Vascular Interstitial Cells in Retinal Arteriolar Annuli Are Altered During Hypertension

David Ramos,[1,2] Joana Catita,[2–4] Mariana López-Luppo,[2,3] Andreia Valença,[1,2] Aina Bonet,[2,3] Ana Carretero,[1–3] Marc Navarro,[1–3] Victor Nacher,[1–3] Simon Mendez-Ferrer,[5] Anna Meseguer,[6–8] Alba Casellas,[2,9,10] Luísa Mendes-Jorge,[1,2] and Jesús Ruberte[1–3]

[1]CIISA–Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, Universidade de Lisboa, Lisbon, Portugal
[2]Center of Animal Biotechnology and Gene Therapy, Universitat Autònoma de Barcelona, Bellaterra, Spain
[3]Department of Animal Health and Anatomy, School of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra, Spain
[4]Department of Anatomy, Faculty of Veterinary Medicine, Universidade Lusófona de Humanidades e Tecnologias, Lisbon, Portugal
[5]Wellcome Trust-Medical Research Council Cambridge Stem Cell Institute and Department of Haematology, University of Cambridge, and NHS-Blood and Transplant, Cambridge, United Kingdom
[6]Renal Physiopathology Group, CIBBM-Nanomedicine, Vall d'Hebron Research Institute (VHIR), Barcelona, Spain
[7]Department of Biochemistry and Molecular Biology, Unitat de Bioquímica de Medicina, Universitat Autònoma de Barcelona, Bellaterra, Spain
[8]Red de Investigación Renal (REDINREN), Instituto Carlos III-FEDER, Madrid, Spain
[9]Department of Biochemistry and Molecular Biology, School of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra, Spain
[10]CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Barcelona, Spain

Correspondence: Jesús Ruberte, Universitat Autònoma de Barcelona, Edifici H, carrer de la Vallmoronta, Bellaterra, Barcelona 08193, Spain; jesus.ruberte@uab.cat.

Submitted: June 11, 2018
Accepted: December 10, 2018

Citation: Ramos D, Catita J, López-Luppo M, et al. Vascular interstitial cells in retinal arteriolar annuli are altered during hypertension. *Invest Ophthalmol Vis Sci*. 2019;60:473–487. https://doi.org/10.1167/iovs.18-25000

Purpose. It has been suggested that arteriolar annuli localized in retinal arterioles regulate retinal blood flow acting as sphincters. Here, the morphology and protein expression profile of arteriolar annuli have been analyzed under physiologic conditions in the retina of wild-type, β-actin–Egfp, and Nestin–gfp transgenic mice. Additionally, to study the effect of hypertension, the KAP transgenic mouse has been used.

Methods. Cellular architecture has been studied using digested whole mount retinas and transmission electron microscopy. The profile of protein expression has been analyzed on paraffin sections and whole mount retinas by immunofluorescence and histochemistry.

Results. The ultrastructural analysis of arteriolar annuli showed a different cell population found between endothelial and muscle cells that matched most of the morphologic criteria established to define interstitial Cajal cells. The profile of protein expression of these vascular interstitial cells (VICs) was similar to that of interstitial Cajal cells and different from the endothelial and smooth muscle cells, because they expressed β-actin, nestin, and CD44, but they did not express CD31 and α-SMA or scarcely express F-actin. Furthermore, VICs share with pericytes the expression of NG2 and platelet-derived growth factor receptor beta (PDGFR-β). The high expression of Ano1 and high activity of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase observed in VICs was diminished during hypertensive retinopathy suggesting that these cells might play a role on the motility of arteriolar annuli and that this function is altered during hypertension.

Conclusions. A novel type of VICs has been described in the arteriolar annuli of mouse retina. Remarkably, these cells undergo important molecular modifications during hypertensive retinopathy and might thus be a therapeutic target against this disease.

Keywords: retina, arteriolar annuli, hypertensive retinopathy

Hypertension is an important public health challenge worldwide. This condition affected 972 million adults in 2000, and its prevalence is predicted to increase by 60% to a total of 1.56 billion people by 2025.[1] Under elevated blood pressure, retinal vasculature undergoes a series of pathologic changes, including generalized and focal arteriolar narrowing, intimal thickening, hyperplasia of the tunica media, and hyaline degeneration.[2] Altogether, these symptoms are referred to as hypertensive retinopathy, which is the second most common type of retinopathy and affects 3% to 14% of the adult population over 40 years of age.[2,3]

Arteriolar annuli have been described as conical accumulations of periodic acid Schiff (PAS)-positive material with increased cellularity surrounding the opening of retinal arteriolar branching sites.[4,5] It has been suggested that these structures regulate retinal blood flow by exerting a sphincter-like activity.[4,6,7] This hypothesis is supported by previous studies demonstrating constrictions at arteriolar branch points.[8,9] However, there is no general agreement concerning

the cellular composition, structure, and function of arteriolar annuli in the retina.

Several cell types cooperate to provide an appropriate vascular tone including endothelial, smooth muscle cells, and pericytes. Recently, multipolar cells with thin processes, which have not been classified within the above mentioned cell types, have been identified in the wall of different blood vessels.[10–15] These cells were called vascular interstitial cells (VICs) because of their structural and molecular similarities with interstitial Cajal cells.

In this work, we aimed to finely define the morphology and the protein expression profile of a new subtype of VICs localized in the annuli of murine retinal arterioles. Because these annuli have been considered as discrete sites for blood flow regulation, the second main objective of this study was to analyze whether VICs are altered during hypertensive retinopathy, becoming thus a possible target for the treatment of this disease.

Materials and Methods

Eyes

Eyes from wild-type CD1 (ICR) mice together with β-actin-Egfp[16] and Nestin-gfp transgenic mice[17] served to analyze the presence of VICs in arteriolar annuli under physiologic conditions. Additionally, to study the effect of hypertension in arteriolar annuli, a model of elevated blood pressure, the kidney androgen-regulated protein (KAP) transgenic mouse, was used.[18] Wild-type and β-actin–Egfp transgenic mice were maintained at the Animal Facility of the Universitat Autònoma de Barcelona (UAB, Barcelona, Spain), whereas Nestin-gfp transgenic mice were kindly gifted by Grigori N. Enikolopov (Stony Brook University, New York, NY, USA). Hypertensive KAP transgenic mice were provided by Anna Meseguer (Vall d'Hebron Institut de Recerca–VHIR and Centre d'Investigacions en Bioquímica i Biologia Molecular–CIBBIM, Barcelona, Spain). Mice were maintained on 12-hour day/night cycles, with free access to food and water. Principles of laboratory animal care were followed. Animal care and experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Ethics Committee in Animal and Human Experimentation of the Universitat Autònoma de Barcelona (CEEAH/UAB).

Retinal Digestion

Retinal digestion was performed according to the technique described by Kuwabara and Cogan[4] and Hammes et al.[19] with minor modifications. After fixation with neutral-buffered formalin, whole mount retinas were washed in filtered water and subjected to a trypsin digestion (3% trypsin in 0.1 M tris buffer) for approximately 90 minutes at 37°C. Once digested, retinas were rinsed several times in filtered water to isolate retinal blood vessels, which were finally stained with hematoxylin and PAS. Digested retinal vasculatures were microscopically analyzed (Nikon Eclipse E800; Nikon, Tokyo, Japan).

Transmission Electron Microscopy Analysis

Retinal fragments from three mice were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde, postfixed in 1% osmium tetroxide, stained in aqueous uranyl acetate, dehydrated, and embedded in epoxy resin. Ultrathin sections (70 nm) of seven different arteriolar side-arm branching sites were stained using lead citrate and examined by a transmission electron microscope (Jeol 1400; Jeol Ltd., Tokyo, Japan).

Immunohistochemistry

Paraffin-embedded eyes were sectioned (3 μm) along the eye axis through the optic disc and cornea, deparaffinized, and rehydrated. Whole mount retinas were fixed in 10% neutral-buffered formalin for 2 hours at 4°C or in cold acetone for 5 minutes, depending on antibodies used. After they were washed in PBS, paraffin sections and whole mount retinas were incubated overnight at 4°C with the following markers: anti-collagen IV (Millipore, Darmstadt, Germany) diluted at 1:20, anti–platelet-derived growth factor-receptor β (PDGF-Rβ) (Abcam, Cambridge, UK) diluted at 1:100, anti–neuron glial 2 chondroitin sulphate (NG2) (Millipore) diluted at 1:200, anti–α-smooth muscle actin (α-SMA) (Abcam) diluted at 1:100, anti–c-Kit (Abcam) diluted at 1:50, anti-anoctamin1 (Ano1) (Abcam) diluted at 1:90, anti-CD44 (Abcam) diluted at 1:100, anti-CD31 (BD Pharmingen, San Jose, CA, USA) diluted at 1:25, anti–glutamine synthetase (GS) (Sigma-Aldrich Corp., St. Louis, MO, USA) diluted at 1:500, anti–enhanced green fluorescent protein (EGFP) (Molecular Probes, Eugene, OR, USA) at 1:300 dilution, and rhodamine-phalloidin (Sigma-Aldrich Corp.) at 5 μg/mL concentration. Retinas were washed several times in PBS and then incubated overnight at 4°C with specific secondary antibodies: anti-rat IgG-biotinylated (Abcam), anti-goat IgG-biotinylated (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-rabbit Alexa Fluor 568 (Invitrogen, Carlsbad, CA, USA) and anti-rabbit Alexa Fluor 488 (Abcam). All secondary antibodies were diluted at 1:100. Once retinas were washed in PBS, Streptavidin Alexa Fluor 488 (Invitrogen) or Streptavidin Alexa Fluor 568 (Invitrogen) were used as fluorochromes at 1:100 dilution. Microscopic analysis was performed with a laser scanning confocal microscope (TCS SP2; Leica Microsystems GmbH, Heidelberg, Germany). To assess retinal morphology, deparaffinized eye sections were stained with hematoxylin and eosin and observed with a Nikon Eclipse E800 microscope (Nikon Corp.).

Measurement of Arterial Pressure

Arterial pressure was measured using tail-cuff equipment adapted for mice (Letica, Barcelona, Spain), at several times between 0900 and 1200 hours, and pressure values were considered acceptable when at least five consecutive measurements provided similar values. To ensure a proper assessment of blood pressure, mice were previously trained to be kept in a plastic restraining chamber for several minutes during several weeks.

Thickness of Arteriolar Tunica Media

The thickness of the arteriolar tunica media was measured in confocal images of α-SMA immunolabeled arterioles. To avoid bias errors, each measure was determined four times per arteriole in four different arterioles per retina. Retinas from three KAP transgenic and three littermate control mice were studied.

