Role of CD44$^+$ Stem Cells in Mural Cell Formation in the Human Choroid: Evidence of Vascular Instability Due to Limited Pericyte Ensheathment

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Purpose. To examine mural cell differentiation and pericyte ensheathment during human choroidal vascular formation and into adulthood.

Methods. Triple- and double-labeled immunohistochemistry (alpha-smooth muscle actin [αSMA], desmin, NG2, calponin, caldesmon, CD44, CD34, and CD39) were applied to human fetal (8–32 weeks' gestation) and adult choroidal and retinal wholemounts and histologic cross-sections. Transmission electron microscopy (TEM) was also undertaken.

Results. Early in development CD44$^+$ stem cells also stained with αSMA and CD39, suggesting a common precursor. At 12 weeks' gestation, αSMA$^+$ mural precursor cells, confirmed by TEM, were found scattered and isolated over the primordial vascular tree. During development, αSMA$^+$ cells formed a continuous sheath around large arterioles; in veins there were gaps in αSMA expression. The choriocapillaris had an extensive vascular bed but limited coverage by αSMA$^+$ and NG2$^+$ mural cells. Calponin was expressed only on large vessels, and no caldesmon was detected. Pericyte ensheathment of adult capillaries was 11% for choroid versus 94% for retina. Remarkably, choroidal pericytes had no visible intermediate filaments (IFs) on TEM, though IFs were present in retinal pericytes. Neither retinal nor choroidal pericytes stained with desmin.

Conclusions. CD44$^+$ stem cells are involved in the formation of mural cells in the human choroidal vasculature. A marked reduction in pericyte ensheathment of human choroidal vessels suggests a permanently open "plasticity window" and a predisposition to vascular instability and poor autoregulatory ability. (*Invest Ophthalmol Vis Sci.* 2011;52:399–410) DOI:10.1167/iovs.10-5403

Blood vessels consist of an endothelial tube ensheathed by mural cells, which are cells embedded within the basal lamina of the vessel wall. The mural cells of arteries, arterioles, and veins are defined as smooth muscle cells (SMCs),[1] and the mural cells of capillaries and venules are defined as pericytes.[2] Mural cells affect the function of blood vessels. In mature vascular plexi, SMCs are primarily contractile, maintaining blood pressure and regulating blood flow. In contrast, the functions of pericytes in the vasculature are less clear, though postulated roles include blood flow regulation, vessel stabilization,[3–6] and VEGF expression.[7]

Structurally, the adult choroid is a unique vascular bed that, unlike the retina, lies outside the central nervous system (CNS). The adult retinal vessels share many characteristics with those of the brain, including blood–brain barrier properties (see Ref. 8 for review), glia[9] and mural cells.[10] In contrast, the adult choroidal vessels differ from CNS vessels in structure (lacking glial ensheathment), flow rate, permeability and oxygen clearance (see Ref. 11 for review). For vision there must be an unimpeded light path through the eye to the photoreceptors. Thus the retinal vasculature only supplies the inner two-thirds of the retina, and the choroidal circulation supplies the outer one-third of the retina through the choriocapillaris. The choriocapillaris is a fenestrated vascular bed that abuts the retina but is physically separated from it by Bruch's membrane. The blood flow rate and the oxygen tension in the choroid are much higher than in any other tissue, and, in general, the choroidal capillaries are larger (see Ref. 11 for review). High blood flow and oxygen tension are required to sustain the metabolic needs of the photoreceptors, though they have also been suggested to help cool the tissue (see Ref. 11 for review). The retinal and choroidal vessels differ in their ability to autoregulate; we have limited understanding of why or how.

A recent study demonstrated that NG-2–positive cells were first present in the developing human choriocapillaris at 11 weeks' gestation, but it was not until 22 weeks' gestation that mature, alpha-smooth muscle actin-positive (αSMA$^+$) cells were present.[12] However, the origin of the choroidal mural cells, the dynamics of their recruitment and differentiation, and the mechanism by which they affect vascular remodeling remain unclear. Different vascular beds are likely to have different sources for mural cells, including the mesoderm, the mesenchyme, the neural crest, epicardial cells, and, in adults, the bone marrow (see Ref. 13 for review). Several mouse studies have suggested that mural and endothelial cells can arise from a common CD44$^+$ precursor,[14,15] and these stem cells can give rise to vascular tubes.[16] CD44, an astrocytic stem cell marker[17] also found in hematopoietic tissue,[18] has been shown to associate with developing blood vessels in the human retina[19] and choroid (Chan-Ling T, Dahlstrom JE, Koina ME, et al., unpublished data, 2009), further implicating a role for CD44$^+$ stem cells in blood vessel development.

Mural cells provide blood vessel stability,[4,5,10] and pericyte ensheathment of a blood vessel can prevent angiogenesis. In the retina, this can be clearly seen during development, when

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Supported by the Baxter Charitable Foundation, the Rebecca Cooper Medical Research Foundation, Financial Markets Foundation for Children, and National Health and Medical Research Council of Australia Grants 571100 and 464859 (all TC-L).

Submitted for publication February 18, 2010; revised June 2 and 28, 2010; accepted June 30, 2010.

Disclosure: **T. Chan-Ling**, None; **M.E. Koina**, None; **J.R. McColm**, None; **J.E. Dahlstrom**, None; **E. Bean**, None; **S. Adamson**, None; **S. Yun**, None; **L. Baxter**, None

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the immature vessels that do not have pericyte coverage can be induced to neovascularization whereas pericyte-covered vessels cannot.[20,21] The reactivity of adult retinal vessels to VEGF, a stimulator of angiogenesis, is highly dependent on pericyte coverage.[21,22] We have also previously shown that the desmin ensheathment ratio is correlated with vessel stability in normal development and in oxygen-induced retinopathy.[20] In adult retinal diseases such as diabetes, pericytes are the earliest cells that are affected.[23] Many cancers are associated with pericyte decrease or, in some cases, pericyte absence (see Ref. 24 for review), and pericytes have been implicated in the pathogenesis of both Alzheimer disease and cerebral ischemia (see Ref. 25 for review). As the disease progresses and blood vessels are left without pericyte ensheathment, the underlying endothelial cells of the blood vessels become reactivated to angiogenic stimulators, and, thus, neovascularization can occur.

Although a number of pericyte markers have been described, none of these constitutes a "pan pericyte marker." Recently, we have made significant progress in the identification of suitable markers of pericytes and SMCs in rat[10] and cat[20] retina. Most commonly, αSMA is used to delineate contractile SMCs and pericytes.[26] Desmin, an intermediate filament (IF) 6 to 12 nm in diameter,[27] has been used extensively in studies to stain for pericytes,[10,20,28–30] and to quantitate pericyte ensheathment of the developing retinal vessels.[20] NG2, an integral membrane proteoglycan, was first described as an oligodendrocyte progenitor cell marker in the CNS but has more recently been identified as a mural cell marker (see Ref. 31 for review).

In contrast to other recent studies,[12,32,33] the aims of our study were to examine the whole of the developing human choroidal blood vessels not just the choriocapillaris, to determine the role of stem cells in the formation of mural cells, to quantify comparatively the pericyte ensheathment of human retinal capillaries and the choriocapillaris in adults, and determine the temporal ensheathment of blood vessels by mural cells. Triple-label immunohistochemistry (αSMA, desmin, NG2, calponin, caldesmon, CD44, CD34, and CD39) were applied to human fetal and adult choroidal wholemounts. In addition, double-labeled histologic cross-sections and transmission electron microscopy were also undertaken, with comparisons made between the retinal and choroidal vascular beds. Our observations lead us to conclude that there is a marked difference in pericyte ensheathment between the choroidal and retinal vessels both during development and in healthy young adults. This limited pericyte ensheathment predisposes the adult choroidal vascular bed to instability, which has relevance to choroidal neovascularization in the pathophysiology of age-related macular degeneration (ARMD). The structural differences between retinal and choroidal vasculature may also underlie the previously reported poor autoregulatory ability of the choroidal vasculature.

Materials and Methods

Collection of Human Fetal Eyes

Human fetal eyes, ranging in age from 8 to 32 weeks' gestational age, were collected from two sources. Eyes younger than 20 weeks' gestation were obtained through a consortium of Sydney-based biomedical scientists for the access of human fetal tissue, headed by Bernie Tuch of the Pancreas Transplantation Unit of the Prince of Wales Hospital. Specimens were collected in accordance with the guidelines set forth in the Declaration of Helsinki and with the approval of the Human Ethics Committee of the University of Sydney. Fetuses were obtained after prostaglandin-induced abortions. The age of each fetus was determined from the date of last menstruation. Eyes older than 20 weeks' gestation were collected in China in accordance with the guidelines set forth in the Declaration of Helsinki, and the study was approved by the Human Ethics Committee of the University of Sydney. These fetuses had died of natural causes after premature or difficult deliveries. The age of each fetus was determined from charts of crown-rump length and crown-heel length.

