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Expression and cellular localization of the Mas receptor in the adult and developing mouse retina

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Purpose: Recent studies have provided evidence that a local renin-angiotensin system (RAS) exists in the retina and plays an important role in retinal neurovascular function. We have recently shown that increased expression of ACE2 and angiotensin (1-7) [Ang (1-7)], two components of the protective axis of the RAS, in the retina via adeno-associated virus (AAV)-mediated gene delivery, conferred protection against diabetes-induced retinopathy. We hypothesized that the protective molecular and cellular mechanisms of Ang (1-7) are mediated by its receptor, Mas, and the expression level and cellular localization dictate the response to Ang (1-7) and activation of subsequent protective signaling pathways. We tested this hypothesis by examining the expression and cellular localization of the Mas receptor in adult and developing mouse retinas.

Methods: The cellular localization of the Mas receptor protein was determined with immunofluorescence of the eyes of adult and postnatal day 1 (P1), P5, P7, P15, and P21 mice using the Mas receptor-specific antibody, and mRNA was detected with in situ hybridization of paraffin-embedded sections. Western blotting and real-time reverse-transcription (RT)–PCR analysis were performed to determine the relative levels of the Mas protein and mRNA in adult and developing retinas, as well as in cultured retinal Müller glial and RPE cells.

Results: In the adult eye, the Mas receptor protein was abundantly present in retinal ganglion cells (RGCs) and photoreceptor cells; a lower level of expression was observed in endothelial cells, Müller glial cells, and other neurons in the inner nuclear layer of the retina. In the developing retina, Mas receptor mRNA and protein expression was detected in the inner retina at P1, and the expression levels increased with age to reach the adult level and pattern by P15. In the adult mouse retina, Mas receptor mRNA was expressed at a much higher level when compared to angiotensin II (Ang II) type I (AT_1R) and type II (AT_2R) receptor mRNA.

Conclusions: The Mas receptor is expressed in developing and adult mouse retinas, and is more abundant in retinal neurons than in endothelial and Müller glial cells. These observations suggest that Mas receptor-mediated signaling may play important roles that extend beyond mediating the vascular effects of Ang (1-7) in developing and adult retinas. In addition, the relatively high expression of the Mas receptor when compared to AT_1R suggests that they may play a more important role in maintaining normal retinal physiology than previously considered.

The renin-angiotensin system (RAS) plays a vital role in regulating the normal physiologic functions of the cardiovascular and renal systems. The RAS was classically viewed as a circulating endocrine system with angiotensin II (Ang II) as the main peptide effector hormone, which mediates its effects primarily through activation of the angiotensin type I receptor (AT_1R). Recent studies have confirmed the presence of an additional local organ-specific RAS in almost all organs including the retina [1-8]. The discovery of the angiotensin-converting enzyme (ACE) homolog ACE2 resulted in the identification of an important pathway responsible for angiotensin (1-7) [Ang (1-7)] synthesis [9-11]. This enzyme can form Ang (1-7) from Ang II or less efficiently through hydrolysis of Ang I to Ang (1-9) with subsequent Ang (1-7) formation by ACE. Ang-(1-7) is now recognized as a biologically active component of the RAS that plays a critical role in counteracting the effects mediated by Ang II. Ang-(1-7) induces vasodilation, improves insulin sensitivity, and has antiproliferative, antioxidative, and anti-inflammatory activities [8,12-15]. In addition, it is now well established that Ang (1-7) is an endogenous ligand for the G protein-coupled receptor Mas [16]. There is growing evidence indicating that this endogenous counter-regulatory axis of the RAS, composed of ACE2, Ang (1-7), and the Mas receptor, has protective effects in many tissues and organs, including the neurovascular system of the retina and the brain [8,15,17-19]. Increasing evidence indicates that a balance between activation of the ACE/Ang II/AT_1R axis and the ACE2/Ang (1-7)/Mas receptor axis plays a critical role in maintaining normal function in different organs and that an imbalance in these opposing pathways toward the ACE/Ang II/AT_1R axis predisposes the organism to many pathological conditions, including retinal vascular diseases such as retinopathy of prematurity, diabetic retinopathy (DR), a common diabetic neurovascular complication, choroidal neovascularization,

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glaucoma, and ocular inflammation [8,17,18,20-23]. Our previous studies have also shown that the increased expression of Ang (1-7) and ACE2 in the retina has a protective role against the development of diabetic retinopathy [17,18] and increased Ang (1-7) levels and ACE2 activities protected ocular inflammation in mice [20,23].

The Mas gene codes for a G-protein-coupled cell surface receptor and was initially discovered by Young et al. in 1986 [24]. They used an RNase protection assay to show high levels of Mas expression in the cerebral cortex and hippocampal regions of the rat brain. The cellular localization and distribution of Mas mRNA in the rat brain was further investigated with in situ hybridization that showed strong specific signals in the dentate gyrus, CA3 and CA4 areas of the hippocampus, piriform cortex, and olfactory bulb, and moderate labeling was observed in the frontal lobe [25,26]. In the mouse, the distribution of Mas mRNA in the brain is comparable to that in the rat, highest in the hippocampus and the piriform cortex. In the adult central nervous system (CNS), Mas mRNA is most abundant in hippocampal pyramidal neurons and dentate granule cells. A study of Mas expression during development showed that Mas is first expressed in the developing rat CNS at postnatal day 1 (P1) [26]. Recently, Mas expression was also discovered in the cardiovascular regions of the brain with western blot and immunofluorescence [27]. However, Mas has a role not only in cardiovascular functions but also in neuronal signaling [19,28-30]. It has been shown by using Mas-deficient mice that Mas has important functions in regulating behavior and the cardiovascular system [31]. Ang (1-7) increases the excitability of CA1 neurons in the hippocampus [28] and enhances hippocampal, long-term potentiation (LTP), whereas Mas-deficient mice did not show Ang (1-7)-induced enhancement of hippocampal LTP [28]. Mas immunoreactivity has also been detected in endothelial cells of blood vessels in the CNS and other organs suggesting that they mediate the vasodilating and vasoprotective actions of Ang (1-7). Recent studies using the Mas-deficient mouse and Mas antagonists have shown that the Mas receptor not only mediates the vascular actions of Ang (1-7) but also is a critical element needed for nitric oxide-mediated vasodilation induced by RAS-dependent and RAS-independent agonists [32].

In this study, we aimed to investigate the expression and cellular localization of Mas receptor mRNA and protein in developing and adult mouse retinas to better understand the molecular and cellular mechanism by which Ang (1-7) attenuates the deleterious effects of Ang II in retinal pathophysiology. Mas receptor protein expression and cell type specificity were determined with immunofluorescence using an antibody against the Mas receptor in combination with other retinal cell-specific markers. Mas receptor mRNA expression and cell type specificity were determined with in situ hybridization. Real-time reverse-transcription (RT)–PCR was performed to quantify the relative levels of Mas receptor expression in the mouse retina compared to other RAS receptors.

METHODS

Animals: Adult C57BL/6J and CD1 wild-type mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Different ages of mice used for this study were bred at Animal Care Service at University of Florida. Animals were maintained under standardized conditions with an artificial 12 h:12 h light-dark cycle, with free access to food and water. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of animals in Ophthalmic and Vision Research and the guidelines of the Institutional Animal Care and Use committee at the University of Florida.

