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R-Ras deficiency in pericytes causes frequent microphthalmia and perturbs retinal vascular development

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Abstract

Purpose.—The retinal vasculature is heavily invested by pericytes. Small GTPase R-Ras is highly expressed in endothelial cells and pericytes suggesting importance of this Ras homolog for the regulation of blood vessel wall. We investigated the specific contribution of pericyte-expressed R-Ras to the development of the retinal vasculature.

Methods.—The effect of R-Ras deficiency in pericytes was analyzed in pericyte-targeted conditional *Rras* knockout mice at birth and during the capillary plexus formation in the neonatal retina.

Results.—The offspring of these mice frequently exhibited unilateral microphthalmia. Analyses of the developing retinal vasculature in the eyes without microphthalmia revealed excessive endothelial cell proliferation, sprouting, and branching of the capillary plexus in these animals. These vessels were structurally defective with diminished pericyte coverage and basement membrane formation. Furthermore, these vessels showed reduced VE-cadherin staining and significantly elevated plasma leakage indicating the breakdown of blood-retinal barrier. This defect was associated with considerable macrophage infiltration in the retina.

Conclusions.—The normal retinal vascular development is dependent on R-Ras expression in pericytes, and the absence of it leads to un-attenuated angiogenesis and significantly weakens the blood-retinal barrier. Our findings underscore the importance of R-Ras for pericyte function during the normal eye development.

Keywords

retina; angiogenesis; vascular permeability; RRas; pericyte

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Author contributions

Jose L Herrera (JLH) designed and performed all experiments, data acquisition and statistical analysis. JLH and Masanobu Komatsu (MK) wrote the manuscript.

Statement of Ethics

The Institutional Animal Care and Use Committee, at Johns Hopkins University-All Children's Hospital, St. Petersburg, Florida, approved all animal procedures.

Conflict of interest statement

The authors have no conflicts of interest to declare.

INTRODUCTION

The crosstalk between endothelial cells (ECs) and pericytes is crucial to the maturation and stability of newly forming blood vessels [1]. Abnormal interactions between the two cell types are associated with a myriad of pathological conditions including the conditions in the eye such as diabetic retinopathy, macular degeneration, and ischemic retinopathy [2–5]. R-Ras is a small GTPase of the Ras family closely related to the Ras proteins, such as K-Ras and H-Ras [6]. These proteins share an identical sequence of the effector binding region [7] and 55% sequence homology overall [6]. However, unlike K-Ras and H-Ras, R-Ras has little or no transforming activity in fibroblasts and does not activate the Raf-Erk pathway [8, 9]. Oncogenic activation mutations of R-Ras have not been reported in solid cancer whereas such mutations are frequent in K-Ras and H-Ras. An important function of R-Ras is to activate integrins and enhance cell adhesion to the extracellular matrix [10]. Interestingly, H-Ras exerts the opposite effect: H-Ras inhibits integrin adhesion via Raf activation [11]. In cultured neurons, integrin activation by R-Ras is necessary for neurite outgrowth [12], and the inhibition of R-Ras by the GAP activity of plexin-B1 leads to semaphorin-induced growth cone collapse [13]. However, genetic studies in mice showed that developmental functions of plexins are not mediated via R-Ras inactivation. R-Ras can also activate PI3 kinase and support cell survival via Akt activation [9]. R-Ras is a unique Ras homolog to have a putative Src homology-3 (SH3) domain-binding proline motif, which is necessary for the integrin-activating function [14–16]. R-Ras is also unique among Ras proteins in that it can be tyrosine-phosphorylated at Tyr 66 residue near the effector-binding region by Eph receptors and Src [14–16]. This phosphorylation inhibits R-Ras functions [14–16].

R-Ras is highly expressed in ECs, pericytes, and vascular smooth muscle cells of different types of blood vessels in most adult tissues, i.e. microvessels, capillaries, arteries, and veins [17]. R-Ras is expressed in these vascular cells at substantially higher levels than in other cell types including neurons, macrophages, and fibroblasts suggesting a specialized role of this Ras homolog in the maintenance and function of blood vessels. We have previously shown that EC-specific upregulation of R-Ras normalizes pathologically regenerating vasculature and improves vessel structure and blood perfusion through enhanced pericyte-EC interaction and VE-cadherin stabilization, which leads to strong endothelial barrier function [18]. We also showed that R-Ras in ECs promotes the lumenization (tubulogenesis) of vessel sprouts during angiogenesis via Akt-dependent microtubule stabilization [19]. In addition, a recent study demonstrated a role of R-Ras as a key regulator of pathological retinal angiogenesis and vascular permeability in oxygen-induced retinopathy [4].

The importance of R-Ras in ECs is well established by a number of studies [17–23]. However, the gain-of-function of R-Ras in pericytes alone can also significantly contribute to the vessel morphogenesis and maturation process independently from endothelial R-Ras signaling [18]. This suggests a potential bi-directional cross-talk and interdependence between the two vascular cell types where the defects in R-Ras pathways in one cell type have obligated consequences for the other. In the present study, we analyzed the specific role and contribution of pericyte-expressed R-Ras to the retinal vascular development.

METHODS

Mice

The Institutional Animal Care and Use Committee, at Johns Hopkins University-All Children's Hospital, St. Petersburg, Florida, approved experiments with mice. Cre/loxP recombination was used to generate pericyte-conditional *Rras* knockout (PC-cKO) mice. Litters were produced by crossing *Rras*$^{fl/fl}$ female mice (generated by Ingenious targeting laboratory, NY) with platelet-derived growth factor receptor-β (PDGFRβ) Cre male mice (provided by Ralf H Adams, Max Planck Institute for Molecular Biomedicine, University of Münster, Germany), which expresses Cre recombinase under the control of a fragment of the *Pdgfrβ* gene promoter/enhancer [24]. The offspring mice with the *pdgfrb*-Cre^{+}; *Rras*$^{fl/fl}$ genotype are pericyte-conditional KO (PC-cKO), and mice with other genotypes from the same litter (both Cre^{+} and Cre^{-} offspring) were used as control. The PCR primers used for mouse genotyping are listed in Supplementary Methods.

Blood-retinal barrier permeability

Retinal vascular permeability was quantified by Miles assay in retinas [25]. Mice were injected intraperitoneally with 2% Evans Blue (EB, 150μL/20g; E2129 Sigma-Aldrich, MO) and, after 17h of circulation, mice were euthanized, the blood was collected from the left ventricle, and the eyes were enucleated. The retinas were dissected from the eyeballs and weighed. EB dye conjugated to serum albumin in retinas was extracted by solubilization in N, N-dimethylformamide (200μL per retina; Acros organics, Belgium) at 78°C overnight. Retinas were then centrifuged at 4°C at 17,000 *g* for 45 minutes and the supernatants were collected. EB dye in the supernatant was detected spectrophotometrically by absorbance at 620nm (blue signal) and 740nm (background subtracted) and measured in comparison with a standard curve. Blood samples were treated similarly but without solubilization and with centrifugation for 15 minutes at 3,550 *g* at 25°C and at a 1:1000 dilution prior to spectrometric reading. Measuring intraretinal dye accumulation, using the following equation, assessed retinal vascular leakage: [retinal EB concentration (mg/mL) / retinal weight (mg)]/[blood EB concentration (mg/mL) × circulation time (h)].

Gross eye morphology and histology

Control and PC-cKO newborns were weighed and euthanized by decapitation and the heads were fixed in 10% buffered formalin. The Histology Core at Sanford Burnham Prebys (SBP) Medical Discovery Institute at La Jolla (California) processed all samples. Briefly, fixed materials were embedded in paraffin and serially sectioned (coronal) at 5μm thick. Sections were stained with Harris hematoxylin (#3801560, Leica, Germany) and eosin (#3801600, Leica) or 2% toluidine-blue (#93-31-19, Sigma Aldrich) and evaluated by light microscopy (Nikon A1R, Nikon Instruments, Melville, NY). Digital pictures were acquired (×10 lens), imported and merged into Adobe Photoshop CS (Adobe Systems, CA). For gross anatomy studies and characterization of the vasculature in different tissues from adult mice by immunofluorescence, 8-week old control and PC-cKO mice were euthanized, and the tissues of interest were dissected, washed in ice-cold PBS, embedded in OCT compound and froze in liquid nitrogen. 10 μm-frozen sections were obtained using a cryostat and submitted to either immunostaining or hematoxylin-eosin staining.

