Role of c-Kit in Atherosclerosis: A Mechanistic Study

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UNIVERSITY OF MIAMI

ROLE OF c-KIT IN AHEROSCLEROSIS: A MECHANISTIC STUDY

By
Lei Song

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
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ROLE OF c-KIT IN ATHEROSCLEROSIS:
A MECHANISTIC STUDY

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**Rationale:** c-Kit, as a receptor tyrosine kinase (RTK), is present in multiple cell types including vascular smooth muscle cells (SMCs). However, so far, limited progress has been made to understand how c-Kit mediates SMC biology, and whether the presence of this receptor is critical for vascular pathogenesis such as atherosclerosis.

**Objectives:** In this dissertation, I investigated the roles of c-Kit in atherosclerosis and underlying mechanisms.

**Methods:** c-Kit distribution was determined in both murine and human aortae using a combination of Kit$^{Bac}$-eGFP reporter mice, immunofluorescence (IF), and Western blot. c-Kit deficiency studies were performed in c-Kit mutant (Kit$^{W/W^-}$) mice on an ApoE$^{-/-}$ background, which were fed for 16 weeks on a high fat diet (HFD) to induce hyperlipidemia. Cell specific c-Kit roles were investigated using: 1) chimeric mice with global Kit$^{W/W^-}$ ApoE$^{-/-}$ cells but Kit$^{+/+}$ ApoE$^{-/-}$ bone marrow cells, and 2) c-Kit SMC conditional knockout (Kit$^{lox66-71/lox66-71}$ Myh11CreER$^{T2}$ ApoE$^{-/-}$) mice. Mechanistic studies were performed using primary aortic SMCs isolated from c-Kit mutant (Kit$^{W/W^-}$) or wild type control (Kit$^{+/+}$) mice. Rescued c-Kit SMCs were created by transfecting a murine Kit gene expressing plasmid into the c-Kit deficient (Kit$^{W/W^-}$) SMCs. All these
SMCs were treated with various atherosclerotic stimuli (e.g., cholesterol or phospholipid) to determine the roles of c-Kit in the context of hyperlipidemia.

**Results:** IF analysis of the transgenic Kit$^{Bac}$-eGFP reporter mice revealed that c-Kit was present in arteries but not veins. Further confocal microscopy analysis of murine aortic tissue demonstrated that the expression of c-Kit was co-localized with SMC marker smooth muscle actin (SMA). Similarly, c-Kit expression was also found in human arterial samples, as determined by IF and Western blot. In c-Kit loss of function studies, both atherosclerotic burden and the lesion site macrophage-like SMC (SMA$^+$, CD68$^+$) ratio were found significantly elevated in hyperlipidemic c-Kit mutant (Kit$^{W/W^-}$ ApoE$^{-/-}$) mice compared to their control littermates (Kit$^{+/+}$ ApoE$^{-/-}$). c-Kit positive bone marrow transplantation (BMT) to hyperlipidemic c-Kit deficient (Kit$^{W/W^-}$ ApoE$^{-/-}$) mice failed to alleviate the atherosclerotic phenotype. However, cell specific deletion of c-Kit in SMCs led to exacerbated atherosclerosis in hyperlipidemic mice. These findings pointed out that the observed c-Kit protective role in atherosclerosis is SMC specific. In mechanistic studies, primary aortic SMCs from c-Kit-deficient (Kit$^{W/W^-}$) mice exhibited a “synthetic” phenotype with decreased contractile marker expression, abnormally accelerated growth, and unexpected migration compared to the aortic SMCs isolated from c-Kit wild type (Kit$^{+/+}$) mice. Furthermore, when treated with cholesterol, these synthetic SMCs were prone to acquire a “foam cell-like (or macrophage-like)” phenotype with enhanced expression and activity of Kruppel-like factor 4 (KLF4), a transcriptional factor that was previously reported to favor the SMC phenotype transformation. Restoration of c-Kit in mutant (Kit$^{W/W^-}$) SMCs decreased the expression and activity of KLF4, subsequently abolished the “foam cell-like” phenotype. In addition, loss of c-Kit led to an
inflammatory state in SMCs. By using a high-throughput microarray assay and in silico pathway analysis, NF-κB signaling related gene expression was found upregulated in mutant (Kit\(^{W/W-V}\)) SMCs, but not in wild type (Kit\(^{+/+}\)). Upon the stimulation with oxidized phospholipid, these c-Kit mutant (Kit\(^{W/W-V}\)) SMCs presented enhanced NF-κB activity with increased NF-κB signaling regulated pro-inflammatory mediator expressions compared to wild type (Kit\(^{+/+}\)) SMCs. Further, c-Kit suppression of NF-κB activity in SMCs was in a TGFβ-activated kinase 1 (TAK1) and a Nemo-like kinase (NLK) dependent manner.

**Conclusions:** This work demonstrates for the first time that vascular expression of c-Kit protects against atherosclerosis, and provides mechanistic evidence suggesting a fundamental role of c-Kit in preserving SMCs in a contractile state, therefore preventing their synthetic, foam- cell-like, and pro-inflammatory phenotype switch under various atherosclerotic stimuli.
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First of all, I would like to express the deepest appreciation to my mentor, Dr. Roberto Vazquez-Padron, who introduced me to the field of cardiovascular research, for his academic guidance and financial support all through these years of this Ph.D. thesis work. As the first Ph.D. student of Dr. Vazquez-Padron, I think I am the lucky one who received special attention and care. With his guidance, not only I gained prevalent, state-of-art experimental technologies and skills, such as transgenic animal generation, cellular genetic engineering, surgical murine bone marrow transplantation, as well as scientific related abilities as critical thinking, experimental design, and troubleshooting etc., but more importantly, he inspired me and triggered my interest to discover, think, and solve academic problems, independently. Also, I am very impressive about his meticulous way of dealing with science and details. Brainstorm is great, but careful bench work and accurate data analysis make a truly professional and reliable scientist. Besides labs and classrooms, moreover, I was encouraged to broaden my scope of the field and have had the opportunities to present my research in oral talks or posters at scientific conferences during my Ph.D. training, at least annually, since 2013. Listening to the authorities and communicating with the peers, I was able to get access to latest findings and achievements in the field, also to open my mind and re-think my research with a different perspective, which is extremely beneficial for my thesis work and academic maturity. Thus, I feel truly blessed to have him as my Ph.D. advisor and am extremely grateful for given opportunities and training I received. Without his guidance, limited progress I may achieve today. Therefore, I highly believe that all these years of intensive and
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AHA</td>
<td>American heart association</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ART1B</td>
<td>Angiotensin II receptor type 1B</td>
</tr>
<tr>
<td>AVF</td>
<td>Arteriovenous fistula</td>
</tr>
<tr>
<td>AVR1A</td>
<td>Arginine vasopressin receptor 1A</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone marrow cell</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood count</td>
</tr>
<tr>
<td>CETP</td>
<td>Cholesteryl ester transport protein</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated protein kinase</td>
</tr>
</tbody>
</table>
FACS, fluorescence-activated cell sorting
FBS, fetal bovine serum
GIST, gastrointestinal stromal tumor
GPCR, G-protein coupled receptor
HDL, high density lipoprotein
HFD, high fat diet
HSC, hematopoietic stem cell
IB, immunoblotting
IF, immunofluorescence
IP, immunoprecipitation
KD, knockdown
KLF-4, Kruppel-like factor 4
KO, knockout
LDL, low density lipoprotein
MCP-1, monocyte chemotactic protein-1
MHP, myosin heavy chain
MMP-2, 9, matrix metalloproteinase-2, 9
NF-κB, nuclear factor κB
NIH, neo-intimal hyperplasia
NLK, nemo-like kinase
OCT, optimum cutting temperature
ox-LDL, oxidized low-density lipoprotein
PC, progenitor cell
PDGFR, platelet-derived growth factor receptor
PI3K, phosphatidylinositol 3 kinase
PFA, paraformaldehyde
PGK, phosphoglycerate kinase
PMSF, phenylmethylsulfonyl fluoride
POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine
PTB, phosphotyrosine binding
PVD, peripheral vascular disease
RBC, red blood cell
RTK, receptor tyrosine kinase
SCF, stem cell factor
SH-2, Src homology 2
siRNA, small interfering RNA
SMA, smooth muscle actin
SMC, smooth muscle cell
SRF, serum response factor
TAK-1, transforming growth factor (TGF) beta-activated kinase 1
TAM, tamoxifen diet
TG, triglycerides
VEGFR, vascular endothelial growth factor receptor
VLDL, very low density lipoprotein
BACKGROUND

Atherosclerosis and its subsequent complications, including cardiac infarction, cerebral stroke, kidney failure, gangrene, etc., account for the leading cause of death worldwide (Weber & Noels, 2011). Unfortunately, limited progress has been achieved to fundamentally understand the origin of atherosclerosis over decades. To date, novel studies are emerging to improve the treatment for this life-threatening disease.

Atherosclerosis is a cardiovascular disease (CVD) with plaque buildup inside the artery wall. Plaque formation occurs as a consequence of chronic endothelial dysfunction, monocyte recruitment, lipoprotein deposition, and tunica smooth muscle cells (SMCs) migration (Bentzon, Otsuka et al., 2014, Falk, 2006, Singh, Mengi et al., 2002).

Typically, the atherosclerotic lesion is underneath the endothelium, containing oxidized lipid, immune cell debris, calcium, and centered with a necrotic core to form a “fatty streak”. The fatty streak is further covered with a “fibrous cap”, which is made of SMC and extracellular matrix (ECM) to form a plaque (Bentzon et al., 2014, Crowther, 2005).

Plaque formation alters the lesion site micro-environment and hemodynamic cue. For example, it narrows down the luminal area which termed as “stenosis”, resulting in the change of blood flow, shear stress, and blood pressure. In the late stage of atherosclerosis, due to SMCs apoptosis and ECM degradation, the weakened fibrous caps make the plaque vulnerable and prone to rupture, which may cause luminal hemorrhage and thrombosis formation, leading to devastating consequences such as patients’ morbidity or mortality (Gutstein & Fuster, 1999).

Of note, as the main component of the fibrous cap, vascular SMCs determine the plaque formation and rupture. Therefore, they attract intensive research attention. Upon
pathogenic stimuli such as hyperlipidemic (Pidkovka, Cherepanova et al., 2007), pro-inflammatory (Ross & Glomset, 1973), and mechanical (Gomez & Owens, 2012, Orr, Hastings et al., 2010) stress, vascular SMCs transform from a quiescent "contractile" to proliferative "synthetic" phenotype. These synthetic SMCs lose their original contractile marker expression and exhibit hyper-dynamic cellular activities, such as increased cell proliferation and migration. Consequences of this synthetic transformation are complicated. On one hand, SMC proliferation and migration contribute to the plaque formation in the initiation stage of atherogenesis (Doran, Meller et al., 2008); on the other hand, these hyper-dynamic cellular activities paradoxically reinforce the fibrous cap, and then stabilize the plaque when atherosclerosis comes to the end stage (Rosenfeld, 2015). Interestingly, growing evidence suggests that, these cap-forming synthetic SMCs are prone to uptake cholesterol from the hyperlipidemic milieu, therefore gaining a “foam cell-like (or macrophage-like)” phenotype (Pidkovka et al., 2007, Rong, Shapiro et al., 2003, Rosenfeld, 2015, Shankman, Gomez et al., 2015). These signature characters include altered cell morphology, disrupted cytosol architecture, exacerbated cellular lipid deposition and macrophage marker expression. These transformed SMCs retain the lipid within the plaque, meanwhile, they impair the fibrous cap stability due to their synthetic characters.

In addition, these proliferative synthetic SMCs have been found to secrete pro-inflammatory cytokines, chemokines, and adhesion molecules. This secretion profoundly manipulates processes such as monocyte recruitment, macrophage infiltration, and foam cell differentiation (Doran et al., 2008, Raines & Ferri, 2005, Singer, Salinthone et al.,
which may lead to the amplification of the inflammatory response and thus worsened atherogenesis.

As a cell surface receptor super family, receptor tyrosine kinase (RTK) has been identified as therapeutic target for vascular pathogenesis. Various family members, such as platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR), have been investigated for their role in atherosclerosis. Mechanistic studies have linked PDGFR activation to SMC phenotypical transformation, through promoting contractile protein degradation and SMC migration (Kawada, Upadhyay et al., 2009, Salabei, Cummins et al., 2013, Sano, Sudo et al., 2001); meanwhile, increased VEGFR activity induces migration and proliferation of vascular SMCs, and an inflammatory prone state of the vessel due to enhanced permeability of vascular endothelial cells (ECs) (Bates, 2010).

As a member of RTK family, c-Kit (CD117) was previously reported to regulate differentiation, proliferation, and survival in a variety of cell types (Roberts & Govender, 2015) (Lennartsson & Ronnstrand, 2012). Gain and loss of c-Kit function have been implicated in carcinogenesis (Roberts & Govender, 2015) and stem cell biology (Lennartsson & Ronnstrand, 2012, Smith, Lewis et al., 2014). More recently, studies present evidence for the expression of c-Kit and its unique ligand, stem cell factor (SCF), in the vasculature on vascular ECs and SMCs (Hollenbeck, Sakakibara et al., 2004, Matsui, Wakabayashi et al., 2004, Miyamoto, Sasaguri et al., 1997). However, to the best knowledge, studies showing an involvement of c-Kit in vascular disease so far is limited to descriptive data that relate elevated c-Kit presence in SMCs with the progression of
Consistent with a positive role in NIH, c-Kit expression in bone marrow (BM) cells was proposed to increase disease progression by mediating the recruitment of progenitor cells to the injury site (Hollenbeck et al., 2004, Wang, Anderson et al., 2006a, Wang, Verma et al., 2007). However, interestingly, the pathogenic character of circulating c-Kit positive cells in NIH contradicts recent studies demonstrating the lack of contribution from BM derived-progenitor cells to the neointimal lesion (Hoofnagle, Thomas et al., 2006).

Consequently, the contribution of c-Kit in vascular disease remains controversial and not sufficiently explored. Based on these observations, my thesis aims at understanding the role of c-Kit in atherosclerosis. To achieve this, I propose to pursue the following specific aims:
SPECIFIC AIMS

Specific Aim 1: Determine the role of c-Kit in atherosclerosis.

Though the presence of c-Kit in vSMC was previously reported, its role in vascular pathogenesis such as atherosclerosis remains elusive. My central hypothesis was that, similar to other receptor tyrosine kinases (RTKs), c-Kit signaling is pro-atherogenic in mice. To test this hypothesis, I used a c-Kit deficient mouse model with a heterozygous mutation of Kit<sup>W</sup> and Kit<sup>W.v</sup> in both alleles. This compound mutation leads to minimal kinase activity, which allows animals to survive gestation (Nocka, Tan et al., 1990, Reith, Rottapel et al., 1990). Both c-Kit deficient (Kit<sup>W/W-v</sup>) mice and their wild type (Kit<sup>+/+</sup>) control littermates were bred with ApoE knockout (KO) mice. These hyperlipidemic prone (Kit<sup>W/W-v</sup> ApoE<sup>−/−</sup> or Kit<sup>+/+</sup> ApoE<sup>−/−</sup>) mice were fed 16 weeks on high fat diet (HFD) to develop atherosclerotic lesions (Nakashima, Plump et al., 1994). Then I compared the severity of atherosclerosis based on following aspects: (1) atherosclerotic burden in aortae and aortic sinus valves were examined by Sudan IV staining; (2) the lesion site macrophage-like SMC (SMA<sup>+</sup>, CD68<sup>+</sup>) ratio was determined by IF; (3) serum samples were prepared for lipid profile evaluation. I expected that the loss of c-Kit in hyperlipidemic mice alleviates lipid accumulation in murine aortae and sinus valves, decreases the atherosclerotic lesion site macrophage-like SMC ratio, and lowers the circulatory cholesterol and lipoprotein levels.

Specific Aim 2: Demonstrate the role of vascular c-Kit in atherosclerosis.

Preliminary studies have eliminated the possibility that, c-Kit positive hematologic stem cells (HSCs) and progenitors are responsible for its observed athero-protection role by
bone marrow transplantation (BMT) experiment. In which c-Kit deficient mice were engrafted with c-Kit positive BM cells but failed to alleviate atherosclerosis after 16 weeks of HFD (Figure 12 and Table 3). Therefore, I hypothesized that rather than bone marrow derived c-Kit, vascular c-Kit predominately plays a protective role in atherosclerotic mice. To test this hypothesis, a Cre-Lox based SMC specific c-Kit deletion mouse model (Kit^{lox66-71/lox66-71 Myh11CreER^{T2}}) was generated. With Tamoxifen chow (TAM) application, the mutant loxP sites, lox66 and lox71 present in these mice were flipped, limiting c-Kit deletion to Myh11 positive SMCs (Kimura, Ding et al., 2011). These triple transgenic mice were bred with ApoE KO mice and fed for 16 weeks on a HFD. The severity of atherosclerosis was evaluated as mentioned previously in Specific Aim 1. I anticipated c-Kit SMC conditional knockout mice would develop intensified lipid accumulation in aortae and sinus valves, and that the lesional macrophage-like SMC ratio and serum cholesterol and lipoprotein levels would be increased compared to their control littermates.

Specific Aim 3: Investigate the mechanism how c-Kit mediates SMC biology in atherogenesis.

Vascular SMCs are key participants in atherogenesis, involved in multiple processes such as early stage plaque formation, sterile inflammation, and end stage plaque rupture. Therefore, to obtain a comprehensive understanding of c-Kit action in SMCs, this specific aim was divided into three sub aims:
Specific Aim 3.1: Determine if loss of c-Kit facilitates the phenotypic switch of SMC from contractile to synthetic.

Due to the plasticity, SMCs undergo a phenotypic transformation in response to the environmental cue change during atherogenesis. This compromised phenotype is termed “synthetic”, characterized by abnormal cellular activities such as proliferation and migration (Owens, 2007). Synthetic SMCs facilitate the plaque formation in the early stage of atherogenesis. My first mechanistic hypothesis was that loss of c-Kit favors the synthetic phenotype in SMCs. To validate this, I isolated aortic SMCs from both c-Kit mutant (Kit<sup>W/W-<sup>v</sup></sup>) and wild type (Kit<sup>+/+</sup>) mice, then compared: (1) cell morphology and cytosol architecture, (2) contractile marker (e.g. Calponin, SM22α) expression; (3) cell growth rate, and (4) cell migration ability. I expected that, c-Kit deficient (Kit<sup>W/W-<sup>v</sup></sup>) SMCs exhibit a synthetic phenotype with downregulation of contractile marker expression, increased cell proliferation and migration compared to those Kit<sup>+/+</sup> control SMCs.

Specific Aim 3.2: Demonstrate that lack of c-Kit promotes SMC to foam cell-like transformation.

Except for synthetic phenotype switch, SMCs undergo a cell type compromise with lipid phagocytosis and foam cell/ macrophage marker expression (e.g., CD68) in atherogenesis (Bennett, Sinha et al., 2016). I hypothesized that c-Kit inhibits SMC to foam cell-like phenotype transformation in the context of atherosclerosis. To validate this, both c-Kit deficient and wild type control SMCs were exposed to water soluble cholesterol for 72 hrs (Pidkovka et al., 2007). To compare their ability of phagocytosing lipids, I performed:
Sudan IV staining of cultivated cells to evaluate cellular lipid accumulation, and (2) quantitatively determined the cholesterol content in cytosol extracts. Additionally, Kruppel-like factor 4 (KLF4), a transcriptional factor, was previously proven to mediate SMC lipid intake and favors SMC macrophage-like phenotype transformation in atherosclerotic mice (Rosenfeld, 2015, Shankman et al., 2015, Shankman, Gomez et al., 2016). Thus, I further hypothesized that c-Kit prevents SMC cellular lipid intake in a KLF4 dependent manner. Specifically, I knocked down (KD) KLF4 in c-Kit deficient SMCs (Kit<sup>W/W-v</sup>) by siRNA lentivirus transfection, and then tested their ability to uptake lipids using a Sudan IV staining. I expected that loss of c-Kit leads to exacerbated cytosolic lipid deposition, and that the macrophage-like phenotype in c-Kit deficient SMCs would be abolished in KLF4 KD cells.

**Specific Aim 3.3 Validate c-Kit deficient SMC is pro-inflammatory.**

In preliminary studies, our team employed a high-throughput microarray assay to screen gene expression in both c-Kit deficient and wild type SMCs. By using an in silico pathway analysis, I found that the expression of genes related to NF-κB, a classic inflammatory prone signaling, was upregulated in Kit<sup>W/W-v</sup> SMCs (Figure 32 and Table 5, 6). Therefore, I hypothesized that loss of c-Kit determines the pro-inflammatory state in SMC. To confirm this, I loaded both Kit<sup>W/W-v</sup> and Kit<sup>+/+</sup> SMCs with a synthesized phospholipid, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), and then compared the NF-κB signaling transcriptional activity and pro-inflammatory mediator production (e.g. MCP-1, MMP-2) (Pegorier, Stengel et al., 2006, Pidkovka et al., 2007, Vladykovskaya, Ozhegov et al., 2011) between the two groups of SMCs. In
addition, preliminary studies indicated that the expression of TAK1 and NLK was downregulated or even absent in c-Kit deficient SMCs (Figure 34A). Interestingly, literature reveals that NF-κB signaling is activated through the TAK1-NLK axis in multiple cell lines (Ajibade, Wang et al., 2012, Li, Zhang et al., 2014, Yasuda, Yokoo et al., 2004). Therefore, I hypothesized that c-Kit inhibits NF-κB in a TAK1-NLK dependent manner in SMCs. To validate this, I knocked down TAK1 and NLK, respectively in Kit^{+/−} SMCs by siRNA lentivirus transfection, then determined the NF-κB transcriptional signaling activity and the expression of downstream inflammatory mediators. I expected to observe increased NF-κB transcriptional activity and pro-inflammatory mediator production in c-Kit mutant SMCs. Furthermore, knockdown of TAK1 and NLK in c-Kit wild type SMCs would lead to enhanced NF-κB transcriptional activity and pro-inflammatory mediator production.
CHAPTER 1 INTRODUCTION

1.1. Overview of Atherosclerosis

1.1.1 History of Atherosclerosis

Atherosclerosis is a disease in which plaque builds up in the inner layer of the arteries.
The atherosclerotic lesion, also known as atheroma or plaque, is chemically composed of fat, cholesterol, calcium, blood, and extracellular matrix proteins. The cellular composition of the plaque mainly consists of smooth muscle cells (SMCs), macrophages, foam cells, and other inflammatory cells. The cellular and chemical composition of the plaque dynamically alters over time, to harden the lesion and narrow the luminal area of arteries, which limits the flow of blood to the body (Ross, 1986). It is known that atherosclerosis affected humans in ancient Africa (Thompson, Allam et al., 2013), and early North America (Holman, Mc et al., 1958). Besides human and non-human primates (Clarkson, Koritnik et al., 1985), it also affects herbivore animals such as pigeons (Lofland & Clarkson, 1959), rabbits (Bragdon, 1952), and chickens (Orita, Masegi et al., 1994) fed on a fat rich diet. These animals fail to tolerate saturated fat and cholesterol as carnivore animals do, due to the differences in digestive system and immune system (Roberts, 2000).

Historically, understanding of this disease is merely limited to the description of the pathological changes in the vessel, characterized by loss of lumen area and thickening of the vessel wall. The Latin word “athero-” indicates the gruel- or porridge-like lesion; meanwhile “-sclerosis” delineates the thicken and harden of the intima layer in the vessel. From centuries ago, Leonardo da Vinci observed this disease in an old man’s autopsy, and noted his findings as “arteries are very dry, shrunken and withered”, and “the coat of
arteries thicken so much that they close themselves up and stop the movement of the blood” (Boon, 2009), which depicted the end stage of atherosclerosis: the close of the vessel, and resulting death.

Tracing back to the fifties and sixties of the last century, modern medicine defined atherosclerosis as a lipid metabolism dysfunction, resulting in lipid deposition within a degenerative arterial lesion termed as “atheroma” (Vastesaeger & Delcourt, 1962). It was noticed by that time that the atheroma can narrow or even block the arteries and as a result, it restricts the oxygen-rich blood flow feeding the essential organs in the body. It was also postulated that the formation of the plaque could be the consequence of compressive contributing factors, such as diet, physical actives, and hormones (Holman et al., 1958).