Immunofluorescence detection of the Mas receptor in developing and adult mouse retinas: For the immunofluorescence studies, eyes were collected from P1, P5, P7, P15, P21, and P60 mice. Eyes from P1–P7 mice were enucleated and immersion

Table 1. Mouse (ms) primers used for Real-Time RT–PCR analysis.

Gene name	Accession number	Sequences
Actin ms	X03672	Forward: 5′-AGCAGATGTGGATCAGCAAG-3′
		Reverse: 5′-ACAGAAGCAATGCTGTCACC-3′
AT1Ra ms	NM_177322	Forward: 5′-ATCGGACTAAATGGCTCACG-3′
		Reverse: 5′-ACGTGGGTCTCCATTGCTAA-3′
AT1Rb ms	AK087228	Forward: 5′-AGTGGAGTGAGAGGGTTCAA-3′
		Reverse: 5′-GGGCATTGAAGACATGGTAT-3′
MAS receptor ms	NM_008552	Forward: 5′-AGGGTGACTGACTGAGTTTGG-3′
		Reverse: 5′-GAAGGTAAGAGGACAGGAGC-3′

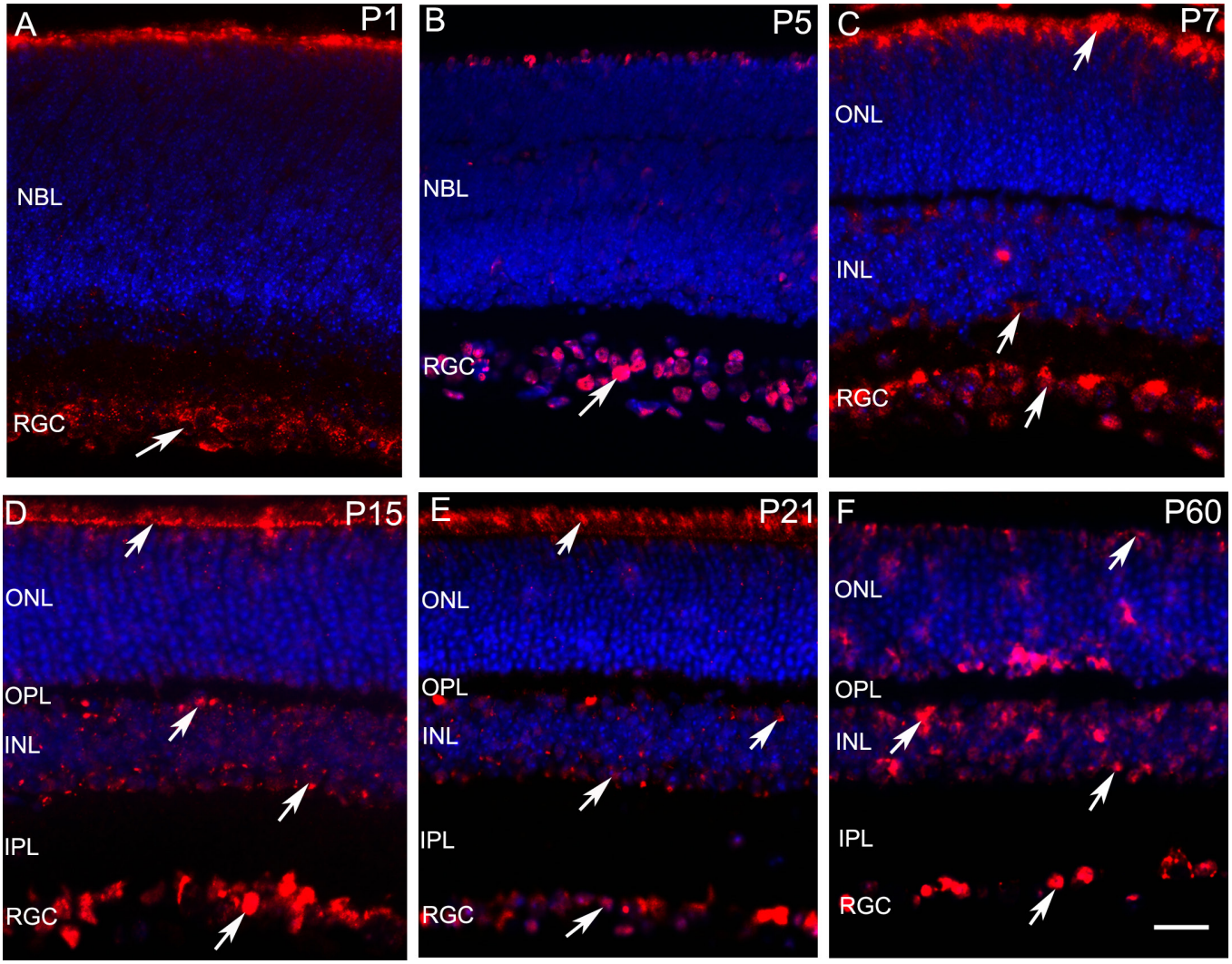

Figure 1. Expression of the Mas receptor protein in the mouse retina at different developmental time points. Immunofluorescence detection of Mas receptor protein expression at (**A**) postnatal day 1 (P1), (**B**) P5, (**C**) P7, (**D**) P15, (**E**) P21, and (**F**) P60 in the C57BL/6J mouse retina. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). NBL=neuroblast layer; ONL=outer nuclear layer; OPL=outer plexiform layer; INL=inner nuclear layer; IPL=inner plexiform layer; RGC=retinal ganglion cell layer. Scale bar=50 µm.

fixed in 4% paraformaldehyde overnight at 4 °C. The P15–P60 mice were first anaesthetized with a ketamine/xylazine mix (ketamine 60 mg/kg; xylazine 10 mg/kg) and then perfused transcardially with 4% paraformaldehyde freshly made in PBS (1X; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Following perfusion, the eyes were enucleated and post-fixed in 4% paraformaldehyde overnight at 4 °C. The eyes were then either embedded in paraffin and 4-µm-thick sections were cut or cryoprotected in 30% sucrose in PBS at 4 °C and frozen in optimum cutting temperature (OCT) compound (Tissue-Tek; Sakura-Finetek, Torrance, CA). Cryostat sections 12 µm thick were cut and mounted on SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, MA). Paraffin sections were first deparaffinized, which was followed by an antigen retrieval step. The antigen retrieval was performed by boiling the sections in antigen retrieval buffer (10 mM sodium citrate; 0.05% Tween-20, pH 6.0) for 15 min. The paraffin and cryosections were then incubated in blocking solution (5% bovine serum albumin [BSA] + 0.3% Triton X-100 in PBS) for 1 h. This was followed by incubation with different primary antibodies—rabbit anti-Mas (1:500, AAR-013, Alomone Laboratories, Jerusalem, Israel), mouse anti-arrestin (1:200), mouse anti-rhodopsin (1:200), mouse anti-glial fibrillary acidic protein (GFAP; 1:500), mouse anti-Brn3a (1:200, MAB1585, Millipore, Billerica, MA), mouse anti-CRALBP (1:500, ab15051, Abcam, Cambridge, MA), mouse anti-RPE65 (1:100, NB100–355, Novus Biologicals, Littleton, CO), biotinylated peanut agglutinin (PNA-biotin

