Human Retinas. Four human retinas—three at 19 weeks’ gestation (WG) and one at 21 WG—were obtained with maternal consent and ethical approval from the Human Ethics Committees of The University of Sydney and The Australian National University, in accordance with the tenets of the Declaration of Helsinki. Ultrasound and postmortem measurements of foot length were used to determine gestational age. The 19 WG retinas were used for RNA extraction, whereas

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Supported by Grant CE0561903 from the Australian Research Council through the ARC Centre of Excellence in Vision Science.

Submitted for publication November 11, 2009; revised January 20 and March 2, 2010; accepted March 3, 2010.

Disclosure: P. Kozulin, None; R. Natoli, None; K.M. Bumsted O’Brien, None; M.C. Madigan, None; J.M. Provis, None

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BNP, brain natriuretic peptide; COL4A2, collagen type IVα2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NPPA, natriuretic peptide receptor A; NPPC, natriuretic peptide receptor C; PEDF, pigment epithelium-derived factor.

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In Situ Hybridization

The genes were cloned with a DNA vector system (pGEM-T; Promega, Madison, WI) and JM109 competent cells (A3600; Promega). A DIG RNA-labeling kit (SP6/T7, 1175025; Roche, Basel, Switzerland) was used to transcribe linearized plasmid and generate DIG-labeled antisense and sense probes. Paraffin-embedded sections of fetal macaque retina were processed as described previously.18 Only sections passing through the optic disc and macular region were used for quantitative analysis in this study.

Immunolabeling

A list of antibodies and their dilutions is shown in Table 2. Immunolabeling of sections was performed by using standard protocols (see Table 2. Antibodies Used).
also Kozulin et al.\(^7\). Sections were incubated in antigen recovery solution (Reveal-It, AR1002; ImmunoSolutions, Newcastle, Australia) for 20 minutes at 85°C to unmask antigens, and then blocked in 10% serum (diluted in PBS) for 1 hour at room temperature, before overnight incubation at 4°C in a mixture of primary antibodies. Binding of one primary antibody was visualized by incubation in a biotinylated secondary antibody for 1.5 hours, followed by incubation in streptavidin Alexa 488. Binding of the second primary antibody was visualized with Alexa 594 conjugated to the appropriate secondary antibody (4 hours incubation at 4°C). All antibodies were diluted in 0.01% Triton PBS.

For the whole mount, the retina was dissected from the RPE and choroid and rinsed in 10% serum in PBS for 1 hour at room temperature before incubation in anti-human collagen type IVα2 diluted in 0.01% Triton PBS, for 5 days at 4°C. After a thorough rinsing in PBS, the retinas were incubated in biotinylated secondary antibody and then streptavidin Alexa 488, each overnight. The retinans were flattened and viewed by confocal microscopy.

**Imaging**

Imaging was performed with a confocal microscope system (LSM 5, PASCAL ver. 4.0 software; Carl Zeiss Meditec, Inc., Jena, Germany) and an HeNe 543 nm and Argon 488 nm scanning lasers (LASOS, Jena, Germany). Scans of ISH were taken with a 40× objective over a field size of 225 × 225 μm with a 1024 × 1024-pixel resolution in 8-bit format (pixel intensity range, 0–255). Optical sections of BNP ISH were taken over varying thickness according to the age of tissue; four sections of Fd 55 tissue (2.4 μm Z-stack), five sections of Fd 70 to P 3m tissue (Z-stack range, 3.2–4.0 μm) and six sections of 11 yo tissue (4.4 μm Z-stack). Optical sections of PEDF ISH were taken over a constant Z-stack size of 3.0 μm (seven sections) except for the specimen at Fd 164 (450 × 450-μm field size, Z-stack size of 5.1 μm, seven sections).

Laser transmission and scan settings were constant in all scans of a single specimen, to allow comparison of labeling intensity between the different regions. Raw images were imported into ImageJ (version 1.41, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html) and compiled by using the Z projection tool (average intensity). For optical densitometry of the Fast Red reaction product, the 594-nm signal channel was isolated, and the images converted to 8-bit grayscale format before compilation in ImageJ. The compiled, grayscale images were inverted (Invert tool, Photoshop CS3, ver. 10.0.1; Adobe Systems Inc., San Jose, CA) so that the color reaction appears dark on a bright background, for better rendition in print. Any adjustments to contrast and brightness were made with identical settings across all images from any one specimen, so that the printed images represent real differences in color reaction intensity.