1.1.2 Current Prevalence of Atherosclerosis

Atherosclerosis and its complications, including coronary heart disease (CHD), cerebrovascular disease (Goto, Baba et al., 2003), peripheral vascular disease (PVD) (Libby, 2009), and kidney disease (Balla, Nusair et al., 2013), cause the leading health concern and a heavy financial burden both nationally and globally.

The annual cost of atherosclerosis treatment, both direct and indirect cost, reaches $316.6 billion currently (compared to cancer, $88.7 billion), and is projected to dramatically increase to ≈$918 billion in 2030 as estimated by American Heart Association (AHA) (Benjamin, Blaha et al., 2017).

According to the latest epidemiology statistics, it is estimated that 92.1 million adults in the United States are affected by atherosclerosis and cardiovascular diseases (CVDs),
which accounts for 36.6% of the national population, and this percentage is projected to climb to 43.9% by the year of 2030. Meanwhile, CVDs are responsible for at least 30.8% deaths nationwide, which is the leading cause of mortality (compared to the second place, cancer 22.5%, and third place, accidents 6.4%), even the rate has been declined since last decades (2004 to 2014). Among all the CVDs, CHD is the leading cause of the death (45.1%), followed by stroke (16.5%), heart failure (8.5%), high blood pressure (9.1%), diseases of the arteries (3.2%), and all other CVDs (Benjamin et al., 2017). By race/ethnicity, African Americans present a significantly higher death rate out of all other races; by sex, males have a slightly greater percentage of both prevalence and mortality (37.4%, 50.6%) than females (35.9%, 49.4%), though the difference is not statistically significant. The association between the occurrence of CVDs and age is clear: at age 20-39, there is only 12.5% prevalence. Remarkably, this percentage increases to 40.4% as at age 40-59, and doubled in the age group over 80 as up to 85.5%. Similarly, the mortality rate climbs as the age increases (250,000 per year < age 85, VS 380,000 ≥ age 85 per year, as of 2014).

Though it is indisputable that aging is a critical factor in atherogenesis, surprisingly, atherosclerosis can develop at early life in childhood or adolescence (Rose, Allen et al., 1976). A nationwide epidemiological study by the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) party reveals that, intimal fatty steaks can be found in approximately one out of three children under age of nine; by puberty, more than half of the teenagers were found to display a pre-mature lesion with extracellular lipid accumulation, macrophages or foam cells, and lipid containing smooth muscle cells; at the age of twenty, about one-third of the population was found to present advanced
plaques with necrotic cores, thickened fibrous cap, and significant loss of the lumen area (McGill, McMahan et al., 2000).

Except from age, risk factors for CVDs include genetics/family history, health concerns such as high blood pressure, diabetes mellitus, metabolism syndrome etc., and unhealthy life styles such as smoking/tobacco use, high calorie diet and physical inactivity (Writing Group, Mozaffarian et al., 2016).

1.1.3 Pathogenesis of Atherosclerosis

The distribution of atherosclerotic lesions is predominantly determined by hemodynamic factors such as shear stress. Plaques are prone to be found in curved arteries and branches where shear stress is low or oscillatory, for example, aortic arch, carotid fork, or aortic bifurcation where common lilac begins. In addition, shear stress affects not only the plaque accumulation, but also the plaque stability: lower shear stress usually leads to vulnerable plaques with a large lesion, while stable plaques are prone to be found in oscillatory shear stress condition (Cheng, Tempel et al., 2006).

Pathologically, atherosclerosis initiates at the endothelium, the innermost layer of the vessel. Under a diversity of insults including pro-inflammatory mediators, hyperlipidemic stress, and hemodynamic alterations, the injured endothelium secrets chemoattractant mediators such as cytokines, chemokines, and adhesion molecules, favoring the immune cell (CD4+ T cell, B cell, NK cell and monocyte, etc.) recruitment from the blood stream to the lesion site. Paralleled, the dysfunctional endothelium with increased permeability and disrupted extracellular matrix (ECM) composition allows the entry and retention of circulatory modified low density lipoprotein (LDL) particles, which will further undergo
endocytosis by immune cells (for example, Ly6C^{hi} monocyte), resulting in lipid deposition underneath the endothelium, which is termed as “fatty streak”. Subsequent changes occur in smooth muscle cells (SMCs), which are the predominant component of the vessel, and mainly reside in the media/adventitia layers. These tunica SMCs proliferate and migrate as response to the pro-inflammatory stimuli, to form a fibrous cap that covers the fatty streak (Stary, Chandler et al., 1994). All these procedures synergically contribute the atheroma formation — a plaque that consists of extracellular lipid, immune cell debris, calcium, underlying a denuded endothelium, with a fibrous cap consist of collagen, ECM and SMCs (Bentzon et al., 2014, Crowther, 2005). Plaque buildup leads to loss of luminal area, accelerated blood flow, with thickening wall of the vessel, which are the hallmarks of atherosclerosis (Falk, 2006).

At the development stage of pathogenesis, the plaque changes its composition dynamically, with monocyte infiltration, lesion site residential macrophage differentiation, and macrophage-derived foam cell accumulation (Libby, Ridker et al., 2011). Meanwhile, the increased extracellular deposition and oxidation causes cellular toxicity, leading to enhanced cell necrosis, thus facilitating necrotic core formation in the center of the plaque. As a result, the architecture of the origin plaque is disrupted. Along with that, the rupture of foam cell leads to the release of pro-inflammatory mediators from the lesion site, thus amplifying the inflammatory response, and worsening atherosclerosis in the lesion site (Singh et al., 2002).

In the end stage of atherosclerosis, the fibrous cap gains vulnerability due to ECM decomposition as a result of proteolytic enzyme activity, and the cap area SMCs transformation from a contractile to a synthetic phenotype (Bennett et al., 2016, Gomez
Rupture of the plaque results in luminal hemorrhage, meanwhile, the deposited calcium in the vessel wall and fibrous cap provides the sites for platelet aggregation, leading to thrombosis, and finally, occlusion of the vessel (Fuster, Stein et al., 1990). Mild atherosclerosis usually companies with no signs or symptoms, until the occurrence of occlusion. Depending on the vessel affected, complications of atherosclerosis include cardiac infarction, cerebral stroke, kidney failure, and gangrene etc.

1.1.4 Atherosclerosis Medication and Intervention

So far, to the best of our knowledge, there is no medication that treats atherosclerosis, but only prevents its progression. Therapeutic strategies target risk factors including high cholesterol, inflammation, hypertension, and platelet aggregation, etc.

Currently, one of the most common therapeutic strategies for atherosclerosis is cholesterol-lowering medication, for example, the statins. As HMG-CoA reductase inhibitors that block cholesterol synthesis (Raal & Santos, 2012), statins have a potent effect and long history as CVD medications. In 2005, a series of randomized clinical trials regarding the efficacy and the safety of statins were delivered, with 90,056 patients involved and five years following-up. One year after the trials, it was found that, statins lowered the LDL level ranging from 0.35 to 1.77 mmol/L (average 1.19mmol/L), leading to a 19% reduction in coronary mortality; moreover, a reduced 5-year incidence of coronary events, coronary revascularization, and stroke was achieved by a ~0.20 per mmol/L decrease in LDL cholesterol, without major safety concerns such as cancer (Baigent, Keech et al., 2005). These results match the outcomes from the “Cholesterol
and Recurrent Events (CARE) trial” in the last century, evaluating the effect of
Pravastatin. Results of CARE indicated that Pravastatin therapy lowered the mean LDL
cholesterol level in patients from 3.6 mmol/L to 2.5 mmol/L during a five year following-
up. Moreover, patients who received Pravastatin presented a 24% decrease in occurrences
of CHDs. Meanwhile, the rate of fatal myocardial infarction was 37% lower in the
Pravastatin group than in the placebo group (Auerbach, Behar et al., 1997). In addition to
lowering cholesterol, statins also exert an anti-inflammatory effect as a substantial benefit
for the patients, by decreasing the serum C-reactive protein (CRP) levels (Jain & Ridker,
2005, Ray & Cannon, 2005). Recently, new approaches emerged as a complementary
approach to the existing ones. For example, Torcetrapib, a cholesteryl ester transport
protein (CETP) inhibitor, has been proven to increase the HDL level in patients (Nissen,
Tsunoda et al., 2003). Another therapeutic strategy is to target HDL, for example,
HDL/ApoA1, an artificial HDL-like Apo lipoprotein A1 complex. ApoA1 is the core
protein which is present in most of the HDL particles, the complex HDL/ApoA1 has a
high lipid affinity. Therefore, it can facilitate the lipid clearance from the circulation
(Nicholls, Tuzcu et al., 2008). Among all these newly developed approaches, it is
worthwhile to mention Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) inhibitor,
a novel application which has emerged recently as an alternative strategy for
atherosclerosis therapy (Lambert, Sjouke et al., 2012). PCSK9 acts when it binds to and
inactivates LDL receptor (LDLR) (Horton, Cohen et al., 2009). As a monoclonal
antibody, PCSK9 inhibitors block PCSK9 activity, therefore release the bound LDLR,
thus lowering the LDL concentration in circulation. Two PCSK9 inhibitors, Alirocumab
and Evolocumab, are now in Phase III clinical trials (Koren, Lundqvist et al., 2014, Roth,
Taskinen et al., 2014), showing a promising future, especially for patients who are statin-intolerant.

Other therapeutic strategies are anti-platelet medications, such as Aspirin. Aspirin is used to prevent the potential platelet aggregation, causing subsequent thrombosis and blockage of the vessel, especially for those patients who have narrowed arteries with plaques.

Other anti-platelet medications include Clopidogrel, Vorapaxar etc. In the Prevent Recurrent Events (CURE) clinical trial, the effect of Clopidogrel on CHDs was evaluated. The outcome indicated that death due to cardiovascular events and stroke dropped from 11.4% to 9.7% by Clopidogrel treatment compared to placebo. Meanwhile, significant decreases of severe cardiac conditions such as recurrent angina, coronary ischemia and heart failure were found in patients with Clopidogrel treatment compared to the placebo group (recurrent angina 20.9% vs 22.9%, severe ischemia 2.8% vs 3.8%, and heart failure 3.7% vs 4.4%), in spite of a major safety concern of bleeding (Yusuf, Zhao et al., 2001); other medications include plaque stabilization with the antidiabetic PPAR agonist Thiazolidinediones (Jandeleit-Dahm, Calkin et al., 2009); or blood pressure lowering with Angiotensin-converting enzyme (ACE) inhibitors, Calcium channel blockers, and Beta blockers. These medications target vessel SMC contraction and epinephrine, thus is currently widely used for the CHDs treatment, to reduce the myocardial infarction, or to relieve angina.

Even with the presence of existing approaches, however, the progressive increase of atherosclerosis-suffering population means that further attention is necessary and merited to conquer this life-threatening disease as a long term goal.
In addition to medication, both surgical and non-surgical interventions are applied when atherosclerosis complications are serious. Percutaneous coronary intervention (PCI), also known as coronary angioplasty, is one of the most prevalent interventions. With an insertion of a catheter tube containing contrast dye and balloon, PCI can locate the blockage and open a coronary artery which is narrowed or blocked due to the plaque buildup. Sometimes, a stent is placed after the procedure to maintain the opening of the compromised vessel (Smith, Dove et al., 2001).

Coronary artery bypass grafting (CABG) is a surgical intervention (Yusuf, Zucker et al., 1994). During CABG, healthy arteries or veins are grafted to the blocked coronary artery, building a bypass to restore the blood flow feeding to the heart. Though CABG is primarily for patients with severe coronary heart disease (CHD), the bypass grafting strategy also can be used towards the blockage in peripheral arteries, where the healthy vessel will be connected, to create a new path for the blood flow in the context of PAD such as gangrene.

Another widely employed surgical intervention is carotid endarterectomy (CEA). In CEA, the carotid, especially in the fork, where the common carotid divides into internal and external carotids, is opened by incision. Then the aggregated atherosclerotic plaques are removed. The carotid is closed using sutures, and a patch is applied to increase the lumen area (Moore, Barnett et al., 1995).

All these interventions are aimed at improving the blood flow to the target organs, such as the heart and brain, to possibly prevent a potential heart attack or stroke. Despite the surgical intervention, a “Heart-Healthy Lifestyle Change” is essential as a long term strategy to improve the quality of life for atherosclerosis and CVD patients. Heart-healthy
lifestyle change includes quitting smoking, managing stress, and body weight control by healthy eating and physical activity as suggested by AHA (Elmer, Obarzanek et al., 2006).

1.2. Smooth Muscle Cells

1.2.1 Smooth Muscle Cell Biology in Vascular Disease

Atherogenesis is a multi-stage process with a variety of types of cell participating. For example, vascular ECs, which compose the innermost layer of vessels, act as an early predictor of pathogenesis (Mudau, Genis et al., 2012). They initiate the local inflammatory in response to then stimuli, and retain lipids, which may trigger further processes such as immune cell recruitment.

Immune cells, including T cells, B cells, lymphocytes, dendritic cells, and mast cells (Galkina & Ley, 2009), contribute predominantly to the inflammation amplification, and therefore, worsen the atherosclerotic lesions. For example, mast cells, upon the activation through Toll-like receptors (TLRs), release histamine to increase the vascular endothelium permeability, to initiate the inflammatory process. Another example is dendritic cells. These CD11c+ immune cells are recruited and adhere to the dysfunctional endothelium by the adhesion molecules E-selectin or P-selectin. Previous investigations unveiled that, depletion of dendritic cells led to a remarkable decrease of intimal lipid accumulation, in-plaque foam cell number, and atherosclerotic plaque size in hyperlipidemic ApoE<sup>−/−</sup> mice (Abraham, Fisher et al., 2002, Chavez-Sanchez, Espinosa-Luna et al., 2014), though the specific mechanism remains elusive.
Compared to those immune cells which exert adverse effects on atherogenesis, the role of macrophages is dual (Moore, Sheedy et al., 2013). On one hand, these monocyte derived mononuclear cells account for the clearance of aggregated lipoproteins on the lesion site by the phagocyte. When they transform into cholesterol-laden foam cells, the trapped lipid will further undergo modifications such as oxidation, or enzymatic cleavage, resulting in cellular toxicity (Dickhout, Basseri et al., 2008).

Among all these cells, vascular SMCs attract intensive research attention. They compose and define the tunica media, which is the central layer of vessel in between intima and adventitia. These tunica SMCs are responsible for the vessel tone maintenance, including control of blood flow, blood pressure, vessel resistance, and shear stress (Mack, 2011). SMCs in the vasculature are not terminally differentiated cells and thereby, possess a remarkable phenotypic plasticity which allows a rapid adaptation to fluctuating pathological cues. SMCs play essential roles in a diversity of vasculature pathogenic processes. For example, they account for the neo-intima hyperplasia (NIH) in post-injury restenosis (Marx, Totary-Jain et al., 2011), or arteriovenous fistulas failure (AVF) (Skartsis, Martinez et al., 2014) due to their proliferative phenotype.

### 1.2.2 Smooth Muscle Cell Biology in Atherosclerosis

In the context of atherosclerosis, arterial SMCs exert paradoxical roles. On one hand, these SMCs are the atherogenic elements since they contribute to atheroma growth and remodeling of the artery during early atherosclerosis. On the other hand, they favor the fibrous cap formation, to cover the fatty steak and facilitate plaque buildup due to their
hyper-proliferative and migration abilities in the latest stage of the disease (Gomez & Owens, 2012).

Currently, two prevalent theories attempt to unveil the SMC cell fate during atherogenesis. One of them is a SMC switch from a quiescent “contractile” to a dynamic “synthetic” phenotype (Gomez & Owens, 2012). Typically, this transformation is accompanied by cell morphology changes from spindle shape to rhomboid, and downregulation of the expression of smooth muscle (SM)-specific markers such as Myocardin, Calponin, and Smooth Muscle Actin (SMA), etc. This cellular phenotypical switch may lead to a compromised function of the vessel, for example, an impaired vascular contraction due to SMC phenotypical transformation was previously observed (Beamish, He et al., 2010). Additionally, these synthetic SMCs present hyper-cellular activities, including cell proliferation and migration (Bennett et al., 2016). Based on these characteristics, SMCs exert a dual function during atherogenesis where they favor the plaque formation in the early stage, whereas paradoxically reinforcing the stability of the fibrous cap in the end stage (Stary, Blankenhorn et al., 1992).

The other theory is the pathological transformation of the SMC to macrophage. With gained marker expression (e.g. CD68, CD11b, F4/80), these macrophage-like SMCs were identified in the atherosclerotic lesion area by various studies (Allahverdian, Chehroudi et al., 2014, Bennett et al., 2016). Further in vitro investigations validated this observation showing that, synthetic SMCs are prone to uptake cholesterol from the hyperlipidemic milieu, and present excessive lipid aggregation in the cytosol (Rong et al., 2003, Shankman et al., 2015), suggesting these SMCs resemble macrophages not merely at a molecular level, but also in cell biological function.
Inflammation/autoimmune is a general pathological mechanism during environmental stress and tissue injury. Except from previous two well-developed theories, investigations regarding SMC inflammatory state also attracted research attention in atherosclerosis medication (Sprague & Khalil, 2009). Growing evidence clearly demonstrated that, transformed synthetic SMCs present a pro-inflammatory phenotype, whereby SMCs secrete cytokines and express cell adhesion molecules (e.g. IL-6, IL-8, and VCAM-1) (Cuneo & Autieri, 2009, Sprague & Khalil, 2009) as response to the environmental cue, thus functionally facilitating the consequent processes such as monocyte adhesion and SMC migration, and resulting in an amplification of inflammatory response during the atherosclerosis development. Remarkably, these factors which drive the inflammatory phenotype are not limited to cytokines, but also include hemodynamic forces imposed on the blood vessel wall, and the immediate interaction between ECs and SMCs (Galkina & Ley, 2009). In addition, strikingly, it has been revealed that these inflamed SMCs are found to disturb the composition of ECM in the fibrous cap area by releasing matrix metallopeptidases (MMPs), which potentially decrease the plaque stability and increase the risk of rupture at the end stage of atherosclerosis (Rudijanto, 2007).

1.3. Receptor Tyrosine Kinase c-Kit

1.3.1 Receptor Tyrosine Kinase in Atherosclerosis

Receptor tyrosine kinase (RTK) is a cell surface receptor super family, with intrinsic protein tyrosine kinase activity. The RTK family consists of approximate 17 classes, and has been studied for over twenty years (Lemmon & Schlessinger, 2010). Ligands for RTKs include cytokines, polypeptide growth factors, and hormones. Typically, RTK
signaling activation is upon the ligand binding and receptor dimerization. This dimer formation engages the intracellular tyrosine residue phosphorylation by transfer of ATP \( \gamma \) phosphate to hydroxyl groups of tyrosine in its counterpart receptor (Hunter, 1998), providing the docking sites for highly conserved Src homology-2 (SH2) domain- and phosphotyrosine binding (PTB) domain- containing proteins. This docking leads to the activation of downstream transduction pathways such as MAP kinase (Zwick, Bange et al., 2001). Members of RTK family present a diversity of biological functions, regulating survival, apoptosis, and differentiation. Also, mutations in RTKs are believed to contribute to the oncogenesis and the progression of certain types of cancer (Lemmon & Schlessinger, 2010).

Unfortunately, to our best knowledge, no medication has been developed so far despite the current understanding of RTKs. Thus, further studies of this receptor super family are necessary to develop strategies which would benefit the anti-atherosclerosis therapy.

Based on the existing knowledge, c-Kit is an important member of type III RTK family, and naturally attracts research attention in atherosclerosis.

### 1.3.2 Receptor Tyrosine Kinase c-Kit (CD117)

c-Kit, also termed as CD117, was initially identified in 1986 as the homolog of viral oncogene \( v-kit \) (Yarden, Kuang et al., 1987). The \( kit \) gene in white spotting (W) locus is located on murine chromosome 5, or on human, chromosome 4q11–q12 (Qiu, Ray et al., 1988). This transmembrane receptor is encoded by 22 exons, and constituted with five extracellular immunoglobulin (Ig)-like domains, a single juxtamembrane domain, and an intracellular kinase domain (Liang, Wu et al., 2013).
So far, over 38 mutations of c-Kit were identified (Abbaspour Babaei, Kamalidehghan et al., 2016, Ashman & Griffith, 2013, Bernstein, Forrester et al., 1991, Hayashi, Kunisada et al., 1991, Reith, Ellis et al., 1991, Ronnstrand, 2004). Among all of these known genotypes, noticeably, mutations occurring in the W/c-kit locus are associated with the severe pleiotropic defects including hematopoiesis, reproduction, pigmentation etc. (Reith et al., 1990, Waskow, Paul et al., 2002), and termed as Kit\textsuperscript{W}. The W mutation causes a G to A nucleotide exchange in intron 9, resulting in a mis-splicing of exon 9 to 11. The produced protein lacks the transmembrane domain, and therefore, fails to express on the cell surface. Due to its absent extracellular expression and ligand binding activity, Kit\textsuperscript{W} serves as a dominant negative mutation which is embryonic lethal when homozygous. Other mutations found in W locus include Kit\textsuperscript{W-f}, Kit\textsuperscript{W-sh}, Kit\textsuperscript{W-s}, Kit\textsuperscript{W-v}, etc. (Grimaldeaston, Chen et al., 2005). Among these, Kit\textsuperscript{W-v} is a missense mutation, with a threonine to methionine substitution (T to M, at 660) in the tyrosine kinase domain (Nocka et al., 1990). Mice with a compound heterozygous mutation of Kit\textsuperscript{W/W-v} present predominant loss of tyrosine kinase activity while surviving gestation, and therefore, have been widely used in c-Kit function studies (Wang, Anderson et al., 2006b, Wang et al., 2007, Wedemeyer & Galli, 2005).

This cell surface receptor, CD117, has been found present in both differentiated and non-differentiated cell lines. The non-differentiated cells include hematopoietic stem cells (HSCs), and a variety of progenitor cells (PCs, e.g. endothelial progenitor cells, EPCs). Meanwhile differentiated cells include mast cell, astrocytes, gastrointestinal (GI) tract Cajal cells (Lennartsson & Ronnstrand, 2012), and vascular ECs (Miyamoto et al., 1997).
Previous investigations of c-Kit function and mutation driven disease were mainly focused on embryogenesis, stem cell differentiation, and oncogenesis. For example, it has been well documented that c-Kit plays an important role in cardiac myocyte final differentiation (Ellison, Vicinanza et al., 2013, Kubo, Berretta et al., 2009), gastrointestinal stromal tumor (GIST) (Heinrich, Blanke et al., 2002), acute myeloid leukemia (AML) (Shen, Tang et al., 2003), melanoma (Garrido & Bastian, 2010), and astroglia oncogenesis (Blom, Fox et al., 2008).

1.3.3 Receptor Tyrosine Kinase c-Kit (CD117) in Atherosclerosis

c-Kit expression has been found in a diversity of stem cells and progenitor cells (PCs) (Sun, Li et al., 2016). According to previous investigations, the role of these c-Kit positive stem cells and PCs in vascular disease is controversial. On one hand, these PCs or stem cells were found to act as a beneficial factor in injury repair. For example, c-Kit has been shown to determine the cardiac stem cell (CSC) differentiation into cardiomyocytes, resulting in myocardial regeneration (Hosoda, 2012). In addition, evidence suggests that, in the context of retina or brain damage, the hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs) are recruited to the lesion sites, giving rise to the local ECs and SMCs. This differentiation is responsible for the injured organ re-vascularization and regeneration (Rafii & Lyden, 2003). On the other hand, investigations by Sata et al. (Sata, Saiura et al., 2002) claim that these stem cells and PCs play a detrimental role in facilitating the plaque formation in the early stage of atherogenesis. However, later studies by Bentzon (Bentzon, Sondergaard et al., 2007) contradicted Sata’s finding and indicated that instead of the bone marrow derived HSCs
and PCs, the local vascular SMCs predominantly accounts for the fibrous cap formation in plaque, based on a hyperlipidemic ApoE\(^{-/-}\) mouse model. Similarly, in the context of neo-intima hyperplasia, SMCs in the hyperplasia area were found with lineage marker expression, indicating the participation of stem cells and PCs in neo-intima formation. (Sata et al., 2002).

Recently, accumulative evidence suggested vascular expression of this receptor on SMCs, in both human and mice (Hollenbeck et al., 2004, Tallini, Greene et al., 2009, Wang et al., 2006b). However, these studies were descriptive and little has been shown to understand how c-Kit influences SMC biology in the context of vascular pathogenesis.