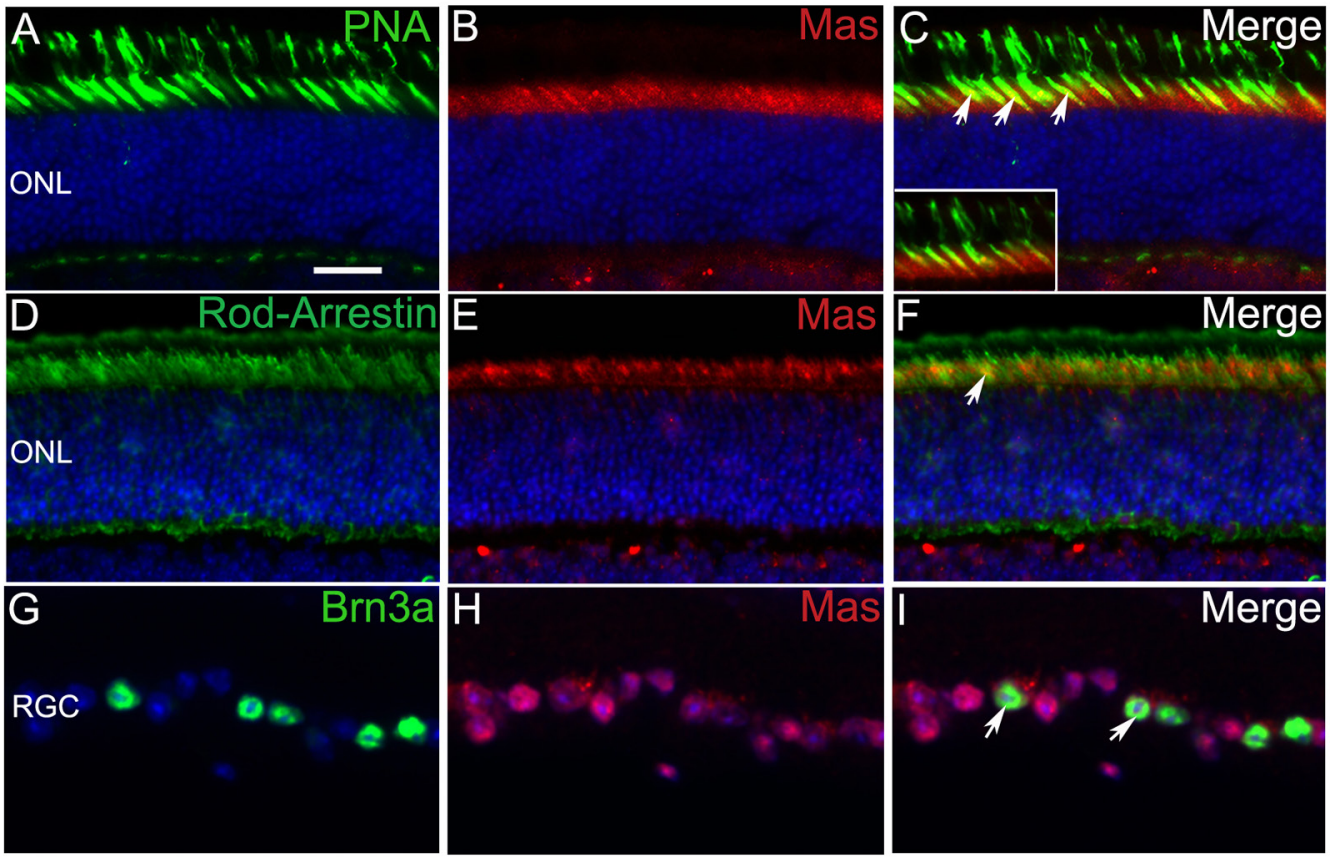

Figure 2. Cellular localization of the Mas receptor protein in mouse the retina. Cell type specificity of the Mas receptor expression in the adult mouse retina was detected with coimmunostaining of the anti-Mas receptor antibody with specific retinal cellular markers for (**A-C**) cone photoreceptors (PNA-biotin), (**D-F**) rod photoreceptors (rod-arrestin), and (**G-I**) ganglion cells (Brn3a). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). ONL=outer nuclear layer; RGC=retinal ganglion cell layer. Representative images at 400X magnification. Scale bar=50 µm.

1:200, B-1075, Vector Laboratories, Burlingame, CA), mouse anti-p27^{kip1} (1:200, BD Transduction laboratories, Lexington KY)—diluted in the same blocking solution (overnight at 4 °C) and then with the appropriate secondary antibodies conjugated to Alexa 488 or 594 (Molecular Probes/Invitrogen, Grand Island, NY) for 1 h at room temperature (RT). Sections were washed in PBS containing the nuclear counterstain 4',6 diamidino-2- phenylindole (DAPI), and mounted in Dako mounting media (Dako North America, Carpinteria, CA). The images were captured on a Keyence confocal microscope (Itasca, IL) or on a spinning disc confocal (UltraVIEW Vox, PerkinElmer, Waltham, MA) using a 20× and/or a 40× objective lens and were prepared for presentation using Adobe Photoshop (San Jose, CA).

Western blot analysis: To quantify the level of Mas receptor protein expression during development, retinal tissue was collected from P1, P15, P21, and P60 wild-type mice in cold RIPA buffer (Sigma, St Louis, MO) supplemented with protease inhibitors and were homogenized by sonication. Homogenized tissues were centrifuged at 12,000 ×*g* for 15 min at 4 °C, and the supernatant was collected. The protein concentration was detected with the Bio-Rad Protein Assay kit (Hercules, CA). Forty microns of protein samples were loaded and separated on 4–12% gradient gels for 1 h at 120 V in Tris-glycine buffer and electrophoretically transferred onto polyvinylidene difluoride membrane. Immunodetection was performed on blots blocked in fluorescence blocking buffer for 1 h (Rockland Immunochemicals, Gilbertsville, PA) and then incubated with primary (rabbit anti-Mas; 1:2,000; Alomone Laboratories), and secondary (goat anti-rabbit IR Dye 800; 1:5,000; Rockland Immunochemicals) antibodies. β-Actin immunodetection was used as a loading control. Immunoblots were visualized by using a Li-Cor odyssey infrared imager (Odyssey; Lincoln, NE). We used Image J software to quantify the relative level of the Mas protein after normalizing it to β-actin.

