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Loss of angiotensin-converting enzyme 2 exacerbates diabetic retinopathy by promoting bone marrow dysfunction

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Abstract

Angiotensin-converting enzyme 2 (ACE2) is the primary enzyme of the vasoprotective axis of the renin angiotensin system (RAS). We tested the hypothesis that loss of ACE2 would exacerbate diabetic retinopathy by promoting bone marrow dysfunction. *ACE2*$^{-/y}$ were crossed with Akita mice, a model of type 1 diabetes. When comparing the bone marrow of the *ACE2*$^{-/y}$-Akita mice to that of Akita mice, we observed a reduction of both short-term and long-term repopulating hematopoietic stem cells, a shift of hematopoiesis towards myelopoiesis, and an impairment of lineage^{-}c-kit^{+} hematopoietic stem/progenitor cell (HS/PC) migration and proliferation. Migratory and proliferative dysfunction of these cells was corrected by exposure to angiotensin-1–7 (Ang-1–7), the protective peptide generated by ACE2. Over the duration of diabetes examined, ACE2 deficiency led to progressive reduction in electrical responses assessed by electroretinography and to increases in neural infarcts observed by fundus photography. Compared to Akita mice, *ACE2*$^{-/y}$-Akita at 9-months of diabetes showed an increased number of acellular capillaries indicative of more severe diabetic retinopathy. In diabetic and control human subjects, CD34^{+} cells, a key bone marrow HS/PC population, were assessed for changes in mRNA levels for MAS, the receptor for Ang-1–7. Levels were highest in CD34^{+} cells from diabetics without retinopathy. Higher serum Ang-1–7 levels predicted protection from development of retinopathy in diabetics. Treatment with Ang-1–7 or alamandine restored the impaired migration function of CD34^{+} cells from subjects with retinopathy. These data support that activation of the protective RAS within HS/PCs may represent a therapeutic strategy for prevention of diabetic retinopathy.

Keywords

hematopoietic progenitors; diabetes; retina; bone marrow; CD34^{+}

Introduction

Hyperactivity of the vasodeleterious arm (Angiotensin-Converting Enzyme (ACE)/angiotensin II (Ang II)/AT1 receptor) of the RAS has been implicated in the pathogenesis of a wide range of diseases that include diabetic retinopathy and nephropathy, as well as cardiovascular disease [1, 2]. ACE2 converts Ang II to Ang-1–7 which, by virtue of its actions on the MAS receptor, opposes the effects of Ang II and is therefore considered the protective arm of RAS (ACE2/Ang-1–7/Mas). Ang-1–7 mediates its beneficial effects by reducing inflammation and oxidative stress [3, 4].

The existence of specific RAS systems in organs including the bone marrow has been well-established [5–13]. Local RAS is active in primitive embryonic hematopoiesis [14, 15] and continues to regulate each stage of physiological and pathological blood cell production in the adult via autocrine, paracrine, and intracrine pathways [16]. ACE degrades the inhibitory tetrapeptide N-Acetyl-Ser-Asp-Lys-Pro and triggers primitive stem cells into S phase [17]. Ang II activates AT1 to stimulate the JAK-STAT pathway promoting hematopoiesis [9, 18].

Local RAS peptides directly regulate myelopoiesis [19], erythropoiesis [20], thrombopoiesis and the development of other cellular lineages [21–23].

Previously, we showed that diabetic individuals who maintain ACE2 mRNA levels in $CD34^+$ cells do not develop diabetic retinopathy [1, 24–29]. We also showed that ACE inhibitors, which are known to increase ACE2 levels, function to hinder the development of diabetes-induced bone marrow dysfunction and myeloidosis in streptozotocin-injected rats [30, 31].

In this study, we examined the impact of chronic ACE2 loss on hematopoiesis and microvascular dysfunction in type 1 diabetes by breeding *ACE2*$^{-/y}$ mice to Akita mice. We hypothesized that chronic loss of ACE2 would dramatically impact hematopoiesis and promote diabetic retinopathy in murine models and in humans.

Materials and methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee (animal protocol # 11165) at the Indiana University School of Medicine. Wild type (WT; genotype: *ACE2*$^{+/y}$-*Ins2*$^{WT/WT}$), Akita (genotype: *ACE2*$^{+/y}$-*Ins2*$^{WT/C96Y}$), ACE2 knockout (*ACE2*$^{-/y}$; genotype: *ACE2*$^{-/y}$-*Ins2*$^{WT/WT}$), and *ACE2*$^{-/y}$-Akita (genotype: *ACE2*$^{-/y}$-*Ins2*$^{WT/C96Y}$) mice were generated as described elsewhere [32]. All mice were on the same C57BL/6J background. Only males were used in the study, because Akita male mice are reported to display a more severe diabetic phenotype than female mice [33]. Mice were housed in the Laboratory Animal Research Center (LARC) at the Indiana University School of Medicine. Blood glucose levels were measured using an Accu-Chek Compact Plus glucose meter (Roche Diagnostics, Indianapolis, IN). *ACE2*$^{-/y}$-Akita, Akita mice showed elevated glucose levels at 6 weeks of age, and were studied at 3 and 9 months after the onset of diabetes. Age-matched *ACE2*$^{-/y}$ and WT were used. The mice were anesthetized by inhalation of isoflurane and euthanized by cervical dislocation.

Bone marrow Lineage$^-$c-Kit$^+$ (LK) cell isolation

Bone marrow cells were flushed from mouse femurs and tibias using PBS. Lineage$^-$c-Kit^+ hematopoietic stem/progenitor cells (HS/PC) were selected by lineage negative and c-Kit positive selection kits (Catalog # 19856 and 18757, STEMCELL Technology, Vancouver, BC, Canada) and EasySep^{TM} magnets following manufacture's instruction.

Migration assay

Migratory function of HS/PCs was analyzed by measuring their ability to migrate towards 100 nM CXCL12 (#460-SD-050, R&D Systems, Minneapolis, MN) using the fluorimetric QCM 5μM 96-well chemotaxis cell migration assay (5 μM pore size, ECM512, Millipore, Temecula, CA) as previously described [25].

Bone marrow LK cell proliferation

Proliferation function was measured using the cell proliferation BrdU ELISA kit (#11647229001, Roche) following incubation for 24 hrs. in serum free stemspan media

(#09650, STEMCELL Technologies) containing cytokine cocktails (IL-3 20 ng/ml, IL-6 20 ng/ml and stem cell factor 50 ng/ml) with or without the supplement of 100 nM Ang-1–7, or alamandine.

Colony forming unit assay

Ammonium Chloride Solution (ACS) was used to lyse red blood cells within the suspensions of bone marrow or blood cells. ACS treated bone marrow or blood cells were plated in MethoCult^{TM} GF M3434 (STEMCELL Technologies) and then identified following manufacturer's instructions and as previously described [34].

Electroretinography

Electroretinography (ERG) was performed on mice after overnight dark-adaption using an UTAS-E 2000 ERG system (LKC Technologies, MD, USA). Scotopic rod signaling was assessed with 10 increasing intensities of white light. Photopic cone signaling was assessed with four increasing light intensities as previously described [35].

Optical coherence tomography

Optical coherence tomography (OCT) was performed using an InVivoVue OCT system (Bioptigen, Inc., NC) as previously described [36]. Three high-resolution lateral B-scan images were obtained in anesthetized mice.

Acellular capillaries quantification

Trypsin digestion of the retina was performed according to a previously published protocol [37].

Flow cytometry

Isolated cells were incubated with 5% rat serum for 15 min at 4 °C. The cells were then incubated with primary antibody cocktails for 30 min at 4°C in the dark (FITC anti-mouse c-Kit, Biolegend, Cat# 105806, 1 μl in 50μl cocktail volume; PE anti-mouse CD34, Biolegend, Cat# 119308, 2 μl in 50μl cocktail volume; BV421™ anti-mouse lineage cocktail, Biolegend, Cat# 133311, 10 μl in 50μl cocktail volume; PE/Cy7 anti-mouse Sca-1, Biolegend, Cat# 108114, 1 μl in 50μl cocktail volume; PerCP-eFluor 710 anti-mouse CD135, eBioscience, Cat# 46–1351-82, 1 μl in 50μl cocktail volume; APC anti-mouse CD16/CD32, eBioscience, Cat# 14–0161-82, 1 μl in 50μl cocktail volume; PE-CF594 anti-mouse CD127, BD Biosciences, Cat# 562419, 1 μl in 50μl cocktail volume). After washing, the cells were then stained with flexible viability dye eFluor 780 (1 ul/ml, eBioscience, San Diego, California, USA) for 30 min at 4°C, washed with PBS twice, and fixed with 1% PFA for flow cytometry.

Human study

All human studies were approved by the Institutional Review Boards at the University of Florida (Study # 535–2011) and Indiana University (Study # 1402550709). Study subjects were recruited as healthy controls (n=13) or diabetics (n=39) with either no retinopathy, or with different stages of diabetic retinopathy. Subjects were enrolled according to the

following criteria: a). Any male or female between the ages of 21– 98 years of age were eligible to participate; b) must have carried the diagnosis of diabetes (either type 1 or type 2 diabetes) or healthy aged control; and c) was willing and able to cooperate with the eye exam protocol. The exclusion criteria included: a) subjects with AMD, glaucoma, uveitis, known hereditary degenerations or other significant ocular complications other than diabetic retinopathy; b) ongoing malignancy; c) cerebral vascular accident or cerebral vascular procedure; d) current pregnancy; e) history of organ transplantation; f) presence of a graft (to avoid any effect of the graft on inflammatory parameters); g) evidence of ongoing acute or chronic infection (HIV, Hepatitis B or C, tuberculosis); and h) subjects with anemia. The degree of diabetic retinopathy was assessed using color fundus imaging and fluorescence angiography. Peripheral blood was obtained for plasma collection and $CD34^+$ cell isolation.

Measurement of plasma Ang-1–7 levels

Peripheral blood was collected in heparin-coated tubes (#367963, BD Biosciences) and placed on ice immediately after collection. Plasma was obtained by centrifugation at 700 g, 4°C for 20 min and then snap frozen in liquid nitrogen. The samples were sent to Attoquant Diagnostics GmbH (Vienna, Austria), where Ang-1–7 levels were quantified using a combination of ultra-pressure-liquid chromatography and mass spectrometry (LC-MS/MS) [38]. Stable-isotope-labeled internal standard controls were used to correct the peptide recovery due to sample preparation for each individual sample.