**1.3.4 SCF/c-Kit Signaling Activation**

c-Kit signaling activation occurs upon the binding of its unique ligand, stem cell factor (SCF), also termed as Kit ligand (Kitl). During the activation process, the extracellular domains 1 to 3 of c-Kit receptor are responsible for SCF binding and ligand-receptor complex formation, meanwhile domains 4 and 5 account for the c-Kit receptor homodimerization. The homodimer formation leads to the intermolecular phosphorylation of the tyrosine residues which are located in the kinase domain by phosphatidylinositol kinase (Ronnstrand, 2004). After the phosphorylation of the tyrosine residues, signals will be passed to the nucleus, thus triggering subsequent biological effects.

The vascular expression of SCF exists in both soluble (sSCF) and membrane-bound (mSCF) forms. Its ability is to promote cell recruitment, and elicit autocrine, paracrine responses, as well as cell-to-cell stimulation (Hollenbeck et al., 2004, Lennartsson & Ronnstrand, 2012, Skartsis et al., 2014). The soluble form of SCF is generated by
alternative splicing or released through the proteolytic action of matrix metallopeptidase 9 (MMP-9) (Bengatta, Arnauld et al., 2009, Hollenbeck et al., 2004, Klein, Schmal et al., 2015, Lennartsson & Ronnstrand, 2012). This latter enzyme is also upregulated in vascular remodeling processes, thereby perpetuating the local effects of the SCF/c-Kit pathway (Hollenbeck et al., 2004, Skartsis et al., 2014). Interestingly, the soluble and membrane-bound SCF isoforms seem to have different effects on c-Kit activation (Miyazawa, Williams et al., 1995). The former causes rapid and transient stimulation and auto-phosphorylation of the receptor as well as fast degradation, whereas the latter leads to sustained activation (Miyazawa et al., 1995). Noticeably, c-Kit signaling is also found constitutively activated in a ligand independent manner in certain cell lines, such as the human mast cell line HMC-1 (Furitsu, Tsujimura et al., 1993), where mutations are found in the kinase domain.

Multiple downstream signaling transduction pathways have been involved upon the activation of SCF/c-Kit, including Ras/ERK, PI3K/Akt, PLC-γ and NF-κB etc., to exert cellular biological function by mediating cell growth, development, and inflammatory state (Da Silva, Heilbock et al., 2003, Hendriks, 2011, Sansal & Sellers, 2004, Summy & Gallick, 2006, Yasuda & Kurosaki, 2008, Young, Torres et al., 2016). For example, SCF/c-Kit signaling is recognized to promote pathological remodeling and pulmonary vascular cell proliferation after hypoxic stimulation via activation of the ERK1/2 pathway (Young et al., 2016). In another study, SCF/c-Kit activation is believed to link with the induction of the Akt-Bcl-2 cascade, which is thought to mediate the anti-apoptotic and migratory SMC phenotype in the context of intimal hyperplasia (IH) (Wang et al., 2007). However, all these pathways mentioned above were investigated in various pathogenic
models. The focus of my thesis was to identify novel pathways, signaling crosstalk, and downstream targets of c-Kit, such as transcriptional factors under the background of atherosclerosis, to better understand the potential mechanisms by which c-Kit regulates the SMC phenotypical switch, cell type transformation, and pro-inflammatory state.

1.4. Significance of the Study

This investigation is highly significant because: (1) it is the first study that rigorously evaluates the contribution of c-Kit in the pathogenesis of atherosclerosis and its complications; (2) outcomes from this study challenge the existing belief that vascular c-Kit expression is pathological (e.g. in a post-injury neo-intima hyperplasia scenario), and instead, suggest a beneficial contribution of this signaling axis on the preservation of SMC’s protective status under adverse conditions; (3) it provides mechanistic evidence that c-Kit has anti-atherosclerotic effects by inhibiting the transformation of SMCs from a physiological contractile to a pathological synthetic, foam-cell like, and pro-inflammatory phenotype; (4) mechanistic explorations further identify novel downstream targets of c-Kit (KLF4, TAK1, NLK), and (5) it furnishes a new therapeutic target – for which there is an existing translatable platform for drug development to prevent and eventually mitigate the devastating effects of atherosclerosis.
CHAPTER 2. METHODS AND MATERIALS

2.1 Methods and Materials for Specific Aim 1

2.1.1 c-Kit reporter mice Kit\textsuperscript{Bac}-eGFP

Kit promoter activity was monitored as previously described in the Kit\textsuperscript{Bac}-eGFP mice (Tallini et al., 2009). This transgenic mouse allows eGFP expression under the Kit promoter. For genotyping, murine genomic DNA was isolated from mouse tail biopsies using an Extract-N-Amp\textsuperscript{Tm} tissue PCR kit (XNAT2, Sigma). Briefly, the tail sample was immersed in the mix of extraction and preparation buffer at 4:1 ratio, heated at 85°C for 10 min. Then an equal amount of neutral buffer was added to finalize the extraction. Genomic DNA was further amplified by using a KAPA2G Fast Hot Start Genotyping Mix (07961316001, Kapa Biosystems, Wilmington, MA) and respective primers (see Supplementary Table 1). DNA was electrophoresed in 2% agarose gel for 40min with a constant voltage of 40. Target bands were visualized using Ethidium Bromide (0.5 ug/mL, 15585011, Thermo Fischer Scientific) under the UV light. The abdominal aortae of these mice were submerged in 0.5% paraformaldehyde (PFA) /0.1% glutaraldehyde/20% sucrose in phosphate buffer 7.0 (w/v) to preserve fluorescence protein activity before embedding in optimum cutting temperature (OCT) medium and cryo-sectioning (Brazelton & Blau, 2005).
2.1.2 c-Kit mutant mice

Kit\(^{W/+}\) and Kit\(^{W-v/+}\) mice (Stock #100410, The Jackson Laboratories, Bar Harbor, ME) (Bernstein, Chabot et al., 1990, Bernstein et al., 1991) were individually cross-bred with the atherosclerosis prone ApoE\(^{-/-}\) mouse (Stock # 002052) (Zhang, Reddick et al., 1992).

The resulting Kit ApoE\(^+/-\) and Kit ApoE\(^+/-\) mice were further crossed with ApoE\(^{W/+}\) ApoE\(^{W-v/+}\) ApoE\(^{W/+}\) ApoE\(^{W-v/+}\) mouse to obtain Kit ApoE\(^{-/-}\) and Kit ApoE\(^{-/-}\) strains. The mating of Kit ApoE\(^{W-v/+}\) ApoE\(^{W-v/+}\) and Kit ApoE\(^{W-v/+}\) ApoE\(^{-/-}\) mice generated c-Kit mutant (Kit ApoE\(^{-/-}\)) and control littermates (Kit ApoE\(^{+/-}\)). Both groups were fed on a high fat diet (HFD, 42% cholesterol, TD 88137, Harlan Laboratories, Indianapolis, IN) for 16 weeks before assessing the atherosclerotic burden in the aorta and aortic sinus.

2.1.3 Human samples collection

Human pulmonary arteries (n=3) and aortae (n=6) were procured from heart transplantation recipients and donors at the University of Miami / Jackson Memorial Health System (Miami, FL).

2.1.4 Study approval

All animal experiments and procedures were performed according to the NIH guidelines (Guide for the Care and Use of Laboratory Animals) after receiving approval from the University of Miami Institutional Animal Care and Use Committee (Protocol 15-114). All human specimen collection was approved by the University of Miami Institutional
Review Board (IRB # 20110443). Informed consent was obtained from all subjects and experiments were performed following the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

2.1.5 Atherosclerotic burden in aorta and aortic Sinus

Murine aorta isolation and excision were performed as previously described (Robbins, Thompson et al., 2014). In brief, mice were anesthetized with 1-5% isoflurane in oxygen, placed in a supine position, and sanitized with 70% ethanol on the abdominal and thoracic regions before removal of the hair. The skin was lifted up and excised with scissors to expose the thoracic cavity, and then locate the heart. Perfuse the left ventricle of the heart with 10 ml of pre-cold PBS, then remove the liver, stomach, spleen etc. to locate the aorta. Carefully remove the surrounding adipocyte tissue by micro-forceps and micro-scissors, then isolate the aorta from the spine dorsally and the esophagus ventrally. All operations were performed under a microscope.

The isolated aortae were preserved in neutral buffered 10% formalin (Sigma, St Louis, MO) were submitted to Eehscience LLC (Pickerington, OH) for Sudan IV staining and independent quantification of atherosclerotic burden (Cornhill, Barrett et al., 1985). The dye Oil Red-O used in Sudan staining represents and visualizes the lipid accumulation due to its fat-soluble and high lipid affinity characters. A digital imaging of the stained aorta was over lapped with a reference template, and the extent of disease was quantified by counting Oil Red-O pixels versus total aortic area. Eehscience remained blinded to experimental groups during atherosclerosis assessment. Once the disease assessment had finished, group information was released to Eehscience for statistical analysis.
Moreover, fixed hearts were sent to the Histology and Light Microscopy Core at the Gladstone Institute (San Francisco, CA) for sectioning and Oil Red-O staining of the aortic sinus (Venegas-Pino, Banko et al., 2013). Serial cryo-sections of 10µm thickness separated by 40-50µm distance were cut through the aortic sinus. Ten sections selected from the most proximal section of the heart to the distal point where the aorta becomes more rounded were examined for morphometric evaluation of intimal lesions using Image Pro morphometry system. The mean lesion size in these 10 sections represented the atherosclerosis disease in the aortic sinus of each animal.

### 2.1.6 Blood chemistry and lipid profile determination

Blood chemistry, lipid profile and complete blood counts (CBC) were determined in heparinized venous blood collected from each mouse before euthanasia. Blood samples were submitted to the University of Miami Comparative Pathology lab for automated analysis. Complete blood count (CBC) was performed in Beckman Coulter MAXM system. Percentages of RBC, Hemoglobin (Hgb), Hct (Hematocrit), and MCV (mean cell volume) were measured. In brief, blood cell suspension was passed through the sensing zone with two electrodes aside, where the particles (cells) were displaced the volume with electrolyte. The electrolyte pulse was recorded by the device, pulses at different wavelength representing different types of particles.

For lipid measurement, the blood samples were further handled with centrifugation at 1,500 x g for 30 min at 4°C, then the serum was carefully isolated. Cholesterol, triglyceride (TG), HDL-, LDL-, VLDL- cholesterol analyses were performed on a Hitachi 704 Analyzer. Detecting reagents for different cholesterols or TG were applied.
Reagents for total cholesterol (0.15 mmol/L 4-Aminophenazone, 4.2 mmol/L Phenol, 0.5 U/mL Cholesterol esterase, 0.15 U/mL Cholesterol oxidase, 0.25 U/mL Peroxidase, 1% Fatty alcohol-polyglycol ether), and for TG (50 mmol/L PIPES buffer, pH 6.8, 40 mmol/L Mg$^{2+}$, 0.20 mmol/L Sodium cholate, 1.4 mmol/L ATP, 0.13 mmol/L 4-Aminophenazone, 4.7 mmol/L 4-Chlorophenol, 1 µmol/L Potassium hexacyanoferrate, 0.65% Fatty alcohol polyglycolether, 5.0 U/mL lipoprotein lipase, 0.19 U/mL glycerol kinase, 2.5 U/mL glycerophosphate oxidase and 0.10 U/mL Peroxidase), and HDL-cholesterol (Cyclodextrin/Buffer, 0.5 mmol/l α-cyclodextrin, 0.5 g/l dextran sulfate, 7.0 mg/ml MgSO$_4$, 0.3 g/l EMSE, and 10 mmol/l MOPS pH7.0).

2.1.7 Western blot and immunoprecipitation (IP)

Protein lysates were prepared in RIPA buffer (10M Tris-Cl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 140 mM NaCl) supplemented with 200mM phenylmethylsulfonyl fluoride (PMSF), 100mM sodium orthovanadate (Santa Cruz Biotechnology, Dallas, TX), and a complete protease inhibitor cocktail (Roche Life Science, IN). The protein concentration was determined using a commercial Bradford’s protein assay kit (BioRad). Proteins were pre-heated at 95~100 °C for 5 min to denature, then resolved in 4-12% SDS-polyacrylamide gels (NuPAGE, Thermo Fisher Scientific) before transferring onto PVDF membranes (GE Healthcare, Marlborough, MA), at a constant voltage of 30. The membrane was further blocked by using 5% (w/v) milk containing TBST incubated at room temperature for 1 hr. Specific proteins were detected using antibodies against c-Kit (1:1000, Cat# sc-1494, Santa Cruz Biotechnology) and β-actin (1:5000, Cat# A5316, Sigma) at 4°C overnight. Bound
antibodies were detected after sequentially incubating the membranes with HRP-conjugated secondary antibodies at room temperature for 1 hr., and developed on the films by using Amersham ECL Western blotting Detection Reagent (GE Healthcare). c-Kit was pulled down from ~200µg of arterial or cellular lysate using 1µg of an anti-c-Kit antibody (Cat# A4502, Dako) and 20µl of Protein A/G PLUS-Agarose microbeads (Santa Cruz Biotechnology). Microbeads were washed with pre-cold RIPA buffer before analysis by Western blot.

2.1.8 Confocal immunofluorescence microscopy

Human arterial tissues were formalin-fixed and paraffin embedded before sectioning. Epitope retrieval was performed in de-paraffinized and rehydrated sections. Signal amplification was not needed in human sections. Nonspecific binding was minimized with blocking solution (DAKO) and primary antibodies (rabbit anti-c-Kit and mouse anti-SMA) were added at a 1:50 dilution overnight at 4°C. Bound antibodies were detected using Alexa Fluor 488 goat anti-mouse and Alexa Fluor 546 goat anti-rabbit (1:1000, Cat # A11035, Thermo Fisher Scientific). Sections were mounted and examined with the confocal scanning laser microscope as described previously.

Arterial tissue in fixative solution (0.5% PFA/0.1% glutaraldehyde/20% sucrose) was washed with 20% sucrose/PBS before embedding and cryo-sectioning. Sections serially immersed in 3% hydrogen peroxide and blocking solution (Perkin Elmer, Waltham, MA) were incubated with rabbit polyclonal antibodies against c-Kit (1:250; Cat # 4502, DAKO Carpinteria, CA) overnight at 4°C. The Tyramide Signal Amplification kit (Perkin Elmer) was used after biotinylated swine anti-rabbit polyclonal antibodies
(1:1000; Cat # E0353, DAKO). The amplified signal was detected with Alexa 546 Streptavidin (1:1000; Cat # S11225, Thermo Fisher Scientific, Waltham, MA). For simultaneous detection of anti-smooth muscle cell actin (SMA) and CD68, sections were incubated overnight with rabbit anti-mouse SMA (1:100, Cat# M0851, DAKO) and rat anti mouse CD68 (1:100, Cat# 137002, Biolegend, San Diego, CA) diluted in blocking solution. The bound antibodies were further detected with donkey anti rabbit Alexa 546 (1:1000, Cat # A21202, Thermo Fisher Scientific), and goat anti rat Alexa 647 (1:1000, Cat# A21202, Thermo Scientific), respectively. Sections were examined by a confocal scanning laser microscope Zeiss LSM 510 META (Carl Zeiss, Thornwood, NY) in an inverted configuration. Data were captured and analyzed with Zeiss LSM 510 Meta and Image Browser software (Carl Zeiss, Oberkochen, Germany). Images were acquired with the use of sequential capture mode to avoid potential fluorescence bleed-through between channels. At least four sections of each animal were analyzed for each immunostaining.

2.2 Methods and Materials for Specific Aim 2

2.2.1 c-Kit mutant chimeric mice

c-Kit deficient (Kit<sup>W/W-v</sup> A<sup>poE</sup><sup>-/-</sup>) and control littermates (Kit<sup>W/V</sup> A<sup>poE</sup> <sup>+/+</sup>) were exposed to a single dose of 1,100 rad (11Gy) irradiation, before receiving an intravenous injection of 2x10<sup>6</sup> BM mononuclear cells from age-matched Kit<sup>W/V</sup> A<sup>poE</sup> <sup>+/+</sup> A<sup>poE</sup> <sup>-/-</sup> donors (An & Kang, 2013). For donor single BM cell suspension preparation, murine femurs and tibia were collected, and bone marrow was flushed from the cavity with cold 2% FBS DMEM. Red blood cells (RBC) were removed by using RBC lysis buffer (155 mM NH<sub>4</sub>Cl, 12 mM NaHCO<sub>3</sub>, and 0.1 mM EDTA). Chimeric mice were recovered for four weeks on normal
chow and antibiotics supplemented water before HFD feeding for an additional period of 16 weeks. Red blood cell counts were used as surrogate marker for BM engraftment and chimerism.

### 2.2.2 Atherosclerosis susceptible vascular e-Kit deficient mice

The Kit conditional knockout mouse (Kit^lox66-71/lox66-71) from Dr. Rafii’s laboratory (Weill Cornel Medical College, Ithaca, NY) was crossed into the ApoE^-/- background until homozygous double transgenic mice were obtained. Simultaneously, male mice carrying the Myh11-CreER^-/- gene that allows for Cre specific recombination in SMCs after tamoxifen injections provided by Dr. Offermanns’ lab (Max-Planck Institute, Berlin, Germany) (Kimura et al., 2011) were bred with female ApoE^-/- animals. Then, male Myh11-CreER^-/- ApoE^-/- mice were cross-bred with female Kit^lox66-71/lox66-71 ApoE^-/- to obtain the Kit^lox66-71/lox66-71 Myh11-CreER^-/- ApoE^-/- triple transgenic mouse. Genotyping was performed by tail-DNA PCR as previously described (Wirth, Benyo et al., 2008). To inactivate the Kit gene specifically in SMCs, male triple transgenic experimental animals were fed a tamoxifen-rich diet (TAM) (TD 130858, Harlan Laboratories) for four weeks; while control mice were fed regular chow. Afterwards, all mice were fed on HFD to accelerate the development of atherosclerosis. Expression of e-Kit was examined before HFD feeding in aortae from TAM treated and control mice by IP-Western blot and confocal immunofluorescence microscopy as previously described in Specific Aim 1 (Western blot and IP, see page #34, and confocal microscopy, see page #35).
2.3 Methods and Materials for Specific Aim 3

2.3.1 Electron microscopy

Murine aortae fixed in 4% PFA in PBS (w/v) were transferred to the University of Miami Transmission Electron Microscopy core facility for further fixation with 1% osmium tetroxide, diluted in 100mM phosphate buffer, dehydrated, plastic embedded and sectioned. The ultrastructure of SMCs was examined in a Phillips CM10 transmission electron microscope (Eindhoven, Netherlands). At least three fields were chosen from each section, and at least four images were captured per field.

2.3.2 Smooth muscle cell isolation and culture

Primary aortic SMC were isolated from c-Kit deficient (Kit$^{W/W}$) mice and control littermate mice (Kit$^{+/+}$) (Stock #100410, The Jackson Laboratories, Bar Harbor, ME) (Bernstein et al., 1990) using the explant technique (Metz, Patterson et al., 2012) with minor modifications. Briefly, mouse aortae were digested with collagenase type II (5 mg/mL, Worthington, Lakewood, NJ) at 37°C for 1 hour, after which they were transferred to 10% FBS and cut with a scalpel into small pieces. Individual SMC migrated out of the explants within one week of culture. Cells were maintained in DMEM-F12-FBS (5:3:2; Thermo Fisher Scientific, Waltham, MA) supplemented with 100U penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, and 0.075% sodium bicarbonate (Metz et al., 2012). Primary cells were maintained at ~90% confluence and used within three passages to avoid fibroblast-like phenotypic compromise. All animal procedures were performed according to the National Institutes
of Health guidelines (Guide for the Care and Use of Laboratory Animals) and approved by the University of Miami Miller School of Medicine Institutional Animal Care and Use Committee (protocol 15-114).

2.3.3 Gene rescue and knockdown

W/W<sup>v</sup> c-Kit mutant (Kit<sup>W/W-v</sup>) SMCs were infected with a lentiviral vector (pRVPG24). This rescue vector was constructed by inserting a blunted BsrBI-NotI DNA fragment (3.6 Kb), containing the coding region of the mouse Kit cDNA under the murine phosphoglycerate kinase (PGK) promoter, into the blunted pLenti CMV PuroDest vector (Addgene Inc, Cambridge, MA) cut with EcoRV and ClaI. The third generation lentiviral stocks were produced in HEK-293 cells co-transfected with the lentiviral vector and the packaging and envelope plasmids psPAX2 and pMD2.G (Addgene Inc.). Transfections were done with a jetPRIME transfection kit (Polyplus, New York, NY). Infected cells (100 MOI) were selected in complete medium containing 10µg/ml puromycin (Sigma). Gene rescue was confirmed by IP- Western blot (see below) and fluorescence-activated cell sorting (FACS) analysis with an anti- cKit antibody (CD117-PE, Cat# 130-091730, Miltenyi Biotec, San Diego, CA).

For silencing KLF4 expression in cultured SMCs, pooled lentiviral particles carrying four different KLF4 siRNAs (Supplementary Table 2) were purchased from Applied Biological Materials (Richmond, Canada). A GFP siRNA was used as control. Transduced cells were puromycin selected (10µg/mL) before being used for experiments. Gene knockdown was confirmed by Western blot.
For knockdown TAK1 or NLK in c-Kit wild type (Kit^{+/+}) SMC was performed using pooled lentiviral particles carrying different target siRNAs (Supplementary Table 3) (Applied Biological Materials, Richmond, Canada). An anti-GFP siRNA was used as control. Transduced cells were puromycin-selected as described above. All gene modifications were confirmed by analytical flow cytometry or Western blot.

2.3.4 Flow cytometry analysis

c-Kit surface expression was evaluated by flow cytometry in SMC stained with an anti-CD117 antibody (CD117-PE, Cat# 130-091730, Miltenyi Biotec, San Diego, CA). Analytical flow cytometry was performed on a BD FACS Canto II (BD Biosciences, San Jose, CA) using the BD FACSDiva software (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using the FlowJo software (Ashland, OR).

2.3.5 Western blot and immunoprecipitation (IP)

Whole cell protein lysates were prepared in RIPA buffer supplemented with 200 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM sodium orthovanadate (Santa Cruz Biotechnology, Dallas, TX), and a complete protease inhibitor cocktail (Roche Life Science, Indianapolis, IN). Lysate concentration was determined using a commercial Bradford’s protein assay kit (BioRad, Hercules, CA). For Western blot analysis, ~50 µg of sample was loaded to a NuPAGE 4-12% Bis-Tris SDS-polyacrylamide gel (Thermo Fisher Scientific) and subsequently transferred to a PVDF membrane (GE Healthcare, Marlborough, MA). Specific proteins were detected using antibodies against c-Kit (1:1000, Cat# sc-1494, Santa Cruz Biotechnology), SM22α, Calponin (1:1000, Cat#
Ab10135 and Cat# Ab46794, Abcam, Cambridge, MA), KLF4 (1:1000, Cat# sc-20691, Santa Cruz Biotechnology), MCP-1, MMP-2, TAK1 (1:500, Cat# sc-1785, sc-1839, and sc-6838, Santa Cruz Biotechnology), NLK (1:1000, Cat# ab26050, Abcam, San Francisco, CA), Src (1:500, Cat# 2108S, Cell Signaling Technology, Danvers, MA), and β-Actin (1:5000, Cat# A5316, Sigma). Bound antibodies were detected after sequentially incubating the membranes with HRP-conjugated secondary antibodies. The Amersham ECL Western blotting Detection Reagent (GE Healthcare) or SuperSignal West Femto Maximum Sensitivity Substrate Reagent (Thermo Fisher Scientific) were used for signal detection. Images were analyzed using ImageJ Pro 5.0.

For IP, c-Kit was pulled down from 200µg of arterial or cellular lysate using 1µg of an anti-c-Kit antibody (Cat# A4502, Dako) and 20µl of Protein A/G PLUS-Agarose microbeads (Santa Cruz Biotechnology). Microbeads were washed with pre-cold RIPA buffer before analysis by Western blot.

For co-IP, ~500 µg of protein lysate was incubated at 4°C for 4 hours with 1 µg of anti-c-Kit (Cat# A4502, Dako, Santa Clara, CA) or TAK1 antibodies and 20 µl of Protein A/G PLUS-Agarose microbeads (Santa Cruz Biotechnologies). Microbeads were washed with cold RIPA buffer before WB analysis for c-Kit, Src, TAK1, or NLK as indicated above.