NADPH Diaphorase Activity Analysis and Quantification

Nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity was analyzed in four KAP transgenic and four control littermate mice. Whole retinas were fixed in 4% paraformaldehyde for 3 hours at room temperature. After several washes in PBS and Tris HCl buffer 0.1 M, pH 8.7, retinas were incubated for 90 minutes at 37°C in a reactive solution containing 15 mM malic acid, 1 mM $MnCl_2$, 1 mM NADP, 0.2 mM nitro blue tetrazolium, and 0.2% Triton X-100 diluted in

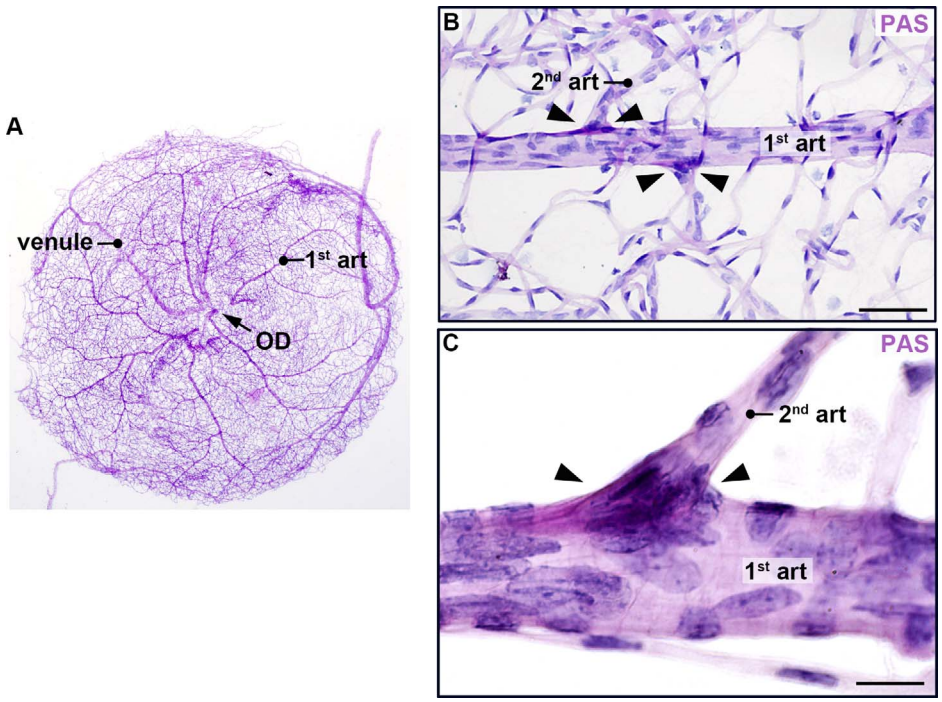

FIGURE 1. Identification of arteriolar annuli in the mouse retina. (**A**, **B**) PAS/hematoxilin staining of pepsin/trypsin-digested retinas evidenced the presence of arteriolar annuli at the origin of side-arm branches of the first order arterioles. (**C**) Arteriolar annuli were characterized by increased PAS stain and hypercellularity. OD, optic disc; 1st art, first-order arteriole; 2nd art, second-order arteriole. *Arrowheads* point to arteriolar annuli. *Scale bars*: (**B**) 57.2 μm; (**C**) 14.9 μm.

Tris buffer 0.1 M, pH 8.7. Then, retinas were washed in Tris buffer HCl 0.1 M, pH 8.7 and mounted in buffered glycerol. Images were acquired with an optic microscope (Nikon Eclipse E800).

To quantify NADPH diaphorase activity, images were converted to black and white, inverted, and binarized. Then, gray intensity was measured in 725.24-μm^2 areas of 35 arteriolar annuli with the Nis Elements Imaging Software (Nikon).

Protein Quantification

To test whether hypertension is capable to affect VICs, Ano1 expression was analyzed in arteriolar annuli from five KAP transgenic and five control littermate mice. Mean fluorescence intensity was measured with Leica LAS AF Lite imaging software (Leica) in 210.77-μm^2 areas of 10 arteriolar annuli per retina.

Statistical Analysis

Results are shown as mean ± SEM. Statistical analyses were performed by unpaired *t*-test. Differences between groups were considered statistically significant at $P < 0.05$. All statistical analyses were performed with the GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Retinal Arteriolar Annuli Topography and Cellular Composition

As previously described, arteriolar annuli in mouse retinas appeared as conical accumulations of PAS-stained material restricted to the origin of side-arm branches of first-order arterioles (Figs. 1B, 1C).[4,5,20,21] An increase in cellularity was observed in mouse arteriolar annuli (Figs. 1B, 1C), similarly to findings reported in other mammals.[5]

Although it was previously suggested that an incompletely characterized cell type might reside in retinal arteriolar branches,[22] the exact nature of cells in arteriolar annuli has remained unknown. Therefore, we used transmission electron microscopy (TEM) and immunohistochemistry to characterize cells in arteriolar annuli.

The ultrastructural analysis of arteriolar annuli evidenced unequivocally three distinct cellular types (Fig. 2A): endothelial cells forming a continuous layer that covered the luminal surface; smooth muscle cells occupying the abluminal surface; and a morphologically distinct VIC-like cell population embedded in a loose extracellular matrix localized between endothelial and smooth muscle cells (Fig. 2A). Moreover, immunohistochemistry against α-SMA (Fig. 2B) and CD31 (Fig. 2C), two well-known markers of smooth muscle and endothelial cells, respectively,[23,24] showed that VIC-like cells were different from endothelial and smooth muscle cells because these cells were CD31 and α-SMA negative (Figs. 2B, 2C).

Morphologic Characterization of VICs in Retinal Arteriolar Annuli

Under TEM analysis, annuli presumptive VICs appeared as irregularly shaped cells with slender processes (Figs. 2, 3A). These observations match previous results reporting the presence of a subset of cells with cytoplasmic processes in arteriolar branches of semi-corroded casts of mouse retinal vasculature.[21] Presumptive VICs in arteriolar annuli also showed nuclei with more than one lobe, numerous large and elongated mitochondria, and many cisternae of smooth and rough endoplasmic reticulum (Fig. 3A). Numerous caveolae were also found all along the nucleated region of presumptive VICs and in their cytoplasmic processes (Figs. 3B, 3D, 3E).

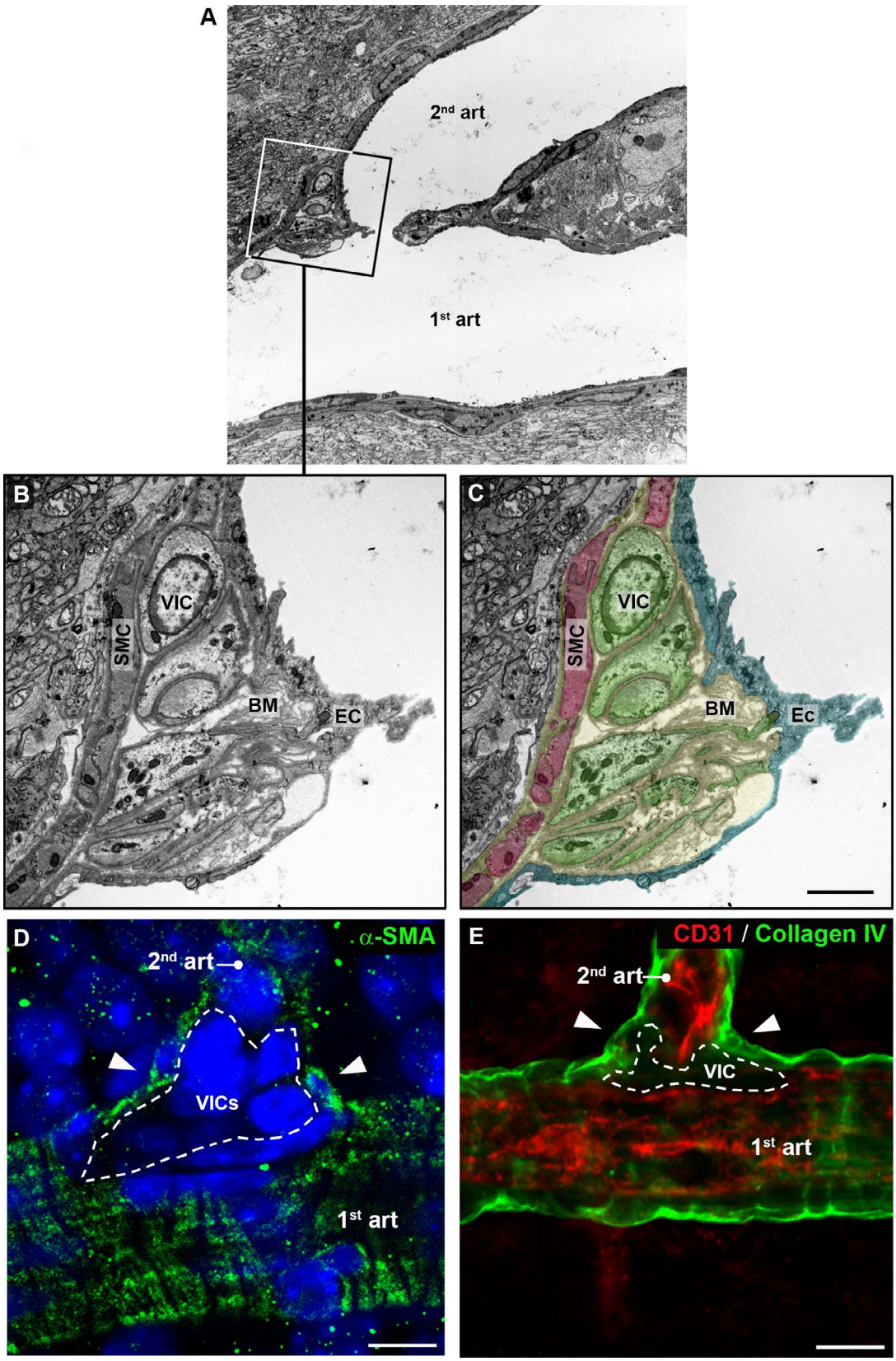

FIGURE 2. Cellular composition of arteriolar annuli. (**A**–**C**) TEM evidenced three distinct cellular types in arteriolar annuli: endothelial cells (EC, *blue*), smooth muscle cells (SMC, *red*), and VICs (*green*). Specific immunostaining of smooth muscle cells with α-SMA (**D**) and endothelial cells with CD31 (**E**) demonstrated that VICs did not belong to these cell types. BM, basement membrane (*yellow*); 1st art, first-order arteriole; 2nd art, second-order arteriole. *Arrowheads* point to arteriolar annuli. *Scale bars*: (**A**) 1.74 μm; (**B**) 5.87 μm; (**C**) 9.32 μm.