Retina whole-mount staining

The postnatal day 5 (P5) eyes were enucleated, washed in cold PBS and fixed in 4% paraformaldehyde 1h at room temperature (RT) in gentle shaking, then transferred to cold PBS in a petri dish where retinas were dissected under a stereoscope microscope (Leica M165 FC). Dissected retinas were fixed for another hour in 4% PFA, washed three times for 10 min in cold PBS and incubated in blocking buffer (20% normal goat serum (NGS, 005-000-121, Jackson ImmunoResearch, PA), 1% BSA and 0.5% Triton X-100 in PBS overnight at 4°C with gentle shaking. If an anti-mouse antibody were to be used, 1 hour blocking at room temperature with mouse IgG Blocking Reagent was performed (MKB-2213, Vector Laboratories, CA). After that, three 20-min washes were performed with fresh Pblec solution (1mM $MgCl_2$, 1mM $CaCl_2$, 0.1mM $MnCl_2$, 1% Triton X-100) at RT to equilibrate retinas. Retinas were then incubated in Pblec solution with desired primary antibodies overnight at 4°C in gentle shaking, including Isolectin B4 (IB4) FITC-conjugated (L2895, Sigma Aldrich) or DyLight® 649 GSL IB4 (DL1208, Vector) to label the retinal endothelium; rabbit anti-NG2 Chondroitin Sulfate Proteoglycan antibody (AB5320, Millipore), mouse anti-PDGFRβ antibody (AF1042 R&D Systems, MN) or rabbit anti-desmin antibody (#5332, Cell Signaling Technology, MA) to label pericytes; anti-Rras monoclonal antibody (M01, clone 2E12, Abnova, Taiwan) to characterize vascular cells, rabbit anti-Ki67 antibody (GTX16667, GeneTex, CA) to label nuclei in proliferation; rabbit anti-ERG monoclonal antibody coupled with Alexa Fluor 647(ab196149) to label endothelial cell nuclei; rabbit anti-Cleaved caspase-3 antibody coupled with Alexa Fluor 488 (#9669, Cell Signaling) to assess apoptosis; rat anti-mouse CD144 (555289, BD Pharmingen™, CA) to label VE-Cadherin in endothelial cells; rabbit anti-mouse collagen IV (2150-1470, Bio-Rad, CA) to label basement membrane; rat monoclonal anti-F4/80 antibody (ab6640, abcam) to label macrophages; goat anti-human fibrinogen antibody (GaHu/Fbg/7S, Nordic MUbio, Netherlands) to assess leakiness. For secondary antibody, retinas were washed in PBS for 2h and then incubated with Alexa Fluor 488, 555 and/or 647 goat anti-rabbit/mouse/rat antibodies (Life Technologies, CA) in PBS containing and 10%NGS at RT for 2 h, and then washed in PBS every hour for 8h. Finally, retinas were mounted on the slide. Phenotypic analysis, imaging and quantitation of filopodia and sprouts were performed as described previously [26]. Briefly, for phenotypic analysis, low-magnification images of IB4-labeled control and PC-cKO retinas were taken using a fluorescence microscope (4× or 10× lens, Eclipse 90i, Nikon, Tokyo, Japan). Pericyte or collagen IV coverage of retinal capillary vessels was determined from 3-D reconstructed confocal images as % fraction of IB_4^+ area that is interacting with $NG2^+$, PDGFRβ, desmin or collagen IV^+ area in the total IB_4^+ area. EC proliferation was assessed by Ki67 immunostaining within IB_4^+ area, and also by Ki67 intensity within ERG^+ area. For a detailed analysis of the central vascular plexus and the angiogenic front (branching points, macrophage number, filopodia, sprout quantitation, vein width and capillary diameter), high-resolution confocal images (60× lenses; 2,048 × 2,048 pixels; Nikon A1R, Nikon Instruments, Melville, NY) with thin Z-sectioning (0.5 μm) were captured and analyzed with Nikon NIS-Elements AR Analysis 4.40 software.

Statistics

Data is presented as Mean ± S.E.M in vertical scatter plot or vertical bar graph. Each variable was compared between groups and the *P*-value was defined using Student's *t*-test with Welch's correction when applicable. For comparison of the mice body weight over time, 2-Way ANOVA was used. Differences were considered to be significant for *P*-value ≤ 0.05. For correlation, Pearson coefficient (r) was calculated and the *P*-value was obtained to assess the significance of the correlation. Linear regression analysis (R^2) was additionally performed. All analysis tests and graphs were done using GraphPad Prism version 5.0b for Mac (GraphPad Software, La Jolla, CA).

RESULTS

Pericyte-targeted ablation of *Rras* causes frequent microphthalmia

We generated conditional knockout (KO) mice with pericyte-targeted *Rras* gene deletion in order to investigate the role of pericyte-expressed R-Ras in neonatal retinal angiogenesis. These mice (PC-cKO) were created in the C57BL/6 background by crossing *pdgfrb*-Cre [14] and $Rras^{f/f}$ lines (Supplementary Fig. 1A). In the developing retina, PDGFRβ is selectively expressed in pericytes of retinal vasculature [27–29], allowing pericyte-specific deletion of *Rras*. The loss of R-Ras in pericytes in these mice was confirmed by immunofluorescence of various tissue sections from adult mice (Supplementary Fig. 2). The absence of R-Ras expression in pericytes was farther demonstrated by western blot of brain microvascular pericytes isolated from the PC-cKO mice (Fig. 1A). These R-Ras-deficient pericytes exhibited abnormal morphology with spindle shape and thin membrane processes, and unlike pericytes from the control mouse brain, they grew without close association with each other (Fig. 1A).

The PC-cKO mice are viable, born in the expected Mendelian ratio, and fertile. No significant difference in the bodyweight was observed at postnatal day 5 (P5) (Fig. 1B). Likewise, the adult bodyweight of PC-cKO mice was not significantly different from that of the control mice. Furthermore, there was no difference in the blood glucose level between the two groups (Fig. 1C). Unexpectedly, however, a significant fraction of the PC-cKO offspring presented microphthalmia in one eye (Fig. 1D). This gross anomaly was observed at birth (Fig. 1E a, b) and persisted in the adulthood, suggesting the importance of pericyte-expressed R-Ras for the eye development. Gross anatomy studies of newborn animals revealed reduced ocular volume with a small lens in the PC-cKO eye with microphthalmia (Fig 1E e–h). The contralateral eyes of these mice appeared normal in size and anatomical structure (Fig. 1E c, d). Despite the frequent cases of microphthalmia, other organs of these animals show normal size, and no obvious sign of abnormality was found by histological examination (Supplementary Fig. 3).