### 2.3.6 Quantitative real-time PCR (qPCR)

Relative gene expression of selected mRNA transcripts was evaluated using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Total RNA was isolated using the Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA). cDNAs were generated
with a high capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Real-time PCR was performed on an ABI Prism 7500 Fast Real-Time PCR System (96-well plate) (Applied Biosystems) using primers/probe sets complementary to the genes of interest: Kit, Mm00445207_m1; Cnn1, Mm00487033_m1; Tagln, Mm00441661_m1; Myocd, Mm00455051_m1; Myh11, Mm00443013_m1; Klf4, Mm00516105_g1; ActB, Mm00607939_m1; Ccl2, Mm00441242_m1; Ikbka, Mm00432529_m1; Ikbkb, Mm01222247_m1; Ikbkg, Mm00494927_m1; Il6, Mm00446190_m1; Kit, Mm00445212_m1; Map3k14, Mm0048444166_m1; Mmp2, Mm00439498_m1; Mmp9, Mm00442991_m1; Nfkia, Mm00479807_m1; Nfkbia, Mm00477798_m1; Nos2, Mm00440502_m1; Ptgs2, Mm00478374_m1; RelB, Mm00485664-m1; Tnf, Mm00443258_m1. Relative gene expression was determined using the ΔΔCT method (Livak & Schmittgen, 2001) and normalized with respect to ActB.

2.3.7 Cell proliferation assay

Primary SMCs were seeded overnight in 24-well plates (5x10^4 cell/well) before starvation with serum-deprived medium to synchronize the cell growth cycle. After that, cells were trypsinized, and 1:10 diluted in PBS. The cell suspension samples were counted by using a Beckman Coulter Z series particle counter, with a detection range of 5-60 µm (Goessl, Bowen-Pope et al., 2001) daily for six constitutive days.

2.3.8 Cell migration assay

Primary SMCs were seeded overnight in the upper compartment of Boyden chambers (8µm pore-size, BD Bioscience, San Jose, CA) coated with fibronectin. Afterwards, cells
were switched to serum deprived medium supplemented with 20 ng/ml platelet-derived growth factor-BB (PDGF-BB, PeproTech, Rocky Hill, NJ) for 24 hr. Mitomycin C (20 µM, Sigma) was added to prevent proliferation. Twenty-four hours later, migrated cells at the bottom well were formalin fixed and DAPI- stained (1:1000, Thermo Fisher). Pictures were taken using an IX71 inverted fluorescence microscope (Olympus, Center Valley, PA). At least three pictures were obtained in each condition. The nuclei of the migrated cells were counted by using Image Pro Plus software (Media Cybernetics, Rockville, MD).

2.3.9 Macrophage-like smooth muscle cell evaluation

The macrophage-like cell phenotype was induced in sub-confluent SMCs cultured with 0.2% BSA medium supplemented with 20 µg/ml water soluble cholesterol (Chol:MβCD, Sigma) for 72hr (Rong et al., 2003). Intracellular lipids were detected in formalin-fixed cells using Oil-red O staining (IHC World, Woodstock, MD). Cellular cholesterol was quantified after hexane/isopropyl (3:2) (Brown, Ho et al., 1980) extraction with an Amplex Red Cholesterol Assay Kit (Thermo Fisher Scientific). The remaining proteins were solubilized with protein lysis buffer and quantified using Bradford’s protein assay (BioRad, Hercules, CA). The results were presented as the amount of cellular lipids per mg of total lysates.

2.3.10 KLF-4 promoter activity assay

Primary SMCs were transfected with a commercial mix of KLF4 Luc-reporter plasmids (Qiagen, Valencia, CA) using an electroporation kit (Lonza, Walkersville, MD), and
electrophoresed by Nucleofector™ 2b Device with program U25, with pre-set parameters uniquely for SMC. Transfected cells were cultured in 24-well plates for 72hr before adding 100 µl of Passive Lysis Buffer (Promega, Madison, WI) for 15 min. Luciferase activity was determined using the double luciferase reporter assay system (Promega) in a Turner Biosystems Luminometer model Glomax 20/20 (Mountain View, CA), and normalized based on the Renilla luciferase activity of the transient transfection control vector. Promoter activity was expressed as folds of control activity.

2.3.11 RNA microarray and pathway analysis

Total RNA was isolated from Kit^{+/+} and Kit^{W/W} SMC using the Quick-RNA Mini-Prep kit (Zymo Research, Irvine, CA). RNA quality was validated in the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) before being sent to Ocean Ridge Biosciences (Palm Beach Gardens, FL) for Mouse MI-Ready Gene Expression Microarray analysis. Once in Ocean Ridge Biosciences, RNA processing included a 30-minute digestion with RNase-free DNase I (Epicentre, Madison, WI) at 37°C followed by purification using the Agen Court RNA Clean XP bead method (Beckman Coulter, Indianapolis, IN). Biotin-labeled complementary RNA (cRNA) was prepared from 2 µg per sample of re-purified RNA by the method of Van Gelder et al. (Van Gelder’s Multi-gene expression profile - US Patent 7049102). 18 µg of biotinylated cRNA per sample was fragmented, diluted in formamide-containing hybridization buffer, and loaded onto the surface of the Mouse MI-Ready microarray slides enclosed in custom hybridization chambers. The slides were hybridized for 16-18 hours under constant rotation in a Model 400 hybridization oven (Scigen, Sunnyvale, CA). After hybridization, the microarray
slides were washed under stringent conditions, stained with Streptavidin-Alexa-647 (Life Technologies, Waltham, MA), and scanned using an Axon Gene Pix 4000B scanner (Molecular Devices, Sunnyvale, CA). Probe intensities were calculated for each feature on each microarray by subtracting the median local background from the median local foreground for each probe. Data for all manufacturer-flagged probes and visually flagged probes impacting >25% of samples were removed. Data for visually flagged probes impacting < 25% of samples were replaced with the sample average for the probe. Probe intensities were transformed by taking the base 2 logarithm of each value. Array-specific detection thresholds (T) were calculated by adding three times the standard deviation of the median local background and the mean negative control probe signal. Probe intensities and T were then normalized by subtracting the 70th percentile of the mouse probe intensities and adding back the mean of the 70th percentile across all samples as a scaling factor. The data were filtered to select for mouse probes showing signal above the normalized T in at least 25% of the samples; data for control sequences and other non-mouse probes were removed. Mouse probe sequences were annotated using a BLAST analysis of the Ensembl Mouse cDNA database version 84 (2016). Gene expression differences between Kit\(^{+/+}\) and Kit\(^{W/W}\) SMC were considered statistically significant if \(p<0.05\) by t-test.

For pathway analysis, genes with statistically significant expression differences in microarray analysis were imported into the Ingenuity Pathway Analysis software (www.ingenuity.com; Ingenuity Systems, Redwood City, CA). The Core Analysis was used to identify the canonical pathways associated with the differentially expressed genes. Pathway overlap and p-value calculations were performed using the reference
gene set in the Ingenuity Knowledge Base, where only molecular relationships (direct and indirect) that have been experimentally observed were considered. The Molecule Activity Predictor tool was used to estimate activation or inhibition of pathway branches based on the observed gene expression fold changes in Kit<sup>W/W</sup>- vs. Kit<sup>+/+</sup> SMCs.

### 2.3.12 NF-κB promoter activity assay

Primary SMCs were transfected with a commercial mix of NF-κB Luc-reporter plasmids (Qiagen, Germantown, MD) using the Axama Basic Nucleofector Primary Smooth Muscle Cells electroporation kit (Cat# VPI-1004, Lonza, Walkersville, MD). Transfected cells were incubated with POVPC (Avanti Polar Lipids, Alabaster, AL) for 24 hours in serum-free medium as previously described (Pidkovka et al., 2007) before lysis using the Passive Lysis Buffer (Promega, Madison, WI). NF-κB promoter activity was determined using the Dual-Luciferase Reporter Assay System (Cat# E1910, Promega) in a Turner Biosystems Luminometer model Glomax 20/20 (Mountain View, CA), and normalized to the Renilla luciferase activity of the kit’s internal control. Promoter activity was expressed as folds of control activity.

### 2.3.13 Enzyme-linked immunosorbent assay (ELISA)

The levels of cellular NF-κB p65 and phosphorylated protein (p-p65) were measured in SMC treated with POVPC as described above. Cells were lysed using the 1X Cell Extraction Buffer PTR provided in the ELISA kit (Abcam, Cambridge, MA). The ELISA
was performed using the NF-κB p65 (pS536 + Total) Simple-Step Kit (Abcam) following the manufacturer’s protocol. Protein levels were measured using an endpoint reading at OD 455 nm in an Ultramark Microplate Reader (BioRad).

2.3.14 Statistics

All results were presented as the mean ± standard deviation (S.D). A two-tailed student t-test was used to compare the difference between two groups. For independent multiple group comparison, data were examined to meet the criteria of parametric analysis (Kruglyak, Daly et al., 1996), before a one-way ANOVA followed by a Newman-Keuls test was applied. A $p$ value <0.05 or less was considered significant.
CHAPTER 3. RESULTS

3.1 Loss of c-Kit exacerbates atherosclerosis in hyperlipidemic mice

3.1.1 c-Kit is present in the murine aorta

c-Kit is expressed in a diversity of cell types among various organs. In specific, this receptor was found in glial cells and astrocytes (Zhang & Fedoroff, 1999) within the brain; in immune cells, hematopoietic stem cells, and progenitor cells within the bone marrow (Sata et al., 2002); and in ECs (Matsui et al., 2004), SMCs (Miyamoto et al., 1997) within the stratified layers of vasculature. I first confirmed c-Kit vascular expression by tracing this gene in the aorta using a Kit\textsuperscript{Bac}-eGFP reporter mouse (Tallini et al., 2009), which was generously provided by Dr. Rafii from Weill Cornell School of Medical Sciences, Cornell University (New York, NY). In this model, the eGFP gene expression was driven by a c-Kit promoter. I found c-Kit positive (eGFP\textsuperscript{+}) cells were enriched in the boundary of endothelium-intima in aorta, but not in vena cava (Figure 1A), suggesting the presence of c-Kit in murine arteries but not in veins. These arterial cells also stained positive for SMA (red), indicating their SMC lineage (Figure 1B, C). Noticeably, these SMA and c-Kit double positive cells were enriched in the intima or media layers adjacent to the thin endothelial layer, which implied the endothelium contingency for the tunica expression of c-Kit.

3.1.2 c-Kit is expressed in human aorta at different levels

Next, I sought to find out if the c-Kit expression can be found in human vasculature. The samples that I used to assess the expression of c-Kit in human vasculature were obtained from heart transplant patients during surgeon, and generously provided by Dr. SiM. Pham
at UHealth system, Jackson Memorial Hospital heart transplantation center (Miami, FL). In total, nine samples including pulmonary arteries and aortae were harvested from both donors and heart recipient patients. Western blot analysis was performed to determine the expression of c-Kit, its active form p-c-Kit (at Y719), and its unique ligand, stem cell factor (SCF). Despite the inter-patient variation, I found c-Kit and its ligand SCF were detected in all human arterial tissues (Figure 2). Additionally, c-Kit expression was further confirmed by immunofluorescence (IF) microscopy. I observed that the majority of medial SMCs in the human aorta expressed c-Kit (Figure 3). Similarly to what was found in mice, most of the human c-Kit positive cells (red) were also expressed SMA (green), suggesting a vascular c-Kit expression in SMC. Taken together, the above data confirmed the presence of c-Kit and SCF in arterial SMCs, therefore prompted a further research on the role of this signaling axis in atherosclerosis.

3.1.3 c-Kit deficiency increases atherosclerotic burden in hyperlipidemic mice

Receptor tyrosine kinase (RTK) is a cell surface receptor super family, with various members previously identified as critical therapeutic targets in atherosclerosis. It is known that other RTKs such as PDGFR, VEGFR, and EGFR increase the endothelium permeability, or facilitate the vascular SMC synthetic phenotypical transformation during this pathogenic process. Therefore, here I hypothesized that c-Kit is a pro-atherogenic receptor. To test this hypothesis, a c-Kit loss of function mouse model was employed. In this study, all ApoE knockout mice, single mutation strain Kit\(^\text{W/+}\) and Kit\(^{W-v/+}\) mice were purchased from Jackson Laboratory (Bay Harbor, ME). Phenotypically, the Kit\(^{W/+}\) heterozygous mouse has a white spot on the abdomen, meanwhile, the Kit\(^{W-v/+}\)
heterozygous mouse is gray coating with white paws. I bred the heterogeneous mutant strain Kit\(^{W/W-}\) with ApoE\(^{-/-}\) mice as shown in Figure 4. In brief, single mutation Kit\(^{W/+}\) or Kit\(^{W-v/+}\) mouse was initially crossed with ApoE\(^{-/-}\) respectively to first generate Kit\(^{W/+}\) ApoE\(^{-/-}\) or Kit\(^{W-v/+}\) ApoE\(^{-/-}\) mice and then, the Kit\(^{W/+}\) ApoE\(^{-/-}\) or Kit\(^{W-v/+}\) ApoE\(^{-/-}\) strains. Next, the resulting Kit\(^{W/+}\) ApoE\(^{-/-}\) or Kit\(^{W-v/+}\) ApoE\(^{-/-}\) mice were mated with each other to obtain the compound Kit\(^{W/W-}\) ApoE\(^{-/-}\) mice and control littermates Kit\(^{+/+}\) ApoE\(^{-/-}\). This Kit\(^{W/W-}\) strain has white coating due to the compromised c-Kit function in pigmentation.

Due to the phenotype, no further genotyping other than ApoE knockout was necessary for the strain identification. This c-Kit W/W- mutant combination exhibits minimum tyrosine kinase activity, meanwhile guarantees the animal survival from the gestation (Bernstein et al., 1990). c-Kit expression was compared in both Kit\(^{W/W-}\) ApoE\(^{-/-}\) and control (Kit\(^{+/+}\) ApoE\(^{-/-}\)) littermates. I found that aortae and spleens of mutant animals had 4.5 and 9.7-fold less c-Kit expression ($p<0.05$ or $p<0.01$), respectively, than those of Kit\(^{+/+}\) ApoE\(^{-/-}\) control littermates as determined by Western blot (Figure 5). Concurrently, aortae of Kit\(^{W/W-}\) ApoE\(^{-/-}\) mice presented a decreased number of SMA\(^{+}\) c-Kit\(^{+}\) double positive cells with respect to those of controls as shown in IF microscope (Figure 6). I assumed that the decline of c-Kit expression was due to the W allele, which encodes an incomplete protein lacking both extracellular and transmembrane domains. This structurally compromised protein is prone to undergo a fast degradation after translation, resulting in an observed decreased expression of c-Kit as determined by Western blot and IF, despite using a C-terminal detecting antibody. After examination of c-Kit expression, 20 mice (Kit\(^{W/W-}\) ApoE\(^{-/-}\) n=9, and Kit\(^{+/+}\) ApoE\(^{-/-}\) n=11) were fed on a HFD for 16 weeks. After that, I harvested the aortae from both c-Kit deficient mice (Kit\(^{W/W-}\) ApoE\(^{-/-}\)) and
littermate controls (Kit<sup>+/+</sup> ApoE<sup>−/−</sup>) to assess the atherosclerotic burdens by Sudan IV staining. Lack of c-Kit increased the atherosclerotic burden, which was 2.5 times greater in the mutant versus control group. Accumulated lipid is shown as red due to the high fat-soluble and lipid affinity dye Oil Red-O used in the staining procedure as shown in Figure 7 (p=0.0002). Global c-Kit deficiency was associated with significant atherosclerosis in all three main sections of the aorta (arch, thoracic, and abdominal in Figure 8, compared to Kit<sup>+/+</sup>, p< 0.05), and the aortic sinus (p< 0.05, as shown in Figures 9), suggesting a direct link between this receptor tyrosine kinase expression and vasculoprotection. Interestingly, I observed that the extent of the disease at all aortic positions was greater in compound heterozygous mice (Kit<sup>W/W−</sup> ApoE<sup>−/−</sup>) than in those carrying a single c-Kit mutant allele (Kit<sup>W/+</sup> ApoE<sup>−/−</sup> or Kit<sup>W−/+</sup> ApoE<sup>−/−</sup>)(Figure 8), indicating that c-Kit prevents from atherosclerosis through its kinase activity.

Additionally, c-Kit deficient mice were anemic secondary to the lack of c-Kit function that compromised normal hematopoiesis, as indicated by a 2.01 fold decrease in Red Blood Cell level (Table 1, p<0.05 compared to Kit<sup>+/+</sup>), and a 1.36 fold decline in Hb (p<0.05), 1.35 fold in Ht (p<0.05) as listed in Table 1. Both groups of mice developed hypercholesterolemia far above the threshold values (total cholesterol ~200-250 mg/dL for C57/BL6 mouse), although it was significantly less in mutant mice versus control littermates (p<0.05). My results indicated that c-Kit is not a pro-atherogenic receptor, but that it plays a protective role during the atherogenesis in hyperlipidemic mice.
3.1.4 Lack of c-Kit favors the lesional macrophage-like SMC formation

Due to hemodynamic factors, lipoproteins deposit in low shear stress areas of the arterial system such as bifurcations in coronary and carotid arteries, the iliac to femoral area, the aortic arch and the aortic sinus. Therefore, the upper portion of the heart that contains the aortic root was cryo-sectioned in all experimental animals (Kit\textsuperscript{W/W-v} ApoE\textsuperscript{-/-} n=3, and Kit\textsuperscript{+/+} ApoE\textsuperscript{-/-} n=3) to analyze the plaque composition by IF microscopy. I found that the aortic sinus lesions of c-Kit mutant mice showed increased plaque formation, with thicker fibrous caps and increased necrotic core (NC) area, compared to those of control mice as shown in Figure 10. Additionally, the lesion caps in mutant mice were more populated by SMCs (SMA\textsuperscript{+}) that also stained positively for macrophage marker CD68, than those found in the lesion cap of littermate control animals (double positive cell\%, 17.14\% of Kit\textsuperscript{W/W-v} ApoE\textsuperscript{-/-} vs 9.05\% of Kit\textsuperscript{+/+} ApoE\textsuperscript{-/-}, \(p<0.05\)). The results indicated that loss of c-Kit induces the macrophage-like phenotype in SMCs in hyperlipidemic mice.

3.2 Vascular c-Kit is athero-protective in hyperlipidemic mice

3.2.1 c-Kit positive bone marrow transplantation does not alleviate atherosclerosis in c-Kit deficient mice

Next, I sought to delineate the role of bone marrow derived c-Kit\textsuperscript{+} expressing cells in atherogenesis. The RTK c-Kit is essential for hematopoiesis and its absence has a profound impact on the function of the immune system. c-Kit also controls mast cell survival during atherogenesis (Sun, Sukhova et al., 2007). Therefore, I hypothesized that c-Kit positive BM cells are atheroprotective in hyperlipidemic mice. To prove this, I investigated the contribution of c-Kit\textsuperscript{+} BM derived factors by a bone marrow translation
(BMT) as shown in Figure 11. In brief, both c-Kit mutant (Kit\(^{W/W-v}\) ApoE\(^{-/-}\)) mice and control littermates (Kit\(^{+/+}\) ApoE\(^{-/-}\)) were given 1mL suspension of \(10^6\) c-Kit mononuclear BM cells via intravenous tail injection immediately after a lethal irradiation (11,000 rads). The engrafted BM cells were freshly isolated from the femoral bone marrow of age matched Kit\(^{+/+}\) ApoE\(^{-/-}\) donors. Noticeably, c-Kit deficient BM cells fail to guarantee the survival of any recipient mice after original bone marrow removal due to the low number of hematopoietic progenitors (Kitamura, Shimada et al., 1977). And that was the reason why a Kit\(^{W/W-v}\) BMT experiment was not included in this thesis. Both Kit\(^{+/+}\) ApoE\(^{-/-}\) and Kit\(^{W/W-v}\) ApoE\(^{-/-}\) chimeric mice underwent a four weeks recovery period with normal chow and various antibiotics supplemented water (100 I.U./mL penicillin and 100 µg/mL streptomycin, in sterile water), followed by 16 weeks of HFD. After that, I harvested aortae from chimeric mice for Sudan IV staining as explained in Specific Aim 1. I found, c-Kit positive BM cells failed to alleviate the atherosclerotic plaque accumulation in mutant chimeric mice, as indicated by a 1.8 fold greater atherosclerotic burden within the aorta of chimeric Kit\(^{W/W-v}\) ApoE\(^{-/-}\), with respect to chimeric Kit\(^{+/+}\) ApoE\(^{-/-}\) (Figure 12, \(p=0.034\)), despite the alleviated anemic condition was achieved after the successful engraftment of c-Kit BM cells (\(p\) values of RBC, Hb, Ht, MCV were <0.05 pre-BMT as shown in Table 1, whereas all the values were >0.05 in post-BMT as shown in Table 2). Taken together, these results indicated that BM c-Kit is not athero-protective, thus in implied a key role of vascular local c-Kit signaling in the prevention of lesion formation.
3.2.2 c-Kit expression in SMCs is vasculoprotective

As I excluded the vasculoprotective role of BM derived c-Kit+ cells in atherogenesis, I next pursued the hypothesis that expression of c-Kit in SMC protects hyperlipidemic mice against atherosclerosis. To evaluate the role of vascular c-Kit in atherogenesis, I generated a novel SMC specific c-Kit conditional knockout mouse model \([\text{Kit}^{\text{lox66-lox71}}/\text{lox66-lox71}} \text{Myh11-CreER}^{T2} \text{ApoE}^{\text{lox66-lox71,lox66-lox71-ApoE}^{\text{lox66-lox71,lox66-lox71}}}]\]. Briefly, I crossed the Kit\(^{\text{lox66-lox71}}\) mouse that carries a Kit allele with a stop codon located between exon 8 and 9 of Kit gene with a Myh11-CreER\(^{T2}\) ApoE\(^{\text{lox66-lox71,lox66-lox71}}\) transgenic mouse as shown in Figure 13. In the former mouse model, both lox66 and lox71 are mutant loxP sites with variant sequences. Upon the Cre recombinase expression, the sequence between the lox66-lox71 pair is inverted. Meanwhile, this Cre-mediated recombination results in one lox72 and one wild type loxP site, thus disabling the possibility of further inversion (Oberdoerffer, Otipoby et al., 2003). As shown in Figure 14A, male mice with Kit\(^{\text{lox66-lox71}}\), Myh11 CreER\(^{T2}\), and ApoE null were selected for further experiments. Noticeably, only male mice could be used in this study because the Myh11 allele is located on the Y chromosome. After Tamoxifen (TAM) treatment for four weeks, the inversion of the c-Kit lox66-lox71 cassette in this triple transgenic mouse was achieved. The TAM fed mice, but not the vehicle chow fed control animals, had inverted (inactive) Kit\(^{\text{lox66-lox71}}\) alleles (Figure 14B). As a result, TAM fed mice presented a decreased c-Kit protein content (47%) in the vasculature (Figure 15A, \(p<0.01\)). Immunofluorescence microscopy (IF) further confirmed c-Kit depletion in the majority of medial SMCs (Figure 16). Nonetheless, as expected, TAM did not affect splenic c-Kit levels in conditional mice (\(p>0.05\), Figure 15B). In total, thirteen of these triple transgenic mice (Vehicle chow n=6, and TAM chow
n=7) were put for 16 weeks on a HFD chow challenge as described in **Specific Aim 1** for hyperlipidemia introduction. After 16 weeks, I found those TAM-treated conditional knockout mice exhibited a 2.47 fold increase in the atherosclerotic burden in the aorta (Figure 17, \( p = 0.0012 \)). Similarly, more lipid deposition was observed in the aortic sinus of TAM mice (Figure 18, \( p < 0.05 \)) compared to those consuming normal diet (Vehicle). Additionally, no differences in terms of atherosclerosis burden between TAM and vehicle treated \( ^{+/-} \) \& \( ^{-/-} \) ApoE mice were found (\( p > 0.05 \) as in Figure 19, \( n=5 \) per group), which rules out any significant drug related protective effects of TAM on atherogenesis as we expected (Fontaine, Abot et al., 2013) in this animal model. The loss of c-Kit facilitated plaque pathogenesis, as shown in the lesion site within aortic sinus, where TAM treated conditional knockout mice had lesions with 2.23 fold more SMC-derived macrophages in the cap area (SMA and CD68 double positive cells, Figure 20, \( p < 0.05 \)) than those in control mice. Taken together, I confirmed the importance of c-Kit signaling in SMCs to prevent atherosclerosis in mice. Interestingly, in spite of an exacerbated atherosclerotic plaque deposition in murine aorta and aortic sinus valve, a significantly decreased in serum cholesterol was observed in TAM led mice with respect to Vehicle chow controls (1022.83 ± 44.61mg/dL vs 560.50 ± 130.21mg/dL, \( p < 0.05 \)) and lipoprotein levels (HDL 97.17 ± 19.03 mg/dL vs 58.00 ± 20.65 mg/dL, \( p < 0.01 \) and LDL 898.50 ± 57.42 mg/dL vs 425.75 ± 78.69 mg/dL, \( p < 0.05 \)) as listed in Table 3. Noticeably, both cholesterol and lipoprotein levels in control mice consumed normal chow (Vehicle) or TAM led mice were significantly increased compared to the physiological range for mouse species, indicating even this difference is statistically significant, however, both mice were identically at a high risk of hyperlipidemia and resulting atherosclerosis.
3.3 Role of c-Kit in the smooth muscle cell phenotype switch