Investigative Ophthalmology & Visual Science

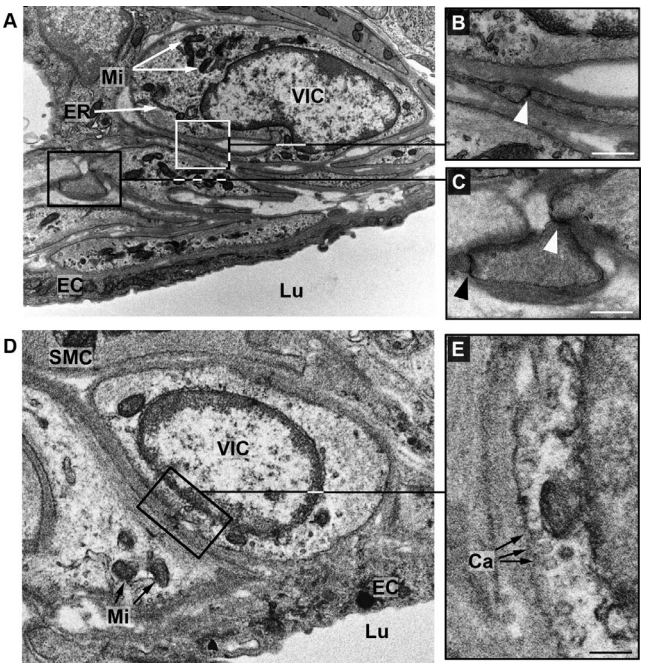

FIGURE 3. Ultrastructural characterization of VICs. (**A**–**C**) TEM evidenced that VICs were characterized by multilobulated nuclei, elongated mitochondria (mi), cytoplasmic processes, and gap junctions (*arrowheads*). (**D**, **E**) Numerous caveolae (ca) and endoplasmic reticulum (ER) were also observed in VICs. SMC, smooth muscle cells; EC, endothelial cell; Lu, vascular lumen. *Scale bars*: (**A**) 175.39 nm; (**B**; *upper inset*) 441.86 nm; (**B**; *lower inset*) 347.94 nm.

Moreover, many gap junctions were observed coupling presumptive VICs with other annuli neighboring cells (Figs. 3B, 3C). All these morphologic features are shared with the established TEM criteria to define interstitial Cajal cells[25] and strongly suggest that the uncovered cell population specifically located between endothelial and smooth muscle cells in retinal arteriolar annuli is comprised by VICs.

Characterization of Protein Profile of VICs in Retinal Arteriolar Annuli

We further characterized VICs immunophenotypically through immunofluorescence of arteriolar annuli.

Similarities and Differences With Interstitial Cajal Cells. c-Kit, a proto-oncogen that encodes a transmembrane tyrosine kinase receptor, is one of the most frequently used markers to detect interstitial Cajal cells.[26–28] However, VICs in retinal arteriolar annuli were negative for c-Kit (Fig. 4A). In this regard, several studies dealing with the presence of interstitial Cajal cells in the vasculature have shown that these cells can be both c-Kit positive[15,29] or negative,[11,12,14] depending on arteries and species under study.

The protein Ano1, a calcium-activated chloride channel, has been reported as a highly selective molecular marker for interstitial Cajal cells in the gastrointestinal tract.[30,31] Immunohistochemical detection of Ano1 in the retina demonstrated a high expression of this protein in VICs in arteriolar annuli (Fig. 4B). A weaker Ano1 expression could also be observed in smooth muscle cells located in second-order retinal arterioles, but not in those localized in first-order retinal arterioles (Fig. 4B). Although Ano1 has been observed in smooth muscle cells of several murine blood vessels,[32–35] our results indicated a notable and specific increase in the expression of Ano1 in VICs in retinal arteriolar annuli. In addition to c-Kit and Ano1, the transmembrane glycoprotein CD44 can also be used as a marker to identify murine interstitial Cajal cells.[28] VICs were positive for this marker in the wall of mouse arteriolar annuli (Fig. 4C).

Several studies have evidenced the expression of NADPH diaphorase in gastrointestinal interstitial Cajal cells.[36,37] NADPH diaphorase is an enzyme involved in nitric oxide synthesis and reflects nitric oxide synthase activity.[38] NADPH activity was detected in VICs in retinal arteriolar annuli (Fig. 4D). Moreover, as previously described,[39,40] NADPH diaphorase activity was also detected in endothelial cells of the mouse retina (Fig. 4D).

Altogether, these results suggest similarities between VICs in arteriolar annuli and gastrointestinal interstitial Cajal cells. In particular, the expression of Ano1 and NADPH diaphorase suggest that VICs might play a role in the motility of arteriolar annuli through pacemaker activity and nitrergic regulation of vascular tone, respectively.

Similarities and Differences With Smooth Muscle Cells. VICs observed in other vascular trees from other body localizations share many morphologic characteristics with smooth muscle cells. However, whether VICs are interstitial Cajal cells or belong to the smooth muscle cell lineage remains debatable.[10,11,29,41]

Several authors have reported distinctive expression of actin and intermediate filaments in interstitial Cajal cells, compared with smooth muscle cells.[14,29,37,42,43] As shown (Fig. 2B), VICs in arteriolar annuli did not express α-SMA, the main functional marker of smooth muscle cells.[44] To further differentiate both cellular types, we analyzed the expression of filament proteins F-actin, β-actin, and nestin in mouse arteriolar annuli.

Phalloidin, a toxin from *Amanita phalloides*, can be used to stain filamentous F-actin.[45] VICs in retinal arteriolar annuli displayed a distribution of filamentous actin different from smooth muscle cells (Fig. 5A). F-actin was scarcely expressed and mainly localized in the periphery of VICs. In contrast, F-actin occupied the whole cytoplasm of vascular smooth muscle cells (Fig. 5A).

In addition, it has been demonstrated that arterial VICs show higher expression of β-actin, compared with vascular smooth muscle cells.[14] To analyze β-actin expression in arteriolar annuli, we used the β-actin–Egfp transgenic mouse,[16] which expresses the EGFP under the control of the chicken β-actin promoter. Although previous data suggested that all cells except hair cells and erythrocytes express EGFP in this transgenic mouse,[16] our results showed that green fluorescence was not homogenously distributed through the entire retina. An intense green emission was observed in the inner limiting membrane, retinal blood vessels, and the inner segments of photoreceptors (Fig. 5B). Interestingly, a detailed analysis of the retinal vasculature showed an intense green fluorescence emission in VICs of arteriolar annuli (Fig. 5C). A higher expression of β-actin was detected in VICs compared with smooth muscle cells of retinal arteriolar annuli. Definitely, this molecular pattern characterized by low expression of contractile proteins, like α-SMA (Fig. 2B), together with high expression of cytoskeletal proteins, like β-actin (Fig. 6C), suggests a lack of contractility of VICs. This is in accordance with previous studies demonstrating that isolated VICs do not contract in response to vasoactive drugs.[10]

The intermediate filament nestin can also be used as a marker for VICs.[46–48] The Nestin-gfp transgenic mice can be used as a highly sensitive reporter system to detect nestin positive cells.[17] Therefore, we used this model to analyze nestin expression in retinal arteriolar annuli.

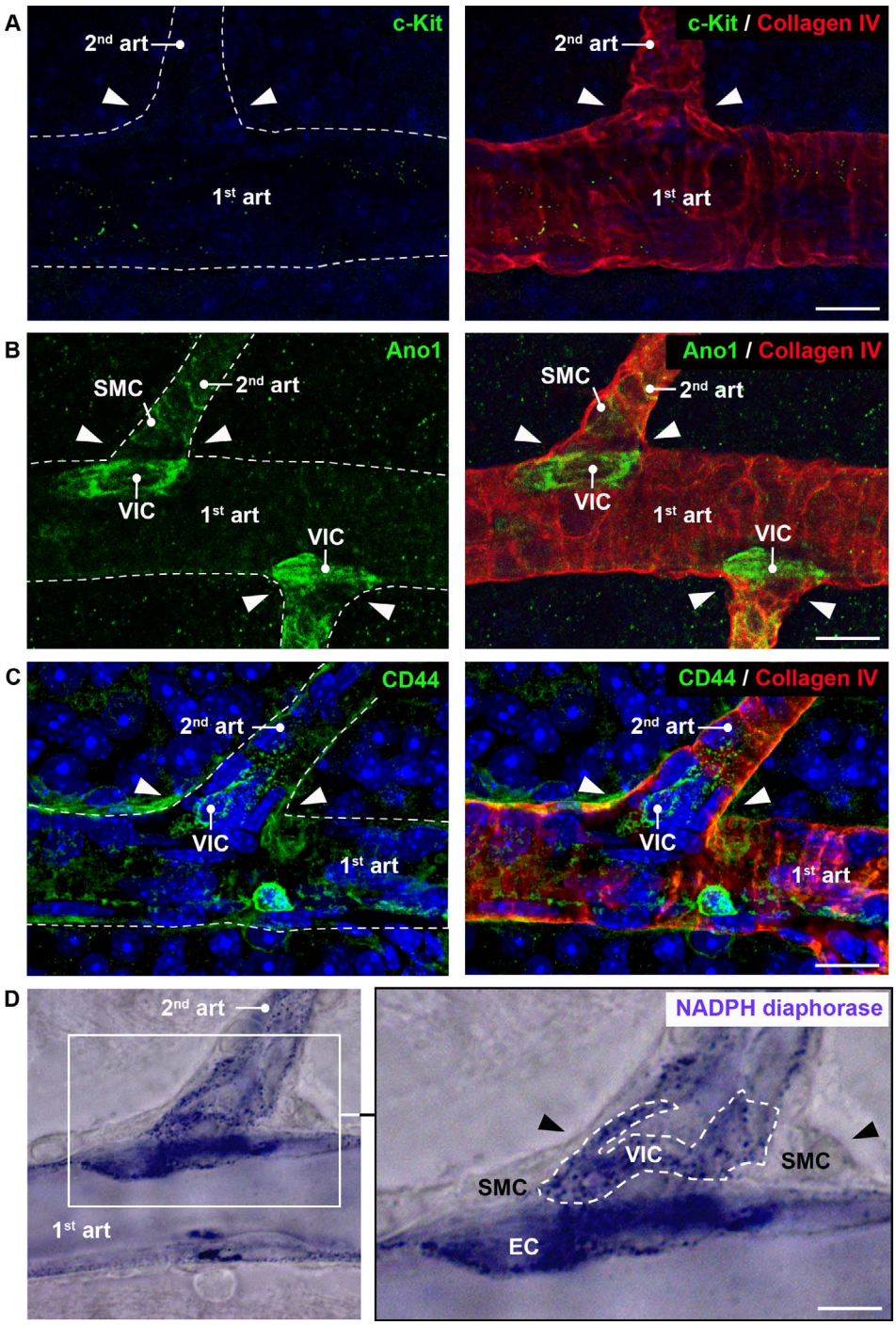

FIGURE 4. Similarities and differences with interstitial Cajal cells. (**A**) No evidence of c-Kit expression was observed in VICs during confocal analysis of whole mount retinas double immunostained against c-Kit (*green*) and collagen IV (*red*). By contrast, confocal microscopy evidenced strong expression of Ano1 (**B**) and CD44 (**C**) in whole mount retinas stained by immunohistochemistry against collagen IV (*red*) and these molecular markers (*green*). (**D**) NADPH diaphorase was increased in VICs and endothelial cells (EC) of arteriolar annuli. SMC, smooth muscle cell. *Arrowheads* point to arteriolar annuli. *Scale bars*: (**A**) 14.4 μm; (**B**) 16.09 μm; (**C**) 14.19 μm; (**D**) 6.87 μm.