**Optical Densitometry Analysis**

For quantitative analysis of hybridized sections, six retinal regions were scanned\(^1\): (1) nasal to the optic disc (nasal), such that the nasal and mid scans are equidistant from the optic disc; (2) immediately temporal to the optic disc (TOD); (3) midway between the macula and optic disc (mid); (4) on the nasal foveal rim (rim); (5) the central fovea (fovea); and (6) temporal to the fovea (temporal), such that the (3) mid and (6) temporal samples are equidistant from the fovea. We used ImageJ and optical densitometry to quantify mRNA expression at the different sample locations at each developmental age. To quantify mRNA expression in the ganglion cell layer (GCL)/nerve fiber layer (NFL) we calculated the mean optical density of mRNA labeling (arbitrary units) in eight sample areas (8.8 μm/1600 pixel\(^2\)) at each location. The sample area approximated the dimensions of a GC body in perinatal retina. In images with a prominent NFL (e.g., TOD location), the sample areas were equally divided between the GCL and NFL. Within each sample area, the mean gray value (pixel intensity on the 256-gray shade scale) was measured (Measure function; ImageJ). Optical densities were calculated by normalizing the mean gray value measures against the maximum pixel intensity in that sample location. Mean pixel intensity ± SD in the GCL/NFL was plotted against retinal location for each age, and the levels of expression in all pair combinations were compared by Kruskal-Wallis test and post hoc Dunn multiple comparison test \((P < 0.01)\).

**RESULTS**

**Quantitative Real-Time PCR**

We detected all genes of interest in each of the biopsy regions of human retina by RT-PCR (Fig. 1A). The differential expression of these genes in the macula relative to the surround and nasal regions by QRT-PCR is illustrated in Figures 1B and 1C, respectively, showing good agreement with data obtained from our previous microarray analysis\(^13\) (asterisks indicate significance at \(P < 0.01\)). Comparison of data in Figures 1B and 1C indicates that expression levels of these genes were similar in the surround and nasal biopsies. The QRT-PCR findings confirm upregulation of both BNP and PEDF in the macula, com-

**FIGURE 1.** QRT-PCR amplification and measurement of differential gene expression. (A) Gel electrophoresis of QRT-PCR products amplified from 19 WG retinal RNA. Each lane was specifically amplified. Left, middle, right lanes of each gene triplet correspond to the surround (S), nasal (N), and macular (M) retinal regions, respectively, from which RNA was extracted. (B, C) Differential gene expression in 19 WG retina shown as percentage change ± SE. (□) Change according to QRT-PCR; (△) changes according to microarray. The microarray shows a significant upregulation of both BNP and PEDF in the macula, compared with the surround (B, \(P < 0.01\) ANOVA), confirmed by QRT-PCR. Differences in levels of expression of COL4A2 and NPRC, between macula and surround, are verified by QRT-PCR although the differences did not reach statistical significance on the microarray. Similar patterns of differential regulation are detected when macular is compared with nasal samples, but also show statistically significant downregulation of COL4A2 for both probe sets (C, \(\ast P < 0.01\) ANOVA). Note that the microarray includes more than one probe set for COL4A2, NPRA, and NPRC, which interrogate different transcript sequences. NPRA was not differentially regulated in the macula versus surround or macula versus nasal comparisons.
pared with that in the surround (Fig. 1B) and nasal (Fig. 1C) samples at 19 WG. In addition, the data indicate upregulation of 
NPRC in the macula, but no differential expression of 
NPRA, consistent with findings from the microarray. COL4A2 is the only gene of the group that showed marked downregulation by QRT-
PCR, which in the microarray data were significant when compared with expression in the nasal region (Fig. 1C, asterisk).

Expression of Pigment Epithelium–Derived Factor (PEDF)

By ISH, we found higher levels of PEDF expression in the GCL and in the RPE at the foveal locations, compared with periph-
ery, at all ages (Fig. 2). At Fd 80, mRNA expression was intense in GCs at the incipient fovea (Fig. 2C), and detected at lower 
levels in GCs at peripheral locations (Fig. 2D). This pattern, showing high PEDF expression in foveal GCs and lower levels of expression in GCs at peripheral locations, was also detected at 
Fd 110 (Figs. 2E, 2F), Fd 145 (Figs. 2G, 2H), Fd 150 (Figs. 2J, 
2K), Fd 164 (Figs. 2M, 2N), and P 5d (Figs. 2O, 2P). We also 
detected higher levels of expression of PEDF in the RPE at the 
fovea compared with peripheral locations, from Fd 55 (Figs. 
2A, 2B) through P 3m (Figs. 2Q, 2R). The sense riboprobe 
showed no specific labeling (Fig. 2I).

