The Role of Krüppel-Like Factor 2 in Mediating the Atheroprotective Functions of Pulsatile Laminar Flow in Vascular Endothelium

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by

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Bioengineering

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In the cardiovascular system, mechanical forces are important modulators of cell functions. Being in constant contact with blood flow, vascular endothelial cells (ECs) are sensitive to hemodynamic forces such as fluid shear stress. Cells in the straight part of the arterial tree are usually resistant to atheroma formation, while cells at vessel bifurcations are athero-prone. These differences in EC phenotype can be attributed mainly to region-specific mechanical forces, i.e., laminar high shear stress in the straight vessel versus disturbed low shear stress at bifurcation points.

Among many genes and proteins that respond to fluid shear stress, Krüppel-Like Factor 2 (KLF2) is a transcription factor that can be induced by laminar flow. It has been noted that KLF2 possesses several anti-thrombotic and athero-protective characteristics similar to those of high laminar shear stress. In the current study, I focused on understanding the regulatory mechanisms of KLF2 under pulsatile laminar flow (PS), and investigated its role in the flow-mediated atheroprotective functions. AMP-activated protein kinase (AMPK) was identified as an upstream signaling
molecule that regulated KLF2 induction by PS flow, and results of my collaborative studies linked AMPK to the previously known ERK5/MEF2 pathway. Our findings provided a complete mechanistic explanation of KLF2 regulation under PS flow via the AMPK/ERK5/MEF2 pathway.

In order to further elucidate the role of KLF2 in the atheroprotective functions of PS flow, I used gene-silencing siRNA to block KLF2 expression and examined its effects on PS flow-mediated cell anti-proliferative and anti-inflammatory responses. My results suggested that KLF2 induction was essential in keeping ECs in a quiescent and non-inflammatory state under PS flow, thus demonstrating KLF2’s role in maintaining vascular homeostasis.

Our investigation has provided new insights on the mechanism of how KLF2 is regulated under the mechanical stimulation of PS flow. By manipulating the expression of KLF2, I also gain understanding of the critical role it plays in PS flow-mediated cell functions. These observations identify KLF2 as an important mechanoo-activated transcription factor that is necessary for the endothelium to remain in an athero-resistant state in the vasculature.
Chapter I

Introduction

A. Atherosclerosis and Vascular Mechanics

Cardiovascular disease (CVD) has been a major health threat in the world, accounting for more than a third of total death. CVD includes heart disease, vascular disease, atherosclerosis, stroke, and hypertension, etc. Being the basic pathophysiological process of most CVD, atherosclerosis is a vascular inflammatory disease characterized by lesion formation and luminal narrowing of the arteries. Atherosclerotic lesions, or “plaques”, result from progressive accumulation of lipids, macrophages and other cellular elements in the subintimal layer of arteries that eventually leads to vessel hardening and occlusion of blood flow. Atherosclerotic plaques are preferentially formed at vessel bifurcation branches, but are usually absent along the straight part of the arterial tree [See Figure 1A and 1B.]. The “site-specificity” of atherosclerosis can be primarily attributed to hemodynamic forces, responsible for deciding which vascular sites are susceptible or resistant to atheroma formation [VanderLaan at el., 2004]. That is, the regional differences in the mechanics of the vasculature may prime the vessels to be either pro- or anti-atherogenic.

The arteries are mainly composed of three layers: intima, media, and adventitia. Endothelial cells (ECs) are the major cell type in the intimal layer and are located at the luminal surface of the vessel. Smooth muscle cells (SMCs) compose the medial layer, while fibroblasts are mainly present in the adventitial tissue. Due to its unique
location in the vessel wall, the endothelium is constantly subjected to hemodynamic and humoral stimulations, and plays a critical role in atherogenesis. Mechanical forces are significant modulators of cellular functions in many tissues, and are particularly important in the vasculature. Being exposed to the circulating blood, ECs experience primarily three types of mechanical forces created by blood flow and the cardiac cycle: (1) shear stress, the tangential frictional force acting on the vessel surface, (2) cyclic stretch and the consequent circumferential strain exerted on the vessel wall, and (3) hydrostatic pressure, which generates the normal force on the vessel wall [Traub et al., 1997]. Among these hemodynamic forces, ECs are particularly sensitive to shear stress, which can significantly modulate endothelial gene expressions and cellular functions [Davies et al., 1995]. As previously mentioned, atherosclerotic lesions are “site-specific” and repeatedly happen at bifurcation points. These high-susceptibility sites are areas with significant curvatures or branches and are usually associated with low shear stress, oscillating turbulence and flow recirculation. In contrast, laminar flow with a pulsatile pattern and high shear stress dominates the “linear” areas that are spared from atherosclerosis [VanderLaan et al., 2004]. ECs in these two distinctive regions sense different flow stimuli and thus have different functional consequences, leading to different vulnerabilities to atherosclerosis. Indeed, vascular mechanics plays a critical role in determining endothelial phenotype and atherosclerotic progression.

The elucidation of the mechanism of atherosclerosis requires the understanding of how the endothelium responds to different types of hemodynamic stimuli and how these mechanical signals are transduced to modulate EC functions. A healthy
endothelium helps maintain vascular homeostasis, while an “activated” endothelium generates atherogenic signals, causing the vasculature to become athero-prone.

A.  

Figure 1. (A) Schematic drawing of the major arteries in the human body. Note atherosclerotic lesions (red shading) are mainly located at bifurcation points. (B) Longitudinal representation of the major arterial vasculature in LDL receptor-deficient mice. The diagram illustrates observed distribution of atherosclerosis (gray shading) in LDL receptor-deficient mice fed a high-fat diet [VanderLaan et al., 2004].

B. Endothelial Responses to Mechanical Forces

Due to the unique location of the endothelium at the blood/vessel wall interface, many studies have focused on endothelial responses to shear stress generated by the flowing blood. A number of in vitro cell culture models have been developed to study the effects of fluid mechanical forces on EC functions. The two major flow systems
used to mimic physiological flow conditions are the parallel-plate flow chambers, which consists of two plates separated by a thin gasket with flow driven by pressure difference across the chambers [Levesque and Nerem., 1985], and the cone-plate viscometer, in which a cone submerged in medium over cultured EC monolayer is rotated at a constant velocity [Bussolari et al., 1982]. Steady laminar shear stress at a constant magnitude has been used widely to mimic flow pattern in the straight sections of arteries in vivo (10 to 20 dyn/cm²) although a pulsatile flow at a controlled mean shear stress level has also been used to more closely mimic the pulsatile nature of blood flow. In addition, an oscillatory flow in which mean shear stress is close to zero [Chappell et al., 1998], has been generated to mimic the low-shear component at vessel bifurcations and branch points. Many other set-ups have been developed to provide improved in vitro flow conditions. These flow models provide the capability to study effects of fluid flows on modulation of EC phenotypes and functions at athero-protective and athero-prone regions in the arteries.

ECs are equipped with many receptors that are sensitive and responsive to fluid shear stress, and thus mediate pro- or anti-atherogenic effects. Several membrane receptors, including integrins, receptor tyrosine kinases (RTKs), ion channels, G-protein coupled receptors (GPCRs), caveolae, and junctional proteins, have been investigated. It has been shown that blocking integrins using antibodies or inhibitory RGD peptides attenuated shear-induced signaling and cellular functions [Ishida et al., 1995; Muller et al., 1996; Bhullar et al., 1998; Liu et al., 2002]. RTKs, such as the VEGF receptor Flk-1 on the EC surface, can be transiently activated by shear stress to associate with adaptor proteins such as Shc [Chen et al., 1999]. Mechanosensitive ion-
channels, including K\(^+\) and Ca\(^{2+}\) channels, can also be activated by shear stress. Blockage of K\(^+\) channels inhibited the shear-mediated NO production [Uematsu et al., 1995]. Inhibition of G-proteins using antisense oligonucleotides can block shear-induced Ras-GTPase activity [Gudi et al., 2003]. Caveolae, which are specialized domains of the membrane lipid bilayer, also play an important role via recruitment and sequestration of other signaling molecules such as G-proteins, tyrosine kinases and others [Couet et al., 1997]. Intercellular junction proteins such as PECAM1 [Tzima et al., 2005] has also been shown to play a role in mechano-sensing. These receptors help in the transduction of extracellular mechano-signals to modulate activities of many intracellular signaling molecules including protein kinase C (PKC), focal adhesion kinase (FAK), c-Src, Rho family GTPases, phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinases (MAPKs), etc. Through these signaling molecules, different flow patterns with distinctive shear stress components can modulate EC gene expressions and cellular functions such as vasomotor tone, proliferation, survival, and remodeling.

Many studies have focused on gene regulations in ECs exposed to atheroprotective steady/pulsatile laminar flow and athero-prone oscillatory flow. Transcriptional profiling using DNA microarray technologies has enabled the identification of genes that are differentially regulated by athero-protective and athero-prone flows [Chen et al., 2001; Garcia-Cardena et al., 2001; McCormick et al., 2001; Brooks et al., 2002; Dekker et al., 2002; Peters et al., 2002]. In particular, Garcia-Cardena et al.’s studies [2001] pointed out that at least 100 genes are differentially regulated by these flow patterns with opposing influences on atherogenesis, including
those with gene products that are of considerable importance in regulating vascular homeostasis, e.g., cell-surface adhesion molecules, and pro-oxidant and anti-oxidant enzymes. Oscillatory, low shear flow up-regulates the expressions of adhesion molecules, e.g., VCAM-1, ICAM-1, MCP-1 and E-selectin, via activation of nuclear factor-κB (NF-κB) [Chappell et al., 1998; Mohan et al., 1997], and down-regulates the expression of endothelial nitric oxide synthase (eNOS). In contrast, laminar flow suppresses the expressions of adhesion molecules, while significantly enhancing the expression of eNOS to prevent leukocyte binding and platelet aggregation, inhibits SMCs growth, and alters lipoprotein metabolism via production of NO [Vanhoutte et al., 1989]. The differential gene regulations by different flow patterns show that laminar and oscillatory flows may exert different functional consequences via modulations of gene expressions. The “site-specificity” of atherogenesis, with atherosclerotic lesions often developing around vessel bifurcations, is related to the distinctive gene regulation pattern by low-shear flow in these regions.