R-Ras deficiency in pericytes retards capillary plexus formation in developing retina

The microphthalmia in PC-cKO mice suggests defective vascular development in the eye, impeding the eye development. The retinal vasculature of normal mice develops postnatally in a tightly regulated spatiotemporal pattern [30]. Superficial vascular plexus develops by extending radially from the optical nerve head during the first 8 days after birth. The

vascular plexus is composed of alternatingly positioned arteries and veins, which are connected by capillary plexus for the continuous blood flow. The vascular plexus outgrows reaching the distal edge of the retina at around P8 [31]. We examined whether the R-Ras deficiency in pericytes has a significant impact on the normal pattern of the retinal vascular development in the postnatal retina at P5, when the formation of the superficial vascular plexus is still incomplete. Since the eyes with microphthalmia were too small and fragile to perform whole-mount staining of retina, the contralateral eyes and the eyes of PC-cKO pups without microphthalmia were used for this study. The size of retina appeared comparable between the PC-cKO and the control eyes. The capillary plexus development was significantly delayed in all of the PC-cKO retinas analyzed. At P5, the angiogenic front of capillary plexus was twice as distant from the edge of the retina compared with the control (Control: 319.3±27.25 μm, PC-cKO: 606.9±32.13 μm; Fig. 2B). When measured from the optic nerve, the distance to the angiogenic front was shorter in the PC-cKO retinas compared with the control group (Control: 1308±21.08 μm, 14 retinas; PC-cKO: 1066±20.66 μm, 9 retinas; Fig. 2B). Sporadically, we also observed abnormal endothelial growth within the capillary plexus of the PC-cKO retina (Fig. 2C, D, Supplementary video 1, 2). These observations demonstrate that R-Ras in pericytes is required for the normal development of retinal vasculature, and its absence leads to aberrant vascular morphogenesis.

Lack of R-Ras in pericytes reduces pericyte coverage and increases capillary branching and sprouting in developing retina

The ratio of the number of pericytes to ECs is the highest (1:1) in the retinal and brain vasculature, highlighting the importance of pericytes for the central nervous system (CNS) vasculature [2]. We found a marked decrease in $NG2^+$ pericyte coverage from 88% of the vessel surface in the normal capillary plexus to 46% in the PC-cKO capillary plexus (Fig. 3A, B; Supplementary Fig. 4A). The loss of $NG2^+$ pericytes was prominent especially at the growing front of the plexus. Immunostaining of alternative pericyte markers such as PDGFRβ or desmin showed a similar result (Fig. 3A, C, D; Supplementary Fig. 4A, B). In addition, we found in the PC-cKO retinas a 75% decrease of N-cadherin, a cadherin protein that mediates the direct interaction of pericytes with ECs at the adherens junction between the two cell types (Fig. 3E). This finding is consistent with the significantly diminished pericyte coverage of the vessels (Fig. 3A–D, Supplementary Fig. 4A, B). To examine whether the decrease in pericyte coverage is due to an increase in apoptotic pericytes, we performed immunostaining of cleaved caspase-3, a critical executioner of apoptosis. In this analysis, apoptotic pericyte strongly stained for cleaved caspase-3 were readily found in different regions of the PC-cKO capillary plexus, sometimes unassociated from the vessels. In contrast, no apoptotic pericytes were found in the control retinas (Fig. 3F).

Pericytes play an important role in suppressing excessive EC proliferation [32]. We next investigated angiogenic parameters such as branching, sprouting, and filopodia formation in the developing capillary plexus. Despite the fact that the radial outgrowth of PC-cKO capillary plexus was delayed (Fig. 2A, B), these vessels showed a 48% increase in the number of branching points compared with the control mice (Fig. 4A). An inverse correlation was found between the extent of vessel branching and pericyte coverage (Pearson's $r = -0.3624$, $P = 0.0123$, $R^2 = 0.1313$), in which PC-cKO and control vessels were

found in separate clusters suggesting two different vessel phenotypes (Fig. 4B). Furthermore, analyses of the angiogenic front demonstrated increased number of EC sprouting and filopodia formation in the PC-cKO retina (30% and 53%, respectively) indicating excessive angiogenic activity (Fig. 4C, D). Consistently with these observations, there was a significant increase of Ki-67$^+$ ECs indicating enhanced EC proliferation in the PC-cKO retina (Fig. 4E, F). This result was confirmed by increased Ki-67 intensity in ERG$^+$ nuclei (a marker for EC nuclei) in the retinal vessels of the PC-cKO mice (Fig. 4E, F). These observations demonstrated the importance of pericyte-expressed R-Ras for attenuating EC proliferation, sprouting, and branching. In addition, pericytes isolated from brain microvasculature of PC-cKO mice exhibited 3-fold increased proliferation in culture compared with the control pericytes (Fig. 4G). Thus, pericyte-expressed R-Ras also attenuates proliferation of pericytes. No differences were observed in the diameters of capillary vessels or major vessels between PC-cKO and control mouse retinas (Supplementary Fig. 5A, B). Taken together, our results underscore the anti-proliferative effects of R-Ras on vascular cells.

Loss of R-Ras in pericytes impairs collagen IV basement membrane formation and disrupts endothelial integrity

Pericytes contribute to the formation of the basement membrane [33]. We analyzed collagen IV, a main constituent of capillary vessel basement membrane, by immunofluorescence staining (Fig. 5A). In the PC-cKO retinas, the vessel surface area covered by collagen IV matrix was decreased by 50%, and the amount of collagen IV deposition (deduced from collagen IV staining intensity per vessel area) was decreased by 40% compared with the control retinas (Fig. 5B). No differences were found in the expression of integrin α1, a collagen-binding integrin that mediates pericyte binding to collagen IV of the basement membrane [34] (Fig. 5C).

The proper pericyte-EC interaction is required for maintaining the endothelial barrier integrity [35]. The accumulation of VE-cadherin at the EC adherens junctions is essential for the endothelial barrier function [36]. Immunostaining detection of VE-cadherin requires clustering of VE-cadherin at EC-EC junction since the antibodies bound to disperse VE-cadherin does not produce fluorescence signal strong enough to be readily detected [18]. In our analysis, we found a 11-fold decrease in the VE-cadherin intensity in the capillary plexus of PC-cKO retina (Fig. 5A, D; Supplementary Fig. 4C). Western blot analyses of whole retina protein extract revealed a 50% decrease in the VE-cadherin protein level while there was no difference in the VE-cadherin mRNA level between the PC-cKO and control retinas (Fig. 5E). These results suggest that the R-Ras deficiency in pericytes results in a significant reduction of VE-cadherin protein expression or clustering leading to VE-cadherin degradation, which in turn, results in the disruption of endothelial integrity.

To directly evaluate the role of pericyte-expressed R-Ras in the endothelial integrity, we conducted Miles assay and analyzed the effect on the blood-retinal barrier. The blood-retinal barrier permeability was quantified by measuring the extravasation of Evans Blue (EB) dye injected intraperitoneally to 6-week old mice. The PC-cKO retina had a 92% increase in EB leakage compared to the control retina (Fig 5F). In addition, immunostaining of P5 retinas

showed a greatly increased accumulation of the plasma protein, fibrinogen, outside the capillary vessels of the PC-cKO retina (Fig. 5G), thus indicating increased vessel leakiness. These data demonstrate the importance of pericyte-expressed R-Ras for the blood-retinal barrier integrity in the developing retina.

Macrophages infiltrate into *Rras* PC-cKO retina

Increased vessel permeability allows extravasation of inflammatory cells from the circulation [37, 38]. There were numerous round $IB4^+$ cells outside of capillary vessels in the PC-cKO retina. IB4 also stains macrophages. We, therefore, stained the retina with another macrophage marker F4/80 [39, 40] and confirmed the identity of these infiltrating cells as macrophages (Fig. 6A). The infiltrating macrophages were frequently present in a large number in the PC-cKO retinas (9.0±2.41 macrophages per observation field) while they were rarely found in the control retinas (0.21±0.10) (Fig. 6A–C). This suggests that the blood leakage from the leaky capillary plexus promoted extravasation of circulating monocytes thereby causing inflammation in the PC-cKO retina. Consistently with this observation, a western blot analysis of pooled retinas demonstrated increased expression of VCAM-1, a cell adhesion molecule upregulated in the inflamed endothelium [41] (Fig. 6D). We also detected $F4/80^+$ cells that are not stained by IB4. These are thought to be activated microglia as they are known to be $F4/80^+$ cells [39,40]. These results suggest that the blood-retinal barrier breakdown by the R-Ras deficiency in pericytes causes inflammation of the retina and activation of microglia.