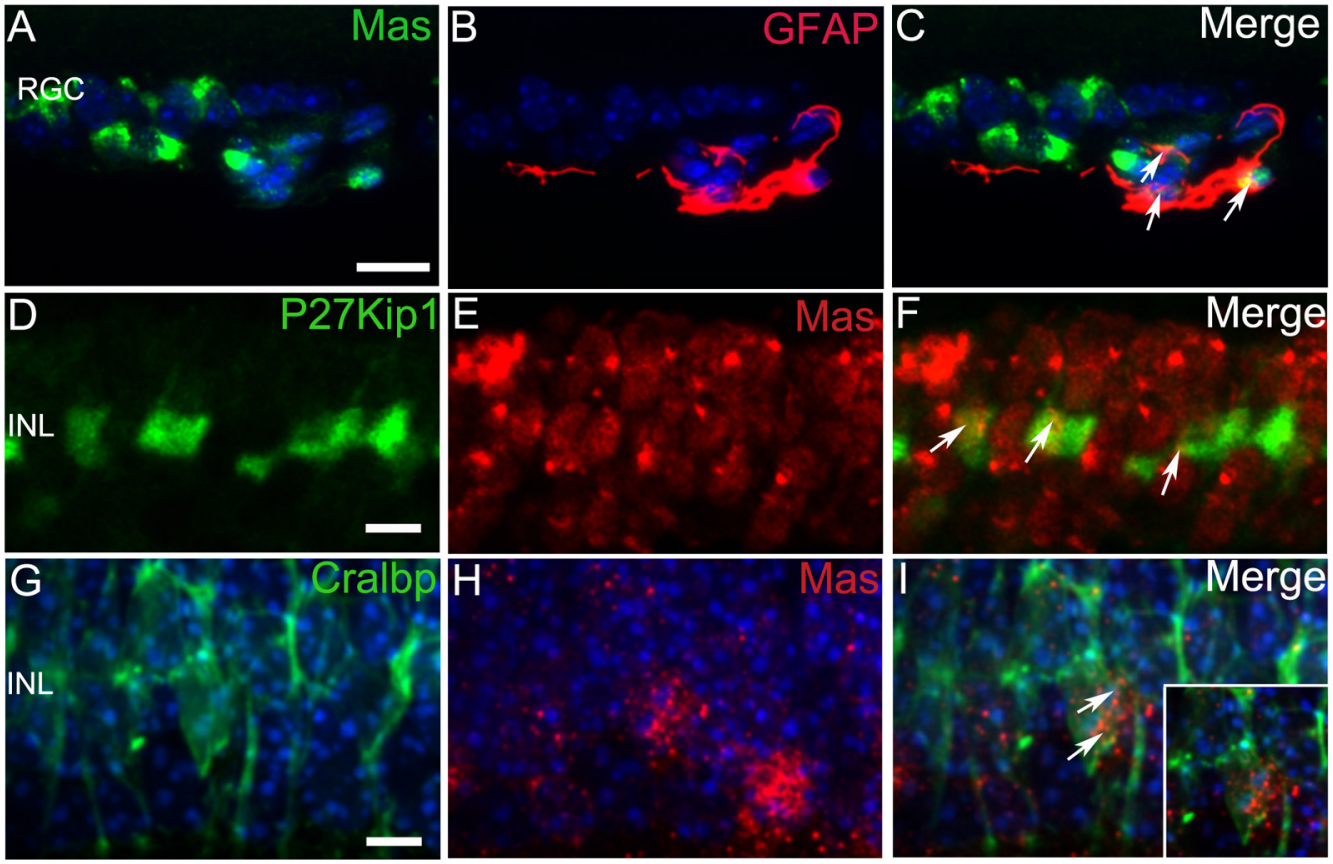

Figure 3. Cellular localization of the Mas receptor protein in retinal glial cells. Double immunostaining for glial cell markers and the Mas receptor in the mouse retina shows a low level expression of Mas in (**A-C**) astrocytes which are glial fibrillary acidic protein (GFAP) positive and in (**D-I**) Müller cell bodies that are $p27^{KIP1}$ and CRALBP positive. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). INL=inner nuclear layer; RGC=retinal ganglion cell layer. Scale bar=50 µm (**A-C**); 20 µm (**D-I**).

Mas receptor mRNA detection with in situ hybridization: Eyes were collected from P1, P5, P7, P15, P21, and P60 mice and immersion fixed in 4% paraformaldehyde, freshly made in phosphate-buffered saline, overnight at RT. The fixed eyes were embedded in paraffin, and 4-µm-thick sections were cut and mounted on SuperFrost Plus slides (Thermo Fisher Scientific). In situ hybridization to look at the Mas receptor gene expression was performed using the RNAscope 2.0 (Red) FFPE Assay kit (Advanced Cell Diagnostics, Hayward, CA) according to the protocol provided by the manufacturer. Briefly, the method involved deparaffinization of the sections by immersion in fresh Xylene (2×5 min) followed by immersion in 100% ethanol (2×3 min) with frequent agitation. The endogenous peroxidase was blocked with the provided pretreatment 1 solution for 10 min at RT, followed by epitope retrieval by boiling in the provided buffer for 10 min, protease digestion for 30 min at 40 °C in a hybridization oven (HybEZ Oven, Advanced Cell Diagnostics) and target hybridization for 2 h at 40 °C followed by six sequential amplification steps each interspersed with washing in the given wash buffer. This was followed by a colorimetric reaction, which involved the addition of fast red substrate on sections for 10 min at RT followed by washing in distilled water (dH_2O). The slides are then baked at 60 °C for 15 min and then mounted in EcoMount mounting media (Biocare Medical, Concord, CA). Control probes against the bacterial gene *DapB* (negative control) and for the housekeeping gene *peptidylprolyl isomerase B* (PIPB, positive control) and arrestin-S, which is expressed exclusively in the photoreceptors in the retina were also included to validate the RNAscope technique. Positive staining was identified as red punctate dots in the section.

Trypsin digest preparations of retinal vasculature: Eyes were enucleated from adult mice and immersion fixed in 4% PFA overnight at 4 °C. The retinas were dissected from the eye cups, washed in water for 1 h, and digested in 3% trypsin for 2–3 h at RT. The tissue was then transferred into water, and the network of vessels was freed from adherent retinal

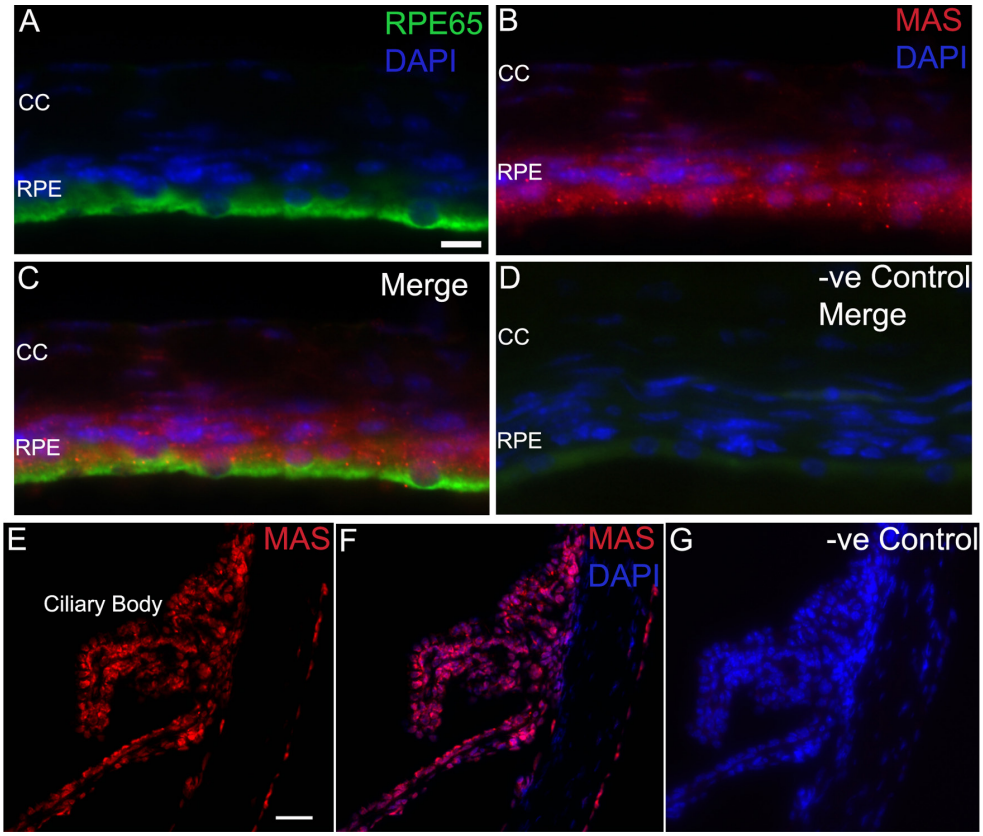

Figure 4. Cellular localization of the Mas receptor protein in the RPE and the ciliary body of the mouse eye. Panel **A-C**: Double immunostaining for the RPE cell marker (RPE 65, green) and Mas (red) in the CD1 mouse retina. **D**: Negative control for RPE65 and Mas staining in the RPE cell layer. **E** and **F**: Immunofluorescence detection of Mas in the ciliary body of the CD1 mouse eye. **G**: Negative control for Mas staining in the ciliary body of the CD1 mice. RPE=retinal pigmented epithelium; CC=choroidal capillaries. Scale bar=20 µm (**A-D**); 50 µm (**E-G**).