With its ability to modulate gene expressions, shear flows can regulate a variety of cellular functions. In relation to vasomotor tone regulation, laminar flow not only increases the release of the vasodilator NO, but also the generation of the anti-coagulant thrombomodulin (TM) to inactivate several clotting factors [Malek et al., 1994]. ECs exposed to oscillatory flow failed to show increases in NO production and TM. For cell growth and survival, several studies have shown that proliferation is significantly reduced by laminar flow and increased by oscillatory flow [Levesque et al., 1990; Akimoto et al., 2000; Lin et al., 2000; Li et al., 2005]. It is also known that laminar flow is beneficial for EC survival by inducing expressions of inhibitors of
apoptosis proteins 1 and 2 [Jin et al., 2002; Taba et al., 2003]. The growth-arrest and pro-survival effects of laminar flow keep ECs in a non-activated state with very low cell turnover. The low cell turnover helps maintain the integrity of EC monolayer. In contrast, oscillatory flow enhances cell turnover by increasing not only EC proliferation, but also EC’s susceptibility to apoptotic stimuli [Li et al., 2005]. The accelerated turnover leads to a loss of monolayer integrity and a more permeable endothelium for macromolecular transport. Studies on experimental animals showed a higher low-density lipoprotein (LDL) accumulation within the vessel wall localized to areas of disturbed low-flow near bifurcation branches [Berceli et al., 1990; Chuang et al., 1990; Herrmann et al., 1994]. Shear flows can also modulate EC morphology. Many studies have shown that laminar flow induces cell elongation and alignment with the direction of flow [Dewey et al., 1981], depending on the shear stress level and exposure duration [Nerem et al., 1981]. Via reorganization of the cytoskeletal structure, notably F-actin filaments [Ookawa et al., 1992], laminar flow an elongated cell morphology, which is in contrast to the cobblestone appearance of cells exposed to disturbed low flow.

I have described a wide range of endothelial responses to shear flows via its membrane receptors and intracellular signaling molecules to modulate gene expressions and cellular functions. The regional differences in the hemodynamic profile between straight arteries and vessel bifurcations prime the endothelial phenotype to respond distinctively to systemic risk factors such as hypercholesterolemia or oxidative stress. To better understand the endothelial regulation by athero-protective laminar flow, I developed a particular interest in a
transcription factor, Krüppel-like factor 2 (KLF2), which has been shown to play an important role in modulating EC functions in response to hemodynamic stimuli. I will discuss next this transcription factor and its role in the vasculature.

C. Introduction to the Krüppel-Like Factor Family

KLF2 belongs to the mammalian KLF family of zinc-finger transcription factors. The nomenclature is based on the DNA-binding domain homology of its founding member, KLF1, which was first found in the *Drosophila* protein Krüppel. “Krüppel”, is the German word for “cripple.” In *Drosophila* embryos with homozygous knockout for the protein Krüppel, there were altered body parts on the anterior, abdominal and thoracic segments, resulting in death [Nusslein-Volhard et al., 1980; Jackle et al., 1985; Preiss et al., 1985]. KLF1, also named erythroid Krüppel-like factor (EKLF), was first cloned in 1993 by Miller and Bieker [1993], and was identified in red blood cells for its regulation of β-globin gene expression and erythrocyte development [Perkins et al., 1995; Nuez et al., 1995]. Based on the homology of its DNA-binding zinc-finger domains to KLF1, KLF2 was identified and cloned by Anderson et al. via hybridization probe analysis [1995]. KLF2 is also termed lung Krüppel-like factor (LKLF) due to its high expression in the lungs when first discovered in mice and rat tissues.

In total, there are 17 members of the KLF family in mammalian cells since the discovery of KLF1 in 1993 [Suske et al., 2005]. Although individual KLFs may be expressed in different cell and tissues types, they share very highly conserved carboxyl terminus where the three zinc-fingers are located. The three Cys2/His2 zinc fingers consist of 25, 25, and 23 residues, respectively, with a highly-conserved inter-finger
spacer sequence of TGEKP(Y/F)X [Dang et al., 2000]. These zinc fingers can bind
GT-box and CACCC elements on DNA binding sites; thus many KLFs can recognize
and bind similar sequences. However, outside of the zinc finger region, KLFs’ non-
DNA-binding domains are highly divergent with relatively little homology [Kaczynski
et al., 2003]. This lack of conservation allows individual KLFs to function in different
tissue types as either activators or suppressors for cellular functions or tissue
development.

Phylogenetic analysis of the 17 KLF members has divided them into a few sub-
groups. In particular, KLF2 is grouped with KLF1 and KLF4 due to their sequence
homology [Bieker, 2001]. With their highly-conserved target DNA binding sequences,
these KLFs exert their specific regulations through tissue-restricted expression. In
addition, via their divergent non-DNA binding activation/repression domains, they can
function in an orchestrated manner even if expressed in the same tissue type.

**D. Overview of Krüppel-Like Factor 2**

Since its first discovery in the lung tissue, KLF2 has been investigated
extensively in several other tissues types, and has been shown to play important roles
in regulating many cellular functions for tissue development.

The human KLF2 transcriptional unit is located in a genomic region of
approximately 3 Kb on chromosome 19p13.1, with three exons interrupted by two
small introns. The translated protein is a 354-amino acid residue that shares greater
than 85% homology to the mouse protein [Anderson et al., 1995]. KLF2 is highly
expressed in the lung, but is also present in erythrocytes, T-lymphocytes, vascular ECs,
and several other tissues [Dang et al., 2000; Kaczynski et al., 2003]. In recent years it
has received most attention for its roles in regulating T-cell differentiation and trafficking and the anti-atherogenic and anti-thrombotic functions of vascular ECs.

Several studies have demonstrated that KLF2 plays a critical role in embryonic development. Anderson et al. [1995] showed that KLF2 is temporally regulated in mouse embryo, with expression starting at embryonic day 7 (E7), decreased at E11, and increased subsequently at E15. Targeted deletion analysis of KLF2 led to embryonic lethality between E12.5 and E14.5 [Kuo et al., 1997; Wani et al., 1998]. KLF2 expression has been found to be essential for normal lung development [Wani et al., 1999]. In KLF2-knockout mice, blood vessel maturation was impaired due to lack of smooth muscle cell recruitment and tunica media formation, which subsequently led to aneurismal dilation and rupture of blood vessels [Kuo et al., 1997]. Using tissue-specific KLF2 knockout mouse models, Lee et al. [2006] showed that endothelial loss of KLF2 resulted in heart failure attributable to high cardiac output. These lines of evidence demonstrate the crucial role of KLF2 in embryonic development related to normal lung formation, vessel generation and heart function.

In addition to being developmentally regulated, KLF2 also plays a role in T-cell differentiation. In the absence of KLF2, mature, single [??] positive T cells exist in an activated state and are more prone to apoptosis [Schober et al., 1999]. Several studies showed that KLF2 inhibits T-cell growth and programs T-cell quiescence via a c-Myc pathway and by up-regulating the cyclin-dependent kinase inhibitor p21^{cip1} [Buckley et al., 2001; Wu et al., 2004]. KLF2 also plays a role in T-cell trafficking, regulating the migration of mature thymocytes from the thymus to control the circulation of peripheral T-cells [Carlson et al., 2006]. A few studies also suggested KLF2 is
involved in adipogenesis, inhibiting pre-adipocytes from differentiating into mature adipocytes via negative regulation of peroxisome proliferator-activated receptor-gamma (PPAR-γ) [Banerjee SS et al., 2003; Wu et al., 2005]. Through its tissue specificity, KLF2 modulates diverse cellular functions in different tissue types to regulate T-lymphocytes development and repress adipocytes differentiation.

I have developed a special interest in studying the role of KLF2 in the vascular endothelium due to its regulation by hemodynamic forces such as shear stress and cyclic stretch. Although several KLFs, including KLF2, KLF4, KLF5 and KLF6, have all been shown to be important players in vascular biology, mounting evidence has demonstrated KLF2 is probably the most critical for the anti-atherogenic responses in the vasculature. In the next section, I will describe the significant role it plays in vascular ECs.

E. Significance of Krüppel-Like Factor 2 in the Vasculature

The role of KLF2 in the vascular endothelium first received notice when Dekker et al. identified KLF2 as a shear-induced gene by prolonged laminar flow using a gene profiling approach [Dekker et al., 2002]. Since then, Lingrel’s group has focused on elucidating the transcriptional regulation of KLF2 by laminar shear stress with the identification of a shear stress response region on its promoter region and the involvement of a few nuclear factors [Huddleson et al., 2004, 2005, 2006; Ahmad et al., 2005]. Meanwhile, Jain’s and Garcia-Cardena’s groups, showed that KLF2 possesses several anti-atherogenic and anti-thrombotic characteristics in vascular ECs, and responds to inflammatory cytokines in addition to shear flow [SenBanerjee et al., 2004; Kumar et al., 2005; Lin et al., 2005, 2006; Parmar et al., 2006]. A great deal of
interests have been developed around KLF2, and many studies have been conducted to better understand its regulation by both anti-atherogenic and pro-atherogenic stimuli, as well as its role in endothelial function and vascular homeostasis.