Retinal paraffin sections stained with hematoxylin/eosin showed that transgene expression did not alter retinal architecture in Nestin-gfp transgenic mice (Fig. 6A). Consistent with previous observations,[49] our immunohistochemical analysis of the retina showed that GFP colocalized with glutamino synthetase, a specific marker of Müller cells[50] (Fig. 6B). These results demonstrate that nestin is predominantly expressed by glial cells in the mature mouse retina.

Within retinal arteriolar annuli, VICs were easily distinguishable due to their strong green fluorescence, confirming a high expression of nestin (Fig. 6C). Given that GFP fluorescence occupied the nucleus, cell body, and cytoplasmic processes of VICs, the morphology and location of these cells within arteriolar annuli could be easily analyzed in Nestin-gfp retinas. VICs presented an elongated cell body, mostly occupied by a multilobulated nucleus, from which ramified

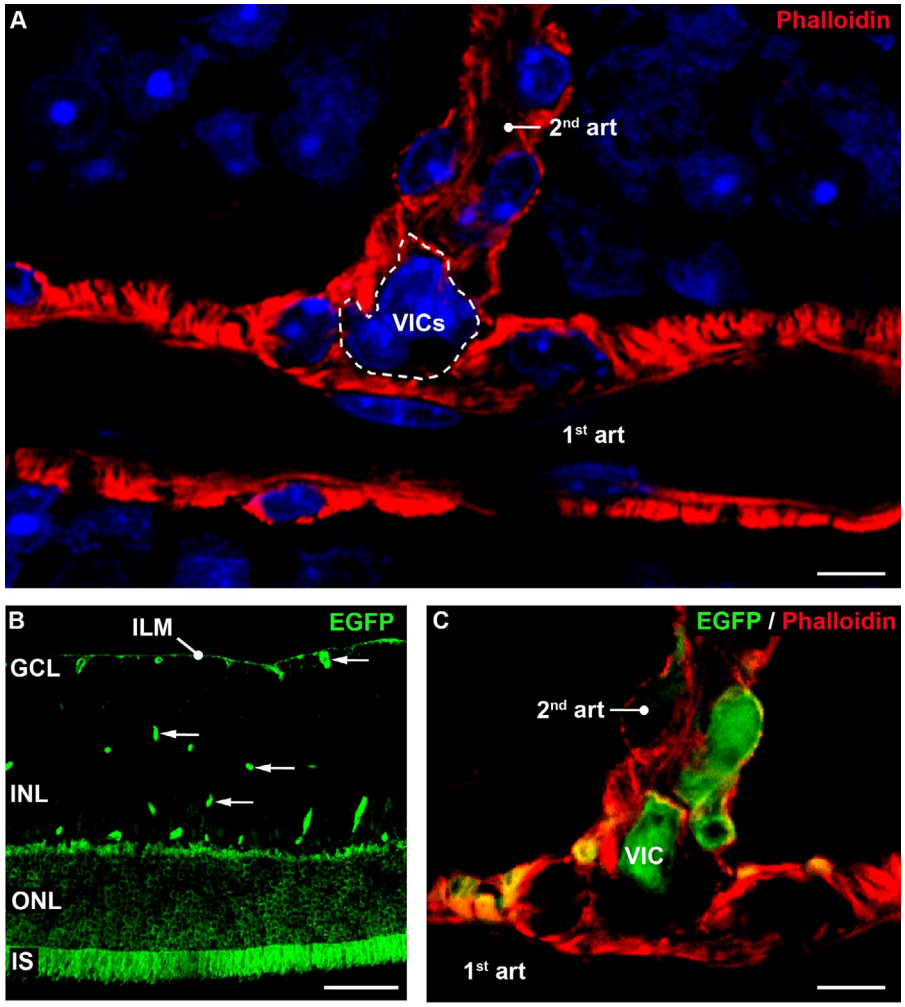

Figure 5. Actin expression differentiates VICs from smooth muscle cells. (**A**) Phalloidin histochemistry evidenced that F-actin was differentially distributed in VICs with respect to smooth muscle cells. (**B**) Retinas from *β-actin–Egfp* mice showed strong green fluorescence in retinal blood vessels (*arrows*), inner limiting membrane (ILM), and the inner segments of photoreceptors (IS). (**C**) A deep analysis of the retinal vasculature of *β-actin–Egfp* mice showed that annuli VICs were characterized by strong expression of *β-actin* (*green*), whereas F-actin (*red*) appeared restricted to the periphery. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. *Scale bars*: (**A**) 5.86 μm; (**B**) 65.79 μm; (**C**) 6.46 μm.

cytoplasmic processes that surrounded the lumen of the arteriolar annuli (Fig. 6C, inset).

Similarities and Differences With Pericytes. Together with smooth muscle cells, pericytes constitute the contractile cell population of blood vessels. Pericytes are completely embedded within the basement membrane of capillaries and postcapillary venules, and it is generally accepted that they are not present in arterioles.[51] We used several molecular markers that can be used to identify pericytes, including NG2, platelet-derived growth factor receptor beta (PDGFR-β), and desmin, among others.[52–55] VICs in retinal arteriolar annuli expressed both NG2 (Fig. 7A) and PDGFR-β (Fig. 7B), but did not express desmin (Fig. 7C). Thus, although VICs do not seem to be contractile cells (based on the lack of α-SMA), these cells exhibit pericyte markers, which are weakly or not expressed in arteriolar smooth muscle cells (Figs. 5A, 5B).

Taken together, these results suggest that VICs located in mouse retinal arteriolar annuli, rather than belonging to the smooth muscle cell lineage, represent a new subtype of interstitial Cajal cells. The Table recapitulates the immunophe-

Table. Immunophenotypes of VICs, Interstitial Cajal Cells, Smooth Muscle Cells, and Pericytes

Protein Marker	VICs	Interstitial Cajal Cells	Smooth Muscle Cells	Pericytes
α-SMA	–	+/–[85]	+++[44]	+[88]
β-Actin	++	+[86]	+/–[14,87]	+[89]
NG2	++	–	–[14]	+++[53]
F-actin	+		+++[24]	++[88]
PDGF-Rβ	+	–	–	++[55]
Ano1	+++	++[30,31]	++[32–35]	–
Nestin	+++	++[46]	–	+[90]
CD44	+	+[28]	–	–
NADPH d	++	++[36,37]	–	–

Results shown refer to normotensive conditions. Superscript numbers are reference sources. –, no expression; +, low expression; ++, moderate expression; +++, strong expression.

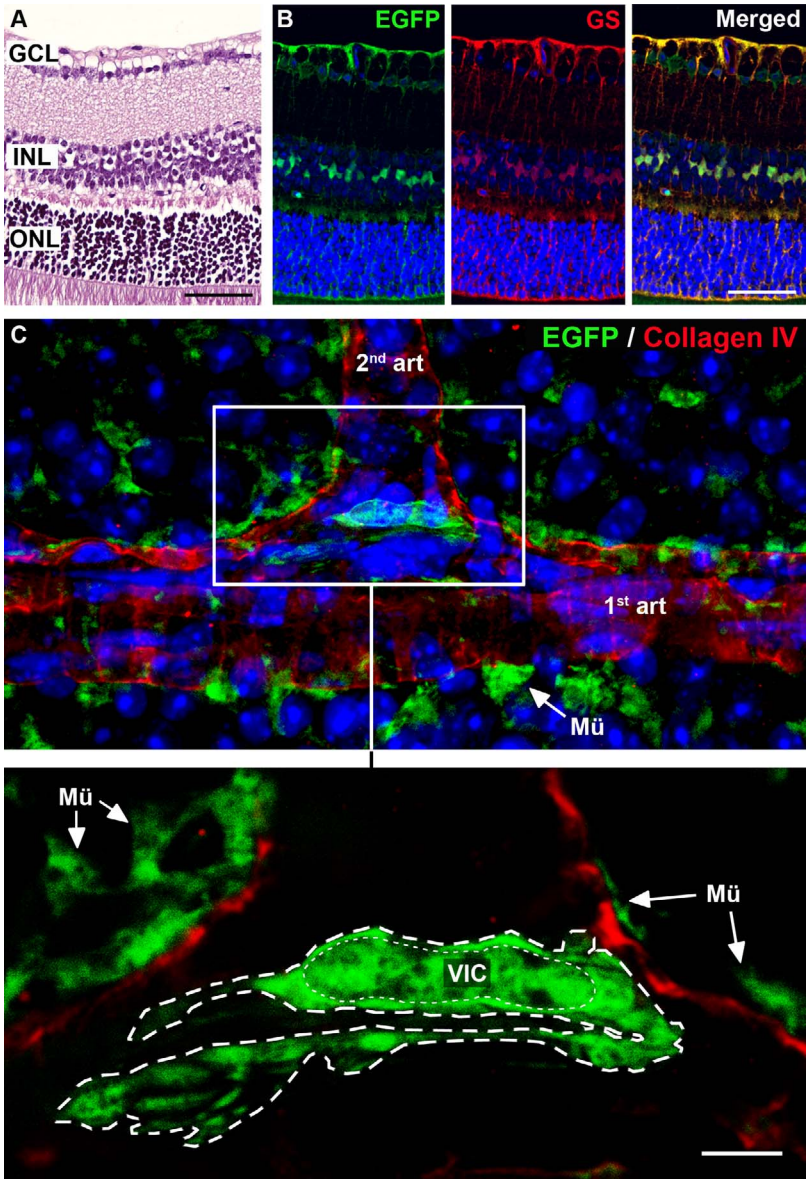

Figure 6. Nestin expression differentiates VICS from smooth muscle cells. (**A**) Hematoxilin/eosin staining of retinal paraffin sections showed that transgene expression did not cause morphologic alterations in retinal architecture of Nestin-gfp mice. (**B**) Confocal microscopy showed high colocalization (*yellow*) between GS (*red*) and EGFP (*green*), indicating that Müller cells highly expressed nestin in the adult retina. (**C**) Analysis of whole mount retinas from Nestin-gfp transgenic mice immunolabeled against collagen IV showed that VICs strongly express nestin. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; 1st art, first-order arteriole; 2nd art, second-order arteriole; Mü, Müller cell. *Scale bars*: (**A**) 60.4 μm; (**B**) 55.6 μm; (**C**) 4.52 μm.

notypic characterization of VICs compared with interstitial Cajal cells, smooth muscle cells, and pericytes under normotensive conditions.

VICs in Retinal Arteriolar Annuli Are Altered During Hypertensive Retinopathy

As a response to elevated blood pressure, the retinal circulation undergoes a series of pathophysiologic changes known as hypertensive retinopathy. This spectrum of vascular signs includes generalized and focal arteriolar narrowing, arteriovenous nicking, retinal hemorrhages, cotton wool spots, and swelling of the optic disk.[56] More specifically, retinal hypertension induces alterations in arteriolar smooth cells, producing hyperplasia of the tunica media.[57] To analyze whether this newly found component of retinal arteriolar annuli (VICs) is affected during hypertension, we analyzed retinas from 8- to 11-month-old KAP transgenic male mice.