tissue by gentle shaking and manipulation under a dissection microscope. The vessels were then mounted on clean slides and allowed to dry. They were then immunostained for CD31 (rat anti-mouse CD31; 1:200; BD PharMingen, San Jose, CA), an endothelial marker, and Mas according to the protocol described.

Mas receptor expression in cultured retinal cells: Four different cell lines were used in this study: a human Müller cell line-MIO-M1 (generously provided by Dr. G. Astrid Limb, University College of Medicine, Institute of Ophthalmology, London, UK) [33], mouse N9-microglial cell line (purchased from American Type Culture Collection, ATCC, Manassas, VA), 661W mouse cone photoreceptor cell line (generously provided by Dr. Muayyad R. Al-Ubaidi, University of Oklahoma Health Science Center, Oklahoma City, OK) [34], and ARPE-19, human RPE cell line (purchased from ATCC). For immunostaining of cultured cells, confluent cells grown in an eight-chambered glass slide were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 10 min followed by incubation with blocking solution (3% BSA+ 0.3% Triton X-100) for 1 h at RT, followed by incubation with primary antibodies in blocking solution (overnight, 4 °C) and secondary antibodies (1 h, RT). Coverslips were washed in PBS containing the nuclear counterstain DAPI and mounted in Dako mounting media.

Real-time RT–PCR analysis of Mas and Ang II receptor expression: Total RNA was isolated from freshly dissected adult mouse retinal samples using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was performed using the Enhanced Avian HS RT–PCR kit (Sigma) following the manufacturer's instructions. Real-time PCR was performed on a real-time thermal cycler (iCycler, Bio-Rad Life Sciences) using iQ SYBR Green Supermix (Bio-Rad Life Sciences). The threshold cycle number (Ct) for real-time PCR was set by the cycler software. Optimal primer concentration for PCR (95°C, 3 min, 1 cycle; 95 °C, 10 s , 56 °C, 30 s, 50 cycles; 72 °C, 10 s, 1 cycle) was determined separately for each primer pair. Each reaction was run in duplicate or triplicate, and reaction tubes with target primers and those with actin primers were always included in the same PCR run. The expression levels of the different genes relative to AT_1Ra were established based on the Ct compared with the control housekeeping gene β-actin in each sample (amount of target=$2^{-\Delta\Delta Ct}$) and presented as fold change. The mouse-specific primers used in this experiment are listed in Table 1.

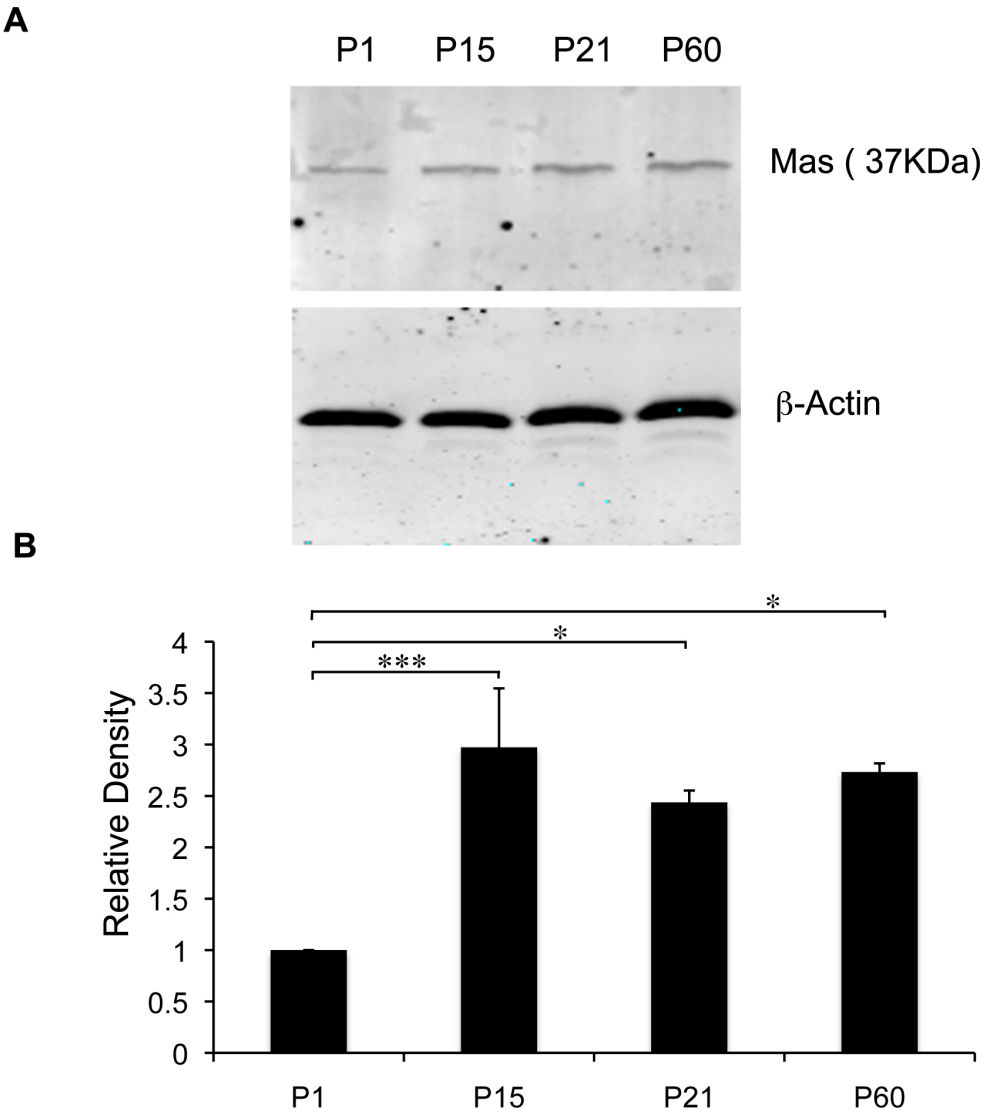

Figure 5. Level of Mas receptor protein expression in the mouse retina at different developmental time points. **A**: Western blot detection of the Mas receptor protein expression in the developing mouse retina. β-actin was used as the loading control. **B**: Quantification of the Mas receptor protein expression level normalized to β-actin. Sample size, n=4/age point, the bars in this graph show the mean ± standard error of the mean (SEM). One-way ANOVA followed by Bonferroni's multiple comparison test was performed to analyze significance; * $p<0.05$ is considered statistically significant.