KLF2 expression can be induced by steady laminar shear stress [Dekker et al., 2002; Huddleson et al., 2004] and pulsatile laminar flow [Parmar et al., 2006]. We have shown that KLF2 is differentially regulated by two opposing flow patterns, i.e., it is induced by pulsatile laminar flow and suppressed by oscillatory low flow [Wang et al., 2006]. These studies demonstrated that KLF2 may very likely be an important molecule in preventing atherosclerosis progression since it is up-regulated by athero-protective flow but down-regulated by athero-prone flow. The cholesterol-lowering drug statin has also been shown to induce KLF2 expression [Parmar et al., 2005]; while proinflammatory stimuli such as interleukin (IL)-1β, tumor necrosis factor (TNF-α), and thrombin suppressed KLF2 expression [SenBanerjee et al., 2004; Lin et al., 2006]. Over-expression of KLF2 in cultured ECs was able to inhibit EC activation by these proinflammatory stimuli, suggesting that KLF2 may inhibit some common proinflammatory pathways to minimize their actions. In particular, it has been shown that KLF2 interacts with p300/CREB-binding protein (CBP)-associated factor (PCAF), a cofactor for optimal NF-κB activity [SenBanerjee et al., 2004]. By competing with NF-κB for this transcriptional coactivator, KLF2 reduces NF-κB’s transcriptional activity and the associated inflammatory responses. Boon et al. also showed that KLF2 strongly inhibits transforming growth factor (TGF-β) signaling via suppressing Smad2 phosphorylation, Smad3/4-dependent transcriptional activation, and nuclear translocation of activated ATF2, a heterodimeric subunit of activating protein-1 (AP-1).
These studies serve to demonstrate KLF2’s role for the anti-inflammatory actions of vascular endothelium.

Many studies suggest that KLF2 is involved with regulations of endothelial thrombotic function and vascular tone. Lin et al. showed that KLF2 potently induces thrombomodulin (TM), an anti-coagulant and suppresses several pro-coagulants such as tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) [2005]. SenBanerjee et al. identified KLF2 as an inducer for eNOS expression and activity [2004], while multiple other studies showed that KLF2 inhibits ET-1, adrenomedullin, and angiotensin-converting enzyme, which are all important genes for regulating vessel tone [Parmar et al., 2006; Dekker et al., 2005, 2006]. Transcriptional profiling studies by both Horrevoets’ and Jain’s groups have also identified many other factors in the thrombotic and vasoactivity pathways that are regulated by KLF2 [Dekker et al., 2006; Parmar et al., 2006]. These studies characterize KLF2 as a key regulator of endothelial thrombotic function to help maintain blood fluidity by mediating anti-coagulation. KLF2 also participates in regulating vascular tone by activating vasodilatory factors and suppressing vasoconstrictive mediators.

In addition to mediating anti-atherogenic and anti-thrombotic responses in the vasculature, KLF2 also regulates endothelial proliferation and migration upon stimulation. Angiogenic responses characterized by increased EC proliferation and migration are important features for pathological states such as chronic inflammation and cancer [Jain 2003]. Bhattacharya et al. showed that KLF2 over-expression inhibits the VEGF-A induced angiogenesis and cell proliferation in their in vivo nude mouse ear model and in vitro cell assay [2005]. Dekker et al. also showed that over-
expression of KLF2 inhibits cell migration using standard wound-healing assays [2006]. Thus, KLF2 can inhibit angiogenesis through reducing EC proliferation and migration, which are processes characterizing the EC phenotype in atherosclerosis.

In summary, KLF2 holds an important role in the vasculature in mediating the anti-inflammatory, anti-thrombotic, and anti-angiogenic responses that help maintain normal vessel tone and protect the endothelium from developing into an athero-prone state. These athero-protective properties are similar to those of laminar flow, suggesting that athero-protective flow pattern and KLF2 share several common characteristics such as mediating inflammation, vasoactivity, and proliferation. However, the mechanisms by which KLF2 senses mechanical signals to modulate cellular functions and the role it plays in shear-mediated cellular responses are not completely elucidated.

G. Hypothesis and Objectives

In this thesis, I focused on elucidating the role of KLF2 in the atheroprotective functions of pulsatile laminar flow (PS) in the vascular endothelium. I hypothesized that KLF2 induction by PS flow is mediated through a novel AMPK pathway, and that KLF2 expression is critical for the anti-inflammatory and anti-proliferative actions of PS flow. Three objectives were outlined in testing my hypotheses (Fig. 2).

In Chapter II the aim was to elucidate the regulatory mechanisms of KLF2 induction under flow. I showed that KLF2 expression and AMPK activity were both induced by PS flow using our in vitro experimental setup. By inhibiting or knocking down AMPK activity, I showed that KLF2 induction by PS flow was significantly reduced. Over-expressing AMPK by an adenovirus which constitutively activated
AMPK increased KLF2 expression in cultured ECs. These results show that AMPK activation is crucial for KLF2 induction under PS flow. In addition, my collaborative work with UC Riverside and Harvard Medical School showed that AMPK is linked to the previously known ERK5/MEF2/KLF2 pathway, from both in vitro and in vivo studies. My findings in this chapter provided a detailed mechanistic explanation of KLF2 regulation by athero-protective flow.

Studies in Chapter III were conducted with the aim to elucidate KLF2’s role in the anti-inflammatory function of PS flow. I showed that PS flow possesses anti-inflammatory properties by up-regulating anti-inflammatory eNOS and down-regulating pro-inflammatory ET-1 and MCP-1, which all serve to be important gene readouts for inflammatory responses. To examine whether KLF2 expression is critical for mediating the anti-inflammatory action of PS flow, I knocked down KLF2 with siRNA and determined the effects on expressions of eNOS, ET-1 and MCP-1. My results showed that KLF2 induction is critical for these inflammation-related genes in their regulation by PS flow. I also demonstrated that KLF2’s upstream regulator AMPK plays an important role in modulating eNOS expression under flow.

Lastly, in Chapter IV, I aimed to elucidate KLF2’s role in the anti-proliferative function of PS flow. PS flow led to cell quiescence in cultured HUVECs. By using siRNAs, we examined the effects of KLF2 and AMPK knockdown on cell proliferation under 24-hour PS flow. Immunofluorescent imaging for BrdU-Labeling Index was used to quantify cell proliferation, and flow cytometry was employed to analyze cell cycle progression. Cells with AMPK- or KLF2-knockdown both exhibited a more mitotic phenotype compared to those transfected with control siRNA.
My results showed that AMPK activation and KLF2 expression were both important for mediating the anti-proliferative function of PS flow.

Studies conducted in this dissertation have identified a novel signaling pathway (AMPK/ERK5/MEF2) through which PS flow induces KLF2 expression. My results also demonstrated the critical roles KLF2 plays in mediating the anti-inflammatory and anti-proliferative functions of PS flow. These findings contribute to a better understanding of the endothelial response to hemodynamic shear flow, particularly via the expression of KLF2 and its upstream regulator AMPK.

Figure 2. An outline for the thesis.
Chapter II

Regulatory Mechanisms of KLF2 Induction by Pulsatile Laminar Flow

II.A Abstract

I have demonstrated that athero-protective pulsatile laminar flow (PS) strongly activates KLF2 gene expression, similar to the effect of high laminar shear stress. To better understand the regulatory mechanisms of KLF2-induction by PS flow, I investigated the signaling pathways that transduce mechanical signals to activate intracellular KLF2 expression. The results identified AMP-activated protein kinase (AMPK) as the upstream signaling molecule that regulates the induction of KLF2 by PS flow. Blocking AMPK phosphorylation activity, using either a pharmacological inhibitor or silencing agent siRNA, caused a significant reduction of PS-induced KLF2 expression. We have also identified that AMPK is the upstream regulator for the previously known ERK5/MEF2 pathway. The findings of our studies provide a detailed mechanistic explanation of KLF2-induction by PS flow via the AMPK/ERK5/MEF2 axis.
II.B Introduction

KLF2 was first isolated and cloned in the lung by Anderson et al. in 1995, but its importance in the vascular endothelium had not been revealed until Dekker et al. demonstrated that KLF2 was induced by prolonged laminar shear stress in ECs using microarray analysis [2002]. Subsequently Lingrel’s group showed that laminar shear stress activated KLF2 transcription in ECs [Huddleson et al., 2004]. In 2006, we and others showed that KLF2 was induced by the atheroprotective pulsatile flow, but suppressed by the atheroprone oscillatory flow [Wang et al., 2006; Parmar et al., 2006]. The differential transcriptional regulations of KLF2 by flow patterns with vs. without a definite direction suggest that KLF2 may be involved in regulating EC phenotype and vascular function in response to the diverse flow stimuli in different arterial sites. Hence, investigations on the mechanotransduction pathways by which endothelial KLF2 is regulated by flow have generated a great deal of research interests.

As a transcription factor, KLF2’s activity can be regulated via interactions with many other proteins through its promoter region, where these proteins bind different sub-domains to either suppress or activate KLF2 transcription [Conkright et al., 2001]. Promoter analysis of KLF2 identified that the region between -138 and -111 base pairs is required for its transcription [Ahmad et al., 2005]. Electrophoretic mobility supershift assay and chromatin immunoprecipitation analysis showed that nuclear factors such as heterogeneous nuclear ribonucleoprotein (hnRNP)-U, hnRNP-D, and P300/CBP associated factor (PCAF) bind to this region of the KLF2 promoter. Furthermore, Lingrel’s group identified a phosphatidylinositol 3-kinase (PI3K)-
dependent/Akt-independent pathway that mediates shear-regulated KLF2 induction, mainly through the recruitment of a regulatory complex containing nucleolin and nuclear factor hnRNP-D and PCAF which acetylates histones H3 and H4 [Huddleson et al., 2005, 2006] to induce KLF2 transcription via binding to its promoter region. In addition to hnRNP-D and PCAF, myocyte enhancer factor 2 (MEF2A and MEF2C) has also been shown to bind and induce KLF2 promoter activity. MEF2 inhibition can reduce the KLF2 response to tumor necrosis factor-alpha (TNF-α) [Kumar et al., 2005]. Sohn et al. identified that ERK5 mitogen-activated protein kinase (MAPK) drives KLF2 transcription by MEF2 activation [2005], and Parmar et al. showed that the flow-dependent activation of KLF2 is mediated via a MEK5/ERK5/MEF2 pathway [2006]. Thus, MEF2 is necessary for the induction of KLF2 by both shear stress and TNF-α, and hence plays a critical role in regulating KLF2 in response to mechanical and chemical stimuli. The involvements of both PI3K-dependent and the ERK5-dependent pathways suggest that regulation of KLF2 by shear stress may be complex events involving balances between different pathways.

