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PROTECTION FROM AORTIC ANEURYSM BY BMAL1 DELETION FROM SMOOTH MUSCLE CELLS

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PROTECTION FROM AORTIC ANEURYSM BY BMAL1 DELETION FROM SMOOTH MUSCLE CELLS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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2017

ABSTRACT

PROTECTION FROM AORTIC ANEURYSM BY BMAL1 DELETION FROM SMOOTH MUSCLE CELLS

Abdominal aortic aneurysm (AAA) is a devastating condition that occurs primarily among older people with high mortality when a rupture occurs. Currently there is no proven pharmacological therapy for AAA due to poor understanding of the underlying pathogenesis. The brain and muscle transcription factor ARNT-like (Bmal1), which is known to regulate circadian rhythm, has been implicated in vascular pathologies including atherosclerosis and vascular remodeling, but its role in AAA has not been explored.

Vascular smooth muscle is a central player in aneurysm formation and development because it is critical in all three aortic aneurysm hallmark processes including (a) degradation of elastin and extracellular matrix protein, (b) loss of medium layer smooth muscle cells, and (c) intense inflammatory cell infiltration.

Here we report that smooth muscle-selective deletion of brain and muscle Arnt-like protein-1 (Bmal1) potently protected mice from AAA induced by mineralocorticoid receptor (MR) agonist deoxycorticosterone acetate (DOCA) or Angiotensin II (ANG II) in the presence of high salt. Bmal1 was upregulated by DOCA-salt in the aorta. Moreover, deletion of Bmal1 in smooth muscle selectively upregulated tissue inhibitor of metalloproteinase 4 (TIMP4) and also abolished DOCA-salt-induced elastin degradation and matrix metalloproteinase (MMP) activation. Mechanistically, Bmal1, when bound to TIMP4 promoter, suppressed the transcription of the promoter. Taken together, these results reveal an important but previously unexplored role of smooth muscle Bmal1 in DOCA plus salt-induced AAA. We suggest that TIMP4 constitutes a novel therapeutic target for AAA treatment.

Keywords: Aortic Aneurysm, Bmal1, Vascular Smooth Muscle Cells, Matrix Metalloproteinases, Tissue Inhibitor of Metalloproteinases.

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PROTECTION FROM AORTIC ANEURYSM BY BMAL1 DELETION FROM SMOOTH MUSCLE CELLS

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Director of Dissertation

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Date

Dedicated to:

My husband Junior and My Son Ethan-David

My Parents

My Father-in-Law Hippolyte Ki Kibutu Mbungu who wanted to be here when I graduate but left us unexpectedly on 9/30/2015

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Getting into this program, I was not sure of what I really wanted to do. I got into research by curiosity and I did not know if it was ever going to be a good fit for me. I became even more reluctant when my course work became more challenging than I would ever imagine. I started asking myself whether I had chosen the right path. Thankfully, I had people around me that were willing to help me succeed however they could. Their help became very important to me when I realized that what started as a curiosity became more than expected. Each individual contribution led me to get to this point today.

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1.1. Aortic Aneurysm

1.1.1. Definition

Aortic Aneurysm (AA) is defined as the permanent and irreversible localized dilation of the aorta[1]. An aortic dilation is considered aneurysmal with an increase of a minimum of 50% of the normal diameter[2].

1.1.2. Type

1.1.2.1. Abdominal Aortic Aneurysm

Abdominal aortic aneurysm (AAA) is the most common form. Here the dilation occurs in the infrarenal region of the aorta. It is most prevalent in men over the age of 65 and is the 14th leading cause of death in the United States. Aortic aneurysm rupture accounts for approximately 4500 deaths not including the 1400 deaths that occur due to surgical repairs to prevent rupture. This chronic enlargement of the aorta is often asymptomatic and frequently leads to rupture and death.

1.1.2.2 Thoracic Aortic Aneurysm

Another form for AA is thoracic aortic aneurysm (TAA); it is less common, occurring in approximately 6-10 per every 100,000 people. The incidence of TAA formation is similar in both genders and it occurs earlier in life than AAA. The majority of TAAs involve the aortic root and/or the ascending aorta. Most patients with TAAs are asymptomatic. Just like AAA, TAA is discovered during imaging screening. However, TAA progression is rather slow; it grows at a rate of approximately 0.1cm/year, and an intervention is

required only when the diameter reaches 5.0 cm to 5.5 cm[3]. The exceptions to this rule are patients with a family history of TAA that later became an acute aortic dissection and those with Loeys-Dietz syndrome. These patients have to undergo a surgical repair when the aortic diameter reaches 4.2 cm[4]. TAA has a strong genetic component, and five different TAAs have been identified. Like AAA, hallmarks of TAA involve changes in the extracellular matrix (ECM).

1) Marfan Syndrome (MFS): was first described in 1896 and later found to be an autosomal dominant genetic disorder in 1936[5]. It is the most common genetic aortic disease, occurring at approximately four to six people per every 100,000. It is best known for its physical features which are pectus excavatum or sunken breastbone, arachnodactyly or long and slender fingers and toes, tall stature, and lens ectopia. MFS is caused by mutations in fibrillin-1(FBN1)[6], a glycoprotein found in the ECM. It has been shown to regulate TGF-β activity. A mutation in FBN1 prevents TGF-β regulation and causing an increase in its activity[7].

2) Loeys-Dietz syndrome (LDS) is the most recent syndrome discovered. It was described in 2005 by Loeys et al[8]. Patients with LDS were initially misdiagnosed as having either MFS or vascular Ehlers-Danlos (vEDS) syndrome because of the similarity in phenotype with those two other syndromes. The most specific features to LDS are hypertelorism and wide uvula. LDS is divided in two different types: 1) facial dysmorphogenic type characterized by a cleft palate, micrognathia and craniosynostosis. 2) vascular EDS-like syndrome characterized by visceral rupture, easy bruising, wide scars, joint laxity and translucent and velvety skin[9]. Other features have been described in LDS with recent research which includes facial milia and an increase in eosinophilic esophagitis as well as an increase inflammatory bowel disease[10]. LDS is caused by mutations in the transforming growth factor receptor 1 and 2 (TGFR1 and TFGR2).

Among the two, the most frequent mutation is the one found in TGFR2[9]. The types of mutations do not lead to a specific type of LDS. Mutations in these two genes affect TGFβ signaling and each mutation affects the pathway differently and has been shown to also phosphorylate SMAD2 and subsequently activate the SMAD pathway[11].

3) vascular Ehlers-Danlos syndrome (vEDS) was first described in 1901 and classified as autosomal dominant in 1949[12]. vEDS is less common than MFS occurring in one individual in every 250,000. Patients with vEDS present the following clinical features: thin skin with visible veins, easy bruising, thin pinched nose, thin lips, prominent ears, hollow cheeks, and tightness of skin of the face. These patients have a short lifespan with 50% risk of mortality at the age of 48[13]. vEDS is caused by mutations in type 3 procollagen (COL3A1) gene which is the major collagen in blood vessels. These mutations cause weaknesses in the vasculature and the heart. In vEDS, the medium and large arteries are susceptible to rupture; they can dissect without any dilation. The aortic root is not affected. Because vEDS also affects other organs, in pregnancy the enlarging uterus is susceptible to rupture in addition to other organs affected by an increase in pressure. The risk of mortality among these pregnant women due to rupture of the uterus is approximately 15%. Therefore, pregnant women with vEDS need to undergo an elective caesarian section at 32 weeks of gestation[13].

4) Turner syndrome was first described in the United States in 1938 and linked to monosomy in 1959[5]. Tuner syndrome is cause by a partial or a complete monosomy of chromosome X (45 Xo). It is quite common occurring in one in every 2000-5000 women. The physical features of Tuner syndrome are: short stature, webbed neck and lymphedema. There are many diseases that are associated with individuals with Turner syndrome including cardiovascular disease which is a main cause of mortality in these patients. Only one percent of Turner conceptions survive. Those that survive are known

to develop aortic diseases as well as aortic aneurysm, elongation of the transverse arch, and aortic coarctation[14]. They also develop abnormalities in the heart such as bicuspid aortic valve and partial anomalous pulmonary venous return to the heart[15].

5) Familial thoracic aneurysm and dissection (FTAAD): About 19% of individuals with aortic dissection have had a first-degree family member affected with the same disease that is not one of the genetic diseases mentioned above. It is believed that these individuals have mutation in genes that have an autosomal dominant inheritance with decreased penetrance. All these individuals are collectively placed in a group called familial thoracic aortic aneurysms and dissection (FTAAD); they present a variety of abnormalities, such that there is a large range of physical features among them[16]. Presently, three different genes with mutations have been identified in the population. First, mutations in the myosin heavy chain 11(MYH11) is expressed by smooth muscle cells and is part of the smooth muscle cell contractile apparatus. Mutations in this gene account for less than 2% of the FTAAD population. Second, mutations in smooth muscle actin alpha2 (ACTA 2) is also a part of the contractile apparatus in smooth muscle cells. Mutations of ACTA2 account for 14% of the FTAAD population[17]. Third are mutations in SMAD3 gene which has been recently described among this population[18]. Mutations in SMAD3 are thought to increase levels of CTGF, TGF-β1, and phosphorylated SMAD2[18]. Patients with this mutation have physical features similar to LDS; however, these patients also have an increase in bone abnormalities. This syndrome is also called aneurysm and osteoarthritis syndrome (AOS), and accounts for approximately 2 % of FTAAD population.

For the purpose of this study, we had focused on AAA rather than TAA, since the former is the most common of both types and it is primarily acquired, rather than genetic. Therefore, we will discuss the risk factors, mechanisms and treatments of AAA.

1.1.3. Risk Factors

The risk factors associated with AAA are: age, sex, ethnicity, smoking and other predisposing factors.

Age, sex and ethnicity: The risk of AAA increases dramatically after the age of 60. Approximately 1% of men between the age of 55 and 65 have a clinically relevant aneurysm. AAA is four to six times more common in men than in women, occurring approximately 10 years later in women. However, once AAA develops in women, the occurrence of AAA is more aggressive, and the aneurysm expands faster with a high probability of rupturing with a small diameter[19]. Lederle et al. showed that Caucasians were more prone to AAA than are African Americans [20].

Smoking: One of the major risk factor of AAA formation is smoking. In a study from Lederle et al, they found that smoking accounted for 75% of all the AAA[20]. The number of years of smoking was found to be directly proportional to the onset of AAA[21].

Other predisposing factors: Hypertension has been shown to be a risk factor of AAA formation and rupture. AAA has been shown to be common in patients with atherosclerosis. It is also prevalent in patients with arteriosclerosis obliterans. Family history is also a risk factor with 4-fold increase in AAA when a first-degree family member is diagnosed with AAA.

1.1.4. Mechanisms

The mechanisms that lead to aortic aneurysm formation are not well understood. However, there have been many hallmarks associated with aortic aneurysm. The hallmarks of AAA are fragmentation of elastin in the aortic media and collagen degradation, leukocytic infiltration and vascular smooth muscle cells depletion[22,

[23].Elastin is a major component of the aortic wall with viscoelastic properties. It is most abundant in the aortic media where it is associated with smooth muscle cells. Collagen, another component of aortic wall, provides tensile strength to maintain structural integrity. Smooth muscle cells are the major cell type in the aorta. Smooth muscle cells and adventitial fibroblasts can synthesize all of the different components of the extracellular matrix[24]. The lack of a definite definition of aortic aneurysm has led to development of different animal models of aortic aneurysm. These models gave new and better perceptive in the pathogenesis of aortic aneurysm. These models have revealed that aortic aneurysm formation involves chronic inflammatory infiltration of macrophages, neutrophils, mast cells, T and B lymphocytes. Different cytokines and extracellular proteases have been shown to increase the inflammatory response and to cause vascular smooth muscle cells apoptosis and extracellular matrix degradation. Macrophages are the most common inflammatory cell in aortic aneurysm tissue. C-C chemokine receptor type 2 (CCR2) interactions with chemokine (C-C motif) ligand1 (CCL2) mediates monocyte chemotaxis. MacTaggart et al investigated the role of CCR2 in aortic aneurysm formation using CCR2-/- mice. When those mice were treated with a periaortic application of $CaCl_2$ for 6 weeks, it was found those mice had an attenuation of aortic aneurysm[25]. These findings were similar to a previous study from Ishibashi et al using the ANGII model[26]. Myeloid differentiation factor 88 (MyD88) which also plays an important role macrophage infiltration, has been found to play a role in aortic aneurysm formation. Deficiency in MyD88 attenuates abdominal aortic aneurysm using the ANGII model[27]. Neutrophils have also been found in human and mice aortic aneurysm. Deletion of L-selectin, a molecule involved in neutrophil recruitment to inflammatory sites[28], decreased aortic diameter after elastase infusion[29].

Many different cytokines and chemokines have been studied in aortic aneurysm and their roles have been established. Transforming growth factor (TGF-β) inhibits inflammation, and stimulates collagen deposition. Systemic neutralization of TGF-β activity was found to increase AngII-induced aortic aneurysm and increase vascular smooth muscle cells death, elastin degradation and aortic rupture in C56BL/6 mice[30]. Administration of cyclosporine A, an immunosuppressant that promotes tissue accumulation and induces TGF-β, attenuates aortic aneurysm formation in an elastase model in rats and $CaCl_2$ model in mice, while administration of anti TGF-β antibody abrogated the protective effects of cyclosporine A[31].

Another critical cytokine that has been associated with aortic aneurysm is tumor necrosis factor (TNF-α). TNF-α has been found to be increased in plasma of patients with AAA[32]. TNF-α as well as other members of the TNF receptor superfamily have been found to be increased in human aortic aneurysm tissues[33-35]. When TNF-α-/- mice underwent periaortic application of $CaCl_2$ for 6 weeks, those mice has shown complete protection from aortic aneurysm. These results has shown that TNF-α is big contributor in the formation of aortic aneurysm.

Several interleukins have been associated with aortic aneurysm including IL1-β, IL-6, IL-17 and IL-23. They have been found to be increased in human aortic aneurysm tissues. IL-6 is significantly increased in patients with AAA and TAA[36]. Using the elastase model to induce aortic aneurysm, deletion of IL-17 or IL-23 in mice attenuates aortic diameter and cytokine production[37]. Similarly, deletion of IL-1β in mice prevented aortic aneurysm formation[38].

Elastin degradation, an early event in AAA, causes dilation whereas collagen degradation causes rupture. Elastin and collagen are degraded by endopeptidases that

are secreted by smooth muscle cells and adventitial fibroblasts within the vascular wall by infiltrated lymphocytes and monocytes. Matrix metalloproteinases (MMPs) are endopetidases associated with AAA and are locally activated. Their actions are inhibited by tissue inhibitor of matrix metalloproteinases (TIMPs) which also increased during aneurysm formation, but TIMPs' action is overwhelmed by a high increase of activated MMPs by other MMPs or plasmin.

Another key event in the formation of an aneurysm is smooth muscle atrophy in the medial layer of the aortic wall. Smooth muscle cells contribute to the maintenance of the structural wall through production of various extracellular matrix proteins. They also play a role in vascular remodeling by expressing proteinases and their inhibitors. In the aneurysm pathology, SMC play a role in matrix synthesis, proteinase and inhibitor elaboration, and inflammatory cell recruitment. The imbalance between matrix metalloproteinases and their inhibitors is observed in aortic aneurysm, with the matrix metalloproteinase overwhelming their inhibitors, is attributed to either an increase in matrix metalloproteinase production from smooth muscle cells or a decrease in tissue inhibitor production from smooth muscle cells[39]. SMCs produce different proteinases in response to different stimuli. Inflammation, shear stress, or injury to vessel walls lead to an increase of MMP3, MMP7, MMP9, and MMP12 in vivo[40, 41].

Studies have shown that smooth muscle cells play a role in matrix degradation in aortic aneurysm[42]. SMCs from aortic aneurysm tissue express a high level of MMP2 compared to non-aneurysmal aorta[43]. SMC constitutively express MMP2 and stimulation with platelet-derived growth factor (PDGF) further increases its expression[44]. PDGF also increases MMP12 expression in SMC[45]. The expression of MMP-9 and MMP-3 in SMC are induced by IL-1β and TNFα[46]. These cytokines have been shown to be increased in human aortic aneurysm tissue[47]. One of the final stages of AAA is medial degeneration.

The decrease in SMC in AAA has led researchers to hypothesize that the decrease in SMC in the media is a key player in the imbalance between the metalloproteinases and their inhibitors. Studies have shown that seeding of SMC prevents AAA formation. In a study conducted in rats, seeding of syngeneic VSMC endoluminally prevented aortic aneurysm increase after 8 weeks of aneurysmal aortic xenografts. The seeding also prevented MMP1, 3, 7, 9 and 12 mRNA increases. TIMPs1, 2, 3 were significantly increased when compare to their control[48]. A previous study from Losy et al. had also shown that seeding of VSMC prevented aortic aneurysm expansion in rats[49]. These studies suggest that SMC play a critical role in aortic aneurysm; therefore, there is a need to better understand the role that smooth muscle cells play in aortic aneurysm formation.

1.1.5. Animal Models

Animal models of aortic aneurysm are classified into three different groups: genetically predisposed animal models, chemical models, and physical models.

1.1.5.1. Genetically predisposed animal models

The blotchy mouse, which has been found to develop spontaneous aneurysms, has a mutation on the X chromosome resulting in a defect in cross-linking of collagen and elastin. The defective cross-liking is a result of an abnormal copper metabolism. These mice have abnormal connective tissue, skin color and neurological function[50], they develop saccular and fusiform aneurysms mostly in the thoracic aorta[51]. These findings have led to the conclusion that copper is involved in aortic aneurysm formation. However, there has not been any evidence from human patients with aortic aneurysm that involved copper since copper levels remained unchanged in aortic aneurysm[52, 53]. Lysyl oxidase (LOX) is an enzyme involved in crosslinkage of elastin and collagen. Deficiency of Lox in mice causes thoracic aortic aneurysm rupture from the perinatal

period[54]. Apolipoprotein E (ApoE) and low-density lipoprotein (LDL) receptor knockout mice develop abdominal aortic aneurysm after being fed with high fat diet[55]. The Tsukuba hypertensive mouse, a transgenic mouse carrying a human gene for renin and angiotensinogen[56], dies from aortic aneurysm rupture after drinking water containing 1% sodium chloride[57].

1.1.5.2. Chemical models

Periarterial application of calcium chloride ($CaCl_2$) solution to the infrarenal aorta causes aortic aneurysm formation. This model was first developed using the rabbit common carotid artery causing the local dilation of the artery. This was associated with loss of endothelial cells, degradation of the elastin lamina, intimal hyperplasia, and inflammatory infiltration. Subsequently, $CaCl_2$ was applied to rabbit aorta. This model consists of applying 0.5M of $CaCl_2$ around the infra-renal aorta for approximately 15 minutes with cotton gauze. This model has been demonstrated to induce medial damage and vascular remodeling, collagen degradation, and an increase in MMP activity. $CaCl_2$ induction does not lead to a spontaneous increase in aortic diameter. However, after 2 weeks, the aortic diameter increased by more than 64%, and increased more than 113% during the third week[58]. Disruption of elastin lamina and an increase in the inflammatory response suggested that this model was clinically relevant since these same features are found in human AAA. Using this model, the role of different MMPs in aortic aneurysm formation has been better understood. Using genetically altered mice, Longo et al. have shown that MMP2-/- and MMP9-/- mice did not develop AAA. MMP9-/- mice developed aneurysm only after being infused with competent macrophages from the wild type mice[59]. These findings showed that MMP2 and MMP9 are both required to develop AAA. Longo et al. also determined the role of MMP12 using MMP12-/- mice; they had demonstrated that MMP12 attenuates dilation of the aorta[60]. Since MMP

activity is tightly regulated by TIMPs, the role of TIMPs has also been studied using this model. TIMP2-/- mice were found to have a smaller aortic diameter than their corresponding control after 6 weeks and had a lower level of MMP2[61], supporting evidence that TIMP2 activates MMP2.

A modified model of $CaCl_2$ was developed in order to accelerate AAA formation: calcium phosphate ($CaPO_4$). In this model, the infrarenal of the aorta is incubated with $CaCl_2$ for 10 minutes followed by phosphate-buffered saline (PBS) for 5 minutes. This model increased aortic diameter 7 weeks after treatment. $CaPO_4$ treatment caused an increase in apoptosis and inflammatory infiltration[62].

The elastase-induced model is also an important model. The procedure involves the insertion of a catheter in the infrarenal aorta through the iliac bifurcation. The aorta is then clamped at the level of the renal vein and the catheter ligated. Type I porcine pancreatic elastase in then introduced to the lumen and incubated for 5 minutes after which the flow is restored. Elastase infusion resulted in immediate dilation of the aorta and an aneurysm developed 2 to 5 days later. This outcome showed the involvement of medial elastolytic activity in aneurysm formation making this model a very useful model. This model has been used to investigate the role of hypertension, gender, and smoking in aortic aneurysm formation. To understand the role of hypertension in aortic aneurysm formation, aortic aneurysm was induced in normotensive Wistar-Kyoto rats (WKY) and hypertensive Wistar Kyoto rats (WKHT) using the elastase infusion model. Around day 7, the aortic diameter was significantly larger within the hypertensive group than the normotensive group. Fourteen days after infusion, the aortic diameter increased even further in hypertensive rats. Although the normotensive rats had an increase in aortic diameter of over 100%, the genetically hypertensive rats had an increase in aortic diameter of over 200%[63]. These results suggested that hypertension contributes to the

expansion of aortic aneurysm. Gender was studied as a risk factor using the elastase-infusion model. It has been demonstrated that when male rats are treated with exogenous estrogen, their aortic diameter was smaller than non-treated male rats. This decrease in aortic diameter was also seen in orchidectomized male rats. Hormone replacement in male rats that were surgically castrated showed an increase in aortic diameter while there was a decrease in aortic diameter in oophorectomized females. This decrease in aortic diameter was associated with a decrease in macrophage infiltration[64]. These data suggest that gonadal hormones regulate aortic aneurysm by altering macrophage infiltration. Smoking being a major risk factor of aortic aneurysm formation was investigated when C57BL/6 mice were exposed to cigarette smoke two weeks prior to elastase infusion and continuously until the aorta was harvested. Mice that were exposed to both cigarette smoke and elastase developed a larger aortic aneurysm compare to those that only were elastase infused. The increase in diameter in mice exposed to both was 60% greater than those that had elastase infusion[65]. The results confirmed the risks of cigarette smoking in aortic aneurysm development. This model was also used to look at the role of TIMPs in aortic aneurysm formation. When TIMP1-/- and their control underwent elastase infusion to induce aortic aneurysm, TIMP1-/- mice developed a larger aortic aneurysm compare to the control 14 days later[66].

Systemic angiotensin II infusion is a model described by Manning et al. and shares the same characteristics as human aortic aneurysm. In this model, AngII in an osmotic mini-pump is implanted subcutaneously into LDL receptor-/- mice or ApoE-/- mice at a dosage of 1000ng/kg/min for 28 days. This model results in accelerated atherosclerosis and abdominal aortic aneurysm in the suprarenal aorta[67]. AngII induced aortic aneurysm results in medial degeneration and remodeling, inflammation, and thrombosis. Data have

shown an increase in inflammation and an increase in MMPs gene expression[68]. These characteristics are also seen in human aortic aneurysm, making this model a good model to study aneurysm. AngII infusion has a modest increase in blood pressure; this increase in blood pressure was shown to be independent of aortic aneurysm formation. When hydralazine was given to lower blood pressure in AngII infused ApoE -/- mice, blood pressure decreased; however, aortic aneurysm formation did not change[69]. The incidence of aortic aneurysm in these mice only decreased with pretreatment with 17β-estradiol[70]. The renin-angiotensin system has been studied to understand its role in this model. When losartan, an AT1 receptor antagonist, was given in conjunction with AngII to ApoE-/- mice, aortic aneurysm formation was completely inhibited[71]. Candesarten, another AT1 receptor antagonist and Lisinopril, an ACE inhibitor, attenuated aortic aneurysm expansion[72]. When given an AT2 receptor antagonist PD123319, the incidence of aortic aneurysm increased as well as the severity[71]. However, spironolactone, an aldosterone receptor antagonist, there was no effect on aortic aneurysm formation[73]. Several drugs have been shown to attenuate aortic aneurysm formation and reduce incidence in this model: Doxycycline[74], Vitamin E[75], simvastatin[76] and rosiglitazone[77].

A mineralocorticoid agonist plus salt induces aortic aneurysm in mice. This model was first developed as a hypertension model and was later found to develop AAA in older mice (8 to 10 months). Subcutaneous implant of a 50 mg Deoxycorticosterone acetate (DOCA) pellet or an osmotic pump containing 200ng/kg/min of aldosterone in conjunction with water containing 0.9% NaCl and 0.2% KCl for 21 days with the DOCA pellet and 28 days with the osmotic pump leads to dilation of the aorta in the suprarenal aorta. This model requires high salt intake. Liu et al. had demonstrated that in the absence of high salt, mice did not develop any aneurysm. This model was shown to be

clinically relevant and to be age-dependent. The incidence and severity of AAA were significantly lower in young (10 weeks old) mice compare to older (10 months old) mice. This model shared the same characteristic as human aortic aneurysm: elastin and collagen degradation, MMP upregulation, vascular smooth muscle cells degeneration and inflammatory cells infiltration.

1.1.5.3. Physical Models

Different physical techniques have been used in order to create aortic aneurysm in animals. However, these techniques do not completely mimic human aortic aneurysm. Therefore they are used to develop new interventions for abdominal aortic aneurysm formation. These models have led to the development of techniques such as stent grafts and the improvement of the endovascular repair[78].

The crude method induces aortic aneurysm by physically damaging the vessel by either intramural injections of chemicals such as acetrizoate or using cryogenic probes, carbon dioxide lasers or resecting the media and adventitia of the aorta[79-82]. The aneurysms formed from these methods are usually saccular aneurysm or pseudoaneurysm. They are unpredictable in size, growth rate and rupture risk.

The synthetic method requires replacing a segment of the aorta with interposition grafts made of synthetic or autologous materials. The aneurysms from this method are more consistent in shape and size[83].

The anterior patch model: in this model an elliptical patch of synthetic materials is placed after a longitudinal incision to the aorta to close the incision. This is one of the successful models of physical model of aortic aneurysm. A study from dogs that underwent the procedure had shown an anterior patch in the infrarenal of the aorta by suturing full thickness of the jejunal patches to the interior of the longitudinal aortic incision,

developed aortic aneurysm. This study was performed in order to determine the efficacy of the endovascular aortic repair[84]. This model was also used to determine the efficacy of a specific stent graft design. In this study a fusiform aortic aneurysm was formed using an iliac vein patch. This technique was shown to be realistic. Twenty-three dogs were used following this technique after developing aneurysm they were treated with Dacron stent grafts and followed up after 6 to 12 months. At 12 months, no leaks or flow to the sac of the aneurysm were detected and there was no further enlargement on the aneurysm[85].