Enrichment of human $CD34^+$ cells

One hundred and twenty ml of peripheral blood was diluted with PBS (1:1). Then, every 25 ml-diluted blood sample was gently overlaid on 12.5 ml Ficoll-Paque Plus (Cat. # 17–1440-02, GE Healthcare) in 50 ml tubes. After centrifugation at 800 g for 30 min, the buffy coat was transferred into a new 50 ml tube and washed three times with PBS supplemented with 2% FBS and 1 mM EDTA. Peripheral blood mononuclear cells were then enriched for $CD34^+$ cells by the human CD34 positive selection Kit (Cat. #18056, StemCell Technologies) using EasySep™ magnets. Enriched $CD34^+$ cells were used either for mRNA isolation or for migration assay.

Quantitative RT-PCR

Total RNAs were extracted from human $CD34^+$ cells using RNeasy Mini Kit (Qiagen, Valenicia, CA). RNA concentration and qualify were determined using Nanodrop (Thermo Scientific, Wilmington, DE). cDNAs were synthesized by iScriptTM cDNA synthsis kit (BioRad, Pleasanton, CA). Quantitative PCR were performed using SYBR Green (Qiagen, Valenicia, CA) with MAS primers (PPH10808A, Qiagen, Germantown, MD). Results were expressed as 2^ (delta Ct) *100 relative to their internal controls.

Data Analysis

Data were initially evaluated for normal distribution using JMP 9 software. One-way or two-way ANOVA followed by an appropriate multiple comparison post hoc test to compare the experimental data sets. If data were not normally distributed, Mann Whitney and Kruskal

Wallace tests were used. The data sets were considered significantly different if the p value amounted to <0.05. Results were expressed as mean ± SE.

Results

Absence of ACE2 caused depletion of short- and long-term repopulating HSCs in diabetic bone marrow

ACE2$^{-/y}$ mice exhibited similar random glucose levels as WT mice at the 3-month time point (Figure 1A). There was a 2.8-fold increase in glucose levels for the Akita group at 3 months of diabetes in comparison with the WT group (Figure 1A). Loss of ACE2 did not increase the glucose levels in Akita mice. At the 9-month time point, both Akita and *ACE2*$^{-/y}$-Akita showed a further increase in glucose levels as compared to 3 months of diabetes; however, no significant difference was detected between these two groups (Figure 1A). Akita and *ACE2*$^{-/y}$-Akita mice showed reduced body weight compared to WT mice at both 3- and 9-month time points (Figure 1B). Non-diabetic groups (WT and *ACE2*$^{-/y}$ mice) had an increase in their body weights over time, while 9-month diabetic mice (Akita and *ACE2*$^{-/y}$-Akita) showed similar body weights as at 3 months of diabetes (Figure 1B).

Both the long- and short-term repopulating hematopoietic stem cells (HSCs) play a key role in maintaining the pool of hematopoietic cells and the balance of differentiated linages. To determine the effect of loss of ACE2 on the diabetic bone marrow, we examined the percent of HSC in bone marrow of *ACE2*$^{-/y}$-Akita mice and age-matched controls using flow cytometry (Figure 1C). Compared to Akita mice with three months' duration of diabetes, the *ACE2*$^{-/y}$-Akita mice exhibited an increase in short-term repopulating (ST)-HSCs (Lin^{-}/CD127^{-}/Sca-1^{+}c-kit^{+}/CD34^{+}CD135^{-}) (Figure 1D). However, the percentage of ST-HSCs was reduced at the 9-month time point in double mutant mice as compared to both WT and Akita mice (Figure 1D) suggesting a possible loss of compensation in the later stages of diabetes.

Long-term repopulating (LT)-HSCs (Lin^{-}/CD127^{-}/Sca-1^{+}c-kit^{+}/CD34^{-}CD135^{-}) represent the most primitive stem cells. Interestingly, we observed an age-related increase in phenotypic LT-HSCs in nondiabetic groups at the 9-month time point when compared to the 3-month time point (Figure 1E), which is consistent with reports from other groups [39, 40]. At 9 months of diabetes, Akita mice showed a 32% reduction in the percentage of phenotypic LT-HSCs as compared to WT mice. *ACE2*$^{-/y}$-Akita mice showed a greater decrease of these cells (~46% reduction versus Akita group) (Figure 1E), supporting our observations that loss of ACE2 intensifies the diabetic defect. While bone marrow-derived HSCs are released into circulation in a circadian pattern and this pattern is disturbed in diabetes [41], we did not detect any difference of lineage^{-}c-kit^{+}sca-1^{+} (LSK) cells between diabetic and nondiabetic groups at the one time point we examined (Supplemental Figure 1).

Impairment of HS/PC migration and proliferation in *ACE2*$^{-/y}$-Akita mice

Proliferation and migration of HS/PCs are critical for their mobilization from the bone marrow and homing to areas of injury. We next asked whether loss of ACE2 affected these key functions of HS/PCs. Akita mice demonstrated reduced lineage^{-} c-Kit^{+} (LK) cell

proliferation at 3 months of diabetes (Figure 2A). Absence of ACE2 worsened the diabetes-mediated impairment of proliferation at both early and late stages of diabetes (Figure 2A). However, when the cells were treated with either Ang-1–7 or alamandine, two vasoactive peptides of the RAS axis, proliferation was restored. Similar beneficial responses were observed in cells from mice with nine months of diabetes (Figure 2B). Migratory function to the mouse chemoattractant CXCL12 was also reduced in LK cells from *ACE2*$^{-/y}$-Akita mice with nine months' duration of diabetes. However, migratory function was restored when cells were pretreated with Ang-1–7 or alamandine and then exposed to CXCL12 (Figure 2C and D).

Loss of ACE2 worsens diabetes-induced myelopoiesis and reduction of lymphopoiesis

Myelopoiesis was observed in the Akita and *ACE2*$^{-/y}$-Akita mice at 9 months of diabetes as demonstrated by an increase in colony forming units (CFU)-Granulocyte/Macrophage/GM (G/M/GM) (Figure 3A). Common lymphoid progenitors (CLPs) are daughter cells of HS/PCs that are able to differentiate into lymphoid lineages (Figure 3). Phenotypic CLP markers are lineage^{-}CD127^{+}Sca-1^{med}c-Kit^{med}. The percentage of CLPs was decreased in Akita mice compared to WT mice at the 9-month time point. A further reduction of CLP percentage was observed in the *ACE2*$^{-/y}$-Akita mice when compared to Akita alone (Figure 3C). These data suggested that diabetes resulted in lymphopoiesis. ACE2 deficiency further worsened the diabetes-mediated changes in hematopoiesis. These data suggest that diabetes results in a shift of hematopoiesis towards myelopoiesis, together with an impairment of lymphopoiesis. ACE2 deficiency further worsened the diabetes-mediated changes in hematopoiesis.

Diabetic mice exhibit reduced retinal thickness

Retinal thickness was assessed by OCT in each of the four cohorts (Figure 4A). Retinas in the Akita and *ACE2*$^{-/y}$-Akita groups (~9.2% reduction) were thinner in comparison to WT and *ACE2*$^{-/y}$ mice, suggesting this effect was not the result of loss of ACE2 (Figure 4B) but rather diabetes.

When examining the central and peripheral retina by fundus photography, we observed that loss of ACE2 led to a dramatic increase in the number of white lesions in the retina, which may represent retinal nerve fiber layer infarcts (Figure 4C and D). These retinal changes occurred irrespective of diabetes suggesting that they were due to loss of ACE2.

ACE2$^{-/y}$-Akita showed a progressive reduction in retinal electrical responses over the duration of diabetes

The electroretinogram (ERG) measures the electrical responses of distinct retinal cell types and provides information about retinal function with scotopic a-waves representing the electrical signal of rod photoreceptors and scotopic b-waves reflecting depolarizing bipolar cells and Müller cells [35]. At 3 months of diabetes, the Akita and the *ACE2*$^{-/y}$-Akita cohorts demonstrated similar scotopic a- and b- wave amplitudes when compared to WT and *ACE2*$^{-/y}$ cohorts with the exception that Akita mice had a slight increase of a-wave amplitude compared to *ACE2*$^{-/y}$ mice. At 9 months of diabetes, Akita mice showed reduced scotopic b-wave but no change in a-wave when compared to WT mice (Figure 5). In

addition, only the loss of ACE2 in the presence of diabetes caused a reduction of both scotopic a- and b-waves, suggesting ACE2 ablation worsen diabetic retinal function.

Retinal microvascular complications in *ACE2*$^{-/y}$-Akita mice

The hallmark feature of diabetic microvascular dysfunction is an increase in the number of acellular capillaries, defined as basal membrane tubes lacking endothelial cell and pericyte nuclei. At 9 months of diabetes, there was a significant increase in acellular capillary numbers in the Akita mice and *ACE2*$^{-/y}$-Akita mice as compared to WT, with the double mutant showing a greater (1.6 fold) increase than Akita. (Figure 6 A, B). These data suggest that the ACE2 loss exacerbates this retinal microvascular defect.

Migratory dysfunction of human bone marrow-wderived CD34^{+} cells is associated with diabetic retinopathy severity

Healthy subjects, diabetic with no microvascular complications or those with retinopathy were assessed for systemic levels of RAS peptides and for CD34^{+} cell function. The clinical characteristics of the healthy and diabetic subjects are summarized in Supplemental Table 1. The levels of Ang II were similar in diabetes with no complications, diabetics with retinopathy and healthy subjects. In healthy controls, Ang-1–7 levels were low (less than 2.4 pg/ml). Ang 1–7 was similarly low in diabetics with retinopathy. However, levels were elevated in diabetic subjects protected from development of retinopathy despite a protracted history of diabetes (Figure 7A).

CD34^{+} cells are bone marrow-derived cells that are considered to be human HS/PCs with vascular reparative potential. We next accessed whether changes in the RAS axis in these cells is associated with development of retinopathy. Gene expression for MAS, the receptor for Ang-1–7, was increased in CD34^{+} cells from diabetics with no retinopathy while significantly reduced in those with retinopathy (Figure 7B).