It is unclear whether the PI3K-dependent and the ERK5-dependent pathways occur in parallel or in series. In addition to PI3K and ERK5, I postulate that another signaling molecule, AMP-activated protein kinase (AMPK), may be involved in the shear-regulation of KLF2. Functioning as a “cellular energy sensor” in multiple organs and systems, AMPK can be activated by a variety of physiological and pathological stresses that result in increased AMP-to-ATP ratio, including exercise, hypoxia, and nutrient depletion. In vascular ECs, AMPK is sensitive to reactive oxygen and nitrogen species, and acts as an important activator of endothelial nitric oxide synthase
Many studies have demonstrated the strong augmentation of eNOS activity by AMPK through phosphorylation of eNOS on its Ser-1177/1179 sites [Chen et al., 1999, 2003; Morrow et al., 2003; Drew et al., 2004; Schulz et al., 2005]. Similar to AMPK’s ability to increase nitric oxide (NO)-bioavailability, KLF2 possesses strong anti-inflammatory characteristics and is also a potent inducer of eNOS [Senbanerjee et al., 2004; Lin et al., 2005; Dekker et al., 2006]. AMPK and KLF2, in addition to being activators of eNOS, are both induced by the cholesterol-lowering drug statin [Samari et al., 1998; Sun et al., 2006; Parmar et al., 2005; Senbanerjee et al., 2005]. It has been shown that both AMPK and KLF2 are necessary for statin-dependent eNOS activation. These common athero-protective effects on endothelial biology shared between AMPK and KLF2 provide the foundation to hypothesize that KLF2 may be functionally linked to AMPK signaling. Several recent papers have shown that AMPK is activated by laminar shear stress in cultured ECs [Fleming et al., 2005; Zhang et al., 2006]. In particular, Shyy and colleagues showed that AMPK is necessary for shear stress-induced eNOS activity, and that it is differentially regulated by laminar versus oscillatory flow [Zhang et al., 2006; Guo et al., 2007]. These findings provide support for the postulation that AMPK may be an upstream signaling molecule in the regulation of KLF2-induction by laminar flow.

Chapter I described the emerging importance of KLF2 as an athero-protective transcription factor in the vasculature that contributes to maintenance of endothelial integrity and vascular homeostasis. The aim of this chapter is to elucidate the regulatory mechanisms for KLF2-induction under flow. An in vitro pulsatile laminar
flow (PS) system was used to provide a more physiologically relevant flow condition that incorporates the pulsatility of the cardiac cycle. I first examined whether KLF2 expression and AMPK activity can be induced by PS flow. I then determined whether AMPK is necessary for KLF2 induction under PS flow by inhibiting or knocking down AMPK activity. In addition, our collaborative work with UC Riverside and Harvard Medical School focused on elucidating whether AMPK is linked to the previously known ERK5/MEF2/KLF2 pathway. These experiments were designed to test the hypotheses that AMPK activation is crucial for inducing KLF2 expression upon PS flow stimulation, and that AMPK is the upstream signaling molecule of the ERK5/MEF2/KLF2 pathway. The findings in this chapter serve to elucidate the role of AMPK in the regulation of vascular endothelial functions and the mechanism of KLF2 regulation by athero-protective flow.
II.C Material and Methods

II.C.1 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase treatment of umbilical cord veins. Cells were cultured on 100-mm plates coated with collagen I (BD Biosciences, Bedford, MA) and maintained in medium M199 (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (Omega, Tarzana, CA), 25% endothelial cell growth medium (Cell Applications, San Diego, CA), 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% penicillin/streptomycin. All cell cultures were kept in a humidified 5% CO\textsubscript{2}-95% air incubator at 37°C. Cells within passages 2 to 6 were used in all experiments.

II.C.2 Pulsatile Laminar Flow Experiment

A modified version of the circulating flow system originally designed by Frangos et al. [1985] was modified and then used to impose fluid shear stress on a confluent monolayer of HUVECs seeded on a collagen I-coated glass slide (75 X 38 mm). A silicone gasket was sandwiched between the EC-containing glass slide and an acrylic plate to create a rectangular flow channel (0.025 cm in height, 2.5 cm in width, and 5.0 cm in length) secured by four large metal clips. The gasket confined the space through which culture medium flows from the inlet to the outlet of the flow channel, exposing the cultured cells to the circulating fluid. Shear flow was generated by the hydrostatic pressure difference between an upper reservoir and a lower reservoir. A reciprocating syringe pump was connected to the circulating system to superimpose a sinusoidal component (frequency = 1 Hz) onto the shear stress, mimicking the pulsatile nature of
physiological blood flow (Figure 3). The flow system was kept at 37°C in a temperature-controlled hood, and the circulating medium was ventilated with humidified 5% CO₂-95% air. Pulsatile laminar flow (PS) was applied to cells with a shear stress magnitude of 12 ± 4 dyn/cm². All static control cells (abbreviated as “0 hr”) were kept in the humidified 5% CO₂-95% air incubator at 37°C while experimental PS cells were sheared on the flow system.

Figure 3. Illustrations of the in vitro PS flow system.
Upper panel shows a schematic drawing of the PS flow system. Bottom panel shows a photographic image of the PS flow system.
II.C.3 RNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction

Total RNA was isolated using the Trizol reagent with a manufacturer-suggested protocol (Invitrogen, Carlsbad, CA). The concentration and quality of RNA were checked by using a spectrophotometer SmartSpec 3000 (Bio-Rad, Hercules, CA). Reverse transcription was carried out with 3 µg of total RNA using the Superscript II reverse transcriptase (Invitrogen), incubated at 42°C for 1 hour. The synthesized cDNA was used to perform real-time quantitative PCR (qPCR) with the iQ SYBR Green supermix (Bio-Rad) on the iCycler real-time PCR detection system (Bio-Rad). The human primers sequences were the following:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: 5’- ATGACATCAAGAAGGTGGTG -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- CATACCAGGAAATGAGCTTG -3’</td>
</tr>
<tr>
<td>KLF2</td>
<td>Forward: 5’- AGACCTACACCAAGAGTTGCATC -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- GATGGGCGCTTGAGTCGACG -3’</td>
</tr>
<tr>
<td>AMPK</td>
<td>Forward: 5’- GAATGGAAGGCTGGATGAAA -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- TTCTGGTCAGCATAGTTGG -3’</td>
</tr>
</tbody>
</table>

Table 1. Primers for qPCR.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a catalytic enzyme in glycolysis, was used as the normalization control for qPCR assay to quantify KLF2 and AMPK gene expressions. The cycling condition was: 95°C for 15 min, followed by 40 cycles of PCR (30 sec at 95°C, 25 sec at 56°C, and 25 sec at 72°C), and then kept at 4°C.
II.C.4 Protein Isolation and Immunoblotting

At the end of the flow experiment, HUVECs were washed with ice-cold PBS twice and lysed with RIPA buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS in 1X PBS) containing a protease inhibitor cocktail [100 µg/mL phenylmethanesulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), 2 mM sodium fluoride (NaF), and 150 KIU/mL aprotinin]. After 30-min incubation on ice and 15-min centrifugation at 13,000 x g at 4°C to remove cell debris, cell lysates were collected as the supernatant. Protein concentrations were determined by Bradford assay on a spectrophotometer Smartspec 3000 (Bio-Rad, Hercules, CA). Equal amounts (~ 20 µg) of denatured proteins were loaded and separated on a 10% SDS polyacrylamide gel by electrophoresis according to standard protocols. Proteins were then transferred from the gel to a nitrocellulose membrane (Bio-Rad) at 4°C. After 1-hour blocking with 5% bovine serum albumin (BSA) in 1X TBSt, the membrane was probed with various primary antibodies and the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP). The chemiluminescent signals from the HRP-labeled protein were revealed by using an Amersham ECL kit or an ECL Plus kit (GE Healthcare, Piscataway, NJ), and densities of the protein bands were quantified by using the ImageJ software (National Institutes of Health).

II.C.5 Antibodies and Reagents

Antibodies used in this study were purchased from the following commercial sources: anti-phospho-AMPKα Thr-172, anti-AMPKα, anti-phospho-ACC Ser-79, and anti-ACC (Cell Signaling Technology, Beverly, MA); anti-beta actin and anti-alpha tubulin (Sigma, St. Louis, MO). Anti-KLF2 was kindly provided by Dr. Guillermo
Garcia-Cardena at Harvard Medical School. Compound C was from Calbiochem (La Jolla, CA). The siRNA targeting AMPK-α1 catalytic-subunit (Hs_PRKAA1_5_HP Validated siRNA) was purchased from QIAGEN (Valencia, CA). A negative control siRNA (Silencer Negative Control #1) was purchased from Ambion (Austin, TX). An adenovirus that expresses constitutively active AMPK (Ad-AMPK-CA) was kindly provided by Dr. John Shyy at University of California, Riverside.

II.C.6 siRNA Transfection and Adenoviral Infection

HUVECs were reverse transfected with siRNA using the siPORT NeoFX transfection reagent according to the manufacturer’s protocol (Ambion, Austin, TX). Cells were incubated with the siRNA-transfection reagent complex in 2 mL of culture media on a glass slide (for flow experiment) for 12 hours. Fresh culture media was then added, and replaced again at 24 hours. 24-48 hours post-transfection, cells were prepared to be used in pulsatile flow experiment.

For adenoviral infection, HUVECs were seeded on culture dish at 50% confluency. 24-hours later, the cells became roughly 80% confluent and appropriate amount of adenovirus was added. Cells were then collected for RNA or protein analyses after 24-hr incubation of virus.

II.C.7 Statistical Analysis

Data for Figure 4, 5 and 6 were analyzed by one-way ANOVA on ranks followed by the Student-Newman-Keuls post hoc test using the SigmaStat 3.5 software (Systat Software, San Jose, CA). Data for Figure 7, 8 and 9 were analyzed by paired Student’s t-test. Results were expressed as mean ± SD from at least 3 independent experiments. *P values < 0.05 were considered to be statistically significant.
II.D Results

II.D.1 Pulsatile Laminar Flow Activates KLF2 Expression at Both Messenger RNA and Protein Levels

Since Dekker et al. [2002] first showed that in HUVECs KLF2 can be induced by prolonged high fluid shear stress using DNA microarray screening, several groups have reported that high laminar steady shear stress (19 ~ 40 dyn/cm²) induces KLF2 expression in HUVECs at the messenger RNA level [Lingrel et al., 2004; Garcia-Cardena et al., 2006]. To test if PS flow (12 ± 4 dyn/cm²) would also regulate the expression of KLF2 in ECs, we sheared HUVECs for various time periods, and isolated total RNA for RT-qPCR analysis. As shown in Fig. 4A, PS flow induced KLF2 gene expression at the mRNA/transcript level as early as 1-hr, with increasing intensity of activation as a function of time (4-hr, 12-hr, and 24-hr).