1.1.6. Treatment

Aortic aneurysms are often discovered after an X-ray, during an ultrasound or echocardiogram. Thereafter, ultrasounds are required to determine the size and the growth rate of the aneurysm. For small aneurysm, an ultrasound is required once every 2 years, while larger aneurysms are observed every 6 to 12 months. A computed tomography (CT) or magnetic resonance angiogram (MRA) may be required for more detailed information, such as determining the position of the aneurysm in relation to the renal arteries or other organs. An angiogram is more useful to determine the size of the aneurysm, whether there is an aortic dissection or a blood clot. The importance of these screening tests is to estimate the risk that an aneurysm has for rupture and to evaluate the risk of rupture to the risk of surgery. The risk of mortality is about 80% for patients with a ruptured aorta by the time they reach the hospital. Those who undergo surgery have a perioperative mortality of approximately 50%. An aortic aneurysm is considered for surgery when the diameter reaches 5.5 cm. The surgery is either an open or endovascular repair (EVAR). An EVAR consists of placing an expandable stent graft in the aorta through the femoral artery. This technique led to a decrease in open surgery and improved patients' survival. Therefore, it was found to be more successful than open

surgery. Candidates suitable for EVAR have a reduced morbidity and mortality. Patients with small aneurysms (less than 5.5 cm) are not at high risk of rupture. These patients are required to undergo pharmacotherapy to reduce the growth rate. Beta-blockers have been used in patients with hypertension and angina, they have been shown to significantly reduce the rate at which aneurysms grow[86]. Those that undergo antibiotic therapy to manage AAA need to have evidence of chronic inflammation in AAA. They also have to show inhibition of proteases and inflammation by antibiotics. These patients are also recommended to stop smoking.

To date, the only therapy for AAA is through mechanical surgical repair.

Therefore, a thorough understanding of the molecular mechanism of aortic aneurysm is a pressing matter.

1.2. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are members of a large family of proteases known as metzincin superfamily. They are also known as matrixins. They are calcium-dependent zinc-containing endopeptitases. Other members belonging to metzincin superfamily are serralysins, astacins, adamalysins or desintegrin metalloproteinases (ADAMS) and reprolysins[87]. Taken together, MMPs are capable of degrading all components of the extracellular matrix (ECM) and the basement membrane. They play an important role in tissue remodeling, angiogenesis, bone development, wound healing, and uterine biology. They have been shown to play a role in regulating the release and the activation of cytokines and chemokines, growth factors and antibiotic peptides and other bioactive molecules[88-90]. MMPs also play a role in different diseases: vascular diseases such as hypertension, atherosclerosis, aortic aneurysm and varicose veins, cancer and different types of inflammatory pathologies.

1.2.1. Classification

To date, there are 28 MMPs classified among which 25 different MMPs have been identified in vertebrates, 24 of which are found in humans including two identical genes encoding for MMP23 called MMP23A and MMP23B[91]. MMPs are synthesized as prepro-enzymes and secreted as pro-enzymes. They can be secreted form the cell or anchored to the plasma membrane which limits the catalytic activity to the extracellular space or to the cell surface. They consist of a pro-peptide domain that is uniquely conserved and contains a cysteine residue within the sequence that interacts with the catalytic zinc in the active site to maintain the latency of the enzyme. However, MMP23 lacks this linker peptide, but possesses proprotein at the C-terminal end of the propeptide, which is activated intracellularly by furin. MMPs are commonly classified based on their structure, their substrate and subcellular localization and are divided as followed: collagenases, stromelysins, matrilysins, gelatinases and furin- activatable MMPs.

Because of their difference in sequence and substrate specificity MMP12, MMP19, MMP20 and MMP27 are not classified. MMP12 also known as macrophage metalloelastase is mainly expressed in macrophages and it is the potent elastolytic enzyme[92]. In humans, MMP19 is expressed in many different tissues and has shown to be a very potent degradative enzyme. It can also degrade gelatin, aggrecan and type IV collagen[93]. First isolated from a porcine enamel organ, MMP20 also known as enamelysin is secreted by odontoblast of the dental papilla and ameloblast[94, 95]. Besides the fact that MMP20 is highly expressed in B-lymphocytes, and that it can degrade gelatin and casein, little information is known about human MMP27. However, it was first identified from chicken embyo fibroblasts[96].

MMP1, MMP8, and MMP13 degrade collagen. They are also called collagenase 1,2 and 3 respectively. They are so called because of their ability to first unwind triple helical

collagen and then to cleave fibrillar collagen type I, II and III into characteristic 3/4 and 1/4 fragments. Their hemopexin domain is essential for the degradation of the native collagen. Once this domain is removed, the MMPs are unable to carry out their catalytic abilities[97].

Matrilysins have 2 members MMP7 and MMP26 also known as matrilysins1 and matrilysins2 respectively. They lack the carboxy-terminal hemopexin domain. The have been involved in the degradation of ECM like laminin, entactin and type IV collagen. MMP7 has been shown to cleave cell surface molecules such as Fas-ligand, syndecan 1 and E-cadherin to generate soluble forms. MMP26 is expressed in breast cancer cells[98] and is an activator of pro-MMP9 under pathological conditions[99].

Stromelysins have 3 members: MMP3, MMP10 and MMP11 also known as stromelysins 1,2 and 3 respectively. They have a structural design similar to the collagenases; they can degrade many different ECM components but cannot cleave native collagen. MMP3 and MMP10 have similar structure and possess the same substrate specificity. They are secreted as inactive proMMP, while MMP11 is distant in its structure and has very weak activity toward ECM. It is secreted as an active enzyme since it is activated intracellularly. MMP3 and MMP10 have the ability to remove the propeptide domain of the three procollagenases as well as proMMP9 and activate them.

Gelatinases have two members: MMP2 or gelatinase A and MMP9 or gelatinase B. They play an important role in the remodeling of collagenous ECM. They possess three repeats of type II fibronectin located inside the catalytic domain, allowing them to degrade denatured collagens and gelatins. They degrade different components of the ECM such as collagen type I, IV, V, VII, IX, X, elastin, fibronectin, aggrecan, vitronectin and laminin[100]. They are also capable of degrading non-ECM molecules such as pro-

TNFα[101], TGFβ[102] and MCP3[103]. They are expressed in fibroblasts, keratinocytes, endothelial cells, chondrocytes and monocytes. Both MMP2 and MMP9 have been shown to play a role in many pathological diseases including inflammation, cancer, vascular disease such as artherosclerosis and aortic aneurysm, cancer and bone diseases.

Furin-activable MMPs have two different subgroups: secreted MMPs and membrane-type MMPs. They are called furin-activatable because they possess a prohormone convertase cleavage site or furin recognition site between the propeptide and the catalytic domain.

Secreted MMPs has two members MMP21 and MMP28. In general, MMPs are secreted in the inactive form and the uniqueness of these enzymes is that they are activated intracellularly by a furin-like protease and secreted as active enzymes. MMP21is expressed in different tissues such as kidney, intestine and skin during embryonic development. MMP28 has not been well documented and its role is not clearly defined. It has been shown to play a role in various diseases such as cancer, multiple sclerosis and certain disease of the central nervous system.

Membrane-type MMPs are classified into two groups: type I transmembrane MT-MMPs and glycosylphosphatidylinositol (GPI) MT-MMPs. These MMPs are located on the cell surface. Type I transmembrane MT-MMP are anchored to the plasma membrane through a transmembrane domain and the GPI MT-MMP through a GPI anchor. Type I transmembrane MT-MMPs members include MMP-14 or MT1-MMP, MMP-15 or MT2-MMP, MMP16 or MT3-MMP and MMP24 or MT5-MMP. These type I transmembrane MT-MMP share the same structural domain. Synthesized a pre-pro enzymes, processing of the signal and prodomain occurs before they are secreted into the cell surface. GPI

MT-MMP include MMP17 or MT4-MMP and MMP25 or MT6-MMP; they also share the same structural domain. The GPI-anchoring peptide is a hydrophobic amino acid stretch at the C-terminus. When GPI-MMPs are secreted, they are secreted as pre-proenzyme anchoring this hydrophobic sequence. While MMP17 has a low enzymatic activity to cleave ECM components, MMP25 has been shown to cleave a broader range of ECM components; this includes gelatin, collagen IV, fibronectin, fibrin and proteoglycans. MMP25 is expressed in leukocytes, lung and spleen and MMP17 in brain, colon, ovary, testis and leukocytes.

1.2.2. Regulation of MMP enzymatic activity

MMPs are considered important regulators for "tissue homeostasis and immunity in the network of multidirectional communication within tissues and cells"[104] because they can degrade a broad spectrum of substrate. Therefore, their activities are tightly regulated. MMP catalytic activity is tightly controlled at four different levels: 1) at the transcriptional and post translational level; 2) their compartmentalization; 3) MMP activation and 4) their inhibition by endogenous inhibitors i.e. tissue inhibitors of matrix metalloproteinases (TIMPs) and other non-specific proteinase inhibitors such as α_2-macroglobulin.

MMP gene expression is primarily regulated at the transcriptional level resulting in a low level of mRNA expression under normal physiological conditions. It has been recently reported that MMP post transcriptional stability is tightly regulated by cytokines, nitric oxide, or micro-RNA. A common cis-element within the promoter region is shared amongst many of the MMP family members; supporting evidence that MMPs are co-expressed or co-repressed by various stimuli, including inflammatory cytokines, growth factors, glucocorticoids, or retinoids[105]. The most surprising finding is the clear distinction in promoters of functionally related MMPs such as MMP2 and MMP9 or gelatinases and MMP1 and MMP8 or collagenases. Based on the composition of the cis-element, MMP

promoters are grouped into three different categories. The first category which includes most MMP promoters contains a TATA box and an AP-1 binding site; most of which have a PEA3-binding site adjacent to the AP-1 binding site. This PEA3 binding site is primarily responsible for controlling MMP transcription by cytokines and growth factors[106]. The second category is a category of MMP promoters that have a TATA box but do not have an AP1-binding site which allows for a more simple and distinct regulation of the promoter. The Third category is a category of MMP promoters that do not contain a TATA resulting with transcription starting at multiple sites. These MMPs include MMP2, MMP14, and MMP28[107]. Other factors that influence the transcriptional control of MMPs are epigenetic mechanisms such as DNA methylation and/or chromatin remodeling with histone acetylation. Hypermethylation represses MMP Transcription[108] while hypomethylation is regulatory for MMP expression[109, 110]. In conclusion, different factors are required for MMP transcription:1) recruitment and cooperation of transcription factors, 2) chromatin remodeling factors, 3) histone-modifying enzymes and 4) basal transcription machinery to a promoter[111].

Recent studies show that posttranscriptional mechanisms are also involved in the control of MMP expression in response to certain cues. Overall et al. showed that in human gingival fibroblasts and prostate cancer, TGFβ extends the half-life of MMP2 and MMP9, and increases their levels[112]. Post-transcriptional mechanisms regulate MMP mRNA stability and require elements with specific sequences: multiple AU-rich elements (ARE) mostly located in the 3' untranslated regions (UTRs). The stability is facilitated by trans-acting RNA-binding proteins that interact with the ARE. Increase binding of HU protein family factors to ARE element enhances mRNA stability while binding of destabilizing proteins promotes mRNA degradation. MMP activity is compartmentalized where and how MMP is released and held in the pericellular environment is also an important

process especially for the regulation of MMP proteolysis. Secreted MMPs are often associated with the cell membrane which leads to substrate specificity in the pericellular space. MMP9 has been shown to be recruited and bind to CD44[102].

MMPs are synthesized as inactive enzymes in the pro-form or zymogens. Their activation consists of removing the pro-domain harboring a cysteine rich motif or cysteine switch. The conformational change in the pro-domain is what determines the activation of the pro-MMPs. This conformational change exposes the cysteine residue which in turn will allow the water to interact with the zinc ion in the active site. There are three different mechanisms known to initiate this event: 1) direct cleavage of another endoproteinase to remove the pro-domain; 2) allosteric reconformation of the pro-domain and 3) modification of the pro-domain through chemical reaction such as with reactive oxygen species or nonphysiological reagents. Allosteric reconformation of the pro-domain and the chemical modification of the pro-domain can allow the enzyme to remove its own pro-domain by autoproteolysis[113]. Activation of pro-MMP is believed to be a process that involves multiple steps and take place in the pericellular membrane. The first step is the conformational change that causes the exposure of the cysteine-switch and its subsequent disruption by interaction with the zinc ion. Partially activated MMP intermediates or other active MMPs remove the pro-domain through intra or intermolecular processing[114]. Pro-MMP2 activation has been the most documented activation, and MT1-MMP or MMP14 plays a very important role. Pro-MMP2 forms a complex with TIMP2; this complex interacts with the catalytic domain of MT1-MMP via the N-terminal domain of TIMP2 and forms a cell membrane-associated ternary complex. The activation of pro-MMP2 is triggered by a second MT1-MMP which would cleave the pro-domain[115]. The activation is completed by autolytic cleavage by MMP-2[116].

In vitro studies have shown that pro-MMP can be activated by thiol-modifying chemical agents such as mercurial compounds, SDS, oxidized glutathione and reactive oxygen species. These agents cause activation of several pro-MMPs. Reactive oxygen species activate pro-MMP via interaction of oxidized thiol and the zinc ion in pro-enzyme and autocatalytic cleavage[117]. Reactive oxygen species have also been shown to inactivate MMPs during increased inflammation[118]. Whether ROS can directly activate or inactivate MMPs in vivo has not been established.

Pro-MMPs can also be activated via the intrinsic allostery of MMP molecule. Fujita et al. have demonstrated that pro-MMP7 can be activated pericellularly by tetraspanin CD151 which is overexpressed in osteoarthritic articular cartilage. This increase in CD151 leads to an excess of MMP7 activity therefore causing cartilage destruction[119]. Later on, Geurts et al demonstrated that pro-MMP9 activation with hemin or beta-hematin, the core constituent of hemozoin, resulted in autocatalysis of the prodomain mediated by allosteric interaction with the hemopexin domain[120].

In vitro studies have shown that pro-MMPs can be activated by a single member of either serine proteinases or other MMPs such as MMP3 and MMP14. However, in vivo studies show that activation of pro-MMPs can be trigerred by more than one mechanism and can involve more than one participant. This has been supported using animal models. MMP3 has been shown to activate pro-MMP9 in vitro. It was suggested that MMP3 may play a key role in pro-MMPs activation[121]. However, complete deletion of MMP3 in mice does not attenuate MMP2 or MMP9 activation after perivascular electric injury either in the carotid artery or the femoral artery[122]. Furthermore, it is well established that MT1-MMP plays a key role in pro-MMP2 activation. MT1-MMP deletion does not prevent pro-MMP2 activation in fibroblasts from MT1-MMP knockout mice after treatment with ascorbic acid[123]. Other supportive studies are: one from Lijnen at al who

have shown that in vivo plasminogen does not necessarily activate pro-MMP2 and pro-MMP9 by using plasminogen deficient mice[124] and another from Tchougounova et al who showed that chymase deficiency partially prevented pro-MMP2 and pro-MMP9 activation[125]. These data strongly suggest that the mechanisms by which pro-MMPs are activated in vivo are still not clear.

It has been well established in vivo that maintaining a balance between active MMPs and their inhibitors is critical in order to avoid diseases caused by uncontrolled ECM turnover, inflammation, dysregulated cell growth, and migration. There are two major inhibitors: tissue inhibitors of metalloproteinases (TIMPs) and α_2-macroglobulin. Human α_2-macroglobulin is a broad spectrum inhibitor. It inhibits almost all endopeptidase in the body fluids and blood by engulfing the entire enzyme; this complex is then cleared by LDL receptor related protein-1 mediated endocytosis[126]. In tissue there are four inhibitors known as TIMPs. Each TIMPs inhibit MMPs with a 1:1 molar ratio. TIMPs are broad spectrum inhibitors of MMPs, but they differ in their specificity. Data have shown that TIMP2, TIMP3 or TIMP4 can interact with pro-MMP2 and TIMP1 or TIMP3 with pro-MMP9[127]. The role that TIMP2 plays when interacting with pro-MMP2 has been well established. However, the role of other TIMPs interaction is not known. Of all the TIMPs, only TIMP3 have shown to be the most relevant in vivo. Since TIMP3 deficient mice were shown to develop pulmonary alveolar enlargement and enhanced apoptosis in mammary gland duct epithelial cells with age[128], while TIMP1 and TIMP2 deficient mice have not been seen with any abnormalities. TIMP4, which is the newest recognized addition to the TIMP family, has not been well studied. TIMP4 knockout mice were recently developed and thus far have shown to be normal [129]. Other molecules have been shown to inhibit some MMPs. Procollagen C proteinase enhancer has been shown to be an inhibitor of MMP2[130]. MMP2, MMP9 and MMP14 activities can also be inhibited

by the glycosylphosphatidylinositol (GPI) anchored angiogenesis suppressor glycoprotein RECK[131].

1.2.3. Matrix Metalloproteinases and AAA

Many MMPs have been implicated in human aortic aneurysms. MMP9 is the most studied. MMP9 is the most abundant elastolytic proteinase produced by human tissues in vitro and is highly expressed in macrophages infiltrated within the aneurysm tissue[132]. Data suggest that the size of the developing aneurysm is determined by the type of MMP within the aortic media[133]. Smaller aneurysms have higher level of MMP2 whereas medium and larger sized aorta and ruptured aneurysm have a higher level of MMP9[132, 134]. The roles of MMP2 and MMP9 in aortic aneurysm have been well established. Longo et al. have reported a relationship between these two gelatinases in aortic aneurysm formation. They found that when MMP2-/- mice and MMP9-/- mice were treated with $CaCl_2$, none of these mice had developed AAA. However, reconstitution of MMP9 by intravenous infusion of wild type mice macrophages into both mice resulted in AAA formation in MMP9-/- mice but not in MMP2-/- mice[59]. These data suggest that both MMP2 and MMP9 are required for AAA formation. Other MMPs have been implicated in AAA formation; these include MMP1, MMP3, MMP12, MMP13 and MT1-MMP. MMP1 and MMP3 have been shown to be elevated in plasma of patients with AAA[135]. MMP12, MMP13 and MT1-MMP are increase in AAA tissue[136-138], with MMP13 been highly expressed in medial smooth muscle cells[136]. Cigarette smoking, a major risk factor in aortic aneurysm, increases MMP1 in rabbits' aortic tissue[139]. While MMP1 has been shown to be increase in sites of in human AAA tissue where collagen generation could be important[140], other data have shown that there is no correlation between MMP1 level in the plasma and the size of the AAA[141]. Immunohistology demonstrated that MMP12 is more localized at the elastin fiber fragments[142]. Deficiency in MMP12 in mice was shown

to attenuate AAA growth after periaortic application of $CaCl_2$, but does not affect MMP2 and MMP9 expression[138]. MMP3 deficient mice were shown to have an attenuated aortic diameter, and the expression of MMP3 in the wildtype mice was co-localized with macrophage infiltrates[143].

Due to the high implication of MMPs in aortic aneurysm formation, many drugs that target MMPs and inhibit their activities have been studied as potential treatment for small aneurysm to prevent AAA expansion. Non-steroidal anti-inflammatory drugs (NSAID) have been widely used for their ability to suppress MMP expression and potentially reduce aortic aneurysm dilation. In a study conducted in rats, indomethacin treated rats had a decrease in MMP9 production and an attenuated aortic diameter after elastase infusion[144]. Marimastat, a synthetic molecule designed to mimic MMPs substrates, prevented MMP2 activation and elastin degradation in aortic organ culture[145]. Investigation of CGS27023A, a broad-spectrum MMP inhibitor, showed a reduction in aortic medial elastin degeneration and ectasia grade, suggesting that MMP inhibition can prevent or slow an aortic aneurysm progression[146]. Many studies have demonstrated that doxycycline treatment inhibits MMP synthesis; therefore, preventing elastin degradation and aortic aneurysm expansion. Doxycycline has the ability to bind to any MMP at the active zinc site causing a conformational change and loss of enzymatic activity[147]. Oral administration of doxycycline in AngII treated mice reduced the incidence of AAA as well as the severity of the aneurysm[74]. In a double-blind, randomized, placebo-controlled study, patients with small AAA that received doxycycline daily for 3 months and were then monitored for 18 months had a decrease in aneurysm expansion[148]. However, this study was criticized at different levels. One point was the size of the diameter in the doxycycline group vs the placebo group. Within the doxycycline group, average diameter was 3.1cm which according to Baxter is

considered dilated but not aneurysmal, and the placebo group had a much larger diameter (3.5cm). The most significant point was that during the whole 18 months period there was no significant difference between the placebo group and the doxycycline group. Significance was only seen when both group were subdivided into different group 6-12 month and 12-18 month[149]. Doxycycline had shown to be beneficiary in animal studies, but in human studies, different trials provided conflicting evidence[150]. Thus, the effectiveness of Doxycycline as a MMP inhibitor to treat small aneurysm is yet to be determined.

1.3. Tissue Inhibitors of Metalloproteinases (TIMPs):

1.3.1. Classification and regulation

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of the matrix metalloproteinases and are consequently important regulators of ECM turnover, tissue remodeling and cellular behavior[151]. There are four human paralogous genes that encodes for TIMPs1-4. The first TIMP was identifying in the 1970s and described as a small collagenase inhibitor because of its ability to inhibit collagenase produced in the media cultured human skin fibroblast[152], human serum[153], bovine cartilage and aorta[154]. It was then called “tissue inhibitor of metalloproteinases” for its ability to not only inhibit collagenases, but gelatinases and proteoglycanase as well[155]. Subsequently, other TIMPs were also discovered with the most recent being TIMP4. All four TIMPs inhibit all identified MMPs; but, their affinity varies with different MMPs. TIMP3 has a broader inhibition spectrum, and it can inhibit some members of the disintegrin metalloproteinases, ADAM and ADAMTs families. TIMPs also have other functions independent of inhibition of metalloproteinase. They can regulate cell proliferation, angiogenesis and plasticity. The structure, activity and biological function of all four TIMPs has been investigated thoroughly.

All four TIMPs are similar in structure; they are about 40% identical in sequence. TIMP2 and TIMP4 share the most similarity; they are about 50% identical in sequence, and TIMP1 shares around 41% of its sequence with other TIMPs[156]. The genes for TIMPs1, 3, and 4 lay within an intron of the synapsin genes. Synapsins are genes that encode for neuro-specific phosphoproteins that coat the cytoplasmic surfaces of synaptic vesicles[157]. TIMP2 is the host for the gene DDC8 (differential display clone 8), a gene that is highly expressed in the testis during spermatogenesis[158]. The four TIMPs have two distinct domains, an N-terminus domain of about 125 amino acid residue and a C-terminus with 65 amino acid residues. There are three disulfide bonds that stabilize the conformation of each domain[159]. The N-terminal domain has the ability to fold and function independently. N-TIMPs, which have the recombinant form of the N-terminal domain, are more stable in structure and are fully active inhibitors of MMPs and ADAMS[160, 161]. Therefore, they are used to investigate different TIMPs properties. Although the four TIMPs share similarities in their structures, they do have differences in expression pattern. TIMPs 1, 3 and 4 are inducible and tissue specific while TIMP2 is more constitutive and ubiquitous. TIMP1 is mainly expressed in the reproductive system, TIMP3 is more seen in the heart, kidney, and thymus and TIMP4 is highly expressed in the cardiovascular system, as well as, kidney pancreas, colon, testes, brain and adipose tissue[162].

The four TIMPs are broad-spectrum inhibitors of all MMPs identified to date. However, their affinity and specificity differ among them. TIMP1 is the most restrictive TIMP in its ability to inhibit MMPs. TIMP1 inhibits MMP1, MMP3, MMP7 and MMP9; it has very low affinity for MMP14, MMP16, MMP19, MMP24 and the membrane-type MMPs. TIMP2 is the only TIMP that interacts on the cell surface with MT1-MMP and pro-MMP2, and is required for the activation of pro-MMP2[115]. TIMP2 can also inhibit MMP2. TIMP2 thus

acts as both an MMP inhibitor and an activator. TIMP3 has a bigger spectrum of inhibition. It can also inhibit members of the ADAM and ADAMTs families while the activity of other TIMPs in this regard are limited[161, 163].

ADAMs (disintegrin and metalloproteinase motif) vary from MMPs in their domain structures and are very different in their catalytic domain sequences. They contain a disintegrin, cysteine-rich, EGF-like transmembrane domains C-terminal in their catalytic site and they are membrane-bound enzyme[164]. ADAMTs (disintegrin and metalloproteinase with thrombospondin motifs), are secreted proteins. They possess the disintegrin domain and different numbers of thrombospondin type1 motifs and other domain in their C-terminal domain[165]. TIMP1 and TIMP3 inhibit ADAM10[163] and TIMP2 inhibits ADAM12[166]. TIMP1 and N-TIMP4 inhibit ADAM 17[167], and TIMP4 inhitbits ADAM28[168]. TIMP3 also inhibit ADAM12, 17, 28, and 33 as well as ADAMTs 1,2,4 and 5. TIMPs usually inhibit MMPs and ADAMs using the N-terminal domain. However, TIMP1 and TIMP3 do not use their N-terminal domain to inhibit ADAM10[169].

1.3.2. TIMPs and AAA

Disruption of the balance between MMPs and TIMPs may result in diseases associated with uncontrolled turnover of matrix, such as aortic aneurysm. The functions of TIMPs have been investigated to understand their role in AAA formation. TIMP1 mRNA has been found to be increased in human AAA tissues[170] and in murine model of AAA[171]. TIMP1-/- mice after a periaortic treatment of $CaCl_2$ were found to have an increase in aortic diameter compare to control[66]. Local overexpression of TIMP1 in the rats' aortas prevented elastin degeneration and therefore prevented aneurysm formation[172]. Three different TIMP1 single nucleotide polymorphisms (SNP) have identified in male Caucasian patients with AAA[173]. TIMP2, a cofactor in MMP2 activation, have been shown to prevent aortic aneurysm increase in genetically deleted TIMP2 mice model

with $CaCl_2$[61]. On the hand, overexpression of TIMP2 by recombinant adenovirus in rat aorta also prevented an increase in aortic diameter by preventing elastin degeneration[174]. These data are consistent with the complex function of TIMP2 in MMP2 activation. An increase in TIMP2 has been shown to activate MMP2 activity; however an overexpression of TIMP2 inhibits MMP2 activity[175]. TIMP3-/- mice, when treated with ANGII, had an increase in aortic aneurysm formation and rupture[176]. While a role of TIMP1/2/3 in aortic aneurysm has been reported[172, 174, 176], whether TIMP4 is involved in aortic aneurysm is unknown. Several lines of evidence implicate a role for TIMP4 in AAA: 1) it non-selectively inhibits MMP activity; 2) TIMP4 is down-regulated in intracranial aneurysms[177]; 3) a Promoter SNP in TIMP4 gene has been associated with Kawasaki disease, a systematic vasculitis[178] and 4) TIMP4 is an inducible gene[179].