CD34^{+} cells isolated from healthy subjects or diabetic patients with no complications migrated toward the chemoattractant, CXCL12, while cells from subjects with retinopathy exhibited impaired migration (Figure 7C). However, pretreatment of the cells with Ang-1–7 and alamandine restored migration from cells of subjects with retinopathy (Figure 7C, D).

Discussion

In this report, we used both murine and human studies to assess the impact of the protective RAS on bone marrow HS/PCs and development of retinopathy. Using *ACE2*$^{-/y}$-Akita mice, we showed that loss of ACE2 expression worsens diabetes-induced bone marrow defects as demonstrated by a reduction in the numbers of the ST-HSCs and LT-HSCs, increased myelopoiesis and diminished common lymphoid progenitors, and impaired migration and proliferation of HS/PCs that was corrected by pretreatment with Ang1–7 or alamandine. *ACE2*$^{-/y}$-Akita mice exhibited a greater number of acellular capillaries compared to Akita mice.

In human subjects, defects were observed in bone marrow-derived CD34^{+}cells isolated from subjects with retinopathy. Reduced migratory function was corrected by the exogenous

supplementation of Ang-1–7 or alamandine. Serum Ang-1–7 levels were elevated in diabetic subjects with no retinopathy, but reduced in subjects with DR. Gene expression for MAS, the receptor for Ang-1–7, was increased in $CD34^+$ cells from diabetics with no retinopathy but was significantly reduced in those with retinopathy. Collectively, these results support the critical role of sustained activation of the protective RAS in bone marrow cells for the prevention of retinopathy.

Bone marrow cells influence vascular repair, blood flow and endothelial barrier integrity by paracrine mechanisms [42]. Extensive literature supports that in chronic diabetes bone marrow cells become dysfunctional and are unable to support the function of the vasculature or repair injury [31, 41, 43–48]. Circulating angiogenic cells, although a small percentage of all circulating cells derived from bone marrow HS/PCs, exert robust vascular protective effects largely mediated by their production of growth factors and cytokines [42]. These factors are vasomodulatory and can mediate changes in vascular reactivity as well as maintain barrier characteristics of the endothelium [49]. However, circulating angiogenic cells of diabetics, such as $CD34^+$ cells, typically lose this ability and secrete proinflammatory and vasoconstrictive factors [42, 50]. Additionally, diabetic circulating angiogenic cells lose their ability to migrate into tissues in response to local injury [31, 51–53].

In this study, we saw that the levels of ACE2 mRNA in $CD34^+$ cells isolated from peripheral blood of diabetic individuals predicted the in vitro function of these cells and directly correlated with the development of retinopathy. As described above, ACE2 generates Ang-1–7, which mediates the beneficial effect of ACE2. Ang-1–7's effect is dependent on MAS receptor expression. We have observed that Ang-1–7 can enhance the migratory function of circulating angiogenic cells in vitro [25]. Ang-1–7 overexpression by lentiviral gene modification restored *in vitro* vasoreparative function of diabetic $CD34^+$ cells and "*in vivo*" homing efficiency to areas of ischemia [25]. Genetic ablation of MAS prevented ischemia-induced mobilization of bone marrow cells thereby impairing blood flow recovery [54, 55].

In the current study, we demonstrated that the peptide alamandine beneficially impacts the migration of dysfunctional $CD34^+$ from diabetics with retinopathy. Thus, these studies and others suggest that activation of the ACE2/Ang-1–7/MAS pathway stimulates the function of dysfunctional murine and human bone marrow cells in diabetes [56]. Here we observed, for the first time, that alamandine has similar beneficial effects on HS/PC migration and proliferation functions as Ang-1–7 in both diabetic mouse model and human subjects. Alamandine is a newly found peptide in the protective RAS axis and is also generated by ACE2 [57]. Alamandine and Ang-1–7 are both composed of 7 amino acids. Alamandine differs only by one N-terminal amino acid from Ang-1–7. Unlike Ang-1–7 binding to MAS receptor, alamandine binds to a different receptor, the MAS-related G protein coupled receptor, membrane D (MRGPRD) [57]. The downstream signaling pathways of MAS involve activation of phosphatidylinositol 3-kinase (PI3K)/Akt and Slit3/ROCK [25, 55]. It is not known whether alamandine/MRGPRD share the same signaling pathway with Ang-1–7/MAS. It is important to investigate in the future the underlying mechanisms of the beneficial effects of almandine on HS/PC functions.

Another novel finding we observed is that loss of ACE2 in diabetes not only caused a profound depletion of ST- and LT-HSCs, but also resulted in an imbalance of myelopoiesis and lymphopoiesis. It is well established that long standing diabetes causes a shift of hematopoiesis towards enhanced myeloid cell production in both type 1 and type 2 diabetic model, which can drive the production and activation of proinflammatory cells [58, 59]. Although several factors are suggested to play a role in this pathological change, including hyperglycemia, leptin deficiency, and impaired cholesterol efflux [58, 60], the underlying molecular mechanisms remain unclear. This study, to our best knowledge, verifies for the first time that the protective RAS serves as a novel mechanism in the maintenance of the balance of myelopoiesis/lymphopoiesis in diabetes, thereby adding new perspectives to the understanding of pathological mechanisms of diabetes on the generation of bone marrow defects.

Diabetes and hyperglycemia result in an increased influx of proinflammatory cells into the retina and secretion of cytokines by the retina [61, 62]. Activation of myelopoiesis in the diabetic bone marrow leads to increased monocytes in the circulation. Myeloid-derived monocytes/macrophages then infiltrate into the diabetic retina, causing leukostasis, which can lead to occlusion of retinal capillaries [63–65]. Bone marrow-derived monocytes can also activate resident microglia, Müller glia and astrocytes in the retina by secreting proinflammatory cytokines, which further exacerbate retinal inflammation and can lead to retinal vaso-degeneration [66–69].

Previously, we showed that bone marrow dysfunction and bone marrow neuropathy precedes the development of retinopathy and that HSC are released in a circadian manner that is altered in diabetes [41]. However, when only measuring one time point as in this study, levels of LSK cells were similar between diabetic and nondiabetic groups. Furthermore, since the blood has considerably fewer LSK cells than the bone marrow, it was not possible to fully characterize these cells into long term- and short term-HSCs as we did for the bone marrow. We also tried to identify the c-kit$^+$ cells using retinal cross sections by immunofluorescence staining. However, the c-kit$^+$ cells are extremely rare in the retina and we were unable to detect them using this approach. Previously, we have used gfp chimeric mice to identify the presence of bone marrow-derived cells in the retina but this approach detects all CD45$^+$ cells not just the c-kit$^+$ cells [51, 70–72].

The impact of the RAS on DR finds support in numerous reports that classic RAS components [prorenin, renin, angiotensinogen, angiotensin-converting enzyme (ACE)-1, angiotensin II (Ang II), and the Ang II type 1 receptor (AGTR1)] are significantly elevated in ocular tissues of diabetic patients [73, 74] and animals [29, 75]. Previously, we showed that retinopathy can be prevented/reversed in streptozotozin-induced diabetes by increasing retinal ACE2 using adenoassociated virus (AAV)-ACE2 [24, 29].

ACE inhibitors and AT1R antagonists attenuate retinal microvascular disease in diabetic rodents by decreasing vascular hyperpermeability, acellular capillaries, and the expression of angiogenic factors, such as vascular endothelial growth factor [76]. AT1R blockade decreased the incidence of diabetic retinopathy in individuals with T1D and improved mild and moderate diabetic retinopathy in those with T2D but not advanced retinopathy [77, 78].

Lack of sufficient penetration of these agents across the blood-retinal barrier was deemed responsible for why suppression of the classic RAS fails to treat advanced disease [29]. These studies led to the notion that restoring the balance between the classic RAS and the vasoprotective RAS represents a viable strategy for controlling diabetic retinopathy in humans. Furthermore, we show, for the first time, that ACE2 plays a critical role in the protection of the neural retina. This protection may be due, in part, to regulation of HS/PCs. ERGs record the electrical responses of the neural retina. Scotopic a-waves represent the neural signal of the rod photoreceptors, while scotopic b-waves are mainly generated by the bipolar cells [79]. In several different retinal injury models, intravitreal administration of HS/PCs have been shown to improve ERG measurements [35, 80]. HS/PCs can have a beneficial effect on the neural retina by i) direct upregulation of genes involved in photoreceptor maintenance, phototransduction, and sensory perception [80, 81]; ii) suppression of proapoptotic gene expression, including caspase-3 [80]; and iii) reduction of oxidative stress [80]. We observed that ACE2 deficiency in the Akita mice resulted in a reduction of both the scotopic a- and b- waves. This change in the scotopic a- and b- waves was associated with a depletion and dysfunction of bone marrow HS/PCs. Moreover, A healthy retinal vasculature is required to maintain the function of the neural retina and HS/PCs serve a critical role in vascular repair.

In addition, ACE2 may affect the neural retina by other mechanisms than modulating HS/PC function. When we examined the mice using color fundus photography, both $ACE2^{-/y}$ and $ACE2^{-/y}$-Akita mice had an increase in white lesions, which we interpret as nerve fiber layer infarcts. No lesions were observed in the Akita mouse. This result suggests that the loss of ACE2 may be responsible for the observed neural layer changes and that ACE2 is needed for protection against development of these lesions.