Since there is no commercially available functional antibody for human KLF2, there had not been any in vitro data showing that laminar shear stress induces KLF2 at the protein level via Western Blotting or immunohistochemistry. With the anti-KLF2 kindly provided by Dr. Garcia-Cardena at Harvard Medical School, we were able to show that PS flow also induced KLF2 expression at the protein/translation level (Fig. 4B). From the quantified data (Fig. 4B bar graph), it was observed that, later than the marked early induction of KLF2 mRNA (2 fold increase) started at 1-hr PS flow (Fig4A), KLF2 protein induction started at 4-hr PS flow (1.8 fold increase). The KLF2 protein induction was highest after 24-hr flow. My results not only validated that PS flow using our flow system leads to a sustained induction of KLF2 expression
at mRNA level, but also demonstrated the corresponding KLF2 protein expression, which has not been shown in the literature.
A. KLF2 Expression Under PS Flow

(* In comparison to “0 hr static control”)

B. PS flow

KLF2

α-tubulin

(* In comparison to “0 hr static control”)

Figure 4. (A) KLF2 is induced by PS flow at mRNA level. Real-time qPCR analysis showed that PS flow (12 ± 4 dyn/cm²) induced KLF2 expression at mRNA level in HUVECs, with increasing intensity as a function of time. (B) PS flow activates KLF2 at protein level. Through Western Blotting, quantified expression levels of KLF2 normalized to α-tubulin showed that KLF2 induction was highest after 24-hr flow.
II.D.2 Pulsatile Laminar Flow Caused AMPK Thr172 phosphorylation in HUVECs

To investigate whether AMPK is the upstream signaling molecule regulating KLF2-induction by PS flow, we first need to know if AMPK can be activated by PS flow. Although Shyy’s group has previously shown that a laminar steady flow activates AMPK at Thr172 in cultured bovine aortic endothelial cells (BAECs) (Guo et al., 2007), the effect of flow with pulsatility (PS flow, which is physiological relevant) on the KLF2 induction in human endothelial cells (HUVECs) has not been examined. As shown in Fig. 5A (gel image), PS flow with a shear stress at 12 ± 4 dyn/cm² and a frequency of pulsatility at 1 Hz increased the phosphorylations of AMPK Thr172 and its target protein ACC Ser79 as early as 5 min. The increased phosphorylations of AMPK and ACC reached their plateaux at 1 hr and sustained for at least 4 hr (Fig. 5A bar graph). It is interesting to note that this sustained pattern of phosphorylation was different from the transient activation seen in BAECs exposed to a steady laminar flow (Fig. 5B) (Zhang, 2006; Guo 2007). The temporal pattern of AMPK activation under PS flow is consistent with the sustained induction of KLF2 expression shown in Figs. 4A and 4B. These results provide evidence in support of the hypothesis that AMPK is the upstream molecule that transduces mechanical stimuli to regulate KLF2 expression.
Figure 5. (A) PS flow of shear stress magnitude $12 \pm 4$ dyn/cm$^2$ and oscillation frequency at 1 Hz activates AMPK in HUVECs. Top left panel is the schematic representation of the PS flow pattern. Bottom left are the western blots showing the activations of phospho-AMPK (T172) and phospho-ACC (S79). Results of quantification of these activations, as shown in the two bar graphs on the right, indicate that they are sustained during the 4 hours of PS flow.
Figure 5. (B) A steady laminar flow at 12 dyn/cm² causes transient activation of AMPK in BAECs. Top panel is the schematic representation of the flow pattern. Bottom panel show the phosphor-activations of AMPK (Thr172) and its downstream target ACC (Ser79). The activations by steady laminar flow were transient and fell back to basal levels after 2-4 hrs of flow, in contrast to the sustained activations after pulsatile flow, as shown in Fig. 5A (From Guo et al., Circ. Res., 100(4), 2007).
II.D.3 Constitutive Activation of AMPK Induces KLF2 Expression

To test the role of AMPK in modulating KLF2 expression, subconfluent HUVECs were infected with Ad-AMPK-CA (adenoviral vector encoding the constitutively activated form of AMPK) with various MOI levels (40-100 MOI) for 4 hours, followed by incubation in fresh culture media for another 24 hours, and then subjected to RNA isolation. KLF2 expression was assayed by real-time qPCR. Constitutively active Ad-AMPK at all MOI levels tested increased KLF2 mRNA expression by 3-4 fold compared to the control virus (Ad-null)-infected cells (Fig. 6). The level of KLF2 expression was independent from the concentration of Ad-AMPK-CA used within the range, indicating that the minimum (40 MOI) was sufficient to induce KLF2 expression. Unpublished data from Dr. John Shyy’s laboratory at UC Riverside [personal communication] showed that treating HUVECs with an AMPK agonist, AICAR, also led to activation of KLF2. These results provide evidence that AMPK is the upstream regulator of KLF2 expression.
Figure 6. Constitutive activation of AMPK induces KLF2 expression. An adenoviral vector over-expressing the constitutively active form of AMPK (Ad-AMPK-CA) was used to infect HUVECs. The effect of Ad-AMPK-CA infection on KLF2 regulation was examined by qPCR analysis. At the three viral concentrations studied (40, 60 and 100 MOI), KLF2 was activated 3~4 fold.
II.D.4 Pharmacological Inhibitor of AMPK Blocks KLF2 Induction by PS Flow

The data from II.D.1, II.D.2 and II.D.3 demonstrated that both AMPK and KLF2 could be activated by PS flow, and that AMPK over-expression led to the activation of KLF2. These studies provide a strong foundation for the hypothesis that PS flow regulates KLF2 expression via AMPK activation. To further test this hypothesis, we used a pharmacological inhibitor of AMPK kinase, Compound C (CC), to examine whether PS-induction of KLF2 could be attenuated if AMPK kinase activity was inhibited.

Confluent HUVECs were pre-treated with either MeOH (solvent control) or CC for 30 minutes before being kept static or subjected to 1-hr PS flow. At the end of 1-hr of PS flow, total proteins were collected for Western Blot analysis of phospho-ACC and total ACC, and RNA was collected for qPCR analysis of KLF2 mRNA expression.

To ensure that CC properly inhibited AMPK kinase activity, we first examined the phosphorylation of the AMPK downstream target ACC (Fig. 7 gel images). The result showed that the strong activation of phospho-ACC by PS flow was significantly attenuated in the presence of CC in a concentration-dependent manner, thus confirming the inhibition of AMPK by CC (under both static and PS flow conditions). Next we investigated the effect of AMPK inhibition on KLF2 induction by PS flow. As shown in Fig. 7 (bar graphs), 1-hr PS flow caused a strong induction of KLF2 in MeOH control cells. In contrast, the KLF2 induction was significantly inhibited in cells treated with CC in a concentration-dependent manner. High concentrations of CC (20 µM and 15 µM) suppressed KLF2 basal expression under static condition, as well as under PS. At 10 µM, CC did not change the basal expression, and therefore the
significant reduction of the KLF2-induction by PS is particularly trustworthy. The result of this study proves our hypothesis that AMPK activation is necessary in the PS-flow induction of KLF2.
Figure 7. Compound C suppresses KLF2 induction by PS flow. Cultured HUVECs were pretreated with various concentrations of CC (20, 15 and 10 µM) for 30 min before being subjected to 1-hr PS flow, with CC in the circulating media during the flow. As shown above, 20 and 15 µM of CC significantly suppressed p-ACC activity and KLF2 expression below basal levels in static condition, while completely abolishing the flow-activation of p-ACC and induction of KLF2. With 10 µM of CC, basal levels of p-ACC and KLF2 were not affected in static conditions, but the activations of p-ACC and induction of KLF2 were reduced under PS flow.

* P < 0.05 (in comparison to MeOH Static).  # P < 0.05 (in comparison to MeOH PS).  § P < 0.05 (in comparison to CC Static).
II.D.5 Specific Silencing Agent of AMPK (small interference RNA) Blocks KLF2 Activation by PS Flow

In II.D.4, we showed that CC, a pharmacological inhibitor of AMPK kinase successfully blocked KLF2-induction by PS flow. To ensure that this result was not due to the possible off-target effects of chemical compounds, I also performed experiments to knock down AMPK expression using a small interference RNA that specifically targeted the alpha 1 catalytic subunit of AMPK (siAMPK).

Reverse transfecting HUVECs with 200 pmoles of siAMPK led to ~ 85% knockdown of AMPK gene expression at mRNA level, as shown by qPCR analysis, in comparison to cells transfected with corresponding amount of negative control siRNA (siNeg). After establishing the knockdown efficiency of the siRNA, transfected cells were either kept in static condition or subjected to 1-hr PS flow. Total proteins were collected for immunoblotting analysis while RNA was isolated for qPCR analysis.

As shown in Fig. 8A, siAMPK transfection knocked down the amount of AMPK at protein level by one half, and reduced the phospho-AMPK activity under PS flow. The phospho-ACC was also attenuated due to the reduction of phospho-AMPK. Having ascertained the knockdown of AMPK total protein and its phosphorylation, I proceeded to examine their effects on KLF2 expression. Using qPCR analysis, Fig. 8B demonstrated that PS-induction of KLF2 was significantly attenuated by siAMPK transfection. These results along with the data from II.D.4 provide solid evidence that PS flow induces KLF2 expression via AMPK pathway.
Figure 8. (A) siAMPK leads to reduced phosphor-AMPK and phosphor-ACC activities under PS flow. siAMPK was used to knockdown AMPK total protein and its phosphorylation (gel-images). Quantified data showed that siAMPK successfully reduced total AMPK level (top right panel). siAMPK suppressed phosphor-AMPK to below basal level in static condition and completely blocked its activation under flow (bottom left panel). Since the downstream pathway of AMPK was partially blocked, the activation of phospho-ACC/total ACC was attenuated (bottom right panel).