1.4. Molecular clock Bmal1

1.4.1. Regulation

Brain and muscle ARNT-like (Bmal1; also known as MOP3 in human or Arnt3 in mouse), is a member of the basic helic-loop-helix period-ARNT period-ARNT-single minded (bHLH-PAS) transcription factor family. It is an obligatory core clock gene and is essential for normal circadian rhythmicity in physiology and behavior[180, 181]. At the cellular level, Bmal1 forms a heterodimer with Clock, another member of the bHLH-PAS family, through their PAS protein-protein interaction domain. The heterodimer constitutes the positive limb of the circadian feedback loop machinery to initiate the transcription of target genes containing E-box cis-regulatory enhancer sequences and are highly selective for those with the sequence CACGTG[182] including the period (per1, per2 and per3) paralogous member of the PAS protein family and cryptochrome (cry1 and cry2)[182-184], members of the vitamin B_2-based blue light photoreceptor/photolyase family. After delays imposed by transcription, translation and posttranslation modification, the

negative limb of the feedback loop is comprised of the heteromultimer formed by per, cry and other proteins that translocate back in the nucleus and directly abrogate the transcriptional activity of the Bmal1:Clock complex[184-186]. This inhibition is believed to be mainly caused by the Cry proteins, probably by repressing histone acetyl transferase (HAT) activity[187, 188] while the translocation of the complex has been attributed to one or more per proteins[184, 187]. Phosphorylation of Per and Cry and subsequential degradation partly by casein kinase I epsilon and delta (CKI ε/δ) result in the Bmal1/Clock heterodimer being released from inhibition and therefore free to initiate transcription again[189-191]. The Bmal1/Clock heterodimer activates another core clock gene creating yet another positive and negative feedback loop. Bmal1/Clock still constitute the positive limb while rev-erbα, which codes for an orphan nuclear receptor[192] constitutes the negative limb. Just like per and cry, rev-erbα contains E-box enhancer in the promoter region which allows binding and activation by Bmal1/Clock[193, 194]. Rev-erbα has been shown to inhibit Bmal1 transcription by binding the retinoic acid-related orphan response elements (ROREs) in the promoter region[193, 195, 196]. It has also been shown that disruption of rev-erbα significantly affects Clock and Cry1[193]. The rev-erbα negative limb is unlike the cry-per negative limb because it only inhibits Bmal1 instead of inhibiting the Bmal1/Clock Complex. The inhibitory effect of Cry-per on Bmal1/clock also acts to inhibit rev-erbα transcription. Competing with rev-erbα at the ROREs is Rorα; whereas rev-erbα acts to inhibit Bmal1 transcription, Rorα activates Bmal1 transcription through two conserved Rorα conserved elements[197]. More ROREs were shown to regulate Bmal1 expression. Since the Rev-erb and the ROR families recognize the similar response element with opposite effect on Bmal1 transcription, Guillaumond et al. investigated the possibility that each member of the both families can regulate Bmal1 expression. Within the Rev-erb family both Rev-erbα and Rev-rebβ were shown to inhibit Bmal1 expression

and the RORs members (RORα, RORβ, RORγ) were shown to activate Bmal1 transcription[198].

At the molecular level, the combination of transcription, translation and posttranslational modification of the core clock genes results in a daily rhythmic expression, or circadian rhythms of those genes. In mammalian physiology, circadian rhythm is driven by a central pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus. The SCN is controled by photic cues from the retino-hypotalamic tract which in turn control and synchronizes clock genes in peripheral tissues. Although the SCN controls rhythmicity in the whole organisms, rhythmicity is also maintained within individual tissues and even at the cellular level[199, 200]. The phases within peripheral tissues have been shown to be delayed by approximately four hours from the SCN. Lesion of the SCN rendered rats behaviorally arrhythmic[201]. Hormonal signals such as glucocorticoids and restricted feeding can uncouple the phase of peripheral tissues from the SCN[202, 203]. In a study by Damiola et al., restricting mice from food during the day on a 12-h light/12-h dark cycle resulted in a 12 hour shift of clock genes expression in the peripheral tissue while the expression in the SCN remained unchanged, and Stocklan et al. demonstrated that food restriction during the light not only shifts the phase of the clock genes expression by 10 hours after 3 days of restricted feeding in rats but it also alters their locomotor activities. Both findings from Damiola and Stocklan suggest that feeding is an important cue to determine clock genes expression pattern in peripheral tissues.

Even though the circadian machinery in the SCN and peripheral tissues is well understood, the mechanisms downstream of the clock machinery that regulate metabolism and physiological processes is still being heavily investigated. Ramsey et al. described in 2007 that there is an approximate of 5% to 10% of the transcriptome has shown to be have a 24 hour variation in the SCN, heart, vasculature and fat. Since then,

more genes have been shown to be under control of the clock core genes now estimated at over 10%. Albumin gene D-site binding protein (DBP) belongs to the PAR leucine zipper transcription factor family; it increases the transcription of many genes containing an insulin-response element in the liver[204]. It is endogenously rhythmic and its expression is not influenced by light. DBP mRNA expression does not change with a short exposure to light. Deletion of DBP does not alter rhythmicity. However, mice lacking DBP have been display significant differences in circadian locomotor activity[205]. (Although DBP is a clock control gene, its regulation requires both Bmal1 and Clock).

Another group of genes that are controlled by clock genes and are involved in metabolic processes such as lipid homeostasis in the vascular system is the peroxisome proliferator-activated receptor (PPAR)[206]. PPAR are member of the nuclear receptor superfamily and there have been three types of PPAR described: PPARα, PPARβ/PPARδ and PPARγ. PPARα, which is highly expressed in the liver and brown adipose tissue, acts as a lipid sensor in the liver. It activates the transcription of specific genes in response to an influx of fatty acids[207]

Bmal1 and Clock are the two transcription factors that set off the circadian rhythm machinery. While the global deletion of Clock in mice does not affect circadian rhythm but affect the response to light[208], global deletion of Bmal1 in mice disrupts behavior and molecular rhythm[183]. Bmal1 null mice have been shown to have a reduced lifespan and pathologies associated with pre-mature aging and an increase in oxidative stress. Bmal1 is involved in vascular pathologies. It has been associated with prothrombotic phenotype[209]. A study from Somanath et al. had shown that when using the mesentery artery to induce thrombosis, the time to thrombotic occlusion in Bmal1 KO mice was shorter than in wildtype control. Bmal1 has been shown to play an important role in vascular pathologies[210]. It has been associated with flow dependent vascular

remodeling[211], aortic vascular stiffness, transplant arteriosclerosis and increased superoxide and endothelial nitric oxide synthase uncoupling in aorta.

1.4.2. Bmal1 and cardiovascular disease

Bmal1 and other core clock genes are expressed in peripheral tissues[212], including cardiovascular organs: heart, aorta and kidney[213, 214]. Data suggest that approximately 10% of genes in peripheral organs exhibit circadian expression in the heart[215, 216] and vascular tissues[217]. These data bring in a new perspective on the function of peripheral clocks in different organs or even at the cellular level. Heart rate, blood pressure, endothelial function, and fibrinolytic activity are all cardiovascular parameters that exhibit circadian variations. An alteration of Bmal1 has been shown to be involved in vascular pathologies[210]. When the germline Bmal1$^{-/-}$ mice underwent a chronic reduction in blood flow in the common carotid artery, those mice developed a wall thickness that was more pronounced then their littermate control. They had also developed vascular remodeling. However, instead of narrowing the lumen as observed with the control, those mice had an increase in lumen diameter which was accompanied with a development of a thrombosis[211]. Another study from Anea et al. had shown that Bmal1-/- mice have an increase in vascular stiffness and a decrease in compliance. They had also shown that Bmal1-/- mice have an increase of MMP2 and MMP9 activities 3 days after of common carotid artery ligation. MMP9 activity was also increase in non-ligated artery[218]. Transplant model demonstrated that a transplant of aorta from Bmal1-/- mice into control mice lead to transplant arteriosclerosis observed by intimal hyperplasia and wall thickness while the transplant had only induced inward remodeling between control and control[219]. Bmal1-/- mice had a reduced endothelial function due to an increase in oxidative stress[220].

Circadian expression of clock genes has been shown to be expressed in vascular endothelial cells and in vascular smooth muscle cells[214]. Data has shown that VSMC stimulation with ANGII induces clock genes expression. The time of stimulation by AngII can be considered as a zeitbeger time in VSMC[214]. Xie et al. had demonstrated that tissue specific deletion of Bmal1 in smooth muscle cells lowered and altered blood pressure circadian rhythm[221]. Although, there are studies that have looked at the role of Bmal1 in vascular diseases, those studies involved a global knockout of Bmal1, making it difficult to assess the role that Bmal1 plays in the vasculature itself without altering its expression in other tissues and cells. Studies from Xie mentioned above are more insightful by looking at the role of smooth muscle Bmal1 in the regulation of blood pressure. Since they have used smooth muscle cells specific Bmal1 KO and blood pressure is regulated in small vessel such as mesenteric arteries and smooth muscle cells are the biggest component of those vessels. Therefore, a specific deletion of Bmal1 in smooth muscle is most appropriate to study the role of Bmal1 in blood pressure regulation. Smooth muscle cells are big player in AAA formation and AAA ruptures have been shown to have time of the day variations. This could imply a role for clock genes in AAA formation. Whether Bmal1 plays a role in aortic aneurysm formation has not been showed yet. Therefore, there is the need to understand the role that smooth muscle Bmal1 could play in the formation of AAA.

1.5. Scope of the dissertation

Abdominal aortic aneurysm (AAA) is a multifactorial disease characterized by a permanent segmental, abnormal dilation of the aorta[1]. This permanent dilation is due to an increase in elastin and ECM degradation; an increase in inflammatory cell infiltration; and smooth muscle cells apoptosis[22, 23]. In human AAA, MMP expression increases in VSMCs[39], and thus play a central role in the development of AAA. MMPs degrade the

ECM of the vasculature including elastin, and the increase in MMPs is induced by an increase in inflammatory cells and cytokines, which are secreted by smooth muscle cells[40, 41, 43, 46, 47]. The precise mechanisms of aortic aneurysm formation are not well understood making it very challenging to develop a therapeutic treatment. Therefore, the only treatment has been an emergency surgery, which has a mortality rate of more than 50%. Therefore, deciphering the molecular mechanism underlying aneurysm formation is urgently needed in order to identify new pharmacological therapeutic targets.

As previously mentioned, Bmal1 is an obligatory core clock gene and a transcriptional factor; it regulates genes essentials for normal circadian rhythm in physiology and behavior[222]. Bmal1 is expressed in the SCN and peripheral tissues including VSMC, and is essential for normal vascular functions[223] . Alteration of Bmal1 has been associated with different vascular pathologies such as vascular stiffness[218], transplant arteriosclerosis[219] and flow-dependent vascular remodeling[211]. However, the role of Bmal1 in AAA formation has yet to be studied. AAA dissection and rupture have been shown to have circadian variability[224], which would suggest an involvement of clock genes, and Bmal1 deletion has been shown to increase oxidative stress and MMPs, which are increased in human AAA VSMC[43, 218, 220]. Therefore, the purpose of this study was to investigate the role that vascular Bmal1 plays in AAA formation. The initial hypothesis was that Bmal1 deletion would exacerbate AAA formation. Therefore, we used smooth muscle cells specific Bmal1 knockout mice to test our hypothesis. However, the hypothesis was rejected because Bmal1 deletion from smooth muscle cells showed a complete protection from AAA formation. We moved to understand the mechanism of protection.

Different experimental approaches were taken to understand and determine the mechanism of protection, and they are presented in chapter 2. The results and their

explanations are presented in chapter 3. The discussion and limitations of the project are presented in chapters 4.

CHAPTER 2

Materials and Methods

2.1. Experimental Design

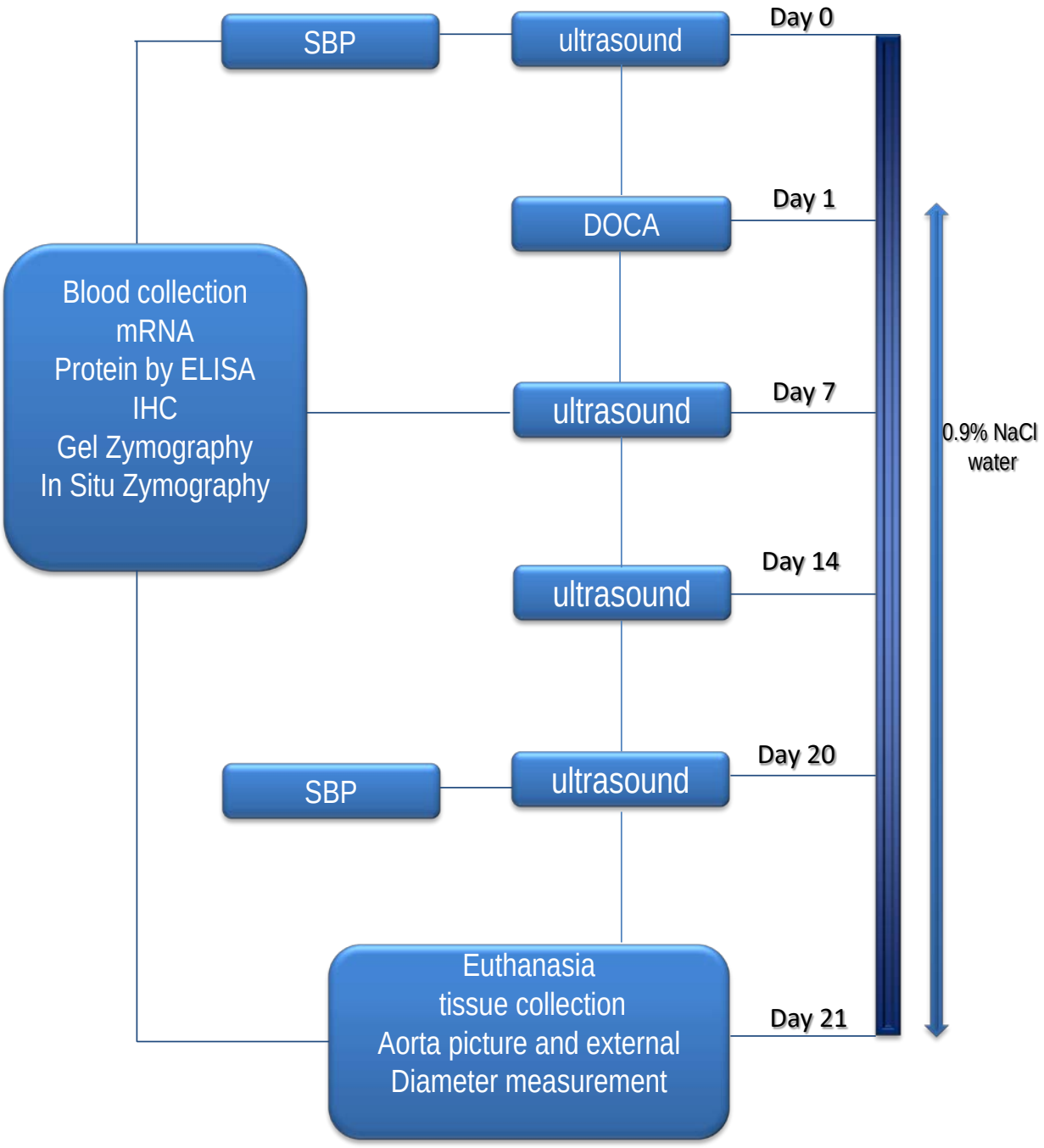

Figure 1: Diagram showing the time line and different methods used

2.2. Animals

Four or eight to nine month old male smooth muscle cells Bmal1 Knockout mice (SM Bmal1$^{-/-}$) and littermate control were used for this study. SM Bmal1$^{-/-}$ mice were generated by crossing Bmal1$^{flox/flox}$ mice with smooth muscle cell specific SM22α-Cre knocked-in mice; both were purchased from Jackson Laboratory. Homozygous Bmal1$^{flox/flox}$ mice possess a lox*P* sites flanking exon 8 which encode for Bmal1 binding site to E-box. To generate the SM22α-Cre knocked-in mice, a modified Cre recombinase under the control of SM22α promoter was introduced into B6SJLF2 donor oocytes. When Bmal1$^{flox/flox}$ mice were crossed with SM22α-Cre transgenic mice, the presence of Cre resulted in the deletion of the flanked exon 8 of Bmal1. Since SM22α has been shown to be smooth muscle cells specific[225], it is expressed in aorta, esophagus, intestine, trachea and uterus. SM22α is also expressed in the heart and skeletal muscles[226]. Bmal1 is specifically deleted in smooth muscle cells including the aorta.

C57B6/J mice used in this study were purchased from Jackson Laboratories. They were left for acclimatization for 2 weeks in light box: The light box is set for 12:12 light cycle: 12 hours of light and 12 hours of dark. The light was set to be turned on at 6:00am and turned off at 6:00pm. The mice were killed at 4 different time point: ZT5, ZT11, ZT17, ZT23 were ZT stand for Zeitgeber Time: the number of hours after the light is turned on; making ZT5 and ZT11 day time and ZT17 and ZT23 night time.

2.3 DOCA/AngiotensinII pump implantation plus salt

To induce Aortic Aneurysm, a 50 mg Deoxycorticosterone Acetate pellet (DOCA, 21-day release, Innovative Research of America, Sarasota, FL) or 1000 ng/kg/day Angiotensin II (Sigma-aldrich, St. Louis, MO) in osmotic minipump (Alzet mini pump 28 days release, Alzet, Cupertino, CA) was administered subcutaneously; the mice were also given drinking water containing 0.9% NaCl and 0.2% KCl for 21 days for DOCA and 28 days

for Angiontensin II plus salt. To perform the surgery, mice were anesthetized by inhalation of isoflurane mixed with oxygen (3% isoflurane and 97% oxygen). Mice were placed in the prone position with continuous anesthesia. Hair from the cervical area was removed using Nair shaving cream and lateral incision was made. There, a pouch was created to insert the pellet or the pump was placed subcutaneously in the thoracic spine area. The wound was closed with suture and the mice monitored for recovery[227, 228].

2.4 Blood Pressure measurement by tail cuff

Prior to DOCA or ANGII implantation, the systolic blood pressure was measured using a non-invasive tail cuff method with automated monitor (CODA6, Kent Scientific Corporation, Connecticut, USA). Mice are placed in a restrainer to keep mice from excessive movements with the tail still on the outside. The holder is then placed in a heated platform at 37^0C then two cuffs: the occlusion cuff (O cuff) and the volume pressure recording cuff (VPR cuff) are placed around the tail to record blood pressure. Blood pressure measurements were recorded for 5 consecutive days at the same time of the day and averaged. Unusually high or low blood pressure collected during the 5 days were considered outliers and therefore excluded from the average blood pressure[73]. The measurements were repeated on the third week for DOCA plus Salt and on the fourth week for ANGII plus Salt.

2.5. Aorta Inner diameter assessments by ultrasound

The aorta inner diameter was first measured prior to DOCA or Angiotensin II pump implantation and monitored weekly thereafter. The inner diameter was measured via ultrasound imaging system using VEVO 2100. In order to perform the ultrasound, mice were first anesthetized by inhalation of isoflurane mixed with oxygen (3% isoflurane and 97% oxygen). Upon loss of total locomotion, mice were placed in the supine position onto 37^oC heated platform. The hair in the abdominal area was removed using Nair

shaving cream. Isoflurane was continuously maintained for the whole procedure. Warmed ultrasound transmission gel was placed in the entire shaved area and the transducer was placed just below the ribs cage. The maximal inner diameter of the aortic suprarenal area was measured from ultrasound image acquired from a cine loop of 100 images. Hepatic artery, hepatic vein, and bile duct were used as marker to acquire image in the same location in each mouse[227].

2.6. Blood Sodium and Potassium Measurement

After the mice were euthanized, freshly drawn blood from a cardiac puncture with a syringe was subsequently placed in an I-STAT E3+ cartridge (cat# SKU: 600-9004) without anti-coagulant. The cartridge was placed into a VetScan I-STAT1 handheld analyzer by ABAXIS and sodium and potassium concentrations were determined.

2.7. mRNA isolation and Real-time PCR

Aorta was removed from mice treated with DOCA plus Salt for 7 or 21 days and from non-treated mice. The aorta was then placed in RNAlater RNA stabilization Reagent (QIAGEN cat # 76106). RNA was extracted using RNeasy plus mini kit (QIAGEN) as follows: The whole aorta was placed in a homogenizer with 300µl of RLT buffer containing β-Mercaptoethanol (for each 1 ml of RLT buffer, we added 10 µl of β-Mercaptoethanol), the tissue was homogenized until complete dissipation into the solution. Only debris of the tissue could be seen through the homogenizer. 580 µl of water and 20 µl of proteinase K (Invitrogen cat # AM2542) were then added followed by pipetting up and down for about 10 times using a syringe and needle. The sample was sucked up into the syringe and transferred in a 1.5 ml Eppendrof tube, then heated in a heating block at 55^0C for 10 minutes then centrifuged at 4^0C at full speed for 5 minutes. The supernatant was removed carefully without touching the pellet and transferred into a 1.5 ml eppendrof tube. Then 400 µl of 100% ethanol was added to the sample and

mixed by pipetting up and down. Then 700 µl of the sample was transferred into RNeasy spin column and centrifuge at 8000xg for 15 seconds and the flow-through was discarded. The remainder of the sample was added to the same spin column and centrifuged. The flow-through was then discarded. 700 µl of RW1 buffer was added to the spin column then centrifuged for 15 seconds at 8000Xg, the flow-through was discarded. Then 500 µl of RPE buffer was added to the RNeasy spin column at 8000xg for 15 seconds. Another 500 µl of RPE buffer was added to the RNeasy spin column and centrifuged at 8000xg for 2 minutes. The flow-through and the collection tube were discarded and the column placed in a new collection tube and centrifuged for 1 minute at full speed to allow the membrane to dry. The collection tube was discarded and the spin column was placed in a new 1.5 ml collection tube and 30 µl of Rnase-free water was added directly to the spin column membrane and centrifuge for 1 min at 8000xg to elute the RNA. Then the RNA concentration was measured using a nanodrop. cDNA was generated by reverse transcriptase PCR as follows:

-RNA, distilled water; random primer and dNTP were mixed together in a PCR tube and placed in a PCR machine:

Step 1: 65^0C for 5 minutes

While step 1 was running, in an epperdorf tube 5x buffer, RNaseout, RT and water were mixed together and placed in the PCR tube while sample was in set 2.

Step 2: 4^0C for 5 minutes

Step 3: 37^0C for 1 hour

Step 4: 70^0C for 15 minutes

Step 5: 4^0C

cDNA generated was then diluted with distilled water for a final concentration of 10ng/µl. The amount of RNA added depended on the concentration of RNA and the total amount of desired cDNA generated with the maximum amount being 5000ng.

For real time PCR, the samples were prepared as followed:

Forward primer, Reverse primer, 5x buffer, $MgCl_2$, dNTP, SYB green, Taq Pol and water were mixed: vortexed and centrifuged together in a 1.5 ml epperdorf tube. 22µl mixture was then aliquoted in 96 well PCR plate (Phenix Research Products, USA lot# 466568) and 3µl or 30ng of the cDNA was added in each well. The plate was centrifuged and placed in PCR machine with the following steps:

Step1: 95^0C for 3 minutes

Step2: 95^0C for 15 seconds

Step3: 60^0C for 1 minute

Step4: Go to step 2 40 times

Step5: Melt curve 60.0 to 95.0 with 0.5 increase for 5 seconds

Step6: 20^0C for 5 minutes

Real-time PCR was performed for quantification of genes expression of tissue inhibitor of metalloproteinases (TIMPs)1, 2, 3 and 4, the mineralocorticoid receptor (MR), Bmal1, Rev-erb, Per1, and Cry1.

2.8. Immunohistochemistry

For immunohistochemistry studies, the aorta was fixed first by incubating the sample in 70% alcohol for 48 hours then in 5% formaldehyde for 24 hours, following which it was embedded in paraffin. With the use of a microtome, the embedded tissues were cut in

series with a thickness of 5µm. Once a section was cut, with the use of a pencil it was held then placed in warmed water and picked up with a slide. Finally the tissue's slides were placed on a heated platform to dry.

The tissue was first stained for elastin with an elastin staining kit (Van Gieson staining, Richard-Allan Scientific CAT # 87017). Samples were deparaffinized by heating at 60^0C for 2 hours, this was followed by a series of incubation for hydration: 2 times in xylene for 15 min each time, 2 times in 100% ethanol for 5 minutes each times then in 95% ethanol for 5 min and 85% ethanol for 5 minutes followed by 70% and 50% ethanol for 5 minutes and water for 5 minutes. Samples were stained in working elastic stain solution for 30 minutes then rinsed in running tap water for 1 minute. The samples were then decolorized in working differentiating solution followed by a rinsed in tap water. The samples were then placed in sodium thiosulfate solution for 1 minute, then rinse with water for 3 minutes. They were then stained with Van Gieson stain solution for 3 minutes followed twice by dehydration in anhydrous alcohol for 1 minute each then cleared in clearing reagent 3 times for 1 minute each and mounted.

For TIMP4 staining, the section were deparaffinized, rehydrated, and treated with low pH antigen retrieve buffer (Vector Laboratory, Burlingame, CA) to retrieve antigen. Sections were treated with 3% H_2O_2 to quench endogenous peroxidases. After blocking endogenous background with Avidin/Biotin blocking kit (Vector/Laboratories) and non-specific binding (normal goat serum, Vectastain ABC Kit), slides were incubated with the TIMP4 primary antibodies (AssayBioTech) overnight at 4^oC. Slides were then incubated with biotinylated secondary antibody (VECTASTAIN, ABC kit, elite). This incubation was followed by signaling detection which required samples to be subjected to the procedure of VECTASTAIN Elite ABC system (Vector Laboratories). Immunoreactivity was

visualized by DAB (DAKO North America Inc, Carpinteria, CA) followed by counterstaining with hematoxylin.

2.9. Gel Zymography

The aorta was freshly dissected and equilibrated in PBS for 30 minutes, then incubated in Krebs buffer for 24 hours at 37^0C. The medium containing MMPs loaded in a 2% gelatin zymogram gel : 10% gel (Acrylamide 29:1, 4x Separate gel buffer pH 8.8, gelatin, 10% APS, TEMED), stacking gel (Stacking buffer, Acrylamide 29:1, 10% APS, TEMED).The gel ran at 100v until the dye front reached the bottom of the gel. It was then incubated 2 times in wash buffer (1M Tris, 1 M $CaCl_2$, 10mM $ZnCl_2$, 2% Na_2N_3, Triton X-100) at 37^0C with shaking, followed by incubation in reaction buffer (1M Tris, 1 M $CaCl_2$, 10mM $ZnCl_2$, 2% Na_2N_3, Triton X-100) at 37^0C overnight. After incubation, the gel was washed 3 times with water at room temperature with shaking, stained with GelCode Blue Safe Protein and destained with water. The gel was scanned for visualization.