KAP transgenic male mice overexpressing KAP driven by its own promoter in a cell- and androgen-restricted manner develop hypertension at 4 months of age.[18] As expected, tail-cuff measurements of systolic and diastolic arterial pressure in KAP transgenic male mice showed that these animals were overtly hypertensive at 7 months of age (KAP-Tg $_{\text{SYSTOLIC}}$ = 154.52 ± 5.63 versus CONTROL $_{\text{SYSTOLIC}}$ = 80.52 ± 3.64, N = 4, $P < 0.0001$; KAP-Tg $_{\text{DIASTOLIC}}$ = 120.76 ± 5.90 versus CONTROL $_{\text{DIASTOLIC}}$ = 61.59 ± 3.25, N = 4, $P < 0.0001$; Fig. 8A).

Retinas from KAP transgenic male mice did not show gross morphologic alterations (Fig. 8B). However, a more detailed analysis of retinal arterioles by immunofluorescent labeling of α-SMA showed a statistically significant increase of the tunica

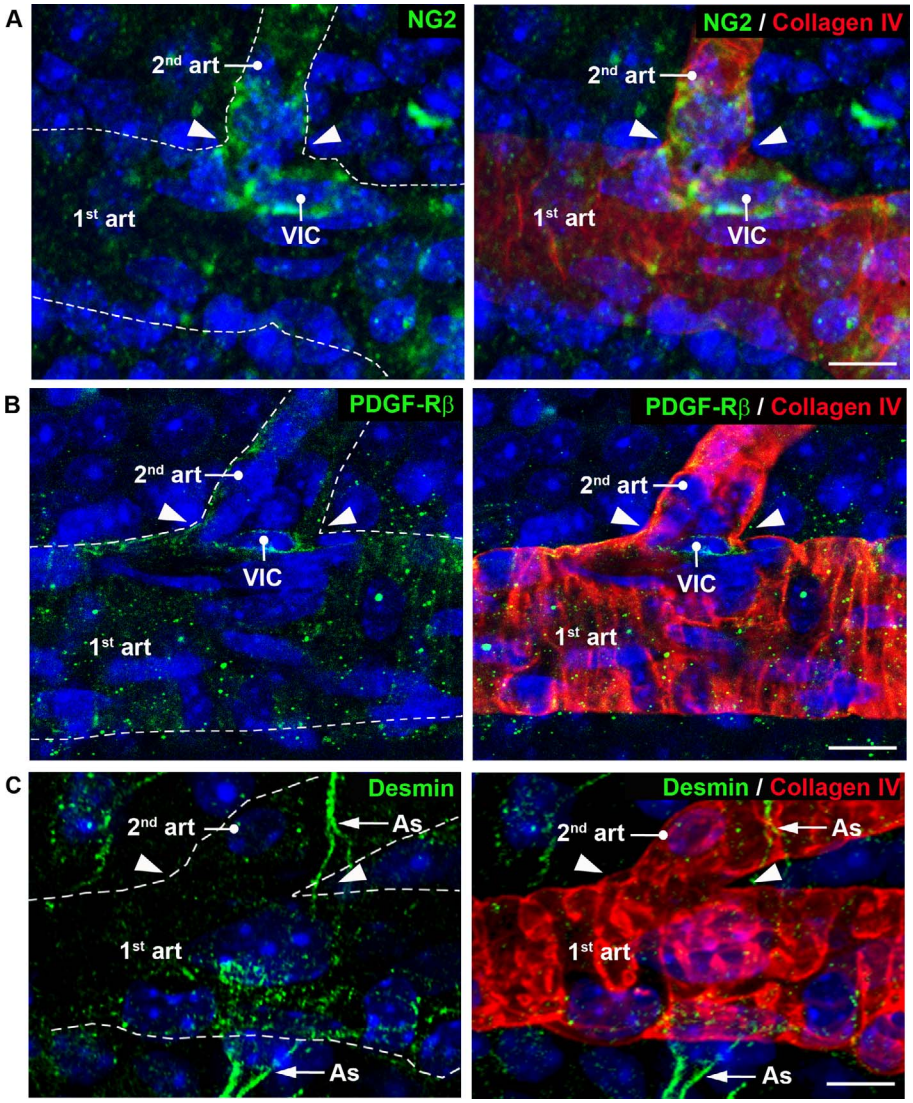

Figure 7. Similarities and differences with pericytes. Confocal microscopy of whole mount retinas immunolabeled against collagen IV (*red*) and pericyte specific markers (*green*) evidenced weak expression of NG2 (**A**) and PDGF-Rβ (**B**). By contrast, desmin was not expressed in VICs (**C**). *Arrowhead* point to arteriolar annuli. *Scale bars*: (**A**) 10.46 μm; (**B**) 12.39 μm; (**C**) 7.63 μm.

media thickness in KAP transgenic retinal arterioles compared with control littermates (1483 ± 0.088 μm in KAP-Tg mice, 12 arterioles measured in three different animals versus 1075 ± 0.061 μm in WT mice, 12 arterioles measured in three different animals; $P = 0.0003$; Figs. 8C, 8D). Focal areas of arteriolar narrowing were also observed in the retina of KAP transgenic mice (Fig. 8E).

Degeneration of photoreceptors has been observed in rats with sustained systemic hypertension.[58,59] However, photoreceptors were not apparently affected histologically in KAP-Tg mice (Fig. 8B). To analyze in more detail photoreceptor morphology, TEM was used. The photoreceptor ultrastructure was maintained in retinas from KAP-Tg mice (Fig. 8F), and only slight mitochondrial alterations were occasionally observed in the photoreceptor inner segments (Fig. 8G).

It is well-known that retinal circulation provides blood to the ganglion cell and inner nuclear layers, whereas photoreceptors are nourished mainly by the choroidal circulation. As retinal and choroidal vascular systems present great anatomical and physiological differences, their responses to hypertension can be markedly different.[60] In fact, it has been reported that photoreceptor degeneration may be caused by hypertensive choroidopathy[61] and that hypertensive changes in choroidal vascularization, including arteriolar narrowing,[62] occur later than changes in retinal vascularization. In this regard, our results would suggest that KAP-Tg mice develop a strong hypertensive retinopathy with a mild hypertensive choroidopathy.

Altogether, these results suggest that KAP transgenic male mice develop vascular lesions compatible with hypertensive retinopathy and can be used as a model for the study of this disease.

It has been previously reported that Ano1 expression is reduced in the basilar artery during hypertension.[63] Therefore, we analyzed the expression of Ano1 in VICs in arteriolar annuli of KAP transgenic mice. Whole mount retinas doubly immunostained for Ano1 and collagen IV showed that Ano1 expression was markedly diminished in arteriolar annuli VICs of KAP transgenic mice compared with age- and sex-matched

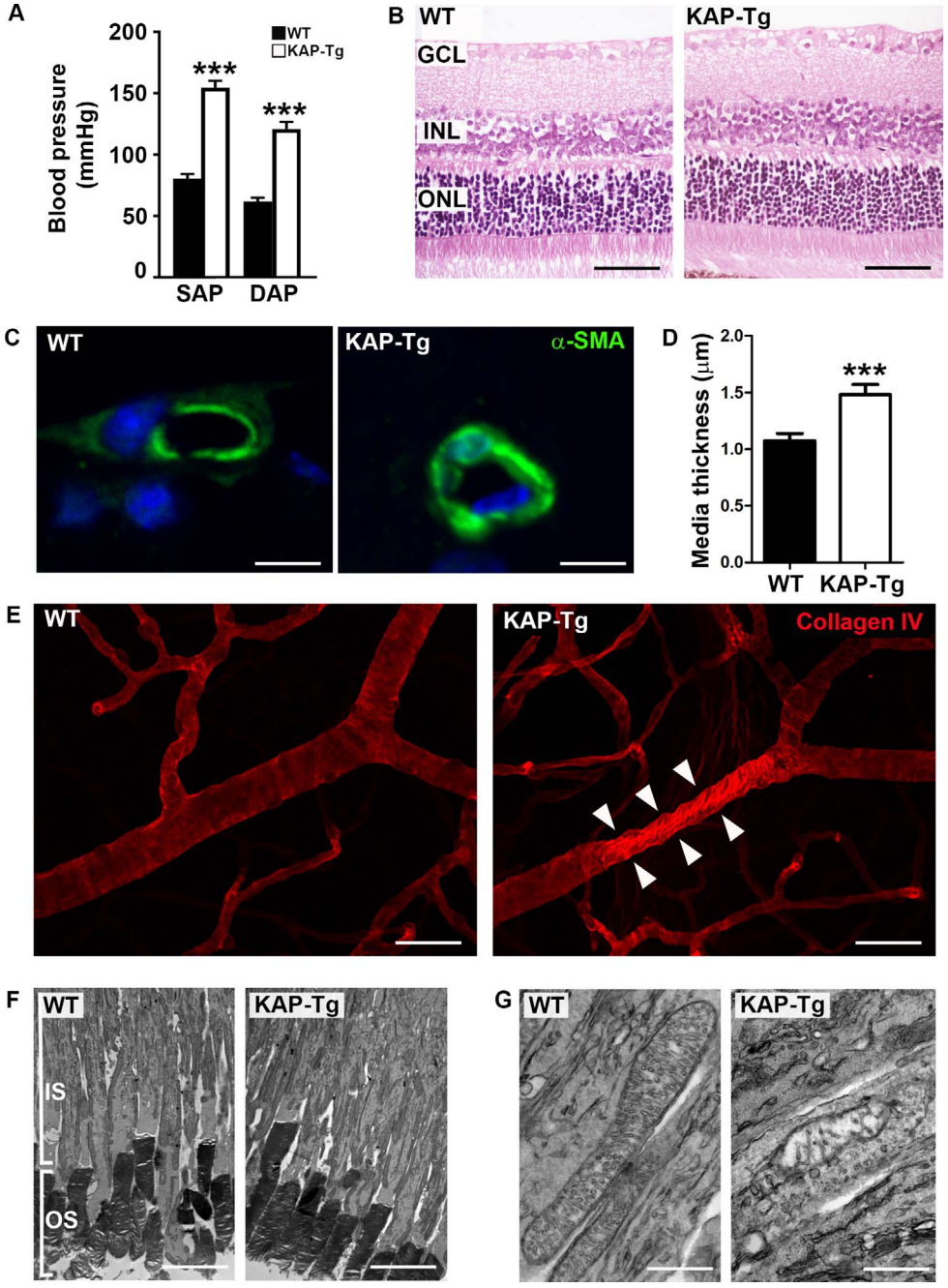

Figure 8. KAP-transgenic (KAP-Tg) mice develop vascular lesions compatible with hypertensive retinopathy. (**A**) Tail-cuff measurements of systolic (SAP) and diastolic (DAP) arterial pressure of 7-month-old KAP-Tg mice and control littermates evidenced that KAP-Tg mice were hypertensive. (**B**) Paraffin sections stained with hematoxylin and eosin evidenced no gross morphologic alterations in retinas from KAP-Tg mice. (**C**, **D**) Immunohistochemistry against α-SMA evidenced a significant increase in the thickness of the tunica media of the retinal arterioles of KAP-Tg mice compared with wild-type (WT) mice. (**E**) Focal narrowing areas (*arrowheads*) were observed in retinal arterioles of KAP-Tg mice during confocal analysis of whole mount retinas immunostained against collagen IV (*red*). (**F**) TEM showed no evident ultrastructural alterations of photoreceptors in retinas from KAP-Tg mice. (**G**) A deeper analysis evidenced slight mitochondrial morphologic alterations in the inner segment of KAT-Tg photorreceptors. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, photoreceptor inner segment; OS, photoreceptor outer segment. $^{***}P < 0.001$. *Scale bars*: (**B**) 34.5 μm; (**C**) 5.78 μm; (**E**) 37.76 μm; (**F**) 4 μm; (**G**) 0.67 μm.

control mice (23.64 ± 6.456 arbitrary units, $n = 5$ versus 64.27 ± 9.443 arbitrary units, $n = 5$; $P = 0.0075$; Fig. 9).