DISCUSSION

In this study, we set out to investigate the precise contribution of pericyte-expressed R-Ras to the neonatal retinal vascular development using pericyte-targeted conditional R-Ras KO mice. Unexpectedly, we found frequent microphthalmia in the offspring of these animals. Further analyses of the neonatal animals showed that normal development of the retinal vasculature requires expression of R-Ras in pericytes. The loss of R-Ras in pericytes caused un-attenuated EC proliferation, sprouting, and branching in the capillary plexus of neonatal retina. Pericyte coverage and basement membrane formation of these vessels were markedly reduced, suggesting that the nascent blood vessels failed to undergo the structural and functional maturation in the absence of R-Ras in pericytes. Moreover, the R-Ras deficiency in pericytes resulted in significant disruption of VE-cadherin in ECs and breakdown of blood-retinal barrier, highlighting the importance of pericyte-expressed R-Ras for the endothelial integrity (Fig. 7).

Plasma leakage induces angiogenic responses in ECs by creating provisional matrix with vitronectin and other leaked plasma proteins [41]. Therefore, the observed EC proliferation in the PC-cKO retina may be explained by increased vessel permeability. In addition, the activated microglia may continue to induce angiogenesis by secreting inflammatory mediators, which in turn stimulates neurons to produce VEGF-A, as described for retinal neovascular diseases [42,43]. In any case, the observations that pericyte-expressed R-Ras controls EC proliferation and endothelial barrier function suggest the existence of one or more mechanisms of intercellular cross-talk between pericytes and ECs mediated by

pericyte-expressed R-Ras. One plausible mechanism is the activation of integrins by R-Ras, which enables the close interaction of the two cell types through the cell adhesion to the shared extracellular matrix. Pericytes share the extracellular matrix with ECs within the basement membrane of the vessels. In normal vasculature, both cell types are stably adhered to the extracellular matrix proteins through integrins. R-Ras activates integrins thereby strengthening cell adhesion to the extracellular matrix [20]. We previously showed that R-Ras reinforces firm adhesion of pericytes to the basement membrane proteins, collagen IV and laminin [18]. The enhanced pericyte adhesion to the extracellular matrix by R-Ras stabilizes pericyte coverage of nascent blood vessels and facilitates proper interaction with ECs [18]. Pericyte association with ECs limits excessive EC proliferation in response to angiogenic stimuli [36]. Therefore, it is likely that pericyte-expressed R-Ras controls ECs by facilitating pericyte-EC interaction via enhanced integrin adhesion.

Other potential mechanism of the cross-talk may involve direct physical contact of pericytes and ECs through adherens junctions between the two cell types. R-Ras expressed in ECs is known to stabilize VE-cadherin clustering at adherens junctions between adjacent ECs thereby enhancing the endothelial barrier function [18]. N-cadherin is the cadherin protein responsible for the adherens junction formation between pericytes and ECs. The effect of R-Ras on N-cadherin clustering, stability, or expression is currently unknown. However, it is noteworthy that N-cadherin protein level was markedly reduced in the PC-cKO mouse retina. Likewise, cultured pericytes isolated from the brain microvasculature of these mice exhibited a decreased level of N-cadherin expression. Other potential cross-talk mechanisms involving paracrine or juxtacrine signaling cannot be ruled out for mediating the effect of R-Ras from pericytes to ECs.

It is puzzling that R-Ras deficiency enhances proliferation of pericytes in culture, yet pericyte coverage of the PC-cKO retinal vasculature is substantially reduced according to the analyses using three different pericyte markers (NG2, PDGFR, and desmin). Pericytes may dissociate from vessels due to the weakened integrin adhesion to the basement membrane. Indeed, we observed the presence of apoptotic pericytes that were unassociated with the vessels. It may be that the dissociated R-Ras-deficient pericytes are unstable without interacting with ECs and undergo apoptosis. The PI3K-mediated activation of Akt is an important downstream pathway of R-Ras [19] and R-Ras promotes survival of ECs [17]. A similar role of R-Ras is expected for the survival of pericytes. Alternatively, these cells may differentiate into other cell types so that they cannot be visualized by the pericyte markers. Pericytes are phenotypically plastic and capable of transdifferentiating to other cell types [45]. Pro-inflammatory cytokines might contribute to pericyte apoptosis as well.

It is unknown at present why a considerable fraction of PC-cKO mice are born with microphthalmia. We suspect that it is also caused by the vascular defect in the eyes of these animals. Previous studies suggested that perturbations in hyaloid vessel formation result in microphthalmia [45]. Hyaloid vessels form in the fetal eyes to provide blood flow to the developing lens, but they regress and disappear after embryonic eye development [46]. Due to the defective pericytes, hyaloid vessels of PC-cKO mice may be too unstable to support adequate blood flow to the developing lens. We did not observe manifestation of microphthalmia in all eyes of PC-cKO mice. This is likely due to varying degrees of the

severity of vascular dysfunction in the embryonic eyes caused by the defective pericytes. Thus, a possible explanation is that the severity of vascular dysfunction is above a certain threshold to develop a severe phenotype (i.e. microphthalmia) in some PC-cKO mice. Unlike PC-cKO mice, global R-Ras KO mice do not exhibit microphthalmia [17]. *Rras* is absent from the very beginning of the embryogenesis in the global KO mice unlike in the conditional KO mice. It appears that other genes compensate the loss of R-Ras in the global KO. This may involve other small GTPases and cytoskeletal machineries that regulate integrin- or cadherin-mediated cell adhesion or PI3 kinase pathways that are normally controlled by R-Ras. In the conditional KO mice, the cell type-targeted, delayed *Rras* deletion may sensitize the developing vasculature to the effect of the genetic manipulation more severely. Microphthalmia in the PC-cKO animals warrants further investigation.

A number of ocular pathologies are characterized by deregulated neovascularization [46]. The global R-Ras KO mice exhibit exaggerated oxygen-induced retinopathy suggesting a potential protective role of R-Ras in human vascular disorders represented by ischemic retinopathies such as retinopathy of prematurity, proliferative diabetic retinopathy, and retinal vein occlusion [47–49]. Our findings with the conditional KO mice highlight the importance of pericytes for the prenatal development of the eye as well as for the postnatal development of the retina. We showed that R-Ras critically supports the role of pericytes in the normal eye development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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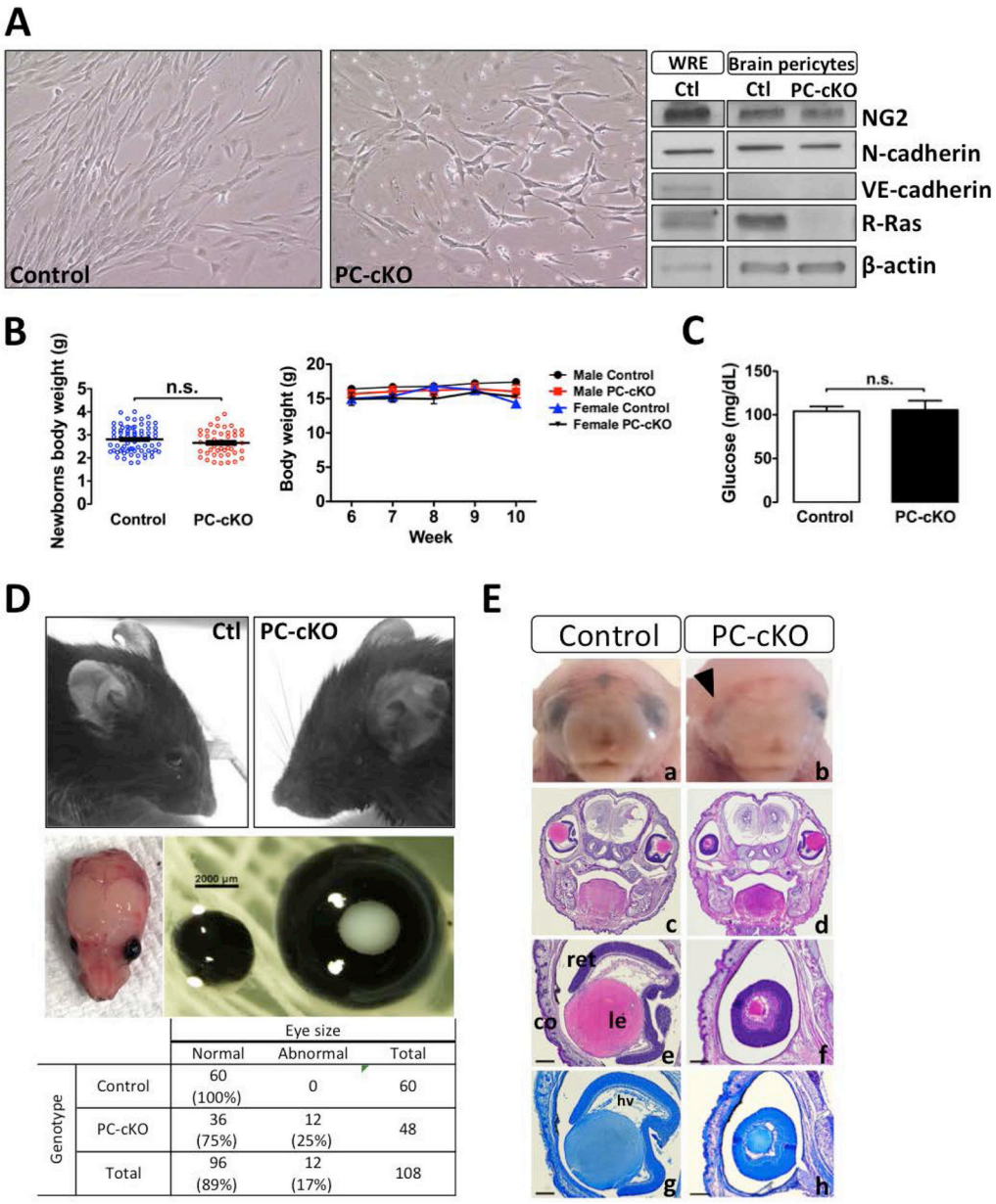

