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The Role Of Cyclin-Dependent Kinase 8 In Vascular Disease

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THE ROLE OF CYCLIN-DEPENDENT KINASE 8 IN VASCULAR DISEASE

by

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ABSTRACT

In response to injury, mature vascular smooth muscle cells (SMCs) undergo dedifferentiation, also known as phenotype modulation or switch. This process is characterized by a downregulation or loss of expression of contractile genes and concomitant with an increase in cell proliferation, migration, and extracellular matrix production, thereby leading to vascular lesion formation. However, the underlying molecular mechanism is not fully understood. The objective of my study was to determine whether cyclin-dependent kinase 8 (CDK8), a transcription-regulating kinase, plays a mediator role in vascular SMC dedifferentiation and lesion formation. Our results from immunochemical staining and Western blot revealed that CDK8 expression was upregulated in vascular SMCs in mouse injured arteries and human arteries with atherosclerosis. In cultured rat aortic SMCs (RASMCs), the dedifferentiated phenotype exhibiting the downregulation of SMC contractile genes such as smooth muscle 22 alpha (SM22α), calponin-1 (CNN1), and alpha smooth muscle actin (αSMA) as well as increased proliferation, migration, and cytokine production was dramatically suppressed and reversed into a more differentiated state by CDK8 inactivation via highly selective CDK8 inhibitor, Senexin A, and shRNA knockdown approaches. Peri-vascular delivery of CDK8 inhibitor, Senexin A attenuated ligation-induced neointima (NI) formation in mouse carotid arteries. At the molecular level, we uncovered that CDK8 facilitated the activation of AKT to inhibit GSK3β which phosphorylates c-MYC for degradation by

proteasomes, thereby promoting vascular SMC dedifferentiation. In addition, activated CDK8 formed signaling complex with AKT thereby activating AKT in vascular SMCs. In conclusion, our results demonstrate that CDK8 is a critical mediator of vascular SMC dedifferentiation at least partly by facilitating the AKT-mediated inhibition of GSK3β to stabilize c-MYC, thereby contributing to NI formation. Targeting CDK8 may be a novel therapeutic approach for the treatment of occlusive vascular disease due to the abnormal growth of vascular SMCs.

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LIST OF ABBREVIATIONS

AKT ..... Protein kinase B

AMP ..... Adenosine monophosphate

AMPK ..... Adenosine monophosphate-activated protein kinase

BMP ..... Bone morphogenetic factor

CCNC ..... Cyclin C

CDK ..... Cyclin-dependent kinase

CTD ..... Carboxy terminal domain

CYLD ..... Cylindromatosis

DIOI ..... Thyroid hormone receptor human type I deiodinase

DMEM ..... Dulbecco's modified Eagle's medium

E2F1 ..... E2F transcription factor 1

EC ..... Endothelial cell

ECM ..... Extracellular matrix

ERK ..... Extracellular signal-activated kinase

FBS ..... Fetal Bovine serum

GAPDH ..... Gylceraldehyde-3-phosphate dehydrogenase

GFP ..... Green fluorescent protein

GSK3β ..... Glycogen synthase kinase-3 beta

HATs ..... Histone acetyltransferases

HDACs ..... Histone deacetylases

IB.....Immunoblotting

ICAM-1..... Intercellular adhesion molecule

IFN-γ..... Interferon-gamma

IP.....Immunoprecipitation

JNK..... c-Jun N-terminal kinase

KLF4.....Krüppel-like factor 4

MAPK.....Mitogen activated protein kinase

Mcp-1..... Monocyte chemotactic protein-1

MED.....Mediator

Mmp2.....Matrix metalloproteinase-2

Msn2..... Multistress response transcriptional factor

mTOR..... Rapamycin

MYH11.....Smooth muscle cell myosin heavy chain

NF-κB..... Nuclear factor kappa B

NI.....Neointimal formation

NO..... Nitric oxide

OPN.....Osteopontin

OxLDL.....Oxidized lipoproteins

p27.....Cyclin-dependent kinase inhibitor 1B ($p27^{kip1}$)

p38.....p38 MAPK

PBS.....Phosphate-buffered saline

PDGF.....Platelet-derived growth factor

PDK-1..... 3-phosphoinositide-dependent protein kinase-1

P-TEFb ..... Transcription elongation factor

RASMC ..... Rat aortic smooth muscle cell

RGM ..... Regular growth medium

RNAPII ..... RNA polymerase II

SD ..... Sprague Dawley

Sen A ..... Senexin A

Ser ..... Serine

SF ..... Serum-free

SM22α ..... Smooth muscle 22 alpha

SMC ..... Smooth muscle cell

SRF ..... Serum response factor

SSh ..... Sonic hedgehog

STAT1 ..... Signal transducer and activator of transcription 1

Ste2 ..... Pheromone alpha factor receptor

TF ..... General transcription initiation factor

TGF-β ..... Transforming growth factor beta

Thr ..... Threonine

TNFα ..... Tumor necrosis factor alpha

VSC ..... Vascular stem cell

VSMC ..... Vascular smooth muscle cell

αSMA ..... Alpha smooth muscle actin

β-actin ..... Beta actin

CHAPTER 1

THE HISTORY OF VASCULAR DISEASE AND CYCLIN-DEPENDENT KINASE 8 FUNCTION

1.1 VASCULAR DISEASE:

Vascular disease is classified as an abnormality associated with the vascular structure and function of blood vessels. There are several types of vascular diseases such as atherosclerosis, restenosis, hypertension, and aneurysm. Although their fate differs, these vascular diseases share are common pathological process, which are vascular remodeling and the development of neointimal formation. This eventually results in the occlusion of the blood vessel, which can lead to several complications. Vascular disease is the world's number one killer, accounting for 17.5 million deaths globally.[1,2] The exact cause of the disease is unknown, however, scientist and clinicians have identified factors that can raise the risk for vascular disease. The major risk factors include unhealthy blood cholesterol level, high blood pressure, smoking, insulin resistance, diabetes, obesity, and physical inactivity. Age and family history have also been found to potentially influence the onset of the disease.

Often, there are no signs or symptoms of vascular disease until the artery is severely narrowed or occluded, by which time can cause heart attack or stroke. The signs and symptoms vary depending on the artery affected, but can range from weakness, paralysis, apnea, unconscious, and overall loss of cognitive function. Doctors diagnose atherosclerosis by a physical examination, diagnostic tests, and/or family and medical

history. Currently, there is no known cure for vascular disease, however there are a number of medications that can be used to slow the progression of the disease. Such medications include cholesterol medications, beta-blockers, angiotensin-converting enzyme inhibitors, calcium channel blockers, and triamterene/hydrochlorothiazide (water pills). In severe cases, the doctor may recommend surgical procedures such as angioplasty, stent placement, or bypass surgery. However, these procedures are not a permanent solution due to the re-narrowing on the artery, known as restenosis, and often require a repeat procedure. Given the inadequacy of current medications and the complications involving surgery, an extensive amount of research has been conducted to develop new therapies for the treatment of vascular disease.

1.2 CELLULAR MECHANISMS OF VASCULAR DISEASE:

The structure and cellular composition of blood vessels are consistent throughout the vascular system. However, there are certain features in the vasculature that vary and reflect distinct functional requirements depending on their location. The basic cellular components of a blood vessel consist of endothelial cells (EC), vascular smooth muscle cells (VSMC), and extracellular matrix (ECM)—which are comprised of collagen, elastin, and proteoglycans.

The vascular system is organized into three concentric layers: the tunica intima, tunica media, and tunica adventitia, and is most distinctive in large vessels such as arteries. The tunica intima is the innermost layer and comprised of ECs lining the lumen of the vessel (known as the endothelium), a subendothelial layer that consist of predominately connective tissue, and the internal elastic lamina, which separates the

intima from the media layer. The tunica media, the middle layer, consist predominately of VSMCs and external elastic lamina. VSMCs are responsible for vasoconstriction and dilation of the blood vessel. The outermost layer, known as the tunica adventitia, predominately consists of loose connective tissue, which is made up of fibroblasts and collagen fibers, and small blood vessels (vasa vasorum).

Vascular disease involves the activation of many cell types including endothelial cells, vascular smooth muscle cells (VSMCs), macrophages, mast cells, lymphocytes, and neutrophils.[3] Endothelial dysfunction is a primary event in vascular disease. Endothelial cells are activated due to metabolic risk factors, thereby causing blood monocytes to permeate the endothelial cell layer and infiltrate the intima and subintima layer of the artery. During this process monocytes mature into macrophages resulting in their uptake of lipids, yielding foam cells. VSMCs migrate from the media to the intima, proliferate, secrete extracellular matrix (ECM), resulting a plaque formation. Necrosis of lipid-containing foam cells and VSMCs result in a necrotic core formation. Dead and dying cells accumulate in the region creating a fibrous cap, predominately composed of smooth muscle cells and macrophages. The last step, thrombosis, involves the rupture of the fibrous cap. Highly thrombogenic components of the necrotic core and tissue factors come into contact with circulating monocytes in the blood, initiating a thrombus in the vessel.

VSMCs exhibit a diverse range of phenotypes. In normal physiological conditions, VSMCs are maintained in a quiescent "contractile" or differentiated phenotypic state, which is necessary for contraction and dilation of the blood vessel. During this quiescent state, VSMCs express a repertoire of VSMC markers, including

smooth muscle cell myosin heavy chain (MYH11), calponin 1 (CNN1), smooth muscle 22 alpha (SM22α), and alpha smooth muscle actin (αSMA). These established VSMC differentiation markers contain a $CC(A/T\text{-rich})_6GG$ (CArG) element and a transforming growth factor β (TGF-β) control element within the promoter/enhancer regions. However, under pathological conditions, VSMCs will undergo a phenotypic transition from quiescent state to a proliferative, non-contractile state or "synthetic" state. These synthetic VSMCs will lose their expression of VSMCs markers, known as dedifferentiation, and exhibit high rates of proliferation, migration and production of ECM proteins and cytokines. This transition is known as a phenotypic modulation or plasticity, and first originated in the 1960s from an ultrastructural characterization of VSMC culture.[4] In their synthetic state, VSMCs can migrate to the intima and proliferate following vascular injury. The phenotypic modulation of VSMCs is a innate property of differentiated VSMCs, which presumably evolved from higher species as an adaptation for survival. However, an exorbitant degree of VSMC plasticity can result in adverse vascular remodeling and disease. The synthetic phenotype of VSMCs, increased proliferation, and increased migration and ECM secretion are key elements in lesion formation. As such, a fine-tuning of VSMC phenotypic transition is necessary to regulate the development and progression of vascular disease.

It is proposed that the dysfunction of vascular stem cells (VSCs) is essential in the pathogenesis of vascular disease. An active state of adult VSCs may be an indication of maladaptive vascular remodeling caused by the loss of quiescence and consequent activation of VSCs. Therefore, a quiescent status of adult resident VSCs may be critical for the repair of a damaged vasculature.[5] The current understanding of adult resident

VSCs is somewhat rudimentary. Adult resident VSCs are believed to originate from embryogenesis. These cells are necessary for vascular development and remain within the vasculature after birth for post-natal growth, aging and disease.[6] These cells remain quiescent until activated by vascular injury or disease. Given their close proximity, they are quickly recruited to the site of injury for self-renewal and differentiation.[7] These stem/progenitor cells have very distinct phenotypes and appear in different compartments in the vessel wall – the intima, media and adventitia. The characterization of these adult resident VSCs remains unclear, due to the discrepancies in multiple reports. However, what is clear is that these adult resident VSCs have the potential to differentiate to SMCs, ECs, and pericytes. This is evident by their upregulation lineage specific cell markers after stimulation with various growth factors and chemokines. In addition, these VSCs have adipogenic, chondrogenic, osteogenic, leiomyogenic and angiogenic potential. Although there are currently no established markers, Sca-1 is a primary marker used to identify adult resident VSCs in the murine vasculature. Multiple reports also indicate that these resident VSCs can contribute to vascular lesion formation rather than repair. Many aspects regarding resident VSCs and progenitor cells remain to be addressed, such as their profile, location, method of isolation, culture conditions, and animal injury models, as well as the mechanisms that control their differentiation and function. A full evaluation of adult resident VSC contribution to vascular remodeling cannot be performed without considering all of these factors. Most importantly, addressing these aspects may connect with the discrepancies in the multiple reports. Further investigation of molecular mechanisms for controlling the functional states of resident VSCs may unravel the nature

of resident VSCs per se as well as the precise contributions of VSC-mediated vascular lesion formation and repair.

1.3 MOLECULAR MECHANISMS REGULATING VASCULAR SMOOTH MUSCLE CELL PLASTICITY:

The phenotype modulation or plasticity of VSMCs plays a crucial role in the development of vascular diseases. Therefore, the elucidation of the molecular mechanisms regulating VSMC phenotypic modulation could provide some insight into the development of vascular diseases. The phenotypic switching of VSMCs in vascular disease is regulated by a multitude of environmental cues/signals, which will be discussed in three categories: 1. Epigenetic mechanisms, 2. Genetic mechanisms, 3. Protein quality control.

1. EPIGENETIC MECHNANISMS

   A preceding level of regulation in SMC gene expression must occur to account for the array of genes involved in the regulation of VSMC phenotype modulation, rather than solely one gene. Transcriptional gene activation is controlled by chromatin remodeling and histone modifications. This hierarchal level of gene activation provides an additional layer of transcriptional control, independent of RNA or DNA sequence, and is referred to as epigenetic mechanism of gene regulation. An extensive amount of research has been conducted to determine the epigenetic mechanism of VSMC phenotypic modulation in vascular disease.

Histones are a group of proteins that package DNA in nucleosomes. The histone complex is an octamer which is assembled by two sets of protein-protein interactions: H2A, H2B, H3, and H4. The acetylation of histones is a major epigenetic mechanism in gene regulation. It involves the addition or removal of acetyl groups from lysine residues on the histone N-terminal tails and is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. HATs add acetyl groups to histones, which promote chromatin unfolding and make DNA more accessible for transcriptional gene activation. HDACs reserve these effects by removal of acetyl groups from histones.

It was reported that HDAC transcription is increased in response to mitogenic stimulation of VSMCs and is required for proliferation by regulating G_1-S phase cell cycle progression. HDAC inhibition induced cell cycle arrest by preventing mitogen-induced retinoblastoma protein phosphorylation, and also repressing mitogen-induced cyclin D1 mRNA expression and cyclin D1 transcription. Furthermore, HDAC inhibition reduced neointimal formation and cyclin D1 expression following vascular injury.[8]

It has also been reported that acetylation at H3 and H4 residues are enriched at SMC gene CArG boxes, and display high levels of H3 Lys4 and Lys79 dimethylation (H3K4dMe and H3K79dMe, respectively) in SMCs relative to non-SMC lineages.[9] In conclusion, epigenetic modifications at the CArG region represent an important site for the phenotypic modulation of VSMCs.

2. Genetic Mechanisms

Serum response factor (SRF), a MADS box transcription factor, and its co-activator myocardin bind to CArG boxes to initiate transcription of VSMC genes in response to pathological environmental cues in culture and in vivo. It was reported that myocardin interacted with H3K4dMe and selectively enhanced SRF binding to CArG box chromatin of SMC genes, resulting in the upregulation of SMC differentiation markers.[9] Therefore, the control of SRF/myocardin binding to CArG boxes represents a major mechanism in the phenotypic modulation of VSMCs in vascular remodeling.

Krüppel-like factor 4 (KLF4) is a transcriptional repressor that has also been found to play a role in the regulation of VSMC phenotype. KLF4 also acts as a potent repressor of VSMC phenotype modulation by binding to the transforming growth factor beta (TGF-β) control element within the promoter/enhancer regions of VSMC differentiation markers.[10] Furthermore, it interacts with SRF and represses the expression of SRF co-activator myocardin and disrupt acetylation of histone H4.[9,11,12]

Platelet-derived growth factor (PDGF) is a potent chemoattractant released by activated platelets and many cell types including, ECs, macrophages, and VSMCs from neointimal lesions. PDGF was shown to be a negative regulator of VSMC markers, in vitro, and also stimulates VSMC proliferation and migration by attenuating SRF/myocardin binding to VSMC CArG boxes. It was demonstrated that KLF-4 is induced after PDGF-BB exposure. Also, suppression of KLF4 by small interfering approach partially blocked PDGF-BB-induced SMC gene repression. It was also determined that morphogen Sonic hedgehog (SSh)

mediates PDGF-induced VSMC phenotypic modulation in cultured VSMCs through regulation of KLF4.[13] Taken together, these results suggest that KLF4 might act as key effector in PDGF-BB-induced repression of VSMC differentiation markers.

Current studies are now aimed at determining the role of KLF4 in VSMC phenotype modulation, in vivo. KLF4, which is not normally expressed in differentiated SMCs, was rapidly upregulated in vivo in response to vascular injury. This was the first evidence suggesting of KLF4 involvement in vascular remodeling.[12] Since KLF4 knockout mice result in early post-natal death[14], SMC-targeted conditional KLF4 knockout mice were developed to determine the role of KLF4 in vivo. It was shown that VSMC-specific conditional knockout of KLF4 markedly reduced lesion size, and increased fibrous cap thickness thereby causing plaque stability.[10] Therefore, the KLF4-dependent SMC phenotypic modulation may be of critical importance the pathogenesis of vascular disease.

The deubiquitinating enzyme cylindromatosis (CYLD) is a critical regulator of many cellular processes, via the regulation of nuclear factor kappa B (NF-κB)-mediated inflammation. Our lab was able to demonstrate a novel role of CYLD in mediating pro-inflammatory responses in VSMCs, via a NF-κB-independent manner. Knockdown of CYLD, by adenoviral approach, decreased tumor necrosis factor alpha (TNFα)-induced pro-inflammatory cytokines monocyte chemotactic protein-1 (Mcp-1), intercellular adhesion molecule (ICAM-1) and interleukin-6 (IL-6) in rat aortic smooth muscle cells (RASMCs). Furthermore, CYLD knockdown also suppressed TNFα-induced activation of

mitogen activated protein kinases (MAPKs), extracellular signal-activated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38. Interestingly, CYLD expression was increased in injured coronary artery afflicted with neointimal hyperplasia.[15] Overall, these results suggest a pro-inflammatory role of CYLD in VSMCs via MAPK activation and NF-κB-independent activation, thereby contributing to the pathogenesis of vascular disease.

3. Protein Quality Control

Apoptosis is a process of programmed cell death that occurs during normal development. Several environmental and endogenous factors have been identified in the apoptosis of VSMCs in the development of vascular disease. These factors include reactive oxygen and nitrogen species, cytokines, growth factors, and oxidized lipoproteins. Other factors that play a role in the initiation and progression of VSMC apoptosis include the Fas/Fas ligand/caspase death-signaling pathway, Bcl-2 protein family/mitochondria, the tumor suppressive gene p53, and the proto-oncogene c-Myc.