KAP gene overexpression induces hypertension partly by activating the renin-angiotensin system.[64] Furthermore, increased production of angiotensin II, the active agent of the renin-angiotensin system, decreases nitric oxide activity by nitric oxide synthase uncoupling.[65] Therefore, to analyze the nitric oxide synthase, we studied NADPH diaphorase activity in retinal arteriolar annuli from KAP transgenic mice. Our results demonstrated that NADPH diaphorase activity was markedly reduced in retinal arteriolar annuli, both in VICs and in endothelial cells from KAP transgenic male mice compared with age- and sex-matched control mice ($0.5153 \times 10^7 + 0.1488 \times 10^7$ arbitrary units, $n = 4$ versus $2.872 \times 10^7 + 0.5902$

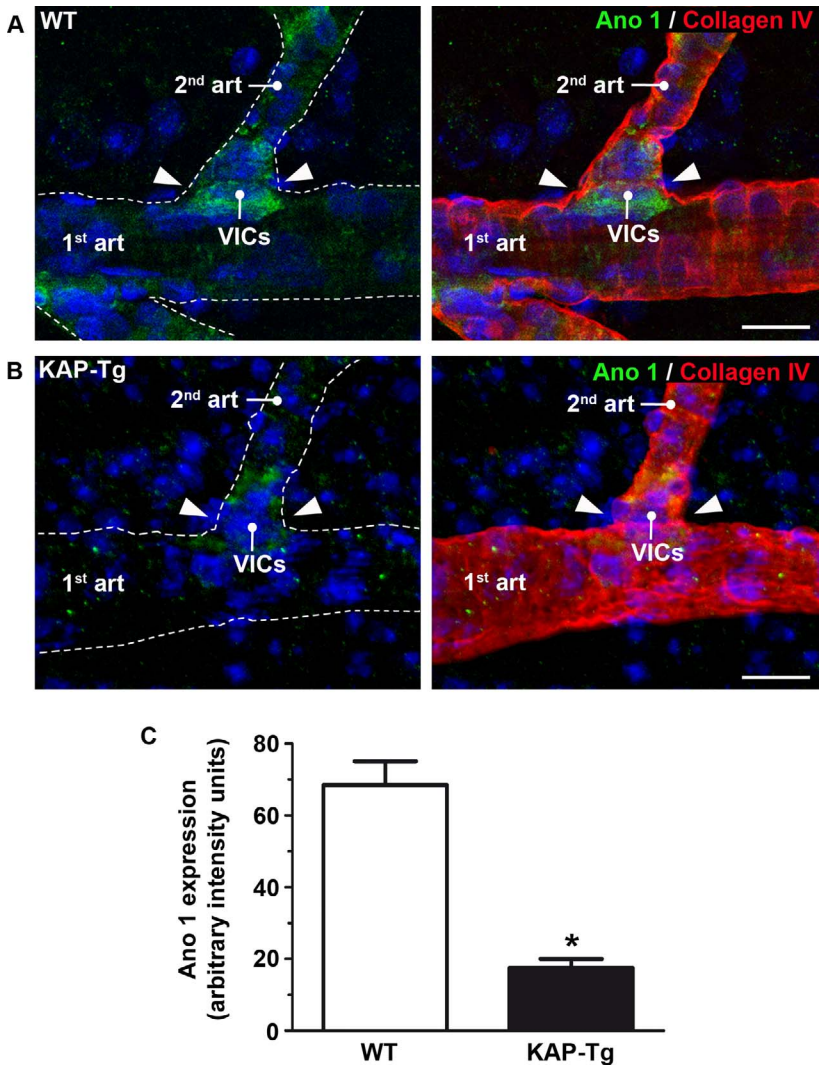

FIGURE 9. Ano1 expression is downregulated in VICs during hypertensive retinopathy. (**A**) Confocal microscopy analysis showed decreased Ano1 expression in whole mount retinas from KAP-Tg mice (**B**). (**C**) Quantification of fluorescence intensity demonstrated that the decrease in Ano1 expression during hypertensive retinopathy was statistically significant. *Arrowheads* point to arteriolar annuli. $^{*}P < 0.05$. *Scale bars*: (**A**) 17.16 μm; (**B**) 14.85 μm.

$\times 10^7$, $n = 4$; $P = 0.0002$; Fig. 10). Thus, our results suggest that nitric oxide synthesis might be reduced in retinal arteriolar annuli during hypertensive retinopathy. Given that nitric oxide is a survival factor for VICs,[65,66] these results suggest a possible alteration of VICs during hypertensive retinopathy.

Altogether, these results suggest a loss of function of calcium-activated chloride channels and nitric oxide synthesis in VICs during hypertensive retinopathy that could affect the control of the retinal vascular tone at the level of arterial annuli.

Discussion

It is now well accepted that blood vessels are far more sophisticated than simple tubes conducting blood. In fact, these highly organized and complex structures are able to autonomously regulate the blood flow to provide oxygen and nutrients according to tissue needs.[67] This autonomous control of blood flow is even more important in the retina, which does not contain sympathetic innervation of blood vessels and where the effect of circulating hormones is almost negligible.[39,68,69]

In this paper, a novel type of vascular cells that could play a role in the motility of retinal arteriolar annuli, and therefore, participate in the retinal blood flow regulation, has been described morphologically and immunophenotypically. According to TEM analysis, these cells were located within the vascular wall, between endothelial and smooth muscle cells. This specific localization and their morphologic features make them compatible with VICs. Furthermore, their ultrastructure and molecular phenotype suggested that VICs in arteriolar annuli, despite sharing some markers with smooth muscle cells and pericytes, belong to a new subtype of interstitial Cajal cells.

The presence of interstitial Cajal cells in the blood vessel wall has been under discussion since 1953, when Meyling[70] described the autonomic interstitial cells. Also Dahl and Nelson[71] reported, in 1964, the presence of cells with irregular nuclei and multipolar processes in several arteries of the central nervous system. Nowadays, interstitial Cajal-like cells have been described in a number of vessels, including portal

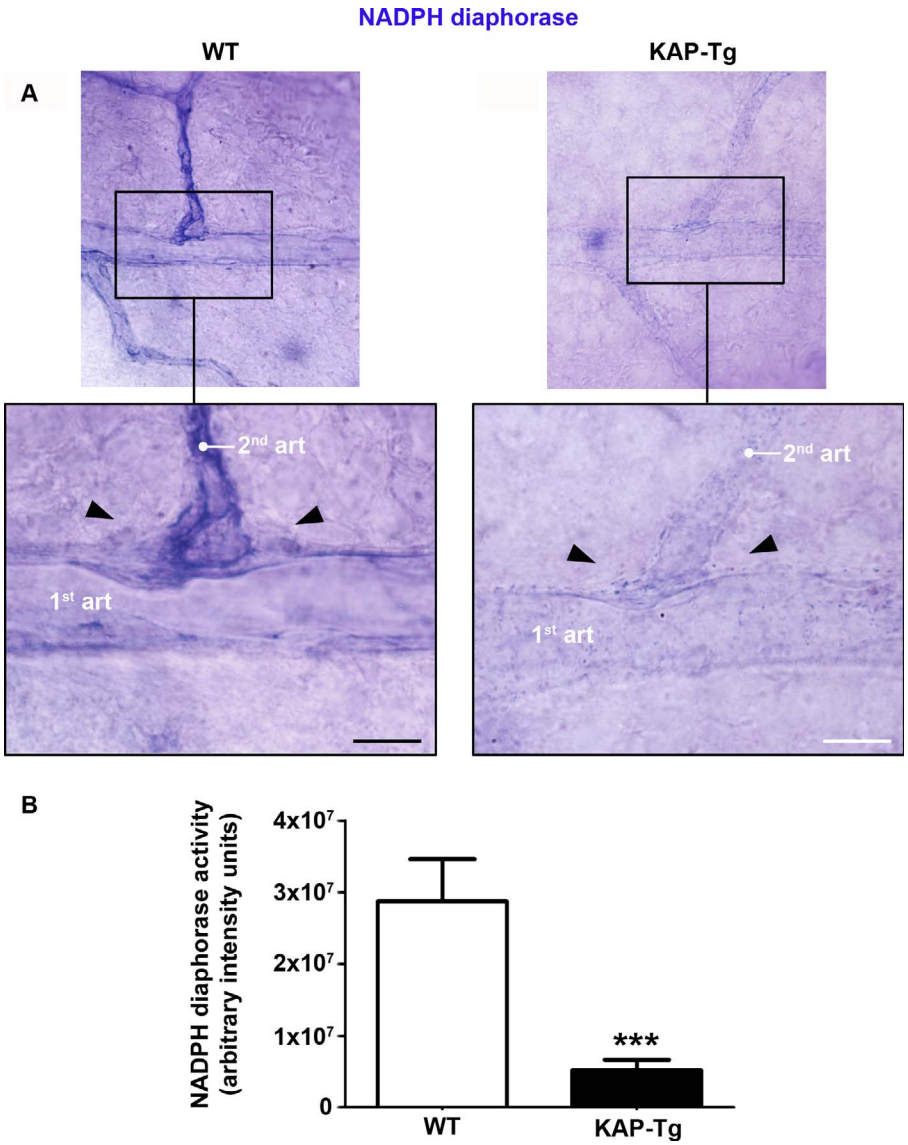

Figure 10. NADPH diaphorase activity is decreased in arteriolar annuli of KAP-Tg mice. (**A**) A great decrease in NADPH diaphorase activity was observed in retinas from KAP-Tg mice compared with wild-type (WT) mice. (**B**) Quantification of NADPH diaphorase activity by measuring staining intensity evidenced statistical significance. *Arrowheads* point to arteriolar annuli. $^{***}P < 0.001$. *Scale bars*: 12.7 μm.

vein, mesenteric arteries, aorta, carotid artery, middle cerebral artery, and pulmonary veins.[10–15]

The nature of interstitial Cajal cells has remained controversial. Cajal believed that interstitial gastrointestinal cells were primitive neurons, whereas other researchers suggested that these cells were specialized smooth muscle cells or even fibroblast-like cells.[72] Developmental studies suggested common precursors for interstitial Cajal and smooth muscle cells.[73,74] Recently, $CD44^{+}$ vascular multipotent stem cells able to differentiate into smooth muscle cells and pericytes have been identified within the adult human arterial adventitia.[75] These multipotent mesenchymal cells show similar molecular phenotype to that of VICs in retinal arteriolar annuli, as they express CD44, NG2, and nestin. Furthermore, it has been reported that interstitial Cajal cells can revert to smooth muscle cell type when the Kit signaling pathway is blocked.[76] Altogether, these results suggest that interstitial Cajal cells, VICs, smooth muscle cells, and pericytes may be part of the same vascular cell lineage.