3.3.1 c-Kit inhibits the synthetic phenotype switch in SMCs

The phenotypic switching of SMCs from the contractile to the synthetic phenotype is considered as one of the essential contributing factors for atherogenesis and progression of atherosclerosis (Owens, 2007). Synthetic SMCs lose the expression of contractile markers such as Calponin, Myocardin, and smooth muscle actin (SMA), while they gain synthetic marker expression such as Caldesmon and Vimentin (Beamish et al., 2010). Functionally, these phenotypically compromised SMCs present hyper-proliferation and migration activities, facilitating the plaque formation in during early atherogenesis. Therefore I hypothesized that c-Kit prevents SMC phenotypic switching towards a synthetic phenotype. To validate this, I investigated if c-Kit modifies the SMC phenotype in vivo, by characterizing aortic SMCs in Kit\(^{W/W-v}\) and Kit\(^{+/+}\) mice using histopathology, electron microscopy (EM), immunoblot (IB) and immunofluorescence (IF). By hematoxylin and eosin (H&E) staining, I observed that aortic SMCs were more abundant in the tunica media and adventitia layers from Kit\(^{W/W-v}\) mice with respect to Kit\(^{+/+}\) mice (Figure 21, \(p< 0.05\)). Further EM examination revealed that SMCs from mutant mice were with disorganized cytosol architecture and giant vacuoles around the nucleus (see Figure 22). Similarly, vacuoles have been observed and reported as a consequence of synthetic activity in SMCs (Salabei et al., 2013). In addition, I found that most of the markers for the contractile phenotype in SMCs were significantly downregulated in the aorta of c-Kit mutant mice with respect to controls (Figure 23, all markers \(p<0.05\)). On the protein level, I found lack of c-Kit activity compromised the expression of contractile markers with a 2.04 fold decrease in Calponin and 2.08 fold in
SM22α ($p<0.05$) compared to wild type control aortae (Figure 24). To validate that loss of c-Kit determines the SMC synthetic phenotype, I further isolated and cultured primary SMCs from both c-Kit mutant (Kit^W/W-v) and control (Kit^+/+) aortae ($n=3$ per group). In addition, a c-Kit rescued SMC line was created by lentivirus transfection, and included to in the following experiments to confirm the critical role of c-Kit in this phenotypical transformation. I observed that c-Kit expression in mutant cells not only restored the receptor expression (top panel, 2.54 times greater vs mutant, Figure 25, $p<0.01$), but also the expression of contractile marker Calponin and SM 22α (2.14 and 2.83 times greater, respectively, see Figure 25, both $p<0.05$). Next, I determined the effect of c-Kit deficiency on cell function employing proliferation and migration assays. PDGF-BB, as a ligand of PDGFR, which is widely used for inducing SMC synthetic phenotype in vitro (Chen, Li et al., 2006, Salabei et al., 2013), was applied to both c-Kit wild type and mutant SMCs. I observed that c-Kit deficient SMCs presented with a higher proliferation rate (Figure 26, starting from day 5, $p<0.05$ or $p<0.01$) compared to Kit^+/+ and rescued SMCs in response to the PDGF-BB (20ng/ml) treatment (Tang, Zhang et al., 2016). Migrated cells were fixed, stained with DAPI and counted. Active migration was observed in c-Kit mutant SMCs, with more cells migrating from the upper chamber inserted into the 24-well plate to the bottom well (2.15 times difference compared to wild type control, $p<0.01$; 2.08 times difference with respect to c-Kit rescue, $p<0.05$), as response to chemo traction. Meanwhile, migration was little in Kit^+/+ SMCs and c-Kit rescued cells (Figure 27). These results supported my hypothesis and showed evidence that c-Kit deficiency facilitates the phenotypic switch of SMCs from a contractile to a synthetic state.
3.3.2 c-Kit prevents the foam-cell like phenotype in synthetic SMCs

Recent investigations indicate that SMCs may undergo a phenotype recession, to a “mesenchymal like” phenotype. With potent plasticity, these mesenchymal like SMCs in pathological conditions may acquire a different cell fate (Bennett et al., 2016). In the scenario of atherosclerosis, Owens group (Shankman et al., 2015) and others (Feil, Fehrenbacher et al., 2014, Rong et al., 2003) showed evidence both in vivo and in vitro, undifferentiated SMCs intake lipid upon a hyperlipidemic stimulus. Therefore, I hypothesized that c-Kit deficiency leads to a phenotypic switch that favors the formation of lipid-loaded cells (foam-like cells). To prove this, I cultured both c-Kit wild type (Kit^{+/+}) and mutant (Kit^{W/W}) SMCs with water soluble cholesterol in serum deprived medium for 72 hrs (Pidkovka et al., 2007), and performed semi-quantitative and quantitative examinations to evaluate the cytosol lipid accumulation. c-Kit deficient SMCs showed significantly more cytoplasmic lipids as determined by Sudan IV staining in line with my in vivo results, which suggested a major contribution of SMC to the pool of lesional macrophages in c-Kit mutant mice (Figure 28A). In addition, these results were confirmed by quantitative determination of the cellular total cholesterol (TC) in c-Kit mutant cells which was significantly elevated compared to c-Kit rescued cells (267.68 ± 7.52 vs 120.74 ± 9.98 ug/mg) and those from controls (267.68 ± 7.52 vs 169.27 ± 8.91 ug/mg) as shown in Figure 28B (p<0.01). Altogether, these data demonstrated that c-Kit is critical for the SMC macrophage-like phenotype switch, thus validating my hypothesis.
3.3.3 c-Kit prevents SMC phenotypic switching and foam-like cell formation via KLF-4

In mechanistic studies, transcriptional factors belonging to the family of Kruppel like factor (KLF) has previously drawn attention due to the dissimilar roles played by its members in atherosclerosis development (Alaiti, Orasanu et al., 2012). KLF2 was found to participate in the inflammation response as a negative regulator (Das, Kumar et al., 2006); while KLF15, to the opposite, activates the inflammatory response in the content of atherosclerosis (Lu, Zhang et al., 2013). Interestingly, despite its detrimental role in inflammation, KLF15 suppresses SMC proliferative phenotype transformation in the scenario of neo-intima hyperplasia (Lu, Haldar et al., 2010), and inhibits lipogenesis under certain conditions (Takeuchi, Yahagi et al., 2016). Among all these KLF family members, KLF4 was reported to suppress the expression of SMC specific markers and promote the phenotypic switching of SMCs toward a foam cell like phenotype (Rong et al., 2003, Shankman et al., 2015). Based on this evidence, I hypothesized that c-Kit deficiency leads to a SMC phenotype switch through a KLF4 dependent mechanism. To verify this hypothesis, KLF4 expression in c-Kit mutant (Kit<sup>W/W-v</sup>) SMCs was examined. I found both protein and mRNA levels of KLF4 were augmented in c-Kit deficient SMCs (Kit<sup>W/W-v</sup>), compared to c-Kit rescued cells and those from littermate controls (Figure 29A, B, $p<0.05$ or $p<0.01$). In addition, the transcriptional activity of KLF4, was increased in mutant versus both control and rescued SMCs (2.72 fold and 1.92 fold, respectively, both $p<0.05$), as determined by a dual luciferase reporter assay, where KLF4 specific transcription activity (Firefly) was standardized with the non-specific internal control (Renilla) as shown in Figure 29C. Finally, I knocked down KLF4 in c-
Kit deficient and control SMCs with >90% efficacy using specific siRNAs (Figure 30, p<0.05, the pool of target siRNA and scramble control, see Supplementary Table 2). As expected, expression of SM22α, as a contractile marker, restored secondary to loss of KLF4 in Kit\(^{W/W^{-}}\) SMCs. However, knockdown of KLF4 in Kit\(^{+/+}\) SMCs did not lead to significant change in both KLF4 and SM22α expressions within these contractile wild type SMCs. Furthermore, both the cellular lipid accumulation and cholesterol levels were attenuated in Kit\(^{W/W^{-}}\) SMCs after KLF4 knockdown compared to the scrambled siRNA negative controls (218.02 ± 27.49 vs 165.26 ± 18.20 ug/mg Figure 31, p<0.05), indicating that lipid deposition in c-Kit deficient cells is dependent on KLF4 expression. In summary, these results indicate that c-Kit prevents SMC phenotype switch by inhibiting KLF4 expression and transcriptional activity, thus validating my hypothesis.

3.3.4 Different gene expression profiles in c-Kit positive and mutant SMCs reveal that the loss of c-Kit determines the pro-inflammatory phenotype in SMCs

Considering the reported contribution of the c-Kit receptor to vascular remodeling processes (Hollenbeck et al., 2004, Skartsis et al., 2014, Wang et al., 2006b, Wang et al., 2007, Young et al., 2016), I sought for differentially expressed genes between c-Kit deficient (Kit\(^{W/W^{-}}\)) and control SMC (Kit\(^{+/+}\)). To achieve this, three independent total RNA samples were isolated from cultured Kit\(^{+/+}\) or Kit\(^{W/W^{-}}\) SMCs, and send to Ocean Ridge bioscience company for a mouse whole genome microarray assay. Out of a total of 34,265 mouse probes queried by microarray, 18,224 yielded a detectable signal above the threshold, and 1,086 genes were found differentially expressed between both experimental groups (Figure 32). Specifically, 564 and 522 transcripts were significantly
up- or downregulated, respectively, with the loss of c-Kit activity compared to control SMC (Figure 32A, B). No statistically significant differences in expression were detected by microarray in the remaining 17,138 genes. Table 4 presented selected differentially expressed genes in c-Kit deficient SMC that are relevant for inflammation such as Ilf2, Ifna14, and Tnfsf9 (Chan, Newman et al., 2006, Crot, 2009, Zhao, Shi et al., 2005). Decreased expression of the anti-inflammatory genes Foxo1, Gdf6, Igf1, Igf2r, and Lpl (Hisamatsu, Ohno-Oishi et al., 2016, Savai, Al-Tamari et al., 2014, Sukhanov, Higashi et al., 2007, Ziouzenkova, Perrey et al., 2003) were also observed. Lipoprotein lipase (Lpl), for example, it was found 14-fold lower in c-Kit deficient cells than in those isolated from littermate controls. Additional changes in c-Kit deficient SMC were associated with a downregulation of the contractile SMC phenotype (increased Tnfaip3 and reduced Sirt1) (Damrauer, Peterson et al., 2010, Huang, Yan et al., 2015) and higher susceptibility to calcification (decreased Foxo1 and Pth1r) (Cheng, Shao et al., 2010, Deng, Huang et al., 2015). Finally, I found significant expression differences in genes that code for cell adhesion proteins, receptors, and enzymes that regulate vasomotor responses (Table 4).

3.3.5 Activation of inflammatory prone NF-κB signaling in c-Kit deficient SMCs by pathway analysis

In silico pathway analysis was used to predict the molecular pathways affected by the loss of c-Kit in SMCs. A total of 71 statistically significant pathways were identified (p<0.05 or p<0.01) by the Ingenuity Pathway Analysis (IPA) software, 42 of which with a biologically relevant function in SMC. These pathways covered cellular processes such
as cell survival and apoptosis, inflammation, cell adhesion, nitric oxide signaling, and lipid metabolism (Table 5). Interestingly, 10 independent molecular pathways were associated with NF-κB signaling, and all of them showed either predicted activation of the entire pathway (5/10; z-scores ranging from 0.258 to 1.265), or of the NF-κB branch (5/10) in c-Kit deficient SMCs (Table 5).

### 3.3.6 Upregulation of NF-κB pathway related genes in c-Kit deficient SMCs

The NF-κB pathway plays a fundamental role in SMC differentiation, inflammation, and response to stress signals (Mack, 2011, Mehrhof, Schmidt-Ullrich et al., 2005, Ramana, Friedrich et al., 2004, Zahradka, Werner et al., 2002). Therefore, I confirmed the upregulation of 7 key components (Ikbka, Ikbkb, Ikbkg, Nfkbia, Map3k14, Nfkb2, RelB) of this pathway in c-Kit deficient cells by real-time PCR (Figure 33A, p<0.05 or p<0.01). As expected, significantly higher expression levels of genes that are part of both the canonical (Ikbka, Ikbkb, Ikbkg, Nfkbia) and alternative (Ikbka, Map3k14, Nfkb2, RelB) NF-κB signaling pathways in Kit<sup>W/W-v</sup> vs. Kit<sup>+/+</sup> SMC was observed. Confirmatory real-time RT-PCR assays were performed for selected inflammation-related genes that showed a non-significant trend by microarray analysis. Tumor necrosis factor (Tnf), interleukin 6 (Il6), C-C motif chemokine ligand 2 (Ccl2), metalloproteinases 2 and 9 (Mmp2, Mmp9), inducible nitric oxide synthase (Nos2), and cyclooxygenase 2 (Ptgs2) were significantly upregulated in c-Kit deficient SMC with respect to controls (Figure 33B, p<0.05).
3.3.7 Increased activity of the canonical NF-κB pathway in stimulated c-Kit deficient SMCs

Given that the NF-κB pathway related genes (Figure 33A) and genes of pro-inflammatory mediators are upregulated in c-Kit deficient SMC (Figure 33B), I hypothesized that this NF-κB activation is associated with c-Kit deficiency in SMCs. Therefore, I investigated the relationship between c-Kit expression and functional activity of NF-κB signaling pathways. To simulate the hyperlipidemic condition in vivo, an artificial phospholipid, 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC), was employed to induce the phenotype in SMCs. As an activator of NF-κB cascade (Pegorier et al., 2006, Vladykovskaya et al., 2011), POVPC induced higher NF-κB transcriptional activity in SMCs with deficient c-Kit expression, compared to wild type with 1.89 fold increase, and c-Kit rescued cells with 1.47 fold increase, as determined by a dual luciferase reporter assay (Figure 34A, \( p<0.05 \)). Additionally, a significantly higher ratio of the S536-phosphorylated/total p65 was detected in Kit\(^{W/W-v}\) SMCs versus wild type (1.93 fold) and rescued cells (1.52 fold), as presented in Figure 34B (\( p<0.05 \)), demonstrating increased availability of active p65 in c-Kit deficient SMC for nuclear translocation and promoter binding (Lawrence, 2009). Finally, I examined the expressions of pro-inflammatory mediators, as the endpoint production of the NF-κB signaling pathway. Among them, MCP-1 is a chemokine responsible for immune cell adhesion to the lesion site in the early stage of atherosclerosis (Song, Kang et al., 2016a); while matrix metalloproteinase MMP-2 accounts for the degradation collagen and ECM, which may result in plaque rupture and subsequent thrombosis formation at the end stage of atherosclerosis (Lee, Seo et al., 2008, Song et al., 2016a). All of these mediator
productions are linked with NF-κB activation. In accordance with the enhanced transcriptional activity shown above, the protein expressions of both MMP-2 (6.50 fold increase compared to control, and 2.84 times compared to rescue), and MCP-1 (4.59 fold increase compared to control, and 3.35 times compared to rescue) were significantly higher in POVPC-stimulated Kit<sub>W/-v</sub> SMCs compared to wild type and rescued cells (Figure 34C, p<0.01). Taken together, these results indicated that c-Kit deficiency leads to NF-κB activation and pro-inflammatory mediator production in c-Kit mutant SMCs, thus validating hypothesis.

3.3.8 c-Kit negatively regulates NF-κB activation through TAK1/NLK axis in SMCs

Src family of tyrosine kinase (SFK) is a non-receptor tyrosine kinases, and involved in c-Kit signaling activation upon SCF binding. Specifically, SFK facilitates the phosphorylation of tyrosine residue Tyr568, and provides binding sites for downstream signaling activations such as Ras/Erk pathway (Ronnstrand, 2004). Previous studies indicated an association between c-Kit, Lyn, and TAK1 (Drube, Weber et al., 2015), a negative regulator of NF-κB signaling in other cell lines such as neutrophils (Ajibade et al., 2012). Therefore, we investigated whether TAK1 or its downstream partner NLK (Li et al., 2014, Yasuda et al., 2004) contribute to the observed inhibition of NF-κB activity in c-Kit expressing SMC. The protein expressions of both TAK1 and NLK were found reduced or even lost in Kit<sub>W/-v</sub> SMCs compared to wild type or c-Kit rescued cells as determined by Western blot (Figure 35A, p<0.05 or p<0.01). Next, I selectively knocked down TAK1 or NLK in Kit<sup>++</sup> SMCs by lentivirus infection as previously described (pool of target siRNA and scramble control, see Supplementary Table 3). Lentiviral infection
led to a dramatic downregulation or even absent expression of the target proteins TAK1 and NLK, respectively, as determined by Western blot (Figure 35B, C, \(p<0.01\)). Further evidence indicated that these genetic manipulations restored the NF-κB transcriptional activity (4.89 fold increase of siTAK1, \(p<0.05\); and 4.47 times increase of siNLK, \(p<0.05\), Figure 36A), phosphorylated/total p65 ratio (1.85 fold increase of siTAK1, \(p<0.01\); and 1.36 times increase of siNLK, \(p<0.05\), Figure 36B), and consequently increased the protein expressions of MMP-2 (13.13 fold increase of siTAK1, and 11.38 times increase of siNLK), and MCP-1 (4.68 fold increase of siTAK1, and 4.36 times increase of siNLK), with respect to control c-Kit wild type SMCs under POVPC treatment (Figure 36C, \(p<0.05\) or \(p<0.01\)). Lastly, I sought to understand how c-Kit mediates TAK1/NLK inactivation in SMC. As in Figure 37A and B, a physical interaction between all c-Kit, Src, TAK1, and NLK was demonstrated by alternative c-Kit- or TAK1- baited co-IPs. Altogether, these experiments demonstrate that c-Kit inhibits NF-κB signaling in SMC through the interactions of TAK1 and NLK as shown in Figure 37C.
CHAPTER 4. DISCUSSION

The c-Kit receptor tyrosine kinase is a proto-oncogene and stem cell marker that has been recently implicated in vascular pathogenesis. Widely recognized for its proliferative and anti-apoptotic role in hematopoietic stem and progenitor cells (Bernstein et al., 1991), c-Kit signaling is now known to increase endothelial permeability (Im, Song et al., 2016, Kim, Choi et al., 2014), and regulate the phenotype of smooth muscle cells (SMC) in the vasculature (Davis, Hilyard et al., 2009, Wang et al., 2007). My research was aimed at demonstrating c-Kit has an important role in the pathogenesis of atherogenesis.

4.1 Distinct roles of c-Kit in atherosclerosis and other vascular disease models

In this dissertation, first, I demonstrated that c-Kit expression was found in human vasculature, especially, in vascular SMCs (Figure 2, 3). Second, I illustrated for the first time that c-Kit signaling protects the vasculature in a hyperlipidemic mouse model of atherosclerosis (Figure 7, 9). Moreover, c-Kit protection depended upon the number of functional alleles, with compound heterozygous animals showing a more extensive plaque formation than mice carrying none or a single mutation (Figure 8).

Interestingly, my finding that c-Kit is athero-protective in hyperlipidemic mice contradicts the existing belief about the noxious effect of SCF/c-Kit signaling to the vasculature.

Before this study, the role of c-Kit in vasculature is controversial. On one hand, animal models indicate that c-Kit activation by its ligand SCF plays an important role in the development of both arterial and venous intimal hyperplasia (IH) (Hollenbeck et al., 2004, Skartsis et al., 2014, Wang et al., 2006b, Wang et al., 2007). Additionally, in
another model of pulmonary artery disease, a small population of c-Kit expressing myofibroblasts/SMCs was also found in pulmonary arteries of patients with idiopathic pulmonary arterial hypertension but not in healthy controls (Montani, Perros et al., 2011). In line with this evidence, both SCF and c-Kit expressions were upregulated in pulmonary arterioles of experimental animals with pulmonary hypertension, where c-Kit co-localized with cells in the endothelium, media, and adventitia (Young et al., 2016). In the latter model, SCF/c-Kit signaling promotes pathological remodeling and pulmonary vascular cell proliferation after hypoxic stimulation via activation of the ERK1/2 pathway (Young et al., 2016); On the other hand, c-Kit expression preserves the SMC contractile phenotype (Davis et al., 2009). In specific, the presence of c-Kit in human primary pulmonary artery SMC upregulates the transcription factor Myocardin and preserves the contractile SMC phenotype, suggesting a protective role of c-Kit under certain vascular conditions. Therefore, whether c-Kit is beneficial or detrimental in vasculature is still under debate, and merits further investigation.

It is noteworthy to recognize that such idea originated from descriptive studies and models of post injury neointimal hyperplasia, in which prior studies have shown reduced neointimal hyperplasia secondary to the loss of c-Kit function in progenitors and SMCs (Hibbert, Chen et al., 2004, Hollenbeck et al., 2004, Wang et al., 2007). I postulate that the SCF/c-Kit axis switches from protective to a pathological context in the setting of post injury neointimal hyperplasia, because it increases the survival of proliferative SMCs through an Akt-dependent mechanism (Wang et al., 2007). This apparent discrepancy can be reconciled on the ground that neointimal hyperplasia (restenosis) and atherosclerosis are different diseases in terms of etiology, natural history, culprit lesions,
and progenitor cell contribution to lesion progression. A defining feature of neointimal hyperplasia is the accumulation of myofibroblasts within the inner layer of an artery or vein in response to injury. In contrast, atherosclerosis is a chronic condition that involves a progressive accumulation of lipid-loaded macrophages in the vessel wall that forms a core encapsulated by layers of SMCs. Progression of this condition is favored by homing of c-Kit positive mast cells and other circulating inflammatory cells to the expanding plaque (Sun et al., 2007). However, these studies confirm that the protective effect of vascular c-Kit expression overrides the negative role of circulating inflammatory cells by suppressing vascular SMCs switching to an athero-prone phenotype.

Another interesting finding of my work is that the serum cholesterol levels are lower in c-Kit deficient mice despite the fact that atherosclerosis is exacerbated (Table 1). Nonetheless, it is noticeable that both cholesterol and lipoprotein levels remained out of the physiological range for mouse species, indicating both c-Kit deficient and wild type control mice were still at an identically high risk to develop atherosclerosis. However, this observation suggests that c-Kit may play a role in modulating cholesterol absorbance or metabolism, a finding which has not yet been described.

In addition, I found that lesional Kit^{W/W-} SMCs may acquire the macrophage marker expression (Figure 10). More SMA and CD68 double positive cells were found in the plaque of Kit^{W/W-} ApoE knockout mice, especially in the fibrous cap area, indicating that the local SMCs may be a key target which is predominately responsible for the pathogenic process. Taken together, my data revealed that the global deficiency of c-Kit
exacerbates atherosclerotic plaque deposition in the arteries, and favors the macrophage-like phenotype transformation in lesional SMCs, in spite of the decline in serum cholesterol and lipoprotein levels.