RESULTS

Developmental expression of the Mas receptor protein in the mouse retina: Immunofluorescence was used to study the pattern of Mas protein expression in developing and adult C57BL/6J mouse retinas as shown in Figure 1. The specificity of the antibody was confirmed by using the control peptide antigen supplied by the manufacturer with the antibody (Alomone Laboratories). At P1, the expression of the Mas protein was mainly confined to the RGC layer. No or low levels of the Mas receptor protein was observed in the neuroblast layer (Figure 1A). At P5, a stronger level of the Mas receptor protein was expressed by the RGCs. In addition, a low level of the Mas receptor protein was expressed in the inner portion of the neuroblast layer (Figure 1B). At P7, expression of the Mas receptor protein increased in the developing inner nuclear layer (INL). Significant levels of the Mas receptor protein continued to be expressed in the retinal ganglion cells; in addition, the Mas receptor protein also seemed to be expressed in the inner segments of the photoreceptors. No protein expression was observed in the outer nuclear layer (ONL; Figure 1C). At P15, strong expression of the Mas receptor protein was evident in the inner segment of the photoreceptor, in the inner nuclear layer where they were expressed by a subset of amacrine cells, bipolar cells, horizontal cells, and in the ganglion cells in the RGC layer (Figure 1D). At P21, the expression pattern remained similar to that seen at P15; however, the Mas receptor expression in the RGCs seemed higher compared to the P15 protein level expression (Figure 1E). At P60, the pattern of the Mas receptor expression remained similar to that observed at P15 and P21 (Figure 1D).

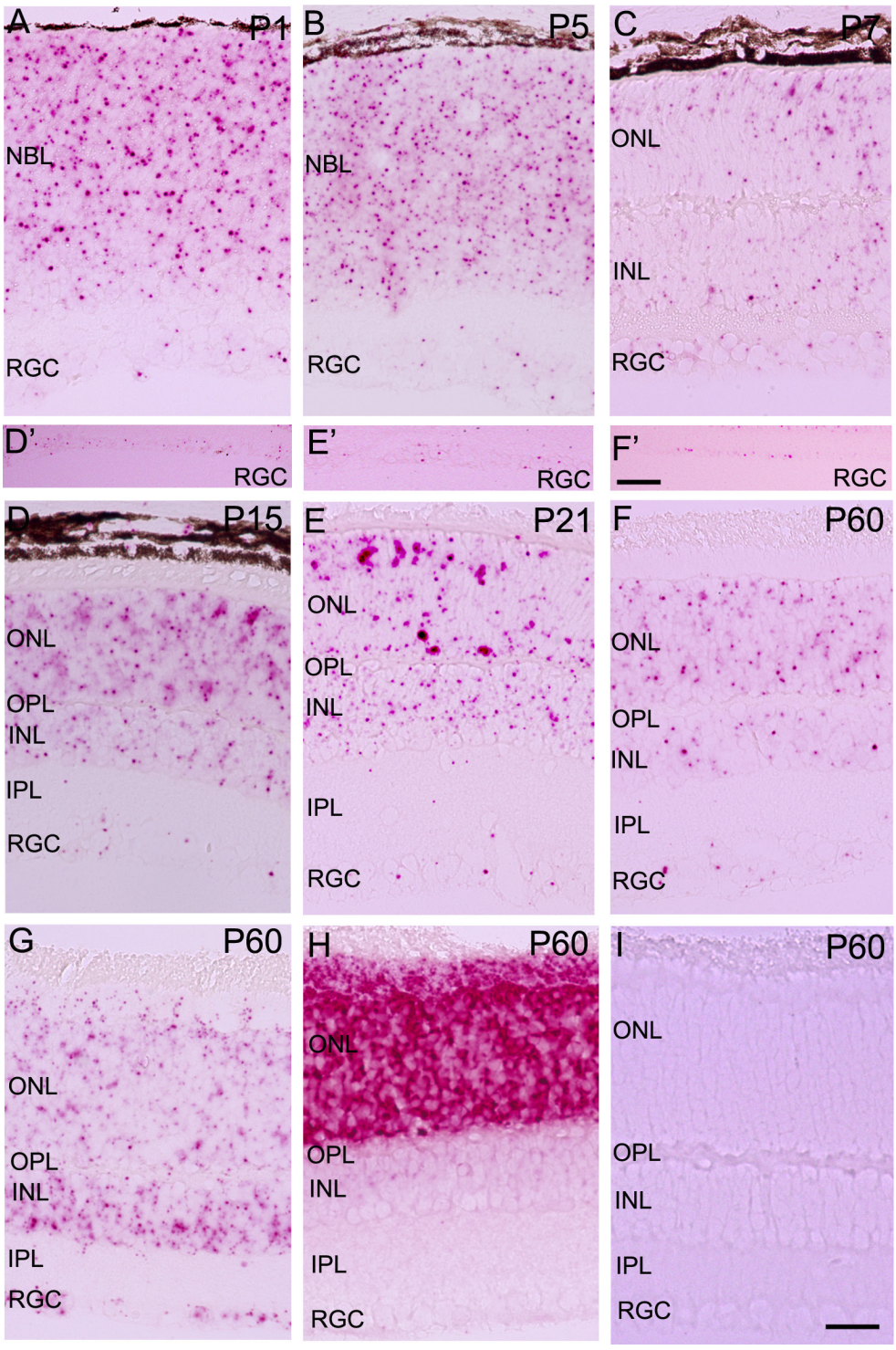

Figure 6. In situ detection of Mas receptor mRNA expression in developing and adult mouse retinas. **A**–**F**: Mas receptor mRNA was detected with in situ hybridization in the retinas of different ages (P1–P60). **D'**–**F'**: Lower magnification (100X) image showing Mas expression in the RGC layer at P15, P21, and P60. **G**: Positive control: In situ detection of peptidylprolyl isomerase B (PIPB) in the P60 mouse retina. **H**: Positive control: In situ detection of arrestin in the P60 mouse retina. **I**: Negative control in the P60 mouse retina. NBL=neuroblast layer; ONL: outer nuclear layer; OPL=outer plexiform layer; INL=inner nuclear layer; IPL=inner plexiform layer; RGC=retinal ganglion cell layer. Scale bar=50 µm (**A**–**D**); **D'**–**F'**=10 µm.