Interstitial Cajal cells regulate gastrointestinal motility through their pacemaker activity,[15] and VICs in retinal arteriolar annuli show morphologic and molecular characteristics (like gap junctions and Ano1 expression) that have been proposed to play a role for pacemaker activity.[77,78] Furthermore, we have shown that VICs have NADPH diaphorase activity and therefore can potentially synthesize nitric oxide, thus suggesting that VICs could play a role in nitrergic regulation of vascular tone. Overall, these findings indicated that VICs could play a role in the motility of arteriolar annuli.

Mechanical distortion of interstitial Cajal cells increases their pacemaker activity.[79] Due to their location, interstitial Cajal cells can monitor the intestine contractility and transmit this information to the enteric nervous system.[79] The precise location of VICs, between endothelial and smooth muscle cells in the arteriolar annuli, could also make these cells able to sense variations in the vascular tone. Furthermore, it has been postulated that adhesion molecules, such as CD44, couple extracellular and nuclear matrix scaffolds by means of cytoskeleton filaments that transduce changes in the basement

membrane to the nucleus.[80] CD44 is expressed by VICs, suggesting that these cells are endowed with a molecular mechanism to transform mechanical distortion into biological responses.

The autonomous control of retinal blood flow operates only within a certain range of perfusion pressure; this autoregulation becomes ineffective above physiologic thresholds. Consequently, arterial hypertension causes derangement of retinal blood flow and arteriosclerosis.[81] Therefore, we studied the effects of hypertension on arteriolar annuli (and, more specifically, on VICs) through the use of a well-established mouse model of arterial hypertension: the KAP transgenic mice.[18,64]

The fine analysis of arteriolar annuli in KAP transgenic mice demonstrated that VICs are altered during hypertensive retinopathy, because there was a marked decrease in Ano1 expression and NADPH diaphorase activity in these cells. Previous reports concerning the status of Ano1 during hypertension are discordant, with some studies arguing for reduced Ano1 expression[63] or altered allostery,[82] whereas other studies reported increased Ano1 activity.[35,83] However, hypertension-related downregulation of Ano1 has been always observed in the vasculature of the central nervous system, both in brain[63] and in the retina.[82] Thus, differences between previous reports may reflect modifications of Ano1 dynamics to adapt the vascular response to the specific needs of each tissue. Furthermore, studies in Ano1 knockout mice have demonstrated that the absence of this protein greatly reduces smooth muscle contraction.[77] Therefore, alterations in Ano1 expression observed in retinas from KAP transgenic mice might indicate a loss of function of calcium-dependent chloride channels that could lead to abnormal vascular motility.

As previously discussed, VICs present NADPH diaphorase activity, suggesting that these cells might play a role in nitrergic regulation of the vascular tone. Nitric oxide is a potent vasodilator that has been implicated in the regulation of blood pressure and regional blood flow, and its expression is altered during hypertension.[84] Therefore, the NADPH abnormalities observed in KAP transgenic mice might diminish nitric oxide synthesis and cause loss of retinal blood flow autoregulation.

In conclusion, a novel type of vascular cells morphologically and molecularly comparable with the interstitial Cajal cell has been described in retinal arteriolar annuli. Remarkably, these cells undergo important molecular modifications during hypertension and might thus be a therapeutic target against hypertensive retinopathy.

Acknowledgments

The authors thank Veronica Melgarejo, Lorena Noya, and Angel Vazquez for technical assistance.

Supported by grants from Instituto de Salud Carlos III (PI12/00605, PI16/00719, SAF2014-59945-R, and Red de Investigación Renal REDinREN 12/0021/0013), Spain; Fundação para a Ciência e a Tecnologia (SFRH/BPD/102573/2014, SFRH/BD/95330/2013), Ministerio da Educação e Ciência, Portugal; and Fondo Europeo de Desarrollo Regional (FEDER).

Disclosure: **D. Ramos**, None; **J. Catita**, None; **M. López-Luppo**, None; **A. Valença**, None; **A. Carretero**, None; **M. Navarro**, None; **V. Nacher**, None; **S. Mendez-Ferrer**, None; **A. Meseguer**, None; **A. Casellas**, None; **L. Mendes-Jorge**, None; **J. Ruberte**, None

References

1. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. *Lancet*. 2005;365:217–223.
2. Wong TY, McIntosh R. Hypertensive retinopathy signs as risk indicators of cardiovascular morbidity and mortality. *Br Med Bull*. 2005;73–74:57–70.
3. Meyer-Rüsenberg B, Pavlidis M, Stupp T, Thanos S. Pathological changes in human retinal ganglion cells associated with diabetic and hypertensive retinopathies. *Graefe's Arch Clin Exp Ophtahlmol*. 2006;245:1009–1018.
4. Kuwabara T, Cogan DG. Studies of retinal vascular patterns part I. Normal architecture. *Arch Ophthalmol*. 1960;64:904–911.
5. Henkind P, De Oliveira LF. Retinal arteriolar annuli. *Invest Ophthalmol Vis Sci*. 1968;7:584–591.
6. Pannarale L, Onori P, Ripani M, Gaudio E. Precapillary patterns and perivascular cells in the retinal microvasculature. A scanning electron microscope study. *J Anat*. 1996;188:693–703.
7. Ninomiya H, Inomata T. Microvasculature of the mouse eye: scanning electron microscopy of vascular corrosion casts. *J Exp Anim Sci*. 2006;43:149–159.
8. Thuránzsky K. *Der Blutkreislauf der Netzhaut: Intravitalmikroskopische und Histologische Studien an der Katzenretina*. Budapest: Ungarische Akademie Wissenchaften; 1957.
9. Friedman E, Smith TR, Kuwabara T. Retinal microcirculation in vivo. *Inv Ophthalmol*. 1964;3:217–226.
10. Povstyan OV, Gordienko DV, Harhun MI, Bolton TB. Identification of interstitial cells of Cajal in the rabbit portal vein. *Cell Calcium*. 2003;33:223–239.
11. Pucovský V, Moss RF, Bolton TB. Non-contractile cells with thin processes resembling interstitial cells of Cajal found in the wall of guinea-pig mesenteric arteries. *J Physiol*. 2003;552:119–133.
12. Bobrishev YV. Subset of cells immunopositive for neurokinin-1 receptor identified as arterial interstitial cells of Cajal in human large arteries. *Cell Tissue Res*. 2005;321:45–55.
13. Morel E, Meynoret D, Thivolet-Bejuy F, Chevalier P. Identification and distribution of interstitial Cajal cells in human pulmonary veins. *Heart Rhythm*. 2008;5:1063–1067.
14. Harhun MI, Szewczyk K, Laux H, et al. Interstitial cells from rat middle cerebral artery belong to smooth muscle cell type. *J Cell Mol Med*. 2009;11–12:4532–4539.
15. Formey A, Buscemi L, Boittin FX, Bény JL, Meister JJ. Identification and functional response of interstitial Cajal-like cells from rat mesenteric artery. *Cell Tissue Res*. 2011;343:509–519.
16. Okabe M. Ikawa M, Kominami K, Nakanishi T, Nishimune Y. "Green mice" as a source of ubiquitous green cells. *FEBS Lett*. 1997;407:313–319.
17. Mignone JL, Kukekov V, Chiang AS, Steindler D, Enikolopov G. Neural stem and progenitor cells in nestin-GFP transgenic mice. *J Comp Neurol*. 2004;469:311–324.
18. Tornavaca O, Pascual G, Barreiro ML, et al. Kidney androgen-regulated protein transgenic mice show hypertension and renal alterations mediated by oxidative stress. *Circulation*. 2009;119:1908–1917.
19. Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M. Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. *Proc Natl Acad Sci U S A*. 1991;88:11555–11558.
20. Agrawal PK, Agarwal LP, Tandon HD. Retinal vasculature in mammalia. *Indian J Ophtalmol*. 1968;16:3–11.
21. Ikebe T, Shimada T, Ina K, Kitamura H, Nakatsuka K. The three-dimensional architecture of retinal blood vessels in kk mice, with special reference to the smooth muscle cells and pericytes. *J Electron Microsc*. 2001;50:125–132.
22. Loewenstein A. Cushion cells in retinal precapillaries and capillaries. *Nature*. 1947;159:229.