		Eye size		
		Normal	Abnormal	Total
Genotype	Control	60 (100%)	0	60
	PC-cKO	36 (75%)	12 (25%)	48
	Total	96 (89%)	12 (17%)	108

Fig. 1. *Rras* PC-cKO mice are frequently born with microphthalmia

(**A**) Cultured brain microvascular pericytes isolated from *Rras* PC-cKO mice exhibit altered morphology. Lysate of these cells was analyzed by western blot for the expression of NG2 (pericyte marker), VE-cadherin (EC marker), R-Ras, N-cadherin, and β-actin (loading control) to demonstrate the lack of R-Ras in pericytes. Pooled whole retina extract (WRE) from control mice was used as a control for antibodies. Eight retinas were pooled. (**B**) Bodyweight at P5 and 6–10 weeks old was compared between *Rras* PC-cKO and control mice. *n*=45 or 73 P5 mice were examined for *Rras* PC-cKO or control group. *n*=5 or 6 mice for 6–10 weeks old bodyweight. Results are shown as Mean ± S.E.M. No significant statistical difference was found (n.s.) (**C**) Blood glucose level analyzed at P5 (**D**) Twenty five percent of *Rras* PC-cKO mice exhibits microphthalmia in one eye at birth. Pictures of

microphthalmia and contralateral eyes of adult *Rras* PC-cKO mice are shown. (**E**) Gross anatomical features of PC-cKO mice. Microphthalmia is present at birth (a, b; arrowhead). H&E (c-f) and toluidine blue (g, h) staining of histological sections of a P0 PC-cKO mouse displays a microphthalmic eye with a small lens surrounded by retina (f, h). The contralateral eyes of the mice displaying microphthalmia did not show any sign of abnormal gross anatomy (d). Ret, retina; le, lens; co, cornea; hv, hyaloid vessels. Scale bar, 100 μm.

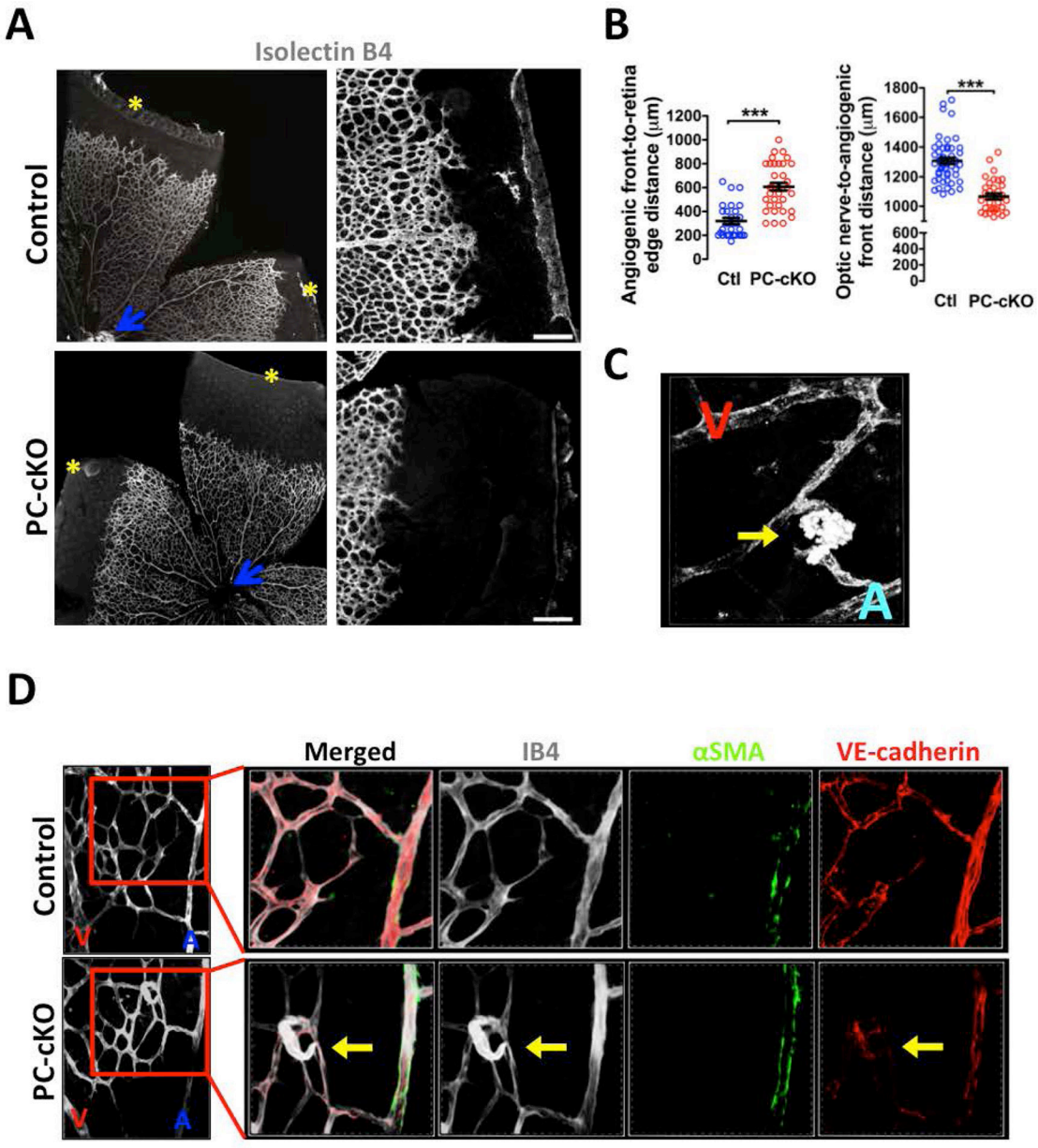

Fig. 2. Abnormal vascular development in *Rras* PC-cKO neonatal retina

(**A**) PC-cKO and control retinas at P5 were cut into four segments, flat-mounted, and stained with isolectin B4 (IB4) to visualize the endothelium of the retinal vasculature. * edge of the retina; blue arrow: central retina. Scale bar, 0.5 μm (**B**) Distance from the angiogenic front to the edge of the retina in all four segments of each retina (Control: 7 retinas, PC-cKO: 8 retinas; left graph) and distance from the optic nerve to the angiogenic front (Control: 14 retinas, PC-cKO: 9 retinas, right graph). Each dot represents the distance in one segment of retina. $P<0.0001$ (**C**) IB4 staining of capillary plexus shows abnormal endothelial growth near capillary branching in the PC-cKO retina (arrow). V, vein; A, artery (**D**) Abnormal endothelial growth in the PC-cKO capillary plexus co-immunostained with IB4 and VE-cadherin antibody (arrows).