Conclusion

In conclusion, we found that loss of ACE2 worsened diabetes-induced bone marrow changes and accelerated the progression of retinopathy. The phenotypic pathology observed in the bone marrow-derived cells of *ACE2*$^{-/y}$-Akita mice was also found in $CD34^{+}$ cells of diabetic human subjects with diabetic retinopathy. Diabetes-induced migratory and proliferative dysfunction could be corrected by exogenous administration of the RAS peptides Ang-1–7 and alamandine. Our studies support that enhancing ACE2 or Ang 1–7 or alamandine could represents a potential therapeutic option for diabetic retinopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Patel VB, Zhong JC, Grant MB et al. Role of the ACE2/Angiotensin 1–7 Axis of the Renin-Angiotensin System in Heart Failure. Circ Res 2016;118:1313–1326. [PubMed: 27081112]
2. Thangaraju P, Chakrabarti A, Banerjee D et al. Dual blockade of Renin Angiotensin system in reducing the early changes of diabetic retinopathy and nephropathy in a diabetic rat model. N Am J Med Sci 2014;6:625–632. [PubMed: 25599050]
3. Fletcher EL, Phipps JA, Ward MM et al. The renin-angiotensin system in retinal health and disease: Its influence on neurons, glia and the vasculature. Prog Retin Eye Res 2010;29:284–311. [PubMed: 20380890]
4. Miller AG, Zhu T, Wilkinson-Berka JL. The renin-angiotensin system and advanced glycation end-products in diabetic retinopathy: impacts and synergies. Curr Clin Pharmacol 2013;8:285–296. [PubMed: 23173957]
5. Beyazit Y, Purnak T, Guven GS et al. Local bone marrow Renin-Angiotensin system and atherosclerosis. Cardiol Res Pract 2010;2011:714515. [PubMed: 21234405]
6. Ferrario CM, Richmond RS, Smith R et al. Renin-angiotensin system as a therapeutic target in managing atherosclerosis. Am J Ther 2004;11:44–53. [PubMed: 14704595]
7. Haznedaroglu IC, Beyazit Y. Pathobiological aspects of the local bone marrow renin-angiotensin system: a review. J Renin Angiotensin Aldosterone Syst 2010;11:205–213. [PubMed: 20807797]
8. Haznedaroglu IC, Malkan UY. Local bone marrow renin-angiotensin system in the genesis of leukemia and other malignancies. Eur Rev Med Pharmacol Sci 2016;20:4089–4111. [PubMed: 27775788]
9. Ozturk MA, Guven GS, Haznedaroglu IC. How hematopoietic stem cells know and act in cardiac microenvironment for stem cell plasticity? Impact of local renin-angiotensin systems. Med Hypotheses 2004;63:866–874. [PubMed: 15488662]
10. Richmond RS, Tallant EA, Gallagher PE et al. Angiotensin II stimulates arachidonic acid release from bone marrow stromal cells. J Renin Angiotensin Aldosterone Syst 2004;5:176–182. [PubMed: 15803435]
11. Strawn WB, Ferrario CM. Angiotensin II AT1 receptor blockade normalizes CD11b+ monocyte production in bone marrow of hypercholesterolemic monkeys. Atherosclerosis 2008;196:624–632. [PubMed: 17692319]
12. Strawn WB, Richmond RS, Ann Tallant E et al. Renin-angiotensin system expression in rat bone marrow haematopoietic and stromal cells. Br J Haematol 2004;126:120–126. [PubMed: 15198742]
13. Uz B, Tatonyan SC, Sayitoglu M et al. Local Renin-Angiotensin system in normal hematopoietic and multiple myeloma-related progenitor cells. Turk J Haematol 2014;31:136–142. [PubMed: 25035670]
14. Jokubaitis VJ, Sinka L, Driessen R et al. Angiotensin-converting enzyme (CD143) marks hematopoietic stem cells in human embryonic, fetal, and adult hematopoietic tissues. Blood 2008;111:4055–4063. [PubMed: 17993616]
15. Sinka L, Biasch K, Khazaal I et al. Angiotensin-converting enzyme (CD143) specifies emerging lympho-hematopoietic progenitors in the human embryo. Blood 2012;119:3712–3723. [PubMed: 22282502]
16. Haznedaroglu IC, Beyazit Y. Local bone marrow renin-angiotensin system in primitive, definitive and neoplastic haematopoiesis. Clin Sci (Lond) 2013;124:307–323. [PubMed: 23157407]
17. Shen XZ, Bernstein KE. The peptide network regulated by angiotensin converting enzyme (ACE) in hematopoiesis. Cell Cycle 2011;10:1363–1369. [PubMed: 21441775]
18. Vrsalovic MM, Pejsa V, Veic TS et al. Bone marrow renin-angiotensin system expression in polycythemia vera and essential thrombocythemia depends on JAK2 mutational status. Cancer Biol Ther 2007;6:1434–1436. [PubMed: 17873518]
19. Lin C, Datta V, Okwan-Duodu D et al. Angiotensin-converting enzyme is required for normal myelopoiesis. FASEB J 2011;25:1145–1155. [PubMed: 21148418]
20. Kato H, Ishida J, Imagawa S et al. Enhanced erythropoiesis mediated by activation of the renin-angiotensin system via angiotensin II type 1a receptor. FASEB J 2005;19:2023–2025. [PubMed: 16223784]

21. Hara M, Ono K, Wada H et al. Preformed angiotensin II is present in human mast cells. Cardiovasc Drugs Ther 2004;18:415–420. [PubMed: 15770428]
22. Huang YL, Kuang J, Hu YZ et al. Bone marrow stromal cell transplantation combined with angiotensin-converting enzyme inhibitor treatment in rat with acute myocardial infarction and the role of insulin-like growth factor-1. Cytotherapy 2012;14:563–569. [PubMed: 22277013]
23. Park TS, Zambidis ET. A role for the renin-angiotensin system in hematopoiesis. Haematologica 2009;94:745–747. [PubMed: 19483149]
24. Dominguez JM, 2nd, Hu P, Caballero S et al. Adeno-Associated Virus Overexpression of Angiotensin-Converting Enzyme-2 Reverses Diabetic Retinopathy in Type 1 Diabetes in Mice. Am J Pathol 2016;186:1688–1700. [PubMed: 27178803]
25. Jarajapu YP, Bhatwadekar AD, Caballero S et al. Activation of the ACE2/angiotensin-(1–7)/Mas receptor axis enhances the reparative function of dysfunctional diabetic endothelial progenitors. Diabetes 2013;62:1258–1269. [PubMed: 23230080]
26. Patel VB, Mori J, McLean BA et al. ACE2 Deficiency Worsens Epicardial Adipose Tissue Inflammation and Cardiac Dysfunction in Response to Diet-Induced Obesity. Diabetes 2016;65:85–95. [PubMed: 26224885]
27. Patel VB, Takawale A, Ramprasath T et al. Antagonism of angiotensin 1–7 prevents the therapeutic effects of recombinant human ACE2. J Mol Med (Berl) 2015;93:1003–1013. [PubMed: 25874965]
28. Shenoy V, Gjymishka A, Jarajapu YP et al. Diminazene attenuates pulmonary hypertension and improves angiogenic progenitor cell functions in experimental models. Am J Respir Crit Care Med 2013;187:648–657. [PubMed: 23370913]
29. Verma A, Shan Z, Lei B et al. ACE2 and Ang-(1–7) confer protection against development of diabetic retinopathy. Mol Ther 2012;20:28–36. [PubMed: 21792177]
30. Dominguez JM, 2nd, Yorek MA, Grant MB. Combination therapies prevent the neuropathic, proinflammatory characteristics of bone marrow in streptozotocin-induced diabetic rats. Diabetes 2015;64:643–653. [PubMed: 25204979]
31. Hazra S, Jarajapu YP, Stepps V et al. Long-term type 1 diabetes influences haematopoietic stem cells by reducing vascular repair potential and increasing inflammatory monocyte generation in a murine model. Diabetologia 2013;56:644–653. [PubMed: 23192694]
32. Patel VB, Bodiga S, Basu R et al. Loss of angiotensin-converting enzyme-2 exacerbates diabetic cardiovascular complications and leads to systolic and vascular dysfunction: a critical role of the angiotensin II/AT1 receptor axis. Circ Res 2012;110:1322–1335. [PubMed: 22474255]
33. Yoshioka M, Kayo T, Ikeda T et al. A novel locus, Mody4, distal to D7Mit189 on chromosome 7 determines early-onset NIDDM in nonobese C57BL/6 (Akita) mutant mice. Diabetes 1997;46:887–894. [PubMed: 9133560]
34. Miller CL, Lai B. Human and mouse hematopoietic colony-forming cell assays. Methods Mol Biol 2005;290:71–89. [PubMed: 15361656]
35. Qi X, Pay SL, Yan Y et al. Systemic Injection of RPE65-Programmed Bone Marrow-Derived Cells Prevents Progression of Chronic Retinal Degeneration. Mol Ther 2017;25:917–927. [PubMed: 28202390]
36. Sulaiman RS, Quigley J, Qi X et al. A Simple Optical Coherence Tomography Quantification Method for Choroidal Neovascularization. J Ocul Pharmacol Ther 2015;31:447–454. [PubMed: 26060878]
37. Bhatwadekar AD, Duan Y, Chakravarthy H et al. Ataxia Telangiectasia Mutated Dysregulation Results in Diabetic Retinopathy. Stem Cells 2016;34:405–417. [PubMed: 26502796]
38. Basu R, Poglitsch M, Yogasundaram H et al. Roles of Angiotensin Peptides and Recombinant Human ACE2 in Heart Failure. J Am Coll Cardiol 2017;69:805–819. [PubMed: 28209222]
39. Jeannet R, Cai Q, Liu H et al. Alcam regulates long-term hematopoietic stem cell engraftment and self-renewal. Stem Cells 2013;31:560–571. [PubMed: 23280653]
40. Kowalczyk MS, Tirosh I, Heckl D et al. Single-cell RNA-seq reveals changes in cell cycle and differentiation programs upon aging of hematopoietic stem cells. Genome Res 2015;25:1860–1872. [PubMed: 26430063]