Preparation of Human Fetal Choroidal Wholemounts

Four to five radial cuts were placed through the entire thickness of the choroid and sclera. The choroid was dissected away from any scleral attachments to ensure continuity of the choroid through the optic nerve head, then immersion fixed in 4% paraformaldehyde for 1 hour at 4°C (Chan-Ling T, Dahlstrom JE, Koina ME, et al., unpublished data, 2009).

Collection of Adult Eyes

Adult eyes were collected from the Sydney Eye Bank. After corneal transplantation, the excess tissue that had been donated was collected for research. Within 24 hours, the eyecup was immersion fixed in 2% paraformaldehyde in 0.1 M phosphate buffer and then stored in this solution until sectioning. Samples came from young adults who had died as a result of motor vehicle accidents and from suicide victims, all of whom had no history of eye disease.

Immunohistochemistry on Choroidal Flatmounts

Multiple labeling was used to covisualize various antigens (see Table 1 for a description of each marker) and the vasculature, with a maximum of three fluorochromes applied to each specimen. A minimum of three specimens were analyzed per described phenomenon, and conclusions reached were representative of all specimens examined. For immunohistochemistry, we looked at a minimum of two fetal eyes from every time point and four eyes for adults. Choroids were incubated overnight at 4°C with primary antibodies (see Table 1), washed with 0.1% nonionic surfactant (Triton X-100; Sigma-Aldrich, St. Louis, MO) in PBS, incubated for 4 hours at room temperature with the appropriate secondary antibodies (see Table 1), and washed again. For double or triple labeling, this procedure was repeated with the different primary antibodies and appropriate secondary antibodies. Negative controls omitting a primary antibody were carried out for each antibody and protocol. All primary and secondary antibodies were diluted with 1% bovine serum albumin in PBS, and all washes were performed with 0.1% nonionic surfactant nonionic surfactant (Triton X-100; Sigma-Aldrich) in PBS. Choroidal wholemounts were mounted in glycerol/PBS (2:1, vol/vol) or antifade reagent (ProLong Antifade; Molecular Probes, Invitrogen, Carlsbad, CA) either RPE up or RPE down, depending on the layer of interest and the findings on analysis.

Microscopy

Choroidal wholemounts were examined by confocal microscopy with an argon-krypton laser mounted on an epifluorescence photomicroscope (DMRBE; Leica Microscope Systems, Wetzlar, Germany). Fluorescence with Alexa488, Cy3, and Cy5 was excited sequentially at 488, 550, and 650 nm, respectively. Images were processed with commercial software (Photoshop CS, version 8.0; Adobe Systems Inc., San Jose, CA). Multiple fields of views were stitched to create a montage (Photoshop CS, version 8.0; Adobe Systems Inc.).

Electron Microscopy

Human fetal eyes were immersion fixed in 4% paraformaldehyde (PFA) for 1 hour at 4°C. They were washed well in 0.1 M sodium phosphate buffer (Sorenson's, pH 7.4), then postfixed with 2% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M sodium phosphate buffer for 2.5 hours. Adult human eyes were immersion fixed in 2% PFA, then postfixed in 2% osmium tetroxide. En bloc staining with 2% uranyl acetate preceded dehydration through a graded series of ethanol steps. Specimens were

TABLE 1. Description of Markers Used to Stain Tissue

Antibody	Description of Marker	Manufacturer	Species	References
NG2	NG2 is the rat homolog of HMW-MAA, a transmembrane chondroitin sulfate proteoglycan expressed by immature human smooth muscle cells and pericytes and by mature smooth muscle cells, but it is expressed only sporadically and at a low level by the capillaries of a quiescent vasculature.	Chemicon International, Temecula, CA	Rabbit polyclonal	28, 29, 30, 34, 35
Desmin	Desmin is expressed by both immature and mature pericytes and by a subpopulation of smooth muscle cells associated with developing or mature arteries.	Dako, Glostrup, Denmark	Mouse monoclonal immunoglobulin G subclass 1 (IgG1)	36–39
		Abcam, Cambridge, UK	Rabbit polyclonal	
SMA	SMA is expressed by immature and mature smooth muscle cells and by a subpopulation of pericytes.	Dako, Glostrup, Denmark	Mouse monoclonal IgG2a	40, 41
Calponin	Calponin expression is restricted to smooth muscle cells and differentiated contractile phenotypes of developing smooth muscle.	Novocastra/Leica Microsystems, Bannockburn, IL	Mouse monoclonal IgG1	
Caldesmon	Caldesmon is a regulatory protein involved in smooth and non-muscle contraction.	Dako, Glostrup, Denmark	Mouse monoclonal IgG (IgG1, κ isotype)	
CD34	CD34 is a single-chain transmembrane glycoprotein selectively expressed on human lymphoid and myeloid hematopoietic progenitor cells and on the filopodial extensions and the luminal membrane of endothelial cells.	Serotec, Raleigh, NC	Mouse monoclonal	42
CD39	CD39 is an ecto-ADPase and a marker of VPCs and human endothelial cells but is also expressed on mature B and microglial cells.	Novocastra/Leica Microsystems, Bannockburn, IL	Mouse monoclonal	
CD44	CD44 is a cell adhesion receptor widely expressed on hematopoietic and nonhematopoietic cells.	Immunotech, Marseille, France	Mouse monoclonal	43

infiltrated with low-viscosity epoxy resin (Spurr's replacement; TAAB Laboratory and Microscopy, Berkshire, UK; 50:50 ethanol/TAAB resin for 2 hours followed by TAAB resin for 3 hours), embedded, and set overnight at 70°C. Multiple levels of thin sections (100 nm) were cut from each block of each eye with a minimum of three levels examined. Thin sections mounted on copper/palladium grids were stained with Reynold's lead citrate and viewed on a transmission electron microscope (JEOL 1011; JEOL Ltd, Tokyo, Japan). Images were captured using a digital camera (MegaView III; Olympus, Tokyo, Japan) and software (AnalySIS; Olympus).

Quantification of Relative Frequency of Pericyte Ensheathment in Adult Human Choroidal versus Retinal Capillaries

Relative frequency of pericyte ensheathment was determined by counting all capillaries present in the sections of choroid or retina and the number of these capillaries with associated pericytes. These numbers were then expressed as a percentage.

RESULTS

Contribution of $CD44^+$ Hematopoietic Stem Cells to Formation of Both Mural Cells and Vascular Endothelial Cells in the Developing Choroid

The earliest sections of human choroid at 9 weeks' gestation did not reveal any $CD44^+$ cells in the developing choroid (Fig. 1A); however, by14 weeks' gestation there were $CD44^+$ cells in the stroma (Fig. 1B, arrows), and by 18 weeks' gestation both the stroma (Fig. 1C, arrowheads) and the developing blood vessels (Fig. 1C, arrows) contained $CD44^+$ cells. Double staining of sections at 14 weeks' gestation with CD44 and αSMA revealed some cells that were $CD44^+$ and αSMA^+ (Figs. 1D, 1E, arrows). At both these ages (14 and 18 weeks' gestation), the double-labeled $CD44^+/\alpha SMA^+$ cells appeared to be associating with other αSMA^+ cells and coalescing to form blood vessels. When sections were stained with CD44 and CD34, there were similarly some double-stained $CD44^+$ and $CD34^+$ cells (Fig. 1F, arrows). In all three sections (Figs. 1D–F), not all cells were double stained. These results were confirmed in choroidal wholemounts, where it was also evident that some cells stained for both CD44 and αSMA and some cells stained for both CD44 and CD39 (Figs. 1G–J). These cell populations were visible in the same sections, suggesting that $CD44^+$ stem cells give rise to both SMCs and vascular endothelial cells. The confocal sections in Figures 1H, 1L, and 1P were very thin and were picked to show that $SMA^+/CD44^+$ cells were lining the vessels. An artifact of this thin section was that SMA appeared to stain the outside, not the cytoplasm, of some cells. However, all the cells in these sections did have cytoplasmic staining of SMA.

At 20 weeks' gestation, $CD44^+$ and $CD39^+$ cells are still visible in the stroma (Figs. 1K–N). It is clear that the SMA^+ cells have

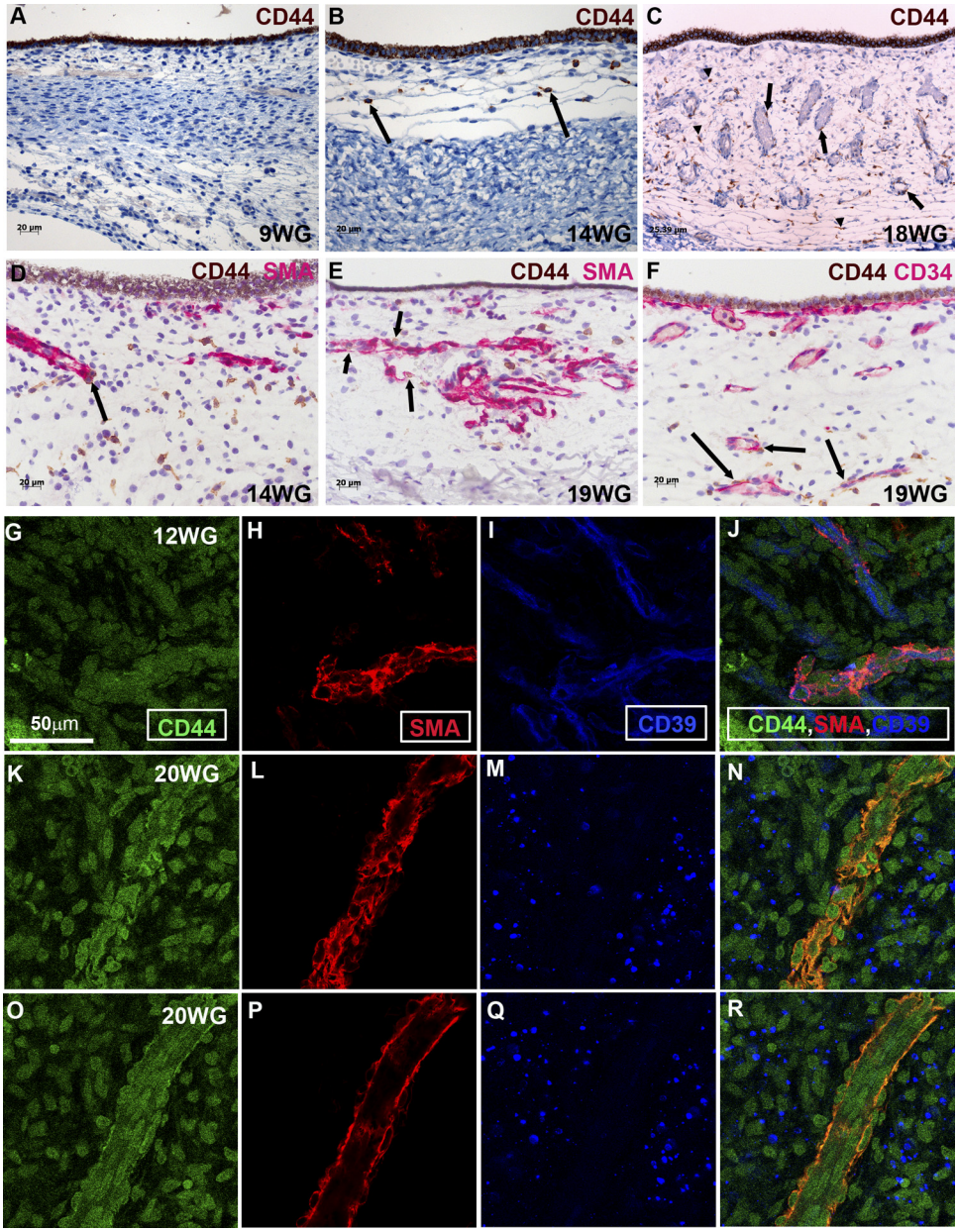

FIGURE 1. $CD44^+$ hematopoietic stem cells give rise to both mural cells and vascular endothelial cells in the choroid. (**A–F**) Cross-sections of human choroid at different ages. At 9 weeks' gestation, CD44 cells are not present in the developing choroid (**A**). By 14 weeks' gestation, $CD44^+$ cells are present in the developing choroidal stroma (**B**, *arrows*). By 18 weeks' gestation, $CD44^+$ cells are throughout the stroma of the choroid (**C**, *arrowheads*) and staining cells are associated with the developing vessels (**C**, *arrows*). *Pink*: SMA. *Brown*: CD44. *Arrows*: double-labeled *brown* and *pink* cells clearly associated with the blood vessels. At 14 and 19 weeks' gestation there were some $CD44^+/SMA^+$ cells lining the vessel walls (**D–E**, *arrows*), but not all cells were double stained. Some single-stained CD44 (*brown*) cells were present in the stroma. At 19 weeks' gestation there were also some $CD44^+/CD34^+$ cells lining the vessel walls (**F**, arrows) but again, not all cells were double stained. (**G–J**) Human choroid at 12 weeks' gestation stained with CD44, SMA, and CD39. Cells staining for both CD44 and SMA suggest that $CD44^+$ stem cells give rise to SMCs, and cells staining for both CD44 and CD39 suggest that $CD44^+$ stem cells can also give rise to $CD39^+$ vascular endothelial cells. (**K–N**) Z section of the top of a vessel in the human choroid at 20 weeks' gestation showing the relationship among CD44, SMA, and CD39. (**O–R**) Mid vessel section of the same vessel.

aligned themselves on the outside of the blood vessel (Figs. 1G–R).

Diffuse NG2 Staining in the Early Human Choroid Compared with the Human Retina

At 12 weeks' gestation, there was extensive staining for NG2 at the choriocapillaris (Fig. 2A) but more sparse staining in the mid choroid (Fig. 2B) and in the vortex veins (Fig. 2D). The same vein in the mid choroid region confirms sparse NG2 coverage but also punctate SMA coverage (Figs. 2B, 2C), whereas the vortex veins are strongly SMA^+ (Fig. 2D). Figures 2E to 2P are the same region of a choroid at 18 weeks' gestation focused on the choriocapillaris (Figs. 2E–H), the midsized vessels (Figs. 2I–L), and the large vessels (Figs. 2M–P) stained with NG2, SMA, and CD34. At 18 weeks' gestation, the SMA staining is still punctate in the choriocapillaris (Fig. 2F) versus the mid layer (Fig. 2J) and the large vessel layer (Fig. 2N), whereas some of the larger vessels are SMA^+. However, NG2 staining at 18 weeks' gestation is punctate and does not visualize the somas of mural cells in human choroid (Figs. 2G, 2K, 2O). This confirms that the choriocapillaris is almost totally devoid of mural cells.

In contrast, the human retina at 18 weeks' gestation has CD34 expression on the filopodial extensions and the luminal membrane of endothelial cells. $NG2^+$ pericytes are located just ahead of the leading edge of $CD34^+$ cells (Figs. 3A–D). The earliest $CD34^+$ vessels at the leading edge of the formation are already ensheathed by $NG2^+$ cells, indicating that all human retinal vessels have pericyte ensheathment. At 17 weeks' gestation, the $NG2^+$ pericytes are located on the abluminal surface (Figs. 3E, 3F), and at 20 weeks' gestation $NG2^+$ precursor cells are scattered mainly ahead of the leading edge of growth of retinal vessels but also within the vascular plexus (Figs. 3G, 3H, arrowhead). At 18 weeks' gestation there are $NG2^+$ precursor cells adjacent to the main vessels (Figs. 3I, 3J , arrows) and on the abluminal surface of an artery (A) (Fig. 3K, arrows) and vein (V) (Fig. 3L, arrows). Note that $NG2^+$ pericyte ensheathment is more continuous in the artery than the vein. Thus

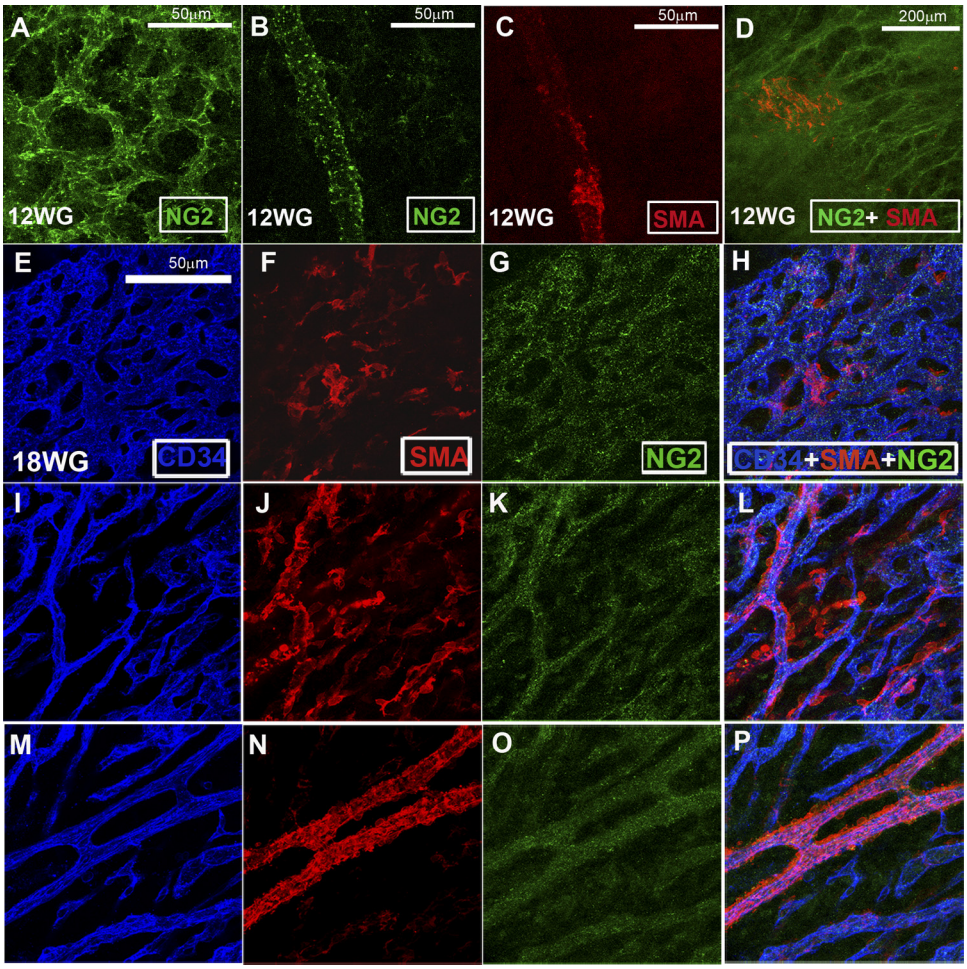

FIGURE 2. NG2 staining in the developing choroidal vasculature. (**A**) Human choroid at 12 weeks' gestation showing the $NG2^+$ choriocapillaris near the optic nerve head. (**B**, **C**) Human choroid at 12 weeks' gestation showing a vein labeled with NG2 and SMA in the mid choroid region. Note the localization and density of the SMA^+ mural cells. (**D**) Human choroid at 12 weeks' gestation showing $NG2^+$ blood vessels and SMA^+ vortex veins. (**E**–**P**) Human choroid at 18 weeks' gestation stained with NG2, SMA, and CD34. (**E**–**H**) Choriocapillaris. (**I**–**L**) Mid vessel layer. (**M**–**P**) Large vessel layer. There is an extensive $CD34^+$ vascular bed in all layers (**E**, **I**, **M**), but only a small proportion of these vessels are ensheathed by SMA^+ and $NG2^+$ mural cells. NG2 staining (**G**, **K**, **O**) is very diffuse and lacking structure. SMCs are barely present in the choriocapillaris (**F**) but are seen slightly more frequently in the mid vessel layer (**H**–**J**). At the large vessel layer (**N**), some vessels are SMA^+, and others are SMA^-.

retinal vessels develop concurrently with their mural cell coverage, there is no lag in mural cell ensheathment, and both arteries and veins have $NG2^+$ mural coverage.

Differences between the Structure of Human Adult Choriocapillaris and Human Retinal Capillaries

This lack of pericyte coverage in the developing choriocapillaris carries through to the adult. Using TEM to image the adult choroid and retina, we almost never noted pericytes on the choriocapillaris (Figs.4A–E). After much searching we were able to locate very rare cases of a choroidal capillary pericyte (Figs. 4C, 4E) but noted that the pericytes that were associated with the choroidal capillaries rarely ensheathed the vessels. This is a marked contrast to the human adult retinal capillaries that have complete pericyte ensheathment (Fig. 4F). These differences in pericyte ensheathment in the choroid versus the retina results in relatively large (7- to 26-μm) lumens in the choroidal capillaries compared with smaller (5- to 7-μm) lumens in the retinal capillaries. Figure 4Cb shows the choroidal fenestrae that were relatively low in number for capillaries but were well formed, and Figure 4Cc shows well-formed junctions that were readily identified in all capillaries.

Markedly Reduced Pericyte Frequency in Human Choriocapillaris versus Retinal Capillaries

We counted the number of pericytes ensheathing the adult choroidal and retinal capillaries and demonstrated that choroidal capillaries were covered only 11% of the time compared with 94.5% coverage in the retina.

Intermediate Filaments Present in the Human Retina But Absent in the Human Choroid

On ultrastructural examination, the pericytes of the choroid contained no IFs (defined as a diameter of 10 nm on average; Fig. 4Ca) whereas in retinal pericytes IFs were readily seen (Fig. 4Fa). We then stained the tissues for desmin, a readily accepted pericyte marker. Surprisingly, neither choroidal nor retinal pericytes stained for desmin; therefore, even though the retinal pericytes contained IF, this was not desmin. Between 8 and 19 weeks' gestation, human choroidal vessels were $desmin^-$ (Figs. 5A–D), even though $desmin^+$ muscle was clearly seen (Figs. 5A, 5C, asterisks). This is in contrast to rat pericytes, which are $NG2^+$ and $desmin^+$.[10] We attempted desmin staining of wholemounted human choroid but were unable to see any staining in these vessels (results not shown), confirming the absence of desmin staining in the developing human choroid. The adult choroidal vessels stained with SMA (Fig. 5E), but, again, no desmin staining was seen (Fig. 5F).

αSMA Staining in the Developing Choroid

Cross-sections of the immature choroid demonstrated that the choriocapillaris had an extensive $CD34^+$ vascular bed, but no αSMA staining was observed (Figs. 6A, 6B). As the choroid matures, however, and in confirmation of our wholemounts (Figs. 2F, 2J, 2N), the vessels in the deeper layers of the choroid express SMA (Fig. 6B).

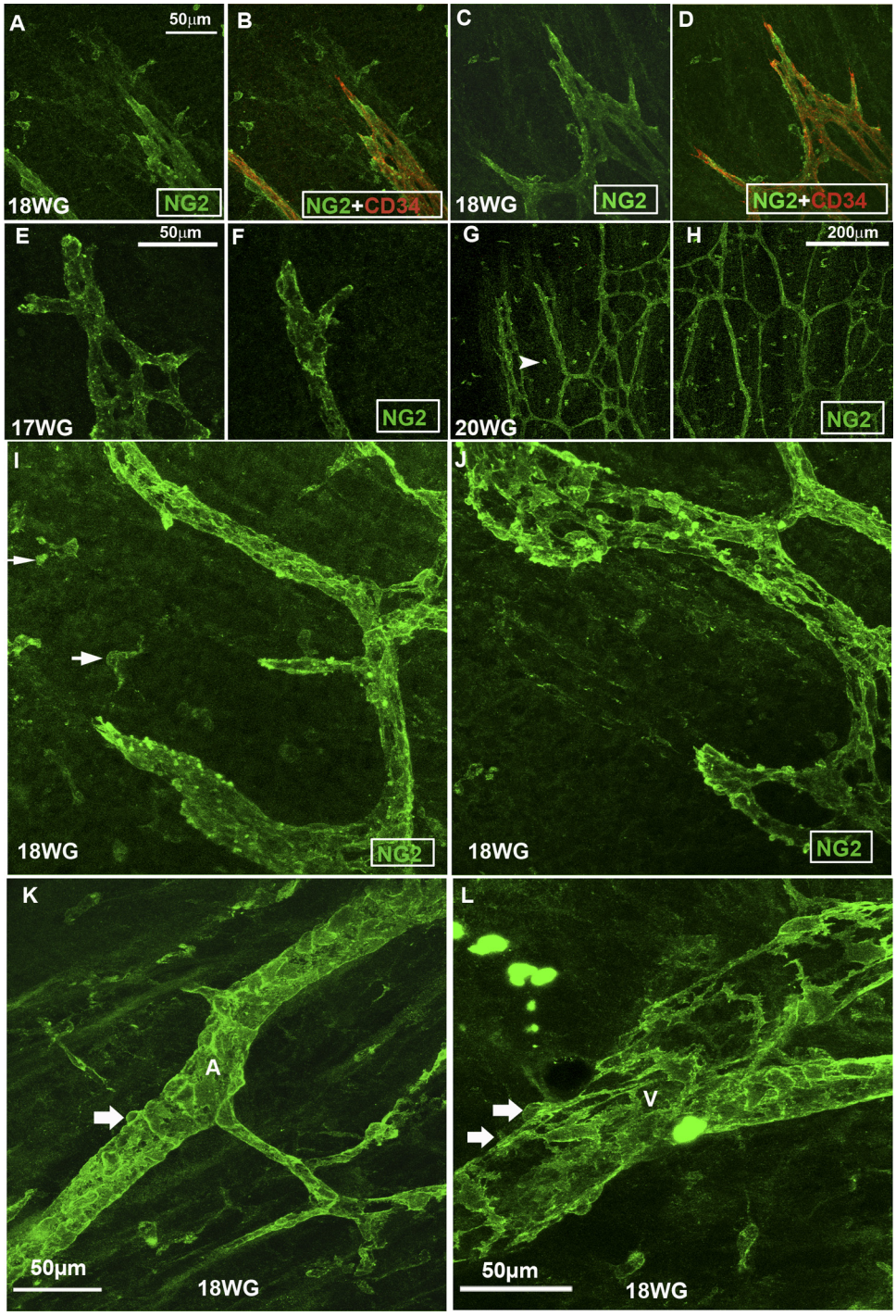

FIGURE 3. $NG2^+$ pericyte cell relationships relative to $CD34^+$ vessels in the human retina. (**A–D**) Human retina at 18 weeks' gestation showing blood vessels at the leading edge of growth labeled with NG2 and CD34. The $NG2^+$ pericytes are located just ahead of the leading edge of $CD34^+$ cells. (**E**, **F**) Human retina at 17 weeks' gestation labeled with NG2. The $NG2^+$ pericytes are located on the abluminal surface. (**G**, **H**) Human retina at 20 weeks' gestation showing $NG2^+$ precursor cells located mainly at the leading edge of growth and throughout the stroma (*arrowhead*). (**I**, **J**) Human retina at 18 weeks' gestation labeled with NG2. There are $NG2^+$ precursor cells adjacent to the main vessels (*arrows*). (**K**, **L**) Human choroid at 18 weeks' gestation showing $NG2^+$ somas on the abluminal surface of an artery (A) and vein (V), respectively (*arrows*). Note that $NG2^+$ pericyte ensheathment is more continuous in the artery than in the vein.

With maturation of the choroid, αSMA staining becomes more intense, staining along the vessel walls is far greater, and a larger proportion of vessels are SMA^+ (Figs. 6C, 6D). At 15.5 weeks' gestation, SMA ensheathment reaches less than half the periphery (Fig. 6C), whereas at 18 weeks' gestation, the SMA^+ main arterioles reach the edge of the choroid (Fig. 6D). There is a disc-to-peripheral development of SMA coverage of the larger vessels. The underlying choriocapillaris still does not stain for SMA, confirming the data from our sections.

We further confirmed a lack of pericyte coverage on the developing choriocapillaris ultrastructurally. At 12 and 20 weeks' gestation, the choriocapillaris is lined with only thick endothelial cells, and their nuclei protrude into the vessel lumen (Figs. 6E, 6F). At neither stage are pericytes present.

Evidence of αSMA Mural Precursor Cells throughout the Developing Human Choroidal Stroma

At 19.5 weeks' gestation, the larger (feeder) vessels have SMA coverage in both the deep (Figs. 7B, 7C) and the middle (Figs. 7E, 7F) layers of the choroid. In the choriocapillaris, the capillaries are still SMA^-, but there are isolated SMA^+ precursor cells between the formed CD34 vasculature (Figs. 7H, 7I). Under TEM, isolated cells morphologically consistent with mu-

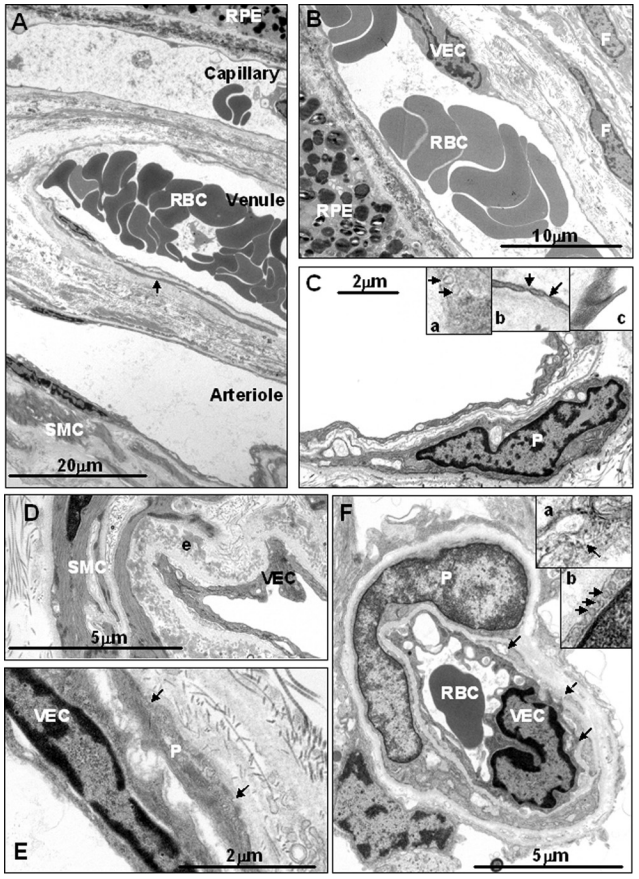

Figure 4. TEM of young healthy adult choroid and retinal capillaries. (**A**) Low magnification of the choroid from an adult showing a capillary, a venule, and an arteriole. The diameters of the capillaries in the choroid ranged from 7 μm to 26 μm. *Arrow*: part of a pericyte on the venule. (**B**) Choroidal capillary. Note the number of RBCs in the lumen and how thin the vascular endothelial cell is compared with the immature vessels (Fig. 6E). The diameter of this capillary is approximately 13 μm. (**C**) Choroidal capillary with an associated pericyte. Association of a pericyte with a choroidal capillary was rare; only 11% of the capillaries in the adult choroid had a pericyte, and those pericytes never exceeded 30% ensheathment of the capillary. (*insets,* **Ca**) High magnification of the cytoplasm of the choroidal pericyte. Note that there are no filaments present. *Arrows*: pinocytotic vesicles. (**Cb**) High magnification of vascular endothelial cell (VEC) showing fenestrae (*arrows*). Fenestrae are not as frequent in choroidal capillaries as in other tissue type capillaries. (**Cc**) High magnification of the junctions present in the VEC. (**D**) Arteriole in the choroid. There are well-developed SMCs with numerous organized thin filaments with focal densities. At higher magnification, pinocytotic vesicles and complete basal lamina are readily identified. An elastin (e) layer is also present in this particular vessel. (**E**) Venule of the choroid with a VEC nucleus present and an associated pericyte (P). *Arrows*: pinocytotic vesicles. (**F**) Retinal capillary in an adult. The maximum diameter of this capillary, like all those examined in the retina, is approximately 5 μm. The pericyte (P) that is visible does not fully ensheathe the capillary, but small fragments of pericyte cytoplasm (*arrows*) can be seen completing the ensheathment. (**Fa**) High magnification of a portion of the pericyte cytoplasm shows intermediate type filaments (*arrow*). (**Fb**) Portion of pericyte shows pinocytotic vesicles (*arrows*).

ral precursor cells are shown in Figures 7J and 7K. These cells were observed either in clusters of two to three or singly in the vicinity of newly formed solid vascular cords and had very poorly defined features consistent with a cell at a very immature stage of differentiation. They were identified by both morphologic characteristics, which included cytoplasm containing rough endoplasmic reticulum, free ribosomes, mitochondria, Golgi, and small immature junctions, and by the lack of features associated with differentiated cells of any other phenotype. Weibel-Palade bodies,[44] present in endothelial cells in recognizable vessels in the choroid and the retina samples examined, were completely absent in the cytoplasm of the mural precursor cells, also indicating that these were unlikely to be endothelial cells.

Artery Vein Differences in αSMA Ensheathment

Various stages of SMC differentiation were identifiable in the arteries and veins of the choroid. At 12 weeks' gestation, weak SMA immunoreactivity was detected on the main choroidal vessels (Fig. 2C). Interestingly, the SMA staining tended to be clumped in clusters in some areas but absent in other areas. At 16 weeks' gestation, SMA had aggregated into concentric filaments, though the blood vessels were relatively amorphous in nature (Figs. 8A, 8B). By 32 weeks' gestation, the αSMA filaments were considerably longer and more organized, forming a continuous concentric sheath around large choroidal arteries. The nature of the αSMA filaments in the artery were striking (Fig. 6C), whereas in the vein, there were gaps in the SMA expression (Fig. 6D). The SMA filaments in the vein also appeared to be significantly smaller than those of the corresponding artery.

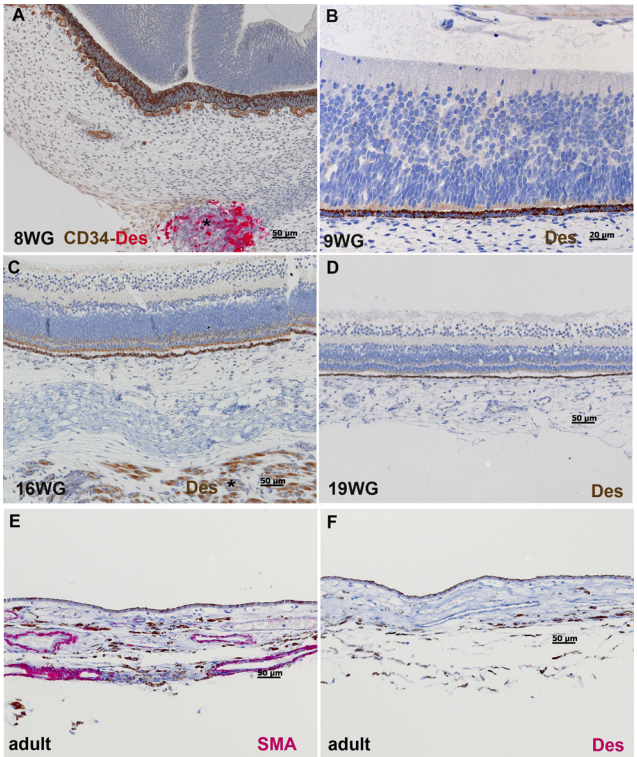

Figure 5. Lack of desmin staining in the retina and choroid. (**A**) Human choroid at 8 weeks' gestation shows desmin^{+} muscle (*asterisks*) and desmin^{-} choroid and retina. (**B**) Higher magnification of a human choroid at 9 weeks' gestation confirms the lack of desmin staining in the retina and choroid. (**C**, **D**) Human choroid at 16 and 19 weeks' gestation labeled with desmin. Despite the presence of desmin staining muscle in an eye at 16 weeks' gestation (**C**, *asterisks*), the vessel walls of the incipient human choroid are devoid of desmin staining. (**B**–**D**) There is nonspecific staining of the nerves and the brush border of the pigmented cells. (**E**, **F**) Adult male eye (age, 56 years) stained with SMA and desmin, respectively. (**E**) SMA staining of most of the choroidal vessels is evident, (**F**) but desmin does not stain any choroidal vessels. The *brown* pigment (**E**, **F**) is melanin.

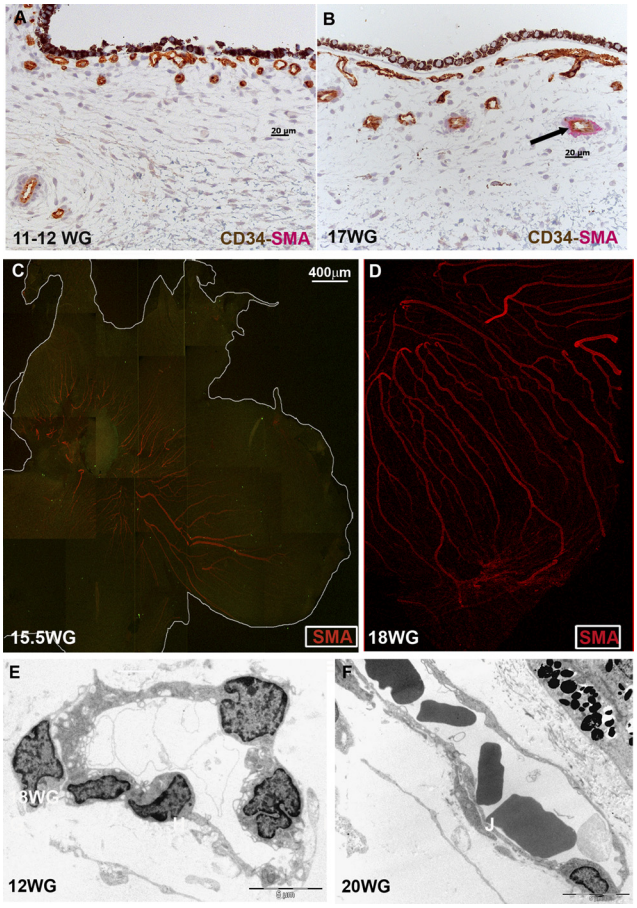

FIGURE 6. SMA staining in the developing choroid; the absence of mural cells in the choriocapillaris. (**A**, **B**) Transverse sections of human choroid. (**A**) Human choroid at 11 to 12 weeks' gestation labeled with CD34 and SMA. $CD34^+$ cells line the vessels of the choriocapillaris and the deeper layers of the choroid. At this stage, all the $CD34^+$ vessels are SMA^-. (**B**) Human choroid at 17 weeks' gestation labeled with CD34 and SMA. With maturation, the mural cells of the deeper choroidal vessels express SMA though the choriocapillaris remains largely SMA^-. (**C**, **D**) Montage of a human choroidal wholemount labeled with SMA at 15.5 weeks' gestation and at 18 weeks' gestation, respectively. With maturation, there is more intense SMA staining, a greater extent of staining along the vessel walls, and a larger proportion of SMA^+ vessels. At 15.5 weeks' gestation, SMA ensheathment reaches less than half the periphery, whereas at 18 weeks' gestation, the SMA^+ main arterioles reach the edge of the choroid. Note that the underlying choriocapillaris does not stain for SMA. (**E**) Capillary in the choroid of a fetus at 12 weeks' gestation. (**F**) Capillary in the choroid of a fetus at 20 weeks. Vascular endothelial cells lining the vessel at both these ages protrude into the lumen, and neither vessel contains any evidence of pericytes. The endothelial cell is thicker (i.e., plumper) than in the adult (compare to Fig. 4C). The *top right-hand corner* of (**D**) contains RPE and is so dark as the contrast was optimized to show other features.

Expression of Calponin, a Calcium Regulating Protein, on Large Vessels in the Choroid

Calponin was expressed only on the large vessels of the choroid (Figs. 8E–K) and colocalized with αSMA. As we have previously demonstrated in the adult rat retina,[10] SMA staining extended just a little further along the vessels than did calponin. Calponin expression was weaker than in the corresponding retinal vessels (results not shown). We could not demonstrate caldesmon staining in any of our wholemounts (results not shown), though we had previously successfully used this marker in our rat wholemount tissues.[10]

A schematic representation of the fields of view and depth of focus for each of the fields shown in the respective data plates is given in Figure 9.

DISCUSSION

We have undertaken an analysis of the development of the mural cell lineage in the human choroid. For the first time we demonstrate the presence of mural precursor cells in the human choroid and show that mural cells and endothelial cells may have a common lineage. To our surprise, we found a quantifiable lack of pericytes both ultrastructurally and in our wholemounts during development and through adulthood. Also remarkable was the absence of IFs in the adult choroidal pericytes. Retinal pericytes had IFs when viewed ultrastructurally, but those IFs were not able to be detected by antibody to desmin. Taken together, these structural observations led us to suggest that the choroidal vasculature is predisposed to vascular instability and has an almost permanently open "plasticity window" because of limited pericyte ensheathment.

In early development of the choroidal vessels, we noted $CD44^+$ stem cells present in both the stroma and the developing blood vessels, in wholemounts and sections. A population of these $CD44^+$ cells stained for CD39, whereas another population of $CD44^+$ cells stained with SMA. These double-labeled cells were present in the same sections, suggesting a common origin. In our previous study, we demonstrated that the incipient choroid had $CD133^+/CD34^+$ hematopoietic stem cells (CD133 is expressed on primitive cell populations, such as CD34 bright hematopoietic stem and progenitor cells, neural, and endothelial stem cells[45–47]) that differentiated into three distinct lineages: vascular precursor cells, monocytes, and mural precursor cells (Chan-Ling T, Dahlstrom JE, Koina ME, et al., unpublished data, 2009). These precursor/stem cells then formed primordial vascular islands/vessels that differentiated in situ to form solid vascular cords that become patent. Other observations also support a common origin for ECs and pericytes. Lutty et al.,[32] in TEM studies of human choroid at 11 weeks' gestation, noted that perivascular and luminal cells had similar ultrastructural characteristics, although studies in rats[10] and mice[14,15] have suggested that mural and endothelial cells can arise from a common precursor.[14,15] Our present study strengthens the argument that vascular and mural cells have a common lineage, though further lineage tracer studies would be needed to confirm this.

The choriocapillaris stained for NG2 at 12 weeks' gestation, confirming previous results seen in sections,[12] but in our wholemounts this staining was diffuse and unlike that seen in the developing retinal vessels.[10] At the same stage, αSMA had previously been shown as scattered cells in the choriocapillaris,[12] and our staining confirmed these results. We noted at 12 weeks' gestation the αSMA staining was punctate and lacking filamentous structure, and by 19.5 weeks' gestation αSMA staining in the choriocapillaris was mostly confined to individual cells in the stroma. We suggest these αSMA^+ isolated cells are mural precursor cells (MPCs) that we have also noted in the developing rat retinal vasculature.[10] These MPCs were observed in the choroid often close to forming vessels, and we confirmed their presence ultrastructurally throughout the developing choroid. We suggest these were MPCs based on previous descriptions.[14,48] They showed no evidence of specific differentiation and contained rough endoplasmic reticulum, free ribosomes, mitochondria, Golgi, and small immature junctions. In addition, they were unlike vascular endothelial cells, astrocytes, fibroblasts, or SMCs. We provide structural evidence that the MPCs were present in the stroma/mesenchyme of the developing human choroid and that these MPCs

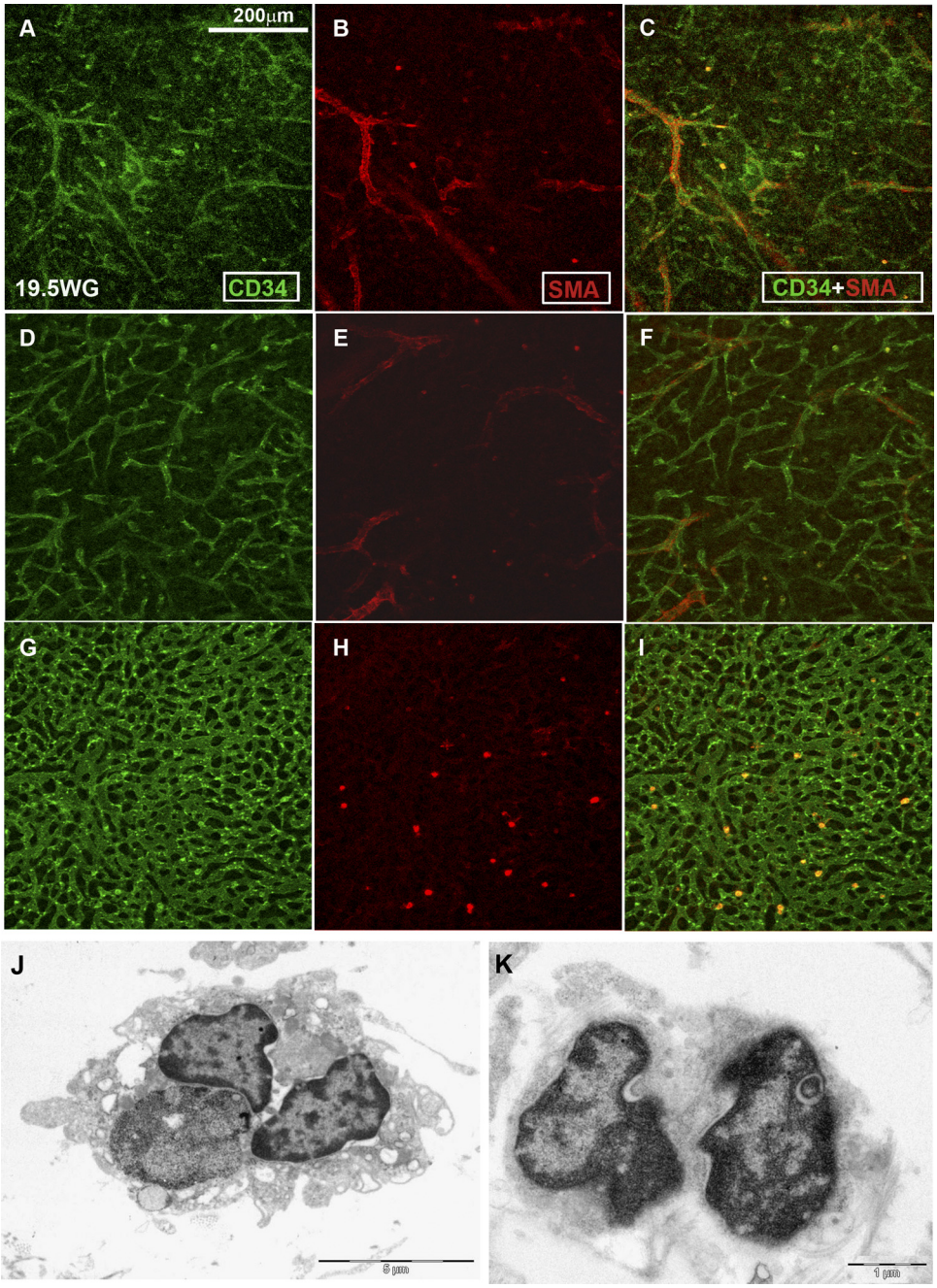

FIGURE 7. Mural precursor cells are present in the stroma of the developing choroid. (**A–I**) Human choroid at 19.5 weeks' gestation stained with CD34 and SMA. Wholemounts confirm the staining seen in the sections in that the deeper vessels have SMA staining (**A–C**), but, as the focus moves through the choroidal vessels layers (**D–F**) to the choriocapillaris (**G–I**), the vessel associated with SMA staining is lost. In the choriocapillaris, there are isolated SMA^+ precursor cells between the formed CD34 vasculature (**H**, **I**). The large vessel layer has SMA^+ feeder vessels (**A–C**). TEM of the immature choriocapillaris identified potential mural cell precursors with very poorly defined features (**J**, **K**). The cytoplasm contains rough endoplasmic reticulum, free ribosomes, mitochondria, Golgi, and small immature junctions.

differentiated to become SMCs and pericyte lineages. These data are consistent with our earlier report that MPCs are present during the formation of the rat retinal vasculature.[10]

αSMA coverage was markedly different on the arteries and veins. The early stages at 12 weeks' gestation demonstrated weak αSMA immunoreactivity on the main choroidal vessels. Interestingly, the αSMA staining tended to be clumped in clusters in some areas but to be absent in other areas. By 16 weeks' gestation, αSMA had aggregated into concentric filaments, though the blood vessels were relatively amorphous. By 32 weeks' gestation, the αSMA filaments were considerably longer and more organized in the choroidal arteries, forming a continuous concentric sheath. In contrast, the αSMA filaments in the vein had gaps and appeared to be significantly smaller than those of the corresponding artery. As the choroidal vasculature matured, αSMA spread in a disc-centered nature to cover most large vessels. Calponin and caldesmon are thought to be involved in regulating SMC tone[40,49–51] with calponin interacting with desmin, suggesting a role in cytoskeletal organization.[52] In our human choroidal samples, calponin appeared as the vessels were maturing and gaining SMA expression but was only present on larger vessels and was lost in the transition from large to smaller vessels. The SMA staining extended just a little further than the calponin staining, confirming our previous observations made in the developing rat retinal vessels.[10] In contrast, caldesmon was not present in our human choroidal samples, though we had previously demonstrated caldesmon on the rat retinal vessels. Thus the human choroidal vessels do not express caldesmon and calponin is not expressed on the capillaries, providing further structural evidence that human choroidal capillaries may be deficient at regulating SMC tone.

The adult choriocapillaris capillary structure was significantly different from that of the retinal capillaries. Capillaries are usually very thin, single-cell–lined vessels approximately 5

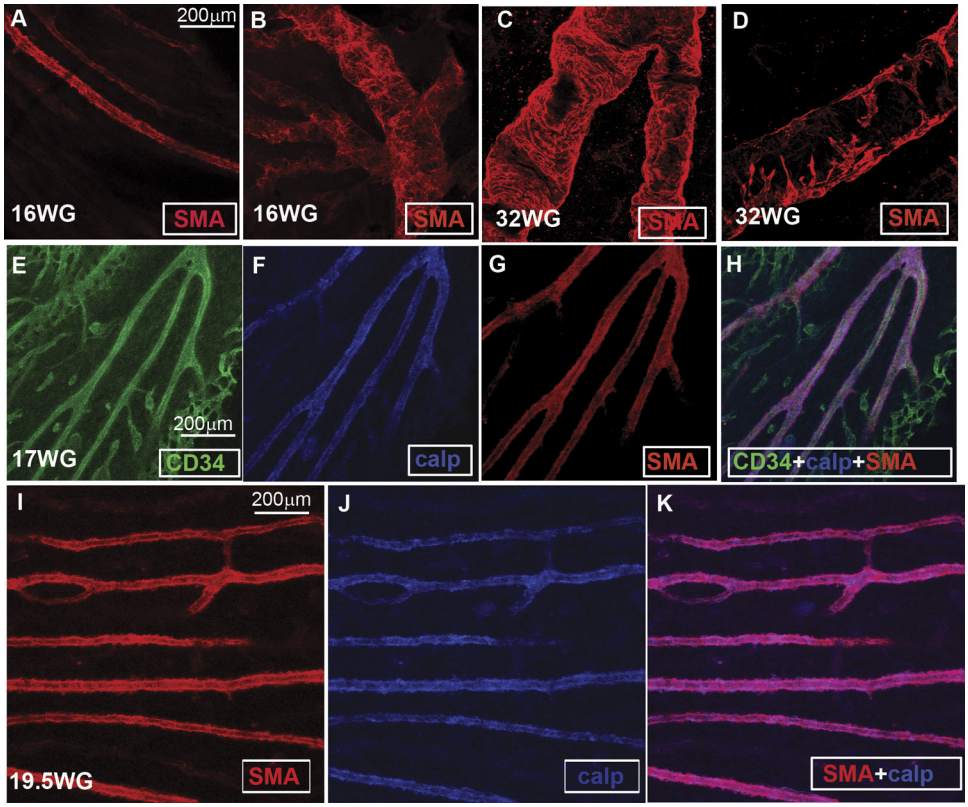

FIGURE 8. SMA staining in arteries and veins and colocalization with calponin. High-magnification artery and vein, respectively, labeled with SMA in human choroid at 16 weeks' gestation (**A**, **B**) and 32 weeks' gestation (**C**, **D**). Note the more organized αSMA filaments and the less amorphous nature of the blood vessels at 32 weeks' gestation compared with 16 weeks' gestation. Note also the incomplete coverage of the artery at 32 weeks' gestation (**C**) with SMA filaments. Calponin and SMA expression predominantly colocalize in the choroid. (**E**–**H**) Human choroid at 17 weeks' gestation labeled with CD34, calponin, and SMA. CD34 labels the full extent of the choroidal vasculature with angiogenic buds and capillary-sized vessels evident in the field of view (**E**). SMA and calponin only label large-caliber vessels (**F**, **G**, respectively). When overlaid, the expressions of calponin and SMA are almost identical, whereas neither stains the smaller capillaries (**H**). (**I**–**K**) Human choroid at 19.5 weeks' gestation labeled with calponin and SMA. In both specimens the calcium regulating protein calponin and SMA are present only on the larger vessels.

to 10 μm wide. A red blood cell (RBC) is 6 to 8 μm wide; therefore, only one RBC can pass through a capillary at any time. However, we noted much larger lumens in our choriocapillaris (7–26 μm) and capillaries with more than one RBC in their lumen. We also noted that the adult capillaries in the choroid were less likely to have pericyte coverage (11% vs. 95% in the retina). The pericytes that were seen on the choroidal vessels rarely wrapped completely around the vessels, unlike the retinal capillaries, where there was typically one pericyte for each endothelial cell and pericytes were clearly closely ensheathing retinal capillaries. The choroidal pericytes were also devoid of IFs, again in contrast to the retinal pericytes, where ultrastructural examination clearly showed IFs.

IFs have a primary role in cell plasticity and as cellular stress absorbers (see Ref. 53 for review). Desmin, an IF 6 to 12 nm in diameter,[27] is an accepted pericyte marker.[10,20,28–30] Two investigators (TCL and JD) working on choroidal wholemounts and sections, respectively, both failed to demonstrate desmin staining in the choroidal mural cells, though desmin was clearly present in the choroidal muscle in our samples, and we have previously demonstrated desmin on rat retinal pericytes.[10] We also failed to see the presence of IFs in our choroidal pericytes by TEM. Surprisingly, we could not demonstrate desmin staining in the human retinal pericytes, though our TEM images clearly demonstrated that IFs were present in these cells. A review of the literature confirmed that other investigators had shown desmin stains retinal pericytes in the mouse,[54] rat,[55] and cat.[20] The only previously reported evidence we found that human retinal pericytes are desmin$^+$ was in cultured cells,[56] and one study demonstrated 2% of SMA$^+$

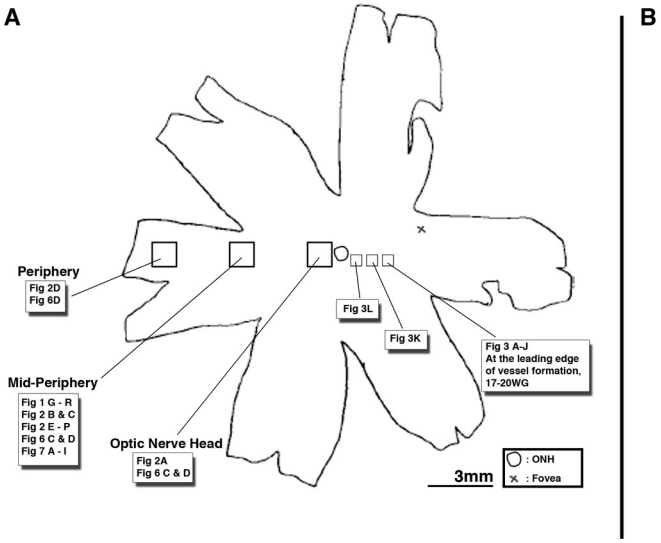

FIGURE 9. Schematic representation of the fields of view and depth of focus for each of the fields shown in the respective data plates. (**A**) Wholemount representation. (**B**) Transverse section from the inner limiting membrane of the retina through to the outer choroid. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; CC, choriocapillaris; MSV, medium-sized vessels; LV, large vessels.

cells were also desmin$^+$.[57] It is possible that the antibodies we used do not detect desmin in human pericytes, though both antibodies clearly stained human muscle, and that we need a more suitable antibody, or indeed a better marker, for human retinal pericytes.

Using desmin knockout mice, Loufrani et al.[58] demonstrated that desmin was required in vascular SMCs and in resistance arteries for efficient control of vascular tone and consequently for optimal blood flow. Desmin was also required for microvascular remodeling.[59] In vimentin knockouts, mice were normal, but their cells lacked structural stability. Functionally, this meant reduced motility and directional migration as well as diminished wound repair.[60] These structural observations lead us to suggest that choroidal pericytes would have very little contractile force compared with retinal pericytes and that choroidal pericytes may be unable to control vascular tone and consequently optimal blood flow supply. These observations may also explain why the blood flow rate and oxygen tension in the choroid is much higher than in other beds (see Ref. 11 for review).

In terms of disease processes, the differences in the pericyte coverage of the retina and choroid have drastic implications. We provide compelling evidence, using ultrastructure and cell-specific markers, for extensive pericyte coverage in human retinal capillaries, making them stable and resistant to proangiogenic stimuli.[20] For neovascularization to occur in the retinal vessels, the pericyte-endothelial cell relationship must be disrupted. This is exactly what is seen in diabetic retinopathy[61] and in the aging retina,[55] where the pericyte coverage of the blood vessels is impaired and pathologic neovascularization can occur. It is also seen during development, when the immature vessels, without pericyte ensheathment, are more prone to neovascularization in retinopathy of prematurity.[20,21] Experimentally, platelet-derived growth factor (PDGF) heterozygous knockout mice have pericyte numbers 30% lower than wild-type that result in a marked increased in angiogenesis when the mutants are subjected to oxygen-induced retinopathy.[61] Similarly, in another PDGF knockout model, a range of phenotypes was generated in which the blood vessels had varying pericyte/endothelial cell ratios.[62] In eyes in which the pericyte density was less than 50% of normal, abnormal vessels were always seen extending into both the vitreous and the choroid. In diseases such as ARMD and polypoidal choroidal vasculopathy,[63] in which blood vessels from the choroid breach Bruch's membrane and grow into the retina, the mechanisms are beginning to be elucidated. Several theories postulate that hypoxia is induced in the retina by, for example, age-induced Bruch's membrane thickening[64] or choriocapillaris atrophy.[65] Irrespective of the stimulus or how it is induced, the result is an increase in the secretion of VEGF from RPE, predominantly on the side of the choriocapillaris,[66] and early proliferation of the choriocapillaris.[67] As we now demonstrate, the adult choroidal vessels have a limited pericyte ensheathment, and this leads us to suggest that this would permit the vessels to be reactive to VEGF. Thus, because the choroid is inherently unstable, new vessel growth is stimulated to alleviate the retinal hypoxia. Previous researchers referred to the time when developing blood vessels have no pericyte coverage, are unstable, and are able to react to VEGF as a plasticity window.[21] In fact, the very limited pericyte coverage of the choroid reported here means that the window is permanently open, and we suggest that this predisposes this adult vascular bed to vascular instability, reactivity to VEGF, and neovascularization.

Acknowledgments

The authors thank Anastasia Korlimbinis for her contributions to the manuscript, Ruth-Ann Sterling for technical assistance, and Margaret Goddall for her generous gift of BU61 (CD39).

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