The Fas/Fas ligand/caspase death-signaling pathway plays an important role in the in the induction of apoptosis in neointimal lesions. Both Fas/Fas ligands have been shown to be expressed in neointimal VSMCs. Notably, overexpression of Fas ligand, by adenovirus approach, was shown to inhibit neointimal formation following balloon injury.[16]

The infiltration of immune cells within neointimal lesions also play a role in the apoptosis of VSMCs. Immune cells, such as macrophages and T lymphocytes, can produce inflammatory cytokines such as TNF and interferon-

gamma (IFN-γ), which can secrete nitric oxide (NO), thereby inducing apoptosis of VSMCs in cell culture. NO can attack iron-containing enzymes involved in DNA synthesis and mitochondrial respiration, thereby leading to apoptosis of target cells, such as VSMCs.[17] It was demonstrated that macrophage-induced NO can activate VSMC apoptosis via Fas ligand pathway. Human plaque derived VSMCS underwent apoptosis when co-cultured with monocytes/macrophages. However, this effect was inhibited by neutralizing antibodies to Fas ligand.[18] In addition, NO inhibitor blocked NO production by macrophages and reduced VSMC apoptosis in response to macrophage induced NO.[19]

Tumor suppressive gene p53, and the proto-oncogene c-Myc were also shown to be involved in VSMC apoptosis. Adenovirus-mediated transfer of p53 induced apoptosis which resulted in atherosclerotic plaque rupture.[20] Fas itself has also been shown to be mediated by p53. In addition, NO-induced apoptosis of VSMCs include downstream mediators p53[21] and Fas.[22] It was also shown that deregulated expression of c-Myc oncogene abolishes inhibition of proliferation of VSMCs and induces apoptosis with various stimuli [23] Moreover, overexpression of p53 induces apoptosis in rat VSMCs infected with c-MYC but not normal cells [24]

Oxidized lipoproteins (OxLDL) can also induce VSMC apoptosis in neointimal lesions thereby contributing to plaque instability and rupture. OxLDL was localized to neointimal lesions as well as medial-derived VSMCs that coexpress proapoptotic protein Bax.[25] Consistent with this study, high OxLDL can induce apoptosis of VSMCs via the Bax/Bcl-2 and lectin-like OxLDL

receptor-1 in VSMCs.[26] As such, the molecular mechanisms causing OxLDL-induced apoptosis may be involved in the destabilization and rupture of atherosclerotic plaques.

Autophagy is another important physiological process, which results in the destruction of cells by lysosomal compartments. While autophagy is necessary for normal vascular function, it has also been found to play a role in vascular disease, due to the dysfunction of SMCs.[27] PDGF induces autophagy in VSMCS independent of AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR). PDGF-induced autophagy also promoted the downregulation of VSMC differentiation markers resulting in a synthetic phenotype, and increased the proliferation and migration of VSMCs.[28] SSh, which has already been identified as a mediator in PDGF-induced VSMC differentiation, also induced autophagy via protein kinase B (AKT) activation. Notably, treatment with autophagy inhibitor 3-methyladenine, AKT inhibitor, or Shh receptor cyclopamine significantly reduced neointimal formation in mouse carotid arteries after ligation.[29] Taken together, these results suggest a critical role of autophagy in the pathogenesis of vascular disease due to the phenotype modulation of VSMCs.

1.4 CYCLIN-DEPENDENT KINASE 8:

Cyclin-dependent kinases (CDKs) are traditionally characterized by their role in cell cycle progression; however, there also exist a set of CDKs that are directly involved in regulation of RNA polymerase II (RNAPII). These CDKs are known as transcriptional

kinases. CDK8 exist within the family of transcriptional kinases. CDK8 is a part of the 30 subunit Mediator (MED) complex, which acts as a molecular bridge between DNA-binding transcription factors and RNAPII. The MED consists of the head, middle, tail, and the CDK8 submodule. The head is composed of subunits MED6, MED8, 11, 17-20, 22, 28, the middle subunits are MED1, 4, 7, 9, 10, 14, 21, 26, 3/27, 31, the tail subunits are MED15, 16, 23-25, 2/29. CDK8 consist within the CDK submodule consisting of CDK8 (or its paralog CDK19), cyclin C, MED12 and MED13. It is well established that MED is important for the pre-initiation complex since it interacts with RNAPII, thereby influencing the transcription initiation process.

The MED complex also joins to transcription factors bound at upstream regulatory elements such as nuclear receptors, and transcription machinery (RNAPII, the general initiation factor TFIIA, -IIB, IID, IIE, IIF, and –IIH, DNA-binding transcription factors) and transcription coactivators, at the promoter region, thereby controlling epigenetic regulation, transcriptional initiation and elongation, transcriptional termination, mRNA processing, noncoding RNA activation, and the chromatin remodeling.[30,31] Given its plethora of functions, the MED complex can be seen as a mass coordinator of development and cell lineage determination. Several components of the MED complex have been shown to cause embryonic lethality, such as MED1, MED12, MED21, MED23, MED24, MED31 and CDK8.[31] As such, gene knockdown approaches of MED subunits have been employed to investigate MED deficiencies. Knockdown mice or targeted inactivation of other subunits, such as MED1, MED12, MED14, MED19, MED25, MED26 and MED28 and CDK8, indicated that these subunits play an essential role in specific gene expression programs during differentiation and

organogenesis. These subunits are also associated with cardiovascular diseases. Specific MED subunits (MED1, MED12, MED13/13L, MED14, MED 15, MED23, MED25, MED30, and CDK8) have been shown to be associated with congenital malformations, such as congenital heart diseases.[31] However, the precise role and interplay of Mediator components and modules in cardiovascular pathologies still remain unclear. More recently CDK8 has been shown to be a key component in the regulation of the MED complex, making it a topic of interest.

CDK8 is a nuclear serine-threonine kinase that can regulate transcription. The majority of evidence regarding CDK8 is based on its association with MED complex; however, only a fraction of CDK8 exists outside of this complex. It has been proposed that up to 30% of CDK8 can exist and function independently of the MED complex. Biochemical analysis of recombinant CDK8 identified RNAPII and TFIIH, as well as histone H3, MED13, and CDK8 as substrates for the CDK8 kinase. It was also revealed that MED12, but not MED13, is essential for human CDK8 kinase activity.[32,33] Furthermore, mass spectrometry analysis revealed CDK8 to be associated with DNA-PK, GCN1L1 and TRiC chaperonin, which may help in controlling its biological function.[32] However, the biological significance of DNA-PK, GCN1L1, and TRiC association requires further elucidation.

Traditionally, CDK8 has been depicted as transcriptional repressor, however much of this knowledge has been limited to research in yeast and metazoan cells. More recent evidence demonstrates that CDK8 is also positive regulator in transcription. Moreover, CDK8 has been shown to be a coactivator in many molecular pathways linked to stem cells and cancer. These pathways include the β-catenin pathway, the p53

pathway, the serum response network, the TGF-β signaling pathway, and thyroid hormone-dependent transcription. Given its dual role in transcription, as a positive and negative regulator, CDK8 can be deemed as a master regulator of transcription.

There are several reports of CDK8 as a negative regulator of transcription. The most initial evidence of CDK8 as a co-repressor was shown in yeast. SRB10, the CDK8 homolog in yeast, was shown to phosphorylate the RNAPII CTD at Ser2 and Ser5 prior to PIC assembly, thereby, inhibiting transcription.[34] However, this has not been confirmed in vivo and the mechanism remains to be elucidated.

SRB10 was also shown to inhibit transcription by promoting nuclear export or degradation of specific transcriptional activators, such as Gcn4, the multistress response transcriptional factor Msn2, and Ste2.[35,36] SRB10 can phosphorylate Gcn4 marking it for degradation by SCF^{Cdc4} for ubiquitin ligase. Msn2 is also a substrate for SRB10 and is translocated to the nucleus by heat-induced stress via hyper-phosphorylation in an SRB10-dependent manner.[35] Another substrate, Ste2, promotes the filamentous growth of budding yeast *Saccharomyces cervisiae* in nutrient limiting conditions. SRB10 inhibits filamentous growth via phosphorylation of Ste2, thereby decreasing its stability. Furthermore, nitrogen limiting conditions resulted in loss of SRB10 protein, as well as kinase activity, and corresponding loss of Ste2 phosphorylation.[36] These results conclude that SRB10 can employ a variety of regulatory mechanisms to coordinate gene expression with growth potential.

Microarray results in yeast revealed that while most MED subunits play a positive role through activator-mediated recruitment and interaction with RNAPII, the CDK-module is predominately a negative regulator of transcription.[37] These results reinforce

this traditional view of CDK8 as an antagonist of transcription, at least in lesser organism such as yeast.

In higher organisms, it was also demonstrated that human CDK8 can inhibit transcription via phosphorylation and inactivation of the cyclin H subunit of TFIIH.[38] Specifically, CDK8 phosphorylation of TFIIH phosphorylates mammalian cyclin H at the amino-terminal and carboxy-terminal alpha-helical domains. This phosphorylation repressed TFIIH to activate transcription and its CTD kinase activity.[38]

In addition, co-activation assays in vitro, demonstrated that human CDK8-MED is less active than the core MED complex, and recombinant CDK module can block the core MED co-activator function.[39–41] However, it's inhibition was independent of CDK8 activation, most likely due to MED12 and MED13 components.[41] Furthermore, compensatory mechanisms for gene activation involved less CDK8-Mediator and more core-Mediator for MAPK-dependent activation of the transcription factor C/EBPβ, and retinoic acid-induced activation of the RARβ2 gene.[42,43]

A previous study by Zhao *et al* reported that CDK8 and cyclin C are negative regulators of lipogenesis in Drosophila and mammalian cells. CDK8 phosphorylation of SREB-1c transcription factor at threonine residue (Thr) resulted in an increase in SREBP-1c ubiquitination and protein turnover, thereby leading to inhibition of lipogenesis.[44]

While several reports indicate SRB10/CDK8 as a negative regulator in yeast, it is also seen as a positive transcriptional regulator in yeast. For example, genome-wide chromatin immunoprecipitation (ChIP) studies demonstrated that CDK8 was present in active and inactivated genes, thus suggesting that CDK8 can promote transcription.[45] It was also shown that SRB10 phosphorylation of GAL4 at serine 699, is necessary for

galactose-inducible transcription, and this transcription occurs concurrently with the transactivation of general initiation factors.[46] Furthermore, yeast CDK7 homolog, KIN28, and SRB10 work together to promote RNAPII transcription and scaffold complex formation by ATP-dependent dissociation of the PIC.[47] This study also demonstrated SRB10 phosphorylates two subunits of TFIID (BDF1 and TAF2) and KIN28 phosphorylates two subunits of MED complex (MED4 and MED14).[47] In summary, the aforementioned studies reveal SRB10/CDK8 is also as a positive regulator of transcription in yeast.

In mammalians, CDK8 is a stimulus specific co-activator of p53-dependent transcription.[48,49] The p53 gene is a tumor suppressive protein involved in cell-cycle arrest and apoptosis. The induction of cell-cycle arrest by p53 is activated by cyclin-dependent kinase inhibitor p21. Several studies have demonstrated that p21 is activated by p53 upon Nutlin 3 treatment. Donner et al demonstrated that CDK8, along with cyclin C and MED12, are recruited to p21 locus exclusively after Nutlin 3 treatment.[49] Additionally, CDK8 recruitment correlates with positive transcriptional activity among p53 target genes, Hdm2 and p21. RNA interference studies further confirmed that CDK8 is indeed a positive regulator of p53 target genes.[49]

CDK8 was also shown to be a positive regulator of thyroid hormone receptor-dependent transcription. Belakavadi demonstrated that CDK8 cooperates with MED complex in thyroid hormone receptor-dependent transcription by promoting RNAPII recruitment and activation of thyroid hormone-receptor target genes.[50] It was further demonstrated that CDK8 kinase activity is important for thyroid hormone dependent transcription in vivo. ChIP assays revealed significant levels of CDK8 at the thyroid

hormone receptor human type I deiodinase (DIOI) gene promotor during active transcription. Additionally, CDK8 knockdown prevented thyroid hormone-dependent activation of DIOI. Finally, CDK8 depletion was rescued with a wild type CDK8, but not a kinase-dead mutant.[50]

It was also shown that CDK8 Enhances SMAD transactivation.[33,48,51] TGF-β and BMP receptor kinases activated SMAD transcription factors by C-terminal phosphorylation, and subsequent translocation to the nucleus with SMAD4 where they form transcriptional complexes of TGF-β- and bone morphogenetic factor (BMP)-regulated genes. In the cytoplasm phosphorylation of receptor-activated SMADs in the linker region led to proteasomal degradation.[48] However, TGF-β and BMP activation led to agonist-induced linker phosphorylation in the nucleus by CDK8 and CDK9. This was due to CDK8 and CDK9 enhancement of receptor-activated SMADs by promoting co-activator recruitment.[51]

Recent studies indicate that CDK8 is a potential oncogene in colon cancer. Wnt/β-Catenin signaling plays and important role in colon cancer.[48,52,53] Upon activation, Wnt signaling leads to stabilization of β-Catenin allowing its translocation to the nucleus and formation of transcription complexes with TCF/LEF at Wnt-responsive genes such as MYC and Cyclin D1. Loss of function studies identified CDK8 to be highly expressed in human colon cancer cells. Suppression of CDK8 expression inhibited proliferation of colon cancer cells while exhibiting high levels of CDK8 and β-Catenin hyperactivity.[52] CDK8 was also found to associate with MYC promoter and knockdown of CDK8-module subunits MED12 and cyclin C had a similar outcome to CDK8 knockdown, suggesting that CDK8 collaborates with the CDK8-module to stimulate β-Catenin

transcription.[52] It still remains unclear whether CDK8 acts directly on the β-Catenin/TCF/LEF complex or on the general transcriptional apparatus. CDK8 can also play a role in β-Catenin activity through transcriptional factor E2F1. E2F1 is a transcriptional regulator, which can promote proliferation and apoptosis. It was reported that E2F1 represses β-Catenin transcription and is antagonized by CDK8 phosphorylation. Furthermore, both CDK8 and E2F1 were at the MYC promoter. [54] Overall, these results demonstrate the positive effects of CDK8 on β-Catenin transcriptional activity, and its inhibitory effect on E2F1.

CDK8 promotes RNAPII elongation within the serum response network. Mitogen-driven activation of the RAS/RAF/MEK/ERK pathway leads to transcription of ELK1-dependent serum responsive genes.[55] CDK8 promotes the recruitment of transcription elongation factor, P-TEFb, providing a mechanism by which CDK8 can positively affect transcription post-RNAPII recruitment steps.[56]

It was also revealed that CDK8 catalyzes histone modifications that correlate with transcriptional activation. Biochemical assays revealed that cdk8-Mediator complex associates with TRRAP and GCN5L polypeptides. Interestingly, the CDK8 and GCN5L subunits within this 'T/G-Mediator' complex work together to catalyze tandem phosphoacetylation of S10/K14 within histone H3.[57] Since tandem H3 phosphoacetylation correlates with active transcription, these results identify a role for CDK8 in transcriptional activation.

The aforementioned studies support the role of CDK8 as positive and negative regulator of transcription, from yeast to mammals. In summary, CDK8 was shown to be involved in the regulation of transcription factor turnover, CTD phosphorylation and

transcriptional activation or repression. CDK8 was also shown to regulate several transcriptional programs involved in nutrient/growth factor sensors and differentiation control, thus making it a potential therapeutic target.[5]

Alder et al demonstrated that CDK8 is required for tumor growth in vivo and maintains tumors in a less differentiated state. Furthermore, CDK8 maintains embryonic stem cell pluripotency, and this effect was partially mediated via the MYC pathway.[58] In this study, CDK8 inhibition resulted in little to no change in MYC mRNA levels, suggesting that MYC is regulated post-transcriptionally. The regulation of MYC activity involves priming the protein for degradation or transcriptional activation by phosphorylation (p) on threonine 58 (T58) and serine 62 (S62), respectively.[59,60] CDK8 inhibition by gene knockdown revealed an increase in the unstable MYC-pT58 and a decrease in the active MYC-pS62, relative to total MYC. Exogenous expression of either wild-type MYC or degradation resistant MYC^{T58A} in embryonic stem cells increased MYC levels compared to control cells and partially rescued the loss of embryonic stem cell pluripotency imparted by CDK8 depletion. Conversely, expression of MYC^{S62A}, which disrupts the active phosphorylation site, increased total MYC levels but was unable to rescue the loss in pluripotency, suggesting that CDK8 regulation of embryonic stem cell pluripotency is partially mediated through MYC activity.[58] Overall, these results revealed that CDK8 might be positioned at a crossroad of tumorigenesis and stem cell fate. It also raises the intriguing possibility that therapeutically targeting CDK8 may inhibit the stem cell-like properties of cancer cells.

Porter et al recently discovered a small molecule inhibitor of CDK8 and its isoform CDK19, though inhibition of p21-induced transcription.[61] The inhibitor, known

as Senexin A, inhibits CDK8 kinase activity by binding to the ATP pocket site. Of note, Senexin A does not affect normal cell growth. Interestingly, the inhibitor was found to suppress chemotherapy-induced tumor promoting paracrine activities of normal and tumor cells. Moreover, the inhibitor also increased the efficacy of chemotherapy against tumor cell xenografts. [61] Overall, these results identify CDK8 as a druggable mediator of disease-promoting paracrine activities involved in DNA damage and senescence. Therefore, CDK8 inhibition offers a potential therapeutic target for the inhibition of these actions in cancer and other related diseases, such as vascular disease.

CDK8 resides upstream for transcription and therefore it might play a specific role in coordinating genome expression in VSMC differentiation and remodeling. As stated previously, some components of the MED complex have already been shown to be involved in cardiovascular disease[53] ; however, the extent of CDK8 involvement remains to be determined. Based on these results, my overall hypothesis is that CDK8 is a critical mediator in SMC dedifferentiation and vascular lesion formation. My aims are as follows:

1. To explore the role of CDK8 in SMC dedifferentiation.
2. To determine the molecular mechanisms by which CDK8 regulates SMC dedifferentiation.
3. To determine the role of CDK8 in SMC-mediated vascular remodeling in mice.

CHAPTER 2

CYCLIN-DEPENDENT KINASE 8 IS A POSITIVE REGULATOR IN VASCULAR SMOOTH MUSCLE CELL DEDIFFERENTIATON

2.1 INTRODUCTION:

Occlusive vascular disease, characterized by abnormal proliferation and accumulation of vascular SMCs in the vascular lesion, is one of the most common causes of morbidity and mortality in USA.[62] Over the last decades, extensive studies including the genetic inducible fate mapping experiments have convincingly supported a working hypothesis that after vascular injury, mature and quiescent vascular SMCs undergo dedifferentiation, also known as phenotype modulation or switch. This process is characterized by a downregulation or loss of expression of contractile genes, such as smooth muscle myosin heavy chain (MYH11), smooth muscle 22 alpha (SM22α), calponin-1 (CNN1), and alpha smooth muscle actin (αSMA), and a concomitant with an increase in cell proliferation, migration, and extracellular matrix (ECM) production, thereby leading to vascular lesion formation.[63–69] At the molecular level, mechanical stress, a number of ligand-receptor interactions, and ECM-sensing machineries drive intricate pathways at multiple levels of the gene expression regulation

[70–73] and/or the protein quality control[74] thus contributing to vascular SMC dedifferentiation. It is becoming clear, however, that these mechanisms are necessary for complete control of vascular SMC physiology and pathophysiology. The master regulators of vascular SMC dedifferentiation, which are druggable for the treatment of vascular disease, remain to be determined.