41. Busik JV, Tikhonenko M, Bhatwadekar A et al. Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. J Exp Med 2009;206:2897–2906. [PubMed: 19934019]
42. Jarajapu YP, Hazra S, Segal M et al. Vasoreparative dysfunction of CD34+ cells in diabetic individuals involves hypoxic desensitization and impaired autocrine/paracrine mechanisms. PLoS One 2014;9:e93965. [PubMed: 24713821]
43. Cogle CR, Wise E, Meacham AM et al. Detailed analysis of bone marrow from patients with ischemic heart disease and left ventricular dysfunction: BM CD34, CD11b, and clonogenic capacity as biomarkers for clinical outcomes. Circ Res 2014;115:867–874. [PubMed: 25136078]
44. Hu P, Thinschmidt JS, Yan Y et al. CNS inflammation and bone marrow neuropathy in type 1 diabetes. Am J Pathol 2013;183:1608–1620. [PubMed: 24160325]
45. Li Calzi S, Purich DL, Chang KH et al. Carbon monoxide and nitric oxide mediate cytoskeletal reorganization in microvascular cells via vasodilator-stimulated phosphoprotein phosphorylation: evidence for blunted responsiveness in diabetes. Diabetes 2008;57:2488–2494. [PubMed: 18559661]
46. Loomans CJ, van Haperen R, Duijs JM et al. Differentiation of bone marrow-derived endothelial progenitor cells is shifted into a proinflammatory phenotype by hyperglycemia. Mol Med 2009;15:152–159. [PubMed: 19295918]
47. Park SS, Moisseiev E, Bauer G et al. Advances in bone marrow stem cell therapy for retinal dysfunction. Prog Retin Eye Res 2017;56:148–165. [PubMed: 27784628]
48. Tikhonenko M, Lydic TA, Opreanu M et al. N-3 polyunsaturated Fatty acids prevent diabetic retinopathy by inhibition of retinal vascular damage and enhanced endothelial progenitor cell reparative function. PLoS One 2013;8:e55177. [PubMed: 23383097]
49. Tasev D, Konijnenberg LS, Amado-Azevedo J et al. CD34 expression modulates tube-forming capacity and barrier properties of peripheral blood-derived endothelial colony-forming cells (ECFCs). Angiogenesis 2016;19:325–338. [PubMed: 27043316]
50. Capla JM, Grogan RH, Callaghan MJ et al. Diabetes impairs endothelial progenitor cell-mediated blood vessel formation in response to hypoxia. Plast Reconstr Surg 2007;119:59–70. [PubMed: 17255657]
51. Chakravarthy H, Beli E, Navitskaya S et al. Imbalances in Mobilization and Activation of Pro-Inflammatory and Vascular Reparative Bone Marrow-Derived Cells in Diabetic Retinopathy. PLoS One 2016;11:e0146829. [PubMed: 26760976]
52. Hazra S, Stepps V, Bhatwadekar AD et al. Enhancing the function of CD34(+) cells by targeting plasminogen activator inhibitor-1. PLoS One 2013;8:e79067. [PubMed: 24223881]
53. Li Calzi S, Neu MB, Shaw LC et al. Endothelial progenitor dysfunction in the pathogenesis of diabetic retinopathy: treatment concept to correct diabetes-associated deficits. EPMA J 2010;1:88–100. [PubMed: 21494317]
54. Pettinato G, De Chiara A, Insabato L. Diagnostic significance of intermediate filament buttons in fine needle aspirates of neuroendocrine (Merkel cell) carcinoma of the skin. Acta Cytol 1989;33:420–421. [PubMed: 2471382]
55. Vasam G, Joshi S, Thatcher SE et al. Reversal of Bone Marrow Mobilopathy and Enhanced Vascular Repair by Angiotensin-(1–7) in Diabetes. Diabetes 2017;66:505–518. [PubMed: 27856608]
56. Singh N, Joshi S, Guo L et al. ACE2/Ang-(1–7)/Mas axis stimulates vascular repair-relevant functions of CD34+ cells. Am J Physiol Heart Circ Physiol 2015;309:H1697–1707. [PubMed: 26386115]
57. Etelvino GM, Peluso AA, Santos RA. New components of the renin-angiotensin system: alamandine and the MAS-related G protein-coupled receptor D. Curr Hypertens Rep 2014;16:433. [PubMed: 24760442]
58. Claycombe K, King LE, Fraker PJ. A role for leptin in sustaining lymphopoiesis and myelopoiesis. Proc Natl Acad Sci U S A 2008;105:2017–2021. [PubMed: 18250302]
59. Vasam G, Joshi S, Jarajapu YP. Impaired Mobilization of Vascular Reparative Bone Marrow Cells in Streptozotocin-Induced Diabetes but not in Leptin Receptor-Deficient db/db Mice. Sci Rep 2016;6:26131. [PubMed: 27188595]

60. Barrett TJ, Murphy AJ, Goldberg IJ et al. Diabetes-mediated myelopoiesis and the relationship to cardiovascular risk. Ann N Y Acad Sci 2017;1402:31–42. [PubMed: 28926114]
61. Hammes HP, Feng Y, Pfister F et al. Diabetic retinopathy: targeting vasoregression. Diabetes 2011;60:9–16. [PubMed: 21193734]
62. Semeraro F, Cancarini A, dell'Omo R et al. Diabetic Retinopathy: Vascular and Inflammatory Disease. J Diabetes Res 2015;2015:582060. [PubMed: 26137497]
63. Abiko T, Abiko A, Clermont AC et al. Characterization of retinal leukostasis and hemodynamics in insulin resistance and diabetes: role of oxidants and protein kinase-C activation. Diabetes 2003;52:829–837. [PubMed: 12606527]
64. Chibber R, Ben-Mahmud BM, Chibber S et al. Leukocytes in diabetic retinopathy. Curr Diabetes Rev 2007;3:3–14. [PubMed: 18220651]
65. Kaji Y, Usui T, Ishida S et al. Inhibition of diabetic leukostasis and blood-retinal barrier breakdown with a soluble form of a receptor for advanced glycation end products. Invest Ophthalmol Vis Sci 2007;48:858–865. [PubMed: 17251488]
66. Abcouwer SF. Angiogenic Factors and Cytokines in Diabetic Retinopathy. J Clin Cell Immunol 2013;Suppl 1.
67. Hinze A, Stolzing A. Differentiation of mouse bone marrow derived stem cells toward microglia-like cells. BMC Cell Biol 2011;12:35. [PubMed: 21854582]
68. Rangasamy S, McGuire PG, Franco Nitta C et al. Chemokine mediated monocyte trafficking into the retina: role of inflammation in alteration of the blood-retinal barrier in diabetic retinopathy. PLoS One 2014;9:e108508. [PubMed: 25329075]
69. Soulet D, Rivest S. Bone-marrow-derived microglia: myth or reality? Curr Opin Pharmacol 2008;8:508–518. [PubMed: 18487084]
70. Grant MB, May WS, Caballero S et al. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. Nat Med 2002;8:607–612. [PubMed: 12042812]
71. Guthrie SM, Curtis LM, Mames RN et al. The nitric oxide pathway modulates hemangioblast activity of adult hematopoietic stem cells. Blood 2005;105:1916–1922. [PubMed: 15546953]
72. Sengupta N, Caballero S, Mames RN et al. Preventing stem cell incorporation into choroidal neovascularization by targeting homing and attachment factors. Invest Ophthalmol Vis Sci 2005;46:343–348. [PubMed: 15623794]
73. Danser AH, Derkx FH, Admiraal PJ et al. Angiotensin levels in the eye. Invest Ophthalmol Vis Sci 1994;35:1008–1018. [PubMed: 8125711]
74. Danser AH, van den Dorpel MA, Deinum J et al. Renin, prorenin, and immunoreactive renin in vitreous fluid from eyes with and without diabetic retinopathy. J Clin Endocrinol Metab 1989;68:160–167. [PubMed: 2642484]
75. Kim JH, Kim JH, Yu YS et al. Blockade of angiotensin II attenuates VEGF-mediated blood-retinal barrier breakdown in diabetic retinopathy. J Cereb Blood Flow Metab 2009;29:621–628. [PubMed: 19107135]
76. Zhang JZ, Xi X, Gao L et al. Captopril inhibits capillary degeneration in the early stages of diabetic retinopathy. Curr Eye Res 2007;32:883–889. [PubMed: 17963108]
77. Chaturvedi N, Porta M, Klein R et al. Effect of candesartan on prevention (DIRECT-Prevent 1) and progression (DIRECT-Protect 1) of retinopathy in type 1 diabetes: randomised, placebo-controlled trials. Lancet 2008;372:1394–1402. [PubMed: 18823656]
78. Sjolie AK, Klein R, Porta M et al. Effect of candesartan on progression and regression of retinopathy in type 2 diabetes (DIRECT-Protect 2): a randomised placebo-controlled trial. Lancet 2008;372:1385–1393. [PubMed: 18823658]
79. Tzekov R, Arden GB. The electroretinogram in diabetic retinopathy. Surv Ophthalmol 1999;44:53–60. [PubMed: 10466588]
80. Machalinska A, Roginska D, Pius-Sadowska E et al. Neuroprotective and antiapoptotic activity of lineage-negative bone marrow cells after intravitreal injection in a mouse model of acute retinal injury. Stem Cells Int 2015;2015:620364. [PubMed: 25810725]

81. Moisseiev E, Smit-McBride Z, Oltjen S et al. Intravitreal Administration of Human Bone Marrow CD34+ Stem Cells in a Murine Model of Retinal Degeneration. Invest Ophthalmol Vis Sci 2016;57:4125–4135. [PubMed: 27537262]

Significance Statement:

Hematopoietic stem/progenitor cells (HS/PC) have garnered attention for their vaso-reparative potential. Longstanding diabetes is associated with HS/PC depletion and dysfunction. Performing both murine and human studies, we tested the hypothesis that the deficiency of ACE2, the key enzyme in the protective axis of the RAS would promote development of diabetic retinopathy by worsening diabetes-induced bone marrow HS/PC dysfunction. Our findings support that sustained activation of the protective RAS in bone marrow cells will prevent of development of retinopathy.