4.2 Bone marrow derived c-Kit archives limited effects in vascular injury repair

To comprehensively understand the role of bone marrow derived factor, a c-Kit positive bone marrow transplantation (BMT) was delivered (Figure 11). I found that, in spite of the recovery of the hematopoiesis (Table 2), these c-Kit positive BM cells were not sufficient to alleviate the atherosclerotic phenotype in hyperlipidemic mice. This result seemed to contradict the widely accepted belief that, the stem cells and progenitor cells (PCs) within the bone marrow are responsible for the atherosclerotic lesion repair. However, this topic attracted intensive research attention, and different hypotheses have been advised in recent years (Frodermann, van Duijn et al., 2015, Liu, Sinha et al., 2005). For example, it was suggested that stem cells and progenitors could be recruited upon the tissue damage to orchestrate repair by direct trans-differentiation or through paracrine affections. A good example is, bone marrow derived endothelial progenitor cells (EPC) were believed to account for the dysfunctional endothelium repair in the context of atherosclerosis (Dotsenko, 2010). Except from the role of injury repair, further studies claimed that these stem cells/progenitors were athero-protective. In specific, the local vascular progenitor cells were found to differentiate into SMCs, thus reinforcing the fibrous cap and stabilizing the vulnerable plaque from rupture (Wang, Hu et al., 2015).
However, it merits attention that instead of the beneficial role of stem cell/progenitors, opposite opinions claim these non-differentiated cells contribute to the early stage lesion formation, thus facilitating the pathogenic progression. For example, the result from Sata’s team (Sata et al., 2002) revealed that instead of vasculature local tunic SMC proliferation and migration, those vascular progenitors are contributing to the fibrous cap formation of the plaque.

More recently, the stem cell contribution to atherosclerosis has been challenged by other studies, in which researchers argued that bone marrow derived cells poses non-essential roles in neither atherogenesis nor lesion repair. For example, one study delivered by Xu’s team claimed those progenitors had limited contribution for the atherogenesis (Xu, 2006). Interestingly, this conclusion contradicts to their own findings, in which they believed the essential role of progenitors in atherogenesis (Xu, 2008). Another investigation challenged Sata’s finding by arguing that these SMCs in atherosclerotic lesions are not progenitor originated, but local derived using an ApoE knockout mouse model (Bentzon, Weile et al., 2006). These opposing opinions were also supported by other groups, who believe in a non-critical role of these stem cells/progenitors in the context of atherosclerosis (Zoll, Fontaine et al., 2008).

Another bone marrow derived factor that deserves attention in a diversity of immune cells originally derived from BM cells, which were further differentiated into monocyte and monocyte-derived macrophage (Lessner, Prado et al., 2002), CD11c+ dendritic cells (Koltsova & Ley, 2011), and CD4+ T cells (Hansson & Hermansson, 2011). These matured immune cells widely participate in atherogenesis and subsequent atherosclerosis
progressions, such as early stage endothelium infiltration, and vascular inflammation amplification during the development stages.

Taken together, though previous investigations suggested both the non-differentiated cells (stem cells and PCs) and BM derived immune cells have their roles that affect atherogenesis, however, in my research, these bone marrow derived factors fail to significantly alleviate atherosclerosis in c-Kit deficient mice.

4.3 Vascular c-Kit expression is athero-protective in hyperlipidemic mice

As discussed above, our bone marrow transplantation experiment excluded the possibility that bone marrow derived c-Kit exerts an essential function in the c-Kit mediated vasculoprotective effects. Instead, it suggested that local c-Kit factor may predominately account for its athero-protective effect.

To validate whether vascular c-Kit is critical for atherosclerosis prevention, a c-Kit SMC conditional knockout mouse model was employed (Figure 13). I found, similarly as the c-Kit global deficiency, that c-Kit SMC deficient mice presented exacerbated atherosclerotic plaque deposition in aortae (Figure 17) and aortic roots (Figure 18). Meanwhile, more macrophage-like SMCs (SMA⁺, CD68⁺) were found in the lesion site, especially enriched in the fibrous cap area (Figure 20). Interestingly, declined serum cholesterol and lipoprotein levels were observed in the c-Kit vascular deficient mice compared to their control littermates (Table 3). Nonetheless, these cholesterol and lipoprotein levels were significantly greater than the physiological range for mouse species. These out-range high cholesterol/lipoprotein levels suggest that both c-Kit vascular deficiency mice and control littermates were identically at high risk of
atherosclerosis. Nonetheless, it merits further study to understand the role of c-Kit receptor in cholesterol turnover.

As a member of RTK, c-Kit plays a protective role in atherosclerosis. Surprisingly, unlike c-Kit, other RTK members such as PDGFR, VEGFR, and EGFR were previously found to play a detrimental role in atherogenesis, via affecting SMC synthetic phenotype transformation (Gomez & Owens, 2012), endothelium permeability (Roberts & Palade, 1995, Sukriti, Tauseef et al., 2014), or monocyte proliferation and macrophage differentiation (Lamb, Modjtahedi et al., 2004). One explanation why we found that c-Kit is distinct from other RTK members might be that different signaling pathways downstream or the transcriptional factors are affected. It is possible that the key factors which determine SMC phenotype such as Myocardin (Wang, Wang et al., 2003) and serum response factor (SRF) are differently regulated by other RTK members than by c-Kit (Liu et al., 2005). This unsolved question triggers me for a further understanding of the underlying mechanism, which has been investigated in the following section.

4.4 c-Kit mediated signaling pathways in SMCs

The mechanism investigation presented the de novo evidence and identified c-Kit as a determinant and/or modulator of the SMC phenotype in the setting of atherosclerosis. In specific, my mechanistic study demonstrated that c-Kit expression preserves the contractile SMC phenotype and hinders their growth (Figure 26), migration (Figure 27), acquisition of a macrophage-like phenotype (Figure 28), and pro-inflammatory characteristics (Figure 34).
SMCs are characterized by tremendous phenotypic diversity during pathogenesis (Yoshida & Owens, 2005). Remarkably, their contributions to vascular diseases greatly depend on their phenotype and state of differentiation (Archer, 1996, Yoshida & Owens, 2005). On one hand, it has been shown that targeted inhibition of c-Kit in SMCs in vitro decreased the expression of SMC-specific contractile markers by reducing the activity of the transcriptional coactivator Myocardin (Davis et al., 2009). Active Myocardin is a potent co-activator of serum response factor (SRF), one of the master MCM1, AGAMOUS, DEFICIENCY and SRF (MADS)-box transcription factors that control SMC gene expression (Wang, Chang et al., 2001). On the other hand, expression of the c-Kit receptor in SMC has been associated with various vascular pathologies such as restenosis and neointimal hyperplasia in both animal models (Skartsis et al., 2014, Wang et al., 2006b, Wang et al., 2007, Young et al., 2016) and human samples (Hollenbeck et al., 2004, Skartsis et al., 2014). However, little achievement has been made to prove its role in the model of atherosclerosis before this thesis.

My research demonstrated that in the setting of atherosclerosis, the SMC synthetic phenotype associated with loss of c-Kit promotes the formation of foam-like cells in a KLF4-dependent manner (Figure 31). These results support the cause and effect relationships between loss of c-Kit actions and enhanced expression of KLF4. These findings are consistent with previous reports stating that contractile gene markers (e.g. SM22α) are selectively down-regulated prior to phenotypic trans-differentiation of SMCs to macrophage-like cells (Feil et al., 2014, Rong et al., 2003, Rosenfeld, 2015, Shankman et al., 2015). Noteworthy, such trans-differentiation is accompanied by increased expression of KLF4 in c-Kit receptor null SMCs (Shankman et al., 2015). KLF4 mediates
the trans-differentiation of contractile SMCs toward a macrophage-like phenotype by binding to and activating G/C repressor elements in the promoters of contractile genes including SMA (\textit{ACTA2}), SM22\alpha (\textit{Tagln}) and SM-MHC (\textit{Myh11}) (Salmon, Gomez et al., 2012, Yu, Zheng et al., 2011, Zheng, Han et al., 2010) (Liu et al., 2005, Mack, 2011, Pidkovka et al., 2007). KLF4 also suppresses Myocardin expression, thereby blocking transcription of contractile genes that contain CA/T-rich-G (CArG) promoter elements (Regan, Adam et al., 2000, Wamhoff, Hoofnagle et al., 2004). Moreover, KLF4 was recently reported to mediate the transformation of contractile SMCs to SMC-derived macrophages (Bennett et al., 2016, Pidkovka et al., 2007, Shankman et al., 2015). It is important to note that the role of KLF4 in atheroprotection highly depends on the cellular context. The expression of KLF4 in endothelial cells and macrophages protects mice against atherosclerosis through dissimilar mechanisms (Sharma, Lu et al., 2012, Zhou, Hamik et al., 2012).

By microarray analysis, I obtained comprehensive information about differential gene expression in pathological SMCs which lack c-Kit receptor compared to control SMCs. Overall, the screening analysis revealed that the absence of c-Kit modified the expression of approximately 6\% of the genes that were detected by microarray in SMC from c-Kit mutant and littermate control mice (\textbf{Figure 32A}). In addition, by application of \textit{in silico} pathway analysis, it was found that 24\% of the differentially regulated pathways identified were associated with NF-\kappa B signaling (\textbf{Table 6}). Furthermore, both the \textit{in silico} analysis and the experimental data demonstrated activation of this pathway in c-Kit deficient SMC with respect to those from littermate controls (\textbf{Figure 34}).

While increased vascular lipoprotein lipase can be pro-atherogenic (Clee, Bissada et al.,
NF-κB signaling is critical for the regulation of proliferation, differentiation, stress responses, and inflammatory processes in vascular SMC (Mack, 2011, Mehrhof et al., 2005, Ramana et al., 2004, Zahradka et al., 2002). Whether NF-κB activation is associated with increased proliferation or apoptosis in SMC is dependent on the upstream stimuli and the type of vessel (Mehrhof et al., 2005, Ogbozor, Opene et al., 2015, Zahradka et al., 2002). A recent study demonstrated that NF-κB activation led to an increased proliferation of fibroblasts, while inducing apoptosis and inflammation in SMC (Mehrhof et al., 2005). Meanwhile, NF-κB was shown to be an important intracellular mediator of angiotensin II (AngII) responses, leading to SMC proliferation and migration under these conditions (Zahradka et al., 2002). In terms of cell differentiation, NF-κB is known to repress myocardin activity and cause downregulation of SMC contractile genes (Tang, Zheng et al., 2008). This molecular interaction has been implicated in the origin of synthetic SMC under inflammatory processes such as atherosclerosis (Mack, 2011).

As we expected, c-Kit deficient cells have decreased expression of the anti-inflammatory and anti-atherogenic factor insulin growth factor-1 (IGF-1) (Sukhanov et al., 2007), and increased susceptibility to calcification due to the downregulation of the Foxo1 and Pth1r genes (Cheng et al., 2010, Deng et al., 2015). It is possible that these changes explain the increased severity of atherosclerosis in c-Kit mutant animals (Song, Selman et al., 2016b). In addition, c-Kit deficient SMC appeared to respond differently to vasomotor stimuli. Their gene expression profile indicates a significantly lower expression of
vasoconstrictive G-protein coupled receptors (GPCR) such as the angiotensin II receptor type 1B (ART1B) (Chiu, Herblin et al., 1989) and the arginine vasopressin receptor 1A (AVR1A) (Holmes, Landry et al., 2003). The response to nitric oxide may also be impaired in these cells due to a lower expression of guanylate cyclase 1 soluble subunit beta (Krumenacker, Hanafy et al., 2004). In the absence of functional experiments, the biological impact of the above described differences in c-Kit deficient SMC compared to their wild type counterparts remains unclear and further investigations were warranted. Interestingly, the reduced expression of Sirt and increased mRNA level of Tnfaip3 in c-Kit deficient cells are independently associated with downregulation of contractile genes in SMC (Damrauer et al., 2010, Huang et al., 2015). These observations are in agreement with the predicted de-differentiated phenotype of c-Kit deficient SMC (Davis et al., 2009) and with the reported atheroprotective role of the c-Kit receptor (Song et al., 2016b). TNFAIP3 normally provides a negative regulatory loop for the NF-κB pathway, including the decreased downstream production of the MCP-1 inflammatory mediator (Giordano, Roncagalli et al., 2014, Patel, Daniel et al., 2006). Downregulation of the Crebbp transcription factor is also thought to reduce NF-κB transcriptional activity (Yang, Jiang et al., 2010). Nonetheless, neither higher Tnfaip3 expression nor less Crebbp in c-Kit deficient SMC seem to have an appreciable inhibitory effect on NF-κB signaling, as demonstrated by followed functional experiments and the increased protein expressions of the pro-inflammatory mediators.

Typical stimuli for NF-κB activation include cytokines, endotoxins, lipids, and mechanical stress (De Martin, Hoeth et al., 2000, Kumar & Boriek, 2003, Maziere, Auclair et al., 1996). For example, the oxidized phospholipid POVPC has been
previously used to induce inflammation in vascular SMC (Lu et al., 2013, Pidkovka et al., 2007) and NF-κB activation (Lu et al., 2013, Pegorier et al., 2006, Vladykovskaya et al., 2011).

As predicted by the in silico analysis, c-Kit deficiency in SMC led to higher levels of NF-κB transcriptional activity, phosphorylation of its key subunit p65, and expression of the NF-κB regulated inflammatory mediators MMP-2,9 and MCP-1 under POVPC challenge (Figure 34). Gene members of the non-canonical NF-κB pathway and other inflammatory mediators were also upregulated in mutant SMC. Similar to our observations, the role of c-Kit as a negative regulator of the NF-κB pathway and related inflammation has been previously described in other cell types and under different stimuli (Jin, Shen et al., 2013, Micheva-Viteva, Shou et al., 2013).

Pharmacological inhibition of c-Kit results in increased activation of NF-κB in HEK293 cells and secretion of TNFα in dendritic cells and the THP-1 monocytic cell line in response to bacterial infection (Micheva-Viteva et al., 2013). Lower expressions of SCF and c-Kit were also associated with increased NF-κB signaling and oxidative stress in gastric smooth muscle (Jin et al., 2013).

Later work further revealed that c-Kit reduces NF-κB mediated inflammation via a direct molecular interaction with the NF-κB negative regulators TAK1 and NLK (Ajibade et al., 2012, Li et al., 2014, Morlon, Munnich et al., 2005, Yasuda et al., 2004). The physical association between c-Kit, Lyn (a member of the Src family of non-receptor tyrosine kinases), and TAK1 has been previously observed in the HEK293T cell line, where these proteins form a signalosome that interacts with IKKβ, one of the catalytic units of the IκB kinase (IKK) complex (Drube et al., 2015). Nonetheless, the inhibitory activity of TAK1
on the NF-κB signaling pathway appears to be cell-specific, since in some cells it can be activating (Ajibade et al., 2012, Israel, 2010). In the inhibitory instances, TAK1 blocks the phosphorylation and inactivates IKK (Ajibade et al., 2012), which in turn is unable to phosphorylate and induce proteasome degradation of the IκB inhibitors of the NF-κB pathway (Israel, 2010, Karin, 1999). When active, IκB proteins prevent the nuclear translocation of p65/RelA complexes (Israel, 2010, Karin, 1999). NLK also functions as an inhibitor of IKK phosphorylation (even in the cells where TAK1 acts as an activator) (Li et al., 2014).

4.5 Limitation of the Studies

Herein this thesis, I demonstrated c-Kit atheroprotective effects in two independent mouse models, where c-Kit deficiency has distant genetic compositions. However, understanding the limitations of the investigations is of equal importance as the striking findings.

Firstly, the characteristics of atherosclerosis in mouse and human species are not identical. Atherosclerotic plaques are frequently found in human coronary arteries, iliac arteries, and carotids. Whereas mice do not develop plaques in coronary arteries. These atherosclerotic lesions are more prone to deposit in murine aortae, especially in aortic arch and root, and innominate arteries (Getz & Reardon, 2012). Besides the lesion distribution, murine atherosclerosis is different in the pathogenic progression compared to human. The most fatal consequence of human atherosclerosis occurs when the plaque ruptures and luminal thrombosis occurs, which leads to the occlusion of the vessel. However, this scenario is scarcely found in mouse (Bentzon & Falk, 2010).
Another limitation that merits attention is, both Kit^W and Kit^{W-v} are the spontaneous mutations specifically found in mouse (Besmer, Manova et al., 1993). So far, no identical or similar genotype of these mutations was reported in human, indicating this Kit^{W/W-v} mouse model merely resembles, but does not identify the c-Kit deficient pattern in human.

Given these two major limitations, it seems that big animals such primates and pigs would be better models to mimic human atherogenesis and atherosclerosis. However, these models carry concerns of high cost and difficulties in genetic modification. In spite of these limitations, the striking findings may be relevant in the setting of atherosclerosis disease development and complications, which may shed light on cell proliferation control, lipid metabolism dysfunction, and inflammation based mechanisms to address other cardiovascular diseases.
CHAPTER 5. SUMMARY AND CONCLUSIONS

5.1 Identification of c-Kit as an athero-protective target in hyperlipidemic mice

In specific aim 1, I attempted to understand the fundamental role of c-Kit in atherogenesis. By using the hyperlipidemic c-Kit global deficiency mouse model (Kit\textsuperscript{W/W-}\textsuperscript{v} ApoE\textsuperscript{-/-}), I found these c-Kit compound mutant mice presented exacerbated atherosclerotic burden in both aorta and sinus valve, with respect to their control littermates (Kit\textsuperscript{+/+} ApoE\textsuperscript{-/-}) after 16 weeks of HFD. This key evidence suggests a protective role of this tyrosine kinase receptor in atherogenesis. In line with that, SMCs in the Kit\textsuperscript{W/W-}\textsuperscript{v} ApoE\textsuperscript{-/-} lesion site were found “macrophage prone”, with an unexpected gain of macrophage marker (CD68) expression, indicating these lesional cell phenotypical compromise may be an essential contributing factor to the pathogenesis.

Serum analyses indicated that the red blood cell level was significantly decreased in Kit\textsuperscript{W/W-}\textsuperscript{v} mice, implicating these c-Kit mutant mice were anemic. This result is in line with one major biological function of c-Kit which indicates an important role in the regeneration and differentiation of hematopoietic stem cell and progenitor cell (Lennartsson & Ronnstrand, 2012, Ronnstrand, 2004).

Furthermore, both c-Kit wild type and mutant mice showed a high level of circulating lipids (LDL, VLDL, and cholesterol) when in an ApoE knockout genetic background, and fed on a HFD for 16 weeks to induce the hyperlipidemia. Elevated cholesterol levels indicated a successful hyperlipidemia introduction. However, cholesterol levels were found higher in c-Kit wild type control compared to in the mutant mice. Nonetheless, cholesterol levels in both wild type and mutant mice were found significantly higher than the physiological range of C57BL/6 mice (Dietschy & Turley, 2002), indicating both
strains were equally at risk of cardiovascular events. In conclusion, my research demonstrated that receptor tyrosine kinase c-Kit is athero-protective in hyperlipidemic mice.

5.2 Vascular c-Kit expression is responsible for the athero-protective effect in hyperlipidemic mice

Based on the findings in Specific Aim 1, I sought to target the specific cell type in which c-Kit expression exerts its essential role for the observed athero-protection. Given the pathogenesis related distribution of c-Kit is within the bone marrow and local vessels, I first investigated the role of bone marrow derived c-Kit.

The c-Kit positive bone marrow transplantation created a chimeric mouse with global c-Kit negative cells, except for c-Kit positive bone marrow (BM) cells. Surprisingly, these transplanted c-Kit positive BM cells failed to significantly alleviate the aortic atherosclerotic burden in c-Kit deficient chimeric mice after 16 weeks of HFD, suggesting that these BM cells contributed little or no effect in atherosclerotic protection, thus pointing to a local contributing factor. Based on this, I investigated the role of vascular c-Kit in atherogenesis.

With the conditional inactivation of c-Kit in SMC (Kit\(^{lox66-71/lox66-71}\) Mhy11Cre\(^{ERT2}\)) after one month of Tamoxifen chow (TAM) feeding, I found these TAM chow fed animals presented more lipid accumulation in aortae and sinus valves compared to their littermate controls, which consumed vehicle chow. Consequently, the lesion site SMCs found in TAM treated (c-Kit vascular deficiency) mice were more macrophage prone as shown by CD68 expression with respect to those found in Vehicle chow treated mice.
In conclusion, c-Kit positive BM transplanted do not protect atherosclerosis in hyperlipidemic mice, indicating that vascular SMC c-Kit may be more important for athero-protection.

5.3 c-Kit inhibits contractile SMCs to synthetic, macro-phage-like and pro-inflammatory phenotypic transformation in vitro

The key question addressed in this Specific Aim pertains to elucidate the mechanism how vascular c-Kit protects atherosclerosis. As a multiple cell type participated process, atherogenesis is along with various contributing factors such as lipid metabolism dysfunction, sterile inflammation, and vascular SMC phenotype switch. Therefore, I mainly pursued to understand the mechanism focusing on three aspects of c-Kit function on conserving the SMC contractile phenotype, from (1) synthetic, (2) foam-cell like, and (3) pro-inflammatory phenotypic transformation.

By using cultivated aortic c-Kit wild type (Kit\textsuperscript{+/+}) or mutant (Kit\textsuperscript{W/W-}) SMCs, I found that c-Kit maintains the SMC physiological morphology and cytosol architecture. Also, it prevents SMCs from switching into a pathological phenotype with contractile marker loss, increased-growth, and migration; In addition, loss of c-Kit leads to SMC cytosol lipid intake from milieu under the water soluble cholesterol loading, in a KLF4 dependent manner. Knockdown of KLF4 in c-Kit mutant SMCs abolished excessive lipid cytosol accumulation; further, c-Kit mutant SMCs were found inflammatory prone with elevated NF-κB signaling related gene expression. Under artificial oxidized lipid stimulation, c-Kit prevents NF-κB activation, and subsequently, of the downstream inflammatory mediator production such as MMP-2, 9 and MCP-1. Further investigation allowed me to
understand that c-Kit suppression of the NF-κB activation is via Src, and TAK1, NLK axis in SMC. Specific inactivation of TAK1 and NLK target genes in c-Kit wild type cell eliminates NF-κB inhibition, therefore activates its downstream inflammatory mediator production.

In conclusion, c-Kit exerts its protective role in maintaining SMCs in their contractile phenotype while suppressing a switch to a proliferative synthetic, pro-inflammatory phenotype, and by suppressing lipid intake in a cholesterol rich milieu.

### 5.4 Significance of thesis work

This study is highly significant because: 1) it is the first study to rigorously evaluate the role of SMC c-Kit in atherosclerosis, 2) it provides the first evidence that c-Kit plays a central role in determining the SMC phenotype, and 3) it furnishes new therapeutic targets – for which there is an existing translatable platform for drug development – to prevent and eventually mitigate the devastating effects of atherosclerosis.

Furthermore, findings from this study may also have applications for other vascular diseases such as restenosis, transplant arteriosclerosis, vein graft failure, and pulmonary vascular occlusive disease.
CHAPTER 6. FUTURE DIRECTIONS

This thesis demonstrates for the first time that vascular expression of c-Kit protects against atherosclerosis, and presents mechanistic evidence suggesting a critical role for c-Kit in preventing SMC synthetic, foam-cell like, and pro-inflammatory phenotypical transformation under atherosclerotic insults. However, to develop a more confirmative and systemic investigation, subsequent and continuous studies are necessary and promising. Thereby, future direction might focus on the following aspects:

6.1. Confirm that vascular c-Kit is athero-protective in hyperlipidemic mice using a c-Kit gain of function model

Present thesis work demonstrated the atheroprotective role of c-Kit by employing two loss of function models. Given that, a c-Kit gain of function study will serve as a piece of supportive evidence for this finding. To achieve this, a novel transgenic mouse model, iKit<sup>+/−</sup> Myh11CreER<sup>T2</sup> ApoE<sup>−/−</sup> might be produced. In this model, c-Kit will be overexpressed specifically in SMC, to see if gain of c-Kit will be protective in hyperlipidemic mice.

6.2. Evaluate the role of endothelial c-Kit in atherogenesis

The vascular c-Kit presents, but not only limits to the SMCs. In fact, endothelium, as the inner layer of the vessel, is a predominant alternative source for vascular c-Kit expression with biological functions (Lennartsson & Ronnstrand, 2012, Tallini et al., 2009). Interestingly, the contribution of the somatic c-Kit positive endothelial cell (EC) to
atherogenesis prevention has never been investigated before. Therefore, it will be interesting to interrogate the role of c-Kit in EC in hyperlipidemic mice as the next move.