Brain Natriuretic Peptide (BNP) and Receptors

BNP mRNA Expression and Immunohistochemistry.

BNP mRNA was detected by ISH in the NFL, GCL, and inner nuclear layer (INL), of fetal and postnatal macaque retinas (Fig. 3). The most intense expression of BNP was in GCs of the central 
macula, although neurons in the inner and outer regions of the 
INL also expressed BNP. Co-localization of BNP mRNA with 
anti-GFAP/vimentin in end-foot-like structures at the retinal 
surface (Figs. 3A–C, arrowheads) and in radial processes in the 
INL and inner plexiform layer (IPL; Figs. 3E–G, arrows) indi-
cates that BNP mRNA is expressed in Müller cells. Co-localiza-
tion of BNP mRNA with vimentin/GFAP-IR in the somas of 
stellate cells deep in the GC layer, indicates that BNP is also 
expressed in the somas of the retinal astrocytes that accomp-
any the developing perifoveal vessels (Figs. 3A–C, oblique 
arrows). However, BNP mRNA was absent from the astrocyte 
processes (Figs. 3A–C, vertical arrows). Similar patterns of BNP 
expression were seen in the foveal regions of an additional six 
animals aged between Fd 55 and 11 yo. The sense riboprobe 
produced no specific labeling (Fig. 3D). Optical densitometry

perinatal macaque retina. The images have been converted to gray 
scale and inverted so that the color reaction appears dark. (A, B) At Fd 
55, PEDF expression was high in the RPE of central retina (A) and 
reduced in retina near the optic nerve (B). (C, D) By Fd 80, a similar 
high-to-low expression pattern was observed in the GCL between 
central retina (C) and retina adjacent to the optic nerve (D). (E–P) An 
upregulation of PEDF in the GCL and RPE was maintained in central 
retina at Fd 110 (E), Fd 145 (G), Fd 150 (J), Fd 164 (M), and P 5d (O), whereas a relative downregulation was observed eccentrically at each 
of these respective ages (F, H, K, N, P). In tissue adjacent to the optic 
nerve the sense riboprobe produced no specific labeling shown by the 
lack of color reaction in the RPE at Fd 150 (L). The equivalent retinal 
location in tissue hybridized to the antisense riboprobe showed posi-
tive RPE labeling (K). (Q–R) Greater PEDF expression in RPE in central 
retina (Q) compared with peripheral retina (R) was observed up to P 
3m. Note that the photoreceptor inner segments, which normally 
show low-grade autofluorescence, are adjacent to the RPE in (R) but 
have been displaced from the RPE in (Q). (C, D, J, and K) are reprinted 
from Peter Kozulin, Riccardo Natoli, Keely M. Bumsted 
O’Brien, Michele C. Madigan, Jan M. Provis. Differential expression of 
anti-angiogenic factors and guidance genes in the developing macula. 
Mol Vis 2009;15:45–59). IS, inner segment; ONL, outer nuclear layer; 
OPL, outer plexiform layer. Scale bar: (M–N) 50 μm; (all other) 25 μm.

FIGURE 2. ISH for PEDF showed an upregulation of expression in the 
RPE and GCL of the central retina, near the site of incipient fovea 
development (A, C, E, G, J, M, O, Q), relative to a more peripheral 
location nasal to the optic nerve (B, D, F, H, K, L, N, P, R) in fetal and
Fig. 3H indicates that, by Fd 55, levels of BNP are higher temporally compared with nasal retina and that a clear peak of BNP expression, which is maintained into adulthood, emerges at the developing fovea by Fd 80. Double labeling using antibodies to BNP and CRALBP (Fig. 4A) showed mild BNP-immunoreactivity (IR) (green) in the inner part of the INL (asterisk), diffuse labeling in the GCL (Fig. 4C), and more intense labeling in association with vessel profiles in the INL and GCL (Figs. 4A–C, arrows). Some CRALBP-immunoreactive Müller cell processes in the INL were mildly immunoreactive to BNP (Fig. 4A, arrowheads). BNP-IR was also present in RPE cells (not shown). Double labeling with anti-vimentin suggests inconsistent co-localization in the glia limitans surrounding the retinal vessels (Fig. 4B, arrow). Similarly, double labeling with anti-CD31 suggests inconsistent co-localization in vascular endothelial cells (Fig. 4C, arrows). Overall, BNP-IR appeared to be within and/or surrounding the vessels (Fig. 4C), rather than localized within specific cellular structures, suggesting that the protein is distributed extracellularly.