* P < 0.05 (in comparison to siNeg Static).  # P < 0.05 (in comparison to siNeg PS).  § P < 0.05 (in comparison to siAMPK Static).
Figure 8. (B) siAMPK reduces KLF2 induction by PS flow. With the AMPK pathway partially blocked, the induction of KLF2 by PS flow was attenuated.

* P < 0.05 (in comparison to siNeg Static).  # P < 0.05 (in comparison to siNeg PS).  § P < 0.05 (in comparison to siAMPK Static).
II.D.6 AMPK Regulates KLF2 Expression via the ERK5/MEF2 Pathway

My results from the previous sections have identified AMPK as the upstream signaling molecule of KLF2 under PS flow. However KLF2 regulation by shear stress may be complex events involving multiple signaling pathways. To further understand the mechanisms by which KLF2 is regulated, I performed work in collaboration with Drs. Shyy at UC Riverside and Garcia-Cardena at Harvard Medical School aiming at elucidating the relationship between AMPK and the previously known KLF2 regulatory network, in particular the ERK5/MEF2 pathway.

To test AMPK-ERK5 interactions, we used the AMPK agonist AICAR and adenoviral vectors encoding dominant negative mutants of ERK5 and MEF2 (Ad-MEK5-DN; Ad-MEF2-DN) to examine the role of AMPK in the ERK5/MEF2/KLF2 pathway. AICAR was able to activate ERK5 and MEF2 phosphorylations, which were reduced by AMPK inhibitor CC or AMPK siRNA (Fig. 9A). Blocking ERK5 and MEF2 strongly suppressed AICAR-induced KLF2 expression (Fig. 9A). Most importantly, the in vivo experiments using AMPKα2 knockout (AMPKα2-/-) mice also showed reduced phosphor-activities of ERK5 and MEF2, and a reduced expression of KLF2 in the aortas of AMPKα2-/- mice (Fig. 9B). These data demonstrated that AMPK is an upstream signaling molecule of the ERK5/MEF2/KLF2 pathway in the vascular endothelium.
A. Young, Wu et al., 2008

Figure 9. (A) AICAR-mediated AMPK activation induces KLF2 expression via ERK5 and MEF2 in HUVECs. On the upper left panel, HUVECs were pretreated with 1 µM Compound C for 30 min. In the upper right panel, HUVECs were transfected with Ctrl siRNA or AMPK siRNA (50 nM) for 48 hr. The cells were then treated with 1 mM AICAR for 1 hr before being lysed. Cell lysates were analyzed by Western blotting with anti-p-AMPK(Thr172), anti-p-ERK5(Thr218) and p-MEF2(Thr312), and anti-α-tubulin. In the lower panels, cells were infected with Ad-GFP, Ad-MEK5-DN, or Ad-MEF2-DN for 24 hr. All cells were then treated with 1 mM AICAR for 1 hr before RNA isolation and qPCR analysis. The levels of KLF2 were normalized against GAPDH.

* indicates p<0.05 between the two compared groups.
Figure 9. (B) Phospho-activities of AMPK, ERK5, MEF2 and protein levels of KLF2 and eNOS are reduced in the aortas of AMPKa2 knockout mice. Phosphorylations of ERK5 and MEF2 and expression of KLF2 are higher in the aorta of the wild-type (Ctrl) mouse in comparison to the AMPKa2-/- . Two aortic extracts from the same line were pooled and the levels of phospho-AMPK, phospho-ERK5, phospho-MEF2, KLF2, eNOS, and α-tubulin were analyzed by immunoblotting.

* indicates p<0.05 between the wild-type and AMPKa2-/- samples.
II.E. Discussion

In order to elucidate the role of KLF2 in shear-stress-mediated vascular homeostasis and vascular tone regulation, I have investigated the mechanotransduction mechanism by which pulsatile laminar flow induces KLF2 expression in vascular ECs. The major finding of this study is the identification of a novel signaling molecule, AMPK, as the upstream regulator of KLF2-induction by PS flow. My experiments have demonstrated that PS flow induces KLF2 expression at the mRNA level by qPCR analysis and at the protein level by Western blotting for the first time, and also the time courses for both mRNA and protein expressions. KLF2 expression at the mRNA level was induced after 1-hr exposure to PS flow, while protein activation was slightly delayed due to the longer time required for protein translation. These results clearly revealed that 1-Hz PS flow significantly induces KLF2 expression.

To show that AMPK is involved in shear-mediated KLF2 induction pathway, I have demonstrated that AMPK was also activated by PS flow. In contrast to what Zhang et al. previously showed that AMPK was transiently activated by steady laminar shear stress [2006], our results showed that PS flow induced sustained AMPK activation in HUVECs. The difference in the activation pattern between Zhang et al.’s studies and mine could result from either the absence/presence of 1-Hz pulsatility, or the differences in cell sources (BAECs versus HUVECs). There are studies showing that in endothelial cells AMPK is activated by two kinases, Peutz-Jeghers syndrome kinase LKB1 and Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (CaMKK) [Hawley et al., 2003; Hurley et al., 2005]. Zhang et al.’s work [2006] identified LKB1
as the upstream kinase causing AMPK Thr172 phosphorylation under steady shear, and I postulate that PS flow may share the same pathway to activate AMPK.

To determine the necessity of AMPK activation for KLF2 induction under PS flow, we tested if AMPK activation alone is sufficient for inducing KLF2 expression. Constitutive activation of AMPK (Ad-AMPK-CA), as well as the AMPK agonist AICAR (data not shown), significantly elicited KLF2 expression. These experiments showed that AMPK activation alone is sufficient for increasing KLF2 expression. We then tested the role of AMPK in PS-induced KLF2 expression. The use of compound C (CC, AMPK kinase inhibitor) and siAMPK (a siRNA specifically targeting AMPK α1-subunit) inhibited the AMPK phosphorylation and significantly attenuated the KLF2 induction by PS flow. These results identified the critical role of AMPK in regulating KLF2 expression under PS flow.

In addition to identifying AMPK as the upstream regulator of PS flow-induced KLF2 expression, my collaborative studies with UC Riverside and Harvard Medical School focused on elucidating the relationship between AMPK and the previously known KLF2 regulatory networks, particularly the ERK5-dependent pathway. Regarding interactions between PI3K and ERK5, although Ramos-Nino et al. showed that hepatic growth factor (HGF) mediates cell proliferation via a PI3K/ERK5/Fra-1 pathway in malignant mesotheliomas hepatocytes [2007], there is no evidence for cross-talk between PI3K and ERK (ERK1/2 and ERK5) pathways under insulin-like growth factor-1 (IGF-1) treatment in ECs [Liu et al., 2001]. Several studies have shown that AMPK-dependent eNOS activation occurs independently of PI3K [Thors et al., 2004; Zhang et al., 2006]. In the study using RAW 264.7 cells, PI3K inhibitor
(Wortmannin) abolished the nicotine-induced AMPK phosphorylation [Cheng et al., 2007], indicating a potential interaction between PI3K and AMPK. However, the interaction between PI3K/Akt pathway and AMPK pathway under PS remains to be determined. From studies using an AMPK-specific activator AICAR and adenoviral vectors encoding dominant negative mutants of ERK5 and MEF2, and studies using AMPKα2/- mice, our results demonstrate that AMPK is an upstream signaling molecule of the ERK5/MEF2/KLF2 pathway in regulating vascular functions both in vitro and in vivo, and strongly support the notion that shear stress-augmentation of AMPK, ERK5, MEF2, and KLF2 plays an important role in physiological regulation of vascular homeostasis.

The results reported here contribute significantly to providing a comprehensive mechanistic explanation of KLF2 regulation by PS flow. PS flow, which mimics the flow pattern along the athero-protective regions of the artery, helps maintain vascular homeostasis through augmentation of AMPK, ERK5, MEF2, and KLF2. In the next two chapters, I further studied the role of KLF2 in the athero-protective effects of PS flow, particularly the anti-inflammatory and anti-proliferative functions.

Chapter II, in full, has been submitted for publication of the material as it may appear in Circulation Research, 2008, Angela Y, Wu W, Sun W, Li YS, Shyy J, Chien S and Garcia-Cardena G. The dissertation author and Ms Wei Wu were the primary investigators and authors of this paper. All materials except results from section II.D.6, Figures 9A and 9B were generated by the dissertation author.
Chapter III

Role of KLF2 in the Anti-inflammatory Function of Pulsatile Laminar Flow

III.A Abstract

It is well known that a normal functioning endothelium is critical for preventing atherosclerotic development. The initiation of atherosclerosis often involves a dysfunctional endothelium with the occurrence of monocyte adhesion and transmigration. A lowered activity of endothelial nitric oxide synthase (eNOS) not only causes vasoconstriction, but also, an increase in endothelial secretion of chemotactic factors, such as monocyte chemoattractant protein-1 (MCP-1), to recruit monocytes. Sustained laminar shear causes a down-regulation of MCP-1 [Shyy et al., 1993]. KLF2, which can be activated by shear stress, possesses anti-inflammatory properties [Atkins et al., 2007]. In the current study, I investigated the role of KLF2 in the anti-inflammatory functions of pulsatile laminar flow (PS). By examining the effects of KLF2 knockdown on the expressions of several inflammation-associated genes, I identified KLF2 as a key player in regulating the anti-inflammatory responses in ECs exposed to flow. My results demonstrated that KLF2 knockdown mitigated the PS-induced decrease in expression of anti-inflammatory gene eNOS, and the PS-induced increases in expressions of pro-inflammatory genes ET-1. These results suggested that PS flow keeps vascular endothelium in an athero-resistant state through the expression of KLF2, which subsequently modulates target genes expressions to exert anti-inflammatory functions.
III.B Introduction

Cardiovascular disease (CVD) has been the leading cause of death worldwide, with atherosclerosis constituting the single most important health threat [Murray et al., 2006]. The views of the pathophysiology of atherosclerosis have evolved substantively over the past century. The scientific community had focused mostly on the link between hyperlipidaemia and atherogenesis until the 1970s, when the attention began to shift to studies on vascular growth control and smooth muscle cells (SMCs) proliferation. Over the past two decades, the role of inflammation in the progression of atherosclerosis has become increasingly appreciated, and atherosclerosis is now recognized as a chronic inflammatory disease.