2.10. In Situ Zymography

To detect MMP/Gelatinases activities, the slides containing sections of paraffin embedded aorta were heated at 59^oC overnight, deparaffinized in xylene and rehydrated in graded alcohol. Samples were incubated with a DQ gelatin fluorescein conjugate (EnzChek Gelatinase/Collagen Assay Kit, Molecular Probes CAT# E12055). Samples were first equilibrated in reaction buffer (0.5 M Tris-HCl, 1.5 M NaCl, 50mM $CaCl_2$, 2 mM sodium azide, pH 7.6) for 5 minutes. Substrate was prepared by dissolving 1mg DQ gelatin in 1.0 ml of water then further diluted (1:200) in reaction buffer to make the reaction mixture. Each sample was incubated with 100µl of the reaction mixture at 37^0C for 2 hours in the dark. The slides were then washed with PBS 3 times for 2 minutes each. The slides were then fixed in 4% formaldehyde for 10 minutes in the dark washed

with PBS 3 times for 5 minutes each. The slides were then counterstain with DAPI then mounted[229].

2.11. ChIP Assay

Freshly dissected aorta was allowed to equilibrate in PBS for 30 minutes and fixed in 1% formaldehyde in PBS for 1 hour, following which 2% glycine in PBS was added in the sample. The tissue was frozen in liquid nitrogen and pulverized until a powder was formed. Then lysis buffer (0.1 M NaCl, 50mm Mop-NaOH pH 7.0, 1M EGTA, 0.1% Tween 20, 2mM $MgCl_2$,0.5mM DTT(TCEP) was added to the sample and allowed to equilibrate at room temperature. The sample was centrifuged and the pellet collected and washed twice with the lysis buffer. The sample was then treated with mung bean nuclease at room temperature followed by sonication (36% power) and centrifugation at 4^oC. The supernatant was diluted 10 fold in CHIP dilution buffer (5% sodium deoxycholate, Triton X-100, 0.5 M EDTA, 1M Tris HCl pH8.0, 5M NaCl) supplemented with proteases and phosphatases inhibitor (PMSF, aprotinin, leupeptin, pepstatin, DTT, NaF, Na_3VO_4). Salmon sperm DNA/protein A/G 50% slurry was added to pre-clear the sample with rotation at 4^0C. Sample was then centrifuged and the supernatant collected and incubated with immunoprecipitating 3ug Bmal1 or non-specific IgG overnight at 4^0C. Salmon sperm DNA/ protein G-agarose slurry was added to collect Ab/Histone complex by rotation at 4^0C. The sample was centrifuged and the pellet was collected. A series of washes followed: 2 times wash with low salt (10% SDS,Triton-X100, 0.5M EDTA, 1M Tris-HCl pH 8.0, 5M Nacl), 2 times High salt (10% SDS,Triton-X100, 0.5M EDTA, 1M Tris-HCl pH 8.0, 5M Nacl), 2 times LiCl (5M LiCl, NP-40, Deoxychloric Acid, 0.5M EDTA, 1MTris-HCl pH 8.0) and 3 times 1X TE(1M Tris-HCl, 0.5 M EDTA). The immunocomplexes were eluted by adding elution buffer (1M Tris-HCl pH 8.0, 5M NaCl, 0.5M EDTA, 10% SDS) to the pelleted complex, then centrifuged following which the

supernatant was collected. 5M NaCl was added to the supernatant and histone crosslink reverse by heating at 65^{0}C. 0.5 M EDTA, 1M Tris-Cl pH 6.5 and 10mg/ml of proteinase K were added to the sample which was followed by incubation at 45^{0}C. DNA was extracted by 1 volume of phenol/chloroform and twice by half volume of straight chloroform. 1/10 Volume of 3M NaOac, 2 volumes of 100% ethanol and 1 ul of glycogen were added to the DNA extract which was stored at -80^{0}C. Then the product centrifuged at 4^{0}C. The pellet was washed with 75% ethanol and resuspended in TE.

1 µl of the sample used for PCR as followed:

Step1: 94^o for 3:00

Step2: 94^o for 0:30

Step3: 55^o for 1:00

Step4: 72^o for 0:30

Step5: Go to step2 for 40 times

Step6: 72^o for 10:00

The product was run in 1.5% agarose gel

2.12. TIMP4 Promoter Cloning

A mouse Bacterial Artificial Chromosome (BAC) clone containing the mouse TIMP4 promoter was purchased from Lifetechnologies. A small portion of the glycerol stock was streaked on a LB plate containing 12.5µg/ml chloramphenicol and incubated at 37^{o}C overnight. A single colony was isolated and incubated in 10ml of LB 12.5µg/ml chloramphenicol at 37^{o}C overnight with shaking. The culture was in 1.5 ml tubes and centrifuged for 1 minute at 14000 rpm. The supernatant was discarded. The pellet was

resuspended in 200 µl of solution I (50 mM Tris pH 8.0 with HCl, 10 mM EDTA, 100µg/ml RNase A). Then 200 µl of solution II (200 mM NaOH, 1%SDS) was added and gently mixed, it was followed by adding 200 µl of solution III (3.0 M Potassium Acetate, pH 5.5). The sample was mixed by inverting the tube gently. A white precipitate had formed. The tube was then centrifuged for 10 minutes at 14,000rpm. The supernatant was transferred to a new tube without transferring any pellets. 900 µl of 100% ethanol was added to the supernatant and mixed well by inverting the tube, followed by centrifugation for 20 minutes at 14,000 rpm. The supernatant was removed and discarded. 100 µl of ice cold 75% ethanol was added to the pellet then centrifuged for 30 sec. The supernatant was completely removed from the pellet and dried for 20 minutes. The pellet was resuspended in 50 µl of distilled water. 1 µl of the sample was used for PCR to amplify the product as followed:

Step1: 94^{o} for 3:00

Step2: 94^{o} for 0:30

Step3: 55^{o} for 1:00

Step4: 72^{o} for 2:00

Step5: Go to step2 for 40 times

Step6: 72^{o} for 10:00

1 µl of the sample was used to verify the product in 1.5% agarose gel.

The PCR product was then ligated to a TA vector as follows: in a 0.5 µl PCR tube, 5 µl of 2x Rapid Ligation Buffer was mixed, 1µl of the vector, 1 µl of the PCR product and 1µl of T4 DNA ligase and nuclease free water was added to a final volume of 10µl. The

reaction was mixed by pipetting and incubated at room temperature for 1 hour at room temperature.

Tube containing E. coli competent cells was placed on ice until thawed. The cells were mixed by gently flicking the tube. 50 µl of cells was transferred in the tube containing the ligation reaction. The tube was then gently flicked and placed on ice for 20 minutes. The tube was placed in a water bath at 42^0C for 50 seconds and placed on ice for 2 minutes. 900µl of SOC medium was added to the reaction and incubated for 1.5 hours at 37^oC with shaking. 100 µl of the transformation culture was plated in a LB plate containing ampicillin/IPTG/X-Gal then incubated overnight at 37^oC. A single colony was picked and incubated in 10ml of LB 12.5µg/ml chloramphenicol at 37^oC overnight with shaking. The culture was in 1.5 ml tubes and centrifuged for 1 minute at 14000 rpm. The supernatant was discarded. The pellet was resuspended in 200 µl of solution I. Then 200 µl of solution II was added and gently mixed, followed by adding 200 µl of solution III. The sample was mixed by inverting the tube gently. A white precipitate had formed. The tube was then centrifuged for 10 minutes at 14,000rpm. The supernatant was transferred to a new tube without transferring any pellets. 900 µl of 100% Ethanol was added to the supernatant and mixed well by inverting the tube, followed by centrifugation for 20 minutes at 14,000 rpm. The supernatant was removed and discarded. 100 µl of ice cold 75% ethanol was added to the pellet then centrifuged for 30 sec. The supernatant was completely removed from the pellet and dried for 20 minutes. The pellet was resuspended in 50 µl of distilled water. One µl of the sample was used to verify the product by PCR as previously discribed. The sample was then digested with KPNI and XHOI and ran in a 1.5% agarose gel to verify the product.

The insert (product) was cut from the gel and placed in a 1.5 ml tube. 10µl of the membrane binding per 10µg of gel solution was added then vortexed and incubated at

65^{o}C until the gel was completely dissolved. The dissolved gel mixture was transferred into SV Minicolumn inserted in a collection tube and incubated at room temperature for 1 minute. The mixture was then centrifuged at 16,000xg for 1 minute. The flow-through was discarded. 700µl of the membrane solution with ethanol was added and centrifuge at 16,000xg for 1 minute. Another 500µl of membrane solution was added and centrifuged at 16,000xg for 5 minutes. The flow-through was discarded and the column was centrifuged for 1 minute with lid open. The minicolumn was transferred into a new 1.5 ml tube and 50 µl of nuclease-free water was added to the column and incubated at room temperature for 1 minute then centrifuged at 16,000xg for 1 minute. While running this experiment, the pGL3 vector was also digested using KPNI and XHOI. Then the DNA purified from the cut gel was ligated to the digested pGL3 vector at 37^{o}C for 1 hour. The ligated product was then transfected into competent E.coli cells as described above.

A single colony was picked from a freshly streaked plate and inoculated in 5 ml LB medium containing chloramphenicol overnight at 37^{0}C with shaking. The culture was diluted 1/500 in LB medium and 200 µl of the diluted culture was inoculated with 100 ml of medium at 37^{0}C overnight with shaking. Culture was transferred into plastic centrifuge tube. The bacterial cells were harvested by centrifugation at 6000 x g for 15 min. at 4^{0}C. The bacteria were resuspended in 10 ml Buffer P1; 10 ml of buffer P2 was added, then mixed vigorously by inverting 6 times, then incubated at room temperature for 5 min. Chilled Buffer P3 was then added to the lysate, and mixed vigorously by inverting 6 times. The lysate was poured into the barrel of the QIAfilter cartridge then incubated at room temperature for 10 min. without inserting the plunger. The cap was then removed from the cartridge and the plunger inserted slowly and the cell lysate was filtered into a 50 ml tube. Then 2.5 ml of buffer ER was added to the filtered lysate and mixed by inverting the tube 10 times, then incubated on ice for 30 min. QIAGEN-tip 500 was

equilibrated by adding 10 ml Buffer QBT and allowed the column to empty by gravity flow. The filtered lysate was placed into the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed 2 times with 30 ml Buffer QC. The DNA was eluted with 15 ml of Buffer QN, then precipitated by adding 10.5 ml of isopropanol. It was then mixed and centrifuged at 15,000 x g for 30 min at 4^0C. The supernatant was removed. The DNA pellet was washed with 5 ml of 70% ethanol and centrifuged at 15,000 x g for 10 min. The supernatant was removed without disturbing the pellet. The pellet was air-dried for 10 minutes and redissolved in 500 µl of Buffer TE. Then the DNA concentration was measured using the nanodrop and the Plasmid aliquoted and stored at -80^0C.

2.13. TIMP4 Promoter Assay

The aorta of SMC Bmal1$^{-/-}$ and their littermate control were used to isolate vascular smooth muscle cells. The cells were transfected with pGL3-TIMP4 luciferase vector and TRL-SV 40 Renillla vector with Lipofectamine-Plus reagent (Life technologies). Cells were first plated in twelve wells plate and incubated for 48 hours until the cells were about 70% confluent. The medium was then changed to OPTI-medium for 3 hours incubation at 37^0C. The DNA was pre-complexed with the plus reagent mixed and incubated at room temperature for 15 min. In a second tube, Lipofectamine reagent was diluted into OPTI-medium; next, this was combined with the precomplexed DNA, mixed and incubated for 15 minutes at room temperature. The DNA-Plus –Lipofectamine Reagent was added to each well containing fresh medium. The complexes was was incubated overnight at 37^0C. The medium was then removed and incubated for 24 hour at 37^0C with DMEM. After transfection was completed, the medium was removed and passive lysis buffer (Promega CAT# E1941) was added to each well followed by shaking

at room temperature. The lysed cells were centrifuge at 4^0C and the supernatant was kept for the reading of the luciferase and renilla.

2.14. TIMP4 ELISA

2.14.1 Sample Preparation

After isolating the aorta from mice, the tissue was snap-frozen with liquid nitrogen and stored at -80^0C until protein extraction. To extract protein from aorta, the frozen tissue was homogenized using rotor homogenizer and RIPA buffer (25mM Tris HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxylatecholate, 0.1% SDS) containing proteases at 4^0C. The samples were centrifuged for 10 minutes at 16000g at 4^0C. The supernatant was aliquoted and the protein concentration was measured by BCA protein assay with Pierce BCA Protein Assay Kit.

2.14.2. ELISA

The capture antibody was diluted to working concentration in PBS. A 96-well microplate was coated with 100 µl of the diluted capture antibody in each well. The plate was sealed and incubated overnight at room temperature. The next day, the capture antibody was aspirate from the wells and washed with wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) 3 times. The plates was then blocked by adding 300 µl of block buffer (1% BSA in PBS, pH 7.2-7.4) and incubated at room temperature for 1 hour; this step was followed by washing the plate with wash buffer 3 times. 100 µl of the sample or standards in reagent diluent (50mM Tris, 10mM $CaCl_2$, 0.15M $NaCl_2$, 0.05 Brij 35, pH 7.45-7.55) per well was added on the plate. The plate was covered and incubated at room temperature for 2 hours. The standards were prepared by making a six point standard curve with 2 fold serial dilutions with a highest concentration being 10,000 pg/mL and the lowest concentration 313 pg/mL. The plate was washed 3 times with wash buffer after incubation. 100 µl of detection antibody diluted in reagent diluent was

added in each well. The plate was covered and incubated for 2 hours at room temperature. The plate was then washed 3 times with was buffer. Then 100µl of the working dilution of streptavidin-HRP was added to each well. The plate was covered first adhesive strip then with aluminum foil to avoid light and incubated for 20 minutes at room temperature. After 20 minutes, the plate was washed three times with wash buffer. 100µl of substrate solution was added to each well and left to incubate for 20 minutes at room temperature, the plate was again covered with aluminum foil to avoid light. 50 µl of stop solution was added to each plate and tapped gently for mixing. The optical density of each well was determined using a microplate reader. To correct imperfections in the plate, 540nm wavelength was subtracted from the readings at 450nm.

2.15. Statistical Analysis

All data were expressed as mean ± S.E.M. Unpaired Student t-test was used for comparison between two groups. One-way ANOVA followed by Newman-Keul's post-hoc analysis was used for the comparison among multiple groups. Two-way ANOVA with repeated measures was used for comparison among multiple groups. Chi Square analysis was used for comparing AAA, TAA, and rupture incidence. A P value of <0.05 was considered significant. A P value of >0.05 was considered non-significant (NS).

2.16. Study approval.

All animal procedures were approved by the Institutional Animal Care and Use Committee of University of Kentucky.

Table 1: List of Primers

Gene	Primer	Sequence	Application
MR	Forward	5'-ATGGAAACCACACGGTGACCT-3'	Real Time PCR
	Reverse	5'-AGCCTCATCTCCACACACCAAG-3'	Real Time PCR
TIMP1	Forward	5'-CCAGAGCCGTCACTTTGCTT-3	Real Time PCR
	Reverse	5'-AGGAAAAGTAGACAGTGTTCAGGCTT-3'	Real Time PCR
TIMP2	Forward	5'-ACGCTTAGCATCACCCAGAAG-3	Real Time PCR
	Reverse	5'TTGGGACAGGGAGTGATCTTG-3'	Real Time PCR
TIMP3	Forward	5'ATCCCCAGGATGCCTTCTG-3'	Real Time PCR
	Reverse	5'-CCCTCCTTCACCAGCTTCTTT-3	Real Time PCR
TIMP4	Forward	5'-TGTGGCTGCCAAATCACCA-3'	Real Time PCR
	Reverse	5'-TCATGCAGACATAGTGCTGGG-3'	Real Time PCR
Bmal1	Forward	5′-CACTGTCCCAGGCATTCCA-3′	Real Time PCR
	Reverse	5′-TTCCTCCGCGATCATTCG-3′	Real Time PCR
Cry1	Forward	5′-TCGCCGGCTCTTCCAA-3′	Real Time PCR
	Reverse	5′-TCAAGACACTGAAGCAAAAATCG-3′	Real Time PCR
Per1	Forward	5′-TCGAAACCAGGACACCTTCTCT-3′	Real Time PCR
	Reverse	5′-GGGCACCCCGAAACACA-3′	Real Time PCR
Rev-erbα	Forward	5′-CCCTGGACTCCAATAACAACACA-3′	Real Time PCR
	Reverse	5′-GCCATTGGAGCTGTCACTGTAG-3′	Real Time PCR
TIMP4	Forward	5'-CCT ACT TGT TTT ACA CAT GGA ACC-3'	CHIP 1
	Reverse	5'-GTT TAC AGA CAG CAA AAT TTA CCC TT-3'	CHIP 1
TIMP4	Forward	5'-CAC CGC TAA GAA GAT TTT TGT TCT-3'	CHIP 2
	Reverse	5'-TGA ACT GGA CAG AGA TAG GCC T-3'	CHIP 2
TIMP4	Forward	5'-TAA GAA AGT AAA CGC TTT CCC AA-3'	CHIP 3
	Reverse	5'-ATC AGC TCA GCC TTG TCA CTT-3'	CHIP 3
TIMP4	Forward	5'-AAG CCA CAC TAG CAG GTG AAG-3'	CHIP 4
	Reverse	5'-CGA ACT CTT TTC TCC ATT AAG TAG GA-3'	CHIP 4
TIMP4	Forward	5'-TGA CAT CCT TCC CTA CCA CC-3'	CHIP 5
	Reverse	5'-CAA GAG CTA GTG AAG GGA AGC A-3'	CHIP 5
TIMP4	Forward	5'-TTT GGT CAA TTT GTC AAA ATA CAA TG-3'	Cloning TIMP4 Promoter
	Reverse	5'-GAC ACT GCA GAG CCC CAG-3'	Cloning TIMP4 Promoter

CHAPTER 3

RESULTS

3.1. Deletion of Bmal1 in vascular smooth muscle cells protects from mineralocorticoid agonist plus salt induced aortic aneurysm

Liu et al. had previously demonstrated that administration of mineralocorticoid receptor agonists, deoxycorticosterone acetate (DOCA) or aldosterone, when combined with high salt, induces aortic aneurysms[227]. To determine the role of vascular smooth muscle Bmal1 in aortic aneurysm formation, we administered DOCA plus salt to 4-month old male SM-Bmal1$^{-/-}$ and littermate control mice for 21 days. The increase in the external diameter of the abdominal and thoracic aorta by DOCA and salt administration was significantly suppressed in the SM-Bmal1$^{-/-}$ mice (Fig.2A). Eight out of 26 control mice had over a 50% dilation in abdominal aorta; however only one out of 25 SM-Bmal1$^{-/-}$ had this. None of the SM-Bmal1$^{-/-}$ mice showed thoracic aorta dilation (Fig.2 A). The incidence of aortic aneurysms was significantly decreased from 30.7% in control mice to 4% in the SM-Bmal1$^{-/-}$ mice ($P<0.01$); the incidence of thoracic aortic aneurysms (TAA) was decreased from 11.5% in control mice to 0% in the SM-Bmal1$^{-/-}$ mice ; and rupture incidence went from 7.6% to 0% (Fig. 2B). Fig. 3 shows photographs of a normal aorta from the SM-Bmal1$^{-/-}$ mice and a typical aneurysmal aorta from the control mice administered with DOCA and high salt.

Mineralocorticoid receptors agonists plus salt induced aortic aneurysm in mice showed that AAA formation and severity was aged dependent; therefore, we investigated whether deletion of Bmal1 in smooth muscle will still afford the same protection in older (8 months) mice. A dramatic protective effect by Bmal1 deletion was observed. Ultrasound quantification of the intraluminal diameter of the abdominal aorta illustrated that DOCA plus salt induced a time- dependent dilation in the control mice and that

dilation was significantly suppressed in the SM-Bmal1$^{-/-}$ mice. The increase in the intraluminal diameter (Fig. 4A; $P<0.001$) and external diameter (Fig. 4B; $P<0.001$) of aorta were much more pronounced in control mice than in SM-Bmal1$^{-/-}$ mice. The aortic aneurysm incidence was drastically decreased from 68.7% in the control to 0% in SM-Bmal1$^{-/-}$ mice ($P<0.001$), and TAAs were decreased from 31.2% in control mice to 0% in SM-Bmal1$^{-/-}$ mice ($P<0.05$) (Fig. 4C). Moreover, no rupture occurred in the 16 SM-Bmal1$^{-/-}$ mice while 2 of 16 control mice died of rupture (Fig. 4C). Fig. 5 shows photographs of a normal aorta from the SM-Bmal1$^{-/-}$ mice and a typical aneurysmal aorta from the control mice administered aldosterone and high salt.

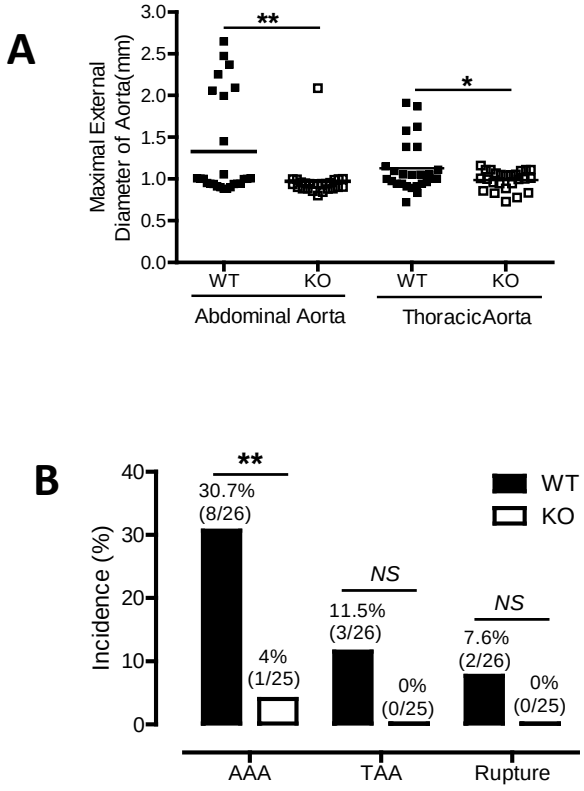

Figure 2: Four month old SM-Bmal1-/- mice are protected from DOCA-salt induced aortic aneurysm.

(A) Quantification of abdominal and thoracic aortic outer diameter in control and SM-Bmal1$^{-/-}$ mice after 3 weeks of DOCA plus salt (B) Incidence of AAA,TAA and aortic rupture after DOCA plus salt administration (3 weeks). Two-way ANOVA followed by Bonferroni's post-hoc analysis was used for statistics in A. Chi Square was used for comparing AA incidence in B. *: P<0.05, **P<0.01, NS: No significance.

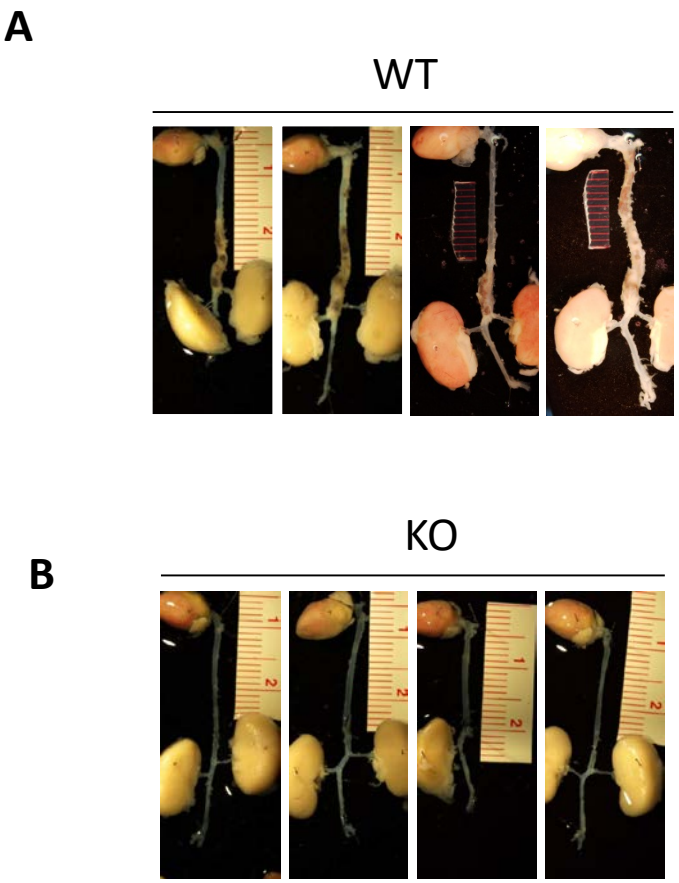

Figure 3: Representative pictures of aortas

Representative pictures of aortas with connected hearts and kidneys from 4 month old (A) WT and (B) KO mice after 3 weeks of DOCA plus salt

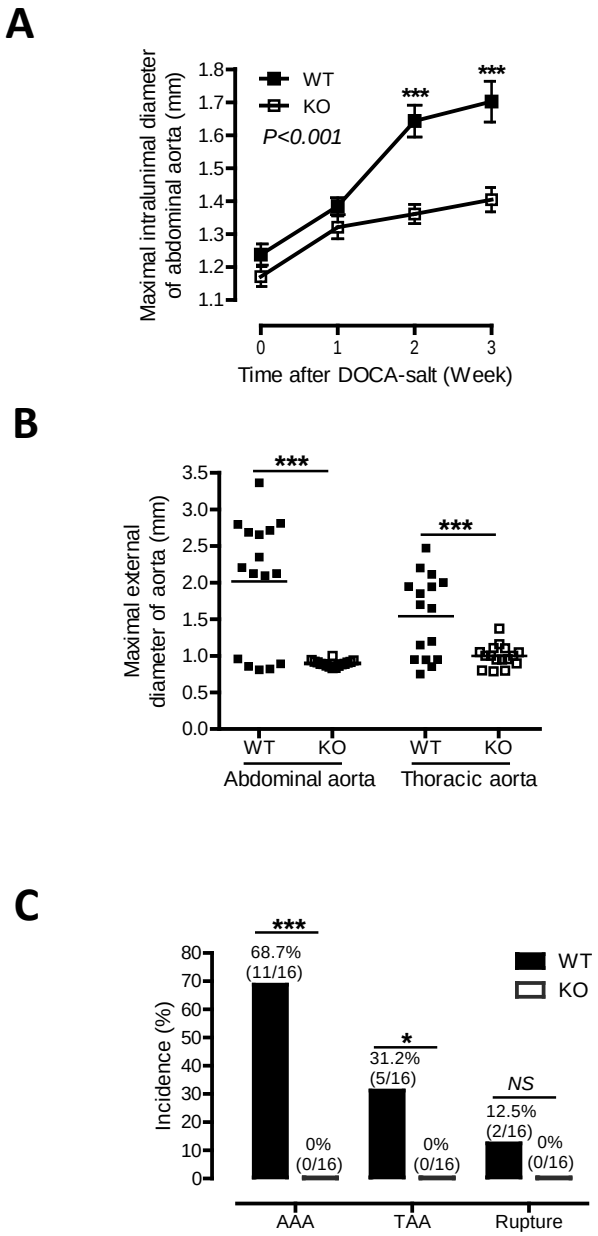

Figure 4: Eight month old SM-Bmal1-/- mice are protected from DOCA- salt induced aortic aneurysm

(A) Quantification of inner abdominal aortic diameter by ultrasound in control and SM-Bmal1$^{-/-}$ mice prior to and after DOCA- salt (B) Quantification of abdominal and thoracic aortic outer diameter in control and SM-Bmal1$^{-/-}$ mice after 3 weeks of DOCA-salt (C) Incidence of TAA, AAA and aortic rupture after DOCA plus salt administration (3 weeks). Two-way ANOVA followed by Bonferroni's post-hoc analysis was used for statistics in A and B. Chi Square was used for comparing AA incidence in C. *: P<0.05, ***P<0.001, NS: No significance.