Natriuretic Peptide Receptors A and C. We were unable to detect specific labeling for NPRC mRNA by ISH and did not detect immunoreactivity for NPRC protein in our series of retinal sections. However, we detected strong NPRA-IR on cones, vascular endothelial cells and GC axons (Figs. 4D, 4F–O, 4Q–R), and at lower levels in presumed bipolar cells in the INL (not shown). NPRA-IR was detected in the cytoplasm of differentiating cones in the foveal region at Fd 55 (Fig. 4D) and between Fd 80, and adulthood NPRA-IR was prominent on the membranes of cone axons (Figs. 4H, 4L, 4O, arrows). In vascular endothelial cells, NPRA-IR was confirmed by co-localization with CD31-IR at all ages (Figs. 4F, 4j, 4M, 4Q). NPRA-IR was present on GC axons in fetal retinas (Figs. 4G, 4K, 4N, arrowheads), but was absent in adult retina (Fig. 4R). Rabbit IgG controls for anti-NPRA showed no specific labeling at all ages tested (Figs. 4E, 4P).

Collagen type Ⅳα2 (COL4A2) Immunohistochemistry. We detected COL4A2-IR in mature retinal vessels in the GCL, IPL, and INL of postnatal macaque retinas (Figs. 5A, 5B) and in well-differentiated retinal vessels in a whole-mount fetal human retina at 21 WG (Figs. 5C, 5D). COL4A2-IR was not detected in the fine, newly formed endothelial cell networks of the 21-WG human retina. Dou-
FIGURE 5. COL4A2-IR (green) was observed predominantly on mature retinal vessels, particularly in peripheral retina. At P 5d COL4A2-IR was present in peripheral retinal vessels in nasal (A) and temporal (B) retina. The smaller COL4A2-positive vessels permeated the retina as deep as the outer portion of the INL. (C, D) On 21-WG wholemount retina, COL4A2-IR was detected in mature, well-differentiated vessels in peripheral retina surrounding the macula. ONL, outer nuclear layer; OPL, outer plexiform layer. Scale bar: (A, B) 50 μm; (C, D) 100 μm.

DISCUSSION

The factors that define and maintain avascularity of the foveal region of the primate retina have not been identified. In our study, the results showed for the first time that the antiangiogenic factor PEDF is highly expressed in the GCL of the incipient fovea during development and that peak levels of expression of PEDF are maintained in the foveal region into adulthood in the macaque retina. We also find higher levels of BNP in the GCL of the foveal region of developing and adult macaque retina. Because the retinal vessels initially form in the GCL and the foveal avascular region is defined in this layer, expression of these factors at high levels in the GCL at the developing fovea during the initial phase of development of the perifoveal capillaries, places them in the optimal location to regulate retinal vascular growth.

Pigment Epithelium–Derived Factor

This is the first study to show a gradient of PEDF mRNA expression in the GCL centered on the developing fovea. PEDF is a serpin with neurotrophic and antiangiogenic functions that are mediated through several pathways. PEDF-IR has been reported in the GC layer of fetal and postnatal primate retina and in mouse retina. In the macaque retina, PEDF-IR was detected in mature, well-differentiated vessels in peripheral retina surrounding the macula. ONL, outer nuclear layer; OPL, outer plexiform layer; Rhod, rhodopsin; Vim, vimentin. Scale bars: (A–C) 25 μm; (D–R) 15 μm.

FIGURE 4. BNP and NPRA immunohistochemistry (green) in macaque retina. (A–C) BNP-IR (green) is associated with developing vasculature. Inconsistent BNP-IR was detected around retinal vessels in INL, IPL, and GCL of Fd 145 retina (arrows) and was prominent around the inner part of the INL (A, *). (A, arrowheads) Low-grade BNP-IR in Müller cell processes in the INL co-labeled with anti-CRALBP (red). Co-localization with vimentin-IR (red) indicates BNP is possibly present within the glia limitans (B, arrow). Double labeling with anti-CD31 (red) shows BNP-IR within and/or surrounding retinal vessels (C, arrows), but no co-localization in Fd 145 retina. (D–R) NPRA immunohistochemistry (green) shown with a range of counterlabels (red) in macaque retina at different stages of development (Fd 55, D–G; Fd 80, H–K; Fd 145, L–N; 11 yo, O–R). Green channel indicates NPRA-IR unless specified otherwise. (D–G) At Fd 55, NPRA-IR was present in the cytoplasm of cones in central retina (D), and remained upregulated in cone axons until 11 yo (H, I, O, arrows). Rhodopsin-IR (red) was present in the outer segments of developing rod photoreceptors (H, L, O, arrowheads). NPRA-IR co-localized with CD31-IR (red) in vascular endothelial cells at all ages tested (F, J, M, Q). NPRA-IR was also present in GC axon bundles in theNFL in fetal retina (G, K, N, arrowheads). The GC axon bundles were surrounded by vimentin/GFAP-positive macroglial processes (red). In adult retina, only very low levels of NPRA-IR were detected in GC axons (R). Rabbit IgG control serum for NPRA (green) showed no specific labeling in fetal (E) or adult (P) retina. ONL, outer nuclear layer; OPL, outer plexiform layer; Rhod, rhodopsin; Vim, vimentin. Scale bars: (A–C) 25 μm; (D–R) 15 μm.
growth of the vessels into the retina on the temporal side of the optic disc.\textsuperscript{2–4} Paradoxically, at this early stage of development, proangiogenic VEGF is also detected at high levels in the avascular GC layer of the developing fovea between \text~Fd 80 and 110.\textsuperscript{30} The present findings suggest that high levels of expression of antiangiogenic PEDF in the GC layer counteract the effects of proangiogenic VEGF,\textsuperscript{30} to prevent retinal vessels from growing into the foveal region, and define the foveal avascular area.

A similar mechanism of balance in expression of these two factors in the RPE has been proposed to prevent spontaneous neovascularization of neural retina from the choroid\textsuperscript{31,32} and overexpression of VEGF in the RPE results in neovascularization of the outer retina.\textsuperscript{33} Localization of peak expression of PEDF to the fovea into adulthood may explain why the foveal region normally remains avascular throughout life.

In the outer retina, the PEDF gene product is secreted by the RPE into the interphotoreceptor matrix in vivo where it is thought to have a role in maintenance of the avascular environment of the outer retina.\textsuperscript{34,35} Consistent with these findings, we detected PEDF expression in the RPE of developing macaque retina, with higher levels of PEDF detected in the more central RPE at early stages of development (Fig. 2). During development, the photoreceptors in the central region are more highly differentiated than in more peripheral locations. The higher levels of PEDF expression detected by ISH may reflect a more advanced state of differentiation of the RPE centrally in fetal retinas. Because the photoreceptor outer segments (OS) and RPE become increasingly autofluorescent with age, it was not possible to further analyze differential expression of PEDF in the RPE in the postnatal period and into adulthood, by using this approach.

**Brain Natriuretic Peptide**

BNP is one of a family of natriuretic peptides produced by the heart and blood vessels that is well known for its role in the regulation of blood pressure.\textsuperscript{36,37} These peptides are cleaved by proteases, and the mature proteins act as hormones that bind natriuretic peptide receptors to regulate sodium and water homeostasis.\textsuperscript{38–40} There is a growing body of evidence indicating that natriuretic peptides and their receptors have even broader functional roles, in that they are expressed dynamically in different regions of the nervous system during development,\textsuperscript{41,42} can directly regulate axon branching, outgrowth, and guidance\textsuperscript{42} and modulate GABA\textsubscript{A} receptor activity.\textsuperscript{43} In addition, atrial natriuretic peptide (ANP) and type-C natriuretic peptide (CNP) have inhibitory effects on VEGF,\textsuperscript{44,45} whereas BNP is sensitive to hypoxia, the primary angiogenic stimulus, via HIF-1\textalpha.\textsuperscript{46} Relatively little detailed information is available, however, concerning the signaling pathways involved. Of the three natriuretic peptide receptors, BNP selectively interacts via HIF-1\textalpha.\textsuperscript{46} Relatively little detailed information is available, however, concerning the signaling pathways involved. Of the three natriuretic peptide receptors, BNP selectively interacts with NPRa and NPRc.