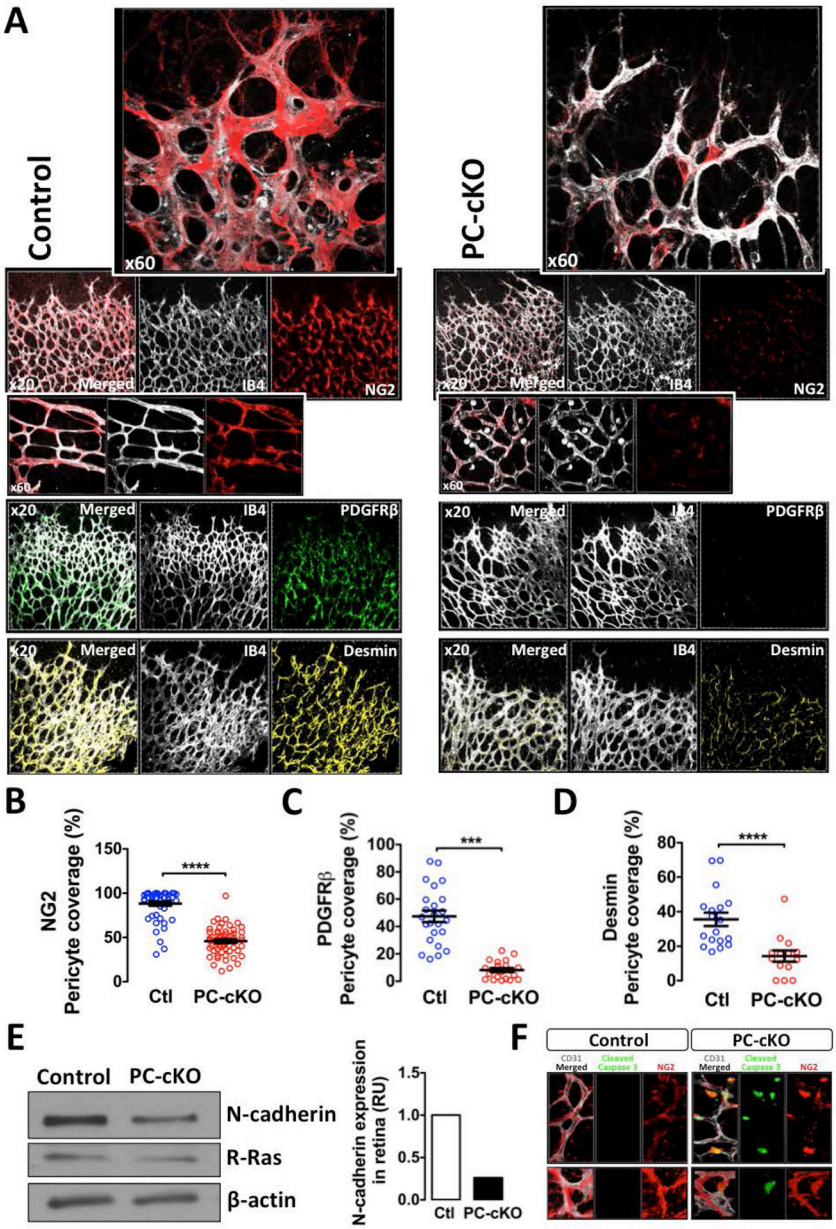

Fig. 3. Absence of R-Ras in pericytes impairs pericyte coverage of capillary plexus

(**A**) Retinas were harvested at P5, whole-mounted and double-stained with Alexa Fluor-conjugated IB4 (white) for EC staining and an antibody for pericyte marker NG2 (red), PDGFRβ (green) or desmin (yellow). 3-D images of the stained vascular plexus are shown (**B-D**) Intersecting areas between ECs ($IB4^+$) and $NG2^+$ (B), $PDGFR\beta^+$ (C) or $desmin^+$ (D) pericytes were quantified in 3-D images and presented as % pericyte coverage. For NG2, at least 52 pictures from 5 retinas were examined for each group. For PDGFRβ, at least 21 pictures from 3 retinas were examined. For desmin, at least 14 pictures from 3 retinas were examined. Results are shown as Mean values ± S.E.M. $P<0.0001$ (**E**) Western blot analysis of N-cadherin in whole retina extract. Twelve retinas were pooled for each group (**F**) Retinas (n=3) were triple-stained with Isolectin B4 (white), a pericyte marker NG2 (red) and an

antibody for apoptosis Cleaved Caspase-3 (green). Confocal 3-D images show the presence of apoptotic pericytes in the PC-cKO retinal vessels. A: Artery.

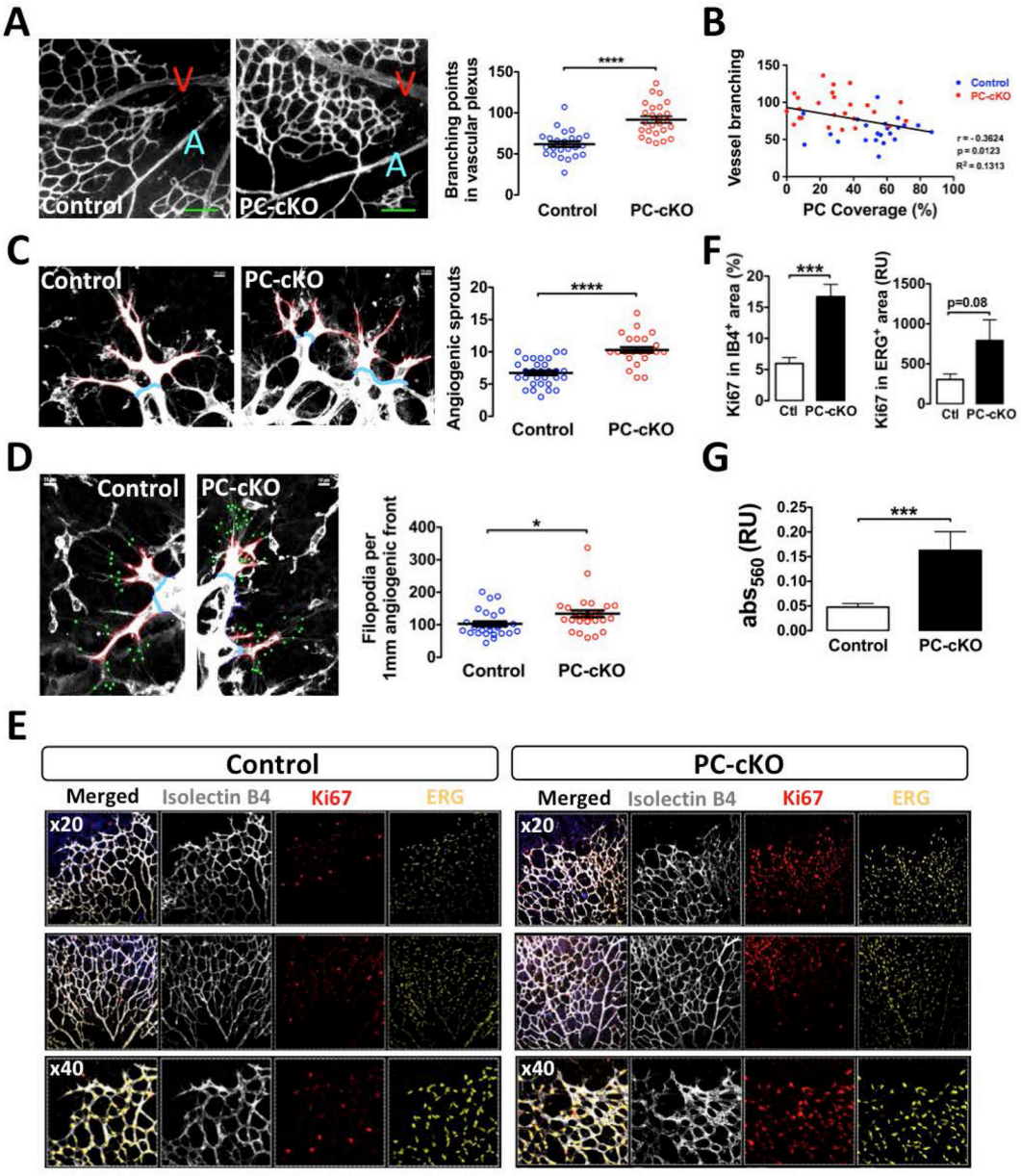

Fig. 4. R-Ras deficiency in pericytes leads to excessive angiogenic activities of ECs