23. Gariano RF, Iruela-Arispe ML, Sage EH, Hendrickson AE. Characterization of developing and mature primate retinal blood vessels. *Invest Ophthalmol Vis Sci*. 1996;37:93–103.
24. Mendes-Jorge L, Llombart C, Ramos D, et al. Intercapillary bridging cells: immunocytochemical characteristics of cells that connect blood vessels in the retina. *Exp Eye Res*. 2012; 98:79–87.
25. Huizinga JD, Thuneberg L, Vanderwinden JM, Rumessen JJ. Interstitial cells of Cajal as targets for pharmacological intervention in gastrointestinal motor disorders. *Trends Pharmacol Sci*. 1997;18:393–403.
26. Maeda H, Yamagata A, Nishikawa S, et al. Requirement of c-kit for development of intestinal pacemaker system. *Development*. 1992;116:369–375.
27. Huizinga JD, Robinson TL, Thomsen L. The search of the origin of rhythmicity in intestinal contraction; from tissue to single cells. *Neurogastrenterol Motil*. 2000;12:3–9.
28. Yu B, Han J, He YT, Guo S, Li SF, Mei F. Immunohistochemical study of CD44 immunopositive cells in the muscular layers of the gastrointestinal tract in adult guinea pigs and mice. *Acta Histochem*. 2009;111:382–390.
29. Harhun MI, Pucovský V, Povstyan OV, Gordienko DV, Bolton TB. Interstitial cells in the vasculature. *J Cell Mol Med*. 2005;9: 232–243.
30. Gomez-Pinilla PJ, Gibbons SJ, Bardsley MR, et al. Ano1 is a selective marker of interstitial cells of Cajal in the human and mouse gastrointestinal tract. *Am J Physiol Gastrointest Liver Physiol*. 2009;296:G1370–G1381.
31. Liu YA, Chung YC, Shen MY, et al. Perivascular interstitial cells of Cajal in human colon. *Cell Mol Gastroenterol Hepatol*. 2014;1:102–119.
32. Davis AJ, Forrest AS, Jepps TA, et al. Expression profile and protein translation of TMEM16A in murine smooth muscle. *Am J Physiol Cell Physiol*. 2010;299:C948–C959.
33. Manoury B, Tamuleviciute A, Tammaro P. TMEM16A/Anoctamin 1 protein mediates calcium-activated chloride currents in pulmonary arterial smooth muscle cells. *J Physiol*. 2010;588: 2305–2314.
34. Thomas-Gatewood C, Neeb ZP, Bulley S, et al. TMEM16A channels generate Ca^{2+}-activated Cl^{-} currents in cerebral artery smooth muscle cells. *Am J Physiol Heart Circ Physiol*. 2011;301:H1819–H1827.
35. Heinze C, Seniuk A, Sokolov MV, et al. Disruption of vascular Ca2+-activated chloride currents lowers blood pressure. *J Clin Invest*. 2014;124:675–686.
36. Xue C1, Pollock J, Schmidt HH, Ward SM, Sanders KM. Expression of nitric oxide synthase immunoreactivity by interstitial cells of the canine proximal colon. *J Auton Nerv Syst*. 1994;49:1–14.
37. Faussone-Pellegrini MS, Thuneberg L. Guide to the identification of interstitial cells of Cajal. *Microsc Res Tech*. 1999;47: 248–266.
38. Vincent SR, Hope BT. Neurons that say NO. *Trends Neurosci*. 1992;15:108–113.
39. Funk RH. Blood supply of the retina. *Ophtalmic Res*. 1997;29: 320–325.
40. Hardy P, Dumont I, Bhattachiarya M, et al. Oxidants, nitric oxide and prostanoids in the developing ocular vasculature: a basis for ischemic retinopathy. *Cardiovasc Res*. 2000;47:489–509.
41. Bolton TB1, Gordienko DV, Povstyan OV, Harhun MI, Pucovsky V. Smooth muscle cells and interstitial cells of blood vessels. *Cell Calcium*. 2004;35:643–657.
42. Pucovský V, Harhun MI, Povstyan OV, Gordienko DV, Moss RF, Bolton TB. Close relation of arterial ICC-like cells to the contractile phenotype of vascular smooth muscle cell. *J Cell Mol Med*. 2007;11:764–775.
43. Popescu LM, Gherghiceanu M, Manole CG, Faussone-Pellegrini MS. Cardiac renewing: interstitial Cajal-like cells nurse cardiomyocyte progenitors in epicardial stem cell niches. *J Cell Mol Med*. 2009;13:866–886.
44. Skalli O, Pelte MF, Peclet MC, et al. Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. *J Histochem Cytochem*. 1989;37:315–321.
45. Prochniewicz-Nakayama E, Yanagida T, Oosawa F. Studies on conformation of F-actin in muscle fibers in the relaxed state, rigor, and Turing contraction using fluorescente phalloidin. *J Cell Biol*. 1983;97:1663–1667.
46. Tsujimura T, Makiishi-Shimobayashi C, Lundkvist J, et al. Expression of the intermediate filament nestin in gastrointestinal stromal tumors and interstitial cells of Cajal. *Am J Pathol*. 2001;158:817–823.
47. Vanderwinden J-M, Gillard K, De Laet M-H, Messam CA, Schiffmann SN. Distribution of the intermediate filament nestin in the muscularis propria of the human gastrointestinal tract. *Cell Tissue Res*. 2002;309:261–268.
48. Hinescu ME, Ardeleanu C, Gherghiceanu M, Popescu LM. Interstitial Cajal-like cells in human gallbladder. *J Mol Histol*. 2007;38:275–284.
49. Lee JH, Park HS, Shin JM, Chun MH, Oh SJ. Nestin expressing progenitor cells during establishment of the neural retina and its vasculature. *Anat cell Biol*. 2012;45:38–46.
50. Haverkamp S, Wässle H. Immunocytochemical analysis of the mouse retina. *J Comp Neurol*. 2000;424:1–23.
51. Armulik A, Genové G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell*. 2011;21:193–215.
52. Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science*. 1997;277:242–245.
53. Ozerdem U, Grako KA, Dahlin-Huppe K, Monosov E, Stallcup WB. NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. *Dev Dyn*. 2001;222:218–227.
54. Chan-Ling T, Page MP, Gardiner T, Baxter L, Rosinova E, Hughes S. Desmin ensheathment ratio as an indicator of vessel stability: evidence in normal development and in retinopathy of prematurity. *Am J Pathol*. 2004;165:1301–1313.
55. Armulik A, Bramsson A, Betsholtz C. Endothelial/pericyte interactions. *Circ Res*. 2005;97:512–523.
56. Wong TY, Mitchell P. Hypertensive retinopathy. *N Engl J Med*. 2004;351:2310–2317.
57. Wong TY, Mitchell P. The eye in hypertension. *Lancet*. 2007; 369:425–435.
58. Sallmann L, Grimes P. Retinal degeneration in mature rats. Comparison of the disease in an Osborne-Mendel and a spontaneously hypertensive Wistar strain. *Invest Ophtalmol*. 1974;13:1010–1015.
59. Ruchoux MM, Droy-Lefaix MT, Bakry F, Guillemain J, Ruchoux P, Autret A. Cicletanine and hypertensive retinopathy. *Ophtalmic Res*. 1990;22:318–329.
60. Tso MO, Jampol LM. Pathophysiology of hypertensive retinopathy. *Ophtalmology*. 1982;89:1132–1145.
61. Li S, Lam TT, Fu J, Tso MOM. Systemic hypertension exaggerates retinal photic injury. *Arch Ophthalmol*. 1995; 113:521–526.
62. Bourke K, Patel MR, Prisant LM, Marcus DM. Hypertensive choroidopathy. *J Clin Hypertens*. 2004;6:417–422.
63. Wang M, Yang H, Zheng LY, et al. Downregulation of TMEM16A calcium-activated chloride channel contributes to cerebrovascular remodeling during hypertension by promoting basilar smooth muscle cell proliferation. *Circulation*. 2012;125:697–707.

64. Grande MT, Pascual G, Riolobos AS, et al. Increased oxidative stress, the renin-angiotensin system, and sympathetic over-activation induce hypertension in kidney androgen-regulated protein transgènic mice. *Free Radic Biol Med*. 2011;51:1831–1841.
65. Touyz RM. Reactive oxygen species and angiotensin II signaling in vascular cells: implications in cardiovascular disease. *Braz J Med Biol Res*. 2004;37:1263–1273.
66. Choi KM, Gibbons SJ, Roeder JL, et al. Regulation of interstitial cells of Cajal in the mouse gastric body by neuronal nítric oxide. *Neurogastroenterol Motil*. 2007;19:585–595.
67. Cantarero I, Luesma MJ, Junquera C. The primary cilium of telocytes in the vasculature: electron microscope imaging. *J Cell Mol Med*. 2011;15:2594–2600.
68. Laties AM. Central retinal artery innervation. Absence of adrenergic innervation to the intraocular branches. *Arch Ophthalmol*. 1967;77:405–409.
69. Delaey C, Van De Voorde J. Regulatory mechanisms in the retinal and choroidal circulation. *Ophthalmic Res*. 2000;32:249–256.
70. Meyling HA. Structure and significance of the peripheral extension of the autonomic nervous system. *J Comp Neurol*. 1953;99:495–543.
71. Dahl E, Nelson E. Electron microscopi observations on human intracranial arteries. Arteries. II. Innervation. *Arch Neurol*. 1964;10:158–164.
72. Sanders KM, Ördög T, Koh SD, Torihashi S, Ward SM. Development and plasticity of interstitial cells of Cajal. *Neurogastroenterol Motil*. 1999;11:311–338.
73. Torihashi S, Ward SM, Sanders KM. Development of c-kit-positive cells and the onset of electrical rhythmicity in murine small intestine. *Gastroenterology*. 1997;112:144–155.
74. Kluppel M, Huizinga JD, Malysz J, Bernstein A. Developmental origin and kit-dependent development of the interstitial cells of Cajal in the mammalian small intestine. *Dev Dyn*. 1998;211:60–71.
75. Klein D, Weisshardt P, Kleff V, Jastrow H, Jakob HG, Ergün S. Vascular wall-resident CD44+ multipotent stem cells give rise to pericytes and smooth muscle cells and contribute to new vessel maturation. *PLoS One*. 2011;6:e20540.
76. Torihashi S, Nishi K, Tokutomi Y, Nishi T, Ward S, Sanders KM. Blockade of kit signaling induces transdifferentiation of interstitial cells of Cajal to a smooth muscle phenotype. *Gastroenterology*. 1999;117:140–148.
77. Huang F, Rock JR, Harfe BD, et al. Studies on expression and function of the TMEM16A calcium-activated chloride channel. *Proc Nat Acad Sci U S A*. 2009;106:21413–21418.
78. Lee H-T, Henning GW, Fleming W, et al. The mechanism and spread of pacemaker activity through myenteric interstitial cells of Cajal in human small intestine. *Gastroenterology*. 2007;132:1852–1856.
79. Huizinga JD, Zarate N, Farrugia G. Physiology, injury, and recovery of interstitial cells of Cajal: basic and clinical science. *Gastroenterology*. 2009;137:1548–1556.
80. Ingber DE. Integrins, tensegrity, and mechanotransduction. *Gravit Space Biol Bull*. 1997;2:49–55.
81. Chaterjee S, Cahttopadhyay S, Hope-Ross M, Lip PL. Hypertension and the eye: changing perspectives. *J Hum Hypertens*. 2002;16:667–675.
82. Gui D, Li Y, Chen X. Alterations of TMEM16a allostery in human retinal microarterioles in long-standing hypertension. *IUBMB Life*. 2015;67:348–354.
83. Wang B, Li C, Huai R, Qu Z. Overexpression of ANO1/TMEM16A, an arterial Ca2+–activated Cl- channel, contributes to spontaneous hypertension. *J Mol Cell Cardiol*. 2015;82:22–32.
84. Huang PL, Huang Z, Mashimo H, et al. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature*. 1995;377:239–242.
85. Bandopadhyay R, Orte C, Lawrenson JG, Reid AR, De Silva S, Allt G. Contractile proteins in pericytes at the blood-brain and blood-retinal barriers. *J Neurocytol*. 2001;30:35–44.
86. Alarcon-Martinez L, Yilmaz-Ozcan S, Yemisci M, et al. Capillary pericytes express α-smooth muscle actin, which requires prevention of filamentous-actin depolymerization for detection. *Neuroscience*. 2018;7:e34861.
87. Lee JH, Park HS, Shin JM, Chun MH, Oh SJ. Nestin expressin progenitor cells during establishment of the neural retina and its vasculature. *Anat Cell Biol*. 2012;45:38–46.
88. Yang B, Zhou X-C, Lan C. Impact of the alterations in the interstitial cells of Cajal on intestinal motility in post-infection irritable bowel syndrome. *Mol Me Reports*. 2014;11:2735–2740.
89. Worth NF, Rolfe BE, Song J, Campbell GR. Vascular smooth muscle cell phenotypic modulation in culture is associated with reorganization of contractile and cytoskeletal proteins. *Cell Motil Cytoskeleton*. 2001;49:130–141.
90. Mei F, Han J, Huang Y, Jiang Z-Y, Xiong C-J, Zhou D-S. Plasticity of interstitial cells of Cajal: a study in the small intestine of adult guinea pigs. *Anat Rec*. 2009;292:985–993.

Investigative Ophthalmology & Visual Science