CDK8 and its closely related paralog CDK19 (80% identical) are transcription-regulating serine/threonine kinases with a functional overlap in the transcriptional regulation.[75] CDK8 is a component of the Mediator (MED) complex, which consists of four distinct modules termed the head, middle, tail and CDK8 kinase module. In mammals, Mediator complex has several subtypes with the most canonical one composed of around 30 subunits to form the head (MED6, MED8, 11, 17-20, 22, 28), middle (MED1, 4, 7, 9, 10, 14, 21, 26, 3/27, 31), tail (MED15, 16, 23-25, 2/29), and the regulatory CDK8 module (CDK8/19, CCNC, MED12, and MED13) and joins transcription factors bound at the upstream regulatory elements and the transcription machinery (PolI, the general initiation factor TFIIA, -IIB, IID, IIE, IIF, and –IIH, DNA-binding transcription factor and transcription coactivators) at the promoter region, thereby controlling epigenetic regulation, transcriptional initiation and elongation, transcriptional termination, mRNA processing, noncoding RNA activation, and the chromatin remodeling.[30,48] Multiple approaches including gene knockout technology in mice have revealed a general requirement for Mediator in coordinating the cell lineage determination, development, and metabolism, which are potentially linked to cardiovascular diseases.[30,31] However, the precise role and interplay of Mediator components and modules in cardiovascular pathologies are unclear. On the other hand,

emerging evidence has indicated that CDK8 is not likely to regulate the expression of stably expressed genes,[48] and instead acts primarily to enable the elongation of transcription of silent genes that become activated by transcription-induced factors.[51,52,56,76] In addition, CDK8 can indirectly regulate gene expression through directly phosphorylating its protein substrates, such as receptor activated SMADs, E2F1, STAT1, and p27, and likely independently of Mediator complex formation.[51,54,76–79] Moreover, the conditional knockout of CDK8 throughout the body of adult mice does not induce gross or histopathological abnormalities.[80] These results indicate that CDK8-operated signaling may not be essential for somatic cellular homeostasis, but play a unique role in the pathological processes, such as the cell transformation for cancer[61], leading to diseases. Therefore, we questioned whether or not CDK8 is a yet unrecognized regulator of vascular SMC dedifferentiation for occlusive vascular remodeling. In this report, we demonstrate that CDK8 is a critical mediator of vascular SMC dedifferentiation.

To investigate the role of CDK8 in SMC dedifferentiation, we used a primary culture of rat aortic SMCs (RASMCs) and tested the effects of CDK8 inhibition by utilizing a specific inhibitor of CDK8, Senexin A[61], and lentivirus of CDK8 shRNA. We also tested the effect of CDK8 overexpression by adenoviral approach. We hypothesized that CDK8 inhibition will promote SMC differentiation by suppressing RASMC proliferation, enhance SMC marker expression, and inhibit SMC migration and ECM secretion. Conversely, CDK8 overexpression will promote SMC dedifferentiation by increasing RASMC proliferation and decrease SMC protein marker expression. In the

current study we demonstrate that CDK8 is a positive regulator of vascular SMC dedifferentiation.

2.2 MATERIALS AND METHODS:

Animals:

Male Sprague Dawley (SD) rats (6-8 weeks old) were purchased from Jackson Laboratories. All animals were housed at the AAALAC-accedited University of South Carolina School of Medicine. All animal procedures were performed in accordance with NIH Guideline for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of University of South Carolina School of Medicine.

Human Specimens:

Tissue sections of arteries were prepared from autopsy organ specimens of humans with cardiovascular disease history (Appendix A.1). This study was approved by the Institutional Review Board for human subject research at Icahn School of Medicine at Mount Sinai, New York.

RASMC Isolation:

Briefly, SD rats were euthanized by intraperitoneal injection by an overdose of pentobarbital (100 mg/kg) before tissue collection. The thoracic aortas were removed, washed in phosphate-buffered saline (PBS) and incubated in 1g/L Dulbecco's modified Eagle's medium (DMEM) containing 7.5 mg Collagenase type II (Worthington Biochemical Corp. Cat. 17101-015) for 30 min. Using a dissection microscope, the surrounding connective tissue and adventitia were removed. The dissected tunica media

tissues were incubated for 2-3 hours with 7.5 mg Collagenase type II and minced periodically throughout incubation. Cells were cultured in conventional regular growth medium (RGM) containing 10% Fetal bovine serum (FBS) in DMEM and 100 μg/mL penicillin/streptomycin in a 37 °C and 5% CO_2 humidifed chamber.

Reagents and Antibodies:

The reagents used in this study were purchased as described below:

Senexin A/B from Senex Biotechnology (Columbia, SC), [H3] Thymidine (Cat #: 240-3905, Millipore, Billerica, MA), Ad-Green fluorescent protein (GFP) (Cat#: 1060, Vector Biolabs, Burlingame, CA), and Ad-c-Myc (Cat#: 1285, Vector Biolabs, Burlingame, CA), Ecolite (Cat#:882475, Millipore, Bilerica, MA).

The following antibodies were purchased from Abcam (Cambridge, MA): c-MYC (ab32072, GFP (ab6673), SM22α (ab14106), CNN1 (ab46974), αSMA (ab5694).

The following antibodies were purchased from Santa Cruz (Santa Cruz, CA): c-MYC (sc40), Ubiquitin (sc-8017), JNK (sc571), CDK8 (sc1521).

The following antibodies were purchased from Sigma Aldrich (St. Louis, MO): B-actin (a1978), GAPDH (a8795), αSMA (A5228), MYH11 (ab125884).

Quantitative real-time PCR (qPCR):

Total cellular RNA was purified with TRIzol (Invitrogen). Gene expression was measured by qPCR, and normalized by concurrent measurement of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA levels. Primers for qPCR included *SM22α* 5'–CCACAAACGACCAAGCCTTTT–3' and 5'–CGGCTCATGCCATAGGATG–3', *Cnn1* 5'–ACATCATTGGCCTACAGATG–3' and 5'– CAAAGATCTGCCGCTTGGTG–3', *Myh11* 5'–CGGTGTTCTCCTGCTAGTCC–3' and 5'–

CTTCTTGGCTACCCAGTCGG–3', *Mmp2* (matrix metalloproteinase-2) 5'–GACCTTGACCAGAACACCATCG–3' and 5'–GCTGTATTCCCGACCGTTGAAC–3- and *Gapdh* 5'–AGTGCCAGCCTCGTCTCATA–3' and 5'–GATGGTGATGGGTTTCCCGT–3', *OPN* (Ostepontin) 5'–AAGGCGCATTACAGCAAACACTCA–3' and 5'–CTCATCGGACTCCTGGCTCTTCAT –3'

Lentiviral Transfection:

pLKO.1 and CDK8 lentivirus was provided by Dr. Igor Roninson (University of South Carolina School of Pharmacy, Columbia, SC). CDK8 lentivirus was generated as previously described (Porter et al., 2012). Knockdown of CDK8 was generated by transduction with pLKO.1 lentiviral vectors expressing the corresponding shRNAs (Open Biosystems), followed by puromycin selection (2.5 μg/ml). The target sequences for CDK8 shRNA was GTCTTATCAGTGGGTTGATTC.

Proliferation Studies:

Isolated RASMCs (p1-p10) were seeded at 10,000 cells/well on 24-well plate in RGM with 10% FBS. Thereafter, RASMCs were serum starved for 16 hr. The following day, RASMCs were treated with vehicle dimethyl sulfoxide (DMSO) 5 μm Senexin A/B for 0 - 9 days and proliferation was measured by a hemocytometer.

[H3]Thymidine Assay:

Isolated RAMCs (p1-p10) were seeded at 40,000 cells/well on a 24-well plate in RGM with 10% FBS. Thereafter, RASMCs were serum starved for 16 hr. RASMCs were treated with vehicle dimethyl sulfoxide (DMSO) 5 μm Senexin A for 24 hours, with [3H]thymidine (1 μCi/ml) being added during the last 4 hours of culture. RASMCs were

washed with ice cold 5% TCA overnight. The following day, RASMCs were washed with ice cold 5% TCA, two times, and washed with 1x PBS, two times. RASMCs were lysed with 0.5 ml of 0.5M NaOH for 30 min in a 37 °C and 5% CO_2 humidifed chamber. Cells were added to Ecolite (2ml/well). DNA synthesis was measured by [3H]thymidine uptake via a liquid scintillation counter.

Differentiation Assay:

Isolated RASMCs (p1-p10) were seeded on 6-cm petri dishes in RGM with 10% FBS and grown to 80-90% confluence. Thereafter, RASMCs were serum starved (DMEM alone) for 16 hr. The following day, RASMCs were treated with the appropriate treatments for 48 hours. After treatment, RASMCs were subjected to Western blot analysis using SMC differentiation markers.

Migration Assay:

Isolated RASMCs (p1-p10) were seeded on 6-cm petri dishes. RASMCs were serum starved for 2 days, treated for the following three days. Treatments included 5 μm Senexin A and 10% FBS. P200 pipet tip was used to create a scratch at the bottom of each well and the cells were incubated further for 3 days. The distance between the edges of the scratch was measured immediately after scratching and again after the time point day 3. Migration was quantified and expressed as follows: % closure = [(distance immediately after scratching – distance after 3 days)/distance immediately after scratching] * 100. Microscopic pictures were taken using Infinity 3 microscope camera.

Western blot:

Protein extracts were prepared by standard procedures. Immunoblotting

was conducted by enhanced chemiluminescence using Western Lightning Plus-ECL (Perkin-Elmer).

Cell Cycle Analysis:

Isolated RASMCs (p1-p10) were seeded on 6-cm petri dishes in RGM with 10% FBS and grown to 80-90% confluence (n=3 per treatment). Thereafter, RASMCs were serum starved (DMEM alone) for 16 hr. The following day, RASMCs were treated with the appropriate treatments for 48 hours. After treatment, cells were harvested by trypsin method and subjected to flow cell cycle analysis. Briefly, RASMCs were centrifuged for 10 min at 1000 rpm at 25 °C in the medium. Media was aspirated. RASMCs were then washed with 5 ml PBS and centrifuged for 10 min at 1000 rpm. Media was aspirated. 500 ul of ice-cold 70% ETOH was added dropwise. Samples were stored in -80 °C until analysis. Cells were centrifuged at 1000 rpm for 5 min and ETOH was aspirated. 1 ml of propidium iodine staining solution was added to cells on ice for 30 min. Samples were then analyzed on Beckson Dickson Flow Cytometer. PI staining solution consisted of 200 ug/ml PI and 2mg/ml DNase-free Rnase A.

Statistics:

Data are shown as mean ± SEM. Differences between 2 groups were evaluated for statistical significance using the Student t test when the sample size was appropriate and the population was distributed normally. When differences among > 3 groups were evaluated, results were compared by one-way ANOVA with Bonferroni test for multiple comparisons. Differences were considered significant at $p < 0.05$.

2.3 RESULTS:

Upregulation of CDK8 in Injured Arteries:

To explore a potential role of CDK8 in the vasculature, we examined the expression pattern of CDK8 in mouse normal and injured arteries. Confocal microscopic analysis revealed that CDK8 is mainly expressed in endothelial cells and media SMCs in the normal carotid artery (Figure 2.1). However, the expression of CDK8 was increased on 3 day and then decreased to near to the basal level on 28 day after complete carotid ligation (Figure 2.2). In addition, the carotid ligation induced CDK8 expression in adventitial cells with a similar pattern aforementioned (Figure 2.2). CDK8 expression was also evident in NI cells on 28 day after the injury, although it was relatively low (Figure IB). Western blot showed that CDK8 expression in the carotid artery was increased on 3 day and then decreased to near to the basal level on 28 day after complete carotid ligation (Figure 2.3). Since previous lineage-tracing studies have demonstrated that the majority of NI cells are not derived from endothelial cells but mature vascular SMCs[68,81] and mature vascular SMCs undergo dramatic dedifferentiation on 3 day[82] in the carotid ligation model, these results suggest that CDK8 may play an important role in the regulation of vascular SMC dedifferentiation and NI formation. Importantly, CDK8 expression was significantly upregulated in SMCs in the atherosclerotic plaque of human carotid arteries (Figure 2.4). These findings highlight a pathological relevance of CDK8 in vascular disease.

A Mediator Role of CDK8 in Vascular SMC Dedifferentiation:

To investigate whether or not CDK8 regulates vascular SMC dedifferentiation, we used a primary culture of rat aortic SMCs (RASMCs), a well-established culture

system of dedifferentiated vascular SMCs.[64] We found that SMC contractile proteins including MYH11, αSMA, and CNN1 were dramatically downregulated in cultured SMCs compared with SMCs in the tunica media, indicating a dedifferentiated state as described elsewhere[64] ; however, the CDK8 expression was significantly upregulated in the dedifferentiated RASMCs (Figure 2.5). Inhibition of CDK8 activity via lentiviral knockdown approach or a highly selective small molecule inhibitor of CDK8, Senexin A[61] attenuated cell number increases of RASMCs cultured in a full growth medium (Figure 2.6) and FBS-induced [^{3}H]-thymidine uptake in RASMCs (Figure 2.7) associated with delayed progression through all phases of the cell cycle including G0/G1, S, and G2/M phases (Figure 2.8) and increased expression of SMC contractile proteins including αSMA, CNN1, and SM22α (Figure 2.9). The specificity of Senexin A was verified in RASMCs (Figure 2.10). Senexin A treatment suppressed migration as well as the expression of matrix metalloproteinase-2 (*Mmp2*) and osteopontin (*Opn*) while upregulating the expression of SMC contractile proteins including *Myh11*, *Cnn1*, and *Sm22α* in cultured RASMCs (Figure 2.11 and 2.12). These results demonstrate that CDK8 is a critical mediator of vascular SMC dedifferentiation with typical synthetic phenotypes including increased proliferation, migration, and secretion.

2.4 DISCUSSION:

CDK8 has been shown to be of importance in cancer cells and embryonic cells.[58,61] However, our results uncover, for the first time that CDK8 is normally expressed in endothelial cells and vascular SMCs, thus suggesting that CDK8 is expressed under normal physiological conditions in the vasculature. Moreover, CDK8

was shown to be upregulated in dedifferentiating vascular SMCs suggesting that CDK8 may play a role in the pathological process of vascular disease. Mature vascular SMCs undergo a dedifferentiation, also known as phenotype modulation or switch. This process plays a critical role in vascular lesion formation and is characterized by a downregulation or loss of expression of contractile genes and a concomitant with an increase in cell proliferation, migration, and extracellular matrix production,.[67,83] CDK8 inhibition was able to regulate all three events by promoting increased proliferation, migration and ECM secretion as well as promoting a synthetic phenotype. Therefore, we can conclude that CDK8 acts as a mediator in SMC dedifferentiation and can potentially be a therapeutic target in the treatment of vascular disease. Further studies are needed to investigate the molecular mechanism by which CDK8 regulates SMC dedifferentiation as well as the pathological relevance of CDK8, in vivo. Nonetheless, these results provide a significant insight of the importance of CDK8 in the vasculature.

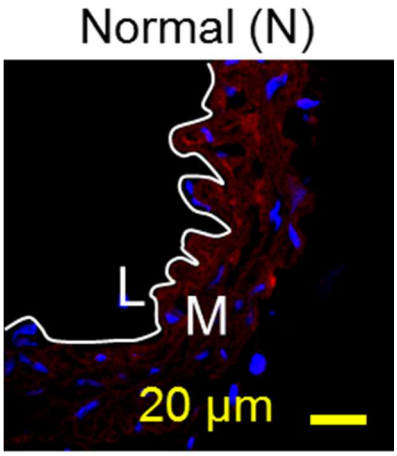

Figure 2.1. CDK8 is expressed in media SMCs in normal carotid artery. Confocal microscopic analysis of CDK8 in normal male adult mice in C57BL/6J background. L, lumen; M, media.

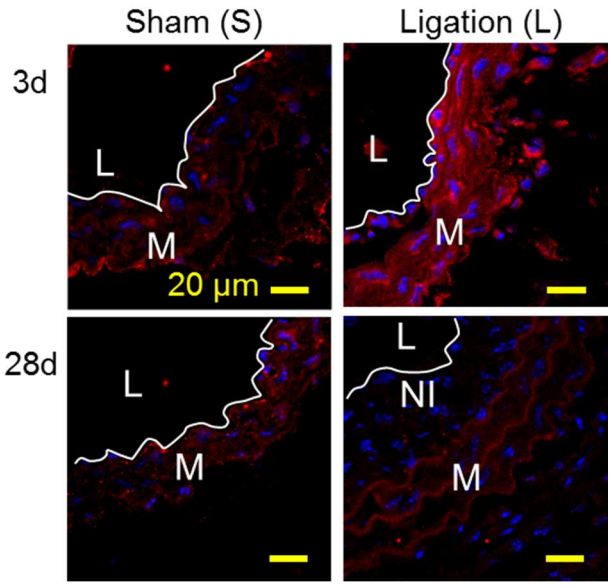

Figure 2.2. CDK8 expression is upregulated after carotid ligation injury in mice. Male adult mice in C57BL/6J background (n=10 for eac h group) were induced with cartoid artery ligation and CDK8 expression was analyzed by confocal microscopy at day 3 and day 28. The representative results are from 3 separate CDK8 stainings. L, lumen; M, media.

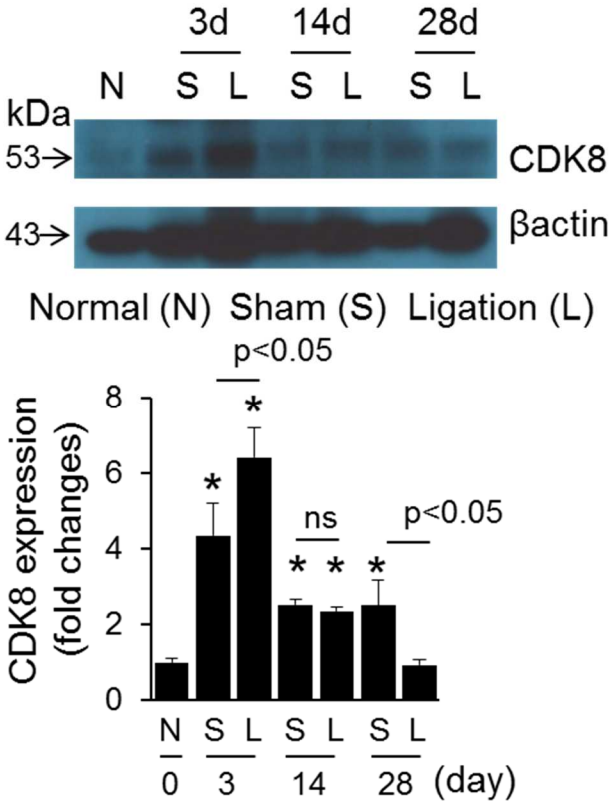

Figure 2.3. CDK8 protein expression is upregulated after carotid ligation injury in mice. Male adult mice in C57BL/6J background (n=10 for each group) were induced with cartoid artery ligation and CDK8 protein expression was analyzed by Western blot analysis (upper panel) and protein bands were quantified using Image J Pro software (lower panel).

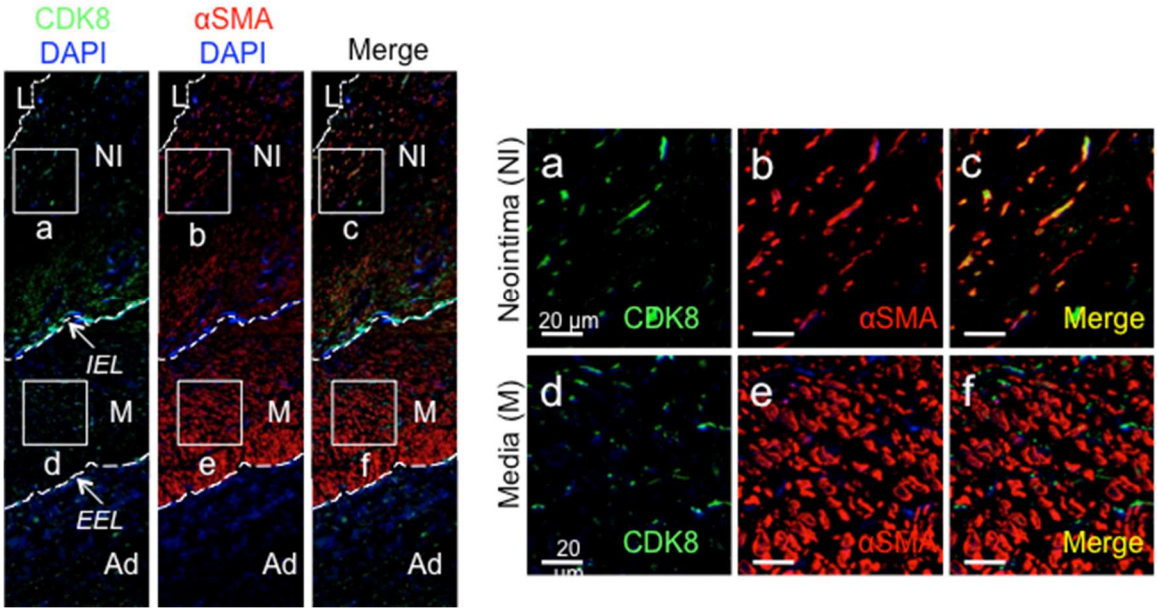

Figure 2.4. CDK8 is expressed in human coronary arteries with atherosclerotic lesions. The images shown are representative of CDK8 staining in tissue sections of human coronary arteries with atherosclerotic lesions (n=6). IEL, internal elastic lamina; EEL, external elastic lamina; Ad, adventitia.

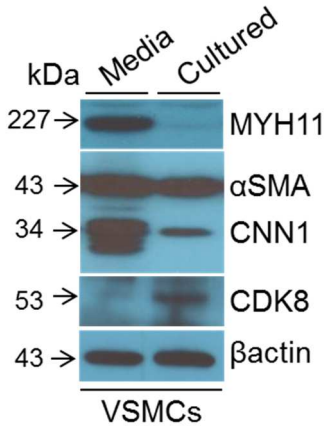

Figure 2.5. CDK8 protein expression is upregulated in RASMC cell culture. RGM-cultured RASMCs at 11 days (passage 3) and the tunica media lysates (isolated from the thoracic aorta of male SD rats at age of 10 weeks) were subjected to Western blot analysis of CDK8 expression and SMC markers MYH11, αSMA, and CNN1. Representative results are from 3 separate experiments.

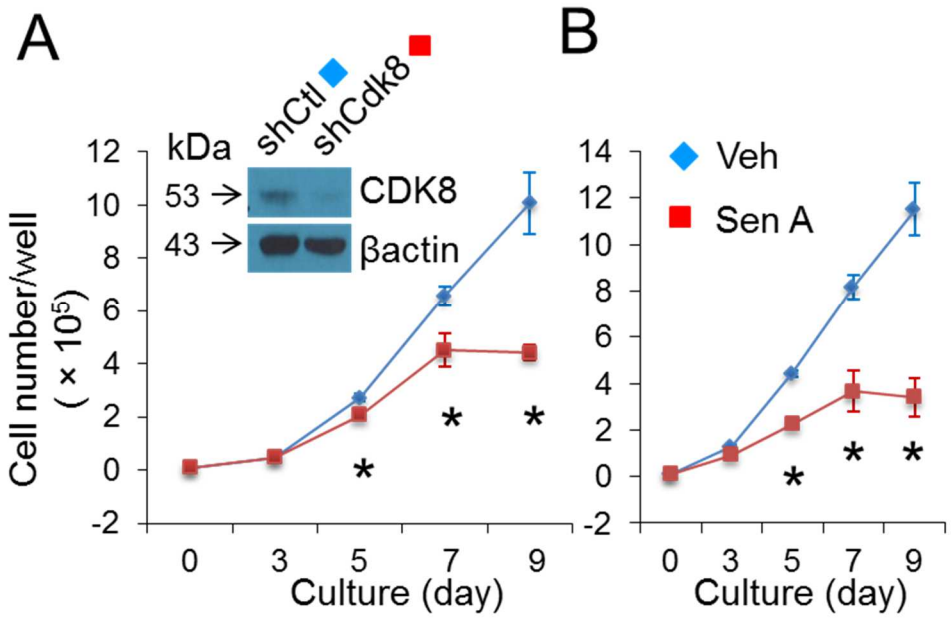

Figure 2.6. CDK8 inhibition reduced RASMC proliferation. A. Serum starved lentiviral RASMCs were cultured in RGM for 0-9 days and proliferation was measured by a hemocytometer (n=3). B. Serum starved RASMCs at early passage were cultured in 10% FBS-supplemented media with 5 μm Senexin A for 0 – 9 days and proliferation was measured by a hemocytometer (n=3).

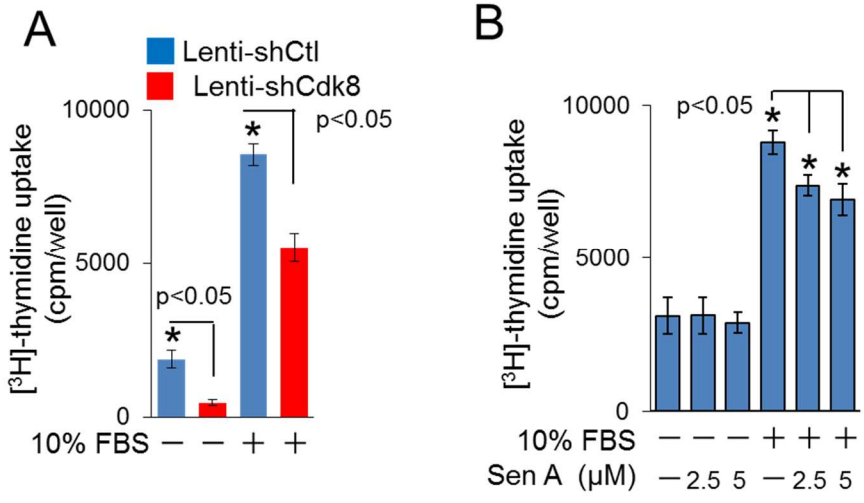

Figure 2.7. CDK8 inhibition reduced DNA synthesis. A. RASMCs were lentivirally transduced with scramble vector or shCDK8 and cultured in RGM. Serum starved lentiviral RASMCs were treated as indicated for 24 h and subjected to [3H]-thymidine uptake assay to measure DNA synthesis. B. RASMCs without lentiviral transduction

were treated with 2.5μM and 5μM Senexin A for 24 h and subjected to [3H]-thymidine uptake assay to measure DNA synthesis. n=6, *p<0.05 vs. control (-).

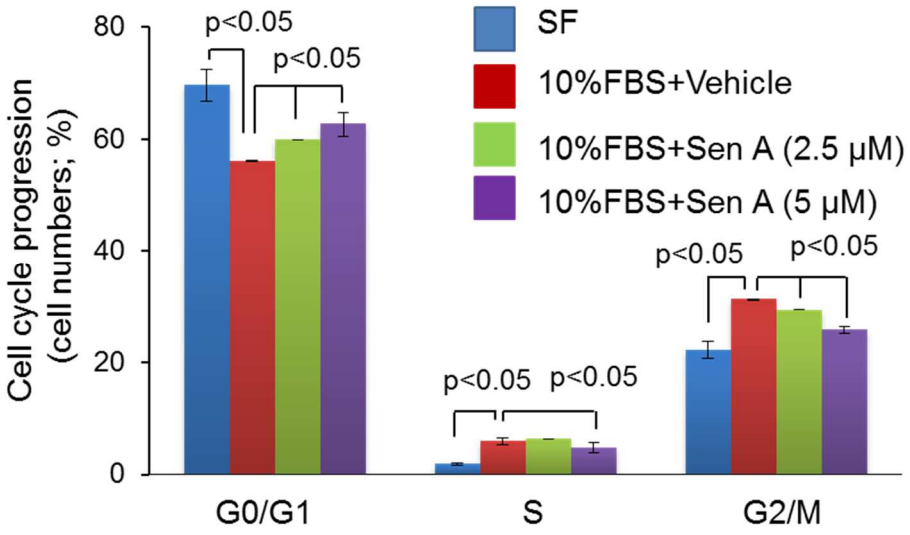

Figure 2.8. CDK8 inhibition prevented RASMC cell cycle progression at g0/g1 phase**.** Serum starved RASMCs at early passage were treated as indicated for 48 h and subjected to flow cytometry cell cycle analysis (n=3).

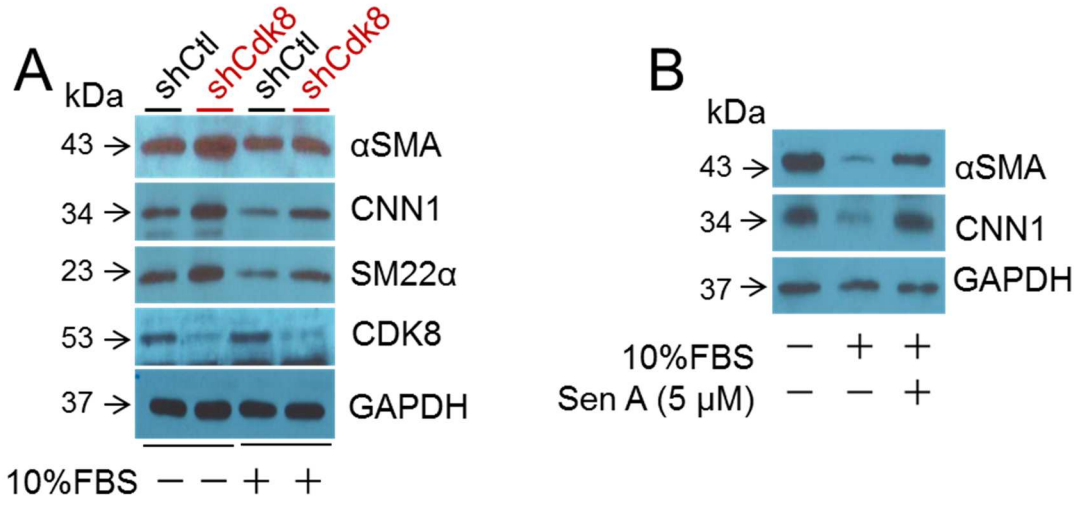

Figure 2.9. CDK8 inhibition promoted RASMC differentiation. A. RASMCs were lentivirally transduced with scramble vector or shCDK8 and cultured in RGM. Serum starved RASMCs were treated with 10% FBS for 10 minutes and subjected to Western blot analysis of SMC protein markers (αSMA, CNN1 and SM22α) and CDK8. B. Serum starved post-confluent RASMCs were stimulated with 10% FBS and 5 μM Senexin A for

48 hours. Western blot was conducted to determine the expression of SMC protein markers (αSMA and CNN1). Representative results are from 3 separate experiments.

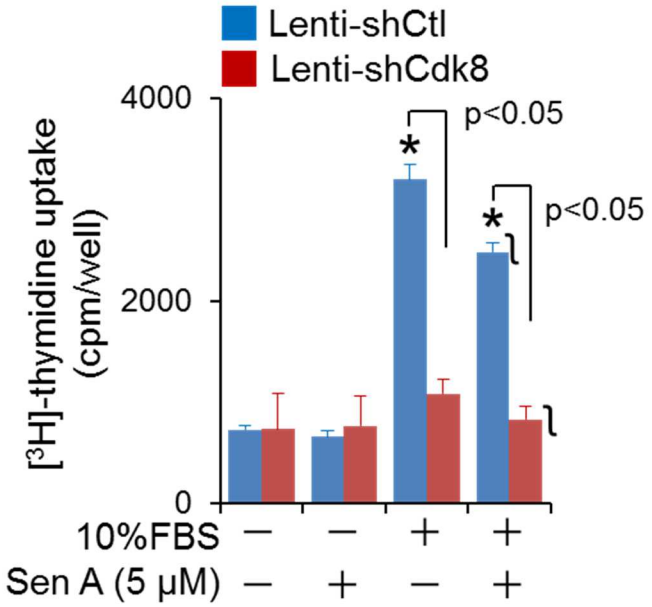

Figure 2.10. Senexin A reduction on DNA synthesis is CDK8 specific. RASMCs were lentivirally transduced with scramble vector or shCDK8 and cultured in RGM. Serum starved lentiviral RASMCs were treated as indicated for 24 h and subjected to [3H]-thymidine uptake assay to measure DNA synthesis. n=6, *p<0.05 vs. control (-).

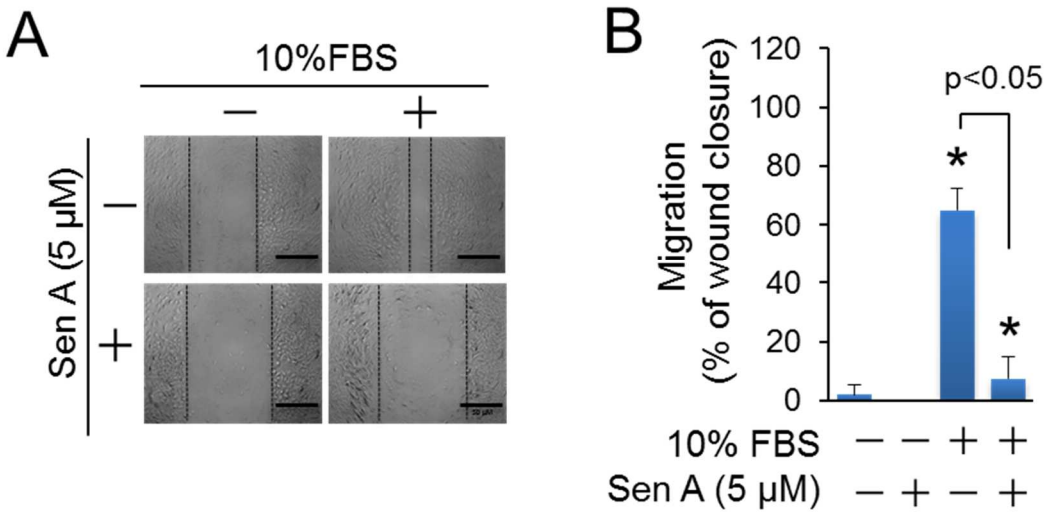

Figure 2.11. CDK8 inhibition attenuated FBS-induced migration of RASMCs. Serum starved RASMCs at early passage were treated as indicated for 72 h and subjected to migration assay. A. Pictures were taken via Infinity 3 microscope software. B. Migration was quantified and expressed as follows: % closure = [(distance immediately after scratching – distance after 72 h)/distance immediately after scratching] * 100 (n=3, * indicates $p<0.05$).

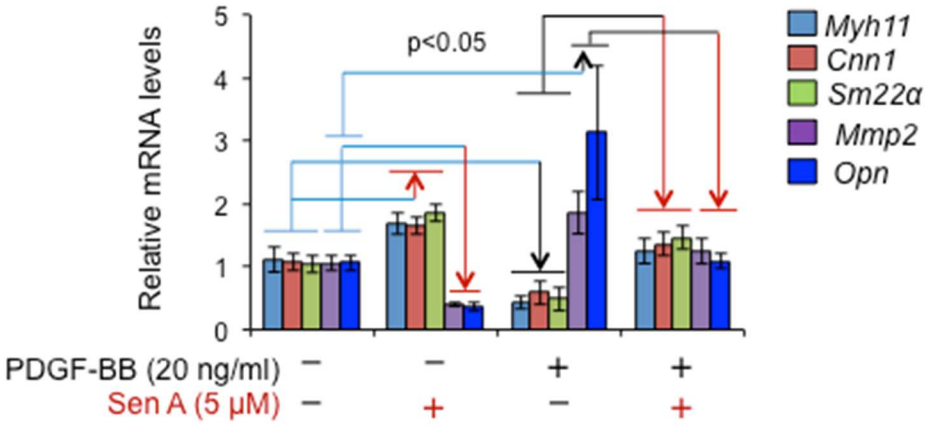

Figure 2.12. Senexin A treatment increased extracellular matrix secretion. Serum starved RASMCs (48 h) were treated with 20 ng/ml PDGF-ββ and 5 μM Senexin A (Sen A) for 24 h and qRT-PCR analysis was conducted to determine the relative mRNA expression of contractile markers *Myh11, Cnn1* and *Sm22α* and secretory markers *Mmp1* and *Opn* (n=3).

CHAPTER 3

CYCLIN-DEPENDENT KINASE 8 IS A POSITIVE REGULATOR IN VASCULAR SMOOTH MUSCLE CELL DEDIFFERENTIATION VIA AKT/GSK3β-MEDIATED ENHANCMENT OF C-MYC STABILIZATION

3.1 INTRODUCTION:

Occlusive vascular disease, characterized by abnormal proliferation and accumulation of vascular SMCs in the vascular lesion, is one of the most common causes of morbidity and mortality in USA.[62] Upon vascular disease, SMCs will dedifferentiate from a quiescent, contractile state with high expression of SMC markers to a proliferative, synthetic state with low expression of SMC markers, thereby causing lesion formation.[63–69] This process is known as SMC dedifferentiation. At the molecular level, mechanical stress, a number of ligand-receptor interactions, and extracellular matrix-sensing machineries drive intricate pathways at multiple levels of the gene expression regulation[70–73] and/or the protein quality control[74] thus contributing to vascular SMC dedifferentiation. It is becoming clear, however, these mechanisms are necessary for complete control of vascular SMC physiology and pathophysiology. The master regulators of vascular SMC dedifferentiation, which are druggable for the treatment of vascular disease, remain to be determined.

CDK8 is a transcriptional kinase that has been involved in several regulatory pathways. A recent study identified CDK8 as a druggable mediator of disease-promoting paracrine activities involved in DNA damage and senescence. Therefore, CDK8 inhibition offers a potential therapeutic target for the inhibition of these actions in cancer and other related diseases, such as vascular disease.

CDK8 inhibition resides upstream for transcription and therefore it might play a specific role in coordinating genome expression in VSMC differentiation and remodeling. In fact, our previous studies demonstrate that CDK8 is a positive regulator in SMC dedifferentiation, resulting in increased cell proliferation and the downregulation of SMC protein marker expression and an increase in FBS-induced migration and ECM secretion. However, the molecular mechanisms that regulate CDK8-mediated SMC dedifferentiation remains to be determined.

CDK8 has been shown to regulate several signaling pathways that are key regulators in embryonic stem cell pluripotency, cancer, and presumably cardiovascular disease.[31,48,58,61] The oncoprotein c-MYC has been identified an important regulator in tumor dedifferentiation and embryonic stem cell pluripotency. c-MYC resides downstream of other target genes, which CDK8 has been shown to regulate. However, upstream target genes of c-MYC have yet to be identified. It is possible that CDK8-mediated SMC dedifferentiation is ultimately regulated by c-MYC. Therefore, we hypothesized that CDK8 is a critical meditator of SMC dedifferentiation via the c-Myc pathway.

To investigate the molecular mechanisms in CDK8-mediated SMC dedifferentiation, we used a primary culture of rat aortic SMCs (RASMCs) and tested the

effects of CDK8 inhibition by utilizing a specific inhibitor of CDK8, Senexin A[61], and lentivirus of CDK8 shRNA, and performed several signaling studies analyzed by Western blot. We also tested the effect of c-MYC overexpression by adenoviral approach and performed another set of signaling studies analyzed by Western blot. We hypothesized that CDK8 plays a role in RASMC dedifferentiation via c-MYC pathway. In this report, we demonstrate that CDK8 is an upstream operator of AKT/GSK3β signaling for c-MYC stabilization leading to vascular RASMC dedifferentiation.

3.2 MATERIALS AND METHODS:

Animals:

Male Sprague Dawley (SD) rats (6-8 weeks old) were purchased from Jackson Laboratories. All animals were housed at the AAALAC-accredited University of South Carolina School of Medicine. All animal procedures were performed in accordance with NIH Guideline for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of University of South Carolina School of Medicine.

RASMC Isolation:

Briefly, SD rats were euthanized by intraperitoneal injection by an overdose of pentobarbital (100 mg/kg) before tissue collection. The thoracic aortas were removed, washed in phosphate-buffered saline (PBS) and incubated in 1g/L Dulbecco's modified Eagle's medium (DMEM) containing 7.5 mg Collagenase type II (Worthington Biochemical Corp. Cat. 17101-015) for 30 min. Using a dissection microscope, the surrounding connective tissue and adventitia were removed. The dissected tunica media

tissues were incubated for 2-3 hours with 7.5 mg Collagenase type II and minced periodically throughout incubation. Cells were cultured in conventional regular growth medium (RGM) containing 10% FBS in DMEM and 100 μg/mL penicillin/streptomycin in a 37 °C and 5% CO_2 humidifed chamber.

Reagents and Antibodies:

The reagents used in this study were purchased as described below:

Senexin A/B from Senex Biotechnology (Columbia, SC), GSK3β inhibitor (SB-216763, Cat#: S3442, Sigma-Aldrich, St. Louis, MO), AKT inhibitor (MK-2206, Cat#: 1032350-13-2, Sigma Aldrich, St. Louis, MO), Epoxomicin (Cat #: E3652, Sigma Aldrich, St. Louis, MO), [H3], Ad-GFP (Cat#: 1060 Vector Biolabs, Burlingame, CA), and Ad-c-Myc (Cat#: 1285, Vector Biolabs, Burlingame, CA).

The following antibodies were purchased from Cell Signaling Technologies (Beverly, MA): pAKT T308 (CST 9275S), p-AKT S473 (CST 9271S), AKT (CST 9272S), AKT (CST 2920), p-GSK3β (CST 9323P), GSK3β (CST 9315), p-ERK Thr202/Tyr04 (CST 9101S), ERK (CST 9102), p-JNK Thr183/Tyr185 (CST 9251), p-p38 Thr180/Tyr182 (CST 9211S), p38 (CST 9212), p-STAT1 Ser727 (9177), STAT1 (CST 9172).

The following antibodies were purchased from Abcam (Cambridge, MA): c-MYC (ab32072), GFP (ab6673), SM22α (ab14106), CNN1 (ab46974), αSMA (ab5694), MYH11 (ab125884).

The following antibodies were purchased from Santa Cruz (Santa Cruz, CA): c-MYC (sc40), Ubiquitin (sc8017), JNK (sc571), CDK8 (sc1521)

The following antibodies were purchased from Sigma Aldrich (St. Louis, MO): B-actin (a1978), GAPDH (a8795), αSMA (a5228).

The following antibody was purchased from ProteinTech Group (Rosemont, IL): CDK8 (22067-1-AP).

Adenoviral Transfection:

Early passage (p) RASMCs (p1-p10) were transfected with Ad-GFP or Ad-cMyc according to manufacturer's protocol. 20 MOI of Ad-GFP or Ad-cMyc was added in SF medium, and cells were incubated for 6 hrs in a 37 °C and 5% CO_2 humidifed chamber. Transfected cells were re-fed fresh media DMEM supplemented with 2% FBS. After 48 hours, the transfected cells were harvested by trypsinization and re-seeded on the appropriate petri dishes for future studies.

Lentiviral Transfection:

pLKO.1 and CDK8 lentivirus was provided by Dr. Igor Roninson (University of South Carolina School of Pharmacy, Columbia, SC). CDK8 lentivirus was generated as previously described (Porter et al., 2012). Knockdown of CDK8 was generated by transduction with pLKO.1 lentiviral vectors expressing the corresponding shRNAs (Open Biosystems), followed by puromycin selection. The target sequences for CDK8 shRNA was GTCTTATCAGTGGGTTGATTC.

Cell Signaling Studies:

Isolated RASMCs (p1-p10) were seeded on 6-cm petri dishes in RGM with 10% FBS and grown to 80-90% confluence. Thereafter, RASMCs were serum starved (DMEM alone) for 16 hr. The following day, RASMCs were treated with the appropriate treatments for 10 or 30 minutes. For some studies RASMCs were pre-treated with 0.1 μM Epoxomicin, 4 nM and 8 nM AKT inhibitor, or 0.01 μM GSK3β inhibitor for 1-2 hours. After

treatment, RASMCs were subjected to Western blot analysis of potential signaling pathways involved.

Proliferation Studies:

Isolated RASMCs (p1-p10) were seeded at 10,000 cells/well on 24-well plate in RGM with 10% FBS. Thereafter, RASMCs were serum starved for 16 hr. The following day, RASMCs were treated with vehicle control, dimethyl sulfoxide (DMSO), and 5 μm Senexin A for 0 - 9 days and proliferation was measured by a hemocytometer.

[H3]Thymidine Assay:

Isolated RAMCs (p1-p10) were seeded at 40,000 cells/well on a 24-well plate in RGM with 10% FBS. Thereafter, RASMCs were serum starved for 16 hr. RASMCs were treated with vehicle dimethyl sulfoxide (DMSO) 5 μm Senexin A/B for 24 hours, with [3H]thymidine (1 μCi/ml) being added during the last 4 hours of culture. RASMCs were washed with ice cold 5% TCA overnight. The following day, RASMCs were washed with ice cold 5% TCA, two times, and washed with 1x PBS, two times. RASMCs were lysed with 0.5 ml of 0.5M NaOH for 30 min in a 37 °C and 5% CO_2 humidifed chamber. Cells were added to Ecolite (2ml/well). DNA synthesis was measured by [3H]thymidine uptake via a liquid scintillation counter.

Differentiation Assay:

Isolated RASMCs (p1-p10) were seeded on 6-cm petri dishes in RGM with 10% FBS and grown to 80-90% confluence. Thereafter, RASMCs were serum starved (DMEM alone) for 48 h. The following day, RASMCs were treated with the appropriate treatments for 48 hours. After treatment, RASMCs were subjected to Western blot analysis using SMC differentiation markers.

Quantitative real-time PCR (qPCR):

Total cellular RNA was purified with TRIzol (Invitrogen). Gene expression was measured by qPCR, and normalized by concurrent measurement of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) mRNA levels. Primers for qPCR include *c-Myc* 5' – TCCTCGCGTTATTTGAAGCCT-3' and 5'–CGAGTCGTAGTCGAGGTCAT–3', and *Gapdh* 5'–AGTGCCAGCCTCGTCTCATA–3' and 5'–GATGGTGATGGGTTTCCCGT–3'.

Immunoprecipitation, Immunoblotting and Immunohistochemical Analyses:

The protein expression in cells and tissues were analyzed by immunoblotting and immunohistochemical staining as we previously reported [83,84]. The protein-protein association in the cell was examined by immunoprecipiation and immunoblotting as well as confocal microscopic analysis as we previously reported [83–85].

Statistics:

Data are shown as mean ± SEM. Differences between 2 groups were evaluated for statistical significance using the Student t test when the sample size was appropriate and the population was distributed normally. When differences among > 3 groups were evaluated, results were compared by one-way ANOVA with Bonferroni test for multiple comparisons. Differences were considered significant at $p < 0.05$.

3.3 RESULTS:

A Critical Role of CDK8 in Activating AKT/GSK3β Pathway and Stabilizing c-MYC in Vascular SMCs:

Recently, c-MYC and STAT1, the established mediators of vascular SMC dedifferentiation,[86–90] have emerged as downstream effectors of CDK8 in stem cells [58] and immune cells, respectively.[78,79] Hence, we extrapolated these findings into cultured RASMCs. We found that Senexin A did not affect STAT1 phosphorylation at Ser727, the previously identified phosphorylation site of CDK8 in immune cells,[78,79] but downregulated c-MYC protein expression (Figure 3.1). The inhibitory effect of Senexin A was further verified by lentiviral knockout of CDK8 (Figure 3.2). These results indicate that c-MYC may be a downstream effector of CDK8 in mediating vascular SMC dedifferentiation. Since CDK8 inhibition did not affect *c-Myc* mRNA expression level (Figure 3.3) and could not lead to the downregulation of c-MYC anymore in the presence of a proteasome inhibitor, Epoxomicin (Figure 3.4 and 3.5), it is most likely that CDK8 upregulates c-MYC expression via a posttranscriptional mechanism that stabilizes c-MYC proteins in vascular SMCs.

It has been demonstrated that in the normal cell, c-MYC protein is rapidly degraded following its synthesis with a half-life of ~20 min and the most prominent route for c-MYC degradation is through the ubiquitin proteasome system (UPS).[91] In addition, the c-MYC degradation is regulated by multiple Ras effector pathways.[91] The most studied pathways are the Raf-MEK-ERK kinase cascade and the PI3K-AKT pathway that inhibits GSK3β. The ERK and GSK3β phosphorylate two sites of c-MYC, i.e., Ser62 and Thr58, respectively. The phosphorylation at Ser62 results in c-MYC stabilization. The phosphorylation at Ser62 is also required for the subsequent phosphorylation of c-MYC at Thr58 by GSK3β, which is associated with c-MYC degradation. Hence, we examined a potential role of CDK8 in regulating the MAPK and AKT/GSK3β signaling for c-MYC

stabilization in cultured RASMCs. Interestingly, not the serum-induced phosphorylation of MAPKs including ERK, JNK and p38, but the phosphorylation of AKT and its downstream effector GSK3β was inhibited by Senexin A (Figure 3.6). In addition, CDK8 inhibition led to increases in ratios of p-c-MYC (Thr58)/β-actin in both serum-starved and serum-stimulated RASMCs (Figure 3.7). These results indicate that CDK8 promotes the activation AKT to suppress GSK3β-mediated phosphorylation of c-MYC at Thr58 for degradation in vascular SMCs.

A Novel Signaling Axis of CDK8-AKT Activation-GSK3β Inhibition-c-MYC Stabilization for Vascular SMC Dedifferentiation:

Previous studies have shown that AKT and GSK3β may either promote or suppress vascular SMC dedifferentiation and NI formation.[92–95] Accordingly, we determined the contribution of AKT and GSK3β to the CDK8 mediated stabilization of c-MYC and phenotype modulation in cultured RASMCs. A dose-response study revealed that AKT specific inhibitor MK-2066 (a highly selective inhibitor of AKT1/2/3 with IC50 of 8 nM/12 nM/65 nM, respectively) at doses from 4 nM to 3 μM dose-dependently suppressed FBS-induced phosphorylation of AKT (Thr308 and Ser473) and GSK3β (Ser9) and upregulation of c-MYC (Figure 3.8 and Figure 3.9). MK-2066 (0.65 μM) also inhibited FBS-induced cell number increases (Figure 3.10) as recently reported.[96] The Senexin A-induced suppression of AKT (Thr308) and GSK3β (Ser9) phosphorylation, c-MYC expression, and increased proliferation were additively enhanced by MK-2066 (Figure 3.9 and 3.10). Like Senexin A, MK-2066 also inhibited FBS-induced downregulation of CNN1 protein expression (Figure 3.11), which is in consistent with a

recent report which shows AKT inhibitor induced suppression of vascular SMC dedifferentiation in vivo.[95] Moreover, MK-2066 and Senexin A additively suppressed the FBS-induced downregulation of SMC contractile protein expression (Figure 3.11). These results indicate that CDK8 is an upstream operator of AKT/GSK3β signaling for c-MYC stabilization leading to vascular SMC dedifferentiation. To verify the notion, we used GSK3β specific inhibitor SB-216763 (IC50, 0.343 μM) at a dose of 0.01 μM, which was verified to maximally promote RASMC proliferation presumably due to the specific suppression of GSK3β-induced c-MYC degradation (Figure 3.12). These results are consistent with a previous report which shows GSK3β–dependent inhibitory effects on vascular SMC proliferation and NI formation.[93] Of note, the GSK3β inhibitor SB-216763 at doses from 0.01 μM to 25 μM was capable of upregulating AKT phosphorylation on both Thr308 and Ser473; however, at effective doses lower than 1 μM, SB-216763 upregulated c-MYC protein levels while at the doses higher than 10 μM, it downregulated c-MYC protein expression (Figure 3.12 upper and Figure 3.13). These results indicate that GSK3β inhibitor SB-216763 at doses higher than 1 μM could not inhibit GSK3β-mediated c-MYC degradation and instead may trigger c-MYC downregulation via yet known off-target effects in vascular SMCs. In addition, GSK3β acts as a negative regulator of AKT phosphorylation in vascular SMCs. Indeed, the pharmacological inhibition of GSK3β-mediated c-MYC degradation attenuated the inhibitory effect of Senexin A on c-MYC expression and cellular proliferation while suppressing the Senexin A-induced upregulation of SMC marker expression (Figure 3.13-3.15). Moreover, the Senexin A-induced growth inhibitor effect and upregulation of SMC contractile gene expression were also largely reversed by overexpression of c-MYC

(Figure 3.16). To this end, our findings collectively demonstrate that CDK8 activates AKT to suppress GSK3β-mediated c-MYC degradation thereby promoting RASMC dedifferentiation.

An Inhibitor Role of CDK8 in AKT Subcellular Compartmental Translocation for Activation in Vascular SMCs:

Although AKT signaling has been well implicated in vascular SMC biology and pathology, the molecular mechanisms for AKT activation in vascular SMCs are largely extrapolated from the other studies.[97] Accordingly, it has been assumed that in response to mitogen stimulation, AKT is translocated to the plasma membrane and then phosphorylated on two key residues, Thr308 and Ser473, by 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and mammalian target of rapamycin complex 2 (mTORC2) respectively, leading to AKT activation. Following activation, AKT detaches from the plasma membrane and translocates into the cytosol and nucleus to trigger its downstream signaling. To explore whether or not CDK8 interrupts the aforementioned signaling events for AKT activation in vascular SMCs, we determined a potential interaction between CDK8 and AKT in cultured RASMCs by confocal microscopy as well as immunoprecipitation (IP) and immunoblotting (IB) analyses. In the serum-starved SMCs, AKT was expressed in the nucleus, cytosol, and membrane, whereas CDK8 is expressed predominantly in the nucleus (Figure 3.17). Upon serum stimulation, AKT was translocated to the plasma membrane and the nucleus; however, CDK8 was translocated to the cytosol (Figure 3.17). Interestingly, inhibiting CDK8 activity via Senexin A (Sen A) enhanced the cytosolic translocation of CDK8 while suppressing AKT membrane and

nuclear translocation associated with AKT peri-nuclear accumulation (Figure 3.17). Notably, CDK8 was co-localized with AKT in the nucleus and peri-nuclear region (Figure 3.17). These results suggest that CDK8 physically associates with AKT to promote its translocation to both the plasma membrane and nucleus thereby facilitating AKT activation and AKT-driven signaling in vascular SMCs. Indeed, IP/IB studies revealed that CDK8 associated with AKT in the serum-starved SMCs and the CDK8 and AKT association was enhanced by serum stimulation; however, this association depended on CDK8 activity (Figure 3.18). Collectively, these results demonstrate that CDK8 kinase activity is critical for its association with AKT thereby activating AKT and AKT-driven signaling in vascular SMCs.

3.4 DISCUSSION:

Our previous studies demonstrate a critical role of CDK8 in SMC dedifferentiation with typical synthetic phenotypes including increased proliferation, migration and ECM secretion; however, the molecular mechanism by which CDK8 regulates SMC dedifferentiation was not addressed. In the present study we identified a novel signaling axis of CDK8-AKT activation-GSK3β inhibition-c-MYC stabilization in mediating SMC dedifferentiation. We uncovered that: activation of CDK8 promotes the activation AKT to suppress GSK3β-mediated phosphorylation of c-MYC at Thr58 for proteasomal degradation in RASMCs; CDK8 activates AKT to suppress GSK3β-mediated c-MYC degradation thereby promoting RASMC dedifferentiation; and CDK8 kinase activity is critical for its association with AKT thereby activating AKT and AKT-driven signaling in vascular SMCs.However, the precise mechanism by which CDK8

mediates vascular SMC dedifferentiation was not completely delineated in this study. Similar to Sen A drug treatment, AKT inhibitor MK-2066 (with IC50 of 8 nM/12 nM/65 nM, respectively) also inhibited FBS-induced downregulation of SMC markers CNN1 at 4nM dosage. Since the selected dosage was much lower than the recommonded IC50 dosage for AKT1 inhbition, it is mostly likely ATK1, can inhibit GSK3β-mediated c-MYC degradation, thereby promoting vascular SMC dedifferentiation. Further studies will need to be conducted to confirm the role of isoform AKT1 in SMC dedifferentation. Moreover, the observed CDK8-c-MYC signaling axis may not be the sole mechanism for facilitating vascular SMC dedifferentiation. Considering the observation that CDK8 primarily enabled the elongation of transcription of silent genes that become activated by transcription-induced factors,[51,52,56,76] usually in the pathological setting [98], it is possible that CDK8 may also control the transcription of a group of genes for vascular SMC dedifferentiation. Further investigation of these subjects will lead to a better understanding of CDK8-mediated vascular remodeling.

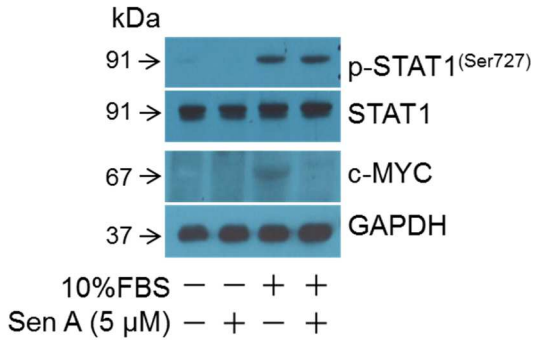

Figure 3.1. Senexin A treatment downregulated c-MYC protein expression but had no effect on STAT1 phosphorylation. Serum starved RASMCs were treated with 10% FBS and 5 μM Sen A for 30 min then subjected to Western blot analysis. Representative results are from 3 separate experiments.

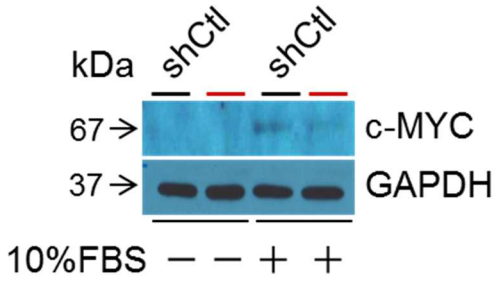

Figure 3.2. CDK8 inhibition by lentiviral approach reduced c-MYC protein expression. Serum starved RASMCs were treated with 10% FBS and 5 μM Sen A for 30 min then subjected to Western blot analysis. Representative results are from 3 separate experiments.

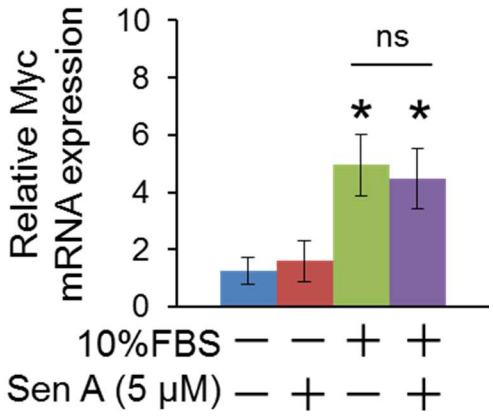

Figure 3.3. CDK8 inhibition did not affect *c-Myc* mRNA expression level. Serum starved RASMCs (24 h) were treated with 10% FBS and 5 μM Sen A for 30 min then subjected to qRT-PCR to determine relative *c-Myc* mRNA expression. Representative results are from 3 separate experiments.

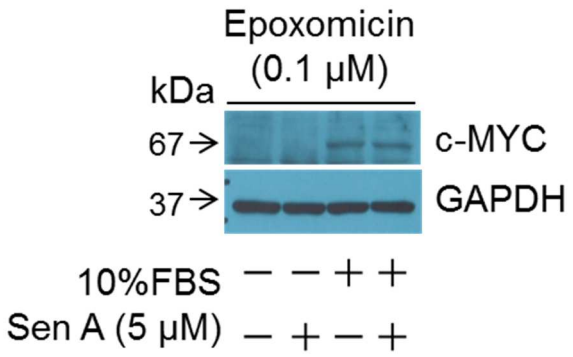

Figure 3.4. Senexin A treatment reduces c-MYC protein level of RASMCs by ubiquitin proteasomal degradation. Serum starved RASMCs were pre-treated with proteasome inhibitor, Epoxomicin at 0.1 μM, for 24 h and treated with 10% FBS and 5 μM Sen A for 30 min then subjected to Western blot analysis for c-MYC protein expression. Representative results are from 3 separate experiments.

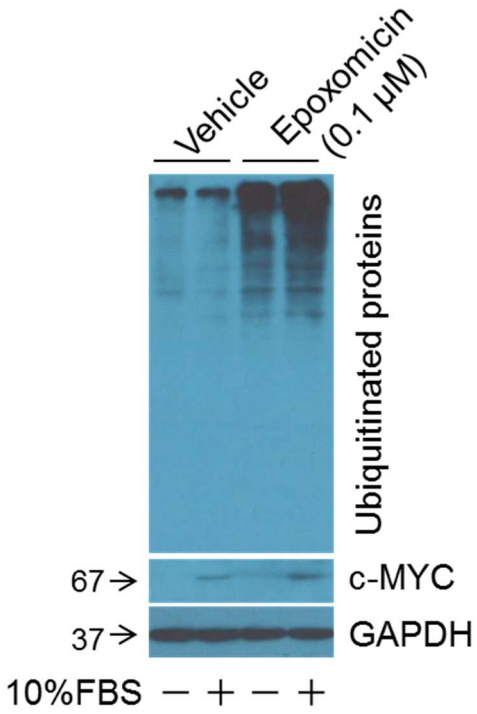

Figure 3.5. The efficacy of Epoxomicin on proteasome function in cultured RASMCs. Serum starved RASMCs were pre-treated with 0.1 μM Epoxomicin and 10% FBS for 24 hours then subjected to Western blot analysis. Representative results are from 3 separate experiments.

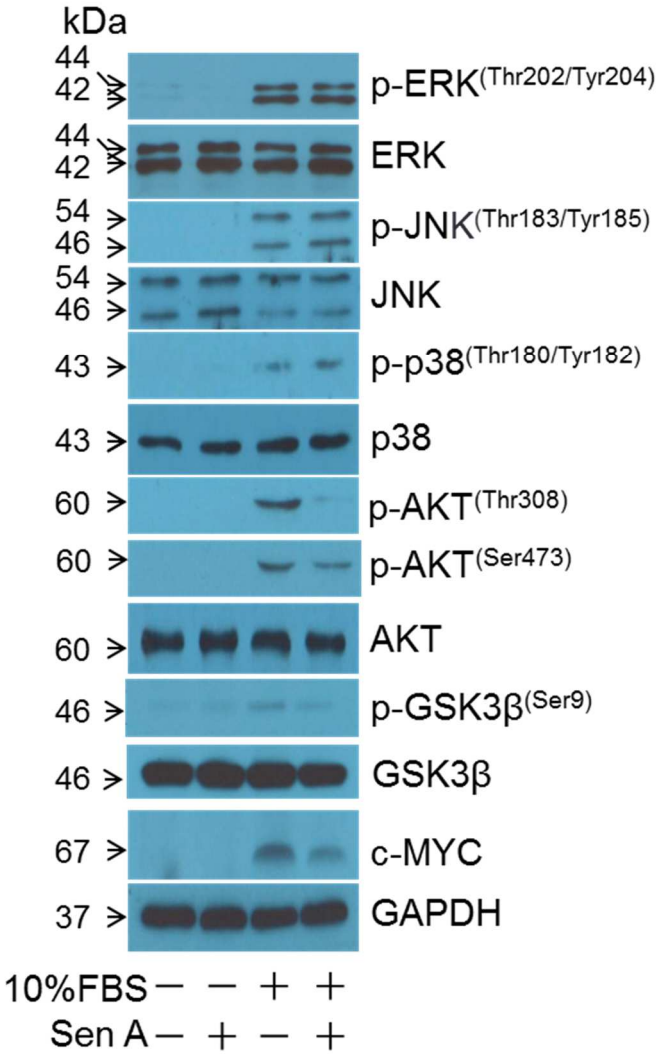

Figure 3.6. The effect of CDK8 inhibitor Senexin A on FBS-induced activation of MAPKs (ERK, JNK and p38) and AKT. Serum starved RASMCs were treated with 10% FBS and 5 μM Sen for 10 minutes and subjected to Western blot analysis of potential signaling pathways involved. Representative results are from 3 separate experiments.

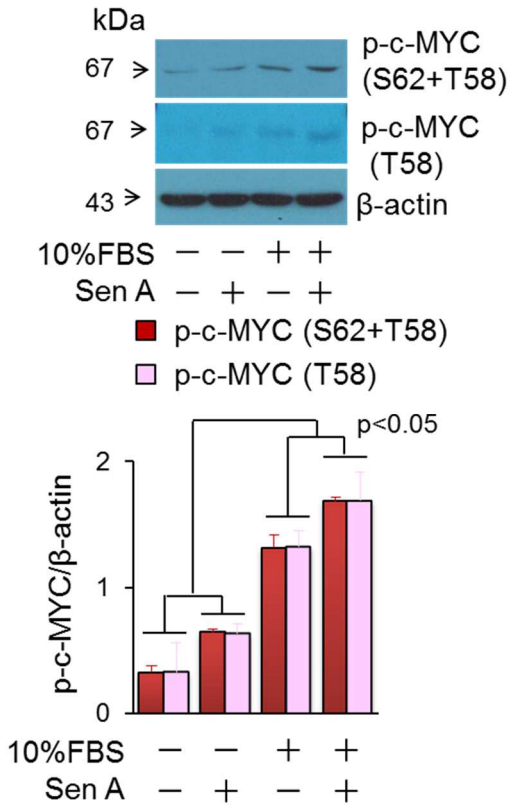

Figure 3.7. CDK8 inhibitor Senexin A increased c-MYC phosphorylation. Serum starved RASMCs were treated with 10% FBS and 5 μM Sen for 30 minutes and subjected to Western blot analysis. Representative results are from 3 separate experiments.

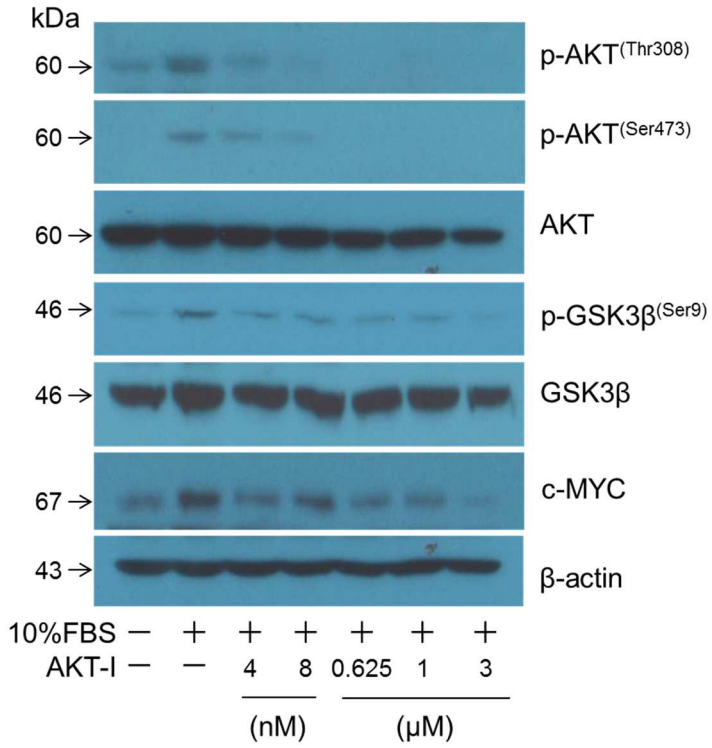

Figure 3.8. The dose-response of AKT inhibitor (AKT-I) MK-2066 on FBS-induced activation of AKT, inhibition of GSK3β, and upregulation of c-MYC. Serum starved RASMCs were treated with 10% FBS and 5 μM Sen for 30 minutes and subjected to Western blot analysis. Representative results are from 3 separate experiments.

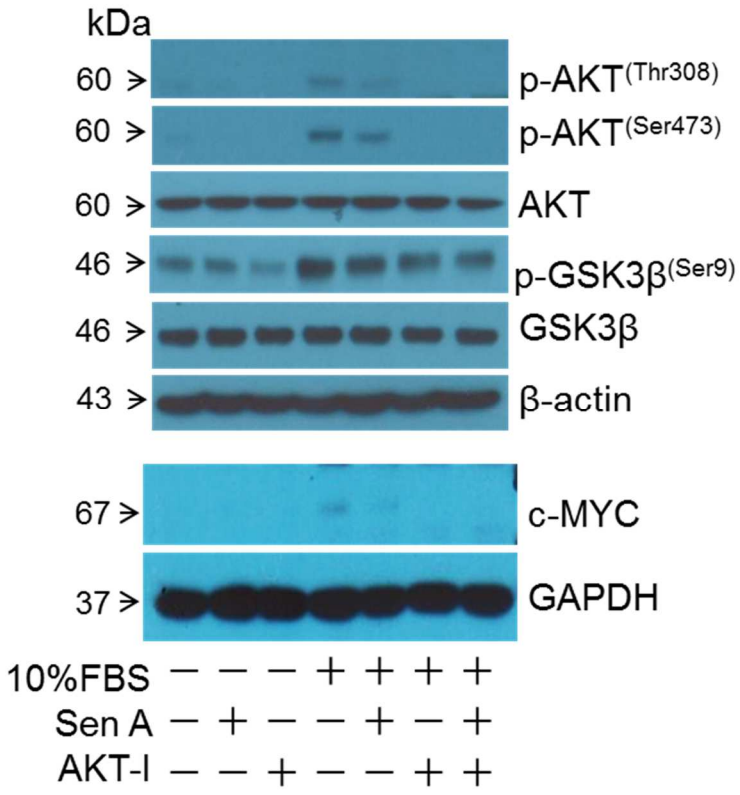

Figure 3.9. CDK8 inhibitor Senexin A and AKT inhibitor MK-2066 behave similarly in FBS-induced activation of AKT/GSK3β pathway for c-MYC stabilization. Serum starved RASMCs were treated with 10% FBS, 5 μM Sen, and 8 μM MK-2066 for 30 min and subjected to Western blot analysis. The representative results are from 3 separate experiments.

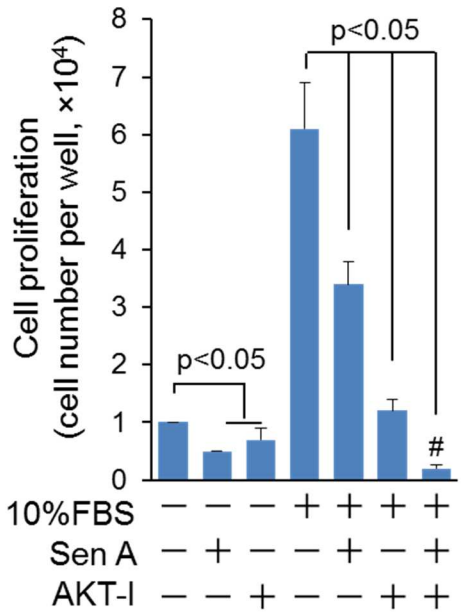

Figure 3.10. AKT inhibitor, MK-2066, inhibited FBS-induced cell proliferation. Serum starved RASMCs at were treated with 10% FBS, 5 um Senexin A, or 0.65 µM MK-2066 for 24 h and subjected to [3H]-thymidine uptake assay to measure DNA synthesis. Representative results are from 3 separate experiments. n=3, #§<0.05 vs. 10%FBS (+) alone.

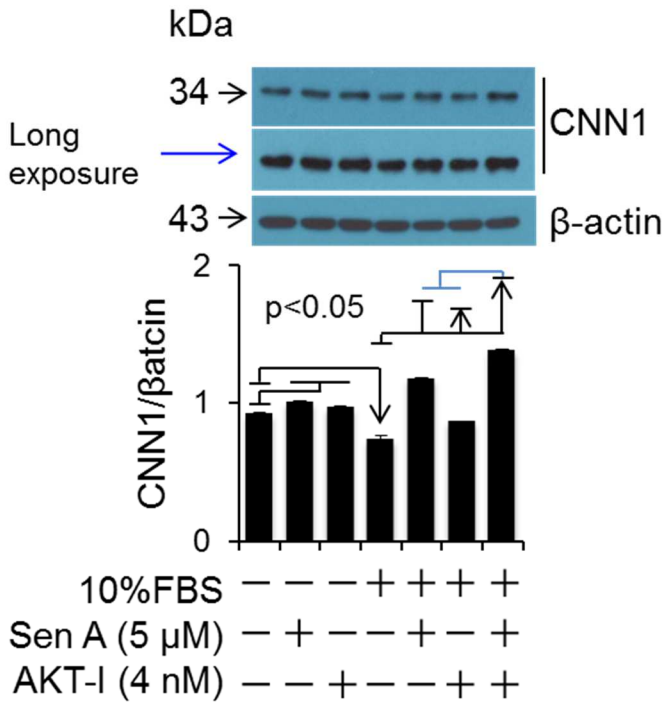

Figure 3.11. The effect of AKT inhibitor (AKT-I) and Senexin A on FBS-induced downregulation of SMC contractile proteins. Serum starved RASMCs at were treated with 10% FBS, 5 um Senexin A, and 4 nM MK-2206 (AKT-I) for 48 h and subjected to Western blot. The upper panel shows representatives of immunoblots from 3 separate experiments. The lower panel shows the semi-quantified results of CNN1 protein expression. * $p<0.05$ vs. the control (-) in the same group.

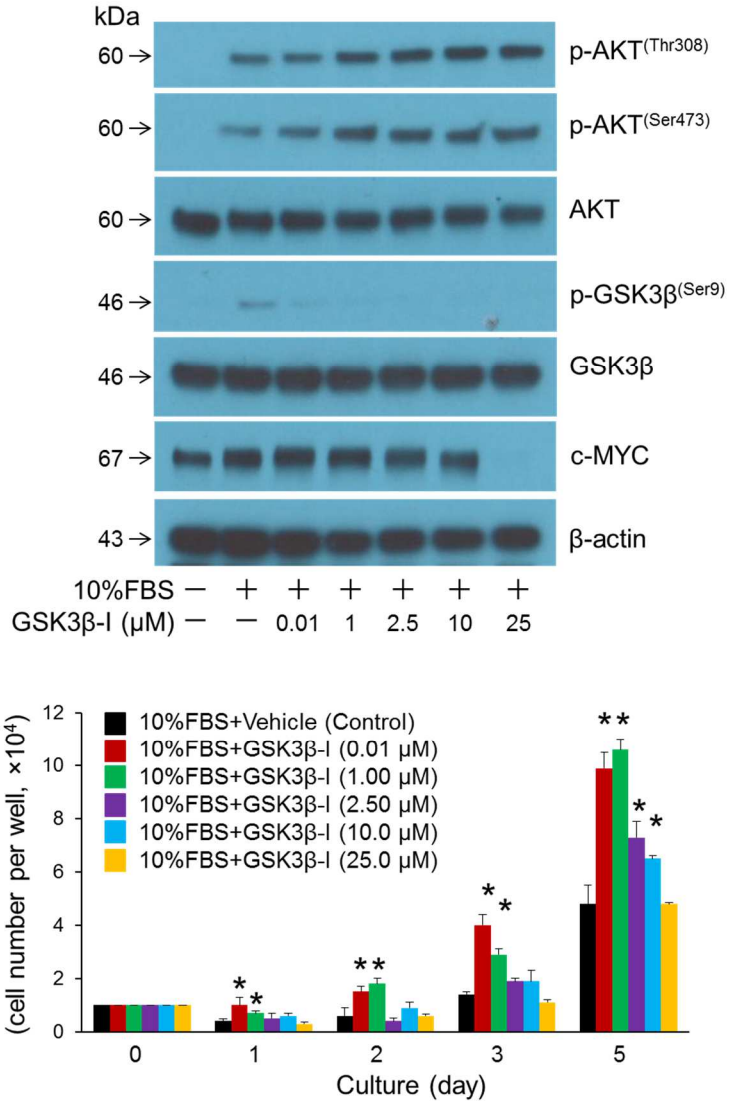

Figure 3.12. GSK3β inhibitor (GSK3β-I) SB-216763 can dose-dependently regulate AKT activity, GSK3 β activity, and c-MYC stability in cultured RASMCs, thereby affecting RASMC proliferation. A. Serum starved RASMCs were treated in 10% FBS with GSK3β-I at various dosages for 30 min and subjected to Western blot anaylsis of PI3K-AKT signaling casade. B. Serum starved RASMCs were treated in RGM with various dosages of GSK3β inhibitor (GSK3β-I) SB-216763 and the proliferation was measured

by a hemocytometer. n=6, * $p<0.05$ vs. the 10%FBS + Vehicle control group at each culture day.

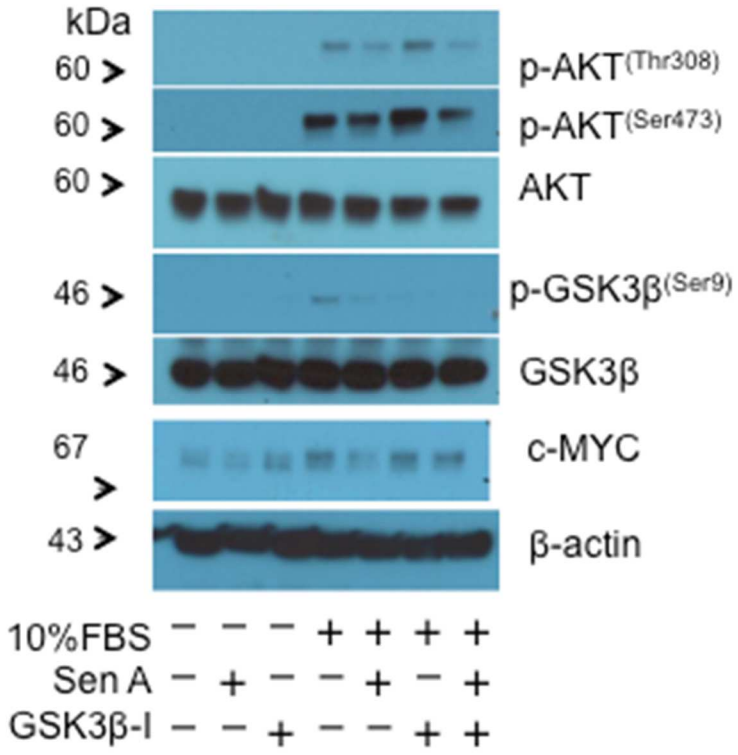

Figure 3.13. CDK8 inhibitor Senexin A and GSK3β-I have opposing effects on FBS-induced activation of AKT/GSK3β pathway for c-MYC stabilization. Serum starved RASMCs were treated with 10% FBS, 5 μM Sen, and 0.01 μM GSK3β-I (SB-216763) for 30 min and subjected to Western blot analysis. Representative results are from 3 separate experiments.

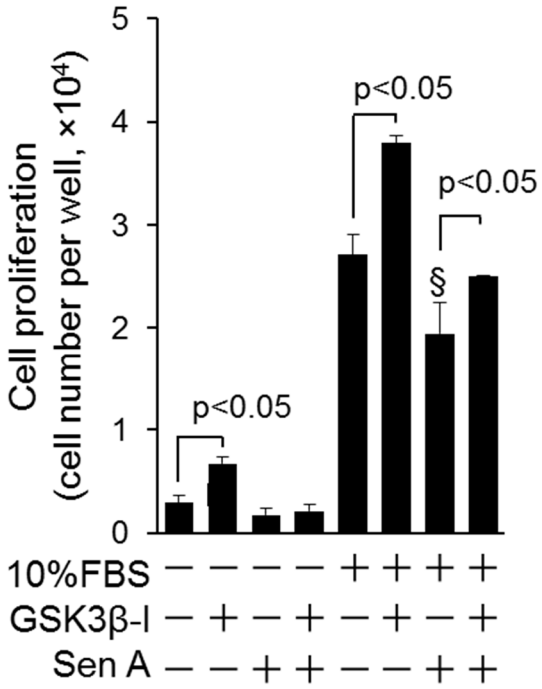

Figure 3.14. GSK3β inhibitor (GSK3β-I) SB-216763 increased FBS-induced cell proliferation. Serum starved RASMCs at were treated with 10% FBS, 5 um Senexin A, and 0.01 μM GSK3β-I (SB-216763) for 24 h and subjected to [3H]-thymidine uptake assay to measure DNA synthesis. n=3, #§<0.05 vs. 10%FBS (+) alone.

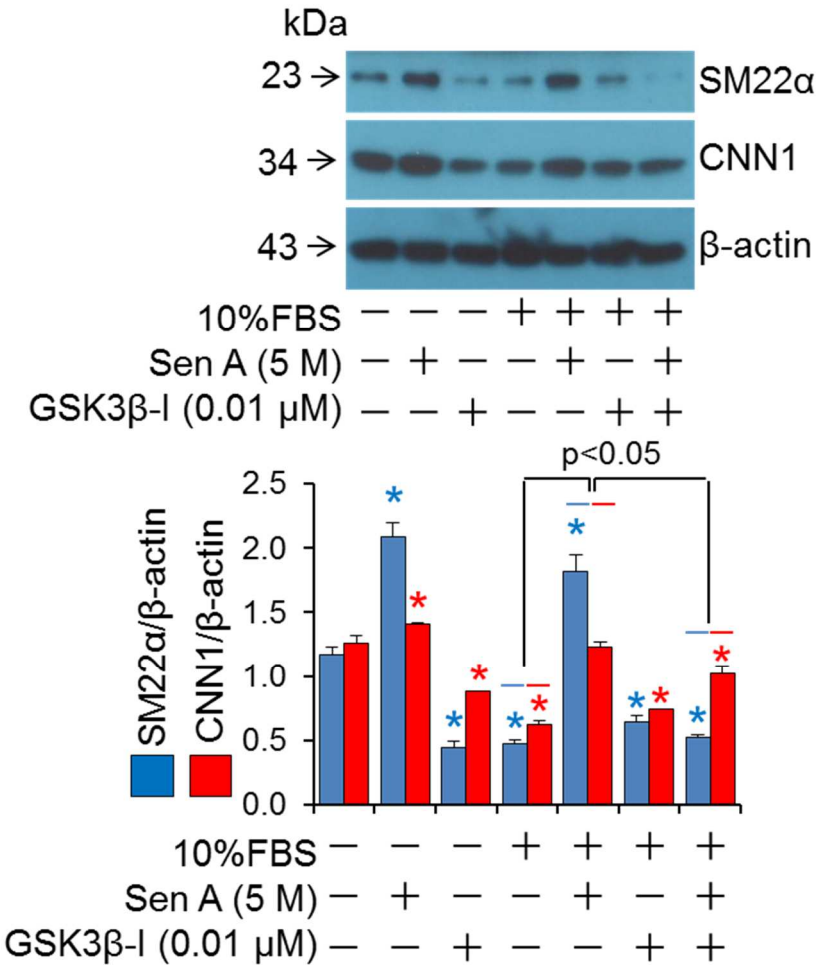

Figure 3.15. GSK3β inhibitor (GSK3β-I) SB-216763 and Senexin A have opposing effects on FBS-induced downregulation of SMC contractile protein markers in cultured RASMCs. Serum starved RASMCs were treated with 10% FBS, 5 μm Senexin A, and 0.01 μM GSK3β-I (SB-216763) for 48 h and subjected to Western blot. The upper panel shows representatives of immunoblots from 3 separate experiments. The lower panel shows the semi-quantified results of SM22α and CNN1 protein expression. * $p<0.05$ vs. the control (-) in the same group.

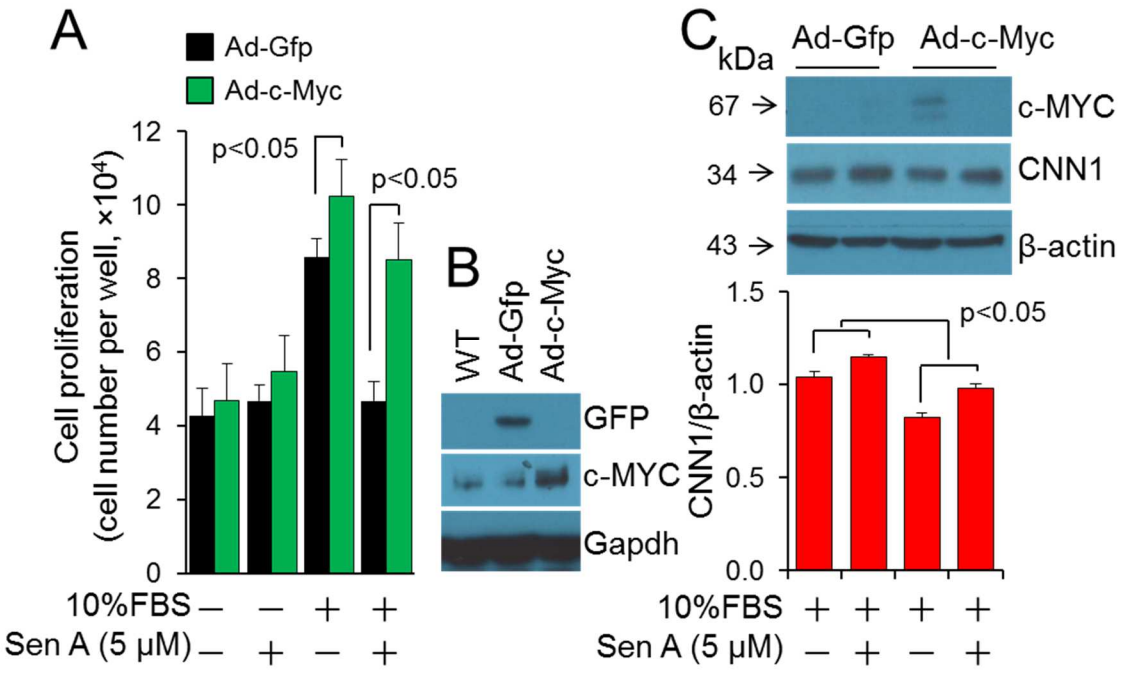

Figure 3.16. Adenoviral overexpression of c-MYC attenuated the effect of Senexin A (Sen A) on RASMC proliferation and SMC contractile protein CNN1 expression. RASMCs were adenovirally transduced with adenoviral GFP or adenoviral c-MYC and cultured in RGM. A. Serum starved adenoviral RASMCs at were treated as indicated 3 days and measured by a hemocytometer (n=3). B. Adenoviral RASMCs were subjected to Western blot analysis of GFP and c-MYC protein markers. C. Adenoviral RASMCs treated with 10% FBS and 5 μM Sen A for 48 h and subjected to Western blot analysis. Representative results are from 3 separate experiments.

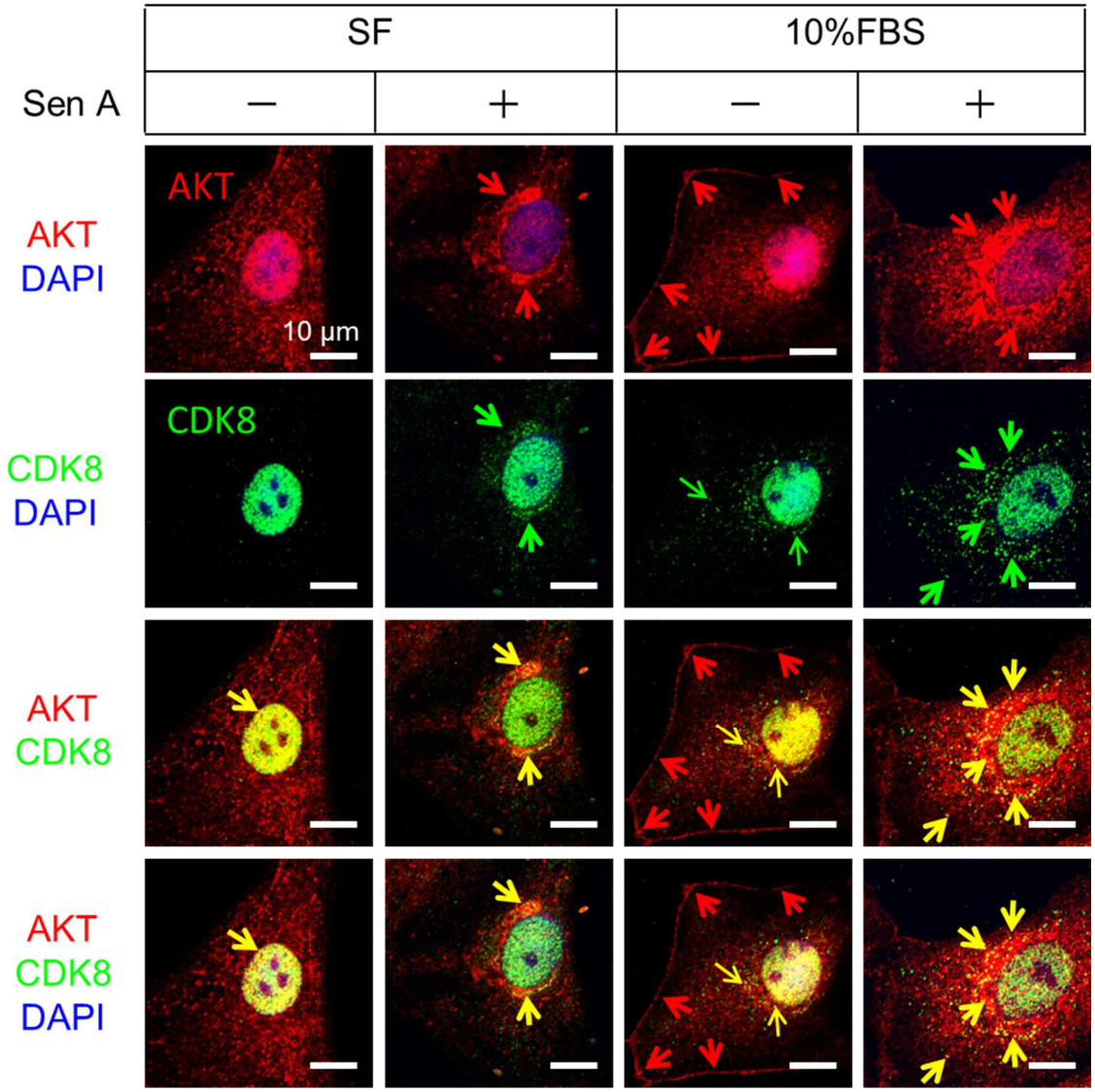

Figure 3.17. The effect of CDK8 inhibitor Senexin A on FBS-induced AKT membrane and nuclear translocation. A. Serum starved RASMCs were treated with 10% FBS and 5 μM Sen A for 30 min and subject to confocal microscopy analysis. Typical confocal microscopic images are representative of 3 separate experiments.

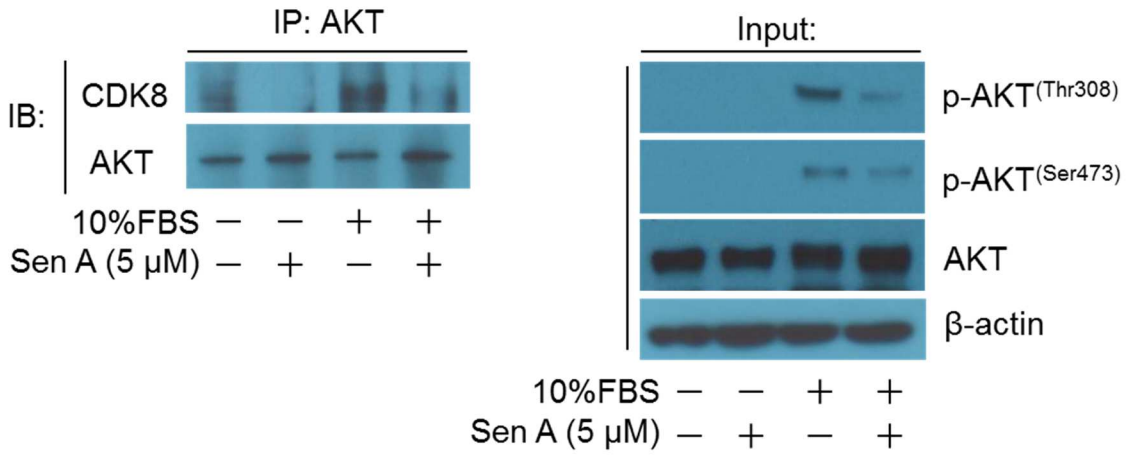

Figure 3.18. The effect of CDK8 inhibitor Senexin A on FBS-induced AKT and CDK8 association. Serum starved RASMCs were treated with 10% FBS and 5 μM Sen A for 30 min then subjected to immunoprecipitation with AKT antibody followed by Western blot analysis. The representative results are from 3 separate experiments.

CHAPTER 4

CYCLIN-DEPENDENT KINASE 8-MEDIATED VASCULAR SMOOTH MUSCLE CELL DEDIFFERENTIATION CONTRIBUTES TO NEOINTIMAL FORMATION

4.1 INTRODUCTION:

Vascular disease remains to the leading cause of mortality in the United States and worldwide.[1,2] Neointimal formation (NI) is a key event in atherosclerosis and several other vascular diseases. NI is caused by an abnormal increase in cell population within the innermost layer of the arterial wall, resulting in a plaque formation that will eventually occlude the vessel. The underlying causes of NI include SMC migration, proliferation, and dedifferentiation.[68,83] The neointima consists mainly of SMCs, however myofibroblasts and fibroblasts have also been reported to contribute to the neointima.[81] The carotid ligation surgery performed in mice has historically been used as a model to study vascular disease.[99] The neointimal SMCs that arise following carotid artery ligation are largely derived from dedifferentiated vascular SMCs.[63–65,67–69,81]

Our previous studies indicate that CDK8 is a positive regulator in SMC dedifferentiation via AKT/GSK3β-mediated enhancement of c-MYC stabilization. Due to these results, we predicted that CDK8 might also play a role in vascular lesion formation.

To investigate the role of CDK8 in vascular lesion formation, we performed carotid ligation surgery on mice and tested the effects of CDK8 inhibition in neointimal

formation by utilizing a specific inhibitor of CDK8, Senexin A.[61] We hypothesized that CDK8 inhibition can reduce neointimal formation after carotid ligation surgery in mice. In this report, we demonstrate that CDK8 is a critical mediator of vascular SMC dedifferentiation and NI formation at least partly via its ability to facilitate activation of the AKT/GSK3β/c-MYC stabilization signaling axis in vascular SMCs. Therefore, targeting CDK8 may be a novel therapeutic approach for the treatment of occlusive vascular disease due to the abnormal growth of vascular SMCs.

4.2 MATERIALS AND METHODS:

Immunoprecipitation, Immunoblotting and Immunohistochemical Analyses:

Immunoprecipitation, immunoblotting, and immunohistochemical staining were performed as we previously reported.[83,85,100] For confocal microscopic analysis of CDK8 and AKT expression and localization in vascular SMCs, RASMCs (P6-10) at 80% confluent state were serum starved overnight prior to the treatment with vehicle DMSO (0.2%, vol/vol) and 5 μM Senexin A (0.2% DMSO, vol/vol) in serum-free (SF) DMEM or full growth medium for 30 min. These cells were then washed with ice-cold PBS twice and fixed in 4% paraformaldehyde for 10 min at room temperature. After treatment with 0.01% triton X 100 for 1 min, all cells were incubated with 1% BSA at room temperature for 1 h. Primary antibodies used were anti-AKT (Cat#: 2920, 1:200 dilution, Cell Signaling), anti-CDK8 (Cat#: SC1521, 1:500 dilution, Santa Cruz Biotechnology) and anti-AKT (Cat#: 2920, 1:200 dilution, Cell Signaling). Secondary antibodies used were Alexa Fluor 488 (Donkey anti-Rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Cat#: A-21206, 1:500 dilution, Invitrogen) and Alexa Fluor 546 (Donkey anti-

Mouse IgG (H+L) highly cross-adsorbed secondary antibody, Cat#: A-10036, 1:500 dilution, Invitrogen). The images were obtained under the same exposure condition. The expression of CDK8 in tissue sections of human coronary arteries with atherosclerotic lesions was also determined by confocal microscopic analysis using the antibodies above mentioned. Fluorescent images were acquired using Zeiss LSM 510 META confocal scanning laser microscope.

Carotid Ligation Model:

Male C57BL/6J mice at age of 12 wks were used for complete carotid artery ligation as previously described.[99] Briefly, the mice were anesthetized by intraperitoneal injection of a solution of xylazine (5 mg/kg body weight) and ketamine (80 mg/kg body weight). The left common carotid artery was dissected and ligated near the carotid bifurcation. At each experimental end point, these mice were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) and euthanized by exsanguination. Blood in the vessel was washed out by 0.9% NaCl perfusion for 5 min. The right carotid artery was not ligated and served as an internal control for morphological measurement. However, sham operated carotid arteries which were subjected to the same procedure except for the ligation were used as the sham control for signaling dissection. This is because in this mouse model, the complete ligation hardly induces neointimal hyperplasia but does have impact on gene expression in the contralateral carotid artery.[99,101] In an effort to establish the complete carotid artery ligation model, we performed several sets of experiments to characterize the complete ligation-induced inward remodeling in carotid arteries. Whole carotid arteries were harvested at the experimental end points. From the ligation point or carotid bifurcation (internal control), serial cross-sections (5-

μm thick per section) were then cut in 5-mm length, resulting in 1000 sections per carotid artery. Fifty sections located at 100-μm intervals from the ligation site or the carotid bifurcation was sampled and numbered (100-μm × 50 = 5-mm) for each carotid artery. We found that 30%~40% of ligated carotid arteries developed neointimal hyperplasia. In addition, the intimal hyperplasia of the ligated carotid arteries (n=10) mainly occurred within 2-3 mm of the vessel distal to the ligation site, which was maximal closest to the ligation site, decreased in the thickness in the direction to the aortic arch, and disappeared within the proximal 2-3 mm of the vessel adjacent to the aortic arch as previously reported. [99]Hence, the segments (~5-mm) from the ligation site or bifurcation to the proximal site of the vessel adjacent to the aortic arch of sham-operated and ligated carotid arteries (n=10) were excised and subject to Western blot analysis as previously described.[100] The contralateral (internal control) and ligated carotid arteries in a length of 1 mm from the ligation site or bifurcation to the aortic arch were subject to morphological measurements of neointima formation, lumen patency, and cellular marker expression. Briefly, after flushing out the blood, these arteries were fixed with 4% paraformaldehyde under physiological pressure of 100 mmHg (13.3 kPa) for 10 min. Whole left and right carotid arteries without the proximal ~2-mm of the vessel adjacent to the aortic arch were harvested and further fixed with 4% phosphate-buffered formaldehyde at 4°C for 24 h. The fixed arteries were embedded in paraffin or OCT for sectioning. All carotid arteries were sectioned (5-μm thick per section) from the distal end. We analyzed 5 sections located at 100-μm intervals from the ligation site or bifurcation to the aortic arch of each control and ligated carotid arteries with or with Senexin A treatment. These sections were subject to hematoxylin and eosin (H&E) or

immunofluorescence staining. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The primary antibody for CDK8 staining was anti-CDK8 (Cat#: 22067-1-AP, 1:500 dilution, Proteintech). The images were obtained under the same exposure condition. Fluorescent images were acquired using Zeiss LSM 510 META confocal scanning laser microscope.

Drug Treatment:

Senexin A was delivered locally as follows: Senexin A (30 μM/L) or vehicle control DMSO (0.3%, vol/vol) in 100 μl 20% Pluronic F-127 gel (Cat: BCBH4538V, Sigma-Aldrich) was delivered to the adventitia of ligated arteries immediately after ligation.

Statistics:

Data are shown as mean ± SEM. Differences between 2 groups were evaluated for statistical significance using the Student t test when the sample size was appropriate and the population was distributed normally. When differences among > 3 groups were evaluated, results were compared by one-way ANOVA with Bonferroni test for multiple comparisons. Differences were considered significant at $p < 0.05$.

4.3 RESULTS:

A Mediator Role of CDK8 in Vascular SMC Dedifferentiation and NI formation in vivo:

Our in vitro results encouraged us to examine the pathological relevance of the CDK8 signaling in vivo. Using a mouse model of NI formation in the carotid artery induced by complete ligation in which the majority (~80%) of NI cells is derived from mature vascular SMCs,[81] we found that peri-vascular delivery of Sen A (30 μM/L) in 20% Pluronic F-127 gel immediately after ligation procedure dramatically suppressed the

activation of AKT and the upregulation of c-MYC while reversing the downregulation of SMC markers such as αSMA, CNN1, and SM22α on 3 day after ligation (Figure 4.1), and significantly inhibited SMC accumulation in the NI (Figure 4.2) and NI formation 28 day post-ligation (Figure 4.3). These findings reveal a potential role of the newly identified CDK8/AKT/GSK3β/c-MYC stabilization-signaling axis in mediating vascular SMC dedifferentiation and NI formation (Figure 4.4).

4.4 DISCUSSION:

Our previous studies uncover for the first time a novel signaling axis of CDK8-AKT activation-GSK3β inhibition-c-MYC stabilization in mediating vascular SMC dedifferentiation, thus providing new insight into the pathogenesis of occlusive vascular disease associated with SMC dedifferentiation. Because of the firmly established concept that the abnormal growth and accumulation of dedifferentiated vascular SMCs largely contribute to vascular lesion formation,[63–65,67–69,81] most of the therapeutic approaches for vascular diseases, in particular occlusive vascular disease, have focused on either direct or indirect inhibition of SMC proliferation in diseased vessels such as failing vein grafts .[102,103] However, none of them are truly effective, thus the 10-year vein graft failure rates (~50%) have remained largely unchanged over the last two decades.[102,103] The underlying reasons may be multifactorial, but a plausible interpretation is that the master regulators of vascular SMC dedifferentiation are still missing. In this regard, our serial findings indicate that CDK8 may be such a regulator. Firstly, we demonstrated that inactivation of CDK8 led to the suppression of vascular SMC dedifferentiation with typical synthetic phenotypes including increased proliferation, migration, and ECM secretion in vitro.

Secondly, we established that inactivation of CDK8 inhibited SMC accumulation in NI and NI formation of ligated carotid arteries, in which the majority (~80%) of NI cells is derived from mature vascular SMCs.[81] Finally, we further confirmed the critical role of CDK8 in mediating the vascular SMC-dependent NI formation in other models of occlusive vascular remodeling, i.e., the transplantation of isologous jugular veins and the transplantation of decellularized aorta scaffolds in which we verified a major contribution of mature vascular SMCs to NI formation by genetic inducible fate mapping approach [104] using myh11-CreERT2::Rosa26Floxed-Stop-eYFP mice (unpublished data). Given the availability of highly selective small molecule inhibitors of CDK8,[105] these findings provide the rationale for the translational study of CDK8 inhibitors on the prevention and/or treatment of vascular lesion formation.

There are several issues requiring future studies. The mediator role of CDK8 in vascular SMC dedifferentiation leading to vascular lesion formation needs to be verified by genetic interrogations including mature vascular SMC specific CDK8 knockout approach in vivo. The potential off-target effects of CDK8 inhibitors also need to be evaluated via aforementioned genetic interrogations. Further investigation of these subjects will lead to a better understanding of CDK8-mediated vascular remodeling and dysfunction in pathological settings, therefore paving the way to develop novel therapeutic approach to target CDK8 for vascular disease.

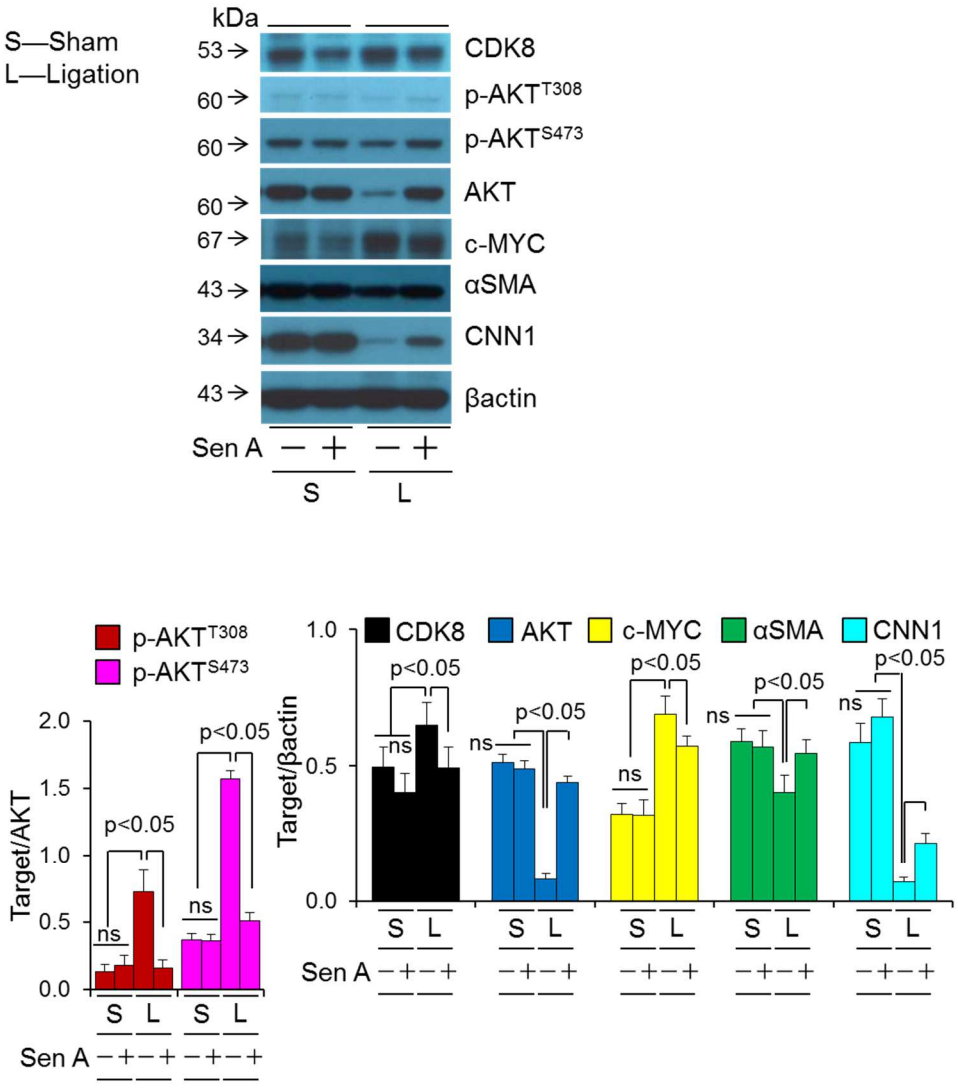

Figure 4.1. Peri-vascular delivery of Senexin A is a mediator of AKT-c-MYC signaling and SMC dedifferentiation. Male adult C57BL/6J mice (n=20) were subjected to sham or complete carotid artery ligation with perivascular delivery of vehicle control or Sen A (30 μM/L) after 3 days. Two pools of carotid arteries (n=10 for each pool of each group) were subjected to Western blot analysis as indicated (upper panel). Lower left panel is the semi-quantified analysis of immunblots (n=4).

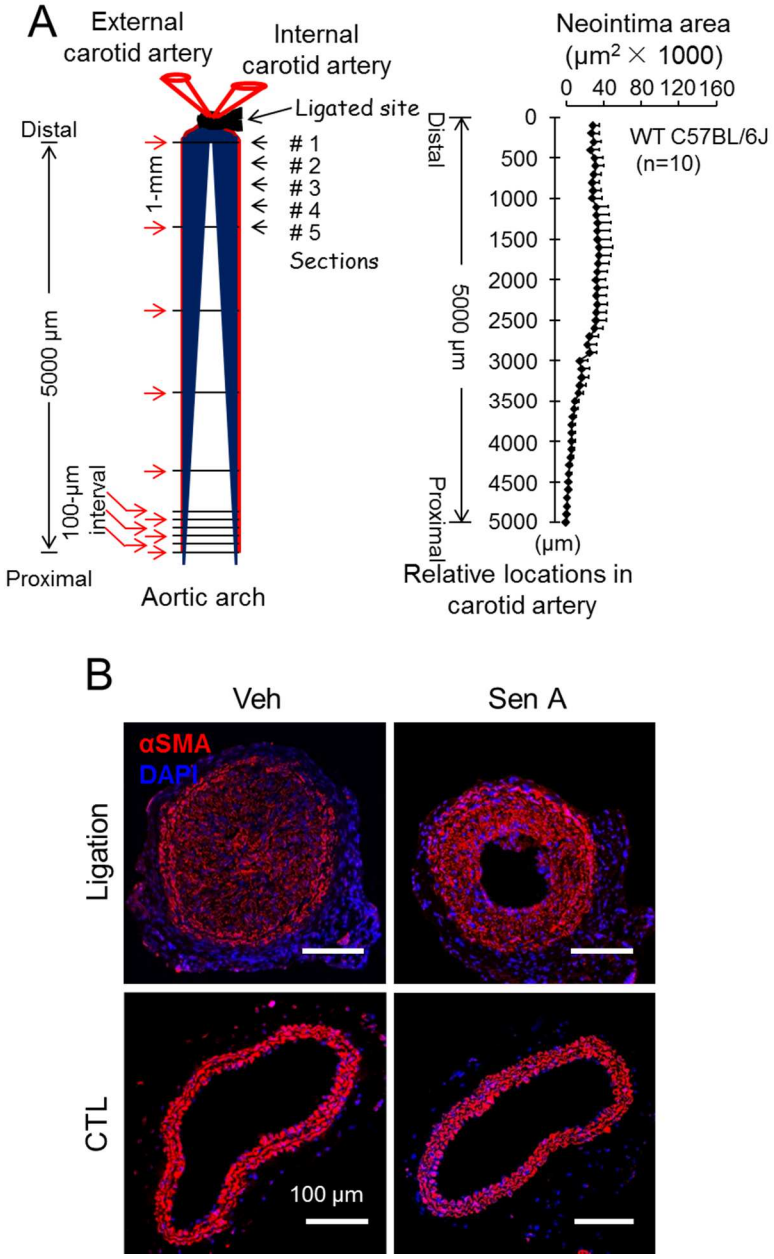

Figure 4.2. Peri-vascular delivery of Senexin A significantly inhibited SMC accumulation in the NI**.** Male adult C57BL/6J mice (n=10) were subjected to sham or complete carotid artery ligation with perivascular delivery of vehicle control or Sen A (30 µM/L) for 28 days. A. Characterization of complete ligation-induced neonitima formation in the carotid artery. B. αSMA expression in unligated and ligated carotid arteries of C57BL/6J mice (n=10) at 28 days after operation was analyzed by confocal microscopy. The representatives of αSMA staining are from 20 sections (n=4, 5 sections from each mouse) from confocal microscopy. Veh, vehicle; Sen A, senexin A; CTL, contralateral unligated carotid arteries.

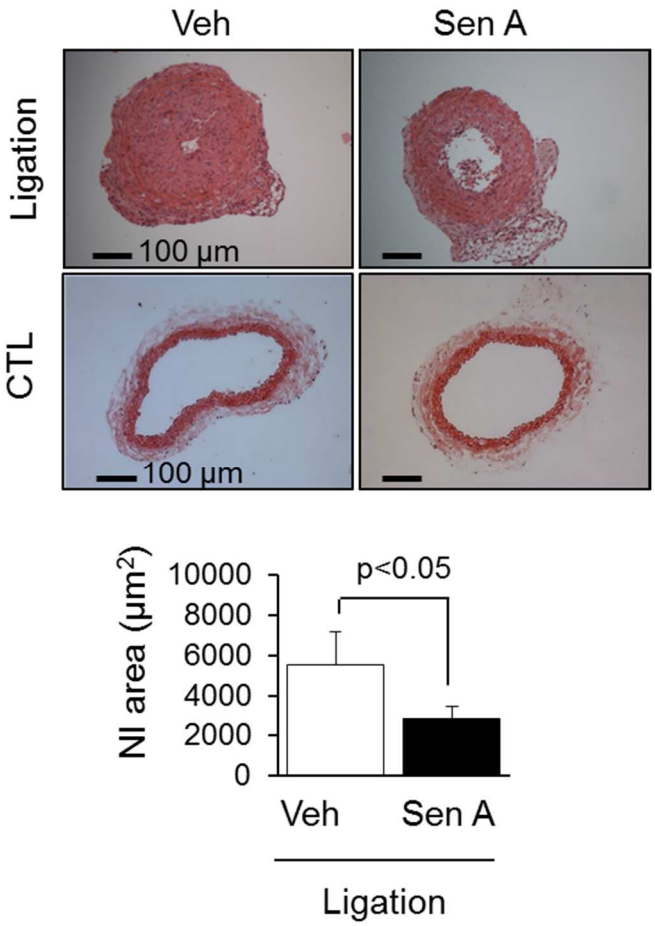

Figure 4.3. Peri-vascular delivery of Senexin A significantly inhibited NI**.** Male adult C57BL/6J mice (n=10) were subjected to sham or complete carotid artery ligation with perivascular delivery of vehicle control or Sen A (30 µM/L) for 28 days. Upper panel is the representative H&E staining and lower panel shows the quantified NI area using Image J Pro software.

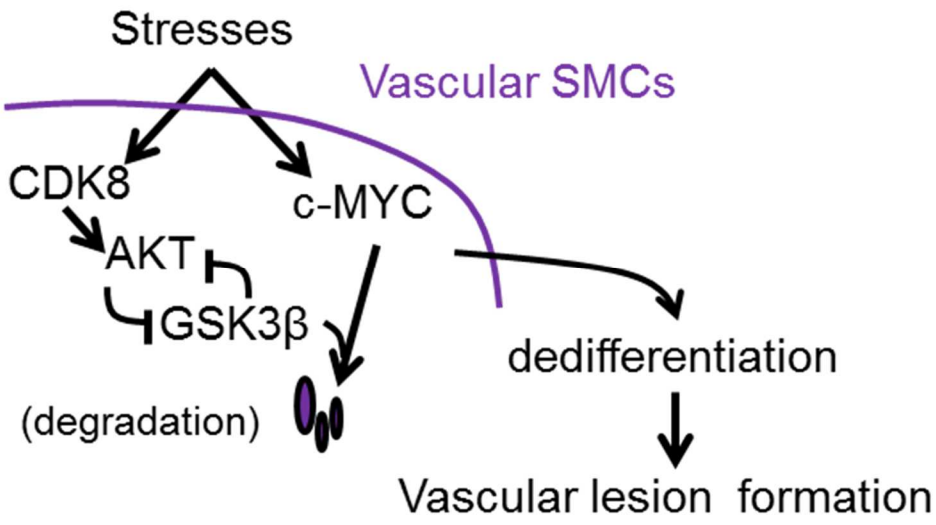

Figure 4.4. The potential role of CDK8/AKT/GSK3β/c-MYC stabilization signaling axis in mediating vascular SMC dedifferentiation and lesion formation. CDK8 is a critical mediator of SMC dedifferentiation at least partly by facilitating the AKT-mediated inhibition of GSK3β to stabilize c-MYC, thereby inducing dedifferentiation and vascular lesion formation.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Occlusive vascular disease, characterized by abnormal proliferation and accumulation of vascular SMCs in the vascular lesion, is one of the most common causes of morbidity and mortality in USA.[62] Upon disease or injury mature and quiescent vascular SMCs undergo dedifferentiation which is characterized by a downregulation of SMC-specific contractile markers, and a concomitant with an increase in cell proliferation, migration, and ECM secretion, thereby leading to vascular lesion formation.[63–69] However, the mechanisms regulating the process of SMC dedifferentiation remain unclear. The current study provides novel findings of a potential role of CDK8 in SMC dedifferentiation. We also characterized the molecular mechanisms through which CDK8 regulates SMC dedifferentiation, as well as the pathophysiological relevance of CDK8 in vivo. We hypothesized that CDK8 is a critical mediator in SMC dedifferentiation and vascular lesion formation.

For Aim 1,we demonstrated for the first time that CDK8 is normally expressed in endothelial cells and vascular SMCs, thus suggesting that CDK8 is expressed under normal physiological conditions in the vasculature. Moreover, CDK8 was shown to be upregulated in dedifferentiating vascular SMCs, suggesting that CDK8 may play a role in the pathological process of vascular disease. CDK8 inhibition was able to regulate all three events by promoting increased proliferation, migration and ECM secretion as well as promoting a synthetic phenotype.

For Aim 2, we identified a novel signaling axis of CDK8-AKT activation-GSK3β inhibition-c-MYC stabilization in mediating SMC dedifferentiation. We uncovered that: activation of CDK8 promotes the activation AKT to suppress GSK3β-mediated phosphorylation of c-MYC at Thr58 for proteasomal degradation in RASMCs; CDK8 activates AKT to suppress GSK3β-mediated c-MYC degradation thereby promoting RASMC dedifferentiation; and CDK8 kinase activity is critical for its association with AKT thereby activating AKT and AKT-driven signaling in vascular SMCs.

For Aim 3, we demonstrated that inactivation of CDK8 led to the suppression of vascular SMC dedifferentiation with typical synthetic phenotypes including increased proliferation, migration, and ECM secretion in vitro. Secondly, we established that inactivation of CDK8 inhibited SMC accumulation in NI.

In conclusion, our results demonstrate that CDK8 is a critical mediator of vascular SMC dedifferentiation at least partly by facilitating the AKT-mediated inhibition of GSK3β to stabilize c-MYC, thereby contributing to NI formation. Therefore, targeting CDK8 may be a novel therapeutic approach for the treatment of occlusive vascular disease due to the abnormal growth of vascular SMCs.

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APPENDIX A. PATIENT MEDICAL HISTORY

Table A.1. Tissue sections of arteries were prepared from autopsy organ specimens of humans with a cardiovascular disease history.

Specimen #	Sex	Age	Disease history	Arterial occlusion
14607	Male	97	Hypertension; Prostate cancer	Left coronary artery (moderate atherosclerosis); Left circumflex coronary artery (mild to moderate atherosclerosis); Left anterior descending coronary artery (mild to moderate atherosclerosis)
14608	Male	60	Septic shock; Disseminated intravascular coagulation	Right coronary artery (mild atherosclerosis); Left anterior descending coronary artery (mild atherosclerosis)
14609	Male	71	Coronary artery disease; Bypass graft 2015; Acute Myocardial Infarction (left ventricle interventricular septum)	Left anterior ventricle circumflex coronary artery (sever atherosclerosis); Left anterior descending coronary artery (sever acute and chronic atherosclerosis); Left posterior ventricle posterior descending coronary artery (sever atherosclerosis)
14612	Male	73	Chronic heart failure	Aorta (mild atherosclerosis)
14613	Female	71	Hypertension; Myocardial rupture	Aorta (mild to moderate atherosclerosis)
14610	Female	80	Hypertension; Asthma; Lung (squamous cell carcinoma)	Right coronary artery (minimal to mild atherosclerosis)