(**A**) Analysis of vessel branching in the capillary plexus. Branching points were defined as junctions of capillary segments and counted in the observation field. Total 25 retina pictures from 4 mice were examined for each group. $P<0.0001$, V=vein, A=artery. Scale bar, 100 μm (**B**) Correlation analysis between pericyte coverage and vessel branching in the capillary plexus. An inverse correlation exists between the two parameters (Pearson's r = −0.3624, P= 0.0123, R^2= 0.1313) (**C**) Endothelial sprouts (red lines) were counted in the angiogenic front of the growing capillary plexus. Blue lines indicate the base of the sprouts. Total 30 retina pictures of 4 mice were examined for each group. $P<0.0001$, Scale bar, 10 μm (**D**) The number of filopodia (green dots) was counted per endothelial vessel length (red lines). Total 28 retina pictures of 4 mice were examined per group. $P=0.019$, Scale bar, 10 μm (**E-F**) EC

proliferation was assessed by anti-Ki-67 (red) and IB4 (white; left graph) or anti-ERG (yellow; right graph) double staining of P5 retina to identify Ki-67$^+$ cycling cells in the endothelium. Proliferation of ECs was assessed by the % fraction of Ki-67$^+$IB4$^+$ area in total IB4$^+$ area (left) or by the Ki-67 intensity within ERG$^+$ nuclei normalized for the total ERG$^+$ area in the image (right). Six mice were examined for each group. $P<0.0001$ (**G**) MTT assay to assess proliferation of cultured brain microvascular pericytes isolated from R-Ras PC-cKO or control mice. Mean ± S.D. $P<0.0001$

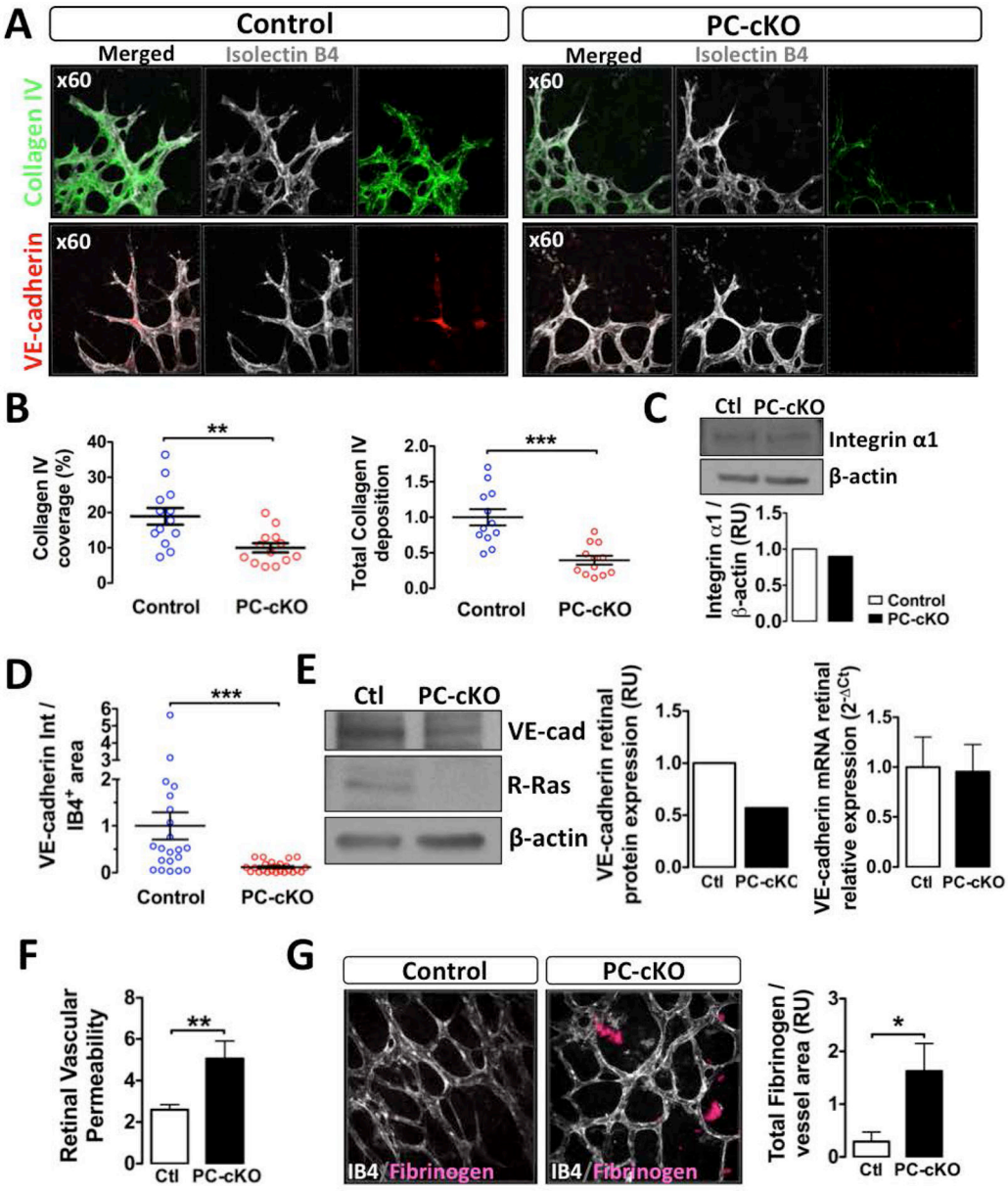

Fig. 5. Impaired basement membrane formation and disruption of endothelial barrier in *Rras* PC-cKO retinal vasculature

(**A**) Representative images of collagen IV (green) and VE-cadherin (red) immunofluorescence of the retina at the angiogenic front. IB4 staining (white) highlights the endothelium. 3-D images were reconstituted by confocal microscopy. (**B**) Quantification of immunostaining in the 3-D images. The vessel surface area covered by collagen IV matrix (coverage) and the amount of collagen IV deposition deduced from the staining intensity per vessel area (relative value compared with the control) are shown. Three to four 3-D pictures were analyzed for each retina. Four or five mice were examined for each group. ** P=0.0031, *** P<0.0001 (**C**) Integrin α1 protein expression level in brain-isolated pericytes analyzed by western blot (**D**) A similar 3-D analysis for VE-cadherin. ** P=0.0067 (**E**) VE-cadherin protein and mRNA expression levels in pooled whole retina extract were analyzed by

western blot and RT-qPCR. Eight to twelve retinas were pooled for each group. (**F**) Permeability of the retinal vasculature was quantified by Miles assay and presented with vascular permeability factor. Five to seven retinas were examined. P=0.0089 (**G**) Vascular permeability was also assessed by the plasma leakage determined by fibrinogen immunostaining of the whole-mounted retina. Data are presented as the total fluorescence intensity of fibrinogen (RU) normalized by the vessel area. Three retinas were examined for each group. P=0.0247

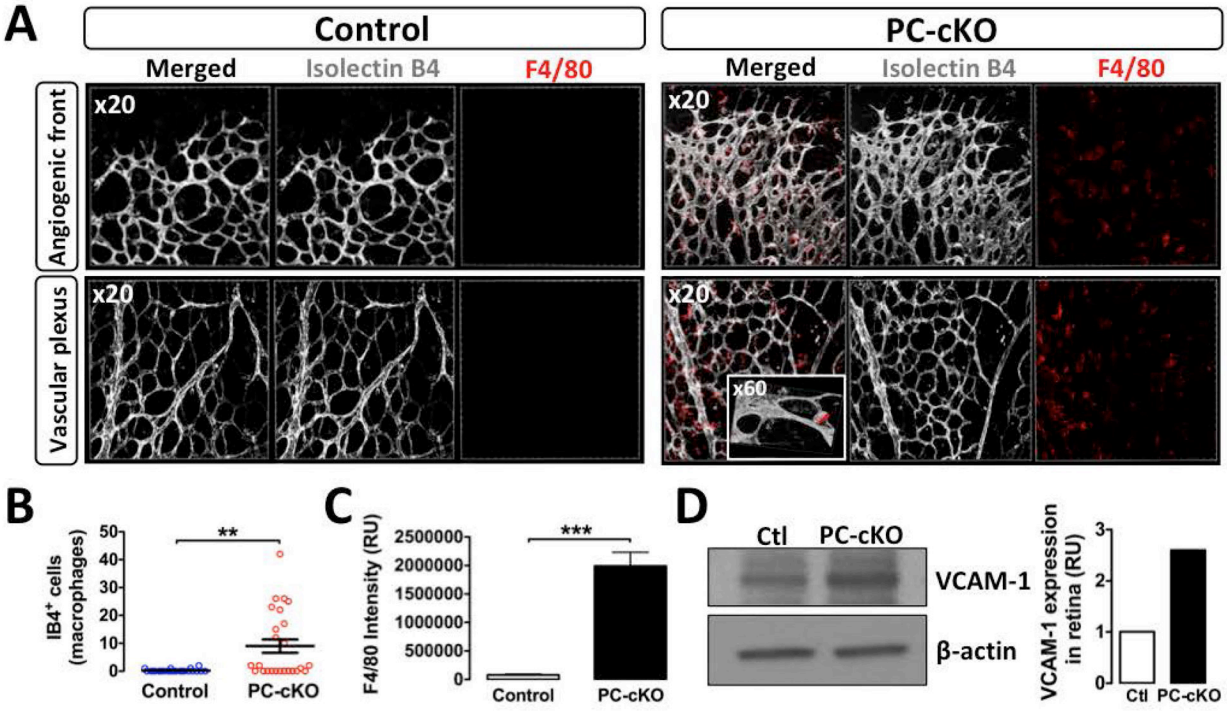

Fig. 6. Infiltrating macrophages accumulate in *Rras* PC-cKO retina

(**A**) Whole-mounted P5 retinas were immunostained for a macrophage marker F4/80, co-stained with IB4, and analyzed by confocal 3-D imaging. Inset, a macrophage doubly stained for F4/80^{+} and IB4^{+} shown in higher magnification (×60) (**B, C**) Quantification of IB4^{+} cell count outside the vessels (macrophages, B) and F4/80 immunofluorescence intensity (C) in the observation. Total of 25 retina pictures of 3 animals were examined for each group. ** P=0.0013, *** P<0.0001 (**D**) Western blot analysis of VCAM-1 in pooled whole retina protein extract. Eight or twelve retinas are pooled for the control or PC-cKO group, respectively.

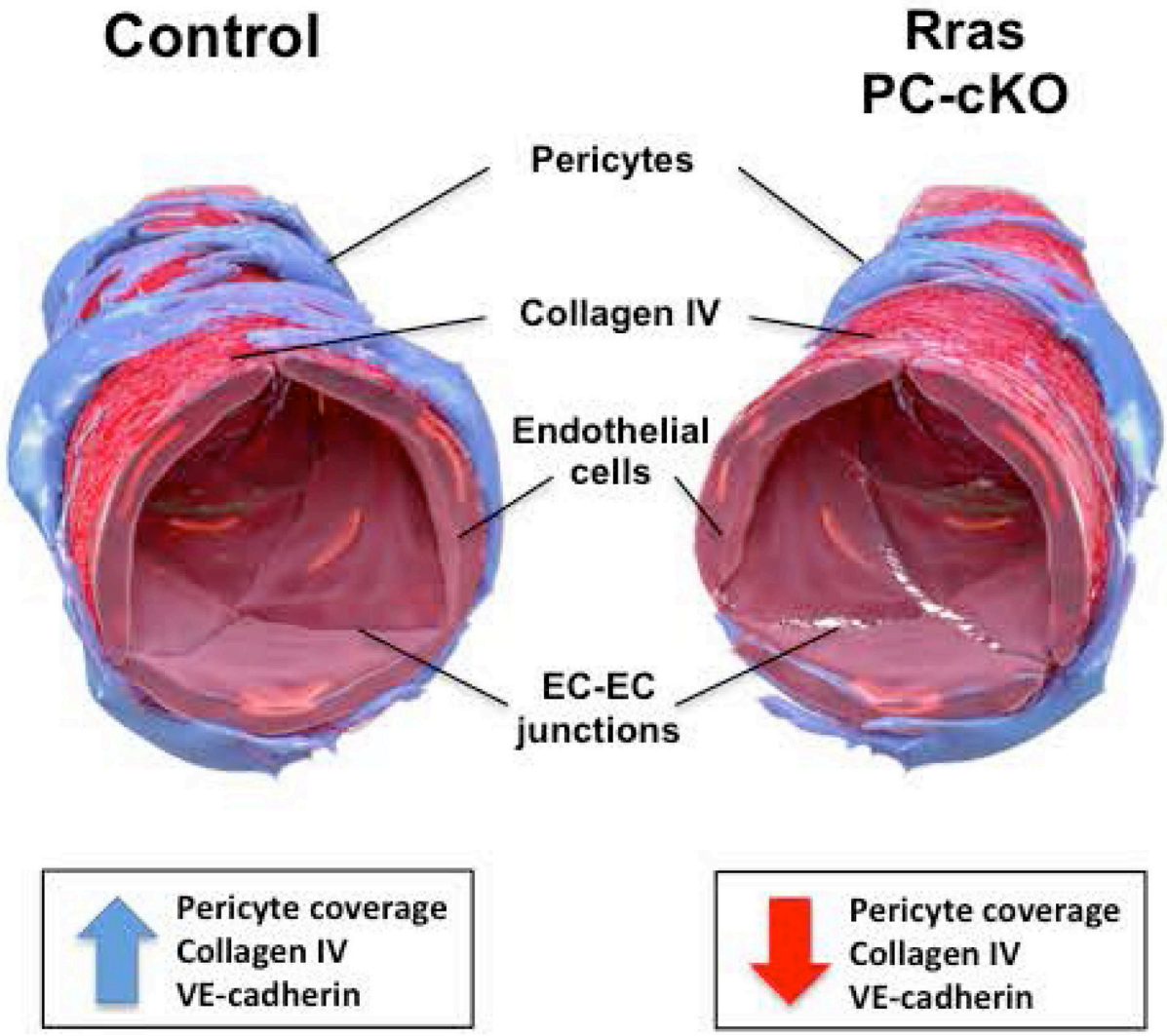

Fig. 7. Schematic diagram depicting the role of pericyte-expressed R-Ras in retinal capillary vessel stability.

R-Ras in pericytes controls endothelial sprouting, branching, and pericyte coverage, and stabilizes the integrity of endothelial lining by VE-cadherin clustering and collagen IV basement membrane formation, leading to the stabilization of the blood-retinal-barrier. EC, endothelial cell; BM, basement membrane; AJ, adherens junctions.