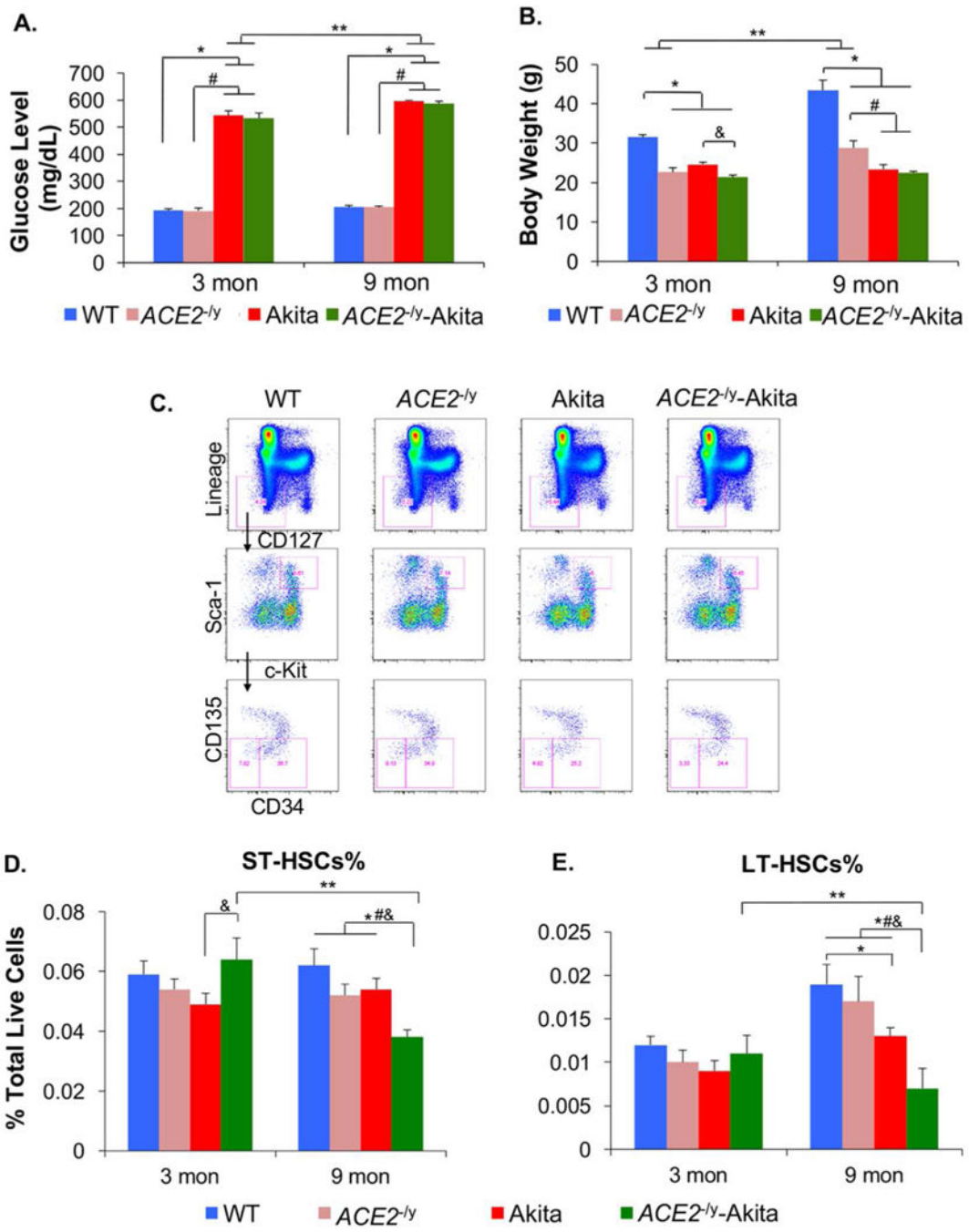


Figure 1. Reduction of both short-term (ST-) and long-term repopulating (LT-) hematopoietic stem cells (HSCs) in bone marrow from ACE2$^{-/y}$-Akita mice at 9 months of diabetes.

(A): Random glucose levels were increased in both Akita and ACE2$^{-/y}$-Akita groups (n = 7–9 per group). (B): Diabetic groups showed consistent low body weight, while the nondiabetic mice had increased body weight over time (n = 9–18 per group). (C): Representative scheme of gating for flow cytometry studies enumerating HSCs in each of the four cohorts, WT, ACE2$^{-/y}$, Akita, ACE2$^{-/y}$-Akita. (D): The percentage of ST-HSCs was decreased in ACE2$^{-/y}$-Akita mice at 9-month time point (n = 12–22). (E): The percentage of LT-HSCs was reduced in the bone marrow of Akita mice compared to WT mice at 9-months.

Loss of ACE2 further decreased the diabetes-induced reduction of LT-HSC enumeration at 9-month time point (n = 12–22). * $p<0.05$, as compared to WT; # $p<0.05$, as compared to $ACE2^{-/y}$; & $p<0.05$, as compared to Akita; ** $p<0.05$, as compared to 3-month time point. Abbreviations: WT, wild type; ST-HSC, short-term repopulating hematopoietic stem cell; LT-HSC, long-term repopulating hematopoietic stem cell; 3 mon, 3 months of diabetes and age-matched control; 9 mon, 9 months of diabetes and age-matched control.

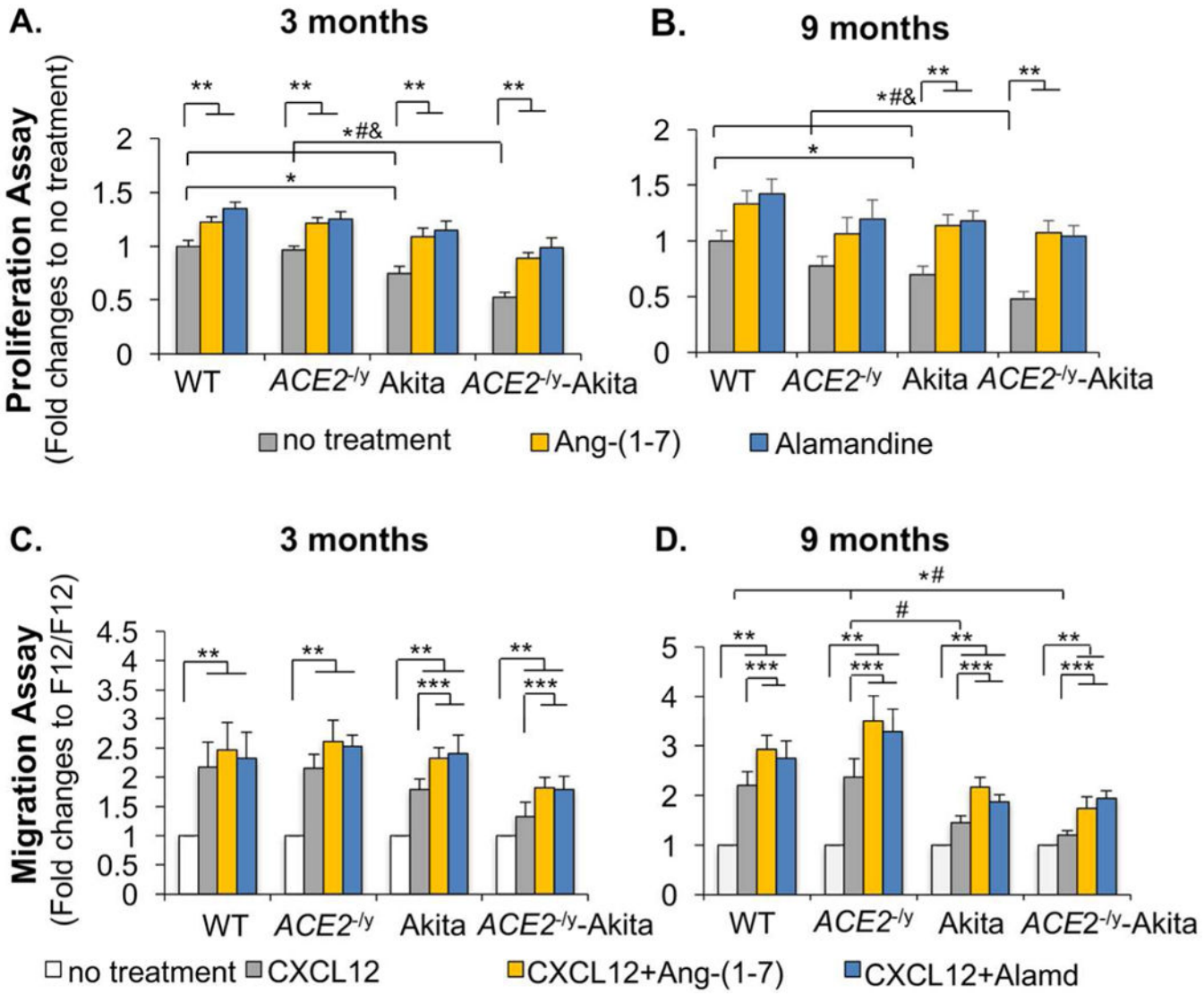


Figure 2. Depletion of ACE2 worsens diabetes-mediated impairment of HS/PC proliferation and migration functions.

(A): Impaired proliferative function of HS/PCs was observed in Akita mice as early as 3 months of diabetes. Loss of ACE2 further reduced proliferation (n = 5–7). (B): Absence of ACE2 exacerbated diabetes-induced impairment of HS/PC proliferation, which was restored by the treatment of Ang-1–7 100nM or alamandine 100nM (n = 5–6). (C): Ang-1–7 or alamandine improved the migration of diabetic HS/PCs toward chemoattractant CXCL12 at 3 months of diabetes (n = 4–6). (D): HS/PCs from ACE2$^{-/y}$-Akita mice had no response to chemoattractant CXCL12. Ang-1–7 or alamandine partially restored the migration of the diabetic HS/PCs (n = 4–6). For A-B, * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2$^{-/y}$; & $p<0.05$, compared to Akita; ** $p<0.05$, as compared to no treatment. For C-D, * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2$^{-/y}$; & $p<0.05$, as compared to Akita; ** $p<0.05$, Compared to no treatment; ***, Compared to CXCL12. Abbreviations: WT, wild type.

A.

	Bone Marrow			Blood
	BFU-E	CFU-G/M/GM	CFU-GEMM	CFUs
WT	14 ± 4	48 ± 8	3 ± 2	35 ± 5
ACE2$^{-/y}$	13 ± 3	48 ± 9	1 ± 1*	30 ± 3*
Akita	11 ± 2$^{*\#}$	61 ± 4$^{*\#}$	1 ± 1*	20 ± 4$^{*\#}$
ACE2$^{-/y}$-Akita	8 ± 2$^{*\#\&}$	73 ± 5$^{*\#\&}$	1 ± 1*	12 ± 6$^{*\#\&}$

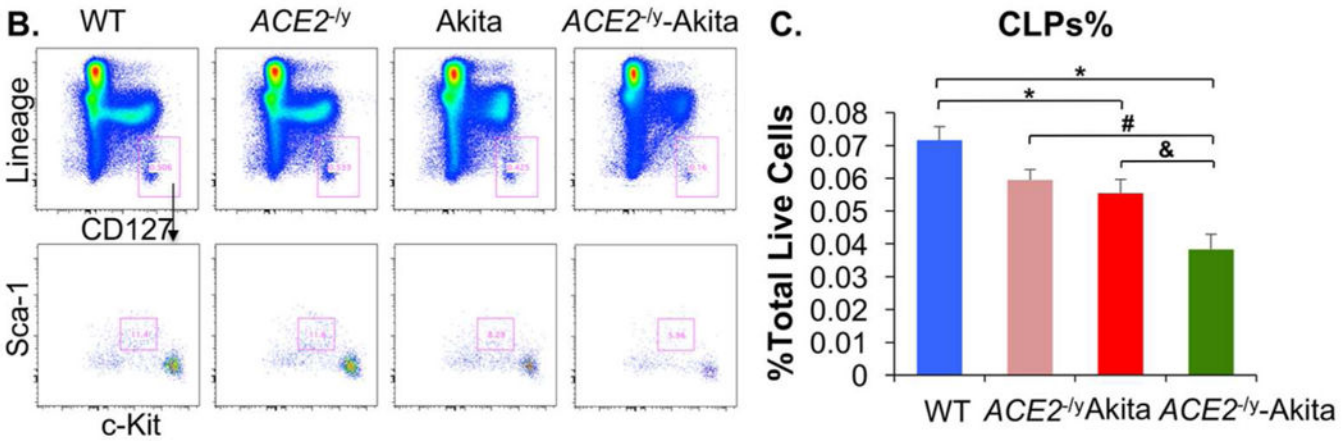
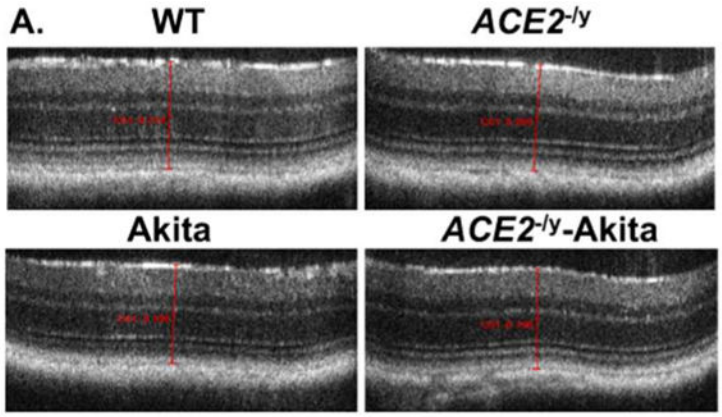


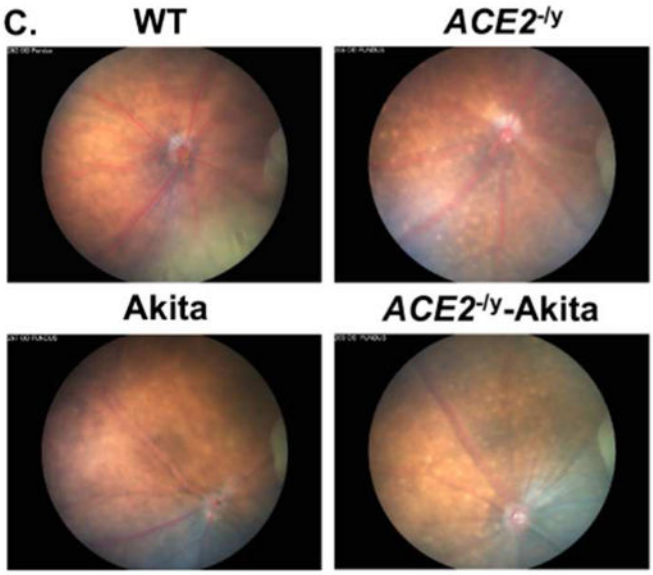
Figure 3. Absence of ACE2 worsens diabetes-mediated imbalance in hematopoiesis.

(A): Colony forming unit (CFU) assay showed an increased number of CFU-G/M/GM when plating bone marrow cells and a decreased number of total CFUs when plating red blood cell-lysed blood cells. Loss of ACE2 exacerbated diabetes-mediated alterations in both bone marrow and blood CFU assay (n = 14–20). (B): Representative gating scheme for enumeration of common lymphoid progenitors (CLP) (Lineage^{-}CD127^{+}Sca-1^{med}c-Kit^{med}). (C): The percentage of CLPs was reduced in Akita mice at 9-month time point. There was a further decrease in CLP percentage in ACE2$^{-/y}$-Akita (as compared to Akita alone) (n = 13–22). * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2$^{-/y}$; & $p<0.05$, as compared to Akita. Abbreviations: CFU, colony forming units; BFU-E, burst-forming unit-erythroid; CFU-G/M/GM, CFU-granulocyte/monocyte/granulocyte-monocyte; CFU-GEMM, CFU-granulocyte/erythrocyte/monocyte/megakaryocyte; CLP, common lymphoid progenitors; WT, wild type.



B.

OCT - Retinal Thickness (μm)	
Types	Mean
WT	219.3±4.2
ACE2$^{-/y}$	221.1±4.2
Akita	201.9±5.9$^{*\#}$
ACE2$^{-/y}$-Akita	203.1±4.4$^{*\#}$



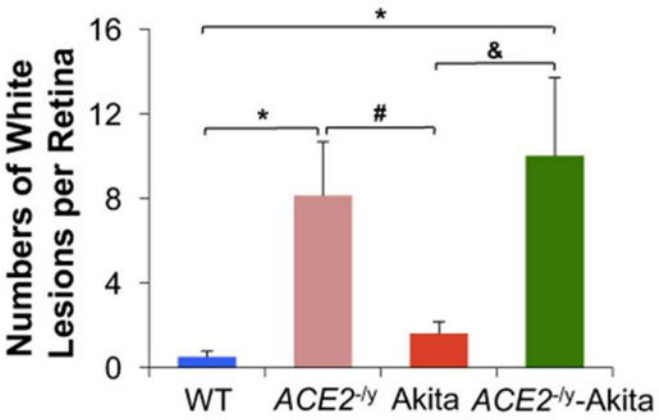


Figure 4. Reduced retinal thickness in Akita groups at 9 months of diabetes.

(A): Representative images of retina near the optic nerve by OCT. (B): Reduced retinal thickness was observed in both Akita and ACE2$^{-/y}$-Akita mice at the 9-month time point when compared to non-diabetic groups (n = 8 per group). (C): Color fundus photography showed increased white lesions in both ACE2$^{-/y}$ and ACE2$^{-/y}$-Akita groups (n = 4–5 per group). (D): Quantification of the number of white lesions per retina. * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2$^{-/y}$; & $p<0.05$, as compared to Akita. Abbreviations: OCT, optical coherence tomography; WT, wild type.

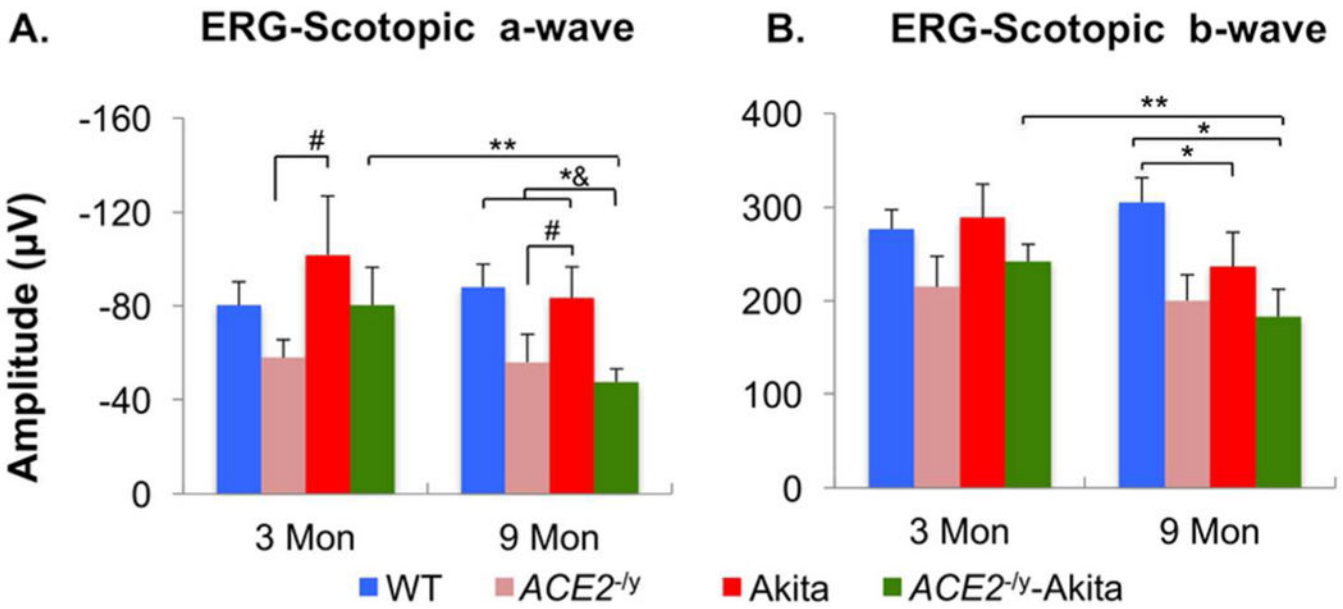


Figure 5. Reduced electrical responses of retinal cells to scotopic light flash in ACE2$^{-/y}$-Akita mice.

(A): scotopic a-waves, which reflects the signal of rod photocurrents, did not change in Akita alone at 9 months of diabetes. Loss of ACE2 caused a decrease in scotopic a-wave amplitude in Akita mice at 9 months of diabetes (n = 5–7). (B): Both Akita mice and ACE2$^{-/y}$-Akita mice exhibited a reduction of scotopic b-wave, indicating low electrical responses of depolarizing bipolar cells and Müller cells. Only ACE2$^{-/y}$-Akita mice showed a reduction of both scotopic a- and b- wave over the duration of diabetes (n = 5–7). * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2$^{-/y}$; & $p<0.05$, as compared to Akita; ** $p<0.05$, as compared to 3-month time point. Abbreviations: ERG, electroretinogram; WT, wild type.

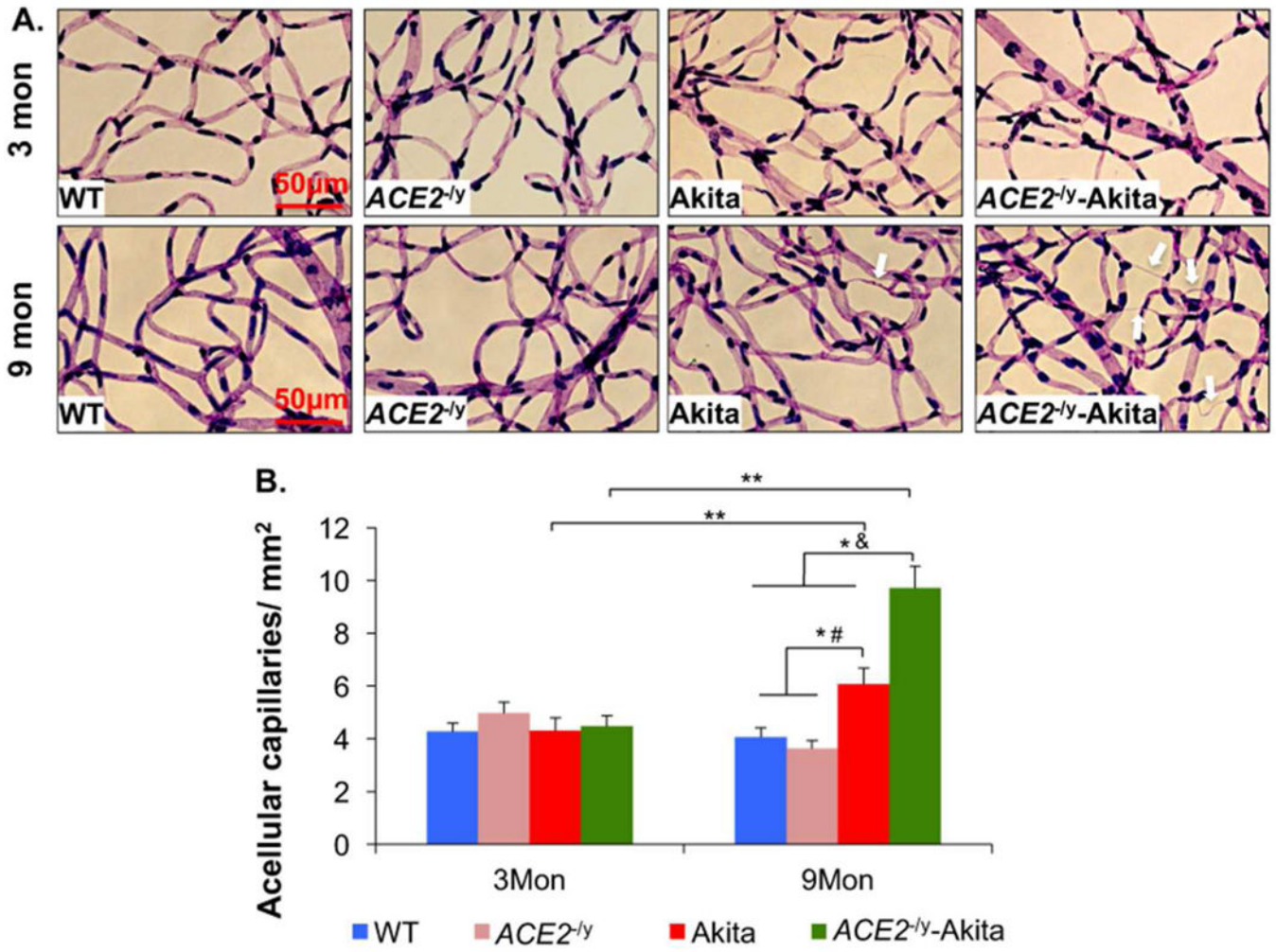


Figure 6. ACE2 ablation worsens diabetes-induced increase in acellular capillaries at 9 months of diabetes.

(A): representative images of retinal vasculature and acellular capillaries (arrow) from the different cohorts at both 3-month and 9-month time points. (B): Increased number of retinal acellular capillaries was observed in Akita mice. ACE2$^{-/y}$-Akita mice had a further increase in acellular capillary number (as compared to Akita mice), suggesting more advanced retinopathy (n = 7–12 per group). * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2$^{-/y}$; & $p<0.05$, as compared to Akita; ** $p<0.05$, as compared to 3-month time point. Abbreviations: WT, wild type.

A.

Groups	Ang II (pg/ml)	Ang-(1-7) (pg/ml)
Control	95.4±18.15	<2.4
DM-NC	105.7±56.20	8.0±3.13*
NPDR	88.0±14.85	2.7±0.57&

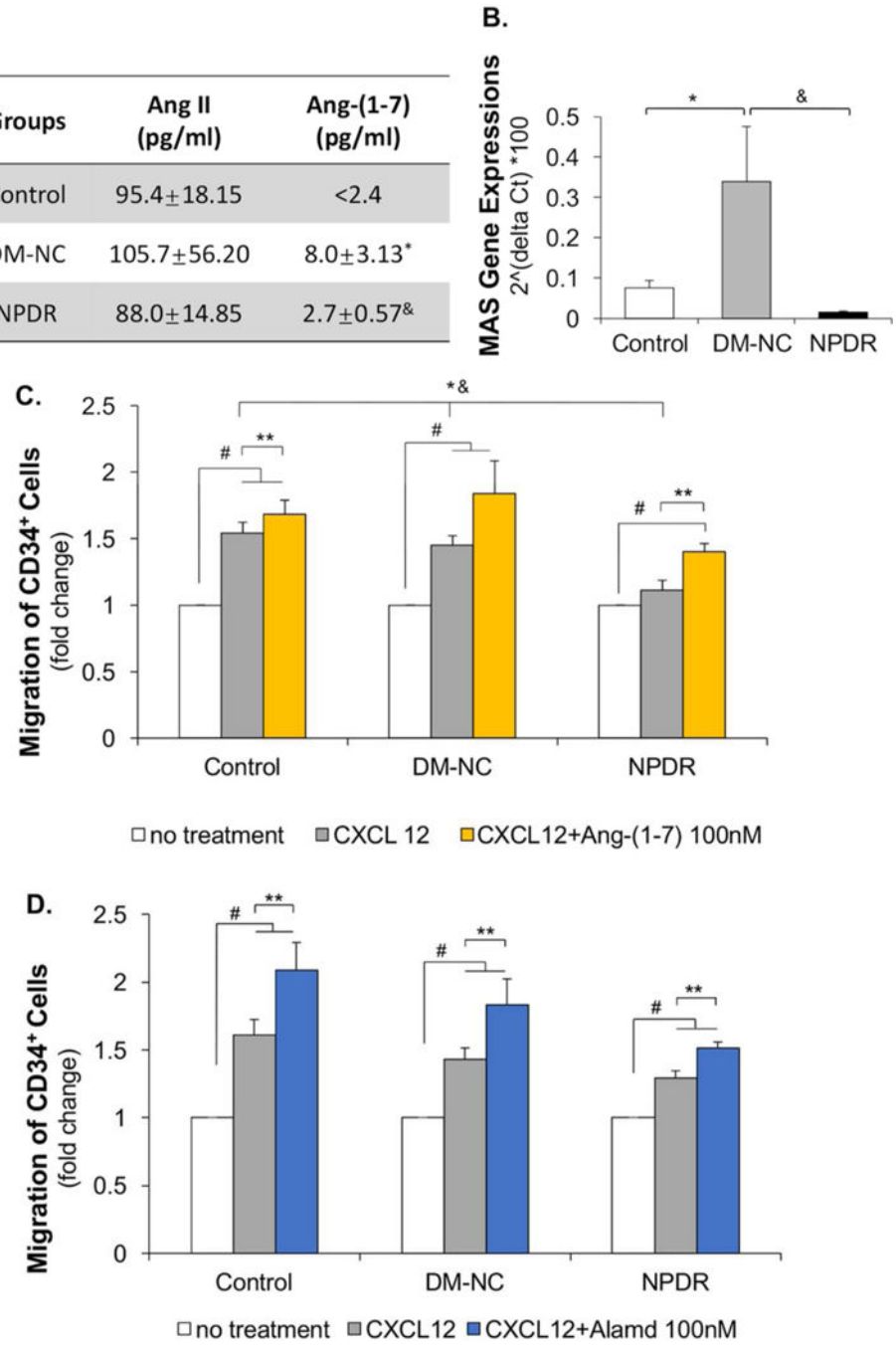


Figure 7. Loss of protective RAS axis and impaired migration function of HSPCs in diabetic subjects with non-proliferative diabetic retinopathy (NPDR).

(A): plasma Ang II and Ang-1–7 peptide levels in healthy control, diabetes with no complications (DM-NC), and diabetes with NPDR. Ang-1–7 level was increased in DM-NC followed by a reduction at NPDR stage (n = 5–10). (B): MAS, receptor for Ang-1–7 was also increased in $CD34^+$ cells from DM-NC patients, but reduced in those from NPDR subjects (n = 5–17). (C): Impaired migration ability of $CD34^+$ cells were observed at the NPDR stage, which can be restored by Ang-1–7 treatment (n = 6–14). (D): Alamandine treatment can also restore the migration function of $CD34^+$ cells at both no complication and

NPDR stages (n = 4–8). For A and B, * $p<0.05$ as compared to control; & $P<0.05$ as compared to DM-NC. For C and D, * $p<0.05$ as compared to control; & $P<0.05$ as compared to DM-NC; ** $p<0.05$ as compared to CXCL12+Ang-1–7 or CXCL12+Alamd group; # $p<0.05$ compared to no treatment group. Abbreviations: DM-NC, diabetes with no complications; NPDR, non-proliferative retinopathy; Alamd, alamandine.