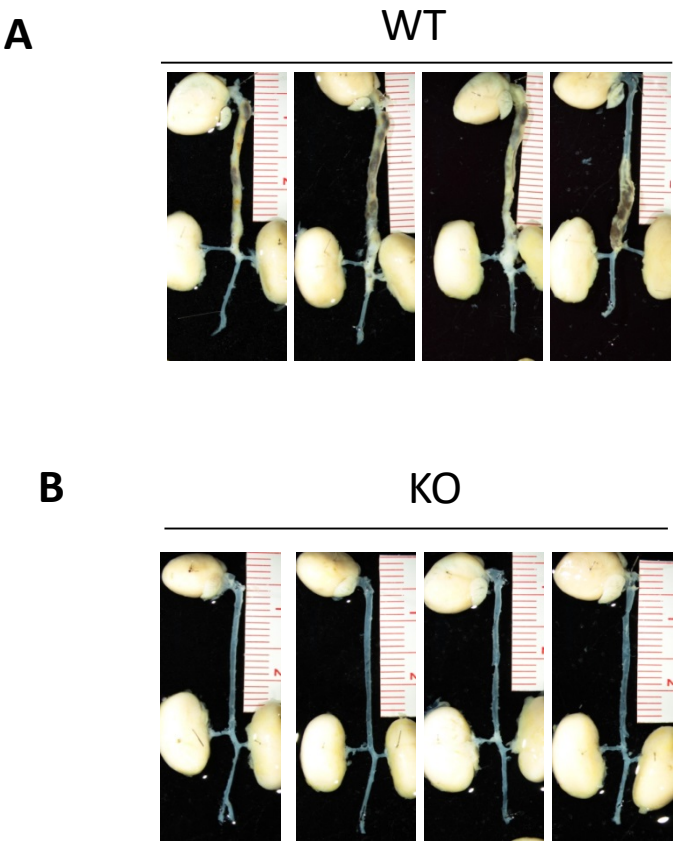

Figure 5: Representative pictures of aortas

Representative pictures of aortas with connected hearts and kidneys from 8 month old (A) WT and (B) KO mice after 3 weeks of DOCA plus salt

3.2. Deletion of Bmal1 in vascular smooth muscle cells has no effect on plasma sodium, mineralocorticoid receptor mRNA, and blood pressure.

Since DOCA plus salt model of aortic aneurysm was shown to be dependent on high salt intake and to work through the mineralocorticoid receptor, we first investigated whether Bmal1 deletion in smooth muscle cell prevents plasma sodium increase in response to DOCA plus salt. We measured plasma sodium before and after DOCA plus salt administration in control and SM-Bmal1$^{-/-}$ mice. The plasma sodium concentration was similar in control and SM-Bmal1$^{-/-}$ mice after DOCA plus salt treatment (Fig 6). We also measured plasma potassium and found similar decrease in plasma potassium in both control ($P<0.05$) and SM-Bmal1$^{-/-}$ mice (Fig.7; $P<0.01$). These results suggest that Bmal1 deletion from smooth muscle cells does not affect sodium retention or potassium excretion. Therefore, this is not a mechanism to account for the protection observed in SM-Bmal1$^{-/-}$ mice. Second, we investigated the possibility that deletion of Bmal1 in smooth muscle cells may decrease mineralocorticoid receptor (MR) expression in the aorta since DOCA plus salt model of AAA is dependent on MR[227] . We therefore looked at both Bmal1 and MR mRNA expression in different regions of the aorta including the arch, descending, suprarenal, and infrarenal aorta in wild type mice ZT5, ZT11, ZT17 and ZT23 (Fig.8). There was no significant difference in Bmal1 and MR mRNA expression among the different regions. Even in the suprarenal aorta, where AAA occurs in mice, Bmal1 and MR mRNA was unchanged. Bmal1 and MR mRNA had a trend towards a time-dependent variation in their mRNA expression, and, in particular, there was an inverse temporal correlation between Bmal1 and MR mRNA expression, indicating that Bmal1 may negatively regulate MR expression. This hypothesis was investigated by looking at aortic MR mRNA expression in control and SM-Bmal1$^{-/-}$ mice before and after 7 days of DOCA and salt treatment at ZT17 (Fig.9). DOCA and salt suppressed MR mRNA expression in the aortic arch ($P<0.05$) and descending aorta of

control mice ($P<0.001$). A trend for downregulation of MR mRNA was found in suprarenal and infrarenal aorta. Deletion of smooth muscle Bmal1 had little effect on MR mRNA expression in the aorta before and after DOCA-salt treatment except for the descending aorta where deletion of smooth muscle Bmal1 significantly decreased MR mRNA expression (Fig.9; $P<0.01$).

As DOCA plus salt administration increases blood pressure and hypertensive patients have high prevalence of aortic aneurysms[230, 231] , we examined blood pressure in control and SM-Bmal1$^{-/-}$ mice by the tail cuff method to investigate whether the protective effects of Bmal1 deletion were associated with inhibition of DOCA plus salt induced increases in blood pressure. First, 4-month-old SM-Bmal1$^{-/-}$ mice had lower blood pressure than control mice (Fig.10A; $P<0.001$), consistent with our previous telemetry results. Interestingly, there was no difference in basal blood pressure between 8-month-old SM-Bmal1$^{-/-}$ mice and control mice (Fig 10B). Second, perhaps more important, both SM-Bmal1$^{-/-}$ and control mice , regardless of their ages, increased their blood pressure to a similar extent in response to DOCA or Aldo plus salt, suggesting that deletion of Bmal1 in smooth muscle has little effect on DOCA or Aldo plus salt-induced hypertension.

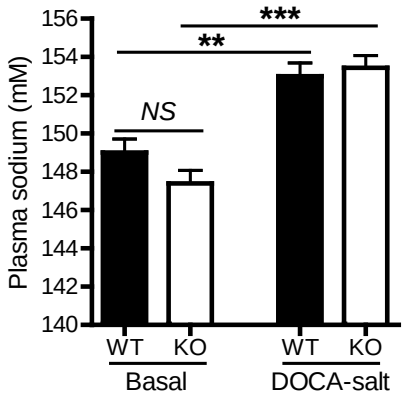

Figure 6: Deletion of Bmal1 from smooth muscle cells does not affect plasma sodium level

Sodium content in plasma from control and SM-Bmal1$^{-/-}$ mice at basal and after 21 days of DOCA plus salt treatment. Two-way ANOVA was followed by Bonferroni's post-hoc analysis**P<0.01,***P<0.001, NS: No Significance

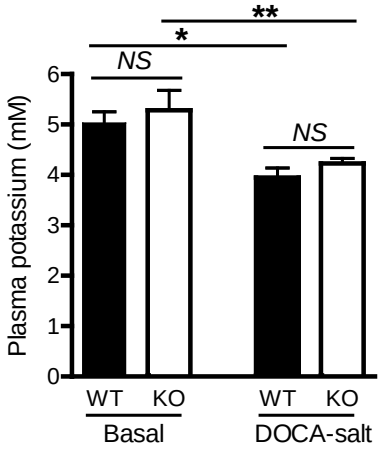

Figure 7: Deletion of Bmal1 from smooth muscle cells does not affect plasma potassium level

Potassium content in plasma from control and SM-Bmal1$^{-/-}$ mice at basal and after 21 days of DOCA plus salt treatment. Two-way ANOVA was followed by Bonferroni's post-hoc analysis*P<0.05,**P<0.01, NS: No Significance

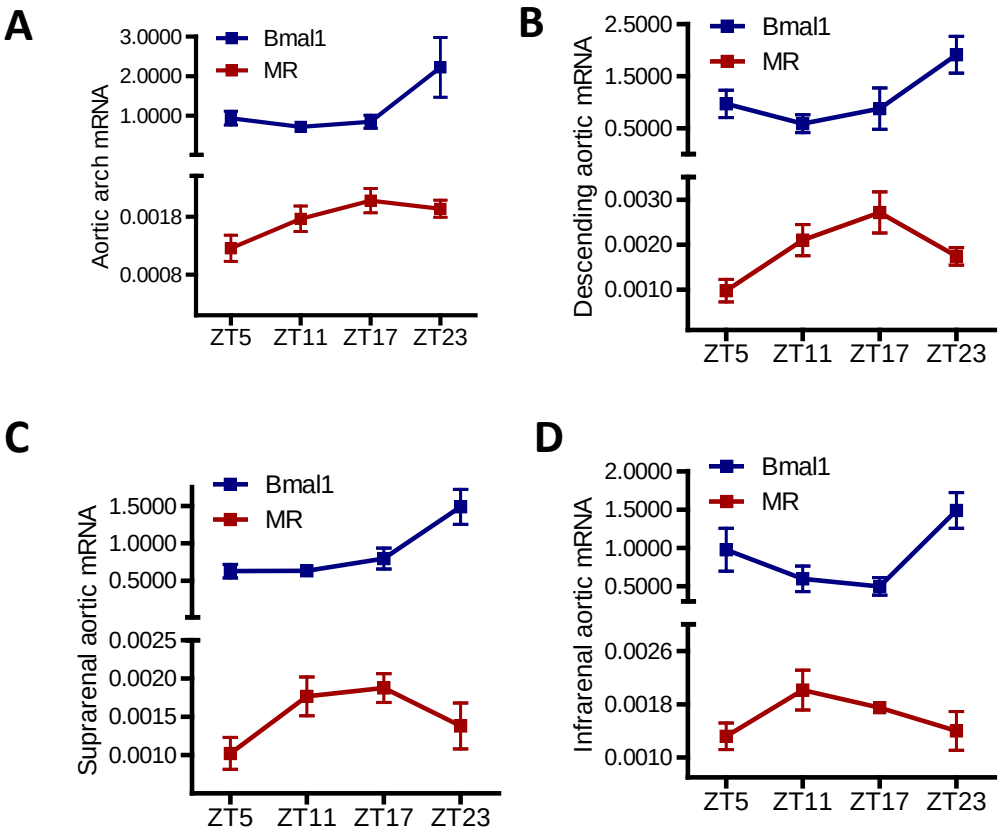

Figure 8: Deletion of Bmal1 in smooth muscle has little effect on basal and DOCA-salt-induced MR expression in suprarenal aorta

Aortas were isolated at ZT5, 11, 17, and 23 from ten-week-old C57BL/6J mice (A through D) Aortas were then cut into arch, thoracic, suprarenal, and infernal aorta. Relative expressions of MR and Bmal1 mRNA were normalized to 36B4 mRNA expression. Two-way ANOVA followed by Bonferroni's post-hoc analysis was used for statistics in (A through D; N=4).

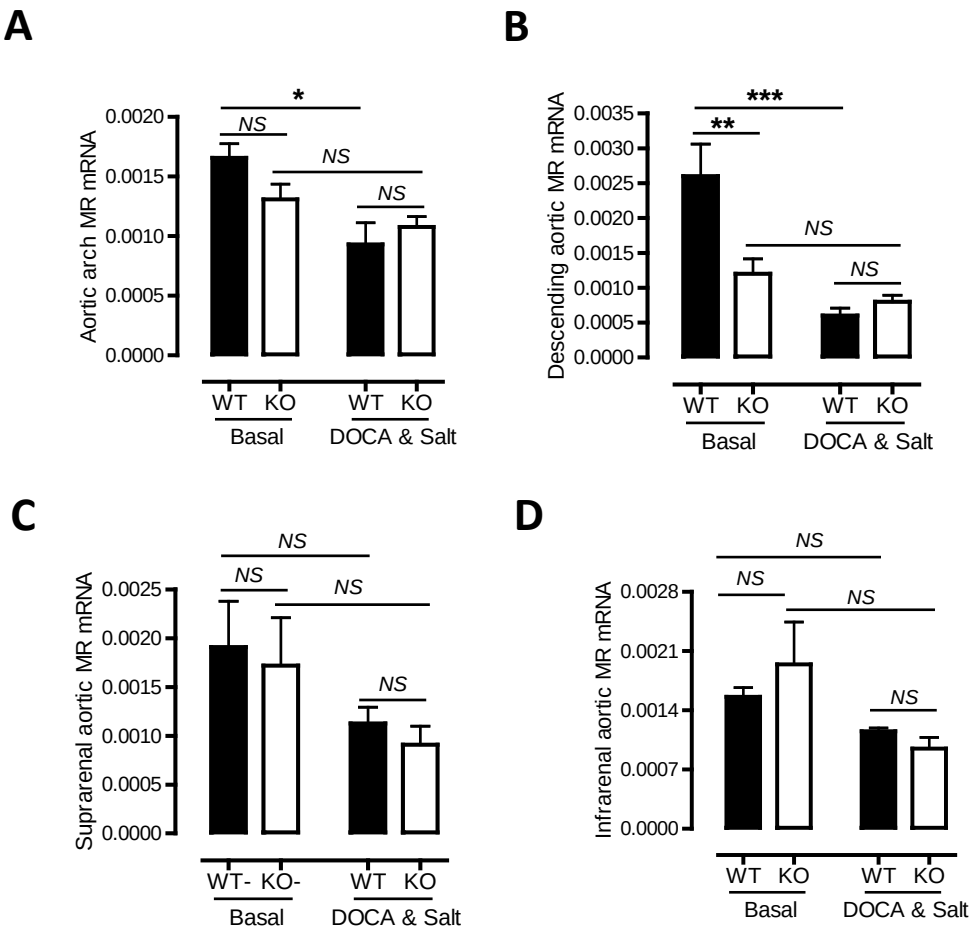

Figure 9: Deletion of Bmal1 in smooth muscle has little effect on basal and DOCA-salt-induced MR expression in suprarenal aorta

Aortas were isolated at ZT5 from ten-week-old SM-Bmal1$^{-/-}$ and WT littermates administered with DOCA-salt for 7 days (A through D). Aortas were then cut into arch, thoracic, suprarenal, and infernal aorta. Relative expressions of MR mRNA were normalized to 36B4 mRNA expression. One-way ANOVA followed by Newman-Keuls' post-hoc analysis was used for statistics in (A through D; N=3 to 4). **P<0.05; **P<0.01; ***P<0.001. NS*: not statistically significant.

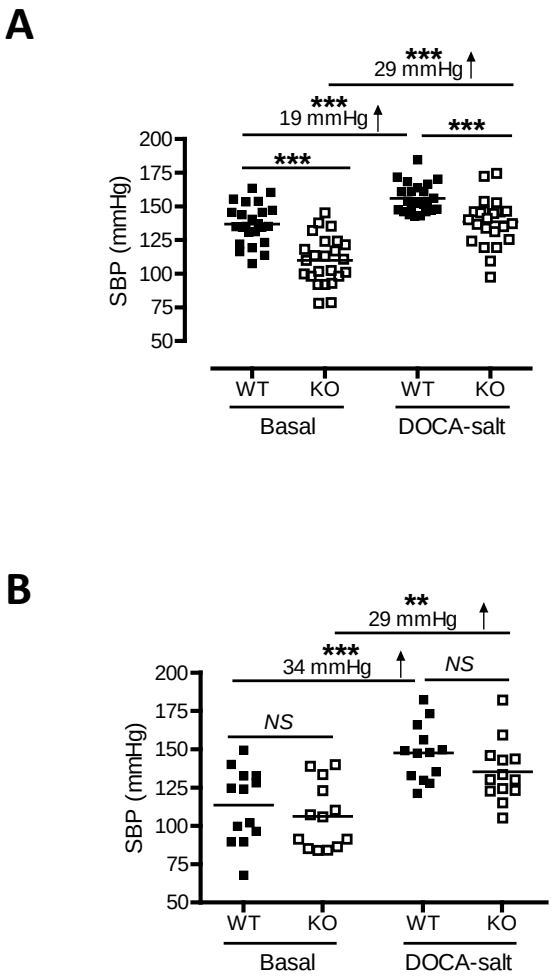

Figure 10: Bmal1 deletion does not affect DOCA plus salt induced hypertension

(A) Systolic blood pressure from 4 months old control and SM-Bmal1$^{-/-}$ mice before and after 21days after DOCA plus Salt treatment (B) Systolic blood pressure from 8 months old control and SM-Bmal1$^{-/-}$ mice before and after 21 days of DOCA plus Salt treatment . Two-way ANOVA was used by Bonferroni's post-hoc correction.**P<0.01, ***P<0.001,NS: No significance

3.3. Deletion of Bmal1 from smooth muscles cells prevents MMP activation triggered by DOCA plus salt administration and elastin degradation

One hallmark of aortic aneurysm is elastin degradation which leads to aortic dilation and subsequently to aortic rupture. We therefore investigated whether Bmal1 deletion eased DOCA plus salt induced Verhoeff's Van Gieson (EVG) elastin staining. DOCA-salt induced elastin degradation (Fig 11; $P<0.001$), and deletion of BMAL1 in smooth muscle effectively prevented mice from DOCA-salt induced elastin degradation (Fig 11).

Matrix Metalloproteinases (MMPs), in particular MMP2 and MMP9, have been demonstrated to play a major role in elastin degradation in aortic aneurysms[232] . Therefore, we investigated the effect of deletion of BMAL1 in smooth muscle on DOCA plus salt-induced MMP activation. We used in situ zymography to measure MMP activity in abdominal aortic cryosections from SM-Bmal1$^{-/-}$ and control mice administered with DOCA and salt for 7 days (Fig.12). In the absence of DOCA and salt, little MMP activity was detected in aorta from both SM-Bmal1$^{-/-}$ mice and controls. In the presence of DOCA and salt, however, a large MMP activity was readily detected in aorta from control mice, but not in SM-Bmal1$^{-/-}$ mice (Fig.12), suggesting that deletion of Bmal1 in smooth muscle prevents mice from DOCA-salt-induced elastin degradation through inhibiting MMP. We also used in situ zymography to measure MMP activity in paraffin-embedded abdominal aortas from mice administered DOCA and salt for 21 days. A similar dramatic protective effect of deletion of Bmal1 in smooth muscle on DOCA-salt-induced MMP activation was also observed in paraffin-embedded aortas (Fig. 13). It should be pointed out that using in situ zymography to detect MMP activity in fixed, paraffin-embedded tissue has been well described[229] . However, the detected MMP activity in paraffin-embedded aortic section was only found in aortic thrombosis area, which is different from what we reported in aortic cryosections where MMP could be readily detected in the media of smooth muscle layer[227] , probably reflecting that less MMP activity was

preserved in paraffin-embedded tissue[229]. Regardless of these differences, it is clear that both data indicate a critical role of smooth muscle BMAL1 in DOCA-salt-induced MMP activation.

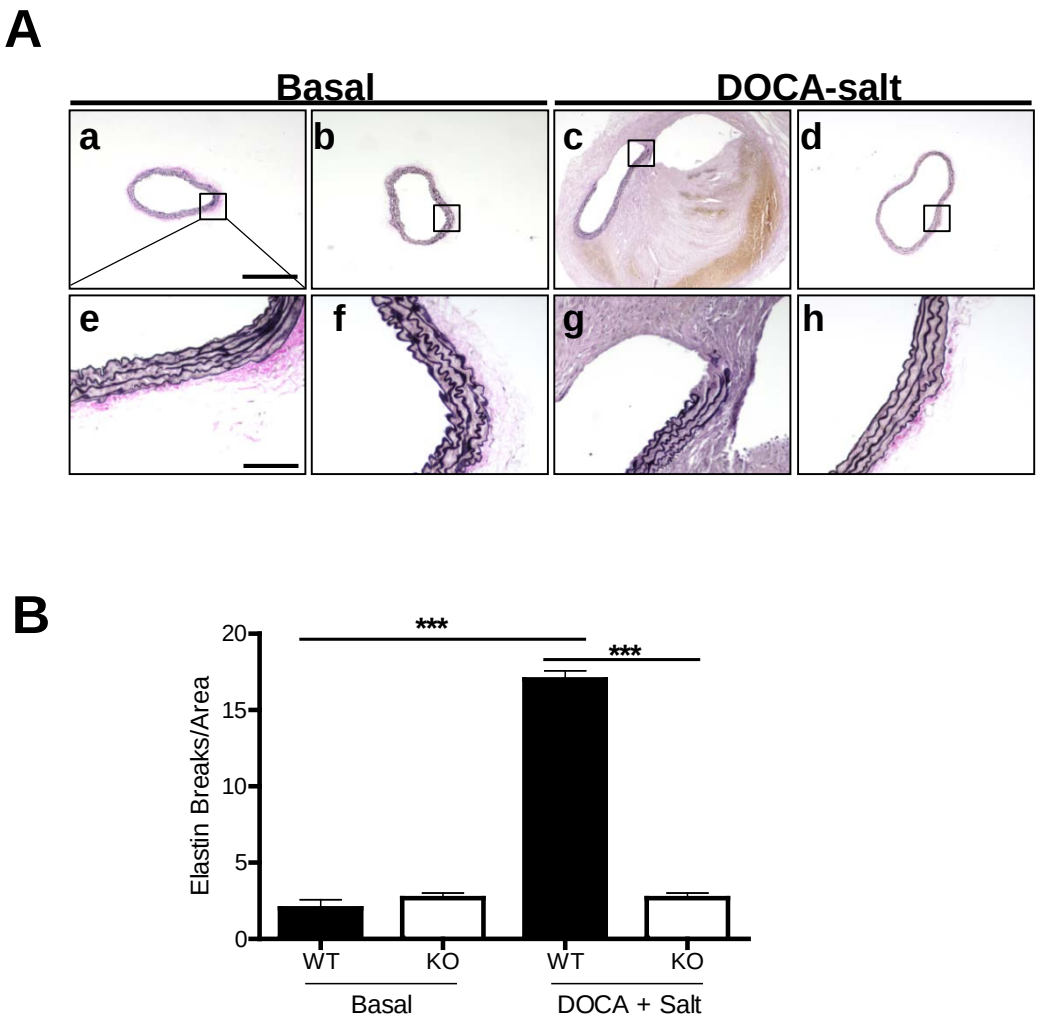

Figure 11: Bmal1 deletion from smooth muscle cells prevents DOCA plus salt induced elastin degradation

(A) Elastin integrity in control and SM-Bmal1$^{-/-}$ mice after 21 days of DOCA plus salt administration. (B): Quantification of elastin breaks per surface area, Two-way ANOVA followed by Bonferroni's post-hoc analysis. ***P<0.001 .Scale bar: at lower magnification: 500µm, higher magnification 100µm

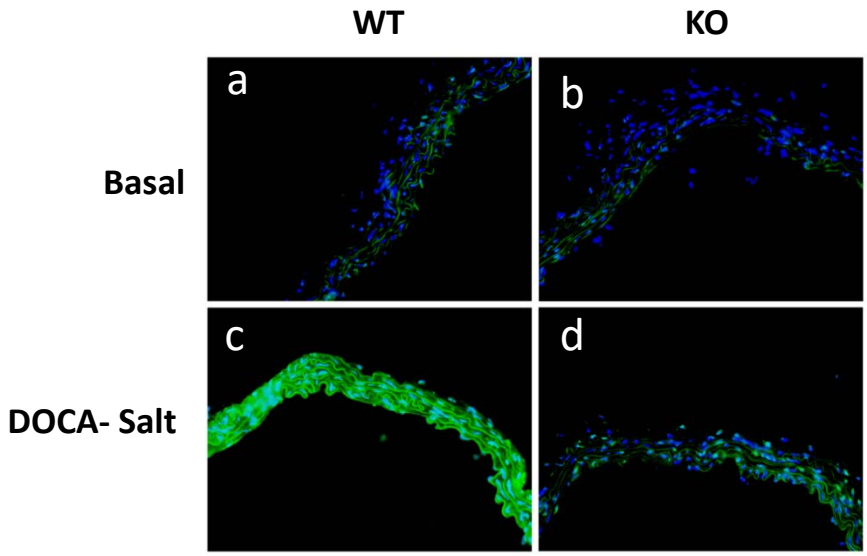

Figure 12: Bmal1 deletion from smooth muscle cells prevents DOCA plus salt induced gelatinases activation in the aorta

In situ zymography of cryosections of abdominal aorta from SM-Bmal1$^{-/-}$ mice and WT littermates administered with DOCA-salt for 7 days. Scale bar: 100µm.