Vascular endothelium plays a critical role in the initiation of atherosclerosis. In healthy normal state, blood leukocytes do not have prolonged contact with endothelial cells (ECs). Upon exposure to activating stimuli such as modified lipoprotein, microbial constituents or pro-inflammatory cytokines [Libby et al., 2002], however, ECs express molecules such as vascular cell adhesion molecule-1 (VCAM-1), P-selectin, and E-selectin [Cybulsky et al., 1991; Geng et al., 1990; Bevilacqua et al., 1989] to recruit monocytes, T-lymphocytes and neutrophils [Larsen et al., 1989] to the endothelium. Chemokines such as MCP-1 are also expressed in the dysfunctioned ECs to direct the transmigration of blood monocytes into the arterial wall [Gerard et al., 2001]. Once in the arterial intima, monocytes acquire macrophage-like characteristics, expressing scavenger receptors to internalize modified lipoproteins such as cholesteryl esters, leading to the formation of foam cells and marking the early atherosclerotic
lesion [Gerrity et al., 1980; Faggiotto et al., 1984]. As atheroma progresses, foam cells replicate and secret more pro-inflammatory cytokines to amplify the local inflammatory responses, recruiting proliferative SMCs to form atherosclerotic, thrombotic plaques. Inside these plaques, SMCs lay down an abundant extracellular matrix, while the formation of microvessels can lead to intra-plaque haemorrhage. As macrophages and ECs secrete matrix metalloproteinases (MMPs), enzymes that break down subendothelial basement membrane and collagens in the plaques, the fibrous cap that protects the lipid core of the plaque becomes thinner, and eventually bursts [Richardson et al., 1989]. The ruptured plaque releases its thrombogenic core to circulating blood, and eventually leads to arterial clotting due to effects of coagulation factors [Wilcox et al., 1989]. Thus, inflammation plays a central role in the pathogenesis of atherosclerosis.

As mentioned in Chapter I, atherosclerotic lesions are “site-specific,” usually located at vessel bifurcations or curvatures where ECs experience a disturbed flow pattern with flow reattachment. In contrast, ECs along the straight part of the arteries experience a laminar flow pattern and are protected from atheroma formation. Many studies have focused on how laminar flow regulates molecular expressions and cellular functions to prevent atherosclerotic progression [see reviews in Traub et al., 1998; Brooks et al., 2004; Wasserman et al., 2004; Li et al., 2005], especially on examining how laminar shear stress down-regulates inflammatory responses. Microarray studies from several groups [Chen et al., 2001; Garcia-Cardena et al., 2001; McCormick et al., 2001; Brooks et al., 2002; Dekker et al., 2002; Peters et al., 2002] have identified a number of inflammation-related genes responsive to laminar shear
stress. Transcriptional profiling and other functional studies have shown that long-term laminar shear suppresses VCAM-1 (a leukocyte adhesive molecule), MCP-1 (a chemoattractant peptide), tissue factor (TF, a pro-coagulant), endothelin-1 (ET-1, a vasoconstrictor), plasminogen activator inhibitor-1 (PAI-1, an anti-fibrinolytic agent), and others. On the induction side, laminar shear activates endothelial nitric oxide synthase (eNOS, a vasodilator), thrombomodulin (TM, an anti-coagulant), etc. [Brooks et al., 2004]. In summary, the laminar flow along the straight parts of the arteries is able to keep their endothelium in a “non-activated” state and free from leukocyte attachment, thus protecting ECs from the first step of atherosclerosis development. However, the mechanisms by which laminar flow regulates the expressions of these genes remain unclear.

In order to elucidate the molecular basis of the anti-inflammatory function of laminar shear stress, I have focused on KLF2 and assessed its role in the regulation of vascular endothelium. KLF2, which was first identified as a shear-induced transcription factor [Dekker et al., 2002], has been shown to possess anti-inflammatory and anti-thrombotic actions in cultured ECs [see review in, Suzuki et al., 2005; Atkins et al., 2007]. Several in vivo studies from human, mouse, rat, chick, and zebrafish have shown that KLF2 was activated in regions exposed to high shear stress/high blood flow [Dekker et al., 2002; 2005; Wang et al., 2006; Groenendijk et al., 2004; Parmar et al., 2006]. Adenoviral-mediated over-expression of KLF2 led to inductions of both eNOS and TM, and gel-mobility shift and chromatin immunoprecipitation (ChIP) assays revealed that KLF2 activated eNOS and TM through direct DNA-binding on their promoter regions [Senbanerjee et al., 2004; Lin et al., 2005].
Lentiviral-mediated long-term KLF2 over-expression induced eNOS expression, while suppressing MCP-1 and PAI-1 expressions [Dekker et al., 2006]. siRNA-mediated KLF2 knockdown led to an increased attachment of monocytic HL-60 cells to the flow-preconditioned endothelial monolayer treated with IL-1β [Parmar et al., 2006]. The results from these studies suggested that KLF2 is an important anti-inflammatory molecule in modulating downstream target genes to inhibit inflammatory and thrombotic responses in the endothelium.

In the previous chapter, I identified AMP-activated protein kinase (AMPK) as the upstream signaling molecule regulating KLF2 induction upon PS stimulation. In this chapter, I first examined the anti-inflammatory responses in cultured ECs using our \textit{in vitro} PS flow system. I then focused on elucidating the role of KLF2 in the anti-inflammatory function of PS flow. To assess inflammatory actions in ECs, I studied the expressions of three inflammatory markers, eNOS, ET-1, and MCP-1, as the readouts; these marker molecules are not only strongly regulated by laminar shear stress, but are also prominent KLF2 target genes. Lastly, I examined whether KLF2’s upstream regulator AMPK contributes to modulating these inflammatory markers under flow. I hypothesize that KLF2 plays a critical role in regulating the expression levels of these inflammatory-related genes under the effect of PS flow. The aim of this study was to provide better understanding towards the anti-inflammatory function of physiological PS flow and the importance of KLF2 as an athero-protective transcription factor in mediating this effect.
III.C Material and Methods

III.C.1 Cell Culture and Pulsatile Laminar Flow Experiment

Human umbilical vein endothelial cells (HUVECs) between passage 2 to 6 were used, under the same culture condition as described in section II.C.1. The procedures for PS flow experiment were described in section II.C.2.

III.C.2 RNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction

Total RNA was isolated using the procedure described in section II.C.3. cDNA synthesis and real-time quantitative PCR (qPCR) were carried out using the same conditions described in section III.C.2. The human primers sequences were the following:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward: 5’- ATGACATCAAGAAGGTGGTG -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- CATACCAGGAATGAGCTTG -3’</td>
</tr>
<tr>
<td>KLF2</td>
<td>Forward: 5’- AGACCTACACCAAAGTTGATGC -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- CATGTGCCGTTTCACTGAGC -3’</td>
</tr>
<tr>
<td>eNOS</td>
<td>Forward: 5’- TGGTACATGAGAGTTGAGATCG -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- CCACGTGGATTCCACTGCTG -3’</td>
</tr>
<tr>
<td>ET-1</td>
<td>Forward: 5’- TCCTCTGCTGTTCTCCTGACT -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- CAGAAACTCCACCCTCTGT -3’</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Forward: 5’- GATCTCAGTGCAGGCTGCT -3’</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’- TGCTTGCTCAAAGCTGTCAT -3’</td>
</tr>
</tbody>
</table>

Table 2. Primers for qPCR.

III.C.3 Reagents

*Escherichia coli* bacterial endotoxin lipopolysaccharides (LPS) and human recombinant Tumor-Necrosis Factor-alpha (TNF-α) were purchased from Sigma (St. Louis, MO). LPS was re-suspended in cell culture media at a stock concentration of 1 mg/mL (working concentration = 10 µg/mL; 1:100 dilution), while TNF-α was re-
suspended in sterile ddH$_2$O at a stock concentration of 10 µg/mL (working concentration = 20 ng/mL; 1:500 dilution).

**III.C.4 siRNA Transfection**

HUVECs were reverse transfected with siRNA using the siPORT NeoFX transfection reagent as described previously in section II.C.6. A siRNA targeting KLF2 sequence corresponding to nucleotide 1482-1504 (AATTTGTACTGTCTG CGGCAT) of its cDNA (GenBank accession No. NM_016270) was selected to knockdown KLF2 gene expression. A negative control siRNA from Ambion (Silencer Negative Control #1) was used as control (Austin, TX). A positive control siRNA (Silencer FAM labeled GAPDH siRNA) was used for monitoring siRNA delivery efficiency. To visualize the siRNA transfection efficiency, fluorescent images of cells were taken using a Nikon Microphot-FX upright fluorescence/phase contrast/DIC microscope with a CCD camera.

**III.C.5 Statistical Analysis**

Data for Figure 10 were analyzed by one-way ANOVA on ranks followed by the Student-Newman-Keuls post hoc test using the SigmaStat 3.5 software (Systat Software, San Jose, CA). Results were expressed as mean ± SEM from 3 independent experiments. Data for Figure 11, 12, 13 and 14 were analyzed by paired Student’s t-test. Results were expressed as mean ± SD from at least 3 independent experiments. *P values < 0.05 were considered to be statistically significant.
III.D Results

III.D.1 Pulsatile Laminar Flow Exerts Anti-inflammatory Functions by Modulating Expressions of Several Inflammation-associated Genes

It has been widely recognized that high shear stress with a net forward direction possesses “anti-atherogenic” properties by keeping ECs in a “non-activated” state, expressing low levels of chemotactic molecules and increasing NO production for platelet aggregation inhibition [Traub et al., 1998]. To demonstrate that our in vitro PS flow system would exert similar anti-inflammatory functions, I examined the effects of PS flow on the expressions of several inflammation-associated genes (eNOS, ET-1, and MCP-1) at various time points. These genes were selected not only because they serve as major inflammatory response readouts in the vasculature, but also because reports in the literature have shown their modulations by KLF2 [Lin et al., 2005; Dekker et al., 2005, 2006].

HUVECs were subjected to PS flow (12 ± 4 dyn/cm²) for various time periods (1hr, 4hr, 12hr, and 24hr). Total RNA was collected at the end of each time period, and expressions of eNOS, ET-1 and MCP-1 were quantified using qPCR analysis. As shown in Fig. 10A, eNOS expression level was increased by PS flow as a function of time. The expression became significantly higher (p < 0.05) after 4-hr flow, which was later than the increase of KLF2 expression seen after 1-hr flow. The expression levels of ET-1 and MCP-1 were suppressed by flow (Fig. 10B), also in time-dependent manners. Different from the immediate suppression of ET-1 under PS flow, MCP-1 expression did not change much after 1-hr, but decreased to less than half (0.4 x) after 4-hr, with continuing suppression till 24-hr.
In summary, PS flow up-regulated the anti-inflammatory eNOS expression, while down-regulating the pro-inflammatory ET-1 and MCP-1 expressions. These results substantiate that PS flow shares similar anti-inflammatory functions with steady laminar flow. The quantified expression data of eNOS, ET-1, and MCP-1 also provide the bases for the analyses of these inflammatory response markers in later experiments.
Figure 10. HUVECS were subjected to PS flow for 1hr, 4hr, 12hr, and 24hr. Total RNA was collected; cDNA synthesis and qPCR were carried out to assess the expression of KLF2 and eNOS (A), and ET-1 and MCP-1 (B) at mRNA level. **(A) PS flow induces KLF2 and eNOS expressions.** Similar to KLF2, eNOS was activated under PS flow, but the onset of activation was later.
Figure 10. (B) PS flow suppresses ET-1 and MCP-1 expressions. Both ET-1 and MCP-1 were suppressed by PS flow as a function of time.
III.D.2 Suppression of KLF2 and Modulation of Gene Expressions toward Inflammation by Pro-inflammatory Cytokines

I have demonstrated that KLF2 is activated by PS flow, which exerts anti-inflammatory functions as evident by regulations of eNOS, ET-1, and MCP-1 expressions (section III.D.1). As a transcription factor, KLF2 was shown to possess anti-inflammatory characteristics via down-regulation of pro-inflammatory VCAM-1 and E-selectin when it was over-expressed [SenBanerjee et al., 2004]. In addition to studying the role of KLF2 in regulating PS-induced anti-inflammatory responses, I also examined the KLF2 regulation in ECs in response to inflammatory cytokines such as LPS and TNF-α.

Confluent monolayers of HUVECs were cultured in 6-well plates and serum-starved for 24 hr before cytokine treatments. 10 µg/mL of LPS was added to culture media for various time periods (1hr, 2hr, 4hr, 6hr, 8hr, and 12hr). In separate experiments, 20 ng/mL of TNF-α was added to culture media for various time periods (2hr, 4hr, 6hr, 8hr, and 24hr). Total RNA was isolated and expressions of KLF2 in response to cytokine treatments were quantified by qPCR. Gene expressions of cytokine-treated cells were normalized against their time-matched controls. LPS, a gram-negative bacterial endotoxin commonly used for eliciting inflammatory responses, significantly down-regulated KLF2 expression, while up-regulating MCP-1 expression (Fig. 11A). TNF-α, a naturally occurring cytokine involved in systemic inflammation, also suppressed KLF2 and eNOS expression along with MCP-1 activation (Fig. 11B). These results establish KLF2’s role in inflammation, being
responsive to not only anti-inflammatory PS flow but also pro-inflammatory cytokines, through modulations of anti-inflammatory and pro-inflammatory genes.
**Figure 11.** LPS (A) and TNF-α (B) elicited inflammatory action in EC treated with LPS. (A) **LPS suppresses KLF2 expression.** 10 µg/mL treatment of LPS down-regulated expression of KLF2, but up-regulated expression of MCP-1 over the 12-hr period. It is interesting to note that maximal suppression of KLF2 and peaked activation of MCP-1 both occurred at 4-hr LPS treatment.
Figure 11. (B) TNF-α suppresses KLF2 expression. 20 ng/mL treatment of TNF-α down-regulated expressions of KLF2 and eNOS, but up-regulated expression of MCP-1. Both LPS and TNF-α elicited inflammatory action in ECs.
III.D.3 Titration of KLF2 Small Interference RNA for Optimal Knockdown of KLF2 Expression in HUVECs

As demonstrated in the previous sections, KLF2 can be induced by anti-inflammatory, and suppressed by pro-inflammatory, stimuli. I then investigated the effect of knocking down KLF2 on the anti-inflammatory functions of PS flow in ECs. To knockdown KLF2, I used a small interference RNA (siKLF2) previously designed in our group [Wang et al., 2006] and examined its effect on KLF2 expression by qPCR. To determine the optimal knockdown efficiency, a titration study was carried out to test a wide range of siKLF2 concentrations. As shown in Fig. 12A, the optimal knockdown efficiency was identified at 400 pmoles/glass slide of cells (40 nM = 400 pmoles were dissolved in 10 mL), which gave ~80% KLF2 knockdown. To ascertain that there were no off-target effects, we examined expressions of several other KLFs (KLF1, KLF4, KLF5, and KLF8), and found that they were not affected by the siKLF2 transfection (Fig. 12B). In addition, I used a FAM-tagged siRNA that targeted GAPDH sequence (siGAPDH) as a positive control to visualize the delivery of siRNA into the transfected cells. As seen in Fig. 12C, the FAM signal (green fluorescent) indicates that the siGAPDH was delivered into the cytoplasm with very high efficiency. qPCR analysis also indicated that FAM-tagged siGAPDH knocked down 90% of GAPDH expression in cells. These results confirm that I achieved effective KLF2 knockdown in HUVECs by delivery of siRNA into the cell cytoplasm, where post-transcriptional gene silencing occurs.
Figure 12. (A) siKLF2 titration graph. A titration plot for siKLF2 knockdown efficiency. 400 pmole/slide was sufficient to give effective knockdown (~80%) with no noticeable cytotoxicity. (B) siKLF2 does not affect expressions of other KLFs. By qPCR analysis, expressions of several other KLFs (KLF1, KLF4, KLF5, and KLF8) which share some sequence homology with KLF2, were examined in cells transfected with siKLF2. Expression levels were normalized against those in siNeg-transfected cells. KLF2 knockdown did not alter the expressions of other KLFs.
C.

**Figure 12.** (C) siRNA transfection efficiency in HUVECs. Fluorescent image of FAM-siGAPDH transfected HUVECs was taken with an upright microscope. Propidium Iodine (PI) nuclear stain (red) indicates the total number of cell in the field and FAM signals (green) indicates the transfected cells. Almost all cells were successfully transfected in the field of view.
III.D.4 KLF2 Knockdown Diminishes the Anti-inflammatory Functions of PS Flow by Reducing the Levels of eNOS Activation and of ET-1 and MCP-1 suppression

Following my demonstration of the effective knockdown of KLF2 with siKLF2, I investigated whether KLF2 expression was crucial in regulating expression patterns of eNOS, ET-1, and MCP-1 under PS flow.

HUVECs were reverse-transfected with 400 pmoles/glass slide of siNeg (negative control siRNA) or siKLF2 for 32 hr, and then either kept in static condition or subjected to a 24-hr PS flow. Total RNA was isolated at the end of the flow experiment, and expressions of KLF2, eNOS, ET-1, and MCP-1 were quantified by qPCR analysis. siKLF2 transfection knocked down the expression of KLF2 in static condition and significantly attenuated its induction by PS flow, which was seen in siNeg-transfected cells (Fig. 13A). For eNOS, which was activated by PS flow (section III.D.1), siKLF2 transfection suppressed eNOS expression in static condition and its induction by flow (Fig. 13A). The reduction of eNOS by siKLF2 in static cells was consistent with other studies [Lin et al., 2005; Sen-Banerjee et al., 2005]. My results indicate that KLF2 expression is critical in the induction of eNOS expression under PS flow, thus KLF2 serves to maintain the vasodilative status in the athero-protective region of the vasculature.

I also studied the effects of siKLF2 on ET-1 and MCP-1 expressions under 24-hr PS flow (Fig. 13B). siKLF2 transfection did not alter ET-1 expression in static condition, but it significantly reversed the strong suppression of ET-1 (~17%) under PS flow back to its basal level (~89%) observed in static, siNeg-transfected cells.
siKLF2 led to an increase of MCP-1 expression in static condition (~143%), but had only a minimal effect in lessening the PS-induced MCP-1 suppression. Thus, siKLF2 had a substantial impact on ET-1 suppression but not so much on MCP-1 suppression under PS flow. High level of KLF2 in the endothelium exposed to high laminar shear stress spares ECs from vasoconstrictive responses to mitigate the progression of atherosclerosis.
Figure 13. Effects of transfection of HUVECs with siKLF2 or siNeg on expressions of KLF2 and eNOS (A) and ET-1 and MCP-1 (B) under static condition or subjected to 24-hr PS flow. (A) **siKLF2 attenuates KLF2 and eNOS inductions by PS flow.** KLF2 knockdown led to decreases in KLF2 (top graph) and eNOS (bottom graph) expressions in static condition. In sheared cells, KLF2 knockdown attenuated the strong inductions of both KLF2 and eNOS under PS flow.

* P < 0.05 (in comparison to siNeg Static).  # P < 0.05 (in comparison to siNeg PS).  § P < 0.05 (in comparison to siKLF2 Static).
B.

**Figure 13.** (B) siKLF2 reverses ET-1 suppression by PS flow but has marginal effect in reducing MCP-1 suppression by PS flow. The pro-inflammatory adhesion molecule ET-1 was suppressed by PS flow in siNeg-transfected cells. KLF2 knockdown did not alter ET-1 expression in static cells but activated MCP-1 expression. In sheared cells, siKLF2 significