LOSS OF THE PROTECTIVE ARM OF RENIN-ANGIOTENSIN-SYSTEM RESULTS IN HEMATOPOIETIC STEM/PROGENITOR CELL DEFECTS, GUT DYSBIOSIS AND RETINOPATHY IN DIABETES

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Angiotensin-converting enzyme 2 (ACE2) is the primary enzyme of the vasoprotective axis of the renin-angiotensin system (RAS). We tested the hypothesis that the protective arm of the RAS axis can i) act to maintain homeostasis in the diabetic bone marrow stem cell compartment, ii) regulate the reparative function of the hematopoietic stem/progenitor cells (HSPCs) and iii) modulate the gut microbiota composition. All processes could influence the development of diabetic retinopathy.

Diabetic ACE2 knockout (KO)/C57BL/6-*Ins2*$^{WT/C96Y}$ (Akita) mice were examined at 3 and 9 months after the onset of diabetes and compared to age-matched controls. Both ACE2KO-Akita and Akita cohorts showed reduced retinal thickness by optical coherence tomography at 9 months of diabetes. The absence of ACE2 in 9-month diabetic mice led to an accelerated increase in acellular capillaries, a hallmark feature of diabetic retinopathy. The absence of ACE2 also caused a reduction of both long-term and short-term repopulating HSPCs in the diabetic bone marrow at 9 months of diabetes. Reparative function studies showed that ACE2KO exacerbated diabetes-induced impairment of lineage$^-$c-kit$^+$ HSPC migration and

proliferation as early as 3-month of diabetes. HSPCs from both early and late stage diabetic mice, pretreated with Ang-(1-7) or alamandine (two downstream peptides of ACE2) showed restored migration and proliferation. The gut microbiota has been implicated in the pathogenesis of diabetes. Analysis of the gut microbiome also revealed a distinct bacterial profile in ACE2KO-Akita group, with a great diversity of bacterial types that were previously reported to contribute to diabetic pathogenesis, including *Tenericutes* at the phylum level and *Mollicutes* at the class level, and with an activation of peptidoglycan biosynthesis pathways. Flow cytometry analysis showed that loss of ACE2 led to less infiltration of circulating angiogenic cells in the gut which may lead to an increased endothelial cell permeability in the intestinal endothelium. This leakage into the blood may promote systemic inflammation known to contribute to the pathogenesis of diabetic retinopathy. These data suggested a loss of the protective arm of RAS contributes to the impairment of HSPCs and alteration of gut microbiota, both of which may contribute to the development of diabetic retinopathy.

Maria B. Grant, MD, co-chair

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE	Angiotensin I converting enzyme
ACE2	Angiotensin I converting enzyme 2
AGE	Advanced glycation end products
Ang-(1-7)	Angiotensin-(1-7)
Ang II	Angiotensin II
ARB	AT1 receptor blocker (ARB)
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
B^0AT1	Neutral amino acid transporters
CAC	Circulating angiogenic cell
CD	Cluster of differentiation
CFU	Colony forming unit
CFU-G/M/GM	CFU-granulocyte/monocyte/granulocyte, monocyte
cGMP	Cyclic guanosine monophosphate
CLP	Common lymphoid progenitor
COX-2	Cyclooxygenase-2
CXCL 12	stromal cell-derived factor-1
DR	Diabetic retinopathy
ECFC	Endothelial colony forming cell
EPC	Endothelial progenitor cell
EPO	Erythropoietin

ERG	Electroretinogram
FOXO1	Forkhead box protein O1
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GVHD	Graft-versus-host disease
HGF	Hepatocyte growth factor
HSPC	Hematopoietic stem/progenitor cell
IFN	Interferons
IL	Interleukin
IP3	Inositol trisphosphate
JAK	Janus kinase
LK	Lineage$^-$c-Kit$^+$
LPS	Lipopolysaccharide
LP	Lamina propria
LSK	Lineage$^-$sca-1$^+$c-Kit$^+$
LT-HSC	Long term repopulating-hematopoietic stem cell
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MRGPRD	Mas-related G protein coupled receptor, membrane D
MSC	Mesenchymal stem cell
MVC	Microvascular complication
NADPH	Nicotinamide adenine dinucleotide phosphate
NEP	Neutral endopeptidase

NO	Nitric oxide
NOD	Nucleotide oligomerization domain
NOS	Nitric oxide synthetase
NPDR	Non-proliferative diabetic retinopathy
OCT	Optical coherence tomography
PDR	Proliferative diabetic retinopathy
PGN	Peptidoglycan
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PLA2	Phospholipase A2
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SIT1	Signaling threshold regulating transmembrane adaptor 1
Slc6	Solute carrier 6
STAT	Signal transducers and activators of transcription
ST-HSC	Short term repopulating-hematopoietic stem cell
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TLR	Toll-like receptor
TNF	Tumor necrosis factor

VEGF	Vascular endothelial growth factor

Chapter 1: Introduction - RAS balance within bone marrow-gut-retina axis: implications for diabetic retinopathy therapeutics

Renin- angiotensin system

The renin-angiotensin system (RAS) is known as a key regulator of blood pressure and electrolyte balance. There are two main axes composed of eight central components (Figure 1-1): the classic deleterious RAS (angiotensin I converting enzyme (ACE)/ Angiotensin II (Ang II)/ Ang II type 1 receptor (AT1R)) and the protective RAS (ACE2/ Angiotensin-(1-7) (Ang-(1-7))/ alamandine/ MAS/ Mas-related G protein coupled receptor, membrane D (MRGPRD) [1, 2]. Before the discovery of the protective RAS axis, most of the studies focused on the classic RAS which was found to have deleterious effects on the cardiovascular system, including inducing vasoconstriction, fibrosis and inflammation. Moreover, ACE2 inhibitors and AT1R blockers have been used in clinic practice to successfully treated patients with cardiovascular diseases. In the past two decades, a novel RAS axis was found that can counteract the most detrimental effects caused by the classic RAS axis. The “beneficial” axis is composed of ACE2 which can convert deleterious Ang II into beneficial Ang-(1-7) and, thus, this arm of the RAS is considered to play a protective role in maintaining RAS homeostasis [3]. In the last 4 years, another novel peptide, alamandine, was also discovered that can be produced by ACE2 and can have beneficial effects on the cardiovascular system [4].

As a major vasoactive peptide in the canonical RAS, Ang II binds two different cell surface receptors, AT1R and AT2R, which both belong to the G-protein coupled receptor family [5, 6]. AT1R, which is abundantly expressed in the cardiovascular system, is responsible for the major physiological and pathological effects of AngII [7]. The activation of AT1R induces intracellular effects through multiple signaling pathways, primarily adenylyl cyclase / mitogen-activated protein kinase (MAPK)/ phospholipase C (PLC)/phospholipase A2(PLA2)/ Janus kinase (JAK) pathway, PLC/inositol trisphosphate (IP3) pathway, signal transducers and activators of transcription (STAT) pathway and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [8]. In contrast, AT2R is not as widely expressed as AT1R and its expression is typically regulated in a tissue-specific manner [6, 9]. Activation of AT2R mainly induces the nitric oxide (NO)/ cyclic guanosine monophosphate (cGMP) pathways through bradykinin or through activation of endothelial nitric oxide synthetase (NOS) [10], which counteracts the effects of AT1R and reduces inflammation, apoptosis and cardiac remodeling.

In the noncanonical RAS, two key vasoactive components are Ang-(1-7) and alamandine. Ang-(1-7) is mainly produced by converting Ang II by ACE2, and alternatively thought converting Ang I by neutral endopeptidase (NEP) [11, 12]. The receptor for Ang-(1-7) is MAS, a G protein coupled receptor. The binding of Ang-(1-7) to MAS activates the MAPK pathway, phosphoinositide 3-kinase(PI3K)/Akt pathway and NADPH oxidase, and eventually leads to the activation of several downstream effectors, such as vasodilator NO, forkhead box

protein O1 (FOXO1) and cyclooxygenase-2 (COX-2). MAS activation promotes vasodilation, reduces inflammation, reduces oxidative stress, and improves glucose uptake and lipolysis [13-15]. Another vasoactive peptide, alamandine, is generated either from Ang A by the catalytic function of ACE2 or from Ang-(1-7) by a decarboxylation reaction [4]. Alamandine has similar beneficial effects to Ang-(1-7) and promotes vasodilation and reduces fibrosis, however alamandine signals through a different receptor, MRGPRD [2, 16].

Hematopoietic stem/progenitor cells (HSPCs) and diabetes

Bone marrow-derived HSPCs are essential for maintaining hematopoietic homeostasis by self-renewal and differentiation into different lineages. Growing evidence also suggests the important role of HSPCs in angiogenesis and vascular repair. In 1997, Asahara et. al. isolated cluster of differentiation (CD) 34^+ cells from the peripheral blood and considered these cells as “endothelial progenitor cells” or EPCs [17]. Another better defined population, $CD34^+KDR^+CD133^+$ cells, were thought to be EPCs and able to form endothelial cells. However, most recent studies support that $CD34^+KDR^+CD133^+$ cells are hematopoietic cells that express CD45 and are not EPCs, nor do these cells have true vessel forming activity [18, 19]. Another population of cells that are true “EPCs” and have vessel forming activities are $CD34^+CD45^-$ cells, called endothelial colony forming cells (ECFCs) [18, 19]. Both $CD34^+KDR^+CD133^+CD45^+$ cells and $CD34^+CD45^-$ cells have been shown to contribute to vascular repair. While ECFCs promote healing by directly

integrating into injured vessels and forming functional vessels in vivo [20-22], HSPCs promote vascular homeostasis by providing paracrine factors to injured vessels [23-26].

A previous study by Schatteman et. al. showed that local injections of $CD34^+$ cell accelerate the restoration of blood flow in diabetic mice with hindlimb ischemia [27]. Ramos et. al. further demonstrated that the injection $CD34^+CD45^+CD133^+CD38^+$ cells isolated from cord blood improved reperfusion in a hindlimb ischemia model; however, the cells maintained their hematopoietic properties and did not incorporate into blood vessels [28]. Human $CD34^+$ cells release multiple growth factors and cytokines [29]. Studies showed that HSPCs modulated $CD14^+$ cell differentiation and accelerate vascular network formation by releasing paracrine factors, such as interleukin (IL)-8, hepatocyte growth factor (HGF) and monocyte chemoattractant protein-1 (MCP-1) [30-32]. Because of the important roles in vascular repair, HSPCs have become a novel target of autologous cell therapy for diabetes-induced vascular complications, including diabetic retinopathy. However, because HSPCs from diabetic individuals are typically dysfunctional, it is critically important to understand the underlying mechanisms of this impairment before these cells are used for autologous cell therapy.

There is reduced egress of diabetic HSPCs from the bone marrow after injury and decreased vasculogenic potential of these cells in rodent models of type 1 and 2 diabetes [33-35]. Moreover, clinical studies also demonstrated that diabetes

results in a comprehensive remodeling of the bone marrow microenvironment, including a reduction of hematopoietic cells, an increase in fat deposition, and microvascular rarefaction [36]. Poorly controlled glucose levels can cause defects of HSPCs and also alter the microenvironment surrounding the cells [33, 37-40]. Although satisfactory glycemic control helps to restore the number of circulating progenitor cells when compared to those with poor glycemic control, the number is still lower compared to healthy subjects, suggesting other risk factors and mechanisms may also participate in maintaining bone marrow homeostasis in diabetes [41].

The molecular pathways involved in the HSPC dysfunctions include accumulation of reactive oxygen species (ROS), destabilization of hypoxia inducible factor-1 (HIF-1) and inflammation. Sustained exposure of endothelial cells and other cell types to high glucose leads to over-production of ROS, which further activates multiple pathways, including increased advanced glycation end products (AGEs), activation of protein kinase C (PKC) pathways, and increased glucose flux via aldose-reductase pathway, all of which can be blocked by reduction of ROS [42, 43]. In health, the bone marrow niche is well-characterized by the presence of active antioxidant defense systems to maintain ROS at a low level. While a modest generation of ROS promotes stem cell self-renewal and differentiation, and migration to the injured area to help vascular repair [44, 45], diabetes results in a sustained increase in ROS. This results in a sustained inflammatory environment, accumulation of AGEs, and alterations in epigenetic modifications of key genes,

eventually resulting in stem cell senescence, reduced response to hypoxia and decreased angiogenic potential [46-49].

Hypoxia induces activation and stabilization of HIF-1, a transcription factor for a cascade of genes, including stromal cell-derived factor-1 alpha (CXCL12) and vascular endothelial growth factor (VEGF) [39, 50-52]. CXCL12 is important for recruiting HSPCs to the region of damaged vasculature. In diabetes, the accumulation of ROS and toxic glycolytic metabolites impairs the stabilization and activation of HIF-1, therefore leads to impaired homing and deficiencies in angiogenic potential [52-54]. Inflammation is also considered as a key regulator of HSPC fate in diabetes. Under homeostatic conditions, basal niche and immune signaling mediate a balanced lineage output with precise control of the production of myeloid and lymphoid cells. However, in diabetes, the increased proinflammatory signaling, including Toll-like receptors (TLRs), interferons (IFNs), tumor necrosis factor (TNF), and IL-1, adversely impact the long-term repopulating (LT)-HSCs by increasing their apoptosis and results in overproduction of myeloid lineages. Alternatively, lymphoid output (immunosenescence) and erythroid production (anemia) are often diminished [55-62].

RAS within bone marrow hematopoietic stem/ progenitor cells

Even though systemic RAS plays an important role in the homeostasis of the cardiovascular system and electrolyte balance, RAS components are also

reported to present in many other tissues including bone marrow. The notion of RAS involved in the hematopoietic system was identified after ACE inhibitors became widely used in clinics. Two research groups both reported that high dose of ACE inhibitors resulted in anemia and leukopenia [63, 64]. Major components of the RAS were identified in bone marrow cells: HSPCs, stromal cells and mesenchymal stem cells (MSCs) [65]. The findings of a “local RAS” in various tissues have changed the perspective of using RAS as a therapeutic target beyond the cardiovascular system.

The RAS axis affects multiple aspects of hematopoiesis, including erythropoiesis, white blood cells formation as well as the angiogenic potential of HSPCs. Ang II/AT1R has been reported to stimulate and increase early erythroid progenitors [66, 67]. An increased hematocrit was observed in AT1R transgenic mice, while a reduction of hematocrit was found in AT1R knockout mice [68, 69]. Studies suggested that the pro-erythropoiesis effect of the classic RAS may be through JAK/STAT pathway activation that increases the level and activity of erythropoietin (EPO) [70-73]. Interestingly, Ang-(1-7) in the protective RAS also enhances erythropoiesis and reduces anemia in cancer patients following the toxic effects of chemotherapy [74, 75].

Because treatment with ACE inhibitors leads to leukopenia in clinic subjects, the classic RAS is believed involved in the formation of white blood cells [64]. ACE2 depletion results in impairment in normal myelopoiesis [76]. Under physiological

conditions, ACE knockout mice exhibit a decrease in neutrophils, but normal levels of monocytes and macrophages [76-78]. Moreover, under hematopoietic stress, such as irradiation or chemotherapy, Ang II infusion restores the numbers of white blood cells [77]. Ang II mainly plays a facilitating role in myelopoiesis as Ang II increases the colony forming unit (CFU)-granulocyte, monocyte and CFU-granulocyte, erythrocyte, monocyte, megakaryocyte but only in the presence of colony-stimulation factors [79].

Besides myelopoiesis, the classic RAS also plays a role in lymphoid development. AT1R promotes the proinflammatory responses of lymphocytes. Moreover, infusion of Ang II results in an imbalance of $CD4^+$ T-cells (T helper) with reduced Th2 and increased Th1, leading to increased generation of pro-inflammatory cytokines [80]. Accordingly, the classic RAS contributes to the systemic inflammation at least partly through modulation of hematopoiesis. In addition, Ang II recruits monocyte to the vascular wall and activate these cells to secret pro-inflammatory cytokines. Ang II can also increase ROS production and activate NF-kB signaling pathways contributing to an inflammatory phenotype in tissue samples [81, 82].

Studies have reported that in the classic RAS, Ang II/AT1R signaling promotes HSPC recruitment and results in a pro-angiogenic effect as part of an acute response. In contrast, in chronic conditions such as diabetes, it leads to inhibition of HSPC proliferation and functions [83-86]. One mechanism involved in this effect

is activation of NAPDH oxidase activity followed by initiation of JNK/ p38 MAPK/ Bax/ Bcl2 apoptosis pathways [87]. In addition, ROS-mediated cellular senescence may also participate in AT1R-induced deleterious effects on HSPCs [88, 89].

One major effect of Ang-(1-7) on hematopoiesis is improving proliferation and migration functions of HSPCs in response to different stress, such as chemotherapy, irradiation and diabetes [74, 75, 90, 91]. In mononuclear cell cultures from rat or mouse bone marrow, Ang-(1-7) treatment was able to increase the number of angiogenic cells with VEGFR2 expression [92]. Although some of the effects of the protective RAS may due to reduced NADPH oxidase and increased eNOS [93], the molecular mechanisms of Ang-(1-7)/MAS signaling remain largely unknown.

Gut microbiota and diabetes

The gut microbiota is a dynamic ecosystem. Both the environment factors and host conditions can affect its composition at the phylum and class levels, which further influences the gut and other distant organs [94]. Gut microbiota contributes to a large array of human diseases, including obesity and diabetes [95, 96]. An altered composition of the microbiota has been showed in both type 1 (T1D) and type 2 diabetic (T2D) individuals. By studying the gut microbiome from young children that are at high genetic risk for type 1 diabetes and from age-matched healthy

subjects, Adriana *et. al.* showed clear differences in the microbiota between these two cohorts [97]. Firstly, the microbial diversity was reduced over time in the autoimmune subjects. In addition, there was an increase in *Bacteroidetes* level and a decrease in *Firmicutes* level in autoimmune children, suggesting that low ratio of *Firmicutes* to *Bacteroidetes* may be used as a biomarker for development of T1D. Another case-control study in children with T1D [98] observed reduced *Firmicutes* and increased *Bacteroidetes* levels. Therefore, the ratio of *Firmicutes* to *Bacteroidetes* is reduced in T1D and negatively correlates with plasma glucose levels. Interestingly, this study also demonstrated that increased levels of *Clostridium* are positively correlated with hemoglobin A1c (HbA1c) levels.

Consistent with the T1D studies, Larsen *et. al.* also reported reduced *Firmicutes* level in T2D, and the ratio of *Firmicutes* to *Bacteroidetes* negatively correlated with glucose concentrations in plasma [99]. In a cohort of Chinese type 2 diabetic patients, a reduction in butyrate-producing bacteria and an increase in several opportunistic pathogens was observed [100]. In another two independent studies of T2D subjects, the abundance of *Clostridium* species was decreased in diabetic subjects, and the level was negatively correlated with HbA1c and fasting glucose levels [101, 102]. This is contradictory with findings in T1D subjects.

Despite the discrepancies of the results from different groups, all the studies observed clear gut microbial dysbiosis in diabetes when compared to healthy controls. Therefore, it is critical to understand the underlying mechanisms through

which the gut microbiota contributes to the development of diabetes and its complications. Chronic low-grade systemic inflammation has been suggested to contribute to the pathogenesis of diabetes and its vascular complications. Bacteria-derived metabolic endotoxemia, including peptidoglycan (PGN) and lipopolysaccharide (LPS), are both considered as triggers for chronic inflammation [103-106]. The endotoxin, LPS, was associated with both prevalent and incident diabetes [107]. Patrice *et. al.* demonstrated in an animal model that high fat diet chronically increased the proportion of LPS-containing bacteria in the gut and plasma LPS levels. Moreover, continuous subcutaneous infusion of LPS for 4 weeks caused hyperglycemia, hyperinsulinemia and adipose tissue weight gain to a similar level as in mice with high fat diet [103]. Besides LPS, another endotoxin PGN was also found to promote diet-induced insulin resistance and inflammation [106]. LPS initiates inflammatory effects through toll like receptor (TLR)-2/4 activation [108-110]. PGN causes proinflammatory responses mainly via activation of nucleotide oligomerization domain 1 (NOD1) protein in adipocyte and hepatocyte [111, 112]. Unlike NOD1 that mediates PGN-induced inflammation, the intact NOD2 and PGN sensing are important for stabilizing microbiota and preventing bacterial translocation from the gut to liver and adipose tissue [106].

Increased gut permeability in diabetes also provides an opportunity for the bacterial antigens and microbiota to affect the systemic metabolism [113, 114]. The disruption of gut epithelial and vascular barriers lead to increased release of bacterial antigen and result in an increase in systemic inflammatory cytokines [115].

Recent studies suggested an interaction between gut microbiota and immune system in diabetes. Frederico *et. al.* showed that gut microbiota released antigens that drove the differentiation of T helper 1 (Th1) and Th7 cells in pancreatic lymph nodes and contributed to the onset of T1D [116]. Several studies demonstrated that using one or a combination of antibiotics alters the microbiota composition, thereby leading to a modulation of immune system in a T1D mouse model [117-119]. Even in type 2 diabetes, chronic obesity induced-inflammation and beta cell stress can activate the immune system, which further induces NOD and TLR pathways to produce cytokines that destroy beta cells [120-122].

The gut microbiota is able to modulate energy metabolism. The gut microbiota utilizes two sources of nutrients, dietary components and host-derived components, like shed epithelial cells and mucus. During utilization, the microbiota generates several metabolites, such as short-chain fatty acids (SCFA) through carbohydrate fermentation and phenolic metabolites through protein fermentation [123]. Needell *et. al.* reported that maternal treatment with SCFA modulated immunity and ameliorated T1D in offspring [124]. Canfora *et. al.* demonstrated that colonic administration of SCFA mixtures increased fasting fat oxidation and energy expenditure on overweight subjects [125].

Role of RAS in gut microbiota

RAS has been shown to play an important role in the gastrointestinal (GI) tract. Even five decades ago, it was suggested that Ang II regulates the contraction of the gut smooth muscle by a direct effect or by activation of the myenteric plexus cholinergic neurons [126-128]. Most of the components of RAS axis have been identified in the GI tract [129]. In the small intestine, ACE is located abundantly in epithelial cell brush border and in the endothelium of mesenteric microvasculature [130]. ACE2 is present in abundance in brush borders of epithelial cells, muscularis propria and mucosa, as well as, in the mesenteric microvascular endothelium and smooth muscle cells [131]. Remarkably, the GI system, especially ileum, duodenum and jejunum, is reported to have the highest ACE2 mRNA expression levels among 72 human tissue types examined [132].

AT1R is located in epithelial brush order, the myenteric plexus, endothelium in the muscularis propria and muscle layers, while AT2R is mainly expressed in the myenteric plexus [133-136]. Ang I and Ang II have been reported to present in the bottom part of crypts [137]. Angiotensinogen is also widely expressed in multiple locations in the rat small intestines, including epithelial brush border, lamina propria, submucosal blood vessels and muscularis mucosa propria [138]. In the colon, the localization of RAS components is different from the small intestine [129]. ACE is mainly expressed in mesenteric microvasculature, mesenchymal cells as well as lamina propria, and only weakly presents in the epithelial layer in the colon.

ACE2 is localized to the endothelium of the mesenteric microvasculature in the colon and is not expressed in the epithelial layer [131, 139]. Both AT1R and AT2R are detected on surface of epithelium and in crypt bases in the colon. AT1R is also expressed in macrophages, mucosal vessel walls and myofibrolasts [139].

RAS affects multiple pathophysiological functions in the gut. ACE inhibitor or depletion affects epithelial cell apoptosis and turnover [140, 141]. Low doses of Ang II and Ang III stimulate water and sodium absorption in the jejunum possibly via conjugation with sympathetic neuron system. In contrast, high doses of Ang II inhibit water and sodium absorption [142-145]. Ang II also promotes cholesterol absorption through up-regulation of Niemann-Pick C1-like 1 protein expression [146]. In addition, Ang II reduces the expression of sodium-dependent glucose transporter, therefore inhibiting glucose transport across the bush border membrane in the jejunum [138]. Both ACE and ACE2 on brush borders serve as peptidases that regulate mucosal digestion and peptide and amino acid absorption [147-149]. Ang II mediates alkaline secretion through AT2R in the duodenal mucosa [150].

In a mouse model of colitis, both Ang II and AT1R levels are elevated [151]. ACE inhibitors and ARB treatment decrease TGF- α, TGF- β expression and reduce inflammation in colitis [151-155]. Angiotensinogen deficiency also prevent inflammation and development of colitis [156].

Studies in the last several years have highlighted the role of RAS in gut function and microbiota composition. Studies by our group and others showed ACE2 is highly abundant in both mouse and human small intestine, but expressed at a low level in the colon [132, 157, 158]. In 2001, a protein named “collectrin” was discovered in the kidney and interestingly, its sequence was highly homologous to the ACE2 c-terminus [157]. Studies uncovered the role of collectrin as a key subunit for the solute carrier 6 (Slc6) family of neutral amino acid transporters (B^0AT1) and B^0AT3, the Slc1 glutamate and aspartate transporter EAAT3, and the amino transporter, signaling threshold regulating transmembrane adaptor 1 (SIT1) in the kidney [159, 160]. Interestingly, collectrin is not seen in the gut, but B^0AT1 is observed. ACE2, that shares a similar structure with collectrin, plays an identical role as collectrin in gut biology [161, 162]. ACE2 binds and interacts with the neutral amino acid transporter, B^0AT1 in the small intestine, which promotes the absorption of nutrients [149, 162]. ACE2 colocalizes with B^0AT1 on enterocytes of the small intestine but does so with noncatalytic effects. Using an ACE2 knockout model, Tatsuo *et. al.* demonstrated loss of ACE2 resulted in a dramatically reduced plasma tryptophan level. The imbalance of amino acids homeostasis occurred through downregulation of B^0AT1 expression level and led to increased susceptibility to colitis and an altered gut microbiota [161]. Moreover, they also verified that ACE2-induced malnutrition is independent of Ang II and apelin cleavage. The mechanisms by which the ACE2/ B^0AT1 interaction affects microbiota are still unclear; however, one explanation is that ACE2 deficiency-mediates a reduction of tryptophan levels and reduces mTOR pathway activation.

Low mTOR activation leads to reduced antimicrobial peptide secretion by Paneth cells which alter microbial composition [158]. The ACE2 in the small intestine can also stabilize another amino acid transporter, SIT1, that regulates sarcosine, proline, and betaine transfer[160, 163]. It is still largely unknown whether ACE2 contributes to the change of microbiota seen in diabetes and in diabetic complications. The functions of ACE2 in gut biology and the microbiota and the complicated communication between the microbiota and host factors need further exploration.

Diabetic retinopathy

Diabetic retinopathy (DR) is the leading cause of blindness in working age adults in the US [164]. The most common classification for DR is based on a number of photographically detectable features as indicators of disease progression [165-167]. DR is classified into two main forms: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR is further classified into mild, moderate and severe NPDR based on the features, such as microaneurysms, intraretinal hemorrhages, venous beading and other intraretinal microvascular abnormities. PDR is characterized by ocular neovascularization and vitreous/preretinal hemorrhage. The newly formed vessels in PDR stage are often very fragile and leaky. The fibrous connective tissues adherent to and grown around the vessel and the resultant traction finally cause hemorrhage and/or retinal detachment. Both diabetes duration and poor glycemic control contribute to the

development of DR [168-170]. Dyslipidemia is considered another risk factor for DR, which is supported by both the Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) and the Action to Control Cardiovascular Risk in Diabetes (ACCORD) studies [171, 172]. In these clinical trials, fenofibrate and simvastatin, used for lipid control helped ameliorate the progression of DR. Blood pressure control is also important for the development of DR [168, 173]. The United Kingdom Prospective Diabetes Study (UKPDS) group demonstrated that tighter blood pressure control (<150/85 mmHg) significantly reduced the development of microaneurysms, hard exudates and cotton wood signs in the retina, compared to loose blood pressure control (<180/105 mmHg) [174]. The Hoorn study demonstrated that diabetic patients with hypertension had two times higher risk of developing retinopathy after 10 years than diabetic individuals without hypertension [175].

Vascular degeneration is considered an essential feature in the pathogenesis of DR, which directly results in non-perfusion and hypoxia in the injured retina. One early histopathological change observed is thickening of the basement membrane of retinal capillaries. This is observed in both diabetic individuals and animal models. Basement membrane thickening is due to increased extracellular matrix modifications, such as collagen IV, laminin and fibronectin [176-180].

Pericytes are considered a primary target during the early stage of DR [181]. Endothelial cells are also dysfunctional at a very early stage, although they have

adaptive responses and compensation for a period of time, but eventually endothelial cells become dysfunctional [182, 183]. All the alterations, including pericyte and endothelial cell loss and thickening of basement membranes, lead to a central pathological hallmark of DR, acellular capillaries. Acellular capillaries are defined as basal membrane tubes with no nuclei following the procedure of trypsin digestion of the retina. Microaneurysms are another “hallmark” lesion associated with DR and are easily observed on color fundus photography. Microaneurysms represent regions of saccular vascular weakness that are associated with vascular leakage. Clinical pathological studies have shown that non-perfused retina capillaries are often “downstream” of microaneurysms that often occur on the arteriolar side of the microvasculature [176, 184]. This “hallmark” lesion may become infiltrated with monocyte and polymorphonuclear cells [185]. However, it does not occur in rodent models of diabetes, thus limiting research on the pathogenesis of this particular lesion.

Besides vascular degeneration, dysfunction of neural retina is also seen in DR [186]. Neural retinal changes include i) apoptosis and dysfunction of photoreceptors which corresponds to abnormalities of the cone and rod signals on electroretinograms, ii) apoptosis of ganglion cells, and iii) dopaminergic and cholinergic amacrine cell loss [187-191]. Retinal glial cells also play an important role in communication between the blood vasculature and neurons [192, 193]. There are 3 types of glial cells in the retina: two macroglial cell types (astrocytes and Müller cells) and microglial cells. In particular, Müller cells are the structural

and functional link between vasculature and neurons. They process waste products and nutrients and help to maintain the function of both the neurons and blood vessels [193]. In diabetes, an increase in the expression of the glial fibrillary acidic protein (GFAP) is observed. Synthesis of glutamate also occurs in Müller cells of diabetics and eventually leads to excitotoxicity, impaired blood-retina barrier and neuron loss [194-196].

The key cell signaling pathways involved in microvascular complications include increased oxidative stress, inflammation, accumulation of AGEs, activation of PKC and polyol pathway flux [42]. It is worth noting that these cell signaling pathways do not work independently, but work in concert to promote the development of DR. These signaling pathways and their metabolites not only cause the apoptosis of retinal cells and overproduce of the extracellular matrix, but also make the microenvironment unfavorable for vascular repair [197, 198].

Bone marrow derived progenitor cells have been suggested to play an essential role in vascular repair. However, diabetes and poor glycemic control results in low circulating levels of the progenitor cells and cells with impaired vasoreparative potential [199-204]. The impairment in the retinal vasculature and the lack of reparative angiogenesis together contribute to the progression of DR.

Overexpression of VEGF in the ocular fluid of diabetic subjects has been closely related to the development of neovascularization [205]. More recently, a role of

semaphorins in regulation of pathogenic angiogenesis has also been demonstrated [206]. Semaphoring 3A, a protein involved in axonal growth cone guidance, is secreted by retinal neurons in response to hypoxia and is able to guide the neovessel growth toward the vitreous while suppressing the revascularization response in the ischemic retina causing neovascularization.

Current treatment options for DR are still very limited. The control of systemic risk factors, like hyperglycemia, dyslipidemia, blood pressure and smoking will slow the progression of DR. Intravitreal anti-VEGF drugs, laser photocoagulation, corticosteroid agents and vitreoretinal surgery are the current therapies to reduce sight-threatening diabetic macular edema or PDR [197]. Current therapeutic strategies mainly focus on treatment of advanced retinopathy and there is no effective treatment to stop or slow the progression at early stages, beyond treating systemic risk factors.

Role of RAS in diabetic retinopathy

Both mouse and human eyes have their own tissue-specific RAS, which is independent of, and can have interactions with, system RAS [207-210]. Local RAS can be divided into two groups based on whether it depends on the circulating RAS for biosynthesis of the local RAS peptides [211]. Studies have shown that Ang II level is increased in the vitreous fluid of patients with advanced PDR, and promotes neovascularization by potentiating vascular endothelial growth factor-

mediated pathologically angiogenesis [212-214]. However, no studies have examined the levels of Ang-(1-7) at the PDR stage. Unlike human subjects, diabetic mouse models without genetic modifications do not develop PDR, but only reach the non-proliferative stage.

An imbalance of ACE/ACE2 with a reduction of protective RAS was found in the retina from STZ-induced diabetic mice[215, 216]. Administration of ACE2 and Ang-(1-7) in the retina prevents or reverses DR in a T1D model [215]. ACE2 activation also protects retinal ganglion cells in diabetic rats [217]. Interestingly, RAS within HSPCs also plays an essential role in the development of DR. The protective RAS in HSPCs is important for maintaining vasoreparative functions in response to vascular injury [93].

Bone marrow-retina connections

The bone marrow serves as a pool for both HSPCs with angiogenic properties and for monocytes with proinflammatory features. The bone marrow has an influence on many other organs in diabetes, including kidney, brain and retina [218, 219]. Diabetes impairs the bone marrow architecture, function, and the number of reparative cells. Diabetes also impacts bone marrow-derived immune cells. Typical characteristics of the diabetic bone marrow include neuropathy, microangiopathy, stem cell depletion, and an alteration in fat deposition, along with a shift of hematopoiesis [37].

Efficient release of bone marrow-derived circulating angiogenic cells into the circulation and mobilization of these cells into injured tissues is a critical component of vaso-reparative and protective mechanisms that occur in response to vascular ischemia and/or damage. Studies suggest that diabetes-induced bone marrow neuropathy results in trapping of stem/progenitor cells in the bone marrow and decreased the release of these cells into the circulation. Bone marrow neuropathy precedes the development of diabetic retinopathy [220, 221].

One mechanism for HSPC dysfunction is the defective HIF-1α/VEGF/CXCL12 signaling pathway [222]. DPP-4, an enzyme that regulates the concentration of CXCL12 may also contribute to diabetes-induced HSPC mobilopathy. Our group showed that $CD34^+$ cells isolated from diabetics showed impaired migration toward CXCL12 and reduced proliferation, contributing to reduced vascular repair potential. Local injection of adeno-associated virus that over express Ang1-7 restored HSPC migratory function, improve localization to areas of injury and help the repair of injured retinal vasculature by secreting paracrine factors [93].

Hyperglycemia results in an imbalance of hematopoiesis and shifts the fate of progenitor cells towards their differentiation to more inflammotory cells and reduces their differentiation to progenitors with angiogenic potential [219]. Just like HSPC dysfunction, the imbalance of myelopoiesis also plays an essential role in the pathological changes of DR [223, 224]. Diabetes disturbs the distribution and balance of M1 (inflammatory) and M2 (anti-inflammatory) macrophages in the

bone marrow and blood, and affects their response to G-CSF stimulation [218]. Studies showed that diabetes induce accumulation of bone marrow-derived monocytes in the retina and pro-inflammatory myeloid cells from bone marrow play a critical role in endothelial cell loss and vascular degeneration in diabetic retina [223, 225]. Harshini *et. al.* tracked the bone marrow cells by using a STZ-induced DR model with green fluorescent protein (GFP)-labeled bone marrow transplantation, and demonstrated that diabetes significantly increased the filtration and activation of GFP-expressing microglial in the retina while reducing the number of bone marrow-derived circulating angiogenic cells [221]. Prabhakara *et. al.* showed that hyperglycemia increases the production of damage associated molecular patterns, S100A8/S100A9, in neutrophils. Neutrophil S100A8/S100A9 further interacts with receptors for AGE on common myeloid progenitor cells and results in increased myelopoiesis [226]. Interestingly, local administration of ACE2 in the retina reduced diabetes-mediated monocyte infiltration and reverses retinopathy in animal models [215].

Bone marrow-gut crosstalk

The concept of communication between the gut and the bone marrow is supported by evidence that the gut microbiota is an extrinsic regulator of hematopoiesis [227]. Very low levels of microbial antigens set the size of the bone marrow myeloid cell pool which correlates strongly with the complexity of intestinal microbiota [228]. As mentioned before, TLRs, especially TLR-4, are receptors that are essential for

mediating microbial antigens, such as LPS, related functions. Interestingly, TLR-4 is highly expressed in bone marrow-derived cells, including macrophages, providing the possibility that bone marrow-derived cells can be regulated by microbial antigens. Maziyar *et. al.* established a mouse chimera model with hematopoietic cell-specific depletion of TLR-1 (BMT-TLR-4$^{-/-}$). Although BMT-TLR-4$^{-/-}$ mice fed a high fat diet did develop obesity similar to wild time mice, they did not have fasting hyperinsulinemia and were protected from insulin resistance. This study suggested that microbial antigen-mediated modulation of bone marrow macrophage content can contribute to the systemic inflammation and insulin resistance [229].

The microbiota is reported to modulate the proportion and function of HSPCs. In a *Rag*1$^{-/-}$ mouse model lacking all mature lymphocytes, there is an alteration of microbiota composition, which further results in a dramatic reduction of LSK, LT-HSC and short term repopulating (ST)-HSC numbers and proportion, and leads to an increase in myeloid progenitors. *Rag*1$^{-/-}$ mice received fecal transplantation using feces from control mice and show a restoration of HSPC levels in bone marrow [230]. The microbiota of obese mice also alters the number and reconstitution ability of long term HSCs and shifts the hematopoiesis from lymphopoiesis towards myelopoiesis by affecting bone marrow microenvironment [231].

The gut microbiota plays an essential role in modulating immune responses. There are many immune cells, including dendritic cells, lymphocytes and macrophages, in the gut lamina propria, most of which are derived from bone marrow [232]. Commensal gut microbiota regulates proper immune function possibly through microbial antigens, which sustain steady-state recruitment of neutrophils and maintain the functions of the immune system against infections [227, 233-235]. The immune cells in the gut produce a series of extrinsic stimuli, which maintain tonic activity of bone marrow HSPCs, as well as neutrophils, monocytes and lymphocytes [236].

In turn, the bone marrow can also affect the functions of gut in multiple ways [237-245]. Recruitment of bone marrow derived-immune cells to the gut elicits acute inflammation necessary for host defense [246-248] and contributes to inflammation resolution and tissue healing [249-254]. Graft versus host disease (GVHD) represents a good example to explain how bone marrow cells affect the gut microbiota. After bone marrow transplantation, the immune cells from the donor bone marrow may identify the host as “non-self”, therefore causing immune rejection. GVHD is reported to inhibit Paneth cell to produce an antimicrobial molecule, α-defensins, which plays an essential role in modulating gut microbial ecology. The reduced α-defensins cause gut microbiota dysbiosis in GVDH [255, 256]. NOD 2 has been shown to interact with PGN to stabilize the gut bacteria. Alnabhani *et. al.* showed that the bone marrow specific deficiency of NOD 2 disturbed the Peyer’s patches homeostasis and led to increased gut epithelial

permeability [257]. Tao *et. al.* used a chimera mouse with the deficiency of bone marrow beta1/2 adrenergic receptors (b1/2-ARs) expression and investigated the role of the bone marrow sympathetic signaling in gut and microbiota homeostasis [258]. Interestingly, the chimera mice exhibited deceased infiltration of $CD4^+$ T and NK cells in the gut and alterations of gut microbial composition. Taken together, these studies suggested an important role of the bone marrow in the regulation of gut immune system and microbiota composition.

Gut-retina communication

Very interestingly, just like the gut, mouth and skin, the eye also has its own community of bacteria in health which protects the eye from invaders [259-261]. The microbial community harbors on the ocular surface of the eye. For a long time, researchers thought that the ocular surface microbiome had only transiently present organisms and did not have a stable microbiome [262]. However, recently, investigators have identified by 16S rRNA gene sequencing that there is a wide range of homeostatic bacterial species colonized on the ocular surface, confirming the concept of a stable ocular surface microbiome [260, 261]. By deep gene sequencing, 5 phyla and 59 distinct genera were identified in the ocular microbiota. Among those, twelve genera are ubiquitous and abundant among all the subjects analyzed and take in account 96% of the total bacteria [260]. The ocular microbiota may be involved in multiple diseases. Sankaridurg *et. al.* observed a change of microbiota composition in subjects wearing contact lens, which may be

associated with corneal infiltrative events [263]. Lee *et. al.* compared the ocular microbiota between blepharitis patients and healthy subjects, and also observed alterations of microbiota in blepharitis, demonstrating enrichment of *Enhydrobacter*, *Streptophyta*, and *Corynebacterium* species [264].

Recently, studies also suggested a role of non-ocular microbiome in ophthalmic disease. Astafurov *et. al.* compared the oral microbiota between patients with glaucoma and control subjects. There were higher bacterial counts in glaucoma patients compared to controls. In addition, low dose of LPS administration in two separate animal models of glaucoma resulted in activation of microglia in the retina and glaucomatous neural degeneration, suggesting oral microbiome mediated chronic subclinical peripheral inflammation contributes to the pathophysiology in glaucoma [265].

It is gradually established that the impaired immune system and chronic intestinal inflammation may lead to an enhanced permeability of gut epithelial barrier [266]. Research also showed that the microbiota and pathogenic bacteria also damage the gut vascular barrier [267]. Accordingly, increased permeability in the gut provides the opportunity for the microbiota antigens and even pathogenic bacteria themselves to cross the leaky gut into the circulation, and affect distant organs, including the eye. Gut microbiota is required for antibody-mediated adaptive immunity in response to eye infections. Elisabeth *et. al.* showed that high fat diet alters the composition of microbiota with increased *Firmicutes* and decreased

Bacteroidetes and leads to increased permeability of the gut. Pathogen-associated molecular patterns released from the leaky gut into the circulation, resulting in low-grade inflammation and neovascular age-related macular degeneration [268]. Shelton *et. al.* also showed that microbiota plays an essential role in diet-induced choroidal neovascularization. The mice with lower glycemic diet did not develop these ocular changes compared to those on a high glycemic diet. Microbiota analysis revealed that the *acteroidales* order and the microbial metabolite, secrotonin, protective against retinal changes in mice on a low glycemia diet [269].

Interestingly, gut microbiota-derived antigens can stimulate immune cells, and trigger autoimmune uveitis. Horai *et. al.* demonstrated that the gut commensal microbiota-derived antigens bind to autoreactive T cell receptors to activate retina specific T cells and provoke an autoimmunity reaction in the eye [270, 271]. Long term administration of oral antibiotics or germ-free environment are able to prevent experimental autoimmune uveitis from developing [272]. All these studies suggested an existence of gut-retina axis, which provides us a novel direction for studying this gut-retina connection in DR.

Implications for diabetic retinopathy

In summary, all the discussions above suggested that bone marrow, gut microbiota and retina are intertwined. RAS can regulate the functions of these organs and potentially affect diabetic retinopathy through the bone marrow-gut-retina axis.

Here we propose a hypothesis that the bone marrow-gut-retina axis contributes to the development of DR (Figure 1-2). Dysregulated RAS results in reduced ACE2 in the bone marrow and gut and potentially drives the disturbed bone marrow and gut communication seen in diabetes. We postulate that the gut will exhibit decreased circulating angiogenic cell infiltration, increased the permeability of barriers, dysbiosis and increased circulating pathogenic antigens, eventually leading to the progression of DR.

We used a T1D model with ACE2 deficiency (ACE2 knockout-Akita mice) to study the effects of the protective RAS on bone marrow HSPCs, gut microbial dysbiosis and DR. The project is divided into 3 aims: aim 1 focuses on the role of ACE2 in bone marrow hematopoiesis and the function of HSPCs in the context of diabetes; aim 2 examines whether the loss of ACE2 in Akita (spontaneously diabetic) mice accelerates the development of DR; and aim 3 investigates the role of ACE2 in gut microbial dysbiosis and examines the potential links between bone marrow and gut and DR.

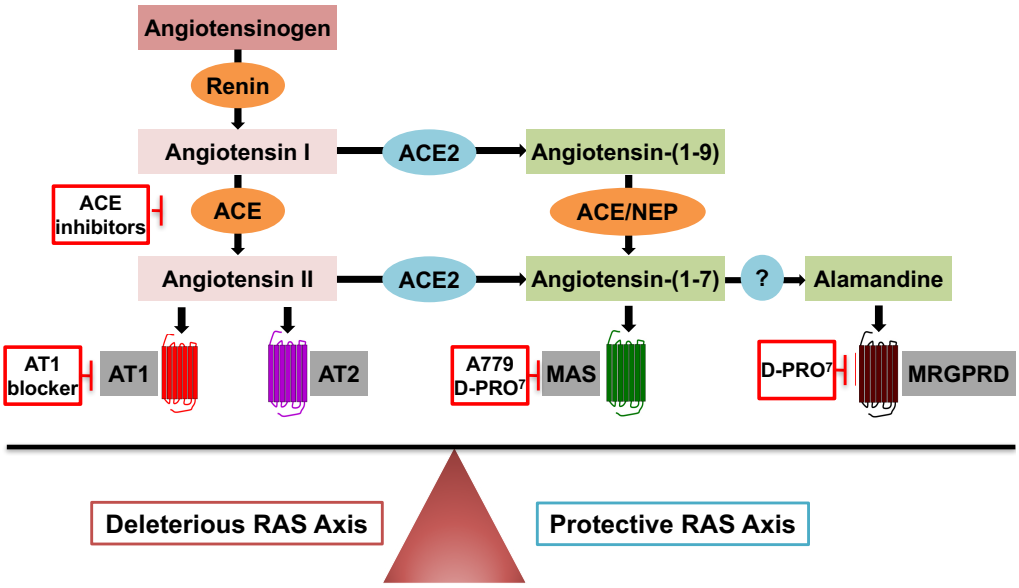


Figure 1-1. Key components of the deleterious and protective axes of renin-angiotensin system.

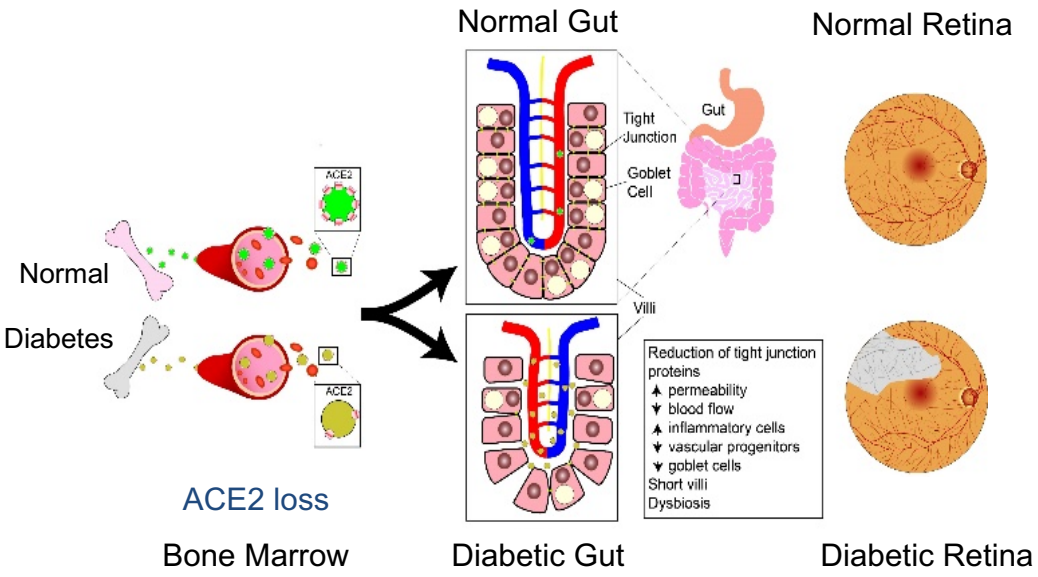


Figure 1-2. Hypothesis: loss of ACE2 disturbs balance to the bone marrow-gut axis and promotes diabetic retinopathy.

Chapter 2: The absence of protective RAS axis exacerbated diabetes-induced impairment of hematopoietic stem and progenitor cells

Introduction

Macro- and micro-vascular complications are the most common complications of diabetes, and lead to markedly increased morbidity and mortality [273, 274]. The molecular pathogenesis of diabetes involves multiple cell signaling pathways, including oxidative stress, inflammation, advanced glycation end products, protein kinase C and polyol pathway, which eventually cause increased endothelial cell damage [275]. However, unlike the pathways involved in endothelial damage, repair mechanisms have been relatively overlooked [222]. Studies have shown that HSPCs play an essential role in vascular repair. Besides maintaining hematopoiesis, bone marrow-derived HSPCs are released into the circulation in response to hypoxia and other stimuli [37, 276]. Circulating hematopoietic cells then home to areas of injury and secret paracrine factors to repair the damaged vasculature. Circulating hematopoietic cells with pro-angiogenic activity are also called circulating angiogenic cells [26, 277].

HSPCs are considered a good source for autologous cell therapy, especially diabetic microvascular complications, because the cells can reach the microvasculature. However, autologous HSPCs from diabetic subjects can be dysfunctional. Studies showed that in diabetic individuals , peripheral blood HSPC

levels are reduced compared to healthy subjects [278, 279]. Data have also suggest that diabetes affects the responses of bone marrow HSPCs to tissue ischemia or to simulation by growth factors and cytokines [26, 227]. In addition, diabetic HSPCs demonstrated a distinct profile of paracrine factor secretory with increased pro-inflammatory factors and reduced stem cell growth factors [26]. HSPCs from diabetics also exhibited impaired proliferation and migration potentials in both preclinical and clinical studies [26, 93]. Therefore, it is important to understand the molecular mechanism of diabetes mediated HSPC defects, and to optimize the cells before their use for cellular therapy.

The classic renin angiotensin system (RAS) axis, composed of angiotensin I converting enzyme (ACE)/angiotensin II (Ang II)/AT1R, is a key regulator of the cardiovascular system, water balance and electrolytes. A novel RAS axis, that mainly comprises angiotensin I converting enzyme 2 (ACE2)/Angiotensin 1-7 (Ang-(1-7))/MAS, counteracts most of the deleterious effects of the classic axis, and is considered the “protective” arm of RAS [280]. Existing evidence from our group and others suggests that the vasoprotective axis of RAS is also a potential target of cell enhancement strategies to restore the function of HSPCs. Our group showed that [93] HSPCs from a unique set of diabetic patients without microvascular complications, despite more than 40 years of poor glycemic control, have higher mRNA levels for vasoprotective RAS genes compared to age, sex and glycemic-matched diabetics with microvascular complications. Moreover, lower expression of the protective RAS genes is associated with impaired function

(migration and proliferation functions) of diabetic HSPCs. Activation of the protective RAS axis, by treating HSPCs with Ang-(1-7), increased the production of bioactive nitric oxide (NO) and reduced reactive oxygen species (ROS), restoring the functions of HSPCs. Similarly, diabetic HSPCs treated with lentivirus-Ang-(1-7) showed restored vasoreparative function in a mouse retinopathy model [216]. These key results suggest that the vasoprotective RAS plays a central role in maintaining HSPC functions and vascular repair in diabetes.

In this study, we generated an ACE2 knockout (ACE2KO)-Akita (*Ins2*$^{WT/C96Y}$) mouse model to test the hypothesis that loss of protective RAS causes a more profound defect in hematopoiesis in diabetes.

Research design and methods

Animals

ACE2 knockout mice were obtained from Dr. Gavin Oudit's laboratory at the University of Alberta. Diabetic heterozygous *Ins2*^{Akita} (Akita) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Male Akita mice were housed in the animal facilities at Indiana University School of Medicine, and bred with female heterozygous ACE2KO (*ACE2*$^{+/-}$) mice to generate ACE2KO-Akita (*ACE2*$^{-/Y}$*Ins2*$^{WT/C96Y}$) male mice, as well as C57BL/6J wild type (WT), Akita and ACE2KO mice. Blood glucose levels were measured using an Accu-Chek Compact Plus

glucose meter (Roche Diagnostics, Indianapolis, IN). ACE2KO-Akita, Akita mice were studied at 3 and 9 months of diabetes. All animal procedures were approved by the Animal Care and Use Committee at the Indiana University School of Medicine. The mice were anesthetized by inhalation of isoflurane and euthanized by decapitation.

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

Bone marrow cells were flushed from mouse femur and tibia bones using phosphate buffered saline (PBS). Lineage$^-$c-Kit$^+$ HSPCs were selected by the lineage negative and c-Kit positive selection kits (Catalog # 19856 and 18757, STEMCELL Technology, Vancouver, BC, Canada) using the EasySep™ magnet following manufacturer's instruction.

Migration assay

Migration function of HSPCs 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). Briefly, 150 μl Ham's F12 medium with or without 100 nM CXCL12 was added to the feeder tray. 20,000 cells were plated in the migration chamber in 100 μl Ham's F12 medium after 30 min pretreatment with Ang-(1-7), alamandine or F12 medium (vehicle). The assay plate was then

incubated at 37°C in a 5% CO2 incubator for 3 hours when using mouse LK cells and 4 hours when using human $CD34^+$ cells. After incubation, the cells were dislodged, lysed in the presence of the CyQuant GR dye solution, and the cell lysates were read by Synergy H1 hybrid plate reader using a 480/520 nm filter set (BioTek, Winooski, VT).

Bone marrow LK cell proliferation

Proliferation function was measured using the cell proliferation BrdU ELISA kit (11647229001, Roche) after 24-hour incubation in serum free stemspan media (#09650, STEMCELL Technologies) containing cytokine cocktails (IL-3 0.02 μg/ml, IL-6 0.02 μg/ml and stem cell factor 0.05 μg/ml) (#403-ML-010, 406-ML-005 and 455-MC respectively, R&D Systems, Minneapolis, MN) with or without the supplement of 100 nM Ang-(1-7), or alamandine.

Colony forming unit assay

Ammonium Chloride Solution (ACS) lysed bone marrow or blood cells were plated in MethoCult™ GF M3434 (STEMCELL Technologies) and then identified following manufacturer's instructions. After vortexing, 1.1 ml MethoCult mixture was dispense into each 35mm petri dish from 3 mL syringes with 16 gauge blunt-end needles. Briefly, cells were diluted in IMDM with 2% FBS to 10× final concentrations of plating. Then, the diluted cells were added into pre-aliquoted

MethoCult at a ratio of 1:10. The final cell concentrations were 5×10^3 per dish for bone marrow and 1×10^5 per dish for peripheral blood cells.

Flow cytometry

Isolated cells were incubated with rat anti-mouse CD16/CD32 (eBioscience, San Diego, California, USA) or 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, Clone 2B8, Biolegend, Cat# 105806; PE anti-mouse CD34, Clone MEC14.7, Biolegend, Cat# 119308; BV421™ anti-mouse lineage cocktail, Biolegend, Cat# 133311; PE/Cy7 anti-mouse Sca-1, Clone D7, Biolegend, Cat# 108114; PerCP-eFluor 710 anti-mouse CD135, Clone A2F10, eBioscience, Cat# 46-1351-82; APC anti-mouse CD16/CD32, Clone 93, eBioscience, Cat# 14-0161-82; PE-CF594 anti-mouse CD127, Clone SB/199, BD Biosciences, Cat# 562419). After washing, the cells were then stained with flexible viability dye eFluor 780 (eBioscience, San Diego, California, USA) for 30 min at 4°C, washed with PBS twice, then fixed with 1% PFA for flow cytometry.

Data analysis

Data were firstly evaluated for normal distribution using JMP 9 software. For multi-group comparisons of normally distributed data, one-way or two-way ANOVA was performed followed by an appropriate post hoc t test. If the data was not normally

distributed, nonparametic multi-comparisons were used. The data sets were considered significantly different if the p value was <0.05. Results were expressed as mean ± SE.

Results

Characteristics of the animals

ACE2KO mice exhibited similar random glucose levels as WT mice at the 3-month time point. 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. Loss of ACE2 did not increase the glucose levels in Akita mice. At the 9-month time point, both Akita and ACE2KO-Akita showed a further increase in glucose levels as compared to 3 months of diabetes, but still, no change was detected in between these two groups (Figure 2-1A). All ACE2KO, Akita and ACE2KO-Akita mice showed reduced body weight compared to WT mice at both 3- and 9-month time points. Non-diabetic groups (WT and ACE2KO mice) had an increase in their body weights over time, while 9 months diabetic mice in both Akita and ACE2KO-Akita groups showed similar body weights as 3 months of diabetes (Figure 2-1B).

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

Both the long-term (LT) and short-term (ST) repopulating HSCs play a key role in maintaining hematopoietic cell pool and balance, and represent a reserve for HSPCs. To test the ACE2 effects on diabetic bone marrow exhaustion, we examined the percentages of HSCs in bone marrow from ACE2KO-Akita mice and age-matched controls by flow cytometry. Figure 2-2A showed the gating schematic and representative dot plots of flow cytometry analysis. Compared to 3 months Akita group, the percentage of bone marrow-derived (ST)-HSCs ($Lin^-/CD127^-/Sca\text{-}1^+c\text{-}kit^+/CD34^+CD135^-$) was increased in ACE2KO-Akita mice. However, ST-HSCs percentage was reduced at the 9-month time point in double mutant mice (Figure 2-2B), suggesting a loss of compensation at the late stage of the disease. (LT)-HSCs ($Lin^-/CD127^-/Sca\text{-}1^+c\text{-}kit^+/CD34^-CD135^-$) represent the true stem cells of the bone marrow and are the most primitive stem cell. Akita mice had a reduction of the percentage of phenotypic LT-HSCs, while ACE2KO-Akita mice had a further decrease of these cells at 9 months of disease (Figure 2-2C), supporting that loss of ACE2 caused a more profound defect of hematopoiesis in diabetes.

Impairment of HSPC migration and proliferation functions in ACE2KO-Akita mice

Proliferation and migration functions of HSPCs are key indicators of their in vivo vaso-reparative ability, because these cells need to self-expand and mobilize from the bone marrow in response to chemoattractants at the site of injury. Therefore, we next examined whether loss of ACE2 also affects the functions of HSPCs

besides the reduction of their reserve pool. The lineage$^-$ c-Kit$^+$ (LK) cells isolated from the bone marrow represent mouse HSPCs. Akita mice demonstrated reduced LK cell proliferation at as early as 3 months of diabetes (Figure 2-3A). The absence of ACE2 exacerbated diabetes-mediated impairment of function at both early and late stage of disease (Figure 2-3A and B). However, when we treated with either 100 nM Ang-(1-7) or alamandine, the two main peptides in the protective RAS axis, the proliferation function of these cells were largely restored in diabetic cohorts even at 9 months of disease (Figure 2-3B). Similar to the proliferation function, LK cells from ACE2KO-Akita mice also showed lower migration ability in response to the chemoattractant, CXCL12. Ang-(1-7) or Alamandine was able to increase their sensitivity to CXCL12 in cells form the 9-month of the diabetic cohort (Figure 2-3C and D). These data suggested that the deletion of ACE2 enhanced diabetesinduced impairment of HSPC functions, which were essential to prevent and repair diabetic retinal vasculature.

ACE2 deficiency led to enhanced myelopoiesis in diabetic bone marrow

Since severe depletion and impaired function of bone marrow HSPCs was observed in ACE2KO-Akita cohort at 9-month of diabetes, we next examined whether colony forming ability was also impaired in both peripheral blood and bone marrow samples using the colony forming assay. After 10 days of culture of ACS-lysis blood cells, Akita mice showed a significant reduction of total blood colony forming units (CFUs) at 9 months of diabetes. The absence of ACE2 led to a further

reduction of blood CFUs compared to Akita mice (Figure 2-4A). In the bone marrow CFU assays, even though there were no changes in total CFU numbers among different genotypes at the 9-month time point, Akita mice exhibited increased numbers of CFU-granulocyte/monocyte/granulocyte, monocyte (CFU-G/M/GM) compared to WT and ACE2KO mice, suggesting enhanced myelopoiesis in diabetic bone marrow. In addition, loss of ACE2 exacerbated this diabetes-mediated pathological change (Figure 2-4A).

Bone marrow-derived HSPCs give rise not only to myeloid lineages, but also lymphoid lineages through the process of hematopoiesis. Next common lymphoid progenitors (CLPs), which are daughter cells of HSPCs and differentiate into lymphoid lineages of blood cells were examined. Figure 2-4B showed the representative dot plot of enumerating CLPs by flow cytometry analysis. Phenotypic CLP markers $Lineage^{-}CD127^{+}Sca\text{-}1^{med}c\text{-}Kit^{med}$ were used. Interestingly, the percentage of CLPs was decreased in Akita mice compared to WT mice at 9-month time point. A further reduction of the CLP percentage in ACE2KO-Akita mice was observed compared to Akita alone (Figure 2-4C). These data suggested that diabetes resulted in a shift of hematopoiesis towards myelopoiesis with a reduction of CLPs. Thus, ACE2 deficiency enhanced the diabetes-mediated imbalance of hematopoiesis.

Discussion

This study reported for the first time that deficiency of ACE2 caused reductions of both phenotypic LT- and ST- HSCs in the diabetic bone marrow, adding additional understanding to the pathological changes in T1D bone marrow. Consistent with our previous findings, ACE2 also affected the migration and proliferation function of diabetic HSPCs. Importantly, not only Ang-(1-7), but also alamandine, a newly found peptide in the protective RAS, can restore diabetic-induced impairment of HSPC functions. Moreover, this study verified that ACE2 plays an important role of the imbalance of hematopoiesis in the diabetic bone marrow and results in enhanced myelopoiesis and reduced lymphospoiesis.

Our previous study showed that Ang-(1-7) treatment can enhance the migration function of $CD34^+$ cells from diabetic patients with microvascular complications [93]. Consistent with that, we observed that Ang-(1-7) also restored Akita HSPC migration and proliferation function. More importantly, we for the first time showed that alamandine has similar beneficial effects on HSPC migration and proliferation functions as Ang-(1-7). Alamandine is a newly found peptide in the protective RAS axis [4]. Alamandine and Ang-(1-7) are both composed of 7 amino acids. Alamandine only has one N-terminal amino acid that is different 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) [4]. The downstream signaling pathways of MAS involve activation of

phosphatidylinositol 3-kinase (PI3K)/Akt and Slit3/ROCK [93, 281]. It is not known whether alamandine/MRGPRD share the same signaling pathway as Ang-(1-7)/MAS. It is important to investigate in the future the underlying mechanisms of the beneficial effects of almandine on HSPC functions.

A recent study observed reduced numbers of bone marrow total LSK (Lineage$^-$ Sca-1$^+$c-Kit$^+$) cells in STZ-induced and db/db diabetic mice model [281]. In our study, no changes were observed in total LSK cells. This may be due to different methodology to assess the numbers or differences in the diabetes models or duration of diabetes at the time of examination. However, when LSK cells were further sub-divided into LT-HSCs, ST-HSCs, and multipotent progenitors (MPP), we observed that Akita mice showed a significant reduction of LT-HSCs as well as a tendency towards a decrease of ST-HSCs. ACE2 ablation contributed to the exhaustion of HSCs in diabetes. LT-HSCs and ST-HSCs are critical for maintaining the hemostasis of the hematopoietic system and for giving rise to all types of blood cells. To further compare the proliferative capabilities of these cell types among different genotypes, we plan to do competitive bone marrow transplantation studies.

One of our major findings in the study is that loss of ACE2 in diabetes-resulted in increased myelopoiesis. By CFU assays, a robust increase in the number of CFU-G/M/GM in ACE2KO-Akita mice bone marrow was observed. Lymphopoiesis, in contrast, was relatively suppressed, as supported by the observations of reduced

bone marrow CLP percentages in the double mutant mice. In both T1D and T2D animal models, previous studies have demonstrated enhanced myelopoiesis, which can drive the production and activation of proinflammatory cells [59, 282]. Therefore, the novel role of protective RAS in hematopoiesis adds a new dimension to the understanding of pathological mechanisms of diabetes on the generation of bone marrow defects. We did not observe a change of total CFUs in bone marrow among different cohorts. Because total bone marrow cells were plated for CFU assays, which includes not only LT-HSCs and ST-HSCs, but also large amounts of progenitors. These progenitors likely contributed to the colony forming units instead of the HSCs.

Studies showed that Ang-(1-7) stimulates the forming of erythroid burst-forming units (BFU-E) and enhances erythropoiesis [74, 75]. In our ACE2KO-Akita mice, we also observed a significant reduction in BFU-E in CFU assay, suggesting a protective role of ACE2/Ang-(1-7) RAS axis in erythropoiesis. Interestingly, Ang II also has a positive effect on erythropoiesis by increasing EPO levels and sensitivity [70, 72]. High-dose of ACE inhibitors also resulted in anemia in human subjects reported by two research group [63, 64]. These data suggest that ACE inhibitor-induced increase in Ang-(1-7) level is not enough to counteract the anaemic effect of Ang II loss.

In summary, our study demonstrated the beneficial effects of the protective RAS on maintaining the numbers and functions of HSPCs and highlights the novel role of ACE2 in maintaining the balance of myelopoiesis and lymphopoiesis.

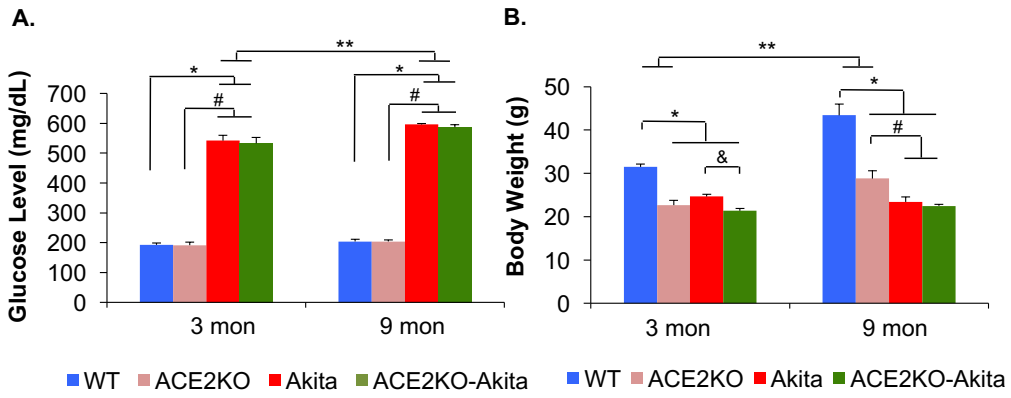


Figure 2-1. Increased glucose levels, reduced body weight in Akita and ACE2KOAkita mice. (A): Random glucose levels were increased in both groups (n = 7-9 per group). (B): Diabetic groups showed consistent low body weight, while the nondiabetic mice had increased body weight over time (n = 9-18 per group). * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2KO; & $p<0.05$, as compared to Akita; ** $p<0.05$, as compared to 3-month time point. Abbreviations: WT, wild type; ACE2KO, ACE2 knockout (ACE2-/Y); ACE2KO-Akita, ACE2 knockout-Akita (ACE2-/YIns2WT/C96Y); 3 mon, 3 months of diabetes and age-matched control; 9 mon, 9 months of diabetes and age-matched control.

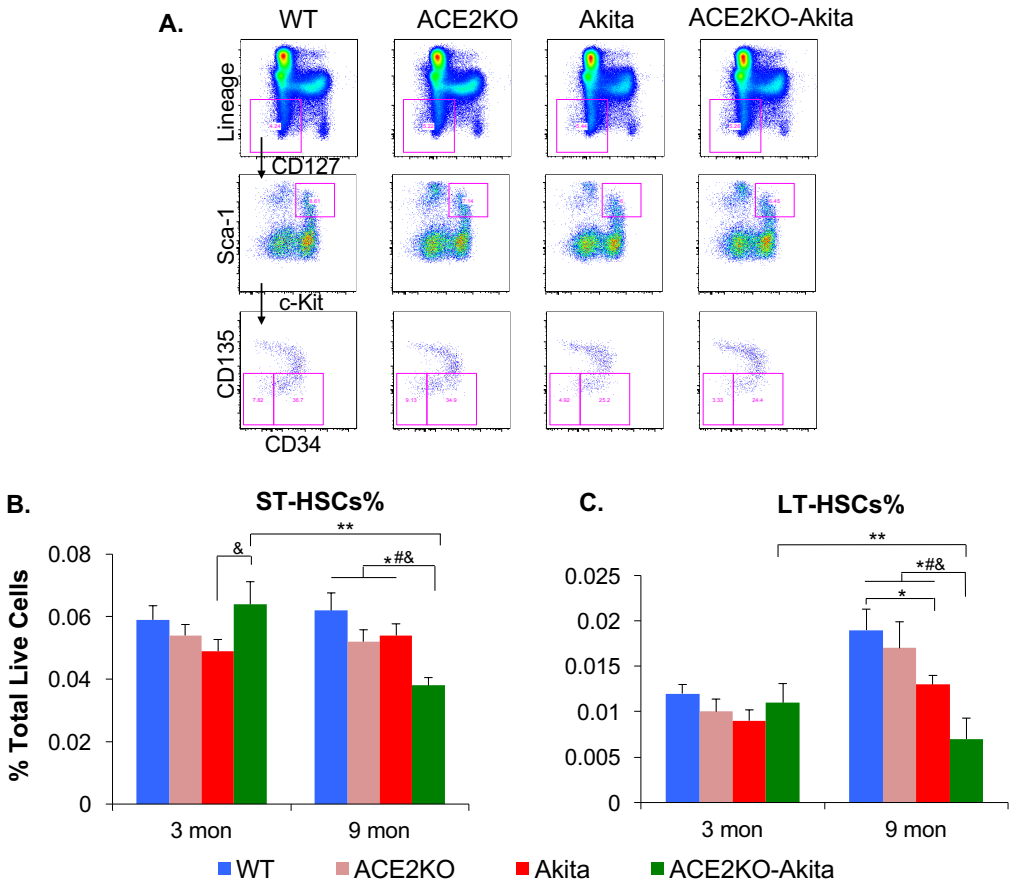


Figure 2-2. Reduction of both short-term (ST-) and long-term repopulating (LT-) hematopoietic stem cells (HSCs) in the bone marrow from ACE2KO-Akita mice at 9 months of diabetes. (A): Representative scheme of gating for flow cytometry enumeration of HSCs. (B): The percentage of ST-HSCs was decreased in ACE2KO-Akita mice at the 9-month time point (n = 12-22). (C): The percentage of LT-HSCs was reduced in the bone marrow of Akita mice (as compared to WT mice). Loss of ACE2 worsened the diabetes-induced reduction of LT-HSC. The enumeration was performed at the 9-month time point (n = 12-22). * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2KO; & $p<0.05$, as compared to Akita; ** $p<0.05$, as compared to 3-month time point. Abbreviations: WT, wild type;

ACE2KO, ACE2 knockout (ACE2-/Y); ACE2KO-Akita, ACE2 knockout-Akita (ACE2-/YIns2WT/C96Y); 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.

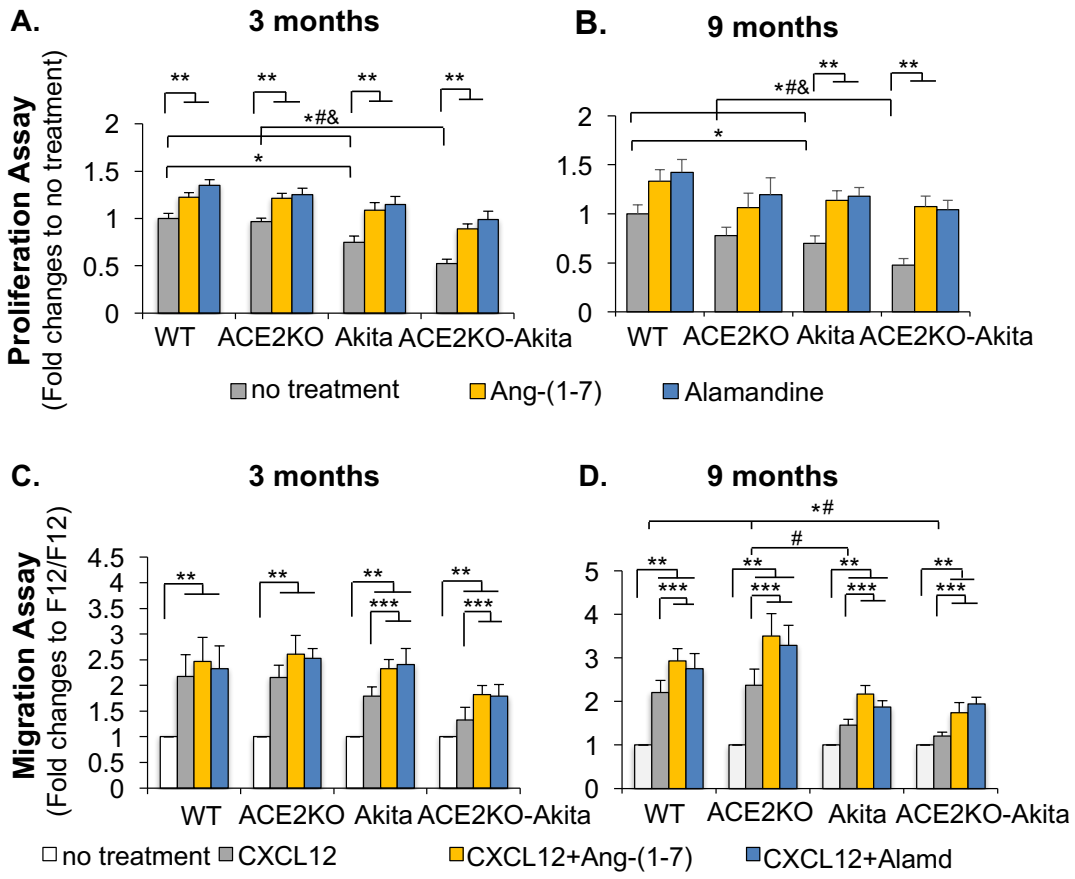


Figure 2-3. Depletion of ACE2 worsens diabetes-mediated impairment of HSPC proliferation and migration functions. (A): Impaired proliferation function of HSPCs was observed in Akita mice as early as 3 months of diabetes. Loss of ACE2 caused a further reduction of proliferation function (n = 5-7). (B): Absence of ACE2 exacerbated diabetes-induced impairment of HSPC proliferation functions, which was restored by the treatment of Ang-(1-7) 100nM or alamandine 100nM (n = 5-6). (C): Akita mice exhibited a trend toward a decrease in HSPC migration toward chemoattractant CXCL12. Ang-(1-7) or alamandine improved the migration of diabetic HSPCs (n = 4-6). (D): HSPCs from ACE2KO-Akita mice had no response to chemoattractant CXCL12. Ang-(1-7) or alamandine partially restored the

migration of diabetic HSPCs (n = 4-6). For A-B, * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2KO; & $p<0.05$, as 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 ACE2KO; & $p<0.05$, as compared to Akita; ** $p<0.05$, Compared to F12/F12; ***, Compared to F12/CXCL12. Abbreviations: WT, wild type; ACE2KO, ACE2 knockout (ACE2-/Y); ACE2KO-Akita, ACE2 knockout-Akita (ACE2-/YIns2WT/C96Y); Alamd, alamandine.

A.

	Bone Marrow			Blood
	BFU-E	CFU-G/M/GM	CFU-GEMM	CFUs
WT	14 ± 4	48 ± 8	3 ± 2	35 ± 5
ACE2KO	13 ± 3	48 ± 9	1 ± 1*	30 ± 3*
Akita	11 ± 2$^{*\#}$	61 ± 4$^{*\#}$	1 ± 1*	20 ± 4$^{*\#}$
ACE2KO-Akita	8 ± 2$^{*\#\&}$	73 ± 5$^{*\#\&}$	1 ± 1*	12 ± 6$^{*\#\&}$

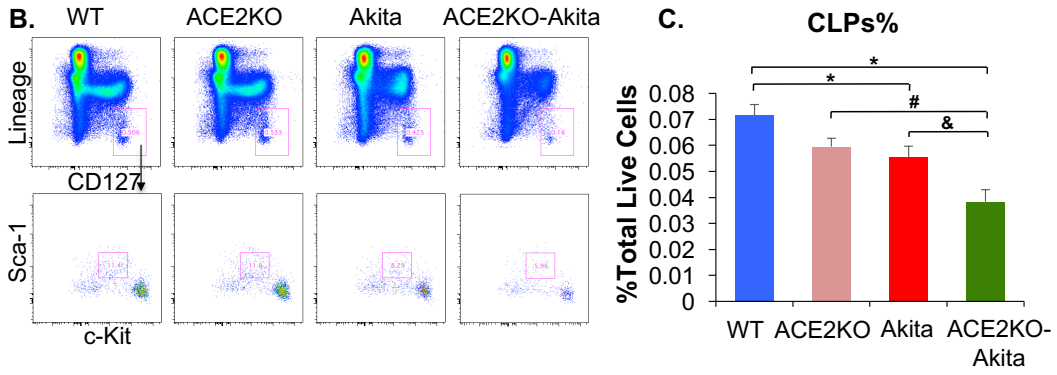


Figure 2-4. Absence of ACE2 worsened the diabetes-mediated imbalance in hematopoiesis. (A): Colony forming unit (CFU) assay showed an increased number of CFU-G/M/GM when plating bone marrow cells from Akita mice as compared to WT mice. A decreased number of total CFUs in Akita group was also observed when plating ACS-lysed cells from peripheral blood. Loss of ACE2 exacerbated diabetes-mediated alterations in both bone marrow and blood CFU assay (n = 14-20). (B): Representative gating schema for enumeration of common lymphoid progenitors (CLP) (Lineage-CD127+Sca-1medc-Kitmed). (C): The percentage of CLPs was reduced in Akita mice at 9-month time point. There was a further decrease in CLP percentage in ACE2KO-Akita (as compared to Akita alone) (n = 13-22). * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2KO; & $p<0.05$, 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; ACE2KO, ACE2 knockout (ACE2-/Y); ACE2KO-Akita, ACE2 knockout-Akita (ACE2-/YIns2WT/C96Y).

Chapter 3: RAS mediated changes in HSPCs is associated with more advanced diabetic retinopathy

Introduction

Diabetic retinopathy (DR) is the most common microvascular complication (MVC) in diabetes and is the leading cause of blindness in American adults [283]. Accelerated endothelial cell apoptosis and pericyte loss are two major cellular changes of retinal capillaries that occur at a relatively early stage, leading to progressive vaso-degeneration [284]. In response to progressive vasoregression, the ischemia retina also mounts a pathological angiogenic responses resulting in continuous vascular remodeling and eventual progression to the vision threatening proliferative DR (PDR) stage [285]. However, the pathological mechanisms underlying progressive vasoregression are still poorly understood. To date there are no specific treatments beside laser photocoagulation and a pharmacological agent that blocks Vascular endothelial growth factor.

Cell therapy is a possible strategy for DR, as it is primarily a small vessel disease. It is well-established that HSPCs play an important role in normal vascular repair, and therefore represent an ideal source for autologous cell therapy [222]. These cells are mobilized mainly from the bone marrow into the circulation, recruited to the injured retinal vasculature in response to ischemia and eventually help to repair via paracrine factors [26, 29, 277]. HSPCs from diabetics with microvascular

complications are usually dysfunctional and exhibit reduced migration and proliferation ability, a different paracrine secretory profile than cells from healthy individuals and desensitization to hypoxia [26, 93]. Therefore, the inherent dysfunctions of the cells are considered as a possible factor in the initiation of vasoregression in DR. However, the underlying mechanisms are still largely unknown.

The protective arm of renin angiotensin system is suggested to have beneficial effects on HSPCs and retinopathy. Firstly, there was an imbalance of ACE/ACE2 ratio in the retina of streptozotocin (STZ)-induced diabetic mice [216]. Ang-(1-7), a major peptide produced from ACE2 was able to correct the migration and proliferation functions of diabetic HSPCs [281]. In addition, gene delivery of ACE2 protected against the development of retinopathy [216]. is Thus, the vasoprotective RAS axis may be a better target than inhibition of the classic/deleterous one Existing pharmacological drugs, ACE inhibitorss and AT1 receptor blockers (ARBs) inhibit the deleterious axis of RAS.

There are four major clinical studies investigating the effects of the classical RAS in DR. The goal of the study “Appropriate Blood Pressure Control in Diabetes (ABCD)” was to establish blood pressure guidelines for improving diabetic complications [286]. The results showed that ACE inhibitor (enalapril) decreased DR progression but showed no difference compared to a calcium channel blocker (nisoldipine), which suggested that the effects was blood pressure dependent. The

Diabetic Retinopathy Candesartan Trial (DIRECT) tested the effect of the ARB (candesartan) on the incidence and progression of DR [287, 288]. It showed that candesartan either prevented development or improved mild/moderate DR, but did not prevent the progression nor did it cause any improvement in the more advanced stages. Another two studies “EURODIAB Controlled Trial of Lisinopril in Insulin-dependent Diabetes” and “Renin-Angiotensin System Study” suggested that the effect of RAS blockers depends on the glucose levels of the subjects [289-292]. Therefore, using RAS blockers for DR treatment was not as successful as expected.

However, ACE inhibitors and ARBs, in addition to inhibiting ACE and AT1R, increase Ang-(1-7) [293, 294]. In addition, ACE inhibitors and ARB increase CAC numbers in both animal models of hypertension and in individuals with heart disease by decreasing senescence, and by increasing proliferation and differentiation of HSPCs [295-300]. Ang-(1-7) is shown to improve HSPCs regenerative capacity in vitro. Therefore, the beneficial effect of ACE inhibitors and ARB on HSPCs may due to the increase of Ang-(1-7). Moreover, ACE2, the key enzyme in the protective RAS arm, can convert the deleterious Ang II into beneficial Ang-(1-7). Thus, we believe that influencing ACE2/Ang-(1-7)/MAS directly would be more efficient as a result of the dual beneficial effect of increasing the vasoprotective and decreasing the vasodeleterious axes of the RAS.

Akita (*Ins2*$^{WT/C96Y}$) mouse is a model of T1D. This autosomal dominant model of T1D carries a point mutation in the insulin 2 gene, resulting in inappropriate folding of proinsulin and toxicity and dysfunctions of primary pancreatic beta-cells [301]. Heterozygous male Akita mice are lean and exhibit hypoinsulinemia and hyperglycemia at around 4 weeks of age [301, 302]. Importantly, these mice develop diabetic complications, such as neuropathy and nephropathy [303, 304]. The Akita mouse is also a good model of retinal complication. Alistair *et. al.* [305] reported that the Akita mouse showed an increase in caspase-3 expression in the retina as early as 1 month of diabetes. Akita mice showed increased vascular permeability in the retina at 3 months of diabetes when compared to their littermate controls. At around 6 months of diabetes, reduced retinal thicknesses of the inner plexiform layer, the inner nuclear layer and the ganglion cell layer were observed in Akita mice. At 9 months of diabetes, a modest increase in retinal acellular capillaries, as well as changes in the morphology of microglia and astrocytes were observed, suggesting that Akita mice represent a suitable model for studying the progression of DR.

In this study, we validated our hypothesis that the absence of ACE2 is a key initial event that leads to the development DR in a genetically modified mouse model. We also tested in human subjects that loss of the protective RAS influences HSPCs migration function which is associated with different stages of DR.

Research design and methods

Animals

Male Akita (*Ins2*$^{WT/C96Y}$) mice (the Jackson Laboratory, Bar Harbor, ME) were bred with female heterozygous ACE2KO (ACE2KO$^{+/-}$) mice (obtained from Dr. Gavin Oudit's laboratory) to generate ACE2KO-Akita male mice, as well as age-matched controls (wild type, ACE2KO and Akita mice). All the mice were housed and bred in the animal facilities at Indiana University School of Medicine (IUSM). All the experiments and procedures were approved by the animal care and use committee at IUSM (animal protocol # 11165). Diabetic mice were studied at 3 months and/or 9 months of diabetes as well as their age-matched controls.

Electroretinogram

Electroretinogram (ERG) were 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.

Optical coherence tomography

Optical coherence tomography (OCT) was performed using a InVivoVue OCT system (Bioptigen, Inc., NC) as previously described [306]. Before the measurement, eyes were dilated by 2.5% phenylephrine hydrochloride and 1% atropine. High-resolution three lateral B-scan images were obtained in anesthetized mice.

Acellular capillaries quantification

Trypsin digestion of retina was performed according to a protocol reported elsewhere (Stem Cells. 2016 Feb; 34(2): 405–417.). Briefly, 4% PFA fixed retinas were isolated and incubated with 3% trypsin 1:250 (Cat. # 27250018, Gibco, Carlsbad, CA) at 37°C for 1-2 hours. After trypsinization, the internal limiting membrane and adjacent tissues were carefully removed under the microscope. Retinas were mounted on slides and stained with the periodic acid-Schiff's base-hematoxylin reagent (Sigma-Aldrich St. Louis, MO).

Human study

All human studies were approved by the Institutional Review Boards at the University of Florida (Study # RAAS 535-2011). Study subjects were recruited as healthy controls or diabetics with either no DR, mild non-proliferative DR (NPDR),

moderate NPDR or severe NPDR from clinics at the University of Florida or the Eugene and Marilyn Glick Eye Institute at IU School of Medicine Indiana University. Diabetic individuals included both males and females with either T1D or T2D. The degree of DR 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 place on ice immediately after collection. Plasma was obtained by centrifugation at 700 g, 4°C for 20 min and snap frozen in liquid nitrogen. The samples were then 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) [307]. 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

120 ml 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) into 50 ml tubes. After centrifugation at 800 g for 30 min, buffy

coat was transferred into a new 50 ml tube and washed three times with PBS containing 2% FBS and 1mM 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 RNAwas extracted from human $CD34^+$ cells using RNeasy Mini Kit (Qiagen, Valenicia, CA). RNA concentration and quality 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.

Blood pressure measurement

After anesthesia with 1.5% isoflurane, mice had a thin catheter placed into their carotid artery. The catheter was connected to a transducer which converted the blood pressure signals into electrical signals (DMD4059, OMEGA Engineering, Nerwalk, CT). Then, the signal of mean blood pressure was recorded by pressure precision T1600 software.

Isometric tension recordings

Isometric tension experiments were performed as previously described [308, 309]. WT, Akita, ACE2KO and ACE2KO-Akita mice were used. The mouse aortas were isolated and cleaned from the connective tissue and fat. The aortic arches were cut into (1-3 mm) rings. The rings were then placed into organ baths, which contained oxygenated (saturated with a gas mixture of 95% O2 and 5% CO2) Krebs buffer maintained at 37°C. The Krebs buffer was a solution containing 131.5 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 25 mM NaHCO3, and 5 mM glucose. The optimum length was determined as described elsewhere [310].

Data analysis

Data were analyzed by JMP 9 software and expressed as mean ± SE. One-way ANOVA was used if comparing multi-group with only one time-point. Two-way ANOVA was used if comparing different genotypes with multiple time points. Then, an appropriate post hoc t test was performed after ANOVA. Nonparametic multi-comparisons were performed if the data was not normally distributed. P value <0.05 was considered as statistically significant.

Results

Diabetic mice exhibited reduced retinal thickness

Color fundus photographs were used to examine the interior surface of eyes from different types of mice. Interestingly, ablation of ACE2 led to a robust increase in the number of white lesions in the retina, irrespective of a diabetic background when comparing Akita alone at 9 months of diabetes and age-matched WT mice (Figure 3-1A). We then examined retinal thickness by OCT in a cohort of mice. Figure 5B showed representative OCT images of each genotypes. Both Akita and ACE2KO-Akita groups exhibited thinner retinal thickness (~9.2% reduction) in comparison with their non-diabetic controls by OCT at 9 months of diabetes (Figure 3-1C), suggesting vasodegeneration in the diabetic retina.

ACE2KO-Akita showed progressive retinal electrical responses over the duration of diabetes

ERGs measure the electrical responses of different retinal cell types and provides information about the retinal function. Scotopic a wave records the signal of rod photocurrents, while scotopic b wave reflects the signal generated by depolarizing bipolar cells and Müller cell currents [311]. In Figure 3-2A and B, diabetic groups demonstrated similar scotopic a- and b- wave amplitudes when compared to nondiabetic groups, except the Akita mice had a slight increase of a-wave

amplitude as compared to ACE2KO mice. At 9 months of diabetes, Akita mice showed reduced scotopic b wave and no change in a wave compared to WT mice. In addition, only loss of ACE2 caused a progressive reduction of both scotopic a- and b- waves over the duration of diabetes, suggesting ACE2 ablation worsen diabetic retinal functions (Figure 3-2A and B).

Deficiency of ACE2 exacerbated diabetes-induced increase in acellular capillaries

Acellular capillaries were quantified from trypsin digests and PAS staining of the retinal vasculature which are defined as basal membrane tubes lacking endothelial cell and pericyte nuclei. Figure 3-3A shows representative retinal vascular images from different cohorts of mice at both 3-month and 9-month time points. No change was observed among the different genotypes at the 3-month time point. A significant increase in acellular capillary numbers was seen in the Akita mice at 9 months of diabetes. Interestingly, the lack of ACE2 resulted in a more robust increase in the pathological phenotype than the Akita alone (Figure 3-3B). These data suggest that ACE2KO-Akita mice had a more advanced retinopathy compared to the Akita mice. Thus, ACE2 loss clearly exacerbated the microvascular defects observed in the diabetic retina.

Deficiency of ACE2 did not worsen diabetes-induced endothelial dysfunction of microvasculature

Classic RAS is well known as a key regulator of cardiovascular function and hypertension. We also examined the effects of protective RAS on hypertension. The mean arterial blood pressure levels were measured in a cohort of mice using carotid artery catheterization. No difference in arterial blood pressure readings was observed in Akita mice as compared to WT group. Unexpectedly, loss of ACE2 also did not result in systemic hypertension in either diabetic ACE2KO-Akita mice at 3 months of diabetes or age matched ACE2KO mice (Figure 3-4A). Similar blood pressure measurements were obtained in these four cohorts at 9 months of diabetes (Figure 3-4A). Endothelial function and reactivity in aortic rings from the same four cohorts of mice was assessed. At 9 months of diabetes, aortic rings from Akita mice exhibited increased sensitivity to phenylephrine, a vasoconstrictor, compare to both WT and ACE2KO groups (Figure 3-4B). Aortic rings of ACE2KO-Akita exhibited a similar level of increased sensitivity to phenylephrine as the Akita mice. Phenylephrine pre-contracted aortas demonstrated that Akita mice showed significant endothelial dysfunction when challenged with acetylcholine as compared to aortas of WT mice. ACE2KO-Akita mice exhibited a similar degree of endothelial dysfunction as aortas from Akita mice (3-4C), whereas endothelial function in aortas of ACE2KO mouse was similar to that of the aortas of WT mice. These data suggest that while the diabetic aortas exhibit profound endothelial

dysfunction and markedly increased sensitivity to catecholamines, loss of ACE2 did not worsen this dysfunction.

Impaired HSPC migration function is associated with different stages of DR and can be corrected by restoring its Ang 1-7 levels in human subjects

$CD34^+$ cells are considered as human HSPCs in the circulation. Inadequate vascular repair of HSPCs plays an important role in the progression of DR. The protective RAS has been reported to help maintain the functions of diabetic HSPCs. Therefore, we next accessed whether human $CD34^+$ cell function is also associated with deficiency of the protective RAS and reflects the severity of DR. Peripheral blood $CD34^+$ cells were isolated from healthy subjects, diabetics with no microvascular complications or those with different stages of DR (Figure 3-5A). Systemic RAS levels were measured in the human cohorts. No change was observed in Ang II levels in diabetics with no complications or with DR as compared to healthy subjects. Interestingly, in healthy controls, Ang-(1-7) levels were all very low (less than 2.4 pg/ml). However, it was elevated in individuals with no microvascular complications and reduced in those with DR, suggesting a loss of Ang-(1-7) compensation in DR (Figure 3-5B). MAS is the receptor for Ang-(1-7). Consistent with plasma Ang-(1-7) levels, we showed that MAS gene expression was also dramatically increased in $CD34^+$ cells from diabetics with no microvascular complications, while significantly reduced in those with DR (Figure 3-5C). Then, we accessed the migration function of human $CD34^+$ cells toward the

chemoattractant CXCL12. $CD34^+$ cells isolated from healthy subjects or diabetic patients with no complications responded well to this chemoattractant. However, even with the onset of DR, $CD34^+$ cells exhibited impaired migratory function. In the severe NPDR subjects, $CD34^+$ cells nearly had no response to CXCL12. When the cells were treated with Ang-(1-7), the migration functions were restored even in those subjects with severe NPDR (Figure 3-5D).

Discussion

In the study, loss of ACE2 resulted in advancement of retinopathy in a diabetic mouse model, as ACE2KO-Akita mice showed a significant increase of retinal acellular capillaries and a reduction of both scotopic a- and b- waves on ERG when compared to Akita alone at 9-months of disease. However, loss of ACE2 did not worsen diabetes-induced endothelial dysfunction of aorta rings. In our clinical study, we provided evidence that the severity of retinopathy is associated with dysfunctional HSPC migration ability. In addition, Ang-(1-7) treatment improved the impaired migratory function of HSPC from NPDR subjects.

It is well known that poor glycemic control and long-standing diabetes typically promotes the development of diabetic microvascular complications, including retinopathy. Consistent with studies by other groups [305, 312], we also observed that Akita mice developed DR at a late stage of diabetes. Currently multiple factors and mechanisms have been suggested as causative in modulating

vasodegeneration in DR, including inflammation, impaired HSPC and increased circulating lipids and fatty acids. We and others have shown that the imbalance of RAS within HSPC may be a causative in their impaired function [93, 281]. These previous studies have mainly focused on the effects of ACE2 on HSPCs. In this study, an ACE2 knockout genetic model was generated to directly address the question that whether ACE2 plays an essential role in the development of DR. We also showed that the beneficial retinal effect of ACE2 may be associated with its favorable effects on HSPCs.

We observed that loss of ACE2 accelerated the diabetes-mediated increase in acellular capillary numbers, suggesting ACE2 plays an important role in diabetes-induced pathological changes. It is worth noting that in rodent models of diabetes, the hallmark, microanuerysms, does not usually occur on fundus images. In addition, it also does not naturally progress to PDR stage. These differences in pathogenesis in rodent models partially limited study on specific features and translation to human subjects.

When we examine the mice using color fundus, only ACE2 knockout mice had an increase of the white lesions while no change was observed in the Akita mouse. Consistent with the fundus data, we observed that ACE2 loss also led to reduced amplitude in both scotopic a- and b- waves while Akita mice only showed reduced scotopic b wave at late stage of diabetes. These results suggest that ACE2 may have some direct effects on the neural retina independent of diabetes. However,

since we did not examine these lesions histologically they may represent something other than neural infarcts and may be small inflammatory lesions composed of macrophages and lymphocytes [313], [314]. To further understand this phenomenon, immunohistochemistry staining should be performed to show the abnormalities at structural and cellular levels. In addition, ganglion cells are an important cell type in the neuron retina. Retina ganglion cell markers, including Brn3b, Math5, Islet1 and γ-synuclein, can be stained on the retinal cross-sections by immunofluorescence staining [315]. Numbers and morphologic abnormalities should be assessed in the retinal ganglion cell layers to observe any changes in the neuron retina in the ACE2 knockout mice.

Another very interesting finding is that both ACE2KO and ACE2KO-Akita mice exhibited normal mean blood pressure level, which was similar to wild-type mice. It is well established that classic RAS is a key regulator of blood pressure. In the clinic, there are several medications that target the classic RAS, including ACE inhibitors and AT1 receptor blocker (ARB) which have a beneficial effect in blood pressure control for hypertensive patients. However, the effects of the protective RAS are still controversial, as contradictory results have occurred in animal studies. At least three different lines of ACE2 knockout models exist [316]. The first group that reported ACE2KO mice described normal blood pressure in on the mice at 3 months of age when compared to their WT littermates [317]. Moreover, even treating those mice with an ACE inhibitor, the ACE2KO mice still exhibited blood pressure reduction to a similar extent as that was observed in WT control

mice. Yamamoto et al. obtained similar results as the previous group using a distinct ACE2KO mouse line, while a third group observed that only their C57/BL6 background ACE2KO mice showed a mild increase in blood pressure (~7 mmHg) [318, 319]. We used carotid artery catheterization to measure the mean blood pressure and we did not observe any change of blood pressure in the ACE2KO mice even with a C57/BL6 background. Therefore, in addition to the genetic background, different methods used for measurement of blood pressure and environmental factors may contribute to different results by various group. Taken together, all the data suggested that, unlike classic RAS, the protective RAS may have very limited effects on blood pressure.

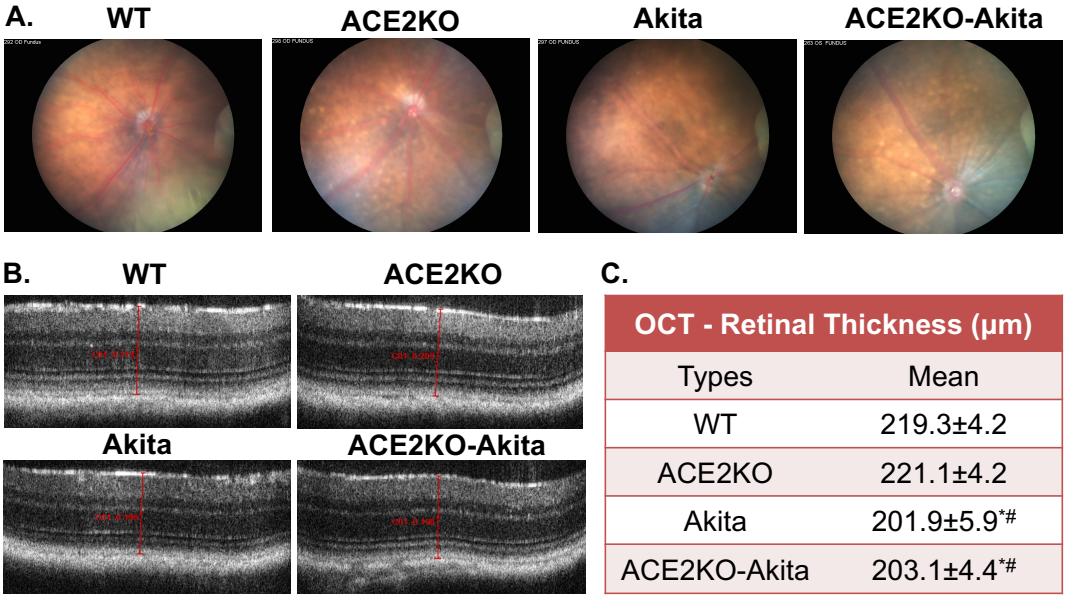
Several independent groups have reported that ACE2KO mice exhibited normal cardiac function, suggesting a non-essential role of ACE2 in regulating physiological cardiovascular function or a compensatory mechanism of the RAS in normal condition [318, 320]. Yemamota et al. reported that ACE2 deficiency caused cardiac abnormalities as accessed by echocardiography after pressure-overload, indicating ACE2 is important in cardiac function modulation under specific stress [319]. Patel et al, observed that ACE2KO mice fed a high fat diet exhibit epicardial adipose tissue inflammation and impaired cardiac diastolic dysfunction by left ventricular pressure-volume analysis [321]. Patel et al. also observed systolic dysfunction in ACE2KO-Akita mice by echocardiographic assessment [322]. In addition, they also showed that Akita mice had a similar response to transient ischemia-reperfusion, while ACE2KO-Akita mice had

changes in flow-mediated vasodilation of the femoral artery in mice with 6 months of diabetes. However, in our study, we also used Akita as a model of chronic stress. Although diabetic mice exhibited increased vaso-contraction in response to PE and impaired vasodilation function of the aorta rings, ACE2 deficiency did not worsen the diabetes-induced alterations of aorta function. Our results suggest that ACE2 may not be necessary for regulation of diabetes-mediated macrovascular dysfunction. The inconsistency of the results in the same animal model may be due to the different ages of mice used. In Patel *et. al*, the diabetic mice had 6 months of disease, while we studied macrovascular function at 9 months of diabetes. In their study, the Akita mice alone at 6 months of diabetes did not show a significant change in flow-mediated dilation. In our study, we observed at 9 months of diabetes, even Akita alone has shown clear differences in PE-induced contraction and ACh-mediated dilation as compared to age-matched wild type.

In our clinical studies, we demonstrated that there was a higher level of plasma Ang-(1-7) and increased MAS gene expression in HSPCs in diabetic patients with no microvascular complications. These findings are consistent with other reports that showed activation of protective RAS in mouse kidney and heart tissues at an early stage of diabetes, suggesting a compensatory response to diabetes-mediated stress [323-326]. It is also worth noting that not only did HSPCs have impaired migratory function in NPDR, but the extent of the dysfunction was associated with the severity of retinopathy. To further establish the role of bone marrow-derived HSPCs and retinopathy, bone marrow cells from ACE2KO mice

could be transplanted into the Akita mice and the mice could be observed for development of more severe DR than mice transplanted with WT cells.

In summary, by using an ACE2KO diabetic model, our data provide direct evidence that the protective RAS plays an essential role in DR. The data from the human subjects demonstrates that the loss of the protective RAS within HSPC may be the causative of the progression of DR and may serve as a novel therapeutic target for autologous cell therapy in DR.



C.

OCT - Retinal Thickness (μm)	
Types	Mean
WT	219.3±4.2
ACE2KO	221.1±4.2
Akita	201.9±5.9*#
ACE2KO-Akita	203.1±4.4*#

Figure 3-1. Reduced retinal thickness in Akita mice at 9 months of diabetes. (A): There were increases in white lesions in both ACE2KO and ACE2KO-Akita groups by color fundus photography (n = 4-5 per group). (B): Representative images of retina near the optic nerve on optical coherence tomography (OCT). (C): Reduced retinal thickness was observed in both Akita and ACE2KO-Akita mice at the 9-month time point, but there was no difference between the groups (n = 8 per group). * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2KO. Abbreviations: OCT, optical coherence tomography; WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akita, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).

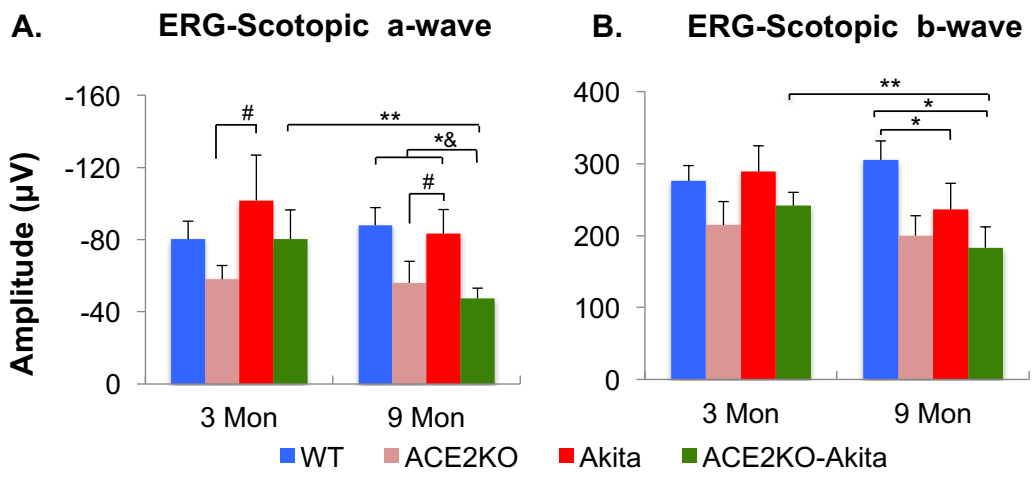


Figure 3-2. Reduced electrical responses of retinal cells in ACE2KO-Aktia mice. (A): There was no change in scotopic a-wave in Akita mice at 9-months of diabetes which reflects the electrical signal of the rod photoreceptors. Loss of ACE2 caused a decrease in scotopic a-wave amplitude in Akita mice at a late stage of diabetes (n = 5-7). (B): Both Akita mice and ACE2KO-Akita mice had a reduction of scotopic b-wave, indicating low electrical responses of depolarizing bipolar cells and Müller cells. Only ACE2KO-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 ACE2KO; & $p<0.05$, as compared to Akita; ** $p<0.05$, as compared to 3-month time point. Abbreviations: ERG, electroretinogram; WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akita, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).

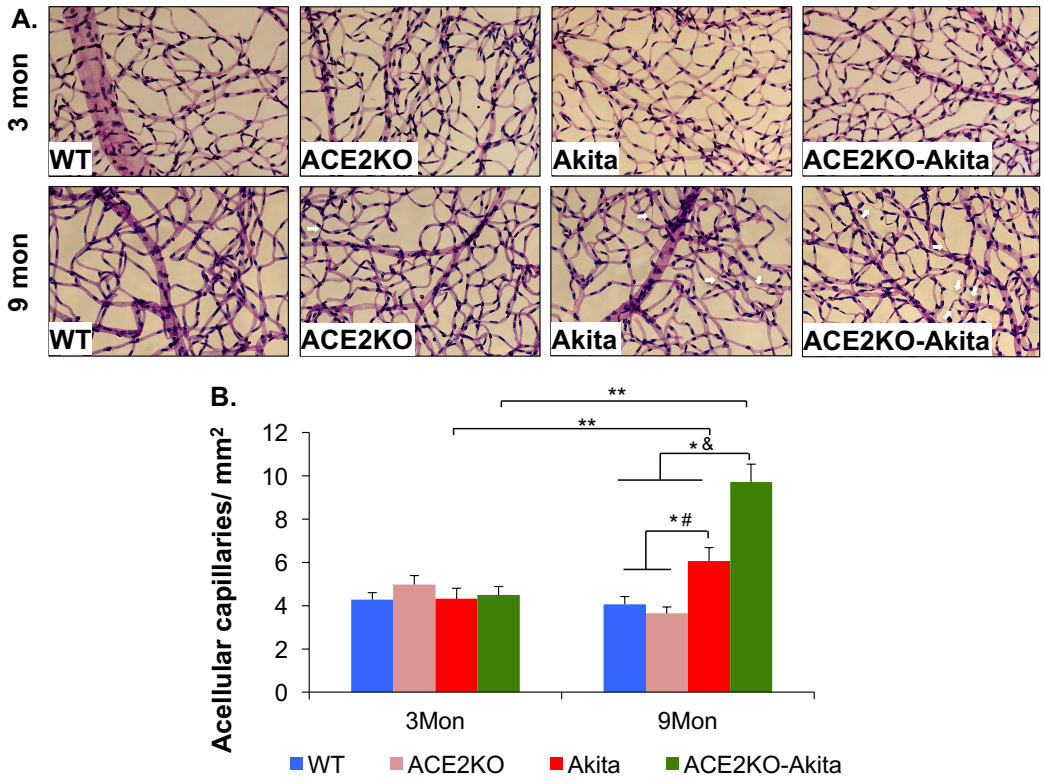
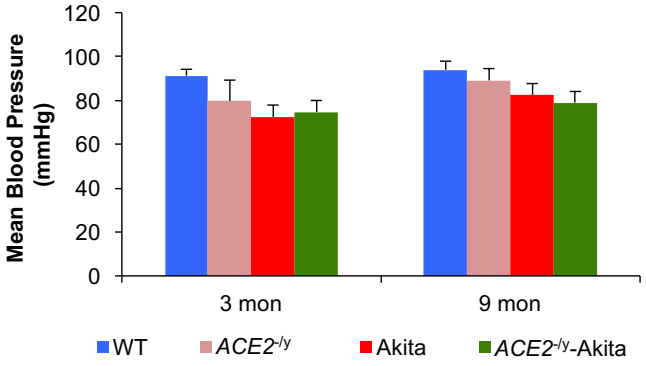
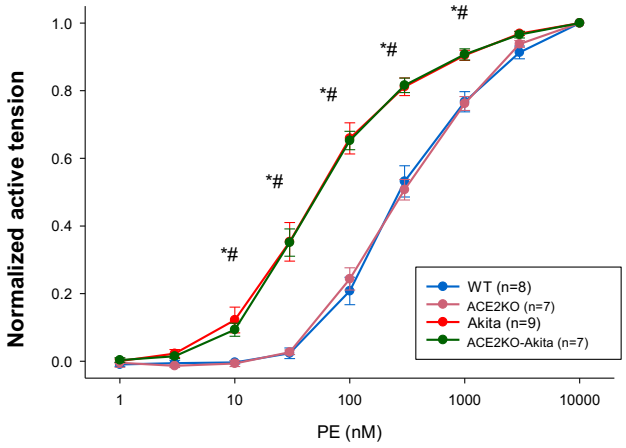


Figure 3-3. ACE2 deletion worsens diabetes-induced acellular capillaries at 9 months of diabetes. (A): representative images of retinal vasculature and acellular capillaries (arrow) in the different genotypes at both 3-month and 9-months. (B): Increased numbers of acellular capillaries was observed in Akita mice. ACE2KO-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 ACE2KO; & $p<0.05$, as compared to Akita; ** $p<0.05$, as compared to 3-month time point. Abbreviations: WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akita, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).

A.



B. Concentration-response curves for PE



C. Concentration-response curves for Ach

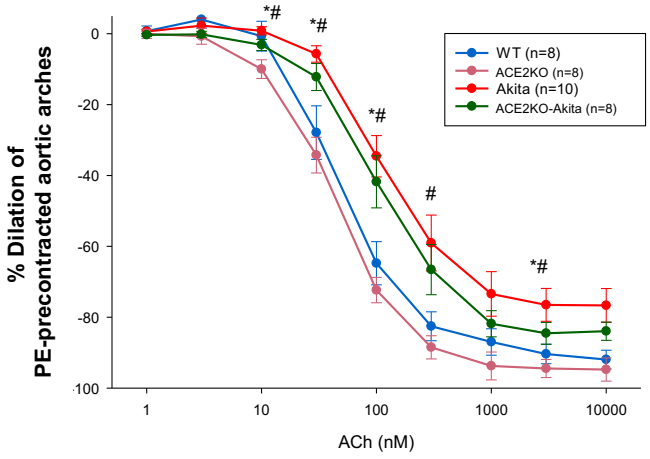


Figure 3-4. Loss of ACE2KO did not exacerbate diabetes-mediated endothelial dysfunctions of macrovasculature. (A): ACE2KO-Akita mice exhibited similar mean blood pressure levels as Akita alone at both 3 or 9 months of diabetes (n = 3-8 per group). (B): Akita aorta rings exhibited increased sensitivity to phenylephrine (PE) at 9 months of diabetes. Aorta rings from ACE2KO-Akita mice responded similarly to PE as Akita alone. (C): Akita aorta rings exhibited impaired vasodilation response to acetylcholine (ACh) at 9 months of diabetes. Loss of ACE2 did not worsen the Akita-induced vascular dysfunction. * $p<0.05$, as compared to WT; # $p<0.05$, as compared to ACE2KO; & $p<0.05$, as compared to Akita; ** $p<0.05$, as compared to 3-month time point. Abbreviations: PE, phenylephrine, Ach, acetylcholine, WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akita, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$); 3 mon, 3 months of diabetes and age-matched control; 9 mon, 9 months of diabetes and age-matched control.

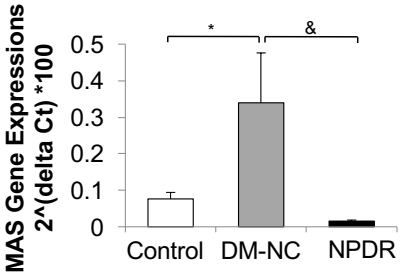
A.

	Control	Diabetes			
Number	13	39			
Gender M/F	6/7	18/21			
Age	39±13	60±11			
HbA1C	4.8	8.6±2.0			
Retinopathy		32			
		mild NPDR 9	Moderate NPDR 15	Severe NPDR 3	PDR 5
Neuropathy		7			
Nephropathy		5			
Hypertension	1	25			
Hypercholesterolemia	1	16			

B.

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&

C.



D.

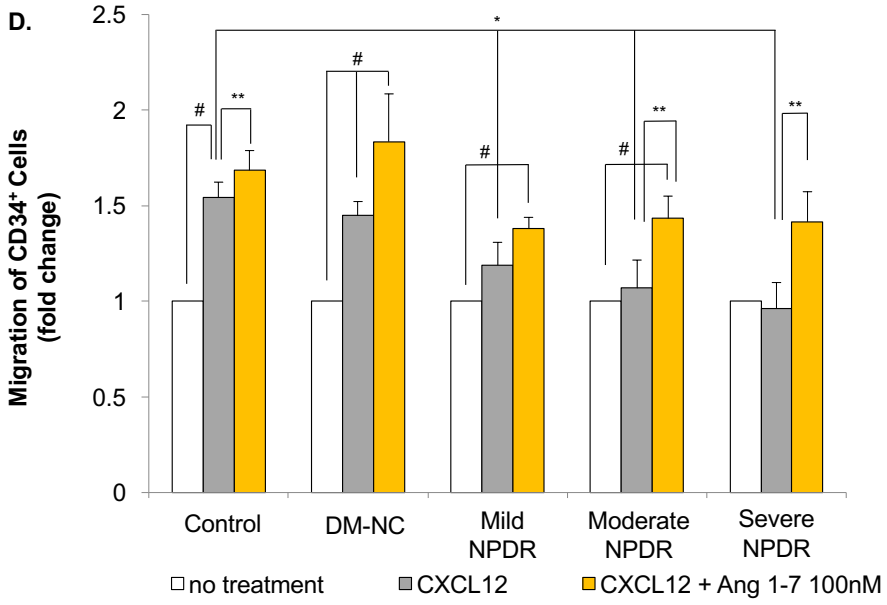


Figure 3-5. Loss of the protective RAS axis and impaired migratory function of HSPCs from diabetic subjects with non-proliferative diabetic retinopathy (NPDR).

(A): Basic characteristics of the control and diabetic subjects recruited. (B): plasma Ang II and Ang-(1-7) peptide levels in health 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). (C): 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). (D): Impaired migration ability of $CD34^+$ cells was observed at the onset of NPDR. The severity of impaired $CD34^+$ cell migration function was associated with different stages of NPDR, which can be restored by Ang-(1-7) treatment (n = 3-12). For A and C, * $P < 0.05$ as compared to control; & $P<0.05$ as compared to DM-NC. For D, * $P < 0.05$ as compared to control; ** $P<0.05$ as compared to CXCL12+Ang (1-7) group; # $P < 0.05$ compared to no treatment group. Abbreviations: DM-NC, diabetes with no complications; NPDR, non-proliferative retinopathy.

Chapter 4: ACE2 deficiency led to alterations in gut microbiota composition and bacterial translocation in type 1 diabetic mouse model

Introduction

The intestinal microbiota describes all living organisms present in the gastrointestinal (GI) tract. These bacteria play an important role in physiological functions, such nutrient digestion and absorption, vitamin biosynthesis, metabolism modulation and immune regulation [327-330]. The intestinal microbes and human host interact with each other and co-evolve, together resulting in the development of intact immune mechanisms for host defense and for maintaining metabolism [331, 332]. Therefore, the disturbance of gut microbiota homeostasis has been considered as an independent risk factor and integral determinant of human diseases.

As the growing evidence and studies about the role of microbiota, our understanding of diabetes pathogenesis is also expanded from the genetic factors and contribution of high fat diet to studies of the effect of microbiota antigens and metabolites. Recent researches on both animal models of T1D and human T1D subjects indicate that gut microbiota is strongly associated with the development of T1D. Reduced ratio of *Firmicutes* to *Bacteroidetes* has been observed in the Bio-Breeding diabetes-prone rats and antibiotic treatments can partially protect from T1D [333]. The alteration of *Firmicutes* and *Bacteroidetes* ratio has also been

found in (pre)diabetic children by 2 independent groups [97, 98]. Alterations of gut microbiota produced metabolites also contribute to the development of diabetes, such as decreased short chain fatty acids (SCFA) [101], increased trimethylamine N-oxide (TMAO) [334], as well as increased LPS and PGN [103, 111]. However, the underlying mechanisms of gut microbiota changes in diabetes remain largely unknown.

RAS plays a vital role in regulating many physiological processes of the vasculature. Ang-(1-7) acting through the Mas receptor mediates its vasoprotective effects by activation of endothelial nitric oxide synthase (eNOS) through an Akt-dependent mechanism and attenuation of NADPH oxidase [93]. Growing evidence has suggested a role of RAS in the alterations of gut microbiota. Using an ACE2 knockout models, Tatsuo *et. al.* demonstrated loss of ACE2 resulted in a dramatically reduced plasma tryptophan level and an imbalance of amino acid homeostasis through downregulation of B^0AT1 expression level leading to a change of gut microbiota and increased susceptibility to colitis [161]. ACE2 deficiency leads to advanced DR and bone marrow defects. But it is still unclear whether ACE2 also play an important role in the change of gut microbiota in the context of diabetes.

In this study, we hypothesized that Akita mice had an alteration of their microbiota composition compared to WT mice. By using ACE2 knockout ($ACE2^{-/Y}$, ACE2KO)

Akita model, we further studied the role of ACE2 in the modulation of microbiota composition and alteration of functional gene pathways in diabetes.

Research design and methods

Animals

ACE2 knockout (ACE2KO) mice were generated and obtained from Dr. Oudit's laboratory at University of Alberta, Canada. Then, ACE2KO heterozygous female mice (*ACE2*$^{-/+}$) were bred with male heterozygous *Ins2*$^{WT/C96Y}$ (Akita) diabetic mice (Bar Harbor, ME) at Indiana University School of Medicine. After breeding, AEC2KO-Akita (*ACE2*$^{-/Y}$/*Ins2*$^{WT/C96Y}$) mice and ACE2KO mice were generated, as well as Akita and WT (wild type) mice that were used as littermate controls. PCR using ear tissues were used to confirm different genotypes. Primers for ACE2 gene: Forward- CCG GCT GCT CTT TGA GAG GAC A; Reverse- CTT CAT TGG CTC CGT TTC TTA GC. Primers for ACE2KO detection: Forward- CCG GCT GCT CTT TGA GAG GAC A; Reverse- CCA GCT CAT TCC TCC CAC TC. Primers for Akita mutation: Forward- TGC TGA TGC CCT GGC CTG CT; Reverse- TGG TCC CAC ATA TGC ACA TG. For Akita mice genotyping, restriction digestion was performed using Fnu4HI after PCR. ACE2KO-Akita mice and Akita mice were studied primarily at 9-months of diabetes and age-matched control groups (WT and ACE2KO). All animal procedures were approved by the animal care and use committee at the Indiana University School of Medicine.

16S rRNA gene sequencing

Fecal, eye, bone marrow supernatant and plasma samples were collected and shipped to Wright Labs, LLC. Genomic DNAs were extracted (approximately 0.25 g per sample) using a MoBio Powerfecal DNA Isolation kit following the manufacturer's instructions (MoBio Carlsbad, CA). The Disruptor Genie cell disruptor (Scientific Industries, Bohemia, NY) was used to vortex the samples. The isolated genomic DNAs were then eluted in 50 µl of 10 mM Tris and quantified using the Qubit 2.0 (Invitrogen) Fluorometer according to the protocol of the dsDNA High Sensitivity option. PCR was performed using 10 ng of template DNA at a total volume of 25 µl per reaction. After PCR amplification, the products were then purified using the Qiagen Gel Purification Kit (Qiagen, Frederick, MD). After quality check using the 2100 Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA), pooled libraries were then shipped to the California State University (North Ridge, CA) for sequencing. After quantification using the Qubit High Sensitivity dsDNA kit (Life Technologies, Carlsbad, CA) and dilution, pooled libraries were loaded on an Illumina MiSeq V2 500 cycle kit cassette with 16S rRNA library sequencing primers and set for 250 base, paired-end reads. Raw sequence data was successfully obtained in all the samples sent. Paired-end sequences were trimmed at a length of 250 bp, and quality control was set as an expected error of less than 0.005 by USEARCH V7. Reads were then analyzed by the QIIME 1.9.0 software. USEARCH61 algorithm was used to identify chimeric sequences and to pick open reference operational taxonomic units (OTUs).

Taxonomy was assigned using the Greengenes 16S rRNA gene database (13-5 release, 97%) and organized into a BIOM formatted OUT table, which was summarized within QIIME 1.9.0.

Alpha diversity analysis

The plots of alpha diversity were generated within the Phyloseq sequence analysis package using a rarified OTU table. Rarefaction was performed on sequences across all samples. Alpha diversity plots were generated using different metrics, including Chao1, ACE, Shannon's Diversity, Simpson, Inverted Simpson and Fisher's richness metrics.

Beta diversity analysis

ANOSIM significance tests and principal coordinates analyses (PCoA) plots were generated from a weighted UniFrac distance matrix from a CSS normalized OTU table within QIIME 1.9.0. The data was then uploaded to the online analysis tool METAGENassist for PLS-DA analysis.

Taxonomic comparisons

BIOM formatted OTU table was used to organize assigned taxonomy. To identify the abundance of prevalent phyla, facet grid bar plots were generated within Rstudio using the Phyloseq package.

LefSe analysis

Relative abundances of taxa were multiplied by 1 million and formatted as described before [335]. Comparisons were made with “Sample Type” as the main categorical variable (“Class”). Alpha levels of 0.05 were used for both the Kruskal–Wallis and pairwise Wilcoxon tests. Linear Discriminant Analysis (LDA) scores greater than 3.0 are displayed.

Metatranscriptome analysis of fecal samples

Fecal samples (n=16) underwent MoBio (Qiagen, CA) RNA extraction. Subsequent Nugen Ovation (Nugen, CA) transcriptome library was prepared for all fecal samples and quality of the library was checked using a high sensitivity bioanalyzer chip (Agilent, CA). Then, the library was purified using QIAquick gel purification kit (Qiagen, CA) after equimolar amounts were pooled. After purification, libraries underwent sequencing on the Illumina HiSeq4000 following a 2 x 150 index run. Quality assessment of the raw sequencing data was performed

using the program FastQC. A sliding window filtration within the program Trimmomatic were utilized to filter the sequence data, then human DNA reads were also removed from the filtered sequence data. The metatranscriptome analysis tool Metaphlan was implemented to quantify the taxonomic profile within each sample. To obtain functional gene profiles, filtered data was annotated using the Uniref90 database within Humann2. Uniref90 annotations were regrouped as KEGG orthology (KO) terms, which consequently underwent CPM (counts per million) normalization within Humann2 for stratified barplot analysis and LefSe enrichment plots. Functional count data was also uploaded to the online analysis tool METAGENassist for PLS-DA analysis.

Small intestine lamina propria (LP) cell isolation

Mouse small intestines were collected and flushed with cold PBS. The intestine was cut open along its length and then into 1.5 cm pieces, after removing Peyer patches. The intestine pieces were washed with cold PBS three times and incubated with 10% FBS in PBS with 20 mM HEPES and 10 mM EDTA in PBS for 25 min at 37°C. After incubation, the tissue was washed with PBS three times while placed on ice, and then digested using collagenase D (2 mg/ml) and DNase II (10 ug/ml) in 10 ml RPMI1640 medium for 1 hour at 37°C. The digested tissues were collected and filtered through a nylon mesh to remove cell aggregates and debris. The resulted filtrate was centrifuged at 500 g to pellet cells. The pellet was resuspended in 10 ml 40% Percoll (GE Healthcare) and overlaid on 10 ml 75%

Percoll. Lamina propria (LP) cells were collected at the interphase of the Percoll layers after centrifuging at 2000 rpm at 23°C for 20 min. The LP cells were washed with 2% FBS in PBS buffer (supplemented with 1mM EDTA) twice. One million cells were used for each FACS analysis.

Gut blood flow recovery measurement

The intestinal ischemia-reperfusion experiments were performed as described elsewhere (cite: shock July 2016 - Volume 46 - Issue 1 - p 75–82). Briefly, anesthetized mice were placed on a heating pad. The abdominal skin was prepared by hair removal lotion. The root of the mesenteric artery was located and occluded for 30 minutes by a microvascular clamp. After ischemia, the clamp was removed for blood flow reperfusion for 60 minutes. Intestinal blood flow was recorded by Laser Doppler imaging (Moor Instruments, DE) at baseline, at the time of ischemia, and immediately the following reperfusion or at 60 minutes after reperfusion.

Flow cytometry analysis

Isolated cells were incubated with rat anti-mouse CD16/CD32 (eBioscience, San Diego, California, USA) for 15 min at 4 °C. The cells were then incubated with primary antibody cocktails for 30 min at 4°C in the dark (For CACs, Alexa700 anti-mouse CD45, BD Biosciences, Cat# 560510; BV650 anti-mouse Flk1, BD

Biosciences, Cat# 740539; PerCP-eFluor710 anti-mouse CD31, eBioscience, Cat# 46-0311-82. For immune cells and monocytes, PerCPCy5.5 anti-mouse NK 1.1, BD Biosciences, Cat# 551114; BV500 anti-mouse CD3, BD Biosciences, Cat# 560771; PE/Cy7 anti-mouse CD11c, BD Biosciences, Cat# 558079; PE anti-mouse Ly6G, BD Biosciences, Cat# 551461; BV421 anti-mouse CD115, Biolegend, Cat# 135513; FITC anti-mouse Ly6C, Biolegend, Cat# 128006). After washing, the cells were then stained with flexible viability dye eFluor 780 (eBioscience, San Diego, California, USA) for 30 min at 4°C, washed with PBS twice, then fixed with 1% PFA for flow cytometry.

Data analysis and statistics

Data were firstly evaluated for normal distribution using JMP software. For multi-group comparisons of normally distributed data, one-way or two-way ANOVA was performed followed by an appropriate post hoc t test. The nonparametic Kruskal–Wallis and pairwise Wilcoxon tests were used for microbiota analysis. The data sets were considered significantly different if the p value amounted to <0.05. Results were expressed as mean ± SE.

Results

No change was observed in the bacterial species richness in fecal samples from ACE2KO-Akita mice

After 16S rRNA gene sequencing, alpha diversity was first performed to assess species richness and evenness in a cohort of mice. Alpha diversity measures the amount of unique bacterial species within each sample (species richness) and the abundance distribution of each species (species evenness). In Figure 4-1, alpha diversity was performed using different calculation metrics, including observed, Chao1, ACE, Shannon, Simpson, InvSimpson, as well as Fisher. It revealed a rich bacterial community in all samples, as a range of 125 to 367 unique bacterial Operational Taxonomic Units (OTUs >97%) was observed within each sample. Interestingly, the wild type group was found to possess the highest average observed species richness when compared to the other groups, however the difference was not statistically significant. In addition, a consistent pattern of difference between Akita and ACE2KO-Akita mice was no observed by alpha diversity analysis.

A distinct clustering of fecal samples was observed among different genotypes

Different from alpha diversity analysis, beta diversity is used to measure the phylogenetic distance between the bacterial community in each sample, therefore this allows you to evaluate bacterial diversity among samples. In Figure 4-2A, each point on the plot is indicative of the entire bacterial community within a sample by PLS-DA plot. Samples that are closer together share similar microbial community makeups, whereas samples that are further apart are less similar. We observed defined clustering of all cohorts. Therefore, taxonomic comparisons were generated to identify taxa driving shifts in microbial community structure.

Phylum-level taxonomic assignments of 16S rRNA gene sequences revealed general microbial community composition within the 46 samples (Figure 4-2B). Slight differences between the cohorts were observed at the phylum taxonomic rank. The *Firmicutes* were observed in greatest abundance across all samples, with average relative abundance of 57.6% across all samples. Marginal differences in phyla relative abundances were observed between the sample cohorts. Therefore, LefSe enrichment tests were calculated to unearth genera significantly enriched within each respective cohort.

Bacterial taxa involved in the development of diabetes were particularly enriched in ACE2KO-Akita mice

Since defined cluster among genotypes was observed, we then generated LefSe plots to display biomarker taxa driving shifts in microbial community structure

among different genotypes. In general, a total of 62 significantly enriched taxa ($p<.01$, LDA > 3.0) were identified within all 4 different types of mice, 29 of which were found to be enriched within the Akita group, 19 within the ACE2KO, 8 within the ACE2-KO Akita mice, and only 6 within the WT group (Figure 4-3A). To better understand the effect of ACE2 on bacterial taxa in diabetes, we further compared the differences of taxa between Akita and ACE2KO-Akita. Very interestingly, when compared to Akita alone group, the most enriched taxa *Ruminococcaceae*, *Tenericutes, Mollicutes*, as well as *Clostridiaceae* in ACE2KO-Akita mice were found to be involved in the development of diabetes (Figure 4-3B). One study showed that Ruminococcaceae and Clostridiaceae were more abundant in bio-breeding diabetes-prone rats than diabetes-resistant ones [336]. Tenericutes was also found to be enriched in STZ-induced type 1 diabetes model [337], while Mollicutes enriched in a high fat diet-induced diabetic mouse model [338]. All the results suggested that loss of ACE2 worsen diabetes-mediated gut dysbiosis at 9 months of disease.

Differential functional gene expression profiles were observed among different genotypes

As we determined a distinct taxa profile in each genotype, we next were interested in identifying the functions of the enriched taxa within each group. Metatranscriptome analysis was performed and PLS-DA analysis was conducted to compare overall differences in functional gene (Kegg Orthology) expression

profiles among different genotypes. Here, we can see defined clustering of samples in 3D-space, indicating differential functional gene expression profiles among groups (Figure 4-4). This serves as a preliminary indication that diabetes and ACE2 deficiency may shape the gene expression profiles of the present microbial consortia. A heatmap of counts per million (CPM) normalized counts of Metaphlan estimated taxonomic read hits was also generated to visualize differences in prominent taxa among different groups (Figure 4-4). Samples are clustered based on similarity of active taxa profiles. While clustering of samples is observed among cohorts, there is overlap among cohorts as well. This further suggests greater differences between the functional expression profiles rather than taxonomic composition.

ACE2KO-Akita group had the most defined functional expression profile among all 4 genotypes

LefSe plot were generated to display enriched expressed functional gene pathway (MetaCyc) within each respective genotype cohort. Figure 4-5 displays all taxa found to be significantly ($p < 0.05$, LDA > 1.0) enriched within each sample cohort. The Y-axis displays each respective enriched functional pathway, whereas the X-axis displays the corresponding LDA enrichment score, which quantifies the strength of enrichment within each respective cohort (separated by color). As indicated by previous PLS_DA analysis, the Akita-ACE2KO cohort appears to have the most defined functional expression profile, as it possesses the highest

number (16) of significantly enriched functional pathways when considering all 4 genotypes. Akita samples were found to possess 3 enriched functional pathways, the ACE2KO cohort was found to possess 1 enriched functional pathway, and the WT cohort was found to possess 0 enriched functional pathways (Figure 4-5).

We further compared enriched functional pathways between WT and Akita and between Akita and ACE2KO-Akita. There were multiple amino acid biosynthesis pathways in microbial community functions from WT, Akita as well as ACE2KO-Akita. The super pathway of aromatic amino acid biosynthesis was enriched in Akita mice as compared to WT and can be a possible biomarker for the progression of diabetes (Figure 4-6 A). In several clinical studies, the serum levels of aromatic amino acid were found to be associated with insulin resistance, obesity and development of diabetes [339-342]. It is worth noting that two major functional pathways in the gut microbiome in ACE2KO-Akita mice both involved peptidoglycan (PGN) biosynthesis when compared to Akita mice (Figure 4-6 B). PGN can penetrate from the gut into the blood and even into bone marrow cells and levels are reduced in germ-free mice [343]. PGN can systemically mediate the innate immune system, augment inflammation and cause profound whole body insulin resistance via the pattern recognition receptor Nod1 signaling pathway [111, 344-346].

ACE2 deficiency mainly affected the infiltration of CACs into the Gut, but not the proinflammatory and immune cell types

Impaired gut-vascular barrier and increased permeability in diabetes provide the opportunity for the gut microbial antigens to cross the gut barrier and cause systemic inflammation. We have previously shown that ACE2 affects bone marrow HSPCs function. CACs are derived from HSPC and play an important role in vascular repair. Studies showed that bone marrow-derived cells migrate to the gut and impact its function. We next evaluated the CAC levels in the four cohorts of mice. As assessed by flow cytometry, there was a decrease in the levels of CACs ($CD45^+CD31^+Flk1^+$) in the bone marrow in Akita mice (Figure 4-7B). Loss of ACE2 led to a more profound reduction of CAC levels compare to the diabetics (Figure 4-7B). Consistent with this observation, we detected less infiltration of CACs into the gut in ACE2KO-Akita mice compare to Akita mice (Figure 4-7C). Interestingly, diabetes did not affect the percentages of bone marrow-derived T cells ($CD45^+CD3^+NK1.1^-$), NK cells ($CD45^+NK1.1^+CD3^-$) or neutrophils ($CD45^+NK1.1^-CD3^-/CD115^+Ly6G^+$) in the small intestine (Figure 4-7E-G). We accessed the monocyte population and showed that diabetes neither changes the percentages of bone marrow derived $Ly6C^+$ monocytes ($CD45^+NK1.1^-CD3^-/Ly6G^-/CD115^+CD11b^+/Ly6C^+$) nor $Ly6C^-$ monocytes ($CD45^+NK1.1^-CD3^-/Ly6G^-/CD115^+CD11b^+/Ly6C^+$) in the gut (Figure 4-7H, I).

ACE2KO deficiency did not worsen diabetes-mediated impairment of gut mesenteric artery flow after ischemia/reperfusion

We observed less infiltration of CACs into the intestine of ACE2KO-Akita mice, which plays an essential role in vascular repair. We access whether loss of ACE2 leads to impaired mesenteric blood flow after ischemia/reperfusion (I/R). We first assessed gut blood flow after occluding the mesenteric arteries for 30 minutes followed by 1-hour reperfusion. Figure 4-8 shows that even at the baseline levels, diabetic mice had reduced blood flow compared to non-diabetic controls. After I/R injury, the Akita group only had 20% blood flow recovery compare to their baseline, while WT mice had almost 90% recovery of their blood flow. Interestingly, ACE2KO-Akita mice reacted similarly as Akita mice both at the baseline and after I/R injury. The absence of ACE2 did not further enhance diabetes-induced impairments of gut blood flow.

Discussion

In this study, we demonstrate for the first time that loss of ACE2 expression changed the composition and functional gene pathway profile of gut microbiota in diabetes. ACE2 deficiency also led to less filtration of bone marrow derived CACs into the small intestine. In contrast, we did not observe alterations in bone marrow derived immune cell types (T cells and NK cells) or $Ly6C^+$ monocytes.

Alterations of gut microbiota in T1D animal models and human subjects have been observed in several studies. However, it is still largely unknown the exact contribution of gut microbiota to development of diabetes or its complications due to multiple reasons, including the environmental effects on the complexity and diversity of gut microbes, large variations between individuals studied, and ethnic differences in studied populations [100, 327, 330, 347]. Beside the environmental effect, how the host precisely affects the gut microbiota and what are the underlying molecular mechanisms that modulate microbiota composition are still unclear. Despite the difficulties of microbiota study, probiotic treatment and gut microbiota modulation, as a therapeutic target, still have tremendous potential for treatment of diabetes [348-350]. In our study, we performed 16S rRNA genes sequencing using bacterial DNA extracted from fecal samples of Akita and ACE2KO-Akita mice at 9 months of diabetes as well as age-matched controls. Neither diabetes nor ACE2 deficiency affected species richness of the phylotypes among the genotypes. Consistent with reports from other researchers, we also observed that the gram-positive *Firmicutes* and the gram-negative *Bacteroidetes* were the most abundant bacterial phyla in all the fecal samples [351, 352]. In addition, there was a slight trend that diabetic Akita mice tend to have more *Bacteroidetes* and less *Firmicutes* as compared to WT mice, however, was not statically significant. We did not observe the same trend in ACE2KO-Akita mice. Although it is suggested an increased *Bacteroidetes* and reduced *Firmicutes* in diabetic human subjects as well as mouse model [97-99], it is difficult to make the conclusion that the ratio of Firmicutes/Bacteroidetes is causative in gut dysbiosis.

There are mainly 2 reasons: Firstly, there is the inconsistency of the results reported by the different research group. While several studies a suggested decreased ratio of *Firmicutes/Bacteroidetes*, other observed no change or increased ratio of that in diabetes [101, 353]. Interestingly, in obese subjects, the trend of *Firmicutes*/*Bacteroidetes* is increased reported by several studies [354, 355]. Therefore, it is hard to just simply conclude which phylum are more beneficial. More importantly, the two dominant bacterial phyla (*Bacteroidetes* and *Firmicutes*) generally represent two large groups of bacteria and can be subdivided into hundreds of genera and thousands of different species [351, 356].

After analysis of general phyla composition in different cohorts, we further performed LefSe enrichment tests to further unearth genera significantly enriched within each group. At the phylum level, we observed increased *Tenericutes* and decreased *Proteobacteria.* Consistent with our data, previous studies also have showed that *Tenericutes* is more abundant in both STZ-induced type 1 diabetes model as well as type 2 diabetic db/db mice [337, 357], and *Proteobacteria* less abundant in type 2 diabetic subjects [353]. At the level of class, *Mollicutes* was increased in ACE2KO-Akita mice, which was also reported to be more abundant in high fat diet-induced diabetes [338]. At the level of order, *RF39* was found to be enriched in ACE2KO-Akita mice as compared Akita alone. Annalouise et al found that *RF39* is positively correlated with plasma trimethylamine N-oxide (TMAO) level, which, as a risk marker of cardiovascular dysfunction, is elevated in diabetes [358, 359]. At a family level, ACE2KO-Akita mice showed a more robust increase

in *Clostridiaceae*, *Ruminococcaceae*, and decrease in *Bacteroidales S24-7* and *Bacteroidaceae* levels when compared to Akita. Supported by the previous study, both *Clostridiaceae* and *Ruminococcaceae* were reported to be more enriched in bio-breeding diabetes-prone rats than diabetes-resistant ones [336]. Decreased *Bacteroidales S24-7* was also reported nonobese type 1 diabetic (NOD) mice, which may protect NOD from developing diabetes through alterations in Treg cells, $CD11b^+$ dendritic cells, and IFN-γ levels [360]. The role of *Bacteroidaceae* in diabetes development is still controversial. *Bacteroidacea*e was more abundant in young children with T1DM associated autoimmune defect [97]. However, in another study, when compared to women with gestational diabetes, normoglycemic women are restricted to the population with a *Bacteroidaceae*-rich microbiome [361]. At the level of genus, an increased *Ruminococcus* and decreased *Blautia* and *Bacteroides* were observed in ACE2KO-Akita. The previous study also showed increased *Ruminococcus* in NOD type 1 diabetic mice, which promote pathogenesis by modulation of immune cell types [360]. Enriched *Blautia* is associated with increased plasma glycerol level and branch-chain amino acids, and may associate with the development of obesity. *Bacteroides* is largely reduced in T2DM patients when compared to pre-diabetes and non-diabetes subjects, and may be negatively associated with glucose intolerance [362]. Taken together, ACE2KO-Akita mice fecal samples are more enriched with the bacteria involving in the development and pathogenesis of diabetes and less abundant with those in favor of protection.

Besides the alterations of microbial composition, it is more important to gain insight into how the microbiota changes affect their functions and interacts with the host. Metatranscriptomics analysis will allow us to examine the genes that are expressed and have active functions in the complex bacterial communities, therefore provides the information of functional alterations of microbiota that shape the gut microbiome toward a disease-driving configuration. LefSe plot of metatransciptome analysis revealed that ACE2KO-Akita has the most enrich functional gene pathways among all the 4 cohorts. Interesting, when compared to Akita mice, ACE2KO-Akita mice are particularly enriched with the biosynthesis pathways of PGN. Gut microbiota-derived PGN can penetrate into circulation, augment systemic inflammation and initiate systemic innate immune responses [343]. Studies showed that PGN induces IL-6 and TLR-2 expression, and trigger inflammation in adipocyte [363]. PGN can also induce insulin resistance during a high fat diet [106]. All these suggesting an important role of PGN in the pathogenesis of diabetes. It is also worth noting that there are lots of amino acid biosynthesis pathways that are active and enriched in ACE2KO-Akita mice, including L_lysine, L_histidine and L_proline biosynthesis. Previous study showed that ACE2 deficiency led to amino acid malnutrition and disturbed microbial ecology the gut [161]. Therefore, the increased amino acid biosynthesis pathways may possibly due to a compensation response of gut microbiota.

In sum, we showed for the first time the ACE2 deficiency contribute to the changes of gut microbiota in the context of diabetes. Loss of ACE2 shaped the complex

microbial community toward a pathogenesis configuration with enriched bacteria that are involved in diabetes development and enriched PGN biosynthesis functional pathways.

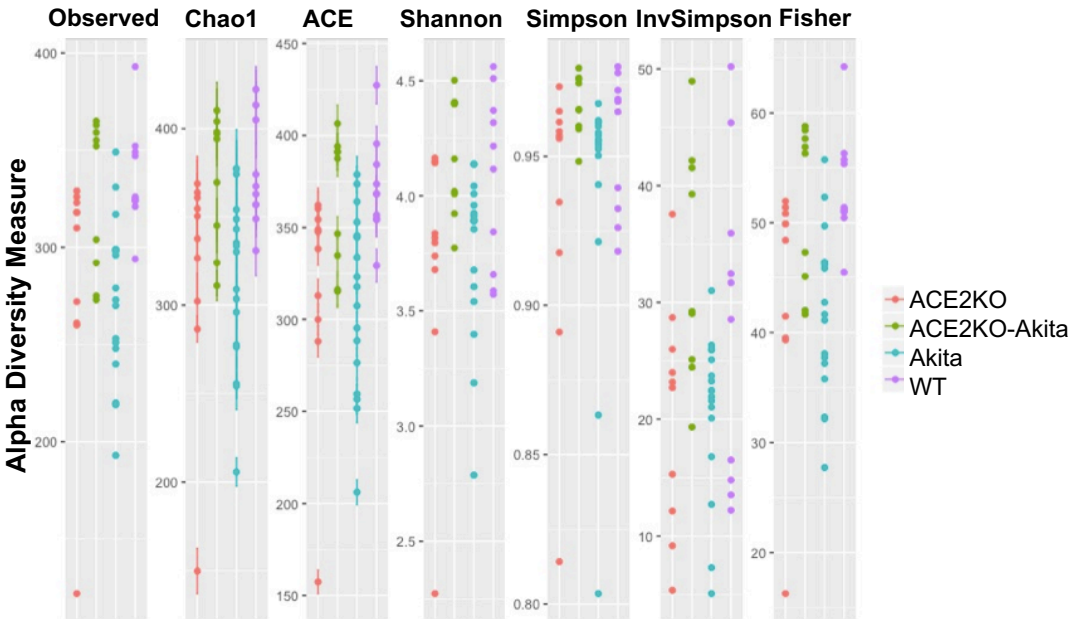
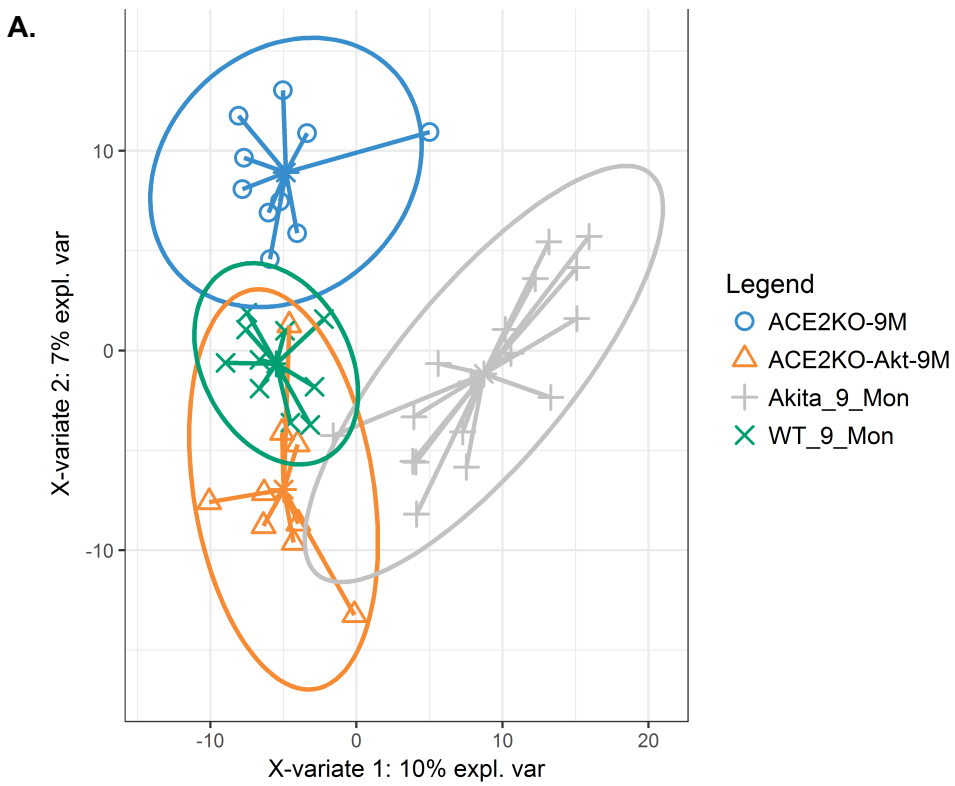


Figure 4-1. A consistent pattern of alpha diversity rarefaction curves among cohorts. No differences in bacterial species richness and evenness were observed in Akita mice and ACE2KO-Akita mice as compared to non-diabetic groups (n = 9-17). Abbreviations: WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akt, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).


A.
10
0
-10
X-variate 2: 7% expl. var
-10
0
10
20
X-variate 1: 10% expl. var
Legend
ACE2KO-9M
ACE2KO-Akt-9M
Akita_9_Mon
WT_9_Mon

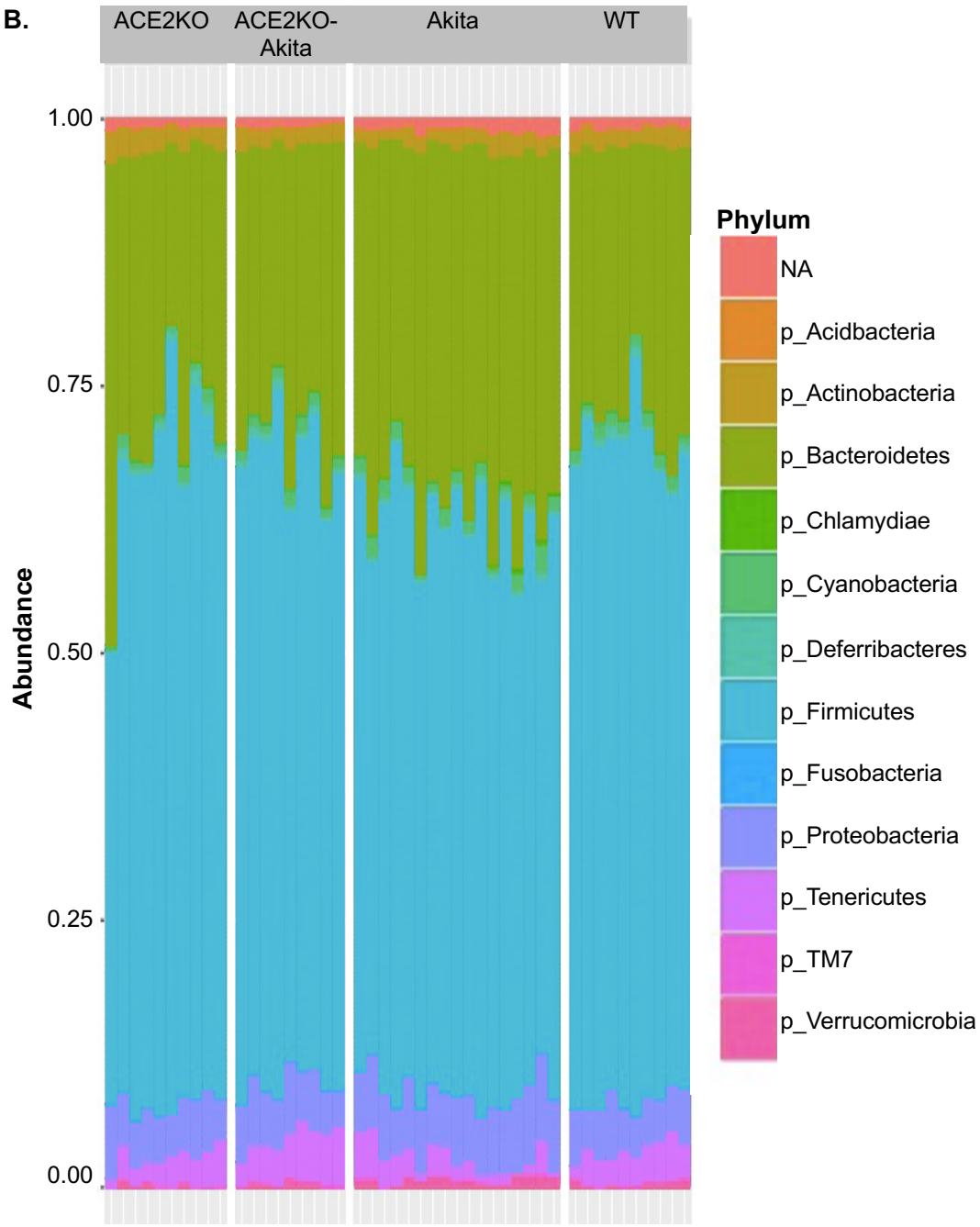
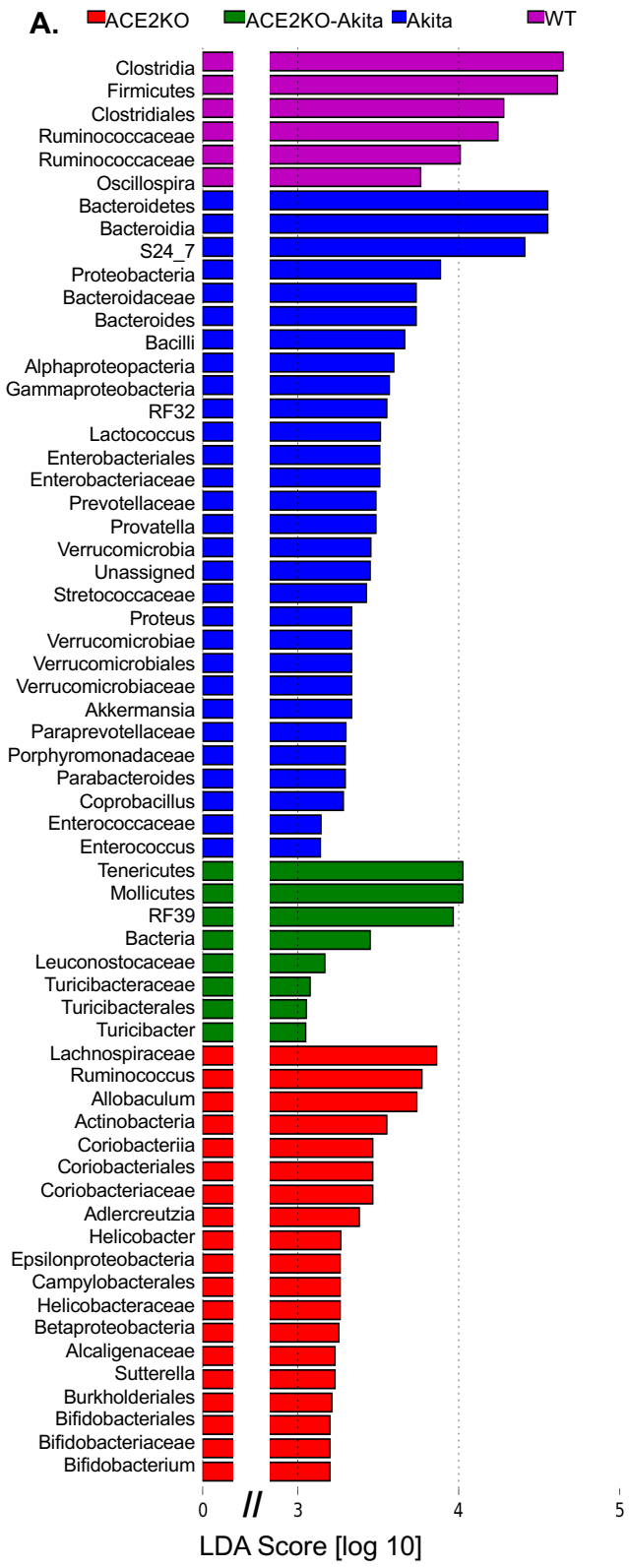


Figure 4-2. Distinct clustering of samples from the four cohorts of mice by beta diversity analysis. A: 2D PLS-DA analysis was performed to compare the overall bacterial community phylogenetic differences (n = 9-17). B. relative abundance plots uncovered 12 identified phyla and their respective relative abundance across

all samples (n = 9-17). The Firmicutes and Bacteroidetes were found dominating microbial community composition within all samples. Abbreviations: WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akt, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).


A.
ACE2KO
ACE2KO-Akita
Akita
WT
Clostridia
Firmicutes
Clostridiales
Ruminococcaceae
Ruminococcaceae
Oscillospira
Bacteroidetes
Bacteroidia
S24_7
Proteobacteria
Bacteroidaceae
Bacteroides
Bacilli
Alphaproteopacteria
Gammaproteobacteria
RF32
Lactococcus
Enterobacteriales
Enterobacteriaceae
Prevotellaceae
Provatella
Verrucomicrobia
Unassigned
Stretococcaceae
Proteus
Verrucomicrobiae
Verrucomicrobiales
Verrucomicrobiaceae
Akkermansia
Paraprevotellaceae
Porphyromonadaceae
Parabacteroides
Coprobacillus
Enterococcaceae
Enterococcus
Tenericutes
Mollicutes
RF39
Bacteria
Leuconostocaceae
Turicibacteraceae
Turicibacterales
Turicibacter
Lachnospiraceae
Ruminococcus
Allobaculum
Actinobacteria
Coriobacteriia
Coriobacteriales
Coriobacteriaceae
Adlercreutzia
Helicobacter
Epsilonproteobacteria
Campylobacterales
Helicobacteraceae
Betaproteobacteria
Alcaligenaceae
Sutterella
Burkholderiales
Bifidobacteriales
Bifidobacteriaceae
Bifidobacterium
0
3
4
5
LDA Score [log 10]

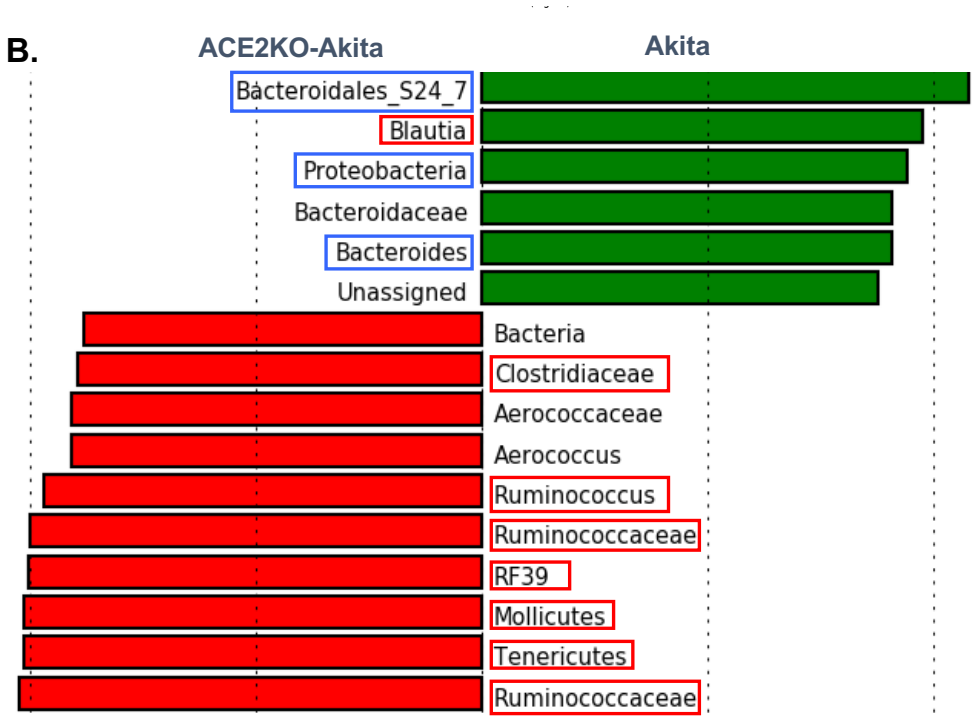
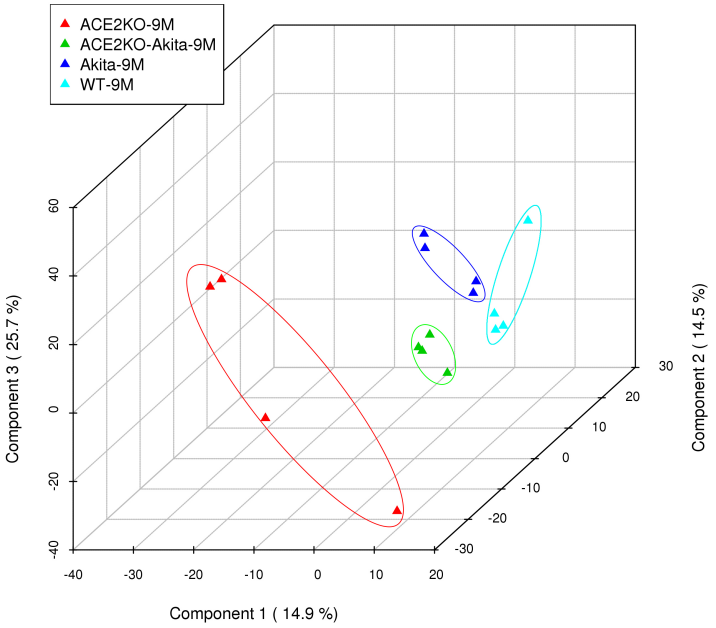


Figure 4-3. Significantly enriched taxa within each genotype. A: 62 enriched taxa found to be significantly ($p < .05$, LDA > 3.0) enriched within each respective sample cohort. The Y-axis displays each respective enriched taxa, whereas the X-axis displays the LDA score, which quantifies the strength of enrichment within each respective cohort (separated by color). Multiple enriched taxa of similar phylogeny can be observed within each respective cohort (n = 9-17). B: 10 enriched taxa found to be significantly enriched in ACE2KO-Akita mice as compared to Akita mice (separated by color), with a lot of them involved in diabetes development (highlighted by red text box) (n = 9-17). Abbreviations: WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akt, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).

PLS-DA analysis



Heatmap of counts of Metaphlan

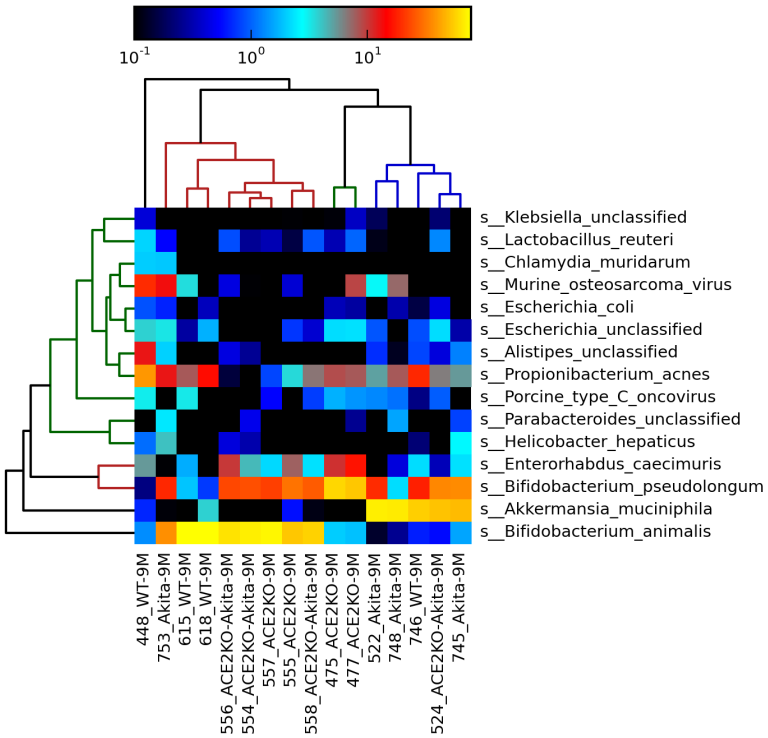


Figure 4-4. Distinct functional gene profile in the bacterial community by metatranscriptome analysis. Left panel： PLS-DA analysis was conducted to compare overall differences in functional gene (Kegg Orthology) expression profiles between genotypes. Defined clustering of samples in 3D-space indicated differential functional gene expression profiles between genotype cohorts. Right panel: A heatmap of CPM normalized counts of Metaphlan displayed differential abundances of several prominent taxa, and observe similarities in the microbiome between samples of similar genotypes. n = 4 per group. Abbreviations: WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akt, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).

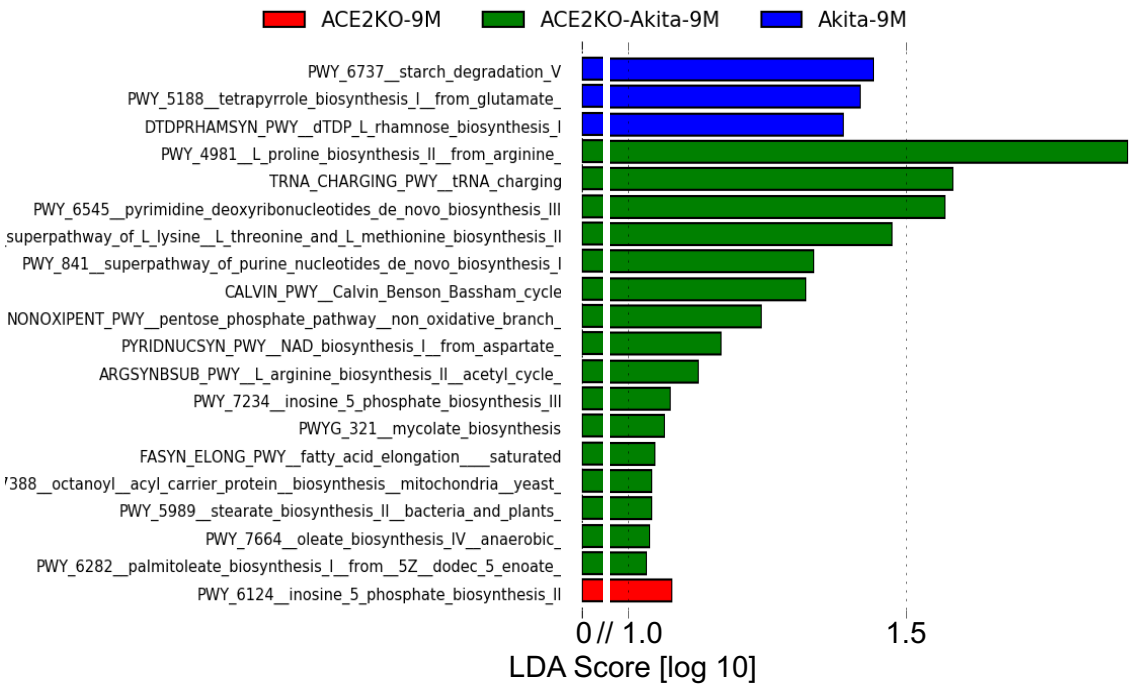
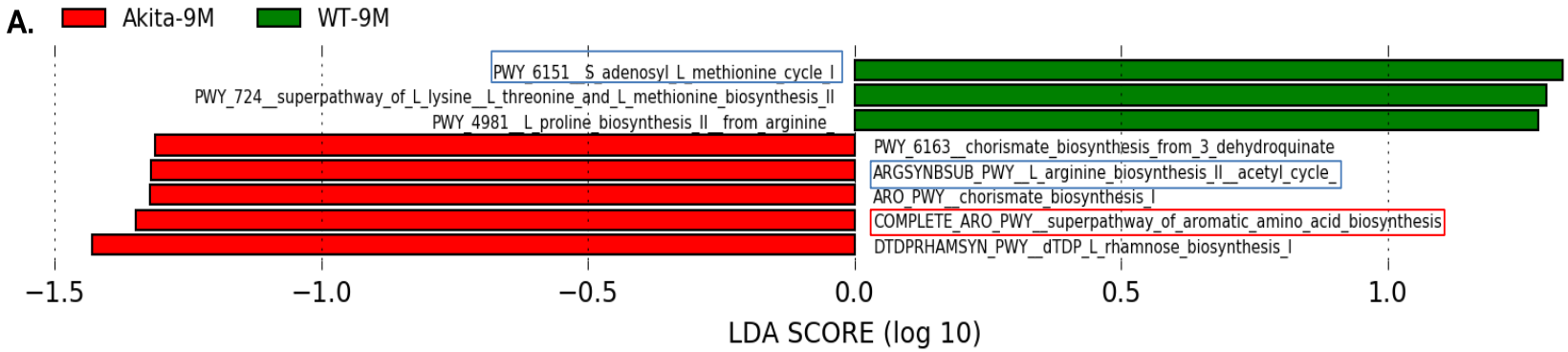


Figure 4-5. ACE2KO-Akita mice displayed the most enriched functional gene pathways in fecal samples by LefSe plots. All pathways found to be significantly ($p < .05$, LDA > 1.0) enriched were shown on the graph. The Y-axis displays enriched functional pathway in each genetype, whereas the X-axis displays the corresponding LDA enrichment score (separated by color). n = 4 per group. Abbreviations: WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akt, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).


A.
Akita-9M
WT-9M
PWY_6151__S_adenosyl_L_methionine_cycle_I
PWY_724__superpathway_of_L_lysine__L_threonine_and_L_methionine_biosynthesis_II
PWY_4981__L_proline_biosynthesis_II__from_arginine_
PWY_6163__chorismate_biosynthesis_from_3_dehydroquinate
ARGSYNBSUB_PWY__L_arginine_biosynthesis_II__acetyl_cycle_
ARO_PWY__chorismate_biosynthesis_I
COMPLETE_ARO_PWY__superpathway_of_aromatic_amino_acid_biosynthesis
DTDPRHAMSYN_PWY__dTDP_L_rhamnose_biosynthesis_I
−1.5
−1.0
−0.5
0.0
0.5
1.0
LDA SCORE (log 10)

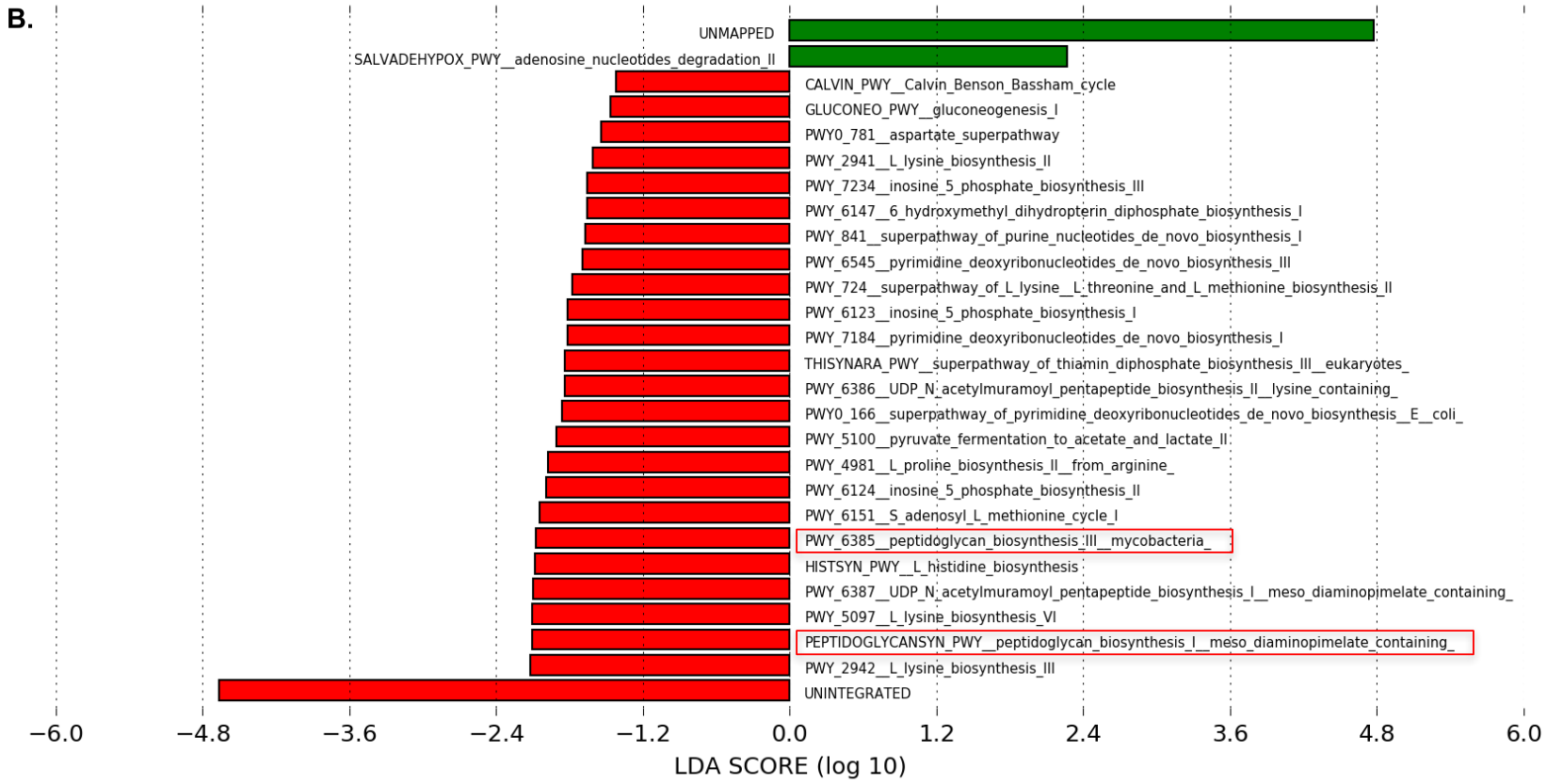
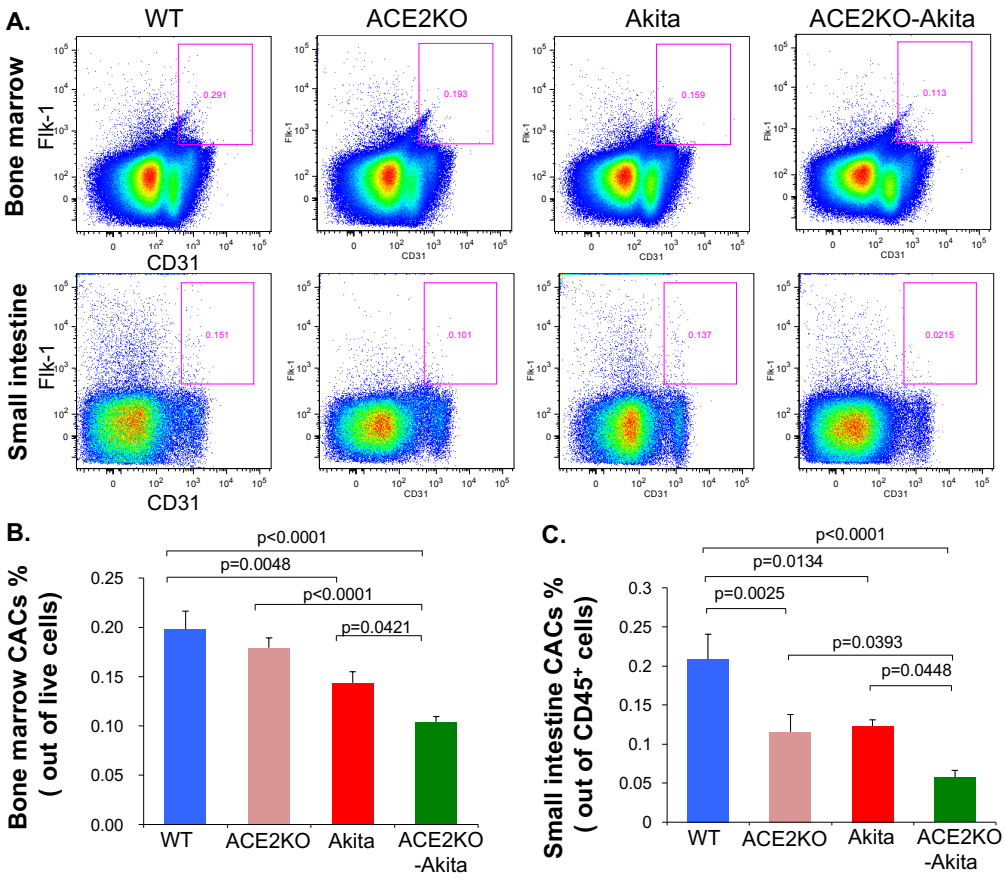

B.
UNMAPPED
SALVADEHYPOX_PWY__adenosine_nucleotides_degradation_II
CALVIN_PWY__Calvin_Benson_Bassham_cycle
GLUCONEO_PWY__gluconeogenesis_I
PWY0_781__aspartate_superpathway
PWY_2941__L_lysine_biosynthesis_II
PWY_7234__inosine_5_phosphate_biosynthesis_III
PWY_6147__6_hydroxymethyl_dihydropterin_diphosphate_biosynthesis_I
PWY_841__superpathway_of_purine_nucleotides_de_novo_biosynthesis_I
PWY_6545__pyrimidine_deoxyribonucleotides_de_novo_biosynthesis_III
PWY_724__superpathway_of_L_lysine__L_threonine_and_L_methionine_biosynthesis_II
PWY_6123__inosine_5_phosphate_biosynthesis_I
PWY_7184__pyrimidine_deoxyribonucleotides_de_novo_biosynthesis_I
THISYNARA_PWY__superpathway_of_thiamin_diphosphate_biosynthesis_III__eukaryotes_
PWY_6386__UDP_N_acetylmuramoyl_pentapeptide_biosynthesis_II__lysine_containing_
PWY0_166__superpathway_of_pyrimidine_deoxyribonucleotides_de_novo_biosynthesis__E__coli_
PWY_5100__pyruvate_fermentation_to_acetate_and_lactate_II
PWY_4981__L_proline_biosynthesis_II__from_arginine_
PWY_6124__inosine_5_phosphate_biosynthesis_II
PWY_6151__S_adenosyl_L_methionine_cycle_I
PWY_6385__peptidoglycan_biosynthesis_III__mycobacteria_
HISTSYN_PWY__L_histidine_biosynthesis
PWY_6387__UDP_N_acetylmuramoyl_pentapeptide_biosynthesis_I__meso_diaminopimelate_containing_
PWY_5097__L_lysine_biosynthesis_VI
PEPTIDOGLYCANSYN_PWY__peptidoglycan_biosynthesis_I__meso_diaminopimelate_containing_
PWY_2942__L_lysine_biosynthesis_III
UNINTEGRATED
−6.0
−4.8
−3.6
−2.4
−1.2
0.0
1.2
2.4
3.6
4.8
6.0
LDA SCORE (log 10)

Figure 4-6. Increased functional pathways of peptidoglycan biosynthesis in ACE2KO-Akita mice compared to Akita mice by LefSe plots. All pathways found to be significantly ($p < .05$, LDA > 1.0) enriched were showed on the graph (red text box, beneficial pathways in diabetes development; red text box, detrimental pathways in diabetes development). A: comparison between WT and Akita; B: comparison between Akita and ACE2KO-Akita. The Y-axis displays enriched functional pathway in each genetype, whereas the X-axis displays the corresponding LDA enrichment score (separated by color). n = 4 per group. Abbreviations: WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akt, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).


A.
WT
ACE2KO
Akita
ACE2KO-Akita
Bone marrow
Small intestine
Flk-1
CD31
0.291
0.193
0.159
0.113
0.151
0.101
0.137
0.0215
B.
Bone marrow CACs %
(out of live cells)
p<0.0001
p=0.0048
p<0.0001
p=0.0421
0.25
0.20
0.15
0.10
0.05
0.00
WT
ACE2KO
Akita
ACE2KO
-Akita
C.
Small intestine CACs %
(out of CD45+ cells)
p<0.0001
p=0.0134
p=0.0025
p=0.0393
p=0.0448
0.3
0.25
0.2
0.15
0.1
0.05
0
WT
ACE2KO
Akita
ACE2KO
-Akita

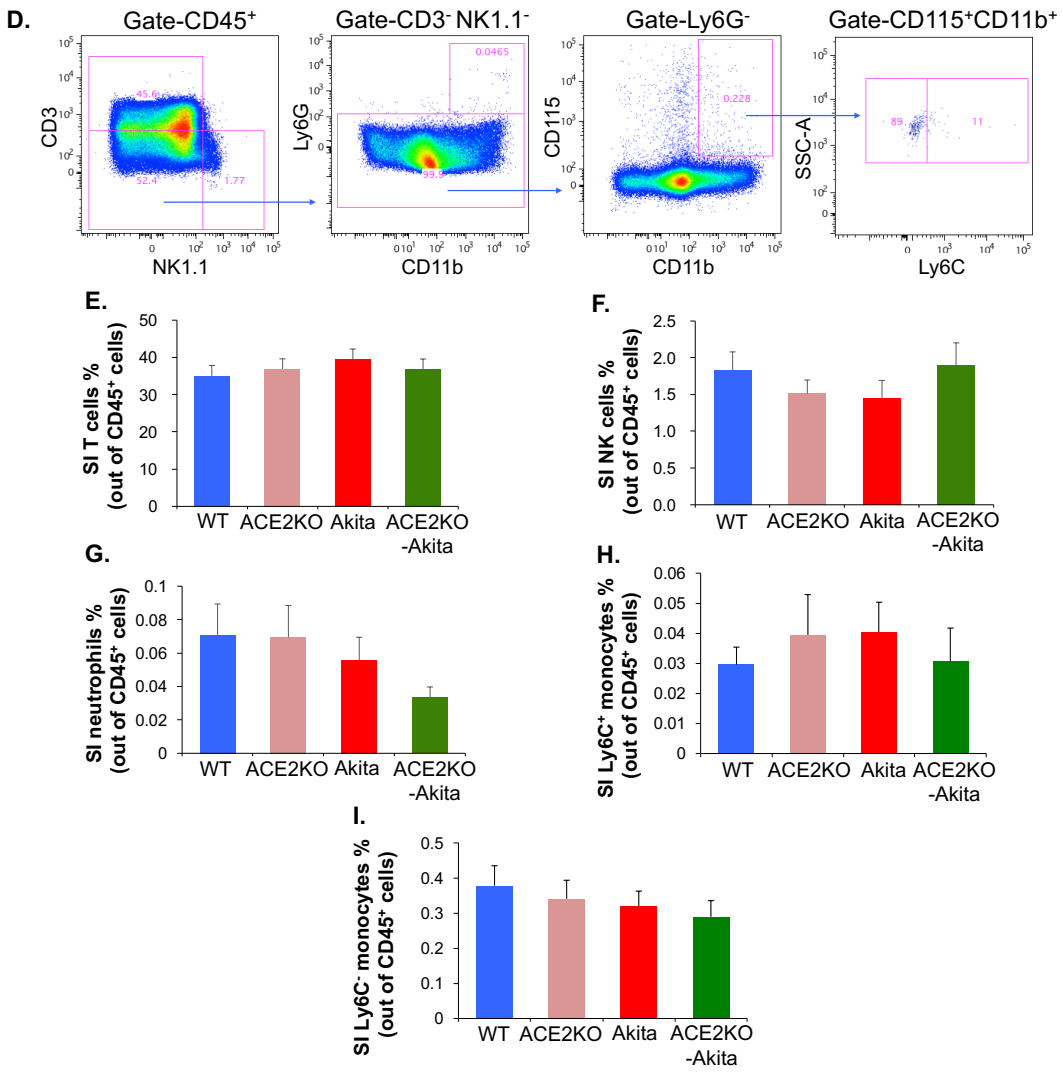


Figure 4-7. Loss of ACE2 worsens diabetes-induced less infiltration of bone marrow-derived CACs into the small intestine. A: Representative flow gating schema of CAC population of each group using small intestine lamina propria cells (LP) (Gates: $CD45^+$). B-C: Quantitative data of percentage of CACs ($CD45^+CD31^+Flk\text{-}1^+$) showed that ACE2KO-Akita had the least percentage of CACs in bone marrow (B) as well as in the small intestine (C) (Bone marrow samples: n = 13-18 per group; LP sampels: n = 8-14 per group). D: Representative flow gating schema for immune and proinflammatory cell types. E-L: No

differences of phenotypic T cells (E), NK cells (F), neutrophils (G), $Ly6C^+$ monocytes (H) and $Ly6C^-$ monocyte (I) in the small intestine LP layer among the 4 different cohorts (n=9-14). Abbreviations: CAC, circulating angiogenic cells; NK cell, natural killer cell; LP, lamina propria; WT, wild type; ACE2KO, ACE2 knockout ($ACE2^{-/Y}$); ACE2KO-Akt, ACE2 knockout-Akita ($ACE2^{-/Y}Ins2^{WT/C96Y}$).

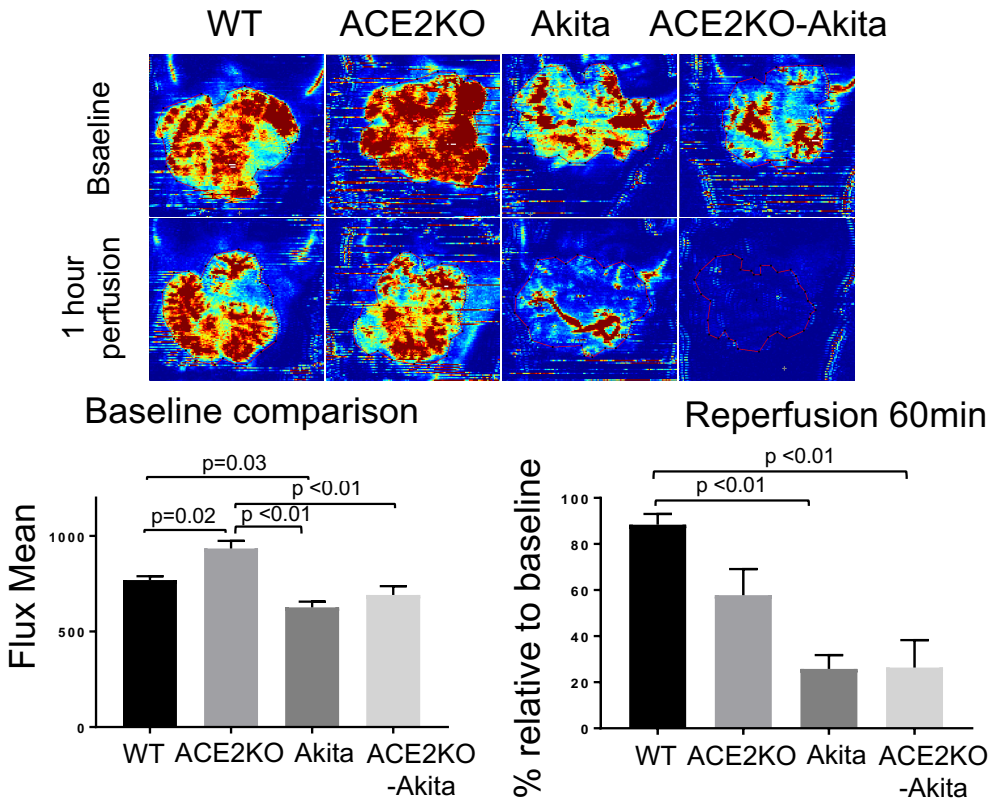


Figure 4-8. ACE2 depletion did not worsen diabetes-induced impairment of mesenteric blood flow after ischemia/reperfusion. Upper panel: Representative images of Laser Doppler blood flow of mesenteric arteries at a baseline level and at 1-hour perfusion after 30 minutes ischemia. Lower panel: quantitative data of baseline blood flow levels before ischemia/reperfusion showed that Akita groups had reduced gut blood flow as compared to their non-diabetic controls. The right graph showed blood flow levels after 30 minutes ischemia followed by 1 hour reperfusion. Both Akita and ACE2KO-Akita showed reduced blood flow recovery when compared to WT control (n = 8-10).

Chapter 5: Conclusions and future studies

In this study, ACE2KO-Akita mice were generated and carefully examined to better understand the role of the protective RAS axis in maintaining bone marrow function, the retina and the gut microbial ecology. As shown in Chapter 2, Akita mice did not change their number of total LSK cells. However, when LSK cell were subdivided into LT-HSC and ST-HSC using CD135 and CD34 phenotypic markers, a reduction of LT-HSCs was observed as well as a tendency towards a decrease of ST-HSCs. Loss of ACE2 expression worsened the diabetes-induced bone marrow defects as a further reduction in the numbers of the ST-HSCs and LT-HSCs was observed.

Migration and proliferation functions of HSPCs are important properties reflecting their vascular repair potential. These cells need to sense the chemoattractants released from the injury site and migrate toward the injured area. The cells need to expand and secrete paracrine factors to supported the impaired vasculature. Impaired migration and proliferation function of HSPCs was observed in Akita mice. ACE2 deficiency exacerbated the diabetes-mediated impairment of HSPC functions. It is worth noting that when the cells were treated with either Ang-(1-7) or alamandine, the migration and proliferation functions were restored in ACE2KO-Akita mice even at the 9 months of diabetes timepoint. ACE2 deficiency not only caused HSC depletion and impaired functions of HSPC, but also affect the balance of hematopoiesis.

By the CFU assay, bone marrow cells isolated from Akita mice formed a higher number of CFU-G/M/GM than those from WT mice. Loss of ACE2 further exacerbated this change. In turn, there was less common lymphoid progenitors in the diabetic bone marrow detected by flow cytometry analysis. ACE2 deficiency worsen the pathological alteration in diabetes, suggesting its important role in maintaining the balance of myelopoiesis and lymphopoiesis. All these results suggest that the protective RAS axis (ACE2/Ang-(1-7)/Alamadine) is important in shaping the phenotype of diabetic bone marrow and may be a promising target for restoring of bone marrow HSPC function and for vascular repair.

In the investigation of the retinal defect caused by ACE2 depletion (Chapter 3), the same cohorts of mice were used. Interestingly, bone marrow changes observed in Chapter 2 were accompanied by a greater number of acellular capillaries in the retina of ACE2KO-Akita mice compared to the Akita mice. Acellular capillaries are considered the “gold standard” for DR and are caused by endothelial cell and pericyte loss. In addition, only the ACE2KO-Akita group exhibited persistent reduction in scotopic a- and b-waves over the duration of diabetes.

Human subjects with different stages of DR were recruited and defects in $CD34^+$ cells were identified in subjects with retinopathy. Diabetic subjects with no complication exhibited increased levels of plasma Ang-(1-7) as well as MAS receptor gene expression in $CD34^+$ cells compared to healthy controls, suggesting a compensatory response in early stages of diabetes. However, the plasma Ang-

(1-7) level and $CD34^+$ cell MAS expression was dramatically reduced with the development of NPDR. Reduced MAS receptor gene expression in $CD34^+$ cells was accompanied by the impaired migratory function of the cells isolated from subjects with NPDR. The reduced migratory function was corrected by the exogenous supplementation of Ang1-7 and alamandine. Collectively, the results suggest more advanced DR in Akita mice with ACE2 deficiency. Our human data provided further support that the deficiency of the protective RAS within HSPC may be associated with the progression of DR.

Chapter 4 focused on the investigation of gut microbial dysbiosis in Akita and ACE2KO-Akita mice. 16S rRNA were isolated from fecal samples of a group of mice and gene sequencing was performed to access the gut microbiota changes in the mice. In general, both Akita and ACE2KO-Akita mice showed similar bacteria species richness as their non-diabetic controls. A difference of diversity in microbial community was observed among different genotypes by beta diversity analysis and the enriched taxa that contribute to the diversity differences were identified. Fecal samples from ACE2KO-Akita were enriched with a great diversity of bacterial types that were all reported to contribute to diabetic pathogenesis, including *Tenericutes* at the phylum level, *Mollicutes* at the class level, *RF39* at the order level, *Ruminococcaceae* and *Clostridiaceae* at the family level, and *Ruminococcus* at the genus level.

Metatranscriptomic analysis revealed that genes involved in PGN biosynthesis pathways and in amino acid biosynthesis were actively expressed in ACE2KO-Akita mice as compared to Akita mice. PGN is considered as a trigger for chronic inflammation. Interestingly, loss of ACE2 resulted in less infiltration of CACs into the LP in small intestine, without affecting bone marrow-derived T cell, NK cells and monocytes. All these results suggested that loss of ACE2 resulted in an alteration of microbiota composition as well as gene signaling pathways. Thus, the RAS may be a potential therapeutic target for diabetes-induced gut dysbiosis.

The 3 studies described in Chapters 2, 3 and 4 may on first glance appear disparate; however, they are interconnected. Bone marrow cells influence vascular repair, blood flow and endothelial barrier integrity by paracrine mechanisms and contribute to the pathological changes in diabetic microvascular complications [26].

An extensive literature supports that in chronic diabetes bone marrow cells become dysfunctional and are unable to support the function of the vasculature or repair injured tissue [219, 220, 225, 364-368]. CACs while representing a small percentage of circulating cells have a robust vascular protective effect largely mediated by their production of growth factors and cytokines [26]. These factors are vasomodulatory and can mediate changes in vascular reactivity as well as maintain the barrier characteristics of the endothelium [369]; however, CACs such as $CD34^{+}$ cells of diabetics typically loose this ability and secrete proinflammatory

and vasoconstrictive factors [26, 38]. Additionally, diabetic CACs lose their ability to migrate into tissues to respond to local injury [221, 225, 370, 371].

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 severity of retinopathy. ACE2 generates Ang-(1-7) and thus Ang-(1-7) mediates the beneficial effect of ACE2. Ang-(1-7) is dependent on MAS receptor expression. Ang-(1-7) can enhance the migratory function of CACs in vitro [93]. Ang-(1-7) overexpression of lentiviral gene modification restored in vitro vasoreparative function of diabetic $CD34^+$ cells and “in vivo” homing efficiency to areas of ischemia [93]. Genetic ablation of MAS prevented ischemia-induced mobilization of bone marrow cells and impairs blood flow recovery [281, 372]. In the current study, the peptide alamandine beneficially impacted the migration of dysfunctional $CD34^+$ from diabetics with retinopathy.

Thus, these studies and others suggest that activation of the ACE2/Ang1-7/MAS pathway stimulates the function of dysfunctional murine and human BM cells in diabetes [373]. The impact of the RAS on DR finds support in numerous studies including that all 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 [374, 375] and animals [216, 376].

Lack of sufficient penetration of these agents across the blood-retinal barrier was deemed responsible for why suppression of the classic RAS failed to treat advanced DR [216]. These studies led to the notion that restoring the balance between the classic RAS and the vasoprotective RAS within HSPCs represented a viable strategy for controlling DR in humans. Strategies focused on restoring the local retinal RAS balance by bolstering the vasoprotective RAS which results in diminished oxidative stress and chemokine production, decreased inflammatory cell infiltration and prevention of retinal vasodegeneration, the key histologic characteristic of DR. Previously, we showed that DR can be prevented/reversed by increasing retinal ACE2 using adenoassociated virus (AAV)-ACE2 [215, 216].

However, in order to further confirm a more important role of RAS within bone marrow HSPC over the systemic RAS, more studies should be performed. Firstly, we showed that loss of ACE2 led to reduced phenotypic ST- and LT-HSCs in mice at 9 months of diabetes and LK cells from ACE2KO-Akita mice showed reduced migration and proliferation functions when compared cells from Akita mice. However, the blood reconstitution and engraftment functions of HSCs from ACE2KO-Akita mice in vivo were not tested under either nondiabetic or diabetic conditions. A competitive bone marrow transplantation can be performed to address this question.

To perform these studies, donor bone marrow cells should contain 50% HSCs from ACE2KO mice (CD45.2) and 50% HSCs from WT mice (CD45.1) and recipients

would be Akita and WT mice (CD45.1) at 2 months of age. After lethal irradiation, the mixed donor HSCs should be transplanted into recipients. Four months later, blood mononuclear cells would be stained with anti-CD45.1 and anti-CD45.2 antibodies and the proportion of these two types of blood cells analyzed by flow cytometry.

If diabetic microenvironment plays an essential role in HSC hematopoiesis irrespective of ACE2 loss, the Akita recipients in general should have lower engraftment rate compared to WT recipients. If ACE2 deficiency caused impaired reconstitution function of HSCs, the percentage of CD45.2 mononuclear cells should be much less than CD45.1 ones in both WT and Akita recipients.

Another important question to address is that whether loss of ACE2 in the bone marrow is the causative for the advancement of DR. Even though, we have already observed that loss of ACE2-mediated bone marrow defects is associated with more advanced retinopathy, we still would have to perform a bone marrow transplantation to further confirm these observations. Either WT bone marrow or ACE2KO bone marrow should be transplanted into lethal irradiated WT and Akita recipients. If bone marrow ACE2 deficiency leads to diabetic retinopathy, we should observe that Akita mice transplanted with ACE2KO bone marrow should have more advanced DR than Akita mice with WT bone marrow.

Another important experiment to perform to address the same question is generating a bone marrow-specific ACE2 overexpressing diabetic mouse model. We have already successfully established a hACE2 knock-in (ACE2KI) mouse model with a EF1α-LoxP-Stop-LoxP cassette in front of hACE2 cDNA sequences. We are planning to breed the ACE2KI mice with vav-1 Cre mice to generate the bone marrow-specific ACE2KI mice, which will be then bred with Akita mice. In our hypothesis, the bone marrow ACE2KI-Akita mice should exhibit less bone marrow defects and be protected from development of DR when compared to the Akita mice. However, there is one limitation of the experiment, as currently none of the vav-1 Cre mice commercially available are inducible, therefore, we cannot control precisely when the ACE2 gene expression occurs. With the current experimental paradigm, ACE2 would be overexpressed in the bone marrow from birth on and could potentially impact bone marrow development and hematopoiesis.

Another connection we observed is the bone marrow and gut microbiota studies. In the case of T1D, an alteration of the intestinal microbiota, changes in intestinal immune responses and increased gut permeability all together form a “perfect storm” for the progression of diabetes [377]. Neu et al. showed that there was an increased permeability of gut epithelial barrier with low expression of tight junction molecules, including claudin, even before the onset of T1D in bio-breeding diabetes-prone rats [378]. Not only in rodent models, abnormal intestinal permeability was also observed in T1D by multiple clinical studies [379-383].

Besides the epithelial barrier, recently, Spadoni et al. verified the existence of gut vascular barrier and demonstrated that the gut vascular barrier may even play a more important role in protecting the interior of the host body from pathogenic bacteria and the aberrant luminal microbial antigens [267]. To the best of our knowledge, no one has examined the gut vascular permeability in diabetes yet.

In Chapter 4, we observed that ACE2 deficiency resulted in diabetes-mediated depletion of bone marrow-derived CACs in the gut lamina propria. Bone marrow cells are present in and affect a variety of other organs, including liver, spleen and gut. Therefore, it is also important to assess the numbers of bone marrow-derived CACs in liver and spleen tissues. This can help us to verify that whether ACE2 deficiency-mediated less infiltration of CACs is a gut tissue specific event or is also happening in other organs.

CACs are known to play an important role in vascular repair. Interestingly, when we checked the gut blood flow of these mice, we only observed that diabetic groups had reduced basal mesenteric blood flow levels, and reduced blood flow recovery after 30 minutes of ischemia followed by 1 hour perfusion. However, loss of ACE2 did not further worsen diabetes-mediated decrease in mesenteric blood flow recovery.

There are two possible explanations for the results of mesenteric blood flow study: firstly, it may just simply due to a short observation time of reperfusion (1 hour)

after ischemia, so that the reparative mechanisms of CACs have not been initiated, or the time is too short to show the vaso-reparative benefits. The other possible reasons may be that ACE2 is more important for maintaining the microvasculature than the macrovasculature. In the chapter 3, we did not observe deleterious effects of ACE2 deficiency on macrovasculature, as ACE2KO-Akita mice exhibited normal mean blood pressure and the impaired endothelial dysfunction of aorta rings, similar to the Akita alone. In contrast, we demonstrated increased acellular capillaries in retinal microvasculature isolated from ACE2KO-Akita mice when compared to Akita mice. Therefore, small vessel density in the small intestine needs to be evaluated in ACE2KO-Akita mice to address this question. We have stained the small intestine for CD31 antibody and are currently quantifying the density differences using Aperio software.

More importantly, it is definitely critical in future studies to assess the vascular permeability of the gut in each of the cohorts of mice to test the hypothesis that ACE2KO deficiency worsens diabetes-induced increases in gut endothelial permeability. In order to examine this, we propose to inject 70KDa FITC-Dextran intravenously. Ten minutes following injection of the FITC dextran, mice will be euthanized and the small intestine samples will be examined under the microscope. Fluorescence outside of the blood vessels will be quantified to show the extent of leakage. Peritoneal fluid will be collected as an alternative way to quantify fluorescence intensity. Eyes will also be collected and examined to

confirm that the increases in gut permeability occur simultaneously with increases in retinal permeability.

We also plan to measure gut epithelial permeability. To accomplish these studies, mice will undergo oral gavage with 40kDa FITC-dextran after 6 hours of fasting. Four hours later, blood will be collected under isoflurane anesthesia via cardiac puncture right before euthanizing. Blood plasma will be isolated and analyzed for FITC fluorescence. Data will be analyzed using a standard curve of FITC-dextran dilution. Examining the expression of the tight junction molecules, including ZO-1, claudin and occludin by immunohistochemistry would also provide insight into the integrity of the endothelium and epithelium. The underlying mechanisms of the "leaky gut" may involve the alteration of microbial antigen and increase in inflammatory mediators, which upregulate zonulin expression and downregulates tight junction proteins, ZO-1 and occludin [113, 384, 385]. Plasma lemma vesicle-associated protein-1 (PV-1) will be stained on paraffin-embedded sections. PV-1, as a marker of endothelial permeability, normally is not expressed in the LP, but can be induced by pathogenic bacteria [267, 386, 387].

In our hypothesis, Akita mice should have increased gut endothelial and epithelial permeability, with reduced expression of tight junction and adherent junction molecules when compared to WT mice. PV-1 expression should also be activated in the LP of the diabetic gut. ACE2 ablation should further worsen those pathological changes.

In our study, we observed reduced CACs in the diabetic bone marrow as well as in the LP of the small intestine. In addition, loss of ACE2 worsen the changes as the least infiltration of bone marrow-derived CACs into the small intestine in ACE2KO-Akita mice when compared to age-matched controls. This is particularly interesting because CACs play an essential role in vascular repair [221, 225, 370, 371]. Gut endothelial integrity plays an important role in preventing intestinal microbes from accessing the bloodstream and distal organs in mice [267]. We raised the question that whether ACE2KO-mediated loss of bone marrow-derived CACs contribute to the pathogenesis of the increase in gut vascular permeability observed in diabetic mice. To address this question, we are planning to isolate CACs from WT bone marrow and treat the Akita and ACE2KO-Akita mice with those “normal” CACs via intraperitoneal injection for up to 7 days. Then, access whether CAC administration can help to restore the gut vascular integrity and increase expression of tight junction and adherent junction molecules, as well as decrease the expression of PV-1 in LP in ACE2KO-Akita mice.

It is interesting that we observed ACE2KO-Akita mice had an alteration of gut microbiota composition, with a robust increase of the types of bacteria that involved in the development of diabetes. However, it is still unknown what is the underlying mechanisms that cause the modulation of gut microbiota. Because we used a global ACE2KO mice model in our study, it is difficult to verify that whether the changes in the gut microbiota are due to the bone marrow defects or the deficiency of ACE2 in the gut itself. One way to test the importance of intestinal ACE2 in

diabetes-induced microbiota modulation is to establish an intestinal specific ACE2KI model, to see if the overexpression of ACE2 in the intestine of Akita mice is able to shift the diabetic microbiota composition toward WT enterotype. To test the contribution of bone marrow cells in the change of gut microbiota, bone marrow transplantation should be performed. Bone marrow cells from ACE2KO mice or WT mice should be transplanted in the WT and Akita recipients after lethal irradiation. If the Akita mice with ACE2KO bone marrow exhibited a more deleterious enterotype when compared to Akita mice with WT bone marrow, it suggests that the bone marrow cells at least partially contribute to the change of the gut microbiota in diabetes.

Not only an alteration of gut microbiota composition but also a change of functional gene expression profile was observed in fecal samples from ACE2KO-Akita mice. Loss of ACE2 resulted in activation of genes that are involved in PGN biosynthesis in diabetes. PGN is an essential component of the bacterial cell wall and is well known as a stimulus for inflammatory responses [388]. Studies showed that PGN promoted high-fat diet-induced insulin resistance [106, 111]. Increased gut permeability in the diabetic gut may provide an opportunity for increased antigens, like PGN, to cross the gut barrier into the circulation. Circulating pathogen-associated molecules, such as PGN and LPS, lead to low-grade endotoxemia and may even affect distal organs, like an eye, through TLRs and NOD signaling pathways [268].

Thus, another important experiment is to assess the PGN levels in both systemic circulation and eye samples in the cohort of mice. In our hypothesis, Akita mice should have increased levels of PGN in the plasma and eye samples, while the loss of ACE2 caused a further increase in PGN in diabetic plasma and eyes. More importantly, we are going to test whether bone marrow-derived CAC administration by protecting the gut vascular barrier will result in a reduction in PGN levels in both the circulation and in the eye. This will allow us to determine whether the cell-mediated protection of gut barrier is sufficient to prevent the translocation of pathogen-associated molecules.

To further establish a causative connection between the microbial antigen, PGN, and DR, WT and Akita mice at 6 months of diabetes can be treated with PGN for 14 days by intravitreal injection. OCT, ERG, fluorescein angiography and acellular capillaries enumeration will be performed to assess whether PGN can initiate the same retinal changes in WT mice as are seen in DR and if PGN can worsen retinopathy in the diabetic mice. If the Akita mice administrated with PGN shows more advanced retinopathy than the Akita mice injected with vehicle, it suggests that PGN plays an essential role in the pathological changes in DR.

In conclusion, we found that the loss of ACE 2 worsened the diabetes-induced bone marrow changes and DR. Of note, the phenotypic changes observed in the bone marrow-derived cells of ACE2KO-Akita mice, found in diabetic human subjects with diabetic retinopathy and could be corrected by RAS peptides, Ang1-

7, and alamandine. Loss of ACE2 also caused less infiltration of bone marrow-derived CACs into the gut and an alteration of intestinal microbiota composition and cell signaling pathways. An increase in bacteria involved in the development of diabetes and active gene expressions in PGN biosynthesis pathway were observed.

More importantly, there is definitely more work to be done in order to understand the integrative physiology and complexity of how all the systems interconnect with each other and work together to contribute to the pathology events in diabetes and its microvascular complications. Our study and future experiments will provide insight into the therapeutic targets for diabetes and its microvascular complications. Hopefully, enhancing ACE2 or Ang 1-7 or alamandine could represent a potential therapeutic option for microvascular complications such as diabetic retinopathy.

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Conferences Attended

- Renin-angiotensin system (RAS) in hematopoietic stem/progenitor cells (HS/PC) regulates vaso-reparative function in diabetic retinopathy. Poster presented at American Diabetes Association's 77th Scientific Sessions, San Diego, US, 2017.

- Renin-angiotensin system (RAS) in hematopoietic stem/progenitor cells (HS/PC) predicts vaso-reparative dysfunction and progression of diabetic retinopathy. Poster presented at ARVO 2017 annual meeting, Baltimore, US, 2017.

- Renin-angiotensin axis in hematopoietic stem/progenitor cells predicts vascular dysfunction and diabetic retinopathy. Poster presented at AHA Scientific Sessions, New Orleans, US, 2016.

- Impact of the protective renin-angiotensin system (RAS) on the vasoreparative function of $CD34^{+}$ CACs in diabetic retinopathy. Poster presented at ARVO 2016 annual meeting, Seattle, US, 2016.

- Hyperglycemia exacerbates endothelial cell injury during hypoxia/reoxygenation via p300-mediated persistent epigenetic regulation of egr-1 gene. Poster presented at American Diabetes Association's 72nd

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