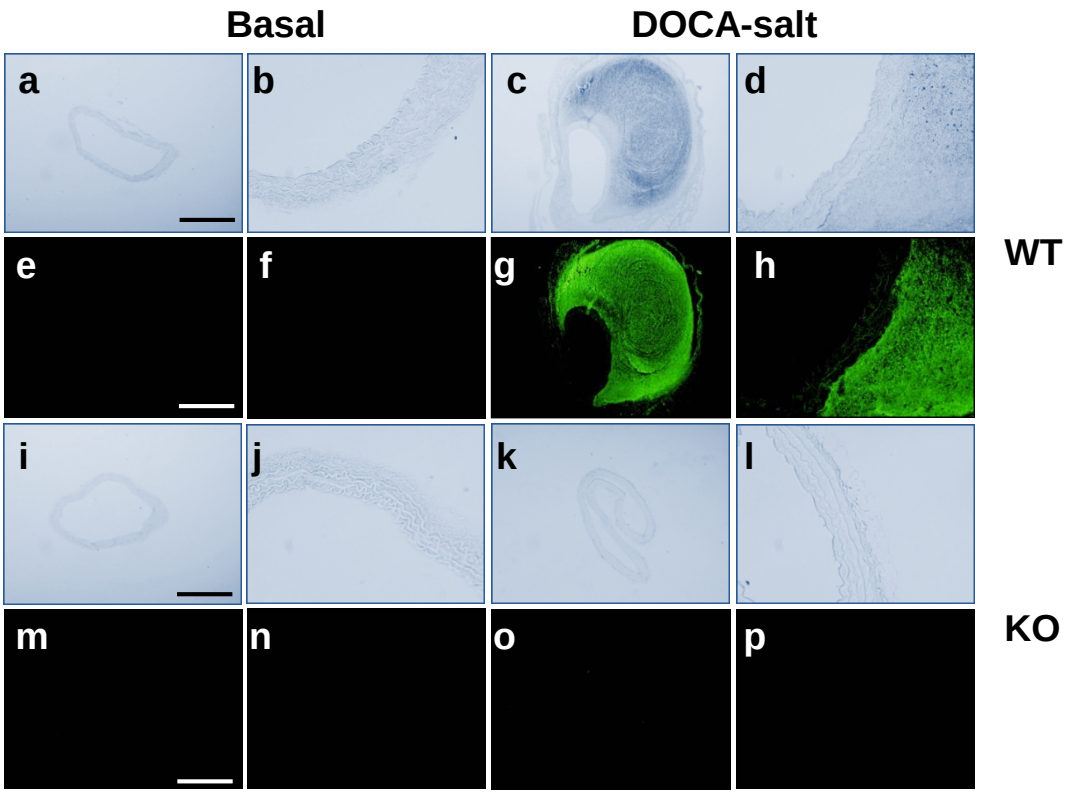

Figure 13: Deletion of Bmal1 in smooth muscle protects mice from DOCA-salt-induced MMP activation

In situ zymography (e through h and m through p) and phase contrast microscopy (a though d and I through l) of abdominal aortic cross-sections from SM-Bmal1$^{-/-}$ mice (I through p) and WT littermates (a through h) with (c, d, g, h, k, l, o, and p) or without DOCA-salt administration (a, b, e, f, i, j, m, and n). Scale bar: at lower magnification: 500μm, higher magnification: 100μm.

3.4. Deletion of Bmal1 in smooth muscle selectively increases TIMP4 expression in abdominal aorta

The protective effect of deletion of Bmal1 in smooth muscle may be attributable to inhibiting/downregulating MMP2/MMP9 and/or to activating/upregulating tissue TIMPs. To investigate which mechanism(s) is (are) operating in SM-Bmal1$^{-/-}$ mice, we determined aortic MMP2 and MMP9 activities in SM-Bmal1$^{-/-}$ and control administered DOCA and salt for 7 days by gel zymography, a simple but sensitive method to detecting MMP activity under a condition at which MMPs are dissociated from bound TIMPs[233]. Consistent with the results of in situ zymography, a significant or a trend towards increase in proMMP2, MMP2, proMMP9, and MMP9 activities was detected in control mice treated with DOCA-salt (Fig. 14A through E). Surprisingly, in sharp contrast to the result of in situ zymography, deletion of BMAL1 in smooth muscle had little effect on DOCA-salt-induced proMMP2, MMP2, proMMP9, and MMP9 activities. Moreover, a significant or a trend towards increase in basal proMMP2, MMP2, proMMP9, and MMP9 activities was found in SM-Bmal1$^{-/-}$ mice relative to that in control mice (Fig. 14A through E). Since in gel zymography is to detect MMP activity under a condition that all MMPs, including proMMPs, are activated by SDS, the activity of MMPs detected by in gel zymography actually is proportional to the level of MMPs protein in samples regardless they are active or inactive[234] . In contrast, in situ zymography is only to detect active MMPs. Thus, these results suggest that the activity of MMPs, but not the level of MMPs, is suppressed by the deletion of BMAL1 in smooth muscle. We then investigated the possibility that the overall inhibition of MMP activity detected by in situ zymography is attributable to enhanced expression of tissue inhibitors of MMPs (TIMPs). TIMPs are specific inhibitors of MMPs that control the local activities of MMPs in tissues[151, 235]. Four TIMPs (TIMP1, TIMP2, TIMP3, and TIMP4) have been identified and characterized so far in mammals, and all four TIMPs can inhibit active forms of all MMPs with distinct

affinity and potency[156, 236]. Five different approaches were taken to rigorously test whether and/or which TIMP(s) in aorta is affected by deletion of Bmal1 in smooth muscle and/or by DOCA or Aldo plus salt in vivo and ex vivo.

First, we determined mRNA expression of all four TIMPs in aorta in SM-Bmal1$^{-/-}$ and control mice administered with DOCA and salt for 7 days. All four TIMP transcripts were found in mouse aorta but among the four TIMPs, Timp4 was the most abundant (Figure 15). The order of relative abundance for the four TIMPs in mouse aorta were TIMP4 > TIMP3 >TIMP2 >TIMP1. The basal level of TIMP4 was 508-, 427-, and 311-fold higher than TIMP1, TIMP2, and TIMP3 in control mouse aorta, respectively. Moreover, among four TIMPs, TIMP4 was selectively upregulated in mouse aorta from SM-Bmal1$^{-/-}$ mice. There was a trend towards increased TIMP4 mRNA expression in the aorta from WT mice but it did not reach statistical significance. Interestingly, a significant increase in TIMP4 mRNA expression was found in aorta from SM-Bmal1$^{-/-}$ mice after DOCA plus salt treatment. (Fig. 15; $P<0.01$). The level of TIMP4 was 1,014-, 2,035-, and 559-fold higher than the levels of TIMP1, TIMP2, and TIMP3 in aorta in SM-Bmal1$^{-/-}$ mice after DOCA-salt administration, suggesting that TIMP4 may play a major role in inhibiting DOCA-salt-induced MMP activation. While little changes were found in TIMP1 and TIMP2 mRNA expression in response to deletion of Bmal1 in smooth muscle and/or DOCA-salt, a significant increase in TIMP3 mRNA was found in both SM-Bmal1$^{-/-}$ mice and WT littermates in response to DOCA-salt treatment. However, deletion of Bmal1 in smooth muscle downregulated TIMP3 mRNA expression before and after DOCA-salt administration, suggesting that TIMP3 unlikely accounts for the protective effect of deletion of BMAL1 in smooth muscle on DOCA-salt-induced AAA.

Second, we determined the mRNA expression of all four TIMPs in an aortic organ culture in the presence of Aldo (10 nM) and high salt (additional 10 mM NaCl) to

investigate whether DOCA and salt directly or indirectly act on the aorta. The mRNA expression pattern of TIMP1, TIMP2, TIMP3, and TIMP4 in response to Aldo and salt observed in ex vivo organ culture (Fig. 16) was similar to that found in vivo although the extent of increases in TIMP4 mRNA expression were somewhere different. These data not only verified that deletion of Bmal1 in smooth muscle selectively upregulated TIMP4 mRNA expression in aorta, but demonstrated that Aldo and salt was able to directly act on aorta ex vivo. Third, we determined TIMP4 mRNA expression in aorta from SM-Bmal1$^{-/-}$ mice and WT littermates administered with DOCA-salt for 21 days (Fig.17). Again, TIMP4 was found to be significantly upregulated by deletion of Bmal1 in smooth muscle ($P<0.01$) and could be further elevated by DOCA-salt in SM-Bmal1$^{-/-}$ mice ($P<0.05$), but not in WT littermates ($P<0.001$). (Fig.17). Interestingly, the level of TIMP4 increase induced by DOCA-salt was higher at 21 days than at 7 days (182% increase at 7 days vs. 85% increase at 7 days), indicating that DOCA-salt-induced TIMP4 upregulation in SM-Bmal1$^{-/-}$ mice is time-dependent.

Fourth, we determined TIMP4 protein expression in aorta by ELISA in SM-Bmal1$^{-/-}$ mice and WT littermates administered with DOCA-salt for 7 days to investigate whether TIMP4 mRNA upregulation leads to its protein upregulation. Quantitative data showed that deletion of Bmal1 in smooth muscle increased TIMP4 protein expression (Fig. 18; $P<0.001$). However, TIMP4 protein was not further increased in aorta by DOCA and salt, which is different from its mRNA upregulation.

Finally, we determined TIMP4 protein expression by immunohistochemistry in aorta from SM-Bmal1$^{-/-}$ mice and WT littermates administered with DOCA and salt for 21 days to investigate the localization of TIMP4 in abdominal aorta. TIMP4 protein was dramatically upregulated in the media smooth muscle layer of the aorta in SM-Bmal1$^{-/-}$ mice before and after DOCA-salt administration (Fig. 19). There was no obvious increase in TIMP4

immunostaining in aorta from WT mice after DOCA-salt treatment, which agrees with quantitative data by real-time PCR and ELISA.

Taken together, these data indicate that selective upregulation of TIMP4 by deletion of Bmal1 in smooth muscle may be responsible for its protective effect on DOCA or Aldo plus salt-induced MMP activation, elastin degradation, and AAA formation.

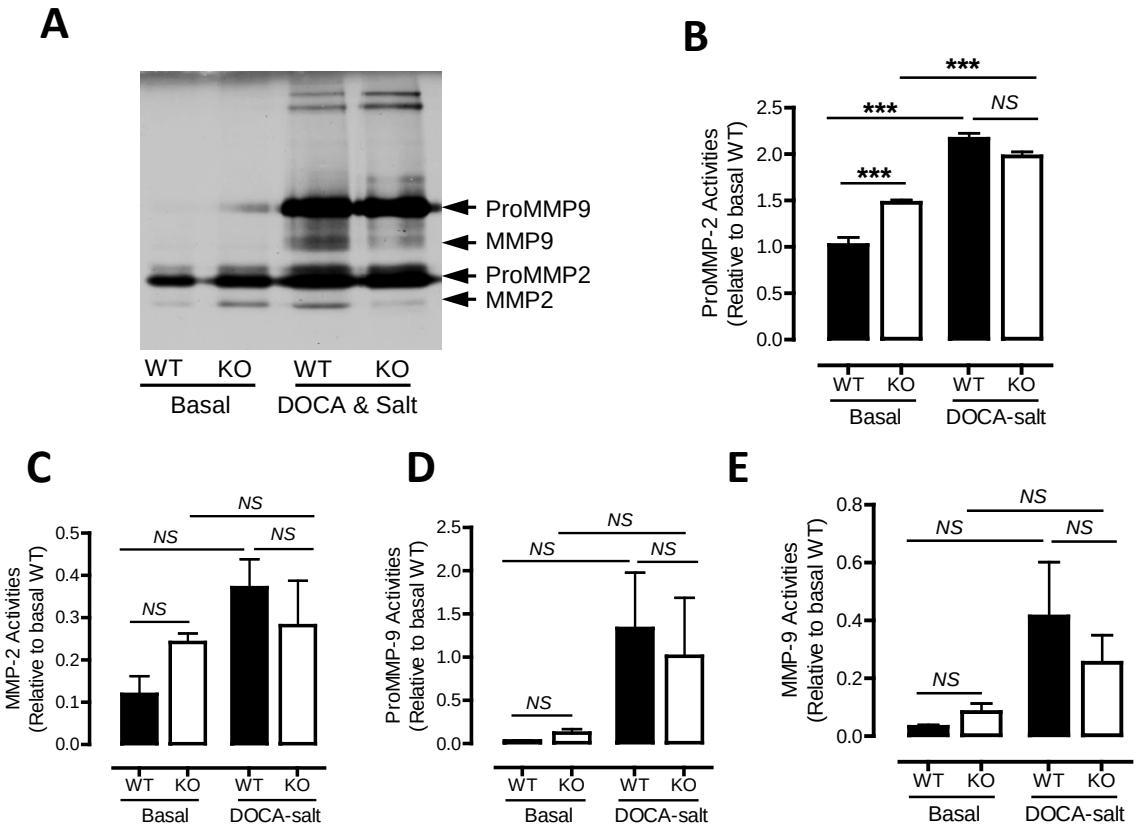

Figure 14: Lack of suppression on the MMP2/9 activities by Bmal1 deletion from smooth muscle cells

(A); Representative gel zymography from the medium in which aortas from control and SM-Bmal1$^{-/-}$ were incubated after 7 days of DOCA plus salt (B): Pro-MMP2 quantification from gel zymography(n=4) (C): Active MMP2 quantification from gel zymography (n=4) (D): Pro-MMP9 quantification from gel zymography (n=4) (E): Active MMP9 quantification from gel zymography . Two-way ANOVA followed by Bonferroni's post-hoc analysis **P<0.01, NS: No Significance

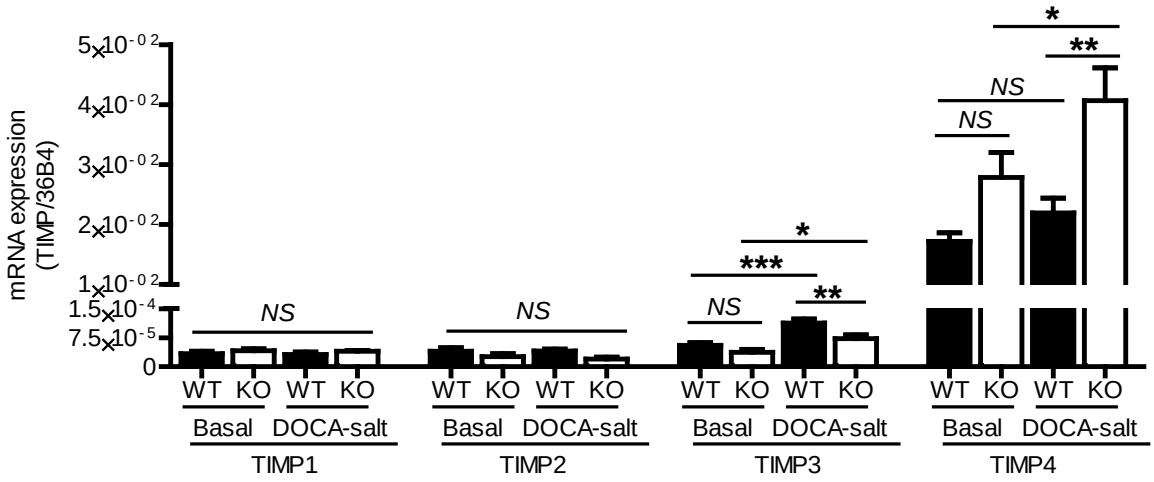

Figure 15: Deletion of Bmal1 in smooth muscle selectively upregulates TIMP4 mRNA expression in aorta

mRNA expressions of TIMP1,TIMP2, TIMP3, and TIMP4 in aortas from SM-Bmal1$^{-/-}$ mice and WT littermates at basal condition and after administered with DOCA-salt for 7 days. One-way ANOVA followed by Newman-Keuls' post-hoc analysis was used for statistics (N=4-6) **P*<0.05, ***P*<0.01, ****P*<0.001. *NS*: not statistically significant.

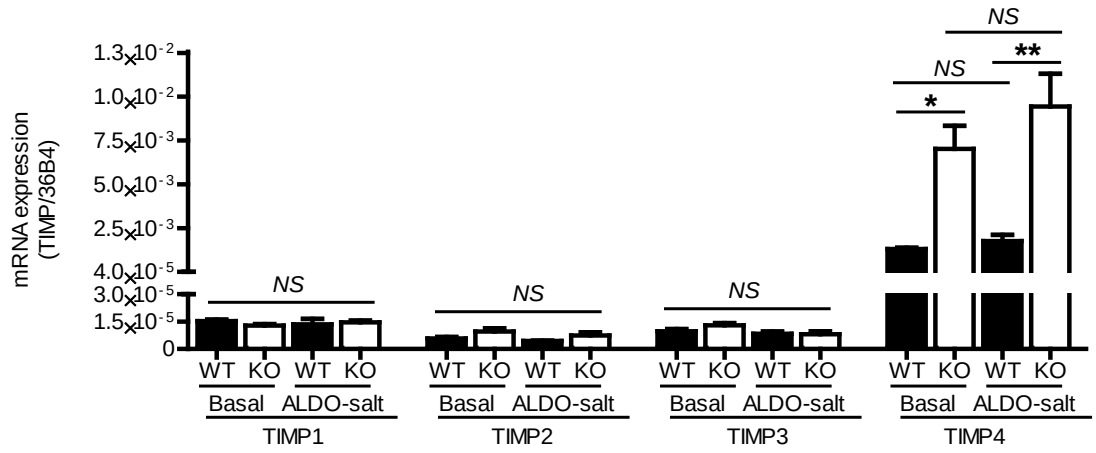

Figure 16: Deletion of Bmal1 in smooth muscle selectively upregulates TIMP4 mRNA expression in aorta.

mRNA expressions of TIMP1, TIMP2, TIMP3, and TIMP4 in aortic organ culture (N=3-4). Aortas were isolated from SM-Bmal1$^{-/-}$ mice and WT littermates and then incubated with Aldo (10 nM) and high salt (10 mM increase) medium for 24 h. One-way ANOVA followed by Newman-Keuls' post-hoc analysis was used for statistics (N=4) **P*<0.05, ***P*<0.01, *NS*, not statistically significant

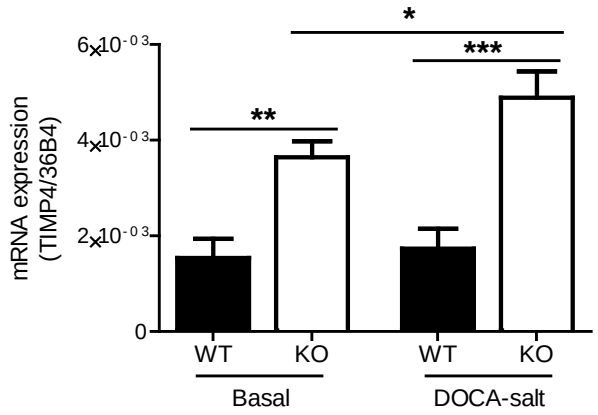

Figure 17: Deletion of Bmal1 in smooth muscle upregulates TIMP4 mRNA expression in aorta

TIMP4 mRNA expression in aortas from SM-Bmal1$^{-/-}$ mice and WT littermates before and after DOCA-salt for 21 days One-way ANOVA followed by Newman-Keuls' post-hoc analysis was used for statistics (N=4) **P*<0.05, ***P*<0.01, ****P*<0.001

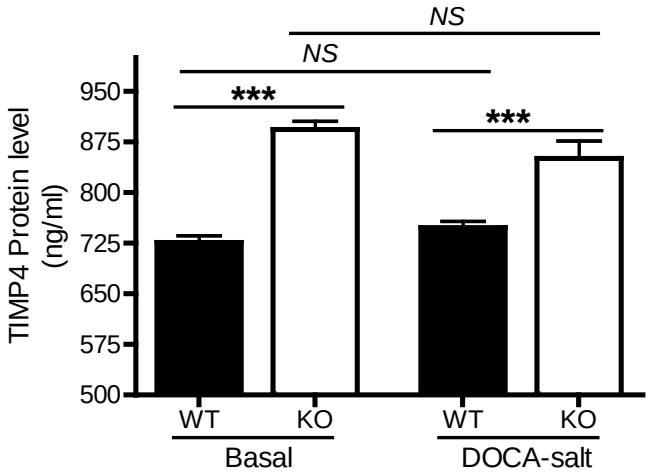

Figure 18: Deletion of Bmal1 in smooth muscle upregulates TIMP4 protein expression in aorta

TIMP4 protein expression was determined by ELISA in aortas from SM-Bmal1$^{-/-}$ mice and WT littermates before and after DOCA-salt for 7 days. One-way ANOVA followed by Newman-Keuls' post-hoc analysis was used for statistics (N=4) ***P<0.001. *NS*: not statistically significant

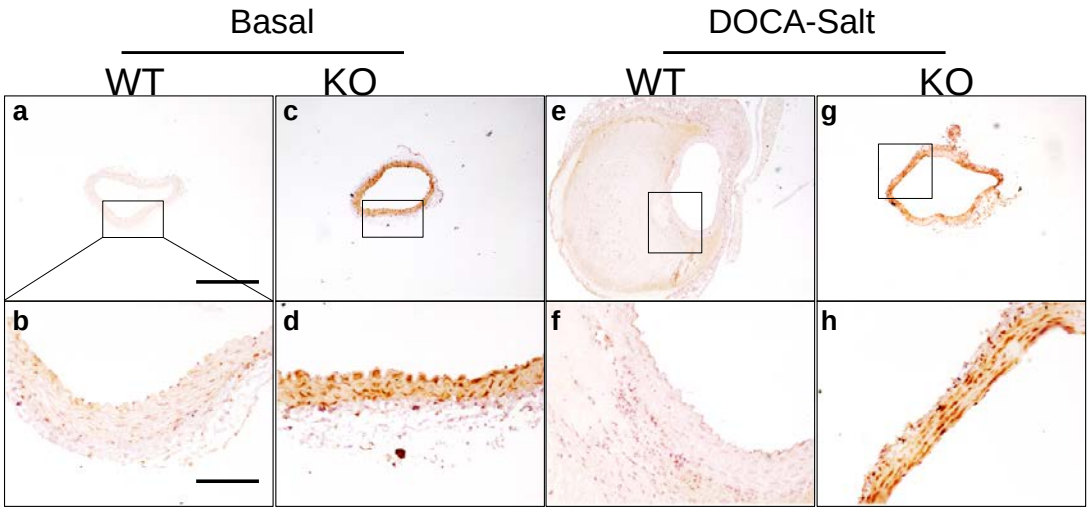

Figure 19: Deletion of Bmal1 in smooth muscle upregulates TIMP4 protein expression in aorta

Representative immunostaining of TIMP4 in paraffin-embedded suprarenal aortic cross-sections in SM-Bmal1$^{-/-}$ mice and WT littermates before and after DOCA-salt for 21 days. Scale bar: at lower magnification (a, c, e, and g): 500 µm, higher magnification (b, d, f, and h): 100µm.

3.5. Bmal1 is upregulated in aorta by DOCA and salt.

It is clear that smooth muscle Bmal1 is critically involved in DOCA plus salt-induced MMP activation, elastin degradation, and AAA formation, which probably involves TIMP4. However it is unclear how smooth muscle Bmal1 is regulated by DOCA plus salt. To address this important mechanistic issue, we first investigated whether smooth muscle Bmal1 mRNA responds to DOCA-salt. An over 6-fold increase in Bmal1 mRNA expression was found in the aorta from mice administered with DOCA-salt for 7 days compared to that in control aorta (Figure 20; $P<0.001$), suggesting that DOCA-salt-induced Bmal1 mRNA upregulation precedes and accounts for its protein upregulation.

To investigate the mechanism by which Bmal1 is upregulated by DOCA-salt, we determined Per1, Cry1, and Rev-erbA mRNA expression in aorta of mice administered with DOCA and salt for 7 days as Bmal1 is negatively regulated by Per1, Cry1, and Rev-erbα under physiological conditions[222]. We found that DOCA-salt administration had little effect on Per1 mRNA (Fig.21), significantly increased Cry1 transcripts (Fig. 22; $P<0.01$), but downregulated Rev-erbα transcript (Fig. 23; $P<0.001$), suggesting that DOCA-salt upregulates Bmal1 through suppressing Rev-erbα in aortic smooth muscle.

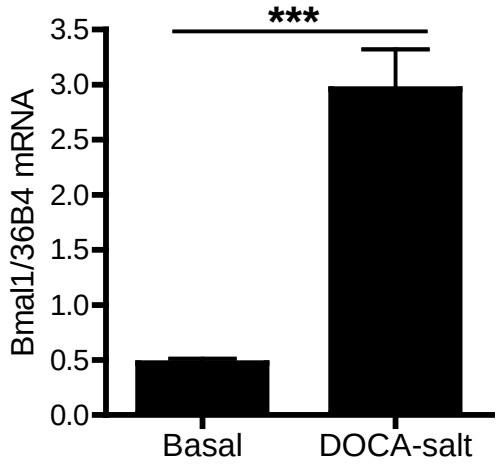

Figure 20: Bmal1 is upregulated after DOCA plus salt treatment

Bmal1 mRNA expression in aortas from control mice before and after DOCA plus salt for 7 days (N= 5-6). Student's *t* test was used for statistics. ***P<0.001

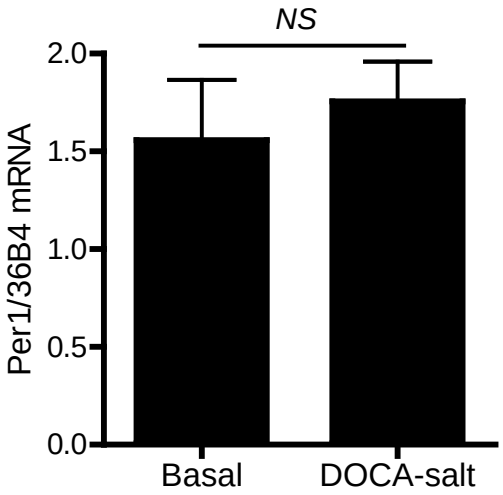

Figure 21: Per1 is not affected by DOCA plus salt treatment.

Per1 mRNA expression in aortas from control mice before and after DOCA plus salt for 7 days (N=5-6). Student's *t* test was used for statistics. NS: not significantly different

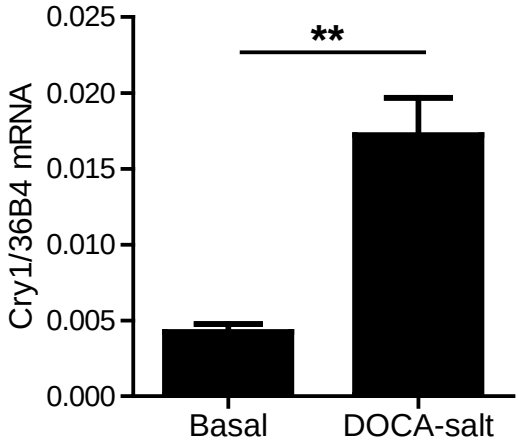

Figure 22: Cry1 mRNA increases with DOCA plus salt treatment

Cry1 mRNA expression in aortas from control mice before and after DOCA plus salt for 7 days (N=5-6). P<0.01 Student's *t* test was used for statistics.

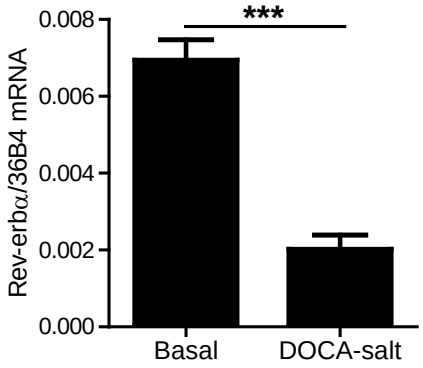

Figure 23: DOCA plus salt treatment negatively affect Rev-erbα mRNA expression

Rev-erbα mRNA expression in aortas from control mice before and after DOCA plus salt for 7 days (N=5-6). Student's *t* test was used for statistics. P<0.001

3.6. Identification of TIMP4 as a new target of Bmal1 in aorta.

To identify the molecular mechanism by which deletion of Bmal1 in smooth muscle upregulates TIMP4 mRNA and protein, we tested the possibility that Bmal1 may directly bind to TIMP4 gene promoter and suppressed its transcription.

First, to determine whether Bmal1, as a transcriptional factor, directly binds TIMP4 promoter, we analyzed the mouse TIMP4 promoter DNA sequence and identified several canonical E-boxes (CANNTG, where N can be any nucleotide) that Bmal1 can potentially bind to (Fig. 24). To determine whether Bmal1 binds to these putative E-boxes, we performed chromatin immunoprecipitation (ChIP) assays in aortas from WT mice. The results showed that Bmal1 bound to the TIMP4 promoter at E-box 2 through E-box 7 except for E-box 5 (Fig. 25).

Second, to investigate whether the binding of Bmal1 to the TIMP4 promoter regulates its activity, we cloned a 2-kb mouse TIMP4 promoter, inserted it into a luciferase reporter vector (pGl3-TIMP4p-luc), and transfected the pGl3-TIMP4P-luc vector into aortic vascular smooth muscle cells isolated from SM-Bmal1$^{-/-}$ and WT littermate mice. In WT cells, the TIMP4 promoter exhibited a 6-fold increase in luciferase activity over the pGL3 luciferase vector (Fig. 26 A). In contrast, when transfected into in Bmal1-deficient cells, the TIMP4 promoter activity was further increased by 9-fold over that in WT cells (Fig.26B), suggesting that TIMP4 transcription is suppressed by Bmal1 in WT cells. The effective deletion of Bmal1 was verified by quantification of its mRNA (Fig. 27).

Third, to investigate whether the observed Bmal1-mediated TIMP4 transcriptional suppression translates to its mRNA suppression, we determined TIMP4 mRNA expression in WT and Bmal1-deficient cells and found that TIMP4 mRNA exhibited a 27-fold increase over that in WT cells (Fig. 28;$P<0.05$), suggesting that Bmal1 suppresses

TIMP4 promoter activity and mRNA expression in cultured vascular smooth muscle cells.

Fourth, to investigate whether Rev-erbA, a negative regulator of Bmal1[222] , is involved in upregulation of TIMP4 by deletion of Bmal1 in smooth muscle in response to DOCA-salt, we determined Rev-erbα mRNA expressions in aorta from SM-Bmal1$^{-/-}$ mice and WT littermates administered with DOCA-salt for 7 days. Deletion of Bmal1 in smooth muscle or administration of mice with DOCA-salt alone had similar potency in suppressing Rev-erbα transcript (Fig.29; $P<0.001$), but combination of both of them further suppressed Rev-erbα transcript (Fig.29; $P<0.05$), suggesting that their inhibitory mechanisms are different. These results also suggest a possibility that Rev-erbα suppresses TIMP4 under physiological conditions and downregulation of Rev-erbα by deletion of Bmal1 in smooth muscle or administration of mice with DOCA-salt abolishes this suppression and results in TIMP4 upregulation. To test whether Rev-erbα binds to the TIMP4 promoter to suppress its transcription, we searched Rev-erbα response elements (AGGTCA) in the 2-kb mouse TIMP4 promoter but we were unable to find one (Z. Guo and M. Gong, unpublished observation), suggesting that it is unlikely that Rev-erbα directly binds to the TIMP4 promoter to suppress its transcription.

Finally, we were intrigued by the fact that Bmal1, generally thought of as a transcriptional activator, suppress TIMP4 transcription. To address this important issue, we determined Cry1 mRNA expression in aorta from in SM-Bmal1$^{-/-}$ mice and WT littermates administered with DOCA and salt for 7 days as it has been shown Cry1 can interact with Bmal1 to switch Bmal1 from a transcriptional activator to a transcriptional repressor[237]. If this is involved in Bmal1 to regulation of TIMP4, the Cry1 transcript should be upregulated. The results supported this possibility (Fig.30).

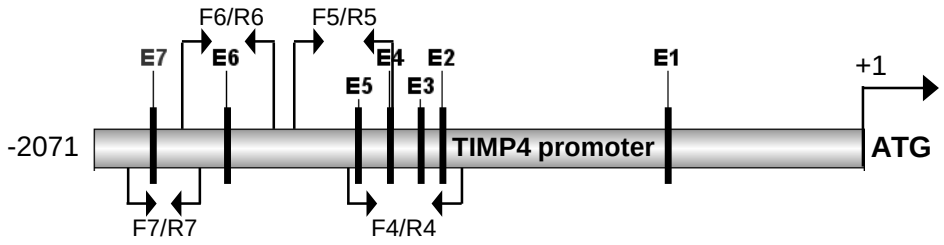

Figure 24: TIMP4 Promoter contains E-boxes

Schematic diagram of 2-kb mouse TIMP*4* promoter showing 7 E-boxes and ChIP-PCR primers. E1-E7: E-box1-7; F (forward)/R (reverse) primers.

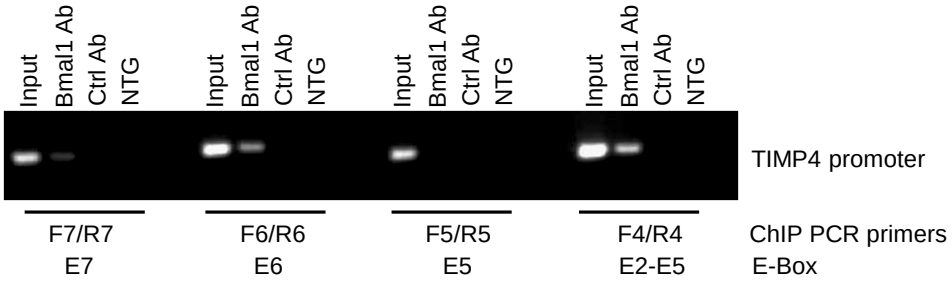

Figure 25: Bmal1 binds to TIMP4 promoter

Representative aortic tissue ChIP PCR showing that Bmal1 binds to TIMP4 promoter at E-box 2, 3, 4, 6, and 7, but not E-box 5.

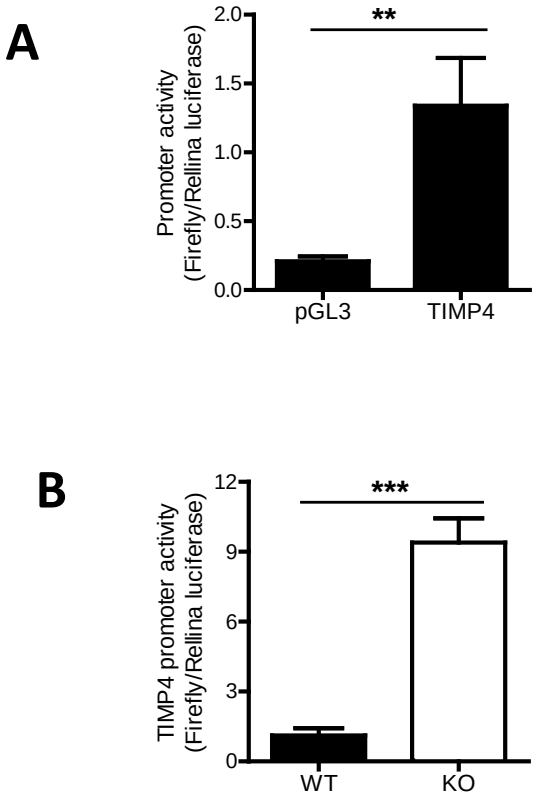

Figure 26: Deletion of Bmal1 increases TIMP4 promoter activity in smooth muscle cells

(A) TIMP4 promoter activity relative to pGL3-basic vector in WT aortic VSMC. (B) TIMP4 promoter activity in aortic VSMC from SM-Bmal1$^{-/-}$ mice and WT littermates. Student's *t* test was used for statistics in A and B. **P<0.01,***P<0.001

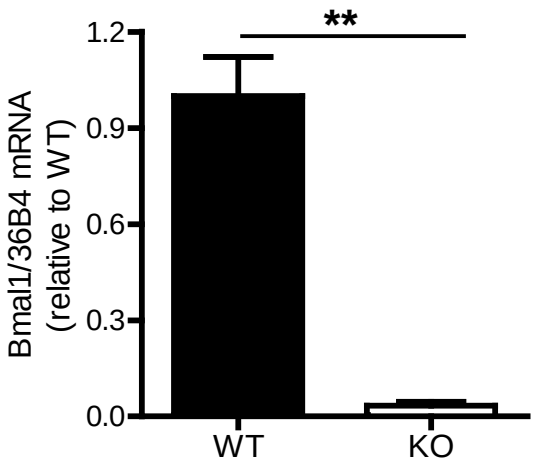

Figure 27: Bmal1 is deleted in Bmal1 KO VSMC

Bmal1 mRNA expression in aortic VSMC from SM-Bmal1$^{-/-}$ mice and WT littermates. (N=3) Student's *t* test was used for statistics **P<0.01

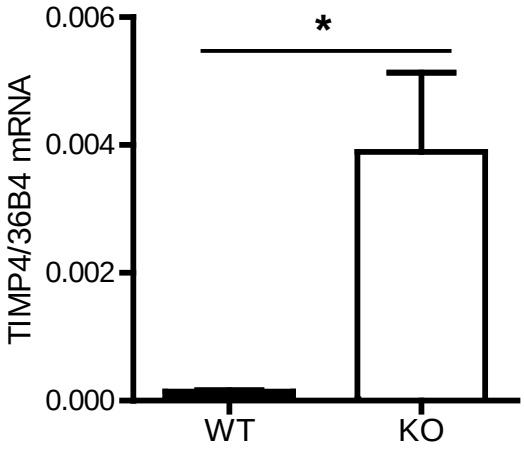

Figure 28: TIMP4 expression is increased in aortic VSMC

TIMP4 mRNA expression in aortic VSMC from SM-Bmal1$^{-/-}$ mice and WT littermates (N=3). Student's *t* test was used for statistics *P<0.05

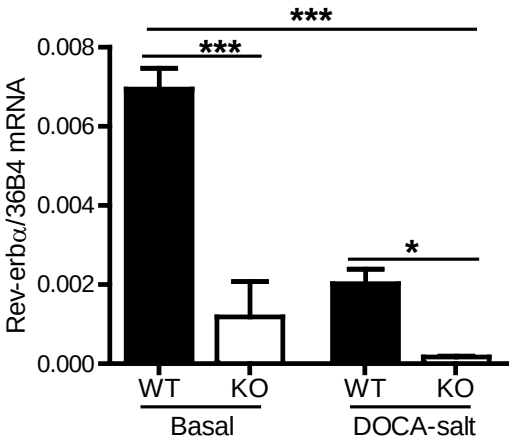

Figure 29: Deletion of Bmal1 in smooth muscle downregulates Rev-erbα mRNA expression in aorta

Rev-erbα mRNA expression in aorta from SM-Bmal1$^{-/-}$ mice and WT littermates before and after DOCA-salt for 7 days. One-way ANOVA followed by Newman-Keuls' post-hoc analysis was used for statistics (N=5-6), *P<0.05,****P*<0.001.

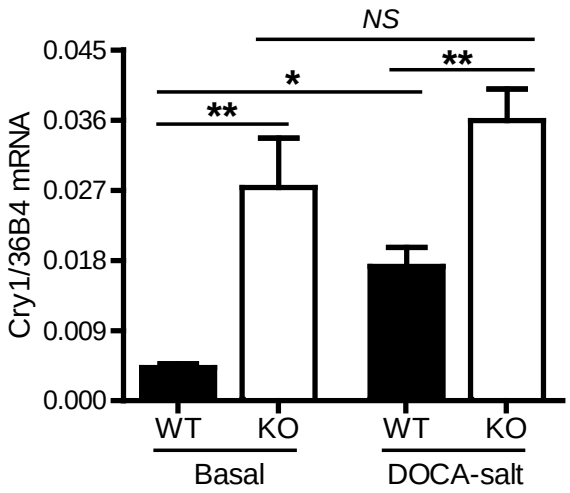

Figure 30: Deletion of Bmal1 in smooth muscle upregulates Cry1 mRNA expression in aorta

Cry1 mRNA expression in aorta from SM-Bmal1$^{-/-}$ mice and WT littermates before and after DOCA-salt for 7 days One-way ANOVA followed by Newman-Keuls' post-hoc analysis was used for statistics (N=5-6) , *P<0.05, **P<0.01, *NS*: not statistically significant

3.7. Deletion Bmal1 in vascular smooth muscle cells protects from Angiotensin II plus salt-induced aortic aneurysm

We have seen that deletion of Bmal1 in smooth muscle showed a dramatic protection from DOCA plus salt induced aortic aneurysm. Moreover, such protective effect was associated with prevention of the massive MMP activation and up-regulation of TIMP4. These results suggest that the up-regulated TIMP4 may inhibit the overall MMP activity to provide the protection. Since the activation of MMP is believed to be responsible for the final degradation of ECM and aortic dilation, a key event can be triggered by divergent up-stream insults. Therefore, we determined whether Bmal1 deletion could provide a broad protection from aortic aneurysm triggered by divergent insults.

To test this possibility, we used a modified model of ANGII induced aortic aneurysm. We administered ANGII for 28 days followed by salt for 14 days in SM-Bmal1$^{-/-}$ and littermate controls. Ultrasound quantification of the intraluminal diameter of the abdominal aorta showed a modest time-dependent dilation after ANGII alone in control mice, this dilation further increased with the presence of high salt. As expected, the abdominal aortic dilation was suppressed in SM-Bmal1$^{-/-}$ (Fig.31 A; $P<0.01$). The maximal external diameter was more pronounced in control mice than SM-Bmal1$^{-/-}$ (Fig. 31 B). The incidence of aortic aneurysm was significantly decrease from 56.2% in control vs 0% in SM-Bmal1$^{-/-}$ ($P<0.01$), and TAAs were decreased from 31.2% in control vs 0% in SM-Bmal1$^{-/-}$ ($P<0.05$). No rupture occurred in SM-Bmal1$^{-/-}$ out of 11 mice while 2 rupture occurred within the control group out of 16 mice (Fig. 31 C). Fig. 32 shows photographs of a normal aorta from the SM-Bmal1$^{-/-}$ mice and a typical aneurysmal aorta from the control mice administered with ANGII and high salt.

We had also examined blood pressure increase in those mice which showed that ANGII alone increases blood pressure in both SM-Bmal1$^{-/-}$and control mice (Fig. 33; $P<0.001$).

However, after ANGII blood pressure increased but remained lower in SM-Bmal1$^{-/-}$ than control mice ($P<0.01$).

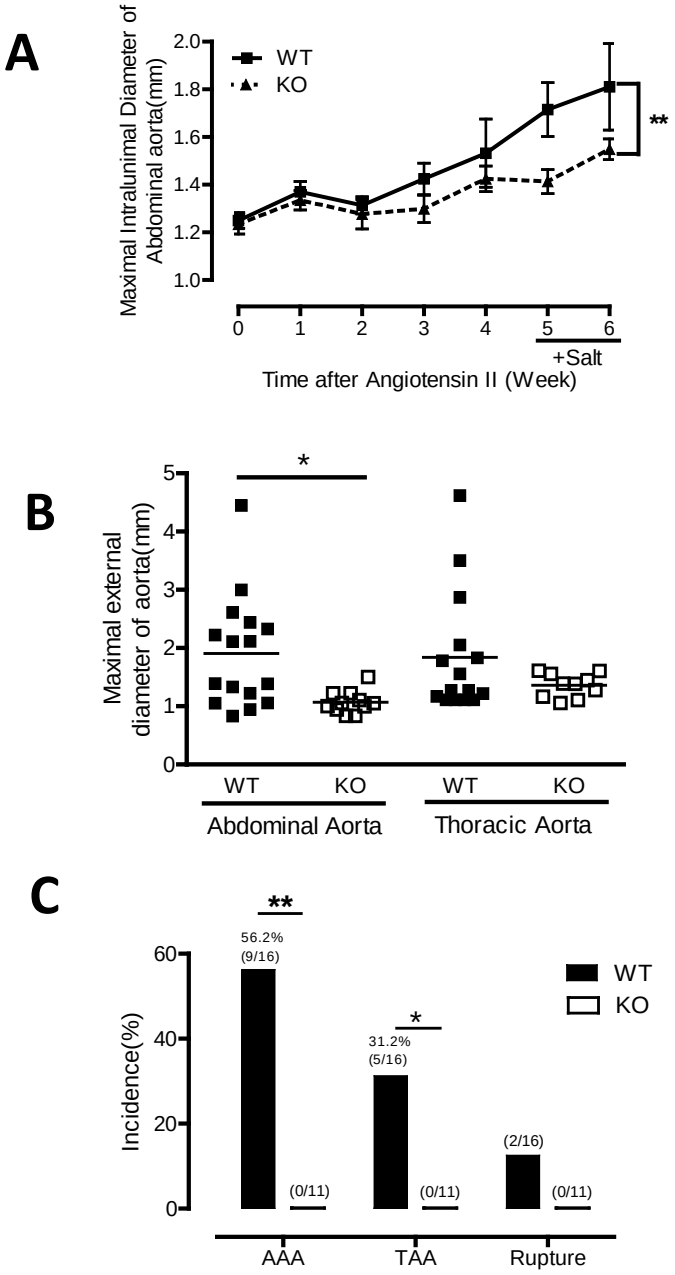

Figure 31: Eight month old SM-Bmal1-/- mice are protected from Angiotensin II followed by salt induced aortic aneurysm

(A) Quantification of inner abdominal aortic diameter by ultrasound in control and SM-Bmal1$^{-/-}$ mice prior to and after ANGII- salt (B) Quantification of abdominal and thoracic aortic outer diameter in control and SM-Bmal1$^{-/-}$ mice after 4 weeks of ANGII followed by 2 weeks salt (C) Incidence of TAA, AAA and aortic rupture after 4 weeks of ANGII followed by 2 weeks salt administration (6 weeks). Two-way ANOVA followed by Bonferroni's post-hoc analysis was used for statistics in A and B. Chi Square was used for comparing AA incidence in D. *: P<0.05, **P<0.01, NS: No significance.

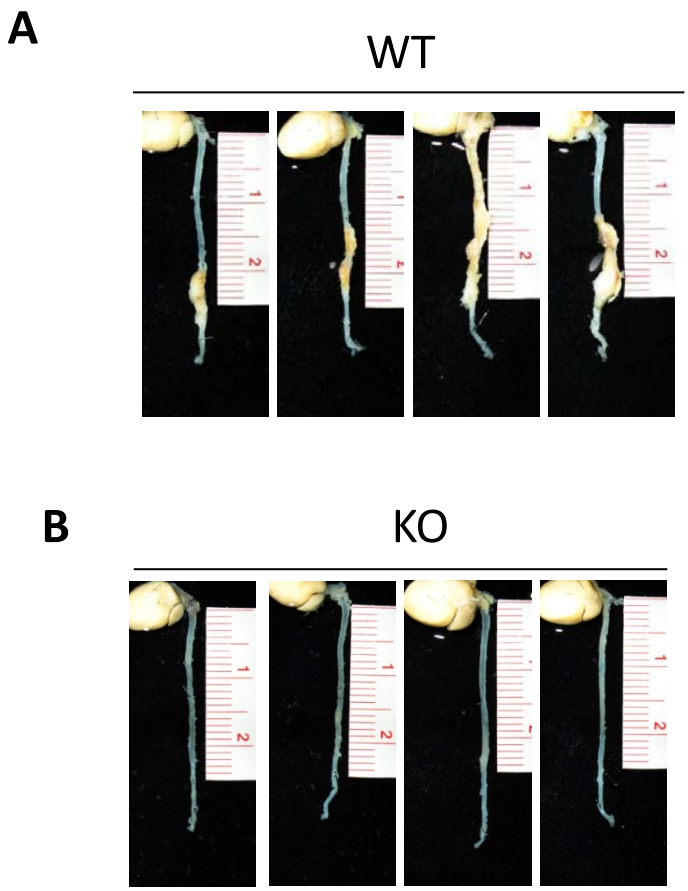

Figure 32: Representative pictures of aortas from 8 month old mice after ANGII

Representative pictures of aortas with connected hearts from 8 month old (A) WT and (B) KO mice after 4 weeks of ANGII followed by 2 weeks of salt

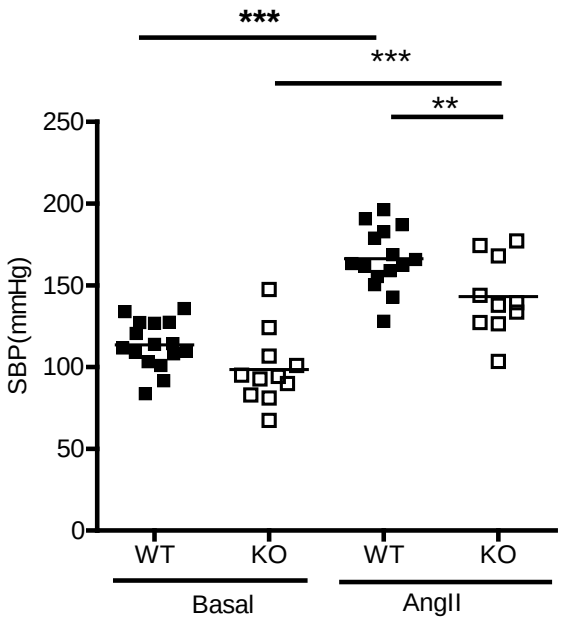

Figure 33: Bmal1 deletion does not affect Angiotensin II induced hypertension

Systolic blood pressure from 8 months old control and SM-Bmal1$^{-/-}$ mice before and after 28 days after ANGII treatment.Two-way ANOVA was used by Bonferroni's post-hoc correction **P<0.01, ***P<0.001.

3.8 SM Bmal1 heterozygous mice are not protected from DOCA plus Salt induced Aortic Aneurysm

We wanted to determine whether both copy of Bmal1 in smooth muscle cells are required for the protection that we have observed in DOCA plus salt induced model of aortic aneurysm and ANGII plus salt induced model of aortic aneurysm. To answer this question, we used 8 month old SM-Bmal1$^{+/-}$ and littermate control mice and administered DOCA plus salt for 21 days. We found that the control mice had a time dependent increase in maximal intraluminal diameter, and the SM-Bmal1$^{+/-}$ mice had a modest but significant decrease (Fig. 34 A; $P<0.05$). The incidence of abdominal aortic aneurysm formation went from 50% in control mice to 35.7% in SM-Bmal1$^{+/-}$ mice and in TAAs from 41.6% to 35.7%. One rupture occurred in the SM-Bmal1$^{+/-}$ out of 14 and 2 occurred in control mice (Fig. 34 B). Although, there is a decrease in maximal intraluminal diameter of the abdominal aorta and a decrease in aortic aneurysm incidence, overall the decrease in incidence did not reach significance. This suggests that to see a complete protection from aortic aneurysm, both copies of Bmal1 have to be deleted in smooth muscle cells. Fig. 35 shows photographs of aortas from the SM-Bmal1$^{+/-}$ and the control mice administered with DOCA and high salt. We had also measured blood pressure which shows no change between SM-Bmal1$^{+/-}$ and the control mice before and after DOCA plus salt (Fig.36).

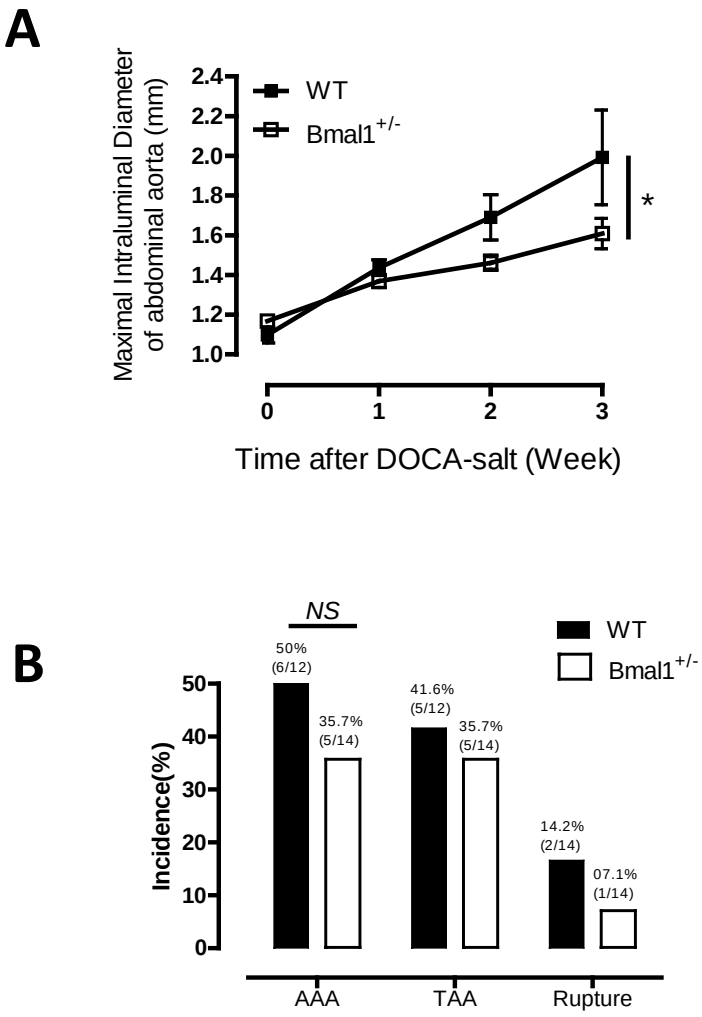

Figure 34: Eight month old SM-Bmal1+/- mice are not protected from DOCA- salt induced aortic aneurysm

(A) Quantification of inner abdominal aortic diameter by ultrasound in control and SM-Bmal1$^{+/-}$ mice prior to and after DOCA- salt (B) Incidence of TAA, AAA and aortic rupture after DOCA plus salt administration (3 weeks). Two-way ANOVA followed by Bonferroni's post-hoc analysis was used for statistics in A. Chi Square was used for comparing AA incidence in B. *: P<0.05, NS: No significance.

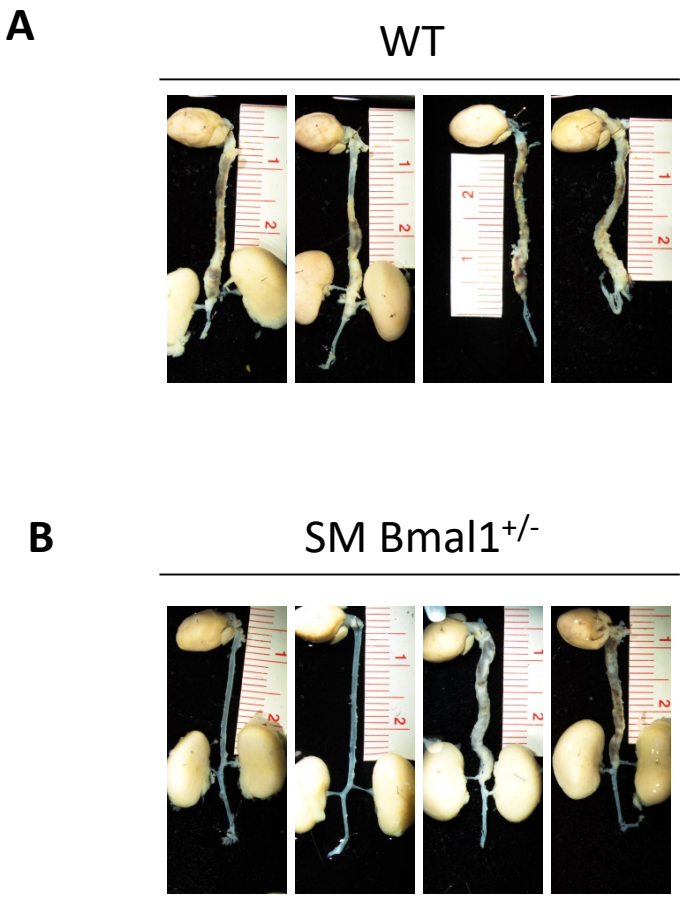

Figure 35: Representative pictures of aortas

Representative pictures of aortas with connected hearts and kidneys from 8 month old (A) WT and (B) SM-Bmal1$^{+/-}$ mice after 3 weeks of DOCA plus salt

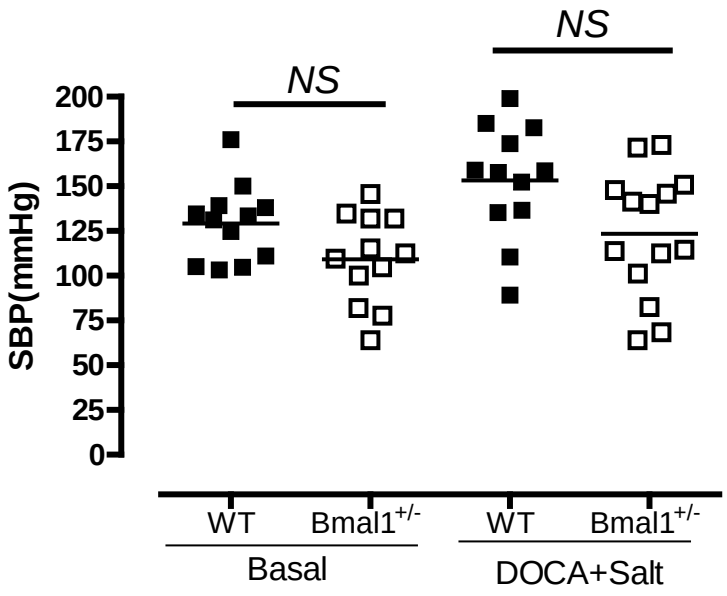

Figure 36: One Copy of Bmal1 does not affect mineralocorticoid receptor agonists induced hypertension

Systolic blood pressure from 8 months old control and SM-Bmal1$^{+/-}$ mice before and after 21days after DOCA plus Salt treatment. Two-way ANOVA was used by Bonferroni's post-hoc correction NS: No significance.

CHAPTER 4

Discussion

4.1. Overview of the study

AAA is a complex multifactorial disease of unknown etiology. AAA is characterized by different features including MMP activation, elastin degradation, inflammation, oxidative stress and smooth muscle cell atrophy. Smooth muscle cells have been considered the epicenter of AAA formation since they modulate early and late events that lead to aortic rupture[238]. Since AAA is so complex and the causative effects are unknown, a direct therapeutic target has proven challenging to develop. Therefore, there is an urgent need to better understand the disease and find a therapeutic target. Disruption of the Bmal1 gene is associated with vascular diseases as well as metabolic diseases. However, the actual role that Bmal1 plays in vascular smooth muscles cells has not been established. We hypothesized that Bmal1 plays a critical role in AAA formation. We deleted Bmal1 in vascular smooth muscle cells and found that this protects from AAA formation. The protection is independent of blood pressure increase. Therefore, we tested other molecular mechanisms and found that Bmal1 deletion increases TIMP4 expression in the vasculature which in turn inhibits MMP2 and MMP9 activities. This inhibition prevents elastin degradation and aortic dilation, protecting against AAA formation. Thus, this study identifies Bmal1 as new key player in the pathogenesis of AAA.

4.2. Bmal1 is ubiquitously expressed and has a specific function in the vasculature

The embryonic global Bmal1 knockout, or conventional, Bmal1 knockout mice have a shorter lifespan and display signs of early aging and age-related pathologies[239]. They also have impaired glucose tolerance, reduced insulin secretion, and are susceptible to diabetes. They have abnormal bone calcification, eye pathologies and

neurodegeneration[240-242]. Finally, they display phenotype such as infertility and increased sensitivity to chemotherapy[243, 244]. Mechanistically, these phenotypes may be caused by an increase in oxidative stress and MMP activities.

Since Bmal1 is expressed in different parts of the brain and in peripheral tissues including smooth muscle cells. Researchers have recently been investigating the role that Bmal1 plays in vascular diseases. Anea et al. have shown that when the carotid arteries of Bmal1 Knockout mice were ligated, Bmal1 knockout mice had higher remodeling than control mice[211]. Other studies have demonstrated that deletion of Bmal1 in mice causes increased vascular superoxide and endothelial NO synthase uncoupling[220]. However, these studies involved the germline global deletion of Bmal1, making it challenging to determine whether these pathologies resulted from Bmal1 function in the vasculature or a systemic disruption from multiple systems. One effective approach to distinguish the tissue specific role of Bmal1 vs. the systemic function of Bmal1 would be tissue specific deletion of Bmal1.

In order to investigate the role that Bmal1 might play in specific tissues, investigators have generated tissue specific Bmal1 knockout mice. Thus far, there have been no reports of early aging and shorter lifespan in these tissue specific knockouts. However, there have been multiple reports that suggest Bmal1 regulates metabolism. Deletion of Bmal1 in skeletal muscle does not lead to loss of locomotor activities as seen in the conventional Bmal1 knockout mice. Relative to WT mice, the skeletal muscle specific Bmal1knockout mice have a normal lifespan, a slight increase in bodyweight, altered muscle glucose metabolism, and muscle insulin resistance[245]. Mice lacking Bmal1 in the liver have normal local insulin sensitivity , normal total body fat content, and hypoglycemia[246]. Mice with Bmal1 deletion in the pancreas have normal bodyweight, fasting hyperglycemia, severe glucose intolerance and diabetes[247]. Mice with a deletion

of Bmal1 in adipose tissue have normal local insulin sensitivity but develop obesity[248]. Mice lacking Bmal1 in the heart have a cardiomyopathy and have early mortality[249]. When smooth muscle cell specific Bmal1 knockout mice were used to determine the role of Bmal1 in blood pressure regulation, Bmal1 in smooth muscle cells was shown to be required for normal amplitude and time-of-day variations of vascular smooth muscle contraction and normal blood pressure circadian rhythms[221]. Thus, these studies suggest that the use of tissue specific Bmal1 abrogates systemic effects of Bmal1 deletion from a prenatal stage. Therefore, in order to determine the role of vascular Bmal1 in AAA pathogenesis, we selected to use smooth muscle specific Bmal1 knockout mice.

4.3. Smooth muscle cells initiate events leading to AAA formation and rupture

In order to avoid the systemic effects of the global Bmal1 knockout mice, we used smooth muscle cell specific Bmal1 knockout mice. Smooth muscle cells are the major cell type in the aorta[250], and they modulate early events of AAA formation[238]. Ailawadi *et al*. had demonstrated that SM22 alpha and smooth muscle alpha actin, which are smooth muscle cell markers, are decreased in the formation of AAA using the elastase model. They had also observed an increase in MMP2 and MMP9 in smooth muscle[238]. Smooth muscle is a source of elastolytic activities, which are thought to be the initial event in AAA formation. Isolated smooth muscle cells from AAA synthesize higher levels of MMP2 and MMP9 than non-aneurysmal tissues[132, 251-253]. Furthermore, they also secrete inhibitors of MMPs. Secretion of TIMP1 from smooth muscle cells has been well described[24]. This increase in metalloproteases by smooth muscle cells and the subsequent degradation of the elastin are part of the initial event of AAA formation. This is followed by an infiltration of inflammatory cells in the vasculature. Lee *et al*. have reported an increase in other metalloproteinases such as MMP1 and MMP3 after smooth muscle cell interaction with monocytes[254] . This increase has been attributed to

an IL-1 dependent mechanism[254]. Increase in inflammatory cytokines such as IL-1 and IL-6 further intensifies the expression of MMPs and their inhibitors[255]. Reactive Oxygen Species (ROS) in smooth muscle cells also play an important role in the AAA formation. ROS also lead to smooth muscle cell apoptosis. Li *et al*. had demonstrated that H_2O_2 is the major species that leads to a depletion of smooth muscle cell population in the aorta[256]. The depletion of smooth muscle cell leads to the expansion and rupture of the aorta. Together, these observations suggest that smooth muscle cells are key players in AAA formation and rupture. Using smooth muscle specific Bmal1 knockout, we demonstrated that deletion of Bmal1 in smooth muscle cells abolishes AAA formation and subsequently we have identified smooth muscle Bmal1 as a new player in the formation of AAA.

4.4. Gelatinases MMP2 and MMP9, and TIMP4 play a role in AAA formation

To gain insight into the protective mechanism from deletion of Bmal1, we focused on MMPs. The degradation of the elastin layer due to an imbalance between MMPs and their endogenous inhibitors is one of the hallmarks of AAA[257]. Our data showed that smooth muscle specific Bmal1 knockout mice are protected from DOCA plus salt induced elastin degradation. Two well characterized elastin degrading enzymes in AAA formation are MMP2 and MMP9[258]. Both MMPs are first secreted as inactive proenzymes and are then activated by other MMPs[259]. MMP2 is predominantly produced locally by smooth muscle cells and adventitial fibroblasts, and to a lesser extent from macrophages, while MMP9 is primarily produced by macrophages[260]. Longo *et al*. investigated the importance of MMP2 and 9 in aortic aneurysm formation using MMP2 Knockout mice and MMP9 knockout mice, and concluded that both MMP2 and MMP9 were necessary for aortic aneurysm formation[59], since inhibition of either MMP2 or MMP9 or both resulted from complete protection from AAA formation. In accordance

with these results, our data demonstrated that MMP2 and MMP9 activities are inhibited in the aorta of smooth muscle cells Bmal1 knockout mice after DOCA plus salt administration. This suggests that MMP inhibition is part of the mechanism by which smooth muscle Bmal1 knockout mice are protected from developing AAA. These results support numerous studies implicating MMP2 and MMP9 as major players in the formation of AAA and rupture.

Under normal physiological conditions, the activities of MMPs are tightly regulated by TIMPs. An imbalance between MMPs and TIMPs favoring MMP activation leads to an increase in vascular remodeling, vascular diseases such as atherosclerosis[261], and AAA formation. This indicates that TIMPs play an important role in aortic aneurysm formation. Among the four TIMPs that have been identified, TIMPs 1, 2, and 3 play an important role in AAA formation. Three polymorphisms in TIMP1 have identified; two of these have been associated with AAA in patients[173]. Mice lacking TIMP1 develop larger aneurysms after elastase infusion than control mice[66], and local overexpression of TIMP1 inhibits elastin degradation, aortic aneurysm formation and rupture in rats. On the other hand, deletion of TIMP2 attenuated aortic aneurysm formation in mice[61] while an overexpression in TIMP2 inhibited aortic aneurysm formation in rats[174]. Whereas the role of TIMP1 is clearly understood as inhibitory for MMPs, the role of TIMP2 remains ambiguous, probably due to the double influence of TIMP2 on MMP2: an increase in TIMP2 activates proMMP2 and an overexpression of TIMP2 inhibits MMP2 activation[175]. Global deletion of TIMP3 in mice causes an increase in aortic aneurysm formation[176].

Our data by in situ zymography clearly showed that MMP activities were inhibited in SMC-Bmal1$^{-/-}$ mice. We had also seen that TIMP4 was the only TIMP upregulated and TIMP4 is a strong inhibitor of gelatinases [179]. Thus, TIMP4 likely inhibited MMP2/9 activity in vivo. Future studies with TIMP4 knockout mice will be necessary to definitively

conclude this. The role of TIMP4 in aortic aneurysm formation has not previously been reported. This study implicates TIMP4 in the protective mechanism of Bmal1deletion in smooth muscle cells against AA formation on the basis of the following observations. In the absence of Bmal1, TIMP4 mRNA and protein increases significantly. TIMP4 is the most abundant among all the TIMPs in the aorta. In response to DOCA or Aldo plus salt, TIMP1, 2, and 4 mRNA do not respond (i.e., increase) to the treatment. DOCA plus salt increases TIMP3 mRNA in vivo. These results are similar with data found in dilated aorta from patients with aortic aneurysm, where the TIMP3 mRNA was the only TIMPs upregulated among the four TIMPs[262], which suggests a compensatory mechanism. TIMP4 is a new target for Bmal1 in the aorta through binding several E-boxes. Within the promoter, TIMP4 does not contain a TATA box, but contains an initiator sequence and relatively few identifiable transcription–factor-binding consensus motifs[263, 264] and we have identified several E-boxes within the promoter. Promoter assays have shown increased TIMP4 promoter activity in the absence of Bmal1 in smooth muscle cells, while the presence of Bmal1 attenuated the promoter activity.

4.5. Hypertension is not a risk factor for AAA formation and does not account for the protection in SMC-Bmal1 Knockout mice

Decreasing blood pressure is unlikely a mechanism underlying the protective effect from aortic aneurysm in SMC-Bmal1$^{-/-}$ mice. Hypertension was widely described as a risk factor for AAA formation. However, recent evidence argues this concept. Using the ANGII model to induce aortic aneurysm, Manning *et. al* had demonstrated that doxycycline, a broad spectrum inhibitor of MMPs, had significantly reduced AAA formation in LDL receptor knockout mice. However, doxycycline had no effect on ANGII induced hypertension[74]. Although vitamin E[75] and 17β estradiol[70] had attenuated AAA formation in APOE knockout mice using ANGII, no difference was observed in systolic

blood pressure with vs. without treatment. DOCA plus salt, a model that has widely been used to induce hypertension, has recently been described as a model that also induced AAA[227]. In this model, losartan and enalapril decreased the systolic blood pressure after DOCA plus salt treatment, but did not alter the rate of aortic aneurysm formation[227]. More studies using genetically deleted genes have supported the evidence that hypertension is not a risk factor for AAA formation. A genetic deletion of uPA[265], osteopontin[266] and BLT[267] in ApoE knockout mice had decrease AAA formation after ANGII infusion, but had no effect on systolic blood pressure.[268] Further investigation in castrated ApoE male mice showed a decrease in AAA formation and no change in systolic blood pressure[268, 269]. The Tsukuba hypertensive mice, which develop AAA after high salt intake, did not differ in systolic blood pressure after high salt intake when compare to the control[57]. Thus in a variety of different experimental settings AAA formation occurs independently of increased blood pressure.

Nevertheless, we determined whether SMC-Bmal1 KO mice had altered blood pressure. Our data showed that the protection we observe in smooth muscle knockout mice is independent of blood pressure increase. Although the knockout mice have a lower basal systolic blood pressure, the blood pressure in response to DOCA plus salt increases in both control and the knockout mice with the same amplitude. Therefore, AAA formation is independent of blood pressure increase and the protection seen in smooth muscle Bmal1 Knockout mice is independent of blood pressure. Indeed, other investigators have demonstrated that lower blood pressure after DOCA plus salt treatment does not protect from AAA formation[227].

4.6. Abdominal aortic aneurysm formation is not a circadian related disease

Our data identified TIMP4, a gene not related to clock genes, as a new target for Bmal1. As a transcription activator, Bmal1 not only regulates the expression of clock genes, but

also regulates the expression of many other genes. Many of the Bmal1 targeted genes are related to metabolism. Through a genome-wide profiling, Hatanaka et al. have found that Bmal1 regulated 10 to 15% of all transcripts including clock genes in different tissues[270]. Therefore, disruption of Bmal1 may result in disruption of regulated genes, leading to different disorders circadian or non-circadian related. Bmal1 has been associated with hypertension[271]. Diseases such as hypertension or stroke are considered to be circadian related diseases, because they disturb a 24 hour pattern and they also have circadian pattern of symptoms. In AAA, the rupture has diurnal variations. Most patients with ruptured AAA are admitted in early morning with a peak between 8:00 am and 10:00 am, the lowest admittance of patients have been between 2:00 pm and 4:00 pm[272]. This pattern had mirrored the circadian rhythm of systolic blood pressure[273]. Here, we see that disruption of Bmal1 in the vasculature leads to protective effects from AAA formation. Since Bmal1 is a core clock gene and regulate other clock genes, diseases associated with Bmal1 could be interpreted as circadian or clock related disease. Although, the rupture of AAA is circadian in pattern, AAA formation will not be considered circadian. In AAA formation, the integrity and structure of the aortic wall is destroyed and is independent of the variation of time. Therefore, AAA formation would not be considered as a circadian related disease.

4.7. Limitations of the study and future directions

By using smooth muscle cell specific Bmal1 knockout mice, we have avoided the systemic effect of the prenatal global deletion of Bmal1 in mice. However, the effect of deleting Bmal1 at the embryonic stage in smooth muscle still remains. Yang et al. have developed an inducible global Bmal1 knockout mouse to overcome the effect of deletion of Bmal1 at the embryonic stage. Although, these mice had a complete loss of rhythmicity in all tissues, they displayed phenotypes that were different from the

conventional Bmal1 knockout mice. These mice had a normal lifespan, normal blood glucose and, though they display some sign of early aging such as ocular abnormalities, they retain fertility, normal body weight, and normal organ size[274]. These mice had additional phenotypes that were opposite to the conventional Bmal1 knockout mice in hair growth and atherogenesis. The inducible Bmal1 knockout mice had increased growing follicles across all ages and a consistent expression of Ccnd1 and Mik67 genes, hair growth promoting genes, in the skin[274]. The difference in phenotypes between the inducible Bmal1 mice and the conventional Bmal1 mice as was suggested by Yang et al. may be attributed to an important role that Bmal1might play during the embryonic stage. Therefore, deletion of Bmal1 during that stage might be the contributing factor to early aging and subsequent phenotypes. Hence, a future goal would be the use of an inducible smooth muscle cell specific Bmal1 to investigate AAA formation.

Our data suggest that the absence of Bmal1 in the vasculature is beneficial since Bmal1 protects from AAA and decreases blood pressure. However, targeting smooth muscle cells Bmal1 for therapeutic treatment would be damaging because blood pressure loses its rhythmicity in such animals[221]. Therefore, the best target may be TIMP4, which our data demonstrated to be downstream of Bmal1 and may contribute to protection from AAA. However, the present study fails to conclusively demonstrate a direct involvement of TIMP4 in aortic aneurysm formation. The other TIMPs 1, 2 and 3 have been demonstrated to play a role in aortic aneurysm formation, but the role of TIMP4 has yet to be adequately explored. The best approach to determine the role that TIMP4 plays in aortic aneurysm formation would be to use TIMP4 knockout mice, TIMP4 transgenic mice, or to locally overexpress TIMP4 on the aorta and administer DOCA plus salt to induce aortic aneurysm. TIMP4 knockout mice have been generated and have been shown to be susceptible to myocardial infraction followed by a right ventricular wall

rupture and death[129]. However, these mice have not yet been available for purchase to the public. Our findings predict that using TIMP4 knockout mice to induce aortic aneurysm, would lead to an increase in rate of AAA formation after DOCA plus salt or Angiotensin II plus salt; while using TIMP4 transgenic mice or local overexpression of TIMP4 would lead to a protection from AAA formation.

We were unable to precisely demonstrate the mechanism by which Bmal1 regulates TIMP4 expression. Multiple lines of evidence show that Bmal1 acts as a transcription factor when bound to an E-box. While we have shown that Bmal1 binds to TIMP4 promoter and the absence of Bmal1 increases TIMP4 promoter activity, we have also shown that deletion of Bmal1selectively increases TIMP4 expression (Fig. 15-19). These data suggest that Bmal1 acts as a break on theTIMP4 promoter. Determining whether or not Bmal1 directly regulates TIMP4 expression has been challenging. One suggested mechanism of regulation was that Rev-erb could act as a repressor. Bmal1 regulates Rev-erb by binding to E-box at the promoter region, and Rev-erb in turn inhibits Bmal1 by binding to a response element. Therefore, we explored the possibility that Rev-erb in the presence of Bmal1 would bind to TIMP4 promoter and repress its expression. However, TIMP4 does not contain a rev-erb response element in its promoter region. Another mechanism envisions the possibility that the interaction between Bmal1 and Cry1, which has been shown to act as a repressor[237], could repress TIMP4 expression. Although, our results have demonstrated an upregulation of Cry1 in the absence of Bmal1, which is consistent with the study from Kondratov et al, more work is needed to confirm that it is the interaction between Bmal1 and Cry1 represses TIMP4 expression.

It will also be important to explore the mechanism for the increase in Bmal1mRNA expression after DOCA plus salt treatment. Aldosterone and angiotensin II have been shown to induce Bmal1 circadian rhythmicity in H9c2 cardiomyoblasts and vascular

smooth muscle cells respectively[214, 275]. Our Data have shown that Bmal1 expression increases in response to DOCA plus salt (Fig. 20), whether this increase is an actual increase, or a shift in rhythmic expression, has not been determined in this study. Bmal1 expression fluctuates during the day and the expression pattern could have shifted after DOCA plus salt treatment. Therefore, to address this question the expression of Bmal1 in the aorta should be examined prior to and after DOCA plus salt/ aldosterone and salt or ANGII plus salt administration in mice at different times of the day. The data could be further tested by looking at Bmal1 expression in VSMC at different times of the day with or without aldosterone and high salt or ANGII plus salt treatment.

One last issue that has not been explored in this study would be to determine whether deletion of Bmal1 in smooth muscle protects from aortic aneurysm across different models of aortic aneurysm. We have shown that smooth muscle cell specific Bmal1 knockout mice are protected from DOCA plus salt induced AAA and from angiotensin II plus salt. The DOCA plus salt model has been used for decades to study hypertension, but it has only been recently shown that it can also induce aortic aneurysm[227]. ANGII plus salt has only been used in our study. Models of aortic aneurysm using angiotensin II have used apoE knockout mice or LDR knockout mice to induced AAA. Therefore, the use of other chemically induced models such as calcium chloride and elastase will determine how broad the protection extends. Both models depend upon different mechanisms but an increase in MMP2 and MMP9 is seen in these models. In this study, we have concluded that the protection is partially due to an increase in TIMP4 expression. Since TIMP4 inhibits both MMP2 and MMP9, we hypothesize that smooth muscle cell Bmal1 knockout mice will be protected or show an attenuation from aortic aneurysm induced by calcium chloride or elastase infusion.

4.8. Conclusions

In this study, we have identified Bmal1 has a key player in the formation of AAA and identified a potential mechanism. We have therefore demonstrated that: 1) Vascular smooth muscle cell deletion of Bmal1 protects from aortic aneurysm formation. 2) Vascular smooth muscle cell deletion of Bmal1 does not affect MR expression before and after DOCA plus salt in the aorta except for the descending aorta. 3) Vascular smooth muscle cell deletion of Bmal1 does not affect plasma sodium. We then explored the mechanism and found the following: 1) Vascular smooth muscle cell deletion of Bmal1 prevents DOCA plus salt induced MMP2/MMP9 increase in vivo. 2) Vascular smooth muscle cell deletion of Bmal1 selectively increases TIMP4 expression. 3) Bmal1 binds to TIMP4 promoter and Bmal1 presence represses TIMP4 expression. 4) The protection seen in this model is independent of blood pressure increase.

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VITA

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Education

2005: High School, Irvington High School

2009: B.S. in Biotechnology, Kean University, NJ

Professional Positions

2006-2009: *Academic Foundation student Assistant*

Exceptional Educational Opportunity Program

Kean University, NJ

2007 : *Scholar*

Summer Medical and Dental Educational program

Howard University, Washington, DC

2008 : *Scholar*

McNAir Summer Research

Kean University, NJ

2008-2009 : *Mathematics tutor*

Kean University, NJ

Spring 2013 : *Facilitator*

PGY206: Elementary Physiology

University of Kentucky, KY

Spring 2014 : *Physiology Tutor*

University of Kentucky, KY

2009-2017 : *Graduate Research Assistant*

Department of Physiology

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Scholastic and Professional honors

2005: Alpha Kappa Alpha scholarship

2006-2009: STEM (Science, Technology, engineering, and Math) scholarship

2007 :- Kaplan Scholarship

-EEO Kaplan scholarship

2007-2008: Dr. Amelia Smith Biotechnology scholarship

2008 : EEO Travel Award

2008-2009: Kean Foundation scholarship

2009 : McNair student of the year

2009-2010: UK Lyman T. Johnson Fellowship

2013 : FASEB MARC travel Award

2011-2013: University of Kentucky T32 Interdisciplinary Cardiovascular Training grant

2014 : Won third place for the Aortic Aneurysm Symposium poster presentation

2014-2016: NIH/NHLBI (Parent F31 – Diversity) Grant#: 5F31HL123315

Publications

2012: Altered Clock Gene Expression and Vascular Smooth Muscle Diurnal Contractile Variations in Type 2 Diabetic db/db Mice. Su W, Xie Z, Guo Z, Duncan MJ, Lutshumba J, Gong MC. AJP Heart and Circulatory Physiology 302(3):H621-33

Abstracts

1. Temperature-tolerance and protein stability assays of Drosophila melanogaster Urielle Marseille Jenny Lutshumba Karl Venescar Karla Bullon David Decicco Michael Enechukwu Rongsun Pu. Abstract for poster presentation for the 67th annual meeting of Society for developmental biology Philadelphia, PA July, 2008

2. Bmal1 deletion from smooth muscle cells protects from Aortic Aneurysm. Jenny Lutshumba, Shu Liu, Ming C. Gong Abstract for poster presentation for Cardiovascular Research day October 2011,

3. Bmal1 deletion from smooth muscle cells protects from Aortic Aneurysm. Jenny Lutshumba, Shu Liu, Ming C. Gong Abstract for oral and poster presentation for Cardiovascular Research day October 2012.

4. A Pivotal Role of Smooth Muscle Bmal1 in DOCA Plus Salt-Induced Mouse Aortic Aneurysm**.** Jenny Lutshumba, Shu Liu, Zhenheng Guo, and Ming C. Gong. Abstract for poster presentation for aortic aneurysm symposium. Lexington, KY August 2014

5. A Pivotal Role of Smooth Muscle Bmal1 in DOCA Plus Salt-Induced Mouse Aortic Aneurysm**.** Jenny Lutshumba, Shu Liu, Zhenheng Guo, and Ming C. Gong. Abstract for poster presentation. ATVB, Nashville, TN. May 2016.

Clubs and Organizations

2012-2014: American Heart Association

2008-2009: McNair Scholar

2006-2009: STEM scholar