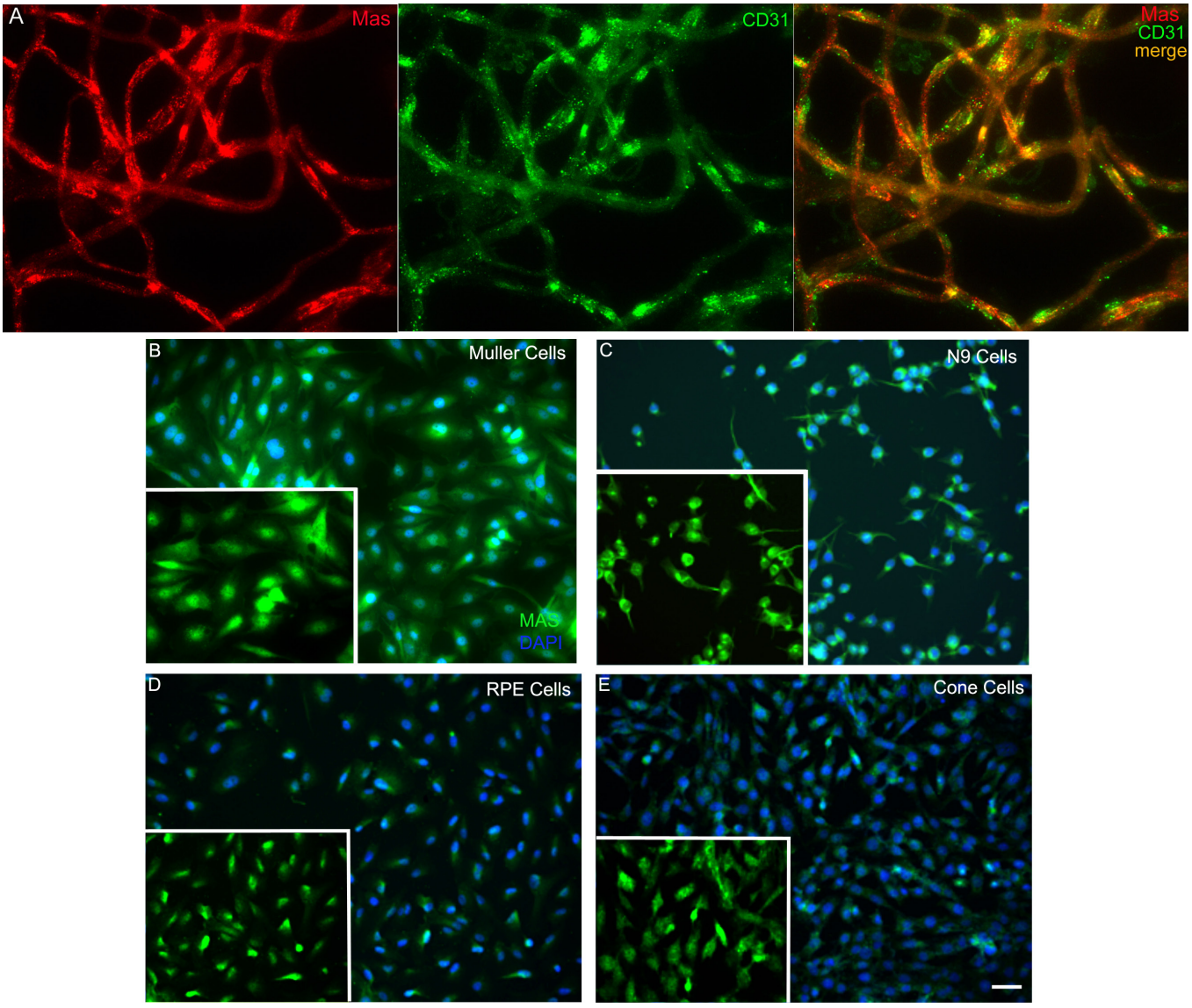

Figure 7. Mas expression in retinal vessels and cultured cell lines. **A**: The Mas receptor protein detected with immunofluorescence and colocalization with endothelial cell marker (CD31) in trypsin digested retinal vasculature preparation. **B**–**E**: Mas expression detected with immunofluorescence in (**B**) human Müller cells, (**C**) mouse N9 microglial cells, (**D**) human retinal pigment epithelial (RPE, ARPE19) cells, and (**E**) cone photoreceptor cells (661W). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). **B**–**E**: Inserts show Mas expression alone. Scale bar=20 µm.

Specific retinal cell markers were used to investigate the cellular colocalization of the Mas receptor protein as shown in Figure 2 and Figure 3. Double immunostaining with PNA, a cone photoreceptor-specific marker, showed that Mas expression was localized to the cone inner segments and was absent from the cone outer segments (Figure 2A-C). Double immunostaining with rod arrestin (Figure 2D-F) and rhodopsin (image not shown), photoreceptor markers, also showed that the Mas receptor protein was localized to the inner segments of the photoreceptors. The level and pattern of expression in photoreceptors suggested that Mas receptor proteins are expressed at higher levels in cone photoreceptors compared to rod photoreceptors. Double immunostaining with Brn3a, a ganglion cell marker, showed that the Mas receptor protein was strongly expressed in the RGCs (Figure 2G-I). Costaining with GFAP, a marker for glial cells and astrocytes, showed that the Mas receptor protein was also expressed in glial cells in addition to neurons in the retina (Figure 3A-C). Coimmunostaining with $p27^{KIP1}$ (Figures 3D-F), which stains only the cell bodies of Müller cells, and

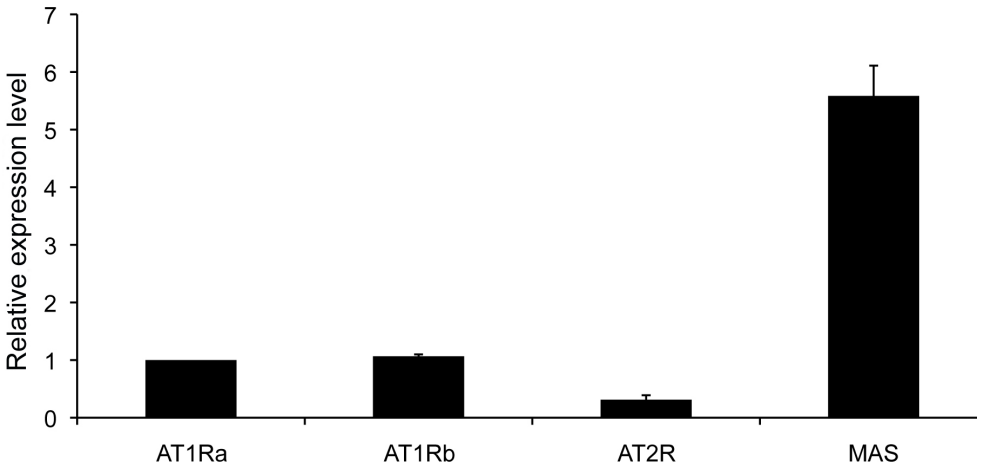

Figure 8. Relative levels of angiotensin receptor expression in the mouse retina. The relative mRNA levels of the AT_1Ra, AT_1Rb, AT_2R, and Mas receptors were examined in adult mouse retinal samples with real-time RT–PCR and normalized to β-actin. Sample size, n=3.

CRALBP (Figures 3G-I), which labels the cell bodies and the processes of Müller cells, suggested that these cells express the Mas receptor protein at low levels in and around their cell bodies compared to the level of expression in retinal neurons. The observed punctate pattern of Mas receptor protein expression in the INL suggests that this protein may be strongly expressed by a subset of INL neurons or microglial cells (Figure 3D-I). Coimmunostaining with the RPE65 antibody in CD1 (non-pigmented) mice eyes allowed us to study Mas receptor protein expression in the RPE and ciliary body, which was difficult to detect in the C57BL/6J mice due to pigmentation and autofluorescence. Figure 4 shows that the Mas receptor protein is expressed in RPE cells, with the protein expressed uniformly in RPE cells, whereas RPE65 is more abundant on the apical side of the RPE cells. The Mas expression pattern also suggested that the protein is expressed in the choroidal capillaries (Figure 4A-D). Strong Mas receptor protein expression was also observed in the ciliary body of the CD1 mouse eye (Figure 4E-G). Western blot data showed that the level of Mas receptor protein expression in the mouse retina increased with age with the lowest expression seen at P1 and the adult steady level of expression reached by P15 (Figure 5A,B).

Expression of Mas receptor mRNA in developing and adult mouse retinas: Mas receptor mRNA was detected with in situ hybridization in paraffin-embedded sections using the RNAscope kit (Advanced Cell Diagnostics). Figure 6 shows the pattern and level of Mas receptor mRNA expression in the developing mouse retina. Expression of Mas receptor mRNA was observed in the mouse retina as early as P1 (Figure 6A) with a significant level of expression seen in the neuroblast layer and the cell bodies of the retinal ganglion cells. A similar level and pattern of expression were seen at P5 (Figure 6B). At P7 and P15 (Figure 6C,D), with the differentiation of the outer and inner nuclear layer in the developing mouse retina, the in situ hybridization expression pattern suggested that Mas receptor mRNA is expressed in photoreceptors, neurons, and/or glial cells in the inner nuclear layer as well as in a subset of RGCs. The adult pattern and level of Mas receptor mRNA expression seemed to be achieved by P15 (Figure 6D). PIPB (Figure 6G) and arrestin (Figure 6H) were used as two positive controls with PIPB expressed moderately throughout the retinal tissue and arrestin confined to the photoreceptor layer. The specificity of the probe binding and the efficacy of the RNAscope assay were also shown by the complete absence of DapB (used as a negative control) signal in the mouse retina (Figure 6I). This technique also provided quantitative information about the level of expression of a particular mRNA. Thus, the strong expression level of arrestin in the photoreceptor layer was evident as expected while PIPB was expressed at a much lower level. Comparatively, a moderate level of Mas mRNA seemed to be expressed in the developing and adult mouse retinas.

Differential levels of Mas expression in retinal blood vessels and cultured retinal cells: Ang (1-7) has a known vasodilatory function that is mediated by the binding to the specific receptor Mas. Previous Mas receptor expression studies in the brain and other organs have shown that Mas receptors are expressed in blood vessels. Here we show for the first time that the Mas receptor is also expressed strongly in retinal blood vessels. Trypsin digestion was used to isolate retinal blood vessels, and coimmunostaining with CD31, an endothelial cell marker, showed that the Mas receptor protein is strongly expressed in the endothelial cells and pericytes of the retinal vessels (Figure 7A). Four cell lines derived from specific retinal cell types were used to examine the presence or absence of Mas receptor expression in different cell types. The immunofluorescence study showed that the Mas receptor

protein is strongly expressed in Müller cells and microglial cells, and a lower level of expression was observed in RPE cells and cone cells (Figure 7B–E).

Quantification of RAS receptor mRNA expression in the mouse retina: Total mRNA was extracted from the adult mouse retina, and a real-time RT–PCR assay was used to determine the relative expression levels of various angiotensin receptor mRNAs in the retina. Real-time RT–PCR data showed that AT_1Ra, AT_1Rb, AT_2R, and Mas receptor mRNA was expressed in the retina. Mas gene expression was about fivefold higher compared to other angiotensin receptor genes tested in the retina (Figure 8).

DISCUSSION

Previous studies that used RT–PCR have shown that Mas receptor mRNA is expressed in the retina and the ciliary body of rodent eyes [18,35-37]. However, the studies did not establish the type of retinal cell in which the Mas receptor is localized. To understand the mechanism by which the protective axis of the RAS comprised of ACE2, Ang (1-7), and Mas receptor functions in the retina, it is essential to identify the specific cell types that express the Mas receptor. In the present study, immunofluorescence and in situ hybridization techniques were used to identify for the first time, to our knowledge, the type of cells in the retina that express the Mas receptor as well as the developmental pattern of the expression.

Our study confirmed that Mas receptor mRNA and protein are expressed in the mouse retina. Studying the expression pattern across developmental time points showed that Mas receptor mRNA and protein expression begins as early as P1 and continues to adulthood, with the adult level and pattern reached by P15. Mas receptor mRNA and protein are highly expressed in retinal ganglion cells and photoreceptors. A much lower level of expression was observed in Müller glial and endothelial cells of the retinal blood vessels since their expression becomes easily evident only when they are isolated from retinal tissue in the form of the Müller cell line and trypsin digest preparation of retinal vasculature. This suggests that the ACE2/Ang (1-7)/Mas receptor axis may play a vital protective role in the neurovascular system in developing and adult retinas. Immunofluorescence studies have shown that the Mas receptor and AT_1R, the main receptor of Ang II, are expressed in the same cells in the retina, consistent with the counteracting effects mediated by the two receptors [38]. Ang (1-7) has been detected in Müller cells in the human retina [39]. The presence of high levels of Mas receptor in retinal neurons thus strongly suggests that the Mas receptor may also be involved in mediating neuron glial interaction. This makes the Mas receptor an important potential therapeutic target for the treatment of retinal diseases characterized by neurovascular dysfunction such as diabetic retinopathy.

The relative expression level and cellular localization of RAS receptors may have an important role in their functionality. Real-time RT–PCR results showed that the Mas receptor is expressed in the retina at a much higher level than AT_1R and AT_2R. The functional significance of these differences in unclear, but may contribute to differential responses in physiologic and pathological conditions. The localization of Mas receptors in specific retinal cells that also express other RAS receptors suggests the possibility of direct interaction at the level of the receptor or an indirect interaction occurring between the corresponding downstream signaling pathways. Previous studies suggested that AT_1R and AT_2R heterodimerize and/or homodimerize. More recent studies show that similar to other G-protein coupled receptors these receptors are more likely to undergo homodimerization and that AT_2R counteracts the AT_1R effect via negative crosstalk in the cytoplasm [40]. A similar interaction is possible between AT_1R and the Mas receptor and must be elucidated in future studies. The relatively high level of Mas mRNA expression when compared to AT_1Ra, AT_1Rb, and AT_2R strongly suggests that the Mas receptor may play a more important role in the pathophysiology of the retina than previously envisaged.

Our previous study showed that intraocular administration of adenoassociated virus (AAV)-mediated gene delivery of ACE2 or Ang (1-7) to diabetic rats and mice attenuated diabetes-induced retinal vascular leakage, infiltrating inflammatory cells and oxidative damage, thus providing protection against diabetic retinopathy [18]. It has also been shown that intravitreal treatment with Ang (1-7) in rabbits decreases intraocular pressure without affecting the aqueous humor outflow [37]. Since these protective effects of ACE2 and Ang (1-7) are mediated through the activation of the Mas receptor, we need to investigate the effect of manipulating the expression or activity level of the Mas receptor on the development of various retinal neurovascular disorders in future studies. In addition, we also need to study the downstream signaling pathway of Mas receptor activation.

Conclusion: Immunocytochemistry, immunofluorescence, and in situ hybridization showed that Mas receptor protein and mRNA are expressed locally in the retina. The Mas receptor protein is specifically expressed in retinal ganglion cells, nerve fibers, Müller cells, and endothelial cells. The Mas receptor protein is expressed at a high level in the mouse inner retina from early development to adulthood suggesting that they may play an important role in neurovascular

function, not only in adult retinas but also in developing retinas. Immunofluorescence in the cell lines showed that the Mas receptor and AT_1R are expressed in the same cells in the retina, consistent with the counteracting effects mediated by the two receptors. The Mas receptor expression level was about five times higher than AT_1Ra and AT_1Rb in the adult mouse retina, thus suggesting that it plays a central role in the pathophysiology of the retinal neurovascular system.

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