의학석사 학위논문

The expression of nestin during the vascular development in mouse retina

쥐 망막의 혈관발생단계별로 살펴 본 nestin의 발현 양상 분석

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The expression of nestin during the vascular development in mouse retina

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Abstract

Introduction: The present study aimed to evaluate the expression of nestin, a neural stem cell marker, in developmental mouse retina. In addition, we aimed to indicate in which kind of retinal cells are related with the expression of nestin during the vascular development in mouse retina.

Methods: Eyes were enucleated from C57BL/6 mice, which were sacrificed postnatally on days P4, P8, P12, P16 and P26, and sectioned to 4㎛ after paraffin embedding. Immunofluorescence and immunohistochemistry were used to identify the expression of nestin and CD31, α-SMA, and GFAP.

Results: The nestin expression was confined to GCL where the end feet of Muller radial glial cell located during P4 to P8. As the intermediate vascular plexus becomes visible on P12, the nestin expression was scarcely observed along the vessel wall located in INL. The nestin expression was observed on GCL and INL with speckled cytoplasmic pattern on P16 and P26. The nestin expression merged with those of CD31 and α-SMA, but not that of GFAP.

Conclusions: The expression of nestin was in close relationship with those of endothelial cells and pericytes, but not with that of astrocytes during the vascular development in mouse retina.

Keywords: Nestin; Retinal vascular development; Endothelial cell; Pericyte; Astrocyte

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Introduction

Nestin, the sixth class of intermediate filament, has been demonstrated to be expressed in neural progenitor cells during development, and known as a neural stem cell marker (1, 2). It is also known to be expressed by immature or progenitor cells in non-neuronal cells in normal tissues (3-5). In addition, the role of nestin as a marker for neovascularization has been highlighted (6-8). It is known to be expressed in vascular endothelial cells from variety of human non-neural tissues, including the pancreas, the corpus luteum, and the full term placenta (8, 9). Its expression has also been reported from various kinds of tumors, and documented to be involved with tumor angiogenesis (10, 11).

Recently, nestin expression has been identified in neonatal rat and human retina (12, 13). It has been shown that Müller cells express the nestin in both differentiated and undifferentiated human fetal retina (14). The role for nestin as a biomarker of retinal injury has been suggested from numerous studies (15-20). However, there are few studies which demonstrated the role of nestin in the development of retinal vasculature.

The mouse retinal vasculature is formed by primary vasculogenesis, which retinal vasculature sprouts radially from the optic nerve via the fine meshwork of astrocytes, and followed by secondary angiogenesis, which the superficial primary vessel plexus sprouts vertically to the deep inner retina (21, 22). Endothelial cells, astrocytes and pericytes are known to closely associated with vascular formation and remodeling during development (23).

The present study aimed to evaluate the expression of nestin in developmental mouse retina. In addition, we aimed to indicate in which kind of retinal cells are related with the expression of nestin during the vascular development in mouse retina using double immunofluorescence of nestin with CD31, α-SMA, and GFAP in mouse retina.

Materials and methods

Animals

C57BL/6 mice were purchased from Samtako (Korea). All animals used in this study were in strict agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 mice were cared in standard 12-hr dark-light cycles and approximately 23℃ room temperature. Eyes were enucleated from C57BL/6 mice sacrificed postnatally on days P4, P8, P12, P16 and P26.

Antibodies

Primary antibody

For the detection of nestin, rat monoclonal IgG1 anti-nestin antibody (1:300, Merck Miilipore, Billerica, MA, USA) was used. For the detection of α-SMA, the marker for pericytes, mouse monoclonal IgG2a antibody (1:300, Sigma-Aldrich, Saint Louis, MO, USA) was used. For the detection of CD31, the marker for endothelial cells, mouse monoclonal IgG1 anti-CD31 antibody (1:200, BD Biosciences, San Jose, CA, USA) was used. For the detection of GFAP, the marker for astrocytes, rabbit polyclonal anti-GFAP antibody (1:600, Dako, Carpinteria, CA, USA) was used. Primary antibodies were diluted with dilution buffer; 1% bovine serum albumin, 0.1% cold fish skin gelatin, 0.05% sodium azide and 0.01M PBS (pH 7.2).

Secondary antibody

For immunofluorescence, Alexa Fluor® 594 donkey anti-mouse IgG (1:500, Invitrogen, Carlsbad, CA, USA), Alexa Fluor® 594 donkey anti-rabbit IgG (1:800, Invitrogen, Carlsbad, CA, USA), and Alexa Fluor® 488 goat anti-rat IgG (1:200, Invitrogen, Carlsbad, CA, USA) were used. For immunohistochemistry, polyclonal rabbit anti-rat antibody (1:100, Dako, Carpinteria, CA, USA) was used.

Immunofluorescence

Paraffin-embedded enucleated eyes were sectioned into 4㎛ sections. Those sections were rehydrated to xylene and graded ethanol; 100%, 100%, 95%, 80%, and 70% ethanol for 5 minutes each. After gently washing in running water for 5 minutes, the sections were washed again in phosphate buffered saline (PBS) for another 5 minutes. Proteinase K was applied to the sections for 10 minutes at 37℃. After washing in PBS for 5 minutes twice, the slides were incubated with 0.1% Triton-X100 for 10 minutes at room temperature. With serial rinsing in PBS for 5 minutes twice, the sections were incubated with blocking solution for 10 minutes, followed by incubation with primary antibodies at 4℃, overnight. After rinsing in PBS for 10 minutes twice, the sections were incubated with secondary antibodies for 1 hour at room temperature, with light hindered. After an additional PBS washing for 10 minutes twice, the sections were mounted on the slide glass using Faramount Aqueous mounting medium (Dako, Glostrup, Denmark) and observed under a fluorescence microscope (Nikon Eclipse 80i, Japan). The digital images were

gained and processed with NIS-Elements Microscope Imaging Software ver. 4.00 (Nikon, Japan).

Immunohistochemistry

After de-paraffinization of the sections according to the method mentioned above, proteinase K and 0.1% Triton-X100 were applied for 10 minutes, consecutively. After PBS washing for 5 minutes, 3% H_2O_2 was applied for 10 minutes. After incubation with blocking solution for 10 minutes, the sections were incubated with primary antibody (anti-nestin, 1:100) for 2 hours at room temperature, and then incubated with secondary antibody for 1 hour at room temperature. After PBS washing for 5 minutes twice, Streptavidin horseradish peroxidase (SA-5704, Vector Laboratories, Inc., Burlingame, CA, USA) was applied for 10 minutes. AEC substrate solution (Invitrogen, Carlsbad, CA, USA) was applied to the section with the microscope monitoring the color change. The sections were thoroughly dried at room temperature and mounted with non-aqueous mounting medium.

Results

1. Normal vascular development in mouse retina

The hyaloid vessels were still persisted on postnatal day 4 (P4), and it started to regress on P8 (**Figure 1A, 1B**). On P4, the superficial vascular plexus originating from the optic nerve head extended radially, which reached to mid-peripheral retina, and it extended to far periphery on P8 (**Figure 1A, 1B**). On P12, the vertical sprouting vessels toward the deep vascular plexus, the secondary angiogenesis, were observed (**Figure 1C**). Further maturation of vessels, especially intermediate vascular plexuses, occurred during P16 to P26 (**Figure 1C**). Taken together, we identified the primary vascular development from optic nerve head extending to peripheral retinal until P8, and secondary angiogenesis which vertically reached to inner nuclear layer (INL) during P12 to P26.

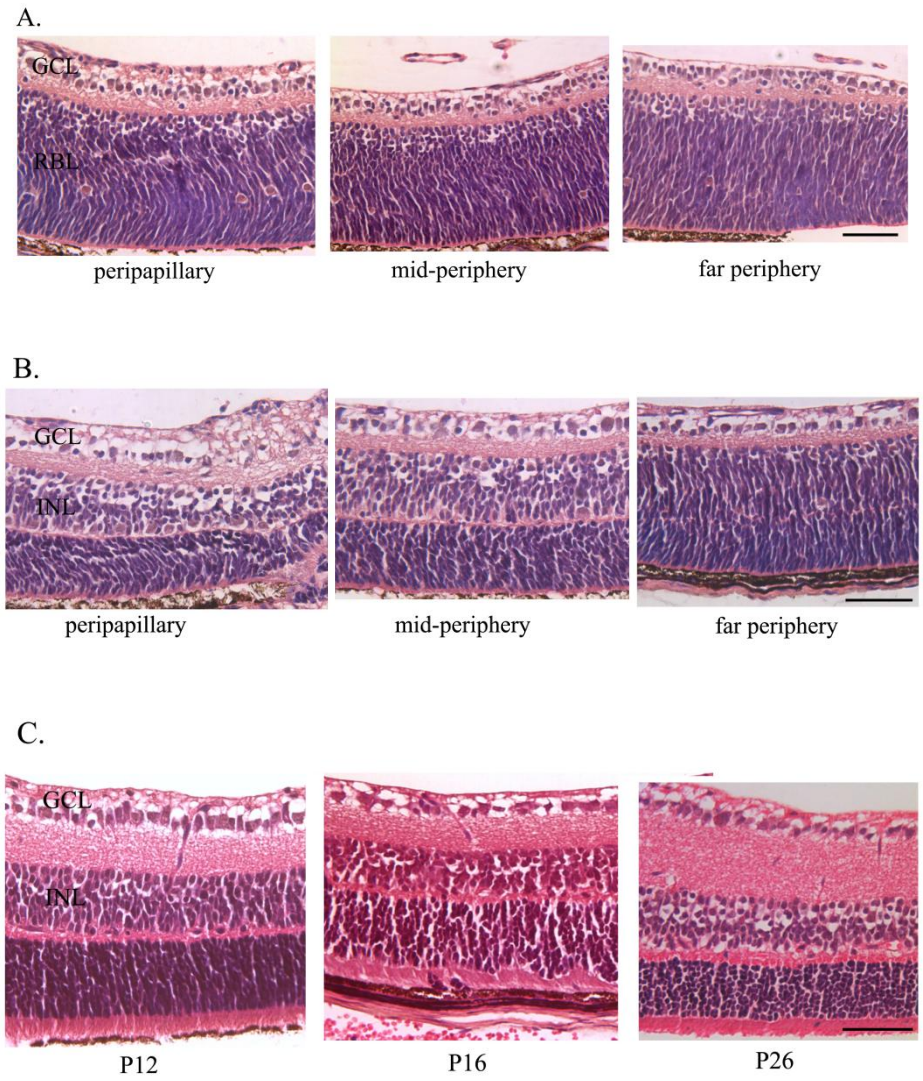


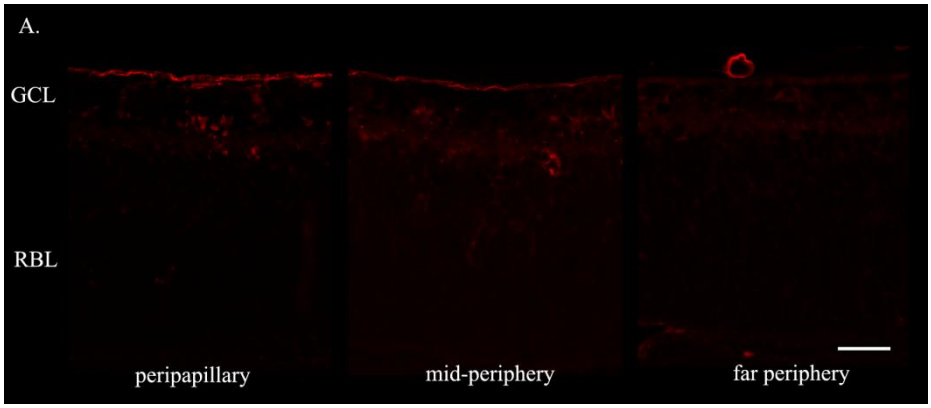
Figure 1. The normal vascular development in mouse retina.

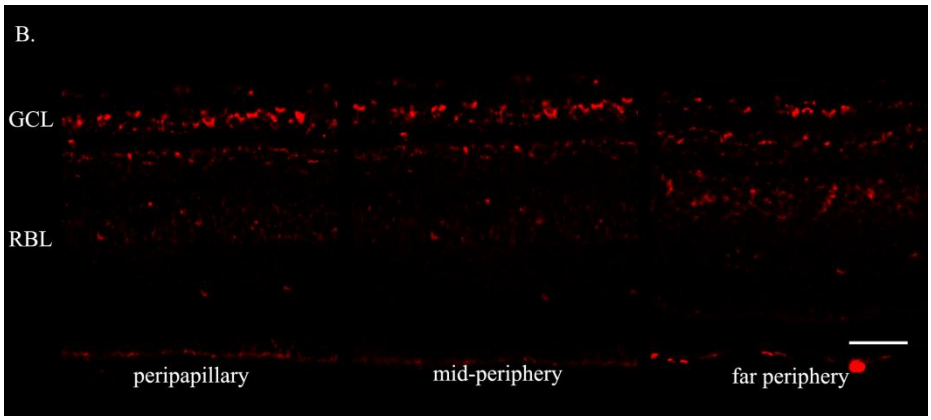
On P4, the superficial vascular plexus originating from the optic nerve head extended radially, which reached to mid-peripheral retina (**A**), and it extended to far periphery on P8 (**B**). The hyaloid vessels still persisted on P4 (**A**), and it started to regress on P8 (**B**). On P12, the vertical sprouting vessels toward the deep vascular plexus, the secondary angiogenesis, were observed (**C**). Further

maturation of vessels, especially intermediate vascular plexuses, occurred during P16 to P26 (**C**). GCL, ganglion cell layer; RBL, retinoblast layer; INL, inner nuclear layer. Scale bar = 50μm.

2. The expression of CD31, α-SMA, and GFAP in developmental mouse retina.

On P4, CD31 was expressed on ganglion cell layer (GCL) at peripapillary and mid-periphery retina, and on hyaloid vessels, but the expression was relatively weak at far periphery retina (**Figure 2A**). GFAP and α-SMA were also expressed on the GCL with relatively weak immunofluorescence at far periphery retina (**Figure 2B, 2C**). On P8, CD31 was expressed on GCL at the entire retina, and the expressions along the vertical vessel wall that sprouting to the deep plexus were observed on peripapillary retina (**Figure 2D**). α-SMA was expressed on GCL and INL at the entire retina (**Figure 2E**). However, GFAP expression was confined to the GCL (**Figure 2F**). On P12, CD31 began to express along the vessels walls on inner plexiform layer (IPL) and INL where secondary angiogenesis occur, and this pattern maintained until P26 (**Figure 2G**). α-SMA expression on INL became obvious at P12, and continued until P26 (**Figure 2H**). GFAP expression was observed only on GCL thorough the whole period of the development (**Figure 2I**). Taken together, we identified the endothelial cells on GCL until P8, and along the vessel walls which reached to INL during P12 to P26. Pericytes were also located on GCL from P4 and extended to INL from P8. However, astrocytes were confined to GCL through the whole period of the development.


A.
GCL
RBL
peripapillary
mid-periphery
far periphery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RBL
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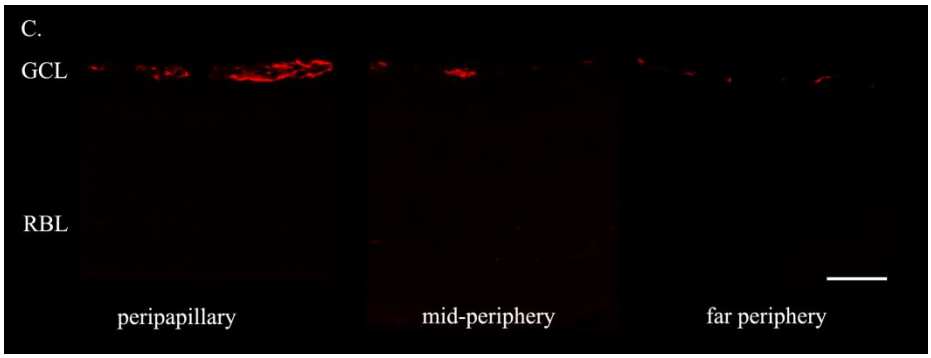
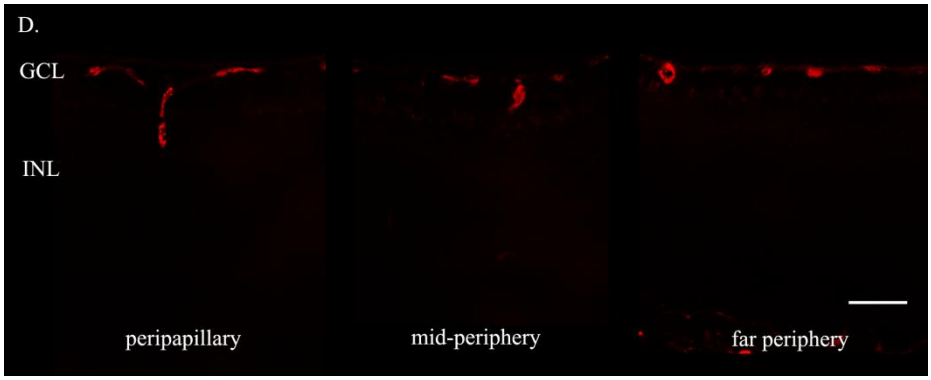
mid-periphery
far periphery


C.
GCL
RBL
peripapillary
mid-periphery
far periphery


D.
GCL
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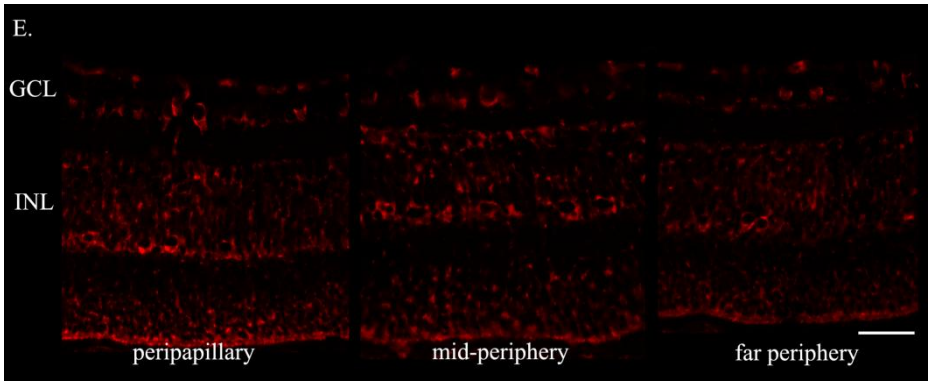
peripapillary
mid-periphery
far periphery


E.
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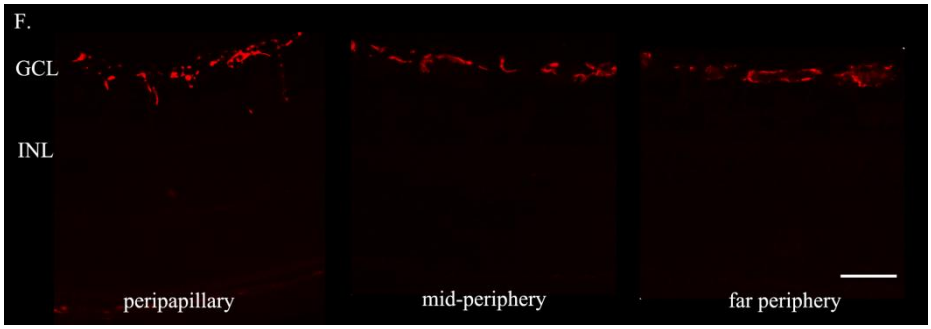
F.
GCL
INL
peripapillary
mid-periphery
far periphery

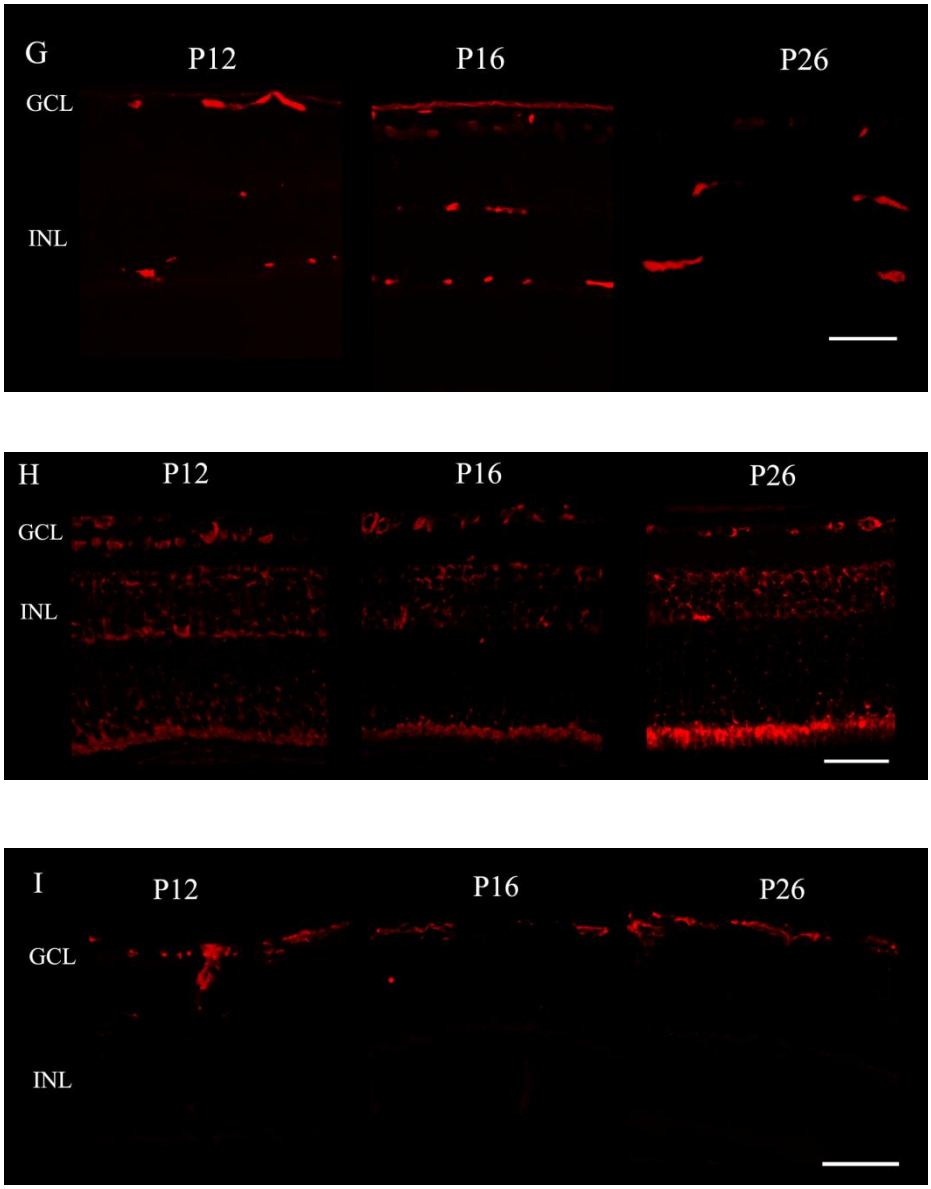

Figure 2. The immunofluorescence expression of CD31, α-SMA, and GFAP in developmental mouse retina.

On P4, CD31 was expressed on ganglion cell layer (GCL) at peripapillary and mid-periphery retina, and on hyaloid vessels, but the expression was relatively weak at far periphery retina (**A**). GFAP (**B**) and α-SMA (**C**) were

also expressed on the GCL with relatively weak immunofluorescence at far periphery retina. On P8, CD31 was expressed on GCL at the entire retina, and the expressions along the vertical vessel wall that sprouting to the deep plexus were observed on peripapillary retina (**D**). α-SMA was expressed on GCL and inner nuclear layer (INL) at the entire retina (**E**). However, GFAP expression was confined to the GCL (**F**). On P12, CD31 began to express along the vessels walls on inner plexiform layer (IPL) and INL where secondary angiogenesis occur, and this pattern maintained until P26 (**G**). α-SMA expression on INL became obvious at P12, and continued until P26 (**H**). GFAP expression was observed only on GCL thorough the whole period of the development (**I**). GCL, ganglion cell layer; RBL, retinoblast layer; INL, inner nuclear layer. Scale bar = 50 μm.

3. The expression of the nestin in developmental mouse retina.

The nestin expression was confined to GCL where the end feet of Muller radial glial cell located during P4 to P8 (**Figure 3A, 3B**). As the intermediate vascular plexus becomes visible on P12, the nestin expression was scarcely observed along the vessel wall located in INL (**Figure 3C**). Although not all retinal cells expressed the nestin, its expression was observed on GCL and INL with speckled cytoplasmic pattern on P16 and P26.

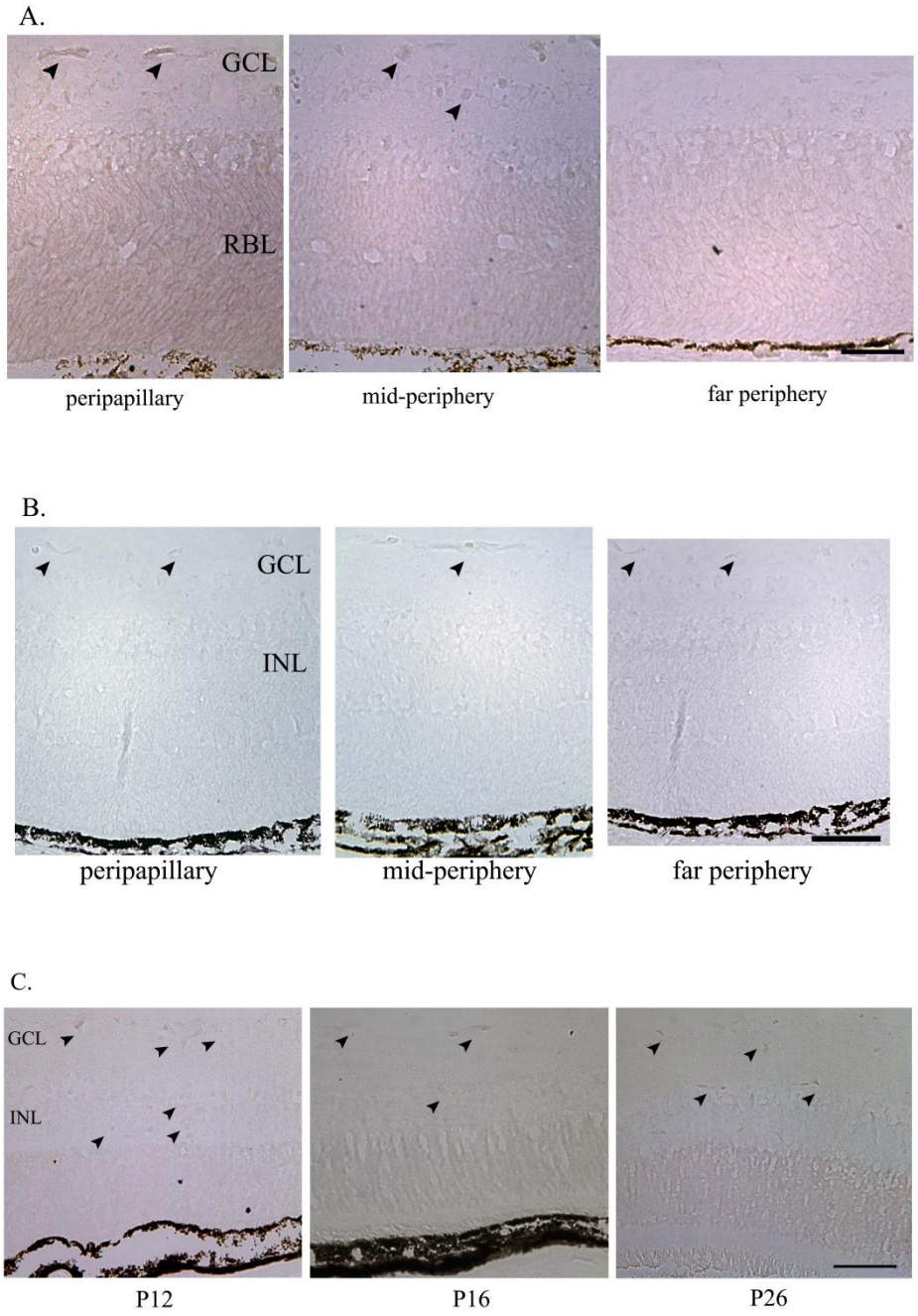

Figure 3. The expression of the nesin in developmental retina

The nestin expression was confined to GCL where the end feet of Muller radial glial cell located during P4 (**A**) to P8 (**B**). As the intermediate vascular

plexus becomes visible on P12, the nestin expression was scarcely observed along the vessel wall located in INL (**C**). Although not all retinal cells expressed the nestin, its expression was observed on GCL and INL with speckled cytoplasmic pattern on P16 and P26 (**C**). GCL, ganglion cell layer; RBL, retinoblast layer; INL, inner nuclear layer. Scale bar = 50㎛.

4. The co-expression of the nestin with CD31 and α-SMA in developmental mouse retina.

From the immunofluorescence study, we observed the expression of the nestin merged with the expression of CD31 and α-SMA, but not that of GFAP. At P4, nestin expression was mainly observed along the vessel walls which merged with CD31, and on GCL and upper portion of RBL which merged with α-SMA (**Figure 4A, 4B**). As the secondary angiogenesis occurred from P12, nestin expressed along the vertical vessel walls that invading the deep plexus, and also on INL. This pattern of merge increased as the postnatal day goes on from P16 to P26 (**Figure 4A, 4B**). GFAP expression was confined to GCL through the whole developmental period. Although nestin expressed on GCL during the vascular development, its expression did not markedly merged with that of GFAP (**Figure 4C**). Taken together, the expression of nestin was in close relationship with those of endothelial cells and pericytes, but not with that of astrocytes.

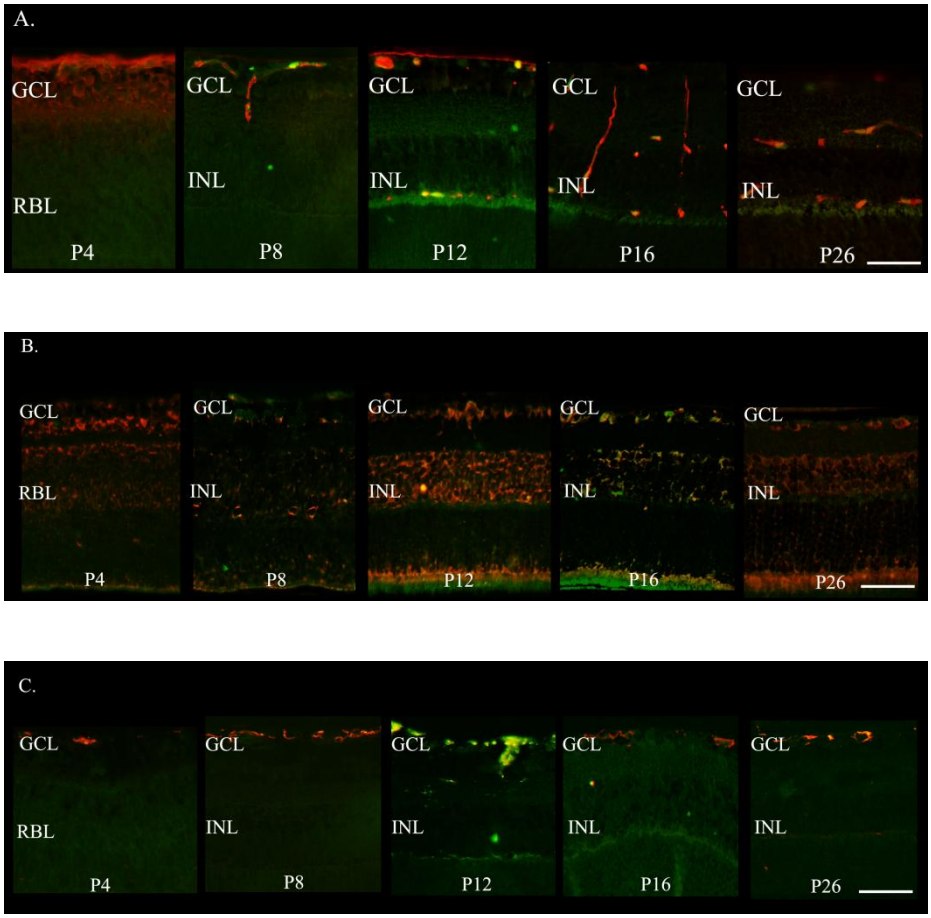

Figure 4. The co-expression of the nestin with CD31 and α-SMA in developmental mouse retina.

At P4, nestin expression was mainly observed along the vessel walls which merged with CD31 (**A**), and on GCL and upper portion of RBL which merged with α-SMA (**B**). At P12, nestin expressed along the vertical vessel walls invading the deep plexus, and also on INL, which merged well with CD31 and α-SMA. This pattern of merge increased as the postnatal day goes on from P16 to P26 (**A, B**). GFAP expression was confined to GCL through the

whole developmental period (**C**). Although nestin expressed on GCL during the vascular development, its expression did not markedly merged with that of GFAP (**C**). GCL, ganglion cell layer; RBL, retinoblast layer; INL, inner nuclear layer. Scale bar = 50㎛.

Discussion

The present study was designed to identify the expression of nestin in developmental mouse retina, and to demonstrate its relationship with endothelial cells, astrocytes and pericytes. The nestin was expressed in GCL where the end feet of Muller radial glial cell located during P4 to P8. Its expression extended to INL as secondary angiogenesis begin from P12. The result of the present study corresponded well with those of the earlier study that identified the expression of the nestin in the retina. Mayer et al (12) reported that nestin expressed in GCL and INL with speckled cytoplasmic staining in adult human retina. Not all retinal ganglion cells showed positivity to the nestin, but the expression dramatically increased in extreme peripheral retina, where proliferative ciliary margin zone located. However, the present study did not find any increase of expression of the nestin according to the location of the retina. Lee et al (24) reported the expression of nestin in developing rat retina, which restricted to the proximal part and finally to the end feet of Muller radial glial cells with aging. Our immunohistochemistry results were in close agreement with those of Lee et al (24).

The immunofluorescence expression of nestin merged with CD31 and α-SMA, which are known as marker for endothelial cells and pericytes, respectively. However, the nestin expression did not merge with that of GFAP, the astrocyte marker. Nestin is well known to be expressed in endothelial cells of developing central nervous system, peripheral nervous system, myogenic and other tissues (6, 8). The expression of nestin in pericytes is also reported

in mouse brain parenchymal vessels and in adult rat retina (25). Wohl et al (20) indicated nestin positive microglia co-expressing NG2, the pericyte marker, but not GFAP in adult rat retina. Lee et al (24) showed electron microscope findings of partially immunoreactive pericytes to nestin in developing rat retina. Taken together, we can infer that nestin is in close relation with endothelial cells and pericytes during vascular development in mouse retina. The intermediate filament nestin would be necessary for the mitosis and migration of the endothelium and pericyte during the active angiogenesis.

From the present study, the nestin expressed at the end feet of Muller glial cells, and did not merge with GFAP expression. The mature Muller cells do not express GFAP in normal atmosphere. However, the earlier studies demonstrated the co-expression of nestin and GFAP on rat retina in pathologic conditions such as glaucoma and optic nerve transection (17, 18, 20). In line with these findings, astrocytes may not express nestin during vascular development but rather in injurious atmosphere.

In conclusion, nestin expression was observed at the end feet of Muller glial cells and along the vessel walls in GCL from P4 to P8, and in INL from P12 when the secondary angiogenesis begins. The expression of nestin was in close relationship with those of endothelial cells and pericytes, but not with that of astrocytes.

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국 문 초 록

서론: 본 연구는 제6형 중간섬유단백으로서, 신경계 줄기세포 마커로 잘 알려져 있고, 최근 신생혈관발생의 마커로도 각광받고 있는 nestin이 망막혈관발생단계에서 어떻게 발현하는지 확인하고, 망막혈관발생단계에서 혈관내피세포, 혈관주위세포, 그리고 성상교세포의 발현과 어떤 연관을 가지는지 확인하고자 하였다.

방법: C57BL/6 쥐의 안구를 출생 후 4일, 8일, 12일, 16일, 그리고 26일 째 적출하였다. 4㎛ 두께 파라핀 절편을 만든 후 면역형광염색과 면역화학염색법을 이용하여 nestin 발현 양상을 확인하고, CD31, α-SMA, 그리고 GFAP 발현 양상과 비교하였다.

결과: Nestin은 출생 후 4일부터 8일째까지 망막 신경절세포층 내 뮐러세포의 발 끝 부분과 혈관벽 주변에서 발현되는 것이 관찰되었다. 출생 후 12일째부터 이차 신생혈관형성이 시작되면서, 망막내핵층과 심층부를 향해 수직으로 뻗어나가는 혈관 주변부에서 발현이 관찰되었고 이런 발현 양상은 출생 후 26일째까지 지속되었다. Nestin의

발현은 CD31과 α-SMA의 발현과 어우러졌으나, GFAP의 발현과는 관련이 없었다.

결론: Nestin은 쥐 망막혈관발생 단계에서 출생 후 8일까지 신경절세포층의 뮐러세포 발 끝 부분과 혈관벽 주위에서, 이차 신생혈관발생이 시작되는 출생 후 12일부터는 내핵층의 혈관벽 주위에서도 발현이 관찰되었다. 망막혈관발생단계에서 Nestin의 발현은 혈관내피세포와 혈관주위세포의 발현과 깊은 관련이 있었으나, 성상교세포의 발현과는 관련이 없었다.

주요어: Nestin; 망막혈관발생; 혈관내피세포; 혈관주위세포; 성상교세포

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