Rollin et al.\textsuperscript{47} reported BNP-IR mainly in the inner layers of adult human retina, including GCL cytoplasm, and in RPE cells. We found that BNP-IR in macaque retina was similar to that in human retinas, although we did not detect intense BNP-IR in GCS, and Rollin et al.\textsuperscript{47} did not report immunoreactivity in the vicinity of retinal blood vessels. These minor differences are most likely due to tissue processing. BNP-IR has also been reported in Müller\textsuperscript{48} and amacrine cells\textsuperscript{49} of rat retina. By ISH, we found that BNP mRNA in macaque retinas was expressed predominantly by GCs, as well as astrocytes and Müller cells, from as early as Fd 55. Because BNP-IR was not strong in GC or astrocytes in macaque retinas, but was predominantly in Müller cells and in association with retinal vessels, our findings confirm that the BNP gene product is secreted in the retina. The observation of mRNA in the inner processes of Müller cells suggests that BNP protein synthesis within these cells is localized to these sites. This extrasomatic expression pattern is not uncommon in retinal cells, having been previously reported for GFAP mRNA in Müller cells\textsuperscript{50} and FGF2, FGFRI, and R4 mRNAs in cone photoreceptor inner segments.\textsuperscript{16,51}

We detected NPRa-IR associated with retinal vessels, GC axons in fetal retina, and cone photoreceptor axons in fetal and mature macaque retinas. Downregulation of NPRa expression in GC axons postnatally is consistent with reports of changes in the expression patterns of these proteins during development\textsuperscript{52,53} and suggests that BNP may have a specific role in the development of GC axons.\textsuperscript{45} We also found low to moderate levels of NPRa-IR associated with bipolar cells in monkey retinas. In the rat, bipolar cells are immunoreactive to both NPRa and NPRb, where it has been shown that BNP modulates activity of rod bipolar cells through the NPRa receptor, leading to suppression of GABA\textsubscript{A} receptor-mediated currents.\textsuperscript{45} Our findings suggest a similar role for BNP and NPRa in the modulation of bipolar cell activity in macaque retina.

**Collagen Type IV α2**

Type IV collagen is a prominent structural component of blood vessel basement membranes\textsuperscript{54} and is a component of the basement membrane of retinal vessels in mice.\textsuperscript{55,56} The antiangiogenic effects occur via the α2 chains, which contain the 24-kDa domain canstatin that inhibits angiogenesis via integrin-mediated, apoptotic pathways. The present findings show that collagen IVα2 is localized to the basement membranes of established retinal vessels; it was not detected in the network of small vessels forming just outside the macula at 21 WG. Thus, the low levels of COL4A2 transcripts detected in the macula are due to the relative absence of retinal vessels in the macula at 19 WG.

**Retinal Vascular Development in Primates: An Update**

VEGF is the major angiogenic factor in the eye, and many studies have shown that VEGF expression, mediated by a relative hypoxia that is sensed by retinal astrocytes, is the predominant factor promoting the formation of the retinal vessels in development\textsuperscript{5,57–60} and disease.\textsuperscript{61,62} Extant models of retinal vascular development, based on animal models in which the macula is not present, have failed to explain satisfactorily why retinal vessels are slow to grow into the macula\textsuperscript{63} and why the foveal region, in most cases, remains avascular.\textsuperscript{4,5} Indeed, we have shown previously that VEGF is highly expressed by GCS in the developing macula,\textsuperscript{50} and yet retinal astrocytes and endothelial cell migration into the developing macula is inhibited.

Our recent findings suggest that migration of astrocytes from the optic nerve head into the temporal retina may be inhibited because those astrocytes express the cell surface proteins ephrin A1 and A4, and their receptor EphA6—with
which they have an inhibitory interaction—is highly expressed in temporal retina, and is at maximum levels in the developing fovea.\(^7\) In the present study, we found peak expression of PEDF in the GCL in the developing fovea that is present early in development. Both the timing of the expression of PEDF by GCs and the localization of peak expression to the foveal region suggest that PEDF is the major factor that defines the primate foveal avascular area. A specific role for BNP in vascular patterning in the macula is yet to be determined.

**Acknowledgments**

The authors thank Anita Hendrickson for sourcing the macaque tissues, Diyat Sajuthi for expert tissue preparation, the staff at the Primate Centre, Bogor, Indonesia, for assistance, the tissue donors and staff in the Department of Endocrinology at the Prince of Wales Hospital for co-ordinating donated human fetal tissues, and John Saari for donating the CRALBP antibody.

**References**


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