Bragdon JH (1952) Spontaneous atherosclerosis in the rabbit. Circulation 5: 641-6


Im JE, Song SH, Suh W (2016) Src tyrosine kinase regulates the stem cell factor-induced breakdown of the blood-retinal barrier. Mol Vis 22: 1213-1220


Lamb DJ, Modjtahedi H, Plant NJ, Ferns GA (2004) EGF mediates monocyte chemotaxis and macrophage proliferation and EGF receptor is expressed in atherosclerotic plaques. Atherosclerosis 176: 21-6


Micheva-Viteva SN, Shou Y, Nowak-Lovato KL, Rector KD, Hong-Geller E (2013) c-KIT signaling is targeted by pathogenic Yersinia to suppress the host immune response. BMC Microbiol 13: 249


Ross R, Glomset JA (1973) Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. Science 180: 1332-9


Wedemeyer J, Galli SJ (2005) Decreased susceptibility of mast cell-deficient Kit(W)/Kit(W-v) mice to the development of 1, 2-dimethylhydrazine-induced intestinal tumors. Lab Invest 85: 388-96


Fig 1. Smooth muscle cells of murine aorta express the receptor tyrosine kinase c-Kit. (A) Immunofluorescence confocal microscopy indicated Green Fluorescent Protein (GFP) expression driven by c-Kit promoter was limited to arterial smooth muscle cells in the vasculature of the KitBac eGFP reporter mice (n=3 independent experiments using different KitBac eGFP murine aortae). (B-C) c-Kit+ cells (green) stain also positively for SMA (red). The section within the box is magnified in C. Merged image shows double positive cells with SMA and c-Kit expressions in yellow. Nuclei were counterstained with DAPI (blue). A, Aorta, V.C., Vena Cava.
Fig 2. Human arteries express c-Kit, p-c-Kit and c-Kit ligand, stem cell factor (SCF). c-Kit, and its ligand SCF can be detected in arteries of heart transplantation donors (D) or recipients (R) (n=9 as presented). Fragments of aortae or pulmonary arteries were collected from heart donor and recipient patients. Proteins were extracted from fresh tissues and Western blot analysis was performed using specific antibodies. Actin was used as loading control. A= aorta; P, pulmonary artery.
Fig 3. **Smooth muscle cells of human aortae express c-Kit.** c-Kit expression in human arterial SMC. The c-Kit (A, green), SMA (B, red) and double positive cells (C, yellow) were abundantly found in the tunica media of a human aorta obtained from a heart transplant donor. (n=3 independent experiments using different human aortae).
Fig 4. A schematic diagram of the how c-Kit deficient mice with c-Kit compound mutation Kit<sup>W/W-v</sup> in the ApoE<sup>-/-</sup> background were generated. Both Kit mutant mice Kit<sup>W/+</sup> and Kit<sup>Wv/+</sup> were crossed with ApoE knockout. Therefore, those resulting transgenic mice (Kit<sup>W/+</sup>ApoE<sup>-/-</sup> or Kit<sup>Wv/+</sup>ApoE<sup>-/-</sup>) were crossed to generate experimental c-Kit deficient mice (Kit<sup>W/W-v</sup>ApoE<sup>-/-</sup>) and their littermate controls (Kit<sup>+/+</sup>ApoE<sup>-/-</sup>).
Fig 5. Spontaneous mutations (W and W-v) in the c-Kit locus significantly decrease the receptor tyrosine kinase expression in the aorta (A), and in the spleen (B) as determined by IP-Western blot. Proteins were extracted from fresh tissues and Western blot was performed using specific antibodies for c-Kit and Actin. Actin was used as loading control. n=3 independent experiments using different murine aortae or spleens. Data were presented as mean ± S.D. * p<0.05, ** p<0.01. p values were calculated using a two-tailed t-test assuming unequal variance.
Fig 6. c-Kit protein can be detected in the tunica media of Kit$^{+/+}$ but not Kit$^{W/W,v}$ mice. Using immunofluorescence confocal microscopy, SMCs were identified using an antibody against smooth muscle actin (SMA). The double positive cells for c-Kit (red) and SMA (green) in the media of Kit$^{+/+}$ mice appear yellow. Nuclei were counterstained with DAPI (blue). L, Lumen. n=3 independent experiments using different aortae from Kit$^{+/+}$ or Kit$^{W/W,v}$ mice.
Fig 7. Loss of c-Kit exacerbates atherosclerosis in hyperlipidemic mice. Mice were fed on a HFD for 16-weeks, then aortae were harvested and stained by Sudan IV for atherosclerosis evaluation. (Left) Aortic atherosclerosis burden in c-Kit deficient (Kit^{W/W-} ApoE^{-/-}) and normal (Kit^{+/+} ApoE^{-/-}) mice. (Right) The percentage of the aorta affected by atherosclerosis in each experimental animal was plotted in the dot blot graphic on the right. The horizontal line indicates the group’s mean. Data are presented as mean ± S.D. of the atherosclerotic lesion in the aortae of c-Kit deficient mice and control littermates. Kit^{+/+}ApoE^{-/-}, 11 mice; Kit^{W/W-}ApoE^{-/-}, 9 mice as plotted. p values were calculated using a two-tailed t-test assuming unequal variance.
Fig 8. Compound heterozygous Kit$^{W/W-v}$ ApoE$^{+/+}$ mice show a more severe atherosclerotic phenotype when compared to Kit$^{+/+}$ ApoE$^{-/-}$ fed 16 weeks on a HFD. Prevalence maps of aortic lesions of ApoE null mice carrying Kit$^{W-v/+}$, Kit$^{W/+}$, or Kit$^{W/W-v}$ mutations. 8 mice were analyzed in each group. Disease incidence in each aortic region was independently plotted per each animal on the right. Data were presented as mean ± S.D. * $p<0.05$. $p$ values were calculated using a two-tailed t-test assuming unequal variance.
Fig 9. Loss of c-Kit exacerbates atherosclerosis in hyperlipidemic mice. Aortic sinus atherosclerosis burden in c-Kit deficient (Kit$^{W/W}$-v ApoE$^{-/-}$) and normal (Kit$^{+/+}$ ApoE$^{-/-}$) mice that fed on a HFD for 16-weeks. The lesion area in each experimental animal was plotted as a bar graph on the right. Kit$^{+/+}$ ApoE$^{-/-}$, 11 mice; Kit$^{W/W}$-v ApoE$^{-/-}$, 9 mice. Data were presented as mean ± S.D. * $p<0.05$. $p$ values were calculated using a two-tailed t-test assuming unequal variance.
Fig 10. Representative image of atherosclerotic plaques in the aortic sinus of c-Kit mutant (Kit\(^{W/W-v}\) ApoE\(^{−/−}\)) and control littermates stained with specific antibodies against smooth muscle cells (anti-SMA, green) and macrophages (anti-CD68, red). (A-B) Double positive cells for SMA (Green) and CD68 (Red) in the media of Kit\(^{W/W-v}\) mice appear in yellow. Nuclei were counterstained with DAPI (blue). (C) Double positive cells in the cap area. NC, necrotic area. n=3 independent experiments using different Kit\(^{+/+}\)ApoE\(^{−/−}\) or Kit\(^{W/W-v}\)ApoE\(^{−/−}\) carotids. Data were presented as mean \(±\) S.D. * \(p<0.05\). \(p\) value was calculated using a two-tailed t-test assuming unequal variance.
Fig 11. A diagram of the c-Kit$^+$ bone marrow transplantation strategy. Both Kit$^{+/+}$ ApoE$^{-/-}$ and Kit$^{+/+}$ ApoE$^{-/-}$ mice were lethally irradiated and grafted with cKit positive bone marrow cells (~2 x 10$^6$ cells) from donors.
Fig 12. Bone marrow transplantation fails to alleviate atherosclerosis in systemic c-Kit mutant mice. Bone marrow cells from Kit^{+/+} ApoE^{-/-} donor transplanted to Kit^{+/+} ApoE^{-/-} recipient mice, or from Kit^{+/+} ApoE^{-/-} donor transplanted to Kit^{W/W-v} ApoE^{-/-} mice. The percentage of the aorta affected by atherosclerosis in each experimental animal was plotted in the dot blot graphic on the right. The horizontal line indicates the group’s mean. Kit^{+/+} ApoE^{-/-} to Kit^{+/+} ApoE^{-/-}, 7 mice; Kit^{+/+} ApoE^{-/-} to Kit^{W/W-v} ApoE^{-/-}, 6 mice as presented. Data were presented as mean ± S.D. p values was calculated using a two-tailed t-test assuming unequal variance.
Fig 13. A diagram for the generation of c-Kit smooth muscle cell conditional knockout mice. Myh11 is a promoter that drives gene expression selectively in smooth muscle cell. In the Myh11CreERT2 transgenic mice, Cre recombinase is activated by Tamoxifen treatment. Cre recombinase expression results in an inversion of the sequence in between these two lox66-71 sites.
Fig 14. The c-Kit conditional and triple transgenic mice are viable and allow the specific deletion of Kit in SMCs after tamoxifen (TAM) feeding. (A) Genotype of a breeding pair used to generate the experimental Kit conditional mice (Kit\textsuperscript{lox66-71}/Kit\textsuperscript{lox66-71}). Both female and male were Kit\textsuperscript{lox66-71}/Kit\textsuperscript{lox66-71} homozygous, Kit\textsuperscript{+/+} (wild type allele) negative and ApoE\textsuperscript{−/−}. The males carried the Myh11-Cre\textsuperscript{ERT2} on the Y-chromosome. Genotyping was performed by tail-DNA PCR. (B) The inactivation of Kit gene only in aortae of TAM treated mice but not in those of controls (Veh). While all mice were positive for the transgene (Kit\textsuperscript{lox66-71}) only those Tamoxifen (TAM) fed showed the aortic PCR product corresponding to gene rearrangement (Kit\textsuperscript{Inverted}).
Fig 15. Tamoxifen diet (TAM) leads to decreased c-Kit expression in aorta, but does not significantly modify c-Kit protein level in the spleen of c-Kit conditional ApoE null mice as determined by IP- Western blot analysis. (A) c-Kit expression in aorta, and (B) in spleen. Proteins were extracted from fresh tissues and Western blot was performed using specific antibodies. Actin was used as loading control. n=3 independent experiments using different aortae or spleens. Data were presented as mean ± S.D. N.S: no significant, ** p<0.01. p values were calculated using a two-tailed t-test assuming unequal variance.
Fig 16. c-Kit is scarcely detected in the tunica media of Kit conditional (Kit$^{lox66-71/lox66-71}$ Myh11-Cre$^{ERT2}$ ApoE$^{-/-}$) mice after feeding a tamoxifen (TAM) rich diet for four weeks. SMCs were identified with an antibody against smooth muscle actin (SMA). Double positive cells for c-Kit (red) and SMA (green) in the media of vehicle treated mice appear in yellow after merging color images. Nuclei were counterstained with DAPI (blue). L, Lumen. n=3 independent experiments using different aortae from Vehicle chow or TAM treated mice.
Fig 17. Lack of vascular c-Kit exacerbates atherosclerosis in aortae of hyperlipidemic mice. Mice were fed on a HFD for 16-weeks, then aortae were harvested and stained by Sudan IV for atherosclerosis evaluation. (Left) Aortic atherosclerosis burden in Kit<sup>lox66-71</sup>/Myh11CreER<sup>T2</sup> mice that received tamoxifen (TAM) or vehicle chow (Vehicle) for 4-weeks followed by 16-weeks of HFD. (Right) The percentage of the aorta affected by atherosclerosis in each experimental animal was plotted in the dot blot graphic on the right. The horizontal line indicates the group’s mean. Data are presented as mean ± S.D. of the atherosclerotic lesion in c-Kit conditional knockout mice and control littermates. Vehicle group 6 mice, TAM group 7 mice as presented. *p* values were calculated using a two-tailed t-test assuming unequal variance.
Fig 18. Lack of vascular c-Kit exacerbates atherosclerosis in the aortic sinus of hyperlipidemic mice. Atherosclerotic burden in the aortic sinus of Kit$^{lox66-71/lox66-71}$Myh11Cre$^{ERT2}$ mice that received tamoxifen (TAM) or vehicle chow (Vehicle) for 4 weeks before 16-weeks of HFD. The lesion area in each experimental animal was plotted as a bar graph on the right. Vehicle group 6 mice, TAM group 7 mice. Data were presented as mean ± S.D. * $p<0.05$. $p$ values were calculated using a two-tailed t-test assuming unequal variance.
Figure 19. Tamoxifen (TAM) has no effect on atherosclerosis development in HFD fed Kit<sup>+/+</sup> ApoE<sup>−/−</sup> mice. Kit<sup>+/+</sup> ApoE<sup>−/−</sup> mice fed for 4 weeks with tamoxifen (TAM) or normal chow (Vehicle) followed by 16-weeks of HFD. Aortae were harvested and Sudan IV staining was performed. (Left) Prevalence maps of aortic lesions of Kit<sup>+/+</sup> ApoE<sup>−/−</sup> mice. (Right) The percentage of the aorta affected by atherosclerosis in each experimental animal was plotted in the dot blot graphic. The horizontal line indicates the group’s mean. Five mice per group as plotted. Data were presented as mean ± S.D. N.S., no significance. <i>p</i> values were assessed using a two-tailed t-test assuming unequal variance.
Fig 20. Representative image of atherosclerotic plaques in the aortic sinus of c-Kit SMC deficient mice (TAM) and control littermates (Veh) stained with specific antibodies against smooth muscle cells (anti-SMA, green) and macrophages (anti-CD68, red). Double positive cells for SMA (Green) and CD68 (Red) in the media appear yellow in (A) control mice (Veh) and (B) c-Kit SMC deficient mice. Nuclei were counterstained with DAPI (blue). (C) Quantifications of double positive cells in the cap area. NC, necrotic core. n=3 independent experiments using different carotids from Vehicle chow or TAM treated mice. Data were presented as mean ± S.D. * p<0.05. p values were calculated using a two-tailed t-test assuming unequal variance.
Fig 21. Loss of c-Kit leads to hypercellularity in medical SMCs. (Left) H&E staining of the Kit$^+/+$ and Kit$^{W/-}$ aortae. (Right) Cell density (cell number/mm$^2$) in media area indicated a hypercellularity in Kit$^{W/-}$ aortae compared to Kit$^{+/+}$ control aortae. Kit$^{+/+}$ n=5, Kit$^{W/-}$ n=4, independent experiments using different aortae from Kit$^{+/+}$ or Kit$^{W/-}$ mice. Data were presented as mean ± S.D. * p<0.05. p values were calculated using a two-tailed t-test assuming unequal variance.
Fig 22. Loss of c-Kit leads to tunica SMCs cytosol vacuoles aggregation. Vacuoles were found in Kit<sup>W/W-v</sup> primary SMCs but not Kit<sup>+/+</sup> SMCs by electron microscopy. The right figure is the magnification of the area which is defined by the red broken line in adjacent figure, vacuoles were indicated by the red arrows. N, nucleus. n=3 independent experiments using different using different aortae from Kit<sup>+/+</sup> or Kit<sup>W/W-v</sup> mice.
Fig 23. Contractile marker gene expression in Kit\textsuperscript{W/W-v} and Kit\textsuperscript{+/+} aortae as determined by real-time qPCR. mRNAs were extracted from fresh tissues and PCR was performed using specific probes. \textbf{Myocd}, Myocardin, \textbf{Calp}, Calponin, \textbf{SMA}, Smooth Muscle Actin, \textbf{MHC-6}, Myosin Heavy Chain-6 (Myosin heavy chain alpha). n=3 independent experiments using different aortae from Kit\textsuperscript{+/+} or Kit\textsuperscript{W/W-v} mice. Data were presented as mean ± S.D. * $p<0.05$. $p$ values were calculated using a two-tailed t-test assuming unequal variance.
Fig 24. Contractile marker gene expressions in Kit$^{W/W-v}$ and control Kit$^{+/+}$ aorta as determined by Western blot. Proteins were extracted from fresh tissues and Western blot was performed using specific antibodies. Actin was used as loading control. n=3 independent experiments using different aortae from Kit$^{+/+}$ or Kit$^{W/W-v}$ mice. Data were presented as mean ± S.D. * $p<0.05$. $p$ values were calculated using a two-tailed t-test assuming unequal variance.
Fig 25. Rescue of c-Kit in Kit^{W/W-v} (c-Kit deficient) aortic SMCs. c-Kit as well as contractile marker expressions in Kit^{+/+}, Kit^{W/W-v} and rescue cells were determined by Western blot. Rescue was achieved by transfection of Kit^{W/W-v} SMCs with lentivirus containing murine c-Kit expression plasmid. Proteins were extracted from cells and Western blot was performed using specific antibodies. Actin was used as loading control. n=3 independent experiments using primary cells from different mice or genetically modified cells from different Kit^{W/W-v} cell cultures. Data were presented as mean ± S.D. * p<0.05, ** p<0.01 using a one-way ANOVA followed by a Newman-Keuls test.
Fig 26. Kit^{W/W-\nu} SMCs proliferate faster compared to Kit^{+/+} and rescue cells. SMCs were seeded in 24 well plate. After synchronized with serum deprived medium overnight, cells were trypnised and counted consecutively for 6 days. n=4 independent experiments using primary cells from different mice or genetically modified cells from different Kit^{W/W-\nu} cell cultures. Data were presented as mean ± S.D. *p<0.05, ** p<0.01 using a one-way ANOVA followed by a Newman-Keuls test.
Fig 27. Kit\textsuperscript{W/W-\textasciitilde} SMCs migrate more than Kit\textsuperscript{+/+} and c-Kit rescued SMCs. SMCs were seeded at the upper chamber of a Corning BioCoat Matrigel Invasion Chamber 24-well plate, and PDGF-BB (20ng/ml) was used as chemo-attractant that facilitates SMCs migration from the upper chamber to the bottom of the well. After 24hrs, the cells migrated into the bottom of the well were stained with DAPI. n=3 independent experiments using primary cells from different mice or genetically modified cells from different Kit\textsuperscript{W/W-\textasciitilde} cell cultures. Data were presented as mean ± S.D. * p<0.05, ** p<0.01, using a one-way ANOVA followed by a Newman-Keuls test.
Fig 28. Cytosolic lipid accumulation in cultured Kit$^{+/+}$, Kit$^{W/W-v}$, and c-Kit rescued smooth muscle cells after cholesterol loading. (A) Accumulation of lipids as detected with the lipophilic dye Oil Red-O. (B) Bar graph representing the mean ± S.D. of the total amount of cellular cholesterol per group after 72hrs of Chol:MβCD (20µg/ml) loading in 0.2% BSA containing medium. n=3 independent experiments using primary cells from different mice or genetically manipulated cells from different Kit$^{W/W-v}$ cell cultures. ** $p<0.01$, using a one-way ANOVA followed by a Newman-Keuls test.
Fig 29. Loss of c-Kit increases KLF4 expression and activity in SMCs. (A) Cultured SMCs isolated from c-Kit deficient (Kit^{W/W-v}) mice had increased KLF4 protein levels (by Western blot), and (B) gene expression (by real time qRT-PCR), with respect to those SMCs isolated from control littermates (Kit^{+/+}). Rescue of c-Kit with a lentiviral vector decreased abnormal expression of KLF4 in c-Kit deficient SMCs (Rescue). (C) Transcription activity was measured by dual-luciferase assay, and presented as the ratio of Firefly/Renilla. Kit^{W/W-v} SMCs presented an elevated transcriptional activity compared to both Kit^{+/+} and rescue SMCs. n=3 independent experiments using primary cells from different mice or genetically modified cells from different Kit^{W/W-v} cell cultures. Data were presented as mean ± S.D. *p<0.05, **p<0.01 using a one-way ANOVA followed by a Newman-Keuls test.
Fig 30. Knockdown of KLF4 in c-Kit deficient SMCs leads to contractile marker SM22α restoration. SMCs from c-Kit deficient and control littermate were transfected with lentivirus carrying small inhibitory RNAs against KLF4 (siKLF4) or GFP (siGFP). The loss of KLF4 was validated by Western blot. In contrast to untreated c-Kit deficient cells, the c-Kit deficient cells treated with siKLF4 displayed a restoration of SM22α in expression. n=3 independent experiments using modified cells from different Kit+/+ or KitW/W-v cell cultures. Data were presented as mean ± S.D. *p<0.05, **p<0.01 using a one-way ANOVA followed by a Newman-Keuls test.
Fig 31. Knockdown KLF4 in c-Kit deficient cells prevent foam-like cell formation in vitro. The SMCs from c-Kit deficient and control littermate were transfected with lentivirus carrying small inhibitory RNAs against KLF4 (siKLF4) or GFP (siGFP). In contrast to untreated c-Kit deficient cells, c-Kit deficient cells treated with siKLF4 did not accumulate cytoplasmatic lipids thus did not transform to a foam cell-like phenotype in vitro. n=3 independent experiments using modified cells from different Kit$^{+/+}$ or Kit$^{W/W}$ cell cultures. Data were presented as mean ± S.D. *p<0.05, ** p<0.01, using a one-way ANOVA followed by a Newman-Keuls test.
**Fig 32. Loss of c-Kit function accounts for significant gene expression differences between c-Kit deficient and wild type SMCs.** (A) Venn diagram indicating the numbers of differentially upregulated genes in primary SMC isolated from c-Kit deficient (blue; Kit^{W/W-}) and littermate control mice (red; Kit^{+/+}) as determined by microarray analysis. The group of genes in the intersection (black area) did not show statistically significant differences by *t*-test between the two strains. *n*=3 per group. (B) Heat map of differentially expressed genes in primary SMC from c-Kit deficient and littermate control mice. *n*=3 independent experiments using primary aortic SMCs from different Kit^{+/+} or Kit^{W/W-} mice.
Fig 33. Genes of pro-inflammatory mediators which are products of the NF-κB signaling cascade are elevated in association with c-Kit loss in mutant SMCs compared to wild type control. Expression of (A) NF-κB related genes and (B) pro-inflammatory mediator genes in c-Kit deficient vs. control SMC as determined by real-time PCR. Values are shown as fold change over expression in Kit^{+/+} SMCs; n=3 independent experiments using primary aortic SMCs from different Kit^{+/+} or Kit^{W/W-v} mice. Data were presented as mean ± S.D. * p<0.05 and ** p<0.01 using a two-tailed t-test assuming unequal variance.
Fig 34. Loss of c-Kit function in primary SMCs is associated with increased NF-κB activity. (A) NF-κB transcriptional activity in c-Kit deficient (Kit\(^{W/W}\)-v), control (Kit\(^{+/+}\)), and c-Kit rescued SMCs (Rescue) after 24-hour treatment with POVPC (50 \(\mu\)g/ml), as determined by dual-luciferase reporter assay. Transcriptional activity is measured in form of the Firefly/Renilla luciferase ratio normalized with respect to the control group (Kit\(^{+/+}\)). (B) Phosphorylated (pS536) protein levels of the NF-κB p65 subunit in POVPC-treated c-Kit deficient, control, and c-Kit rescued SMC as determined by ELISA. Values are expressed as the mean ± SD of the p-p65/total p65 ratio normalized with respect to the control group (Kit\(^{+/+}\)). (C) Protein expression of the NF-κB related pro-inflammatory mediators MMP-2 and MCP-1 in POVPC-treated c-Kit deficient, control, and c-Kit rescued SMC as determined by Western blot. n=3 independent experiments using primary cells from different mice or genetically modified cells from different Kit\(^{W/W}\)-v cell cultures. Data were presented as mean ± S.D. * \(p<0.05\) and ** \(p<0.01\), using a one-way ANOVA followed by a Newman-Keuls test.
Fig 35. TAK-1 and NLK expressions in Kit$^{+/+}$, Kit$^{W/W}$, and c-Kit rescued SMCs. (A) Protein expression of the TAK1 and NLK regulatory proteins in c-Kit deficient (Kit$^{W/W-}$), control (Kit$^{+/+}$), and c-Kit rescued SMC as determined by Western blot. Protein fold change is expressed as the mean ± S.D. of the TAK1/β-actin or NLK/β-actin signal ratios normalized with respect to the control group (Kit$^{+/+}$). (B-C) Protein expression of TAK1 (B) and NLK (C) in Kit$^{+/+}$ cells transduced with lentivirus-encoded siRNAs of the corresponding targets or GFP control. Protein fold change is expressed as the mean ± S.D. of the TAK1/β-actin and NLK/β-actin signal ratios normalized with respect to the siGFP-treated group. n=3 independent experiments using primary cells from different mice or genetically modified cells from different Kit$^{W/W-}$ cell cultures. * p<0.05 and ** p<0.01, using a one-way ANOVA followed by a Newman-Keuls test.
Fig 36. c-Kit inhibits NF-κB activity in SMCs through the actions of TAK1 and NLK. (A) NF-κB transcriptional activity in Kit \(^{+/+}\) SMC transduced with lentivirus-encoded siRNAs complementary to TAK1, NLK, or GFP after 24 hr treatment with POVPC (50 \(\mu\)g/ml), as determined by dual-luciferase assay. Transcriptional activity is represented as the mean ± SD of the Firefly/Renilla luciferase ratio normalized with respect to the siGFP-treated group. (B) Phosphorylated (pS536) protein levels of NF-κB p65 in POVPC-treated Kit \(^{+/+}\) SMC transduced with lentivirus-encoded siRNAs as determined by ELISA. Values are expressed as the mean ± S.D. of the p-p65/total p65 ratio normalized with respect to the siGFP-treated group (n=3 independent experiments). (C) Protein expression of pro-inflammatory mediators MMP-2 and MCP-1 in POVPC-treated Kit \(^{+/+}\) SMC transduced with lentivirus-encoded siRNAs as determined by Western blot. n=3 independent experiments using modified cells from different Kit \(^{+/+}\) cell cultures. * \(p<0.05\) and ** \(p<0.01\) using a one-way ANOVA followed by a Newman-Keuls test.
Fig 37. **c-Kit forms a molecular complex with the regulatory proteins TAK1, Src, and NLK in SMCs.** (A-B) Co-immunoprecipitation experiments in control (Kit^{+/+}) and c-Kit deficient (Kit^{W/W-}) SMC using anti-c-Kit (A) and anti-TAK1 antibodies (B) to pull down protein complexes. Antibodies used to detect eluted proteins are indicated on the left. IP, immunoprecipitation; IB, immunoblot. n=3 independent experiments using primary aortic SMCs from different Kit^{+/+} or Kit^{W/W-} mice. (C) Diagram illustrating the proposed molecular complex between c-Kit, TAK1, Src, and NLK in SMC and their inhibitory function on NF-κB transcriptional activity.
### TABLES

**Table 1. Influence of c-Kit mutations on cell blood count and lipid profile of ApoE\(^{-/-}\) mice**

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Kit(^{+/+})ApoE(^{-/-})</th>
<th>Kit(^{W/W})ApoE(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>X10(^3)/ul</td>
<td>9.72 ± 0.96</td>
<td>4.87 ± 1.09*</td>
</tr>
<tr>
<td>Hb</td>
<td>X10(^6)/ul</td>
<td>12.38 ± 1.60</td>
<td>9.11 ± 1.37*</td>
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<tr>
<td>Ht</td>
<td>%</td>
<td>40.78 ± 4.29</td>
<td>30.43 ± 4.68*</td>
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<tr>
<td>MCV</td>
<td>fL</td>
<td>44.11 ± 2.36</td>
<td>63.42 ± 6.10*</td>
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<tr>
<td>Segs</td>
<td>%</td>
<td>32.55 ± 9.79</td>
<td>25.71 ± 5.02*</td>
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<tr>
<td>Cholesterol</td>
<td>mg/dL</td>
<td>1426.33 ± 424.0</td>
<td>1030.14 ± 211.65*</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>mg/dL</td>
<td>125.77 ± 68.49</td>
<td>119.28 ± 64.38</td>
</tr>
<tr>
<td>HDL</td>
<td>mg/dL</td>
<td>122.77 ± 22.77</td>
<td>93.28 ± 10.09</td>
</tr>
<tr>
<td>VLDL</td>
<td>mg/dL</td>
<td>23.50 ± 13.55</td>
<td>23.71 ± 12.70</td>
</tr>
<tr>
<td>LDL</td>
<td>mg/dL</td>
<td>1278.33 ± 402.7</td>
<td>913.10 ± 211.15*</td>
</tr>
</tbody>
</table>

Kit\(^{+/+}\) n=9, Kit\(^{W/W}\) n=7. All mice were ApoE\(^{-/-}\). Blood was drawn from vena cava before euthanasia. Both CBC and lipid profile were examined. RBC, red blood cell; Hb, hemoglobin, Ht, hematocrit; MCV, mean corpuscular volume; Segs, segmented neutrophils. HDL, high density lipoprotein; VLDL very low density lipoprotein; LDL low density lipoprotein;

**Data Information**: data are presented as mean ± S.D. *p<0.05, **p<0.01. (p values were assessed using a two-tailed t-test assuming unequal variance)
Table 2. Complete Blood Count (CBC) and lipid profile of c-Kit chimeric mice

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Kit&lt;sup&gt;+/+&lt;/sup&gt; ApoE&lt;sup&gt;-/-&lt;/sup&gt; into Kit&lt;sup&gt;+/+&lt;/sup&gt; ApoE&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Kit&lt;sup&gt;+/+&lt;/sup&gt; ApoE&lt;sup&gt;-/-&lt;/sup&gt; into Kit&lt;sup&gt;W/W-v&lt;/sup&gt; ApoE&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>X10&lt;sup&gt;3&lt;/sup&gt;/ul</td>
<td>8.17 ± 0.20</td>
<td>7.88 ± 0.17</td>
</tr>
<tr>
<td>Hb</td>
<td>X10%/ul</td>
<td>11.02 ± 0.23</td>
<td>10.55 ± 0.26</td>
</tr>
<tr>
<td>Ht</td>
<td>%</td>
<td>37.60 ± 0.42</td>
<td>35.00 ± 0.46</td>
</tr>
<tr>
<td>MCV</td>
<td>fL</td>
<td>46.20 ± 0.19</td>
<td>42.67 ± 0.51</td>
</tr>
<tr>
<td>Segs</td>
<td>%</td>
<td>27.00 ± 0.41</td>
<td>45.66 ± 0.51*</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>mg/dL</td>
<td>1485.00 ± 2.55</td>
<td>556.00 ± 2.10*</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>mg/dL</td>
<td>133.00 ± 1.07</td>
<td>153.83 ± 1.01</td>
</tr>
<tr>
<td>HDL</td>
<td>mg/dL</td>
<td>82.20 ± 0.32</td>
<td>72.00 ± 0.44</td>
</tr>
<tr>
<td>VLDL</td>
<td>mg/dL</td>
<td>26.60 ± 0.47</td>
<td>30.66 ± 0.44</td>
</tr>
<tr>
<td>LDL</td>
<td>mg/dL</td>
<td>1378.00 ± 122.54</td>
<td>453.33 ± 102.08**</td>
</tr>
</tbody>
</table>

Lethally irradiated mice were rescued with bone marrow cells from a control littermate mouse. Kit<sup>+/+</sup> into Kit<sup>+/+</sup> n=6, Kit<sup>+/+</sup> into Kit<sup>W/W-v</sup> n=6, all mice were ApoE<sup>-/-</sup>. Blood was drawn from vena cava before euthanasia. Both CBC and lipid profile were examined. RBC, red blood cell; Hb, hemoglobin, Ht, hematocrit; MCV, mean corpuscular volume; Segs, segmented neutrophils. HDL, high density lipoprotein; VLDL very low density lipoprotein; LDL low density lipoprotein.

**Data Information:** data are presented as mean ± S.D. * p<0.05, **p<0.01. (p values were assessed using a two-tailed t-test assuming unequal variance)
Vehicle n=6, TAM n=7. All mice were ApoE^{−/−}. Blood was drawn from vena cava before euthanasia. Both CBC and lipid profile were examined. RBC, red blood cell; Hb, hemoglobin, Ht, hematocrit; MCV, mean corpuscular volume; Segs, segmented neutrophils. HDL, high density lipoprotein; VLDL very low density lipoprotein; LDL low density lipoprotein;

**Data Information**: data are presented as mean ± S.D.  * p<0.05. (p values were assessed using a two-tailed t-test assuming unequal variance)
Table 4. Selected list of differentially expressed genes in c-Kit deficient vs. wild type SMCs

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Product</th>
<th>Fold Change *</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transcription Factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cebpba</td>
<td>CREB binding protein</td>
<td>-1.18</td>
<td>0.010</td>
</tr>
<tr>
<td>Foxo1</td>
<td>Forkhead box O1</td>
<td>-2.05</td>
<td>0.007</td>
</tr>
<tr>
<td>Il1f2</td>
<td>Interleukin enhancer binding factor 2</td>
<td>1.38</td>
<td>0.034</td>
</tr>
<tr>
<td>Irf3</td>
<td>Interferon regulatory factor 3</td>
<td>-1.14</td>
<td>0.029</td>
</tr>
<tr>
<td>Nfatc1</td>
<td>Nuclear factor of activated T-cells 1</td>
<td>-1.36</td>
<td>0.040</td>
</tr>
<tr>
<td>Nfatc2</td>
<td>Nuclear factor of activated T-cells 2</td>
<td>2.01</td>
<td>0.033</td>
</tr>
<tr>
<td>Nfatc4</td>
<td>Nuclear factor of activated T-cells 4</td>
<td>-2.32</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>Cell Adhesion Proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdh5</td>
<td>Cadherin 5</td>
<td>-5.00</td>
<td>0.043</td>
</tr>
<tr>
<td>Itga9</td>
<td>Integrin subunit alpha 9</td>
<td>-2.91</td>
<td>0.047</td>
</tr>
<tr>
<td>Itgai1</td>
<td>Integrin subunit alpha 11</td>
<td>-13.28</td>
<td>0.034</td>
</tr>
<tr>
<td>Pcdh7</td>
<td>Protecadherin 7</td>
<td>-2.71</td>
<td>0.015</td>
</tr>
<tr>
<td>Pcdha1</td>
<td>Protecadherin alpha 1</td>
<td>-1.44</td>
<td>0.042</td>
</tr>
<tr>
<td>Pcdha8</td>
<td>Protecadherin alpha 8</td>
<td>2.26</td>
<td>0.002</td>
</tr>
<tr>
<td>Selp</td>
<td>P-selectin glycoprotein ligand 1</td>
<td>-3.94</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>Cytokines/Growth Factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ccl6</td>
<td>Chemokine (C-C motif) ligand 6</td>
<td>-8.76</td>
<td>0.005</td>
</tr>
<tr>
<td>Gdf6</td>
<td>Growth differentiation factor 6</td>
<td>-4.10</td>
<td>0.019</td>
</tr>
<tr>
<td>Ifna14</td>
<td>Interferon alpha 14</td>
<td>1.27</td>
<td>0.043</td>
</tr>
<tr>
<td>Igf1</td>
<td>Insulin-like growth factor 1</td>
<td>-5.06</td>
<td>0.027</td>
</tr>
<tr>
<td>Pdgfb</td>
<td>Platelet-derived growth factor subunit B</td>
<td>-2.59</td>
<td>0.011</td>
</tr>
<tr>
<td>Pgf</td>
<td>Placental growth factor</td>
<td>-4.96</td>
<td>0.037</td>
</tr>
<tr>
<td>Tnfsf9</td>
<td>Tumor necrosis factor ligand superfamilly member 9</td>
<td>2.97</td>
<td>0.048</td>
</tr>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bmp1</td>
<td>Bone morphogenetic protein 1</td>
<td>-2.36</td>
<td>0.016</td>
</tr>
<tr>
<td>Casp3</td>
<td>Caspase 3</td>
<td>2.06</td>
<td>0.040</td>
</tr>
<tr>
<td>Cend1</td>
<td>Cyclin D1</td>
<td>2.20</td>
<td>0.043</td>
</tr>
<tr>
<td>Gucyl1b3</td>
<td>Guanyl cyclase 1 soluble subunit beta</td>
<td>-8.95</td>
<td>0.033</td>
</tr>
<tr>
<td>Lbpbb</td>
<td>Inhibitor of nuclear factor kappa-B kinase subunit beta</td>
<td>1.34</td>
<td>0.001</td>
</tr>
<tr>
<td>Lpl</td>
<td>Lipoprotein lipase</td>
<td>-14.24</td>
<td>0.022</td>
</tr>
<tr>
<td>Mmp23</td>
<td>Matrix metalloproteinase 23</td>
<td>-6.61</td>
<td>0.049</td>
</tr>
<tr>
<td>Pde1a</td>
<td>Ca2+/calmodulin dependent phosphodiesterase 1A</td>
<td>-4.73</td>
<td>0.028</td>
</tr>
<tr>
<td>Pde2a</td>
<td>Phosphodiesterase 2A</td>
<td>-1.91</td>
<td>0.048</td>
</tr>
<tr>
<td>Sirt1</td>
<td>Sirtuin 1</td>
<td>-1.52</td>
<td>0.034</td>
</tr>
<tr>
<td>Tnfaip3</td>
<td>TNF alpha induced protein 3</td>
<td>3.46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adra2a</td>
<td>Adrenoceptor alpha 2A</td>
<td>-6.49</td>
<td>0.002</td>
</tr>
<tr>
<td>Agtr1b</td>
<td>Angiotensin II type 1b receptor</td>
<td>-9.88</td>
<td>0.043</td>
</tr>
<tr>
<td>Apvr1a</td>
<td>Arginine vasopressin receptor 1A</td>
<td>-6.62</td>
<td>0.010</td>
</tr>
<tr>
<td>Cxcr4</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
<td>-6.55</td>
<td>0.040</td>
</tr>
<tr>
<td>Igrp2</td>
<td>Insulin-like growth factor 2 receptor</td>
<td>-1.48</td>
<td>0.012</td>
</tr>
<tr>
<td>Il3ra</td>
<td>Interleukin 3 receptor subunit alpha</td>
<td>-2.38</td>
<td>0.023</td>
</tr>
<tr>
<td>Il20ra</td>
<td>Interleukin 20 receptor alpha</td>
<td>-3.52</td>
<td>0.003</td>
</tr>
<tr>
<td>Pdgfb</td>
<td>Platelet-derived growth factor receptor beta</td>
<td>-2.89</td>
<td>0.016</td>
</tr>
<tr>
<td>Pth1r</td>
<td>Parathyroid hormone 1 receptor</td>
<td>-5.42</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* Average fold gene expression change in c-Kit deficient smooth muscle cells compared to wild type cells.
Table 5. Selected canonical pathways with differentially expressed genes in c-Kit deficient vs. wild type SMCs

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Biological Function</th>
<th>P-Value</th>
<th>Z-Score</th>
<th>Predicted Status</th>
<th>Differentially Expressed Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN signaling</td>
<td>Proliferation, apoptosis, de-differentiation, cell migration, inflammation</td>
<td>&lt;0.001</td>
<td>0.258</td>
<td>Activation</td>
<td>Akt2, Casp3, Rac1, Cen1, Igl2r, Rac3, Ddr1, Shc1, Ikkb, Inpp5f, Foxo1, Bmpr1a, Mag2, Mag3, Egr2</td>
</tr>
<tr>
<td>Death receptor signaling</td>
<td>Apoptosis</td>
<td>0.003</td>
<td>1.265</td>
<td>Activation</td>
<td>Map2k4, Gas2, Rock1, Diab1, Ihk1, Casp3, Hina2, Tbk1, Parp1, Birc2</td>
</tr>
<tr>
<td>TNFR2 signaling</td>
<td>Proliferation, cell survival, inflammation</td>
<td>0.005</td>
<td>1.000</td>
<td>Activation</td>
<td>Map2k4, Ihk1, Tnfaip3, Tbk1, Birc2</td>
</tr>
<tr>
<td>Wnt/β-catenin signaling</td>
<td>Proliferation, cell survival, cell migration</td>
<td>0.008</td>
<td>0.577</td>
<td>Activation</td>
<td>Sfp4, Akt2, Crobbp, Cenk1a1, Fzd9, Cendid1, Rac1, Fzd8, Cdh5, Dkk3, Sox18, Ppp2r5e, Sftp1, Wnt5b</td>
</tr>
<tr>
<td>IRF activation pathway</td>
<td>Inflammation</td>
<td>0.011</td>
<td>1.134</td>
<td>Activation</td>
<td>Map2k4, Ihk2, Crobbp, Tbk1, Irf3a, Irf2</td>
</tr>
<tr>
<td>ERK/MAPK signaling</td>
<td>Proliferation, cell migration, vasoconstriction</td>
<td>0.028</td>
<td>1.069</td>
<td>Activation</td>
<td>Crobbp, Rac1, Ppp1r1a4a, Mkk3k2, Rac3, Nfatc1, Akt2, Pla2g6e, Shc1, Pla2g6e, Pkrar2b, Pkrar2, Rps6ka1, Ppp2r5e</td>
</tr>
<tr>
<td>TNFR1 signaling</td>
<td>Cell survival, inflammation</td>
<td>0.044</td>
<td>1.000</td>
<td>Activation</td>
<td>Map2k4, Casp3, Tnfaip3, Birc2</td>
</tr>
<tr>
<td>Wnt/Ca++ signaling</td>
<td>Proliferation, cell migration</td>
<td>&lt;0.001</td>
<td>-1.000</td>
<td>Inhibition</td>
<td>Fzd8, Pcle4, Crobbp, Nfatc2, Fzd9, Nfat4, Wnt5b, Nfatc1, Act2</td>
</tr>
<tr>
<td>AMPK signaling</td>
<td>Cellular senescence, anti-inflammatory, differentiation, vasoconstriction</td>
<td>&lt;0.001</td>
<td>-0.535</td>
<td>Inhibition</td>
<td>Pten1, Akt2, Crobbp, Cendid1, Sic2a4, Elavl1, Akt2, Ak6, Pkrar2b, Fox01, Adrn2a, Ppm1b, Sirt1, Pkrar2, Ppm1a, Ppp2r5e, Ppar, Camk2</td>
</tr>
<tr>
<td>Apoptosis signaling</td>
<td>Apoptosis</td>
<td>&lt;0.001</td>
<td>-0.302</td>
<td>Inhibition</td>
<td>Map2k4, Gas2, Rock1, Diab1, Ikkb, Casp3, Hina2, Rps6ka1, Bc2a1, Parp1, Birc2</td>
</tr>
<tr>
<td>Phospholipase C signaling</td>
<td>Vasoconstriction, stress responses</td>
<td>0.001</td>
<td>-0.378</td>
<td>Inhibition</td>
<td>Rala, Argph2f1, Pld3, Fesg2a, Argph15, Crobbp, Rac1, Ppp1r1a4a, Nfatc4, Fesg2b, Rhob, Nfatc1, Akt2, Pla2g6e, Shc1, Pla2g6e, Pcle4, Itp2, Fcer1g, Nfatc2</td>
</tr>
<tr>
<td>Nitric oxide/GC signaling</td>
<td>Vasodilation</td>
<td>0.005</td>
<td>-0.302</td>
<td>Inhibition</td>
<td>Bdkrb2, Kng1, Pdeo2a, Akt2, Pkrar2b, Pkrar2b, Pdeo1a, Gqcy1b3, Paf1</td>
</tr>
<tr>
<td>Integrin signaling</td>
<td>Cell adhesion, cell migration, proliferation, apoptosis, stress responses, differentiation</td>
<td>0.029</td>
<td>-1.387</td>
<td>Inhibition</td>
<td>Map2k4, Akt2, Rala, Rac1, Rac3, Rhob, Bdkrb2, Rock1, Argph5p, Shc1, Igl2a, Igl2b, Act2, Tspan6, Nedd9</td>
</tr>
<tr>
<td>Adipogenesis pathway</td>
<td>Lipid synthesis and storage</td>
<td>&lt;0.001</td>
<td>N.D.</td>
<td>Could not be predicted</td>
<td>Nr2f2, Sln3b, Fzd9, Nfatc4, Rbp1, Slc2a4, Fzd8, Cdk5, Foxo1, Bmpr1a, Lpl, Sirt1, Cib6, Cloc4, Fapb4, Rps6ka1, Stat5b</td>
</tr>
<tr>
<td>Fibroblast inflammatory pathway</td>
<td>Proliferation, cell migration, differentiation, inflammation</td>
<td>0.012</td>
<td>N.D.</td>
<td>Could not be predicted</td>
<td>Map2k4, Sftp4, Akt2, Crobbp, Cenk1a1, Rac1, Fzd9, Nfatc4, Cendid1, Rac1, Pkrar2b, Pkrar2, Fas, Act2, Rock1, Ikkb, Fzd8, Pdeo4, Dkk3, Nfatc2, Sftp1, Wnt5b</td>
</tr>
<tr>
<td>GuG signaling</td>
<td>Proliferation, cell migration, vasoconstriction</td>
<td>0.026</td>
<td>0.000</td>
<td>Could not be predicted</td>
<td>Rock1, Ikkb, Pcle4, Akt2, Pdeo3, Act2, Itp2, Nfatc2, Nfatc4, Avpr1a, Rhob, Nfatc1</td>
</tr>
</tbody>
</table>

* Z-score and predicted functional status in c-Kit deficient smooth muscle cells compared to wild type cells. The z-score measures how well the gene expression data matches the experimentally observed direction of pathway regulation in the literature. A positive z-score predicts activation, while a negative z-score indicates inhibition. N.D., could not be determined.

* NF-κB associated signaling pathway
### SUPPLEMENTARY TABLES

#### Supplementary Table 1. Primers for transgenic animal genotype

<table>
<thead>
<tr>
<th>Target</th>
<th>Wild Type</th>
<th>Mutant</th>
<th>Common</th>
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</thead>
<tbody>
<tr>
<td>Kit-eGFP</td>
<td>5'--TGG TAG CTG TTG GCT TTT CC-3'</td>
<td>5'--ACA AAA GCA TCA CCA AAC TCG-3'</td>
<td>5'--CCA GAC TGC CTT GGG AAA AG-3'</td>
</tr>
<tr>
<td>ApoE</td>
<td>5'--GCC TAG CCG AGG GAG AGC CG-3'</td>
<td>5'--TGT GAC TTA GGA GCT CAG C-3'</td>
<td></td>
</tr>
<tr>
<td>Kit&lt;sup&gt;ins66-71&lt;/sup&gt;</td>
<td></td>
<td>5'--GCC GTC CCC GAC TGC ATC T-3'</td>
<td></td>
</tr>
<tr>
<td>Myh11-CreER&lt;sup&gt;12&lt;/sup&gt;</td>
<td></td>
<td>5'--CCC GGA GCC CAC AAT AGA TTG-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wild Type</td>
<td>5'--AAC CAG CTG GGG CTC GAA ATT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutant</td>
<td>5'--CTG TCC TGG GAA ATT GCT TTA-3'</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5'--AGT CCC TCA CAT CTT GAG CTT-3'</td>
<td></td>
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</tbody>
</table>

#### Supplementary Table 2. siRNA sequence pool for KLF4 and GFP (negative control) knockdown in Kit<sup>W/W<sup>-</sup>y</sup> SMCs

<table>
<thead>
<tr>
<th>Target</th>
<th>siRNA Pool Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLF4</td>
<td>297 5'--CCTCCTGGAGCTAGACTTTATCTTTCCA -3'</td>
</tr>
<tr>
<td></td>
<td>721 5'--AGGCCCTGCTGACATCAGTATGCTAGCAAAGG -3'</td>
</tr>
<tr>
<td></td>
<td>1202 5'--CCTGTGACTATGCAGGGGCTGGGCAAACC -3'</td>
</tr>
<tr>
<td></td>
<td>1419 5'--CCACCTGGCTTTACACATGAAGAGGCAC -3'</td>
</tr>
<tr>
<td>GFP</td>
<td>5'--GGGTGAACCTACGTCAGAA -3'</td>
</tr>
</tbody>
</table>
Supplementary Table 3. siRNA sequence pool for TAK1, NLK and GFP (negative control) knockdown in Kit<sup>+/+</sup> SMCs

<table>
<thead>
<tr>
<th>Target</th>
<th>siRNA Pool Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAK1</td>
<td>771 5'-GCCCATTTGAGAGCTTGATGACACGCTGTT -3'</td>
</tr>
<tr>
<td></td>
<td>880 5'-GCGGATGAGCGCCCTACAGTATCCCTTGCA -3'</td>
</tr>
<tr>
<td></td>
<td>1073 5'-AGCAACAGAGTGAATCTGGACGCCTGAGC -3'</td>
</tr>
<tr>
<td></td>
<td>1474 5'-TCAGATAACTCCATCCCAATGCGTATCT -3'</td>
</tr>
<tr>
<td>NLK</td>
<td>447 5'-TGGCGTTGTCTGGTCAGTAACAGATCCAA -3'</td>
</tr>
<tr>
<td></td>
<td>892 5'-ACTCAGGAAGTAGTTACTCAGTATTACCG -3'</td>
</tr>
<tr>
<td></td>
<td>1103 5'-CTTGTGAAGGGTCAGTAAGGCACACATACTC -3'</td>
</tr>
<tr>
<td></td>
<td>1477 5'-AGAGTGCCCTCTCTGCATCAACCCGAGTC -3'</td>
</tr>
<tr>
<td>GFP</td>
<td>5'-GGGTGAACTCGAAGCTCAGAA -3'</td>
</tr>
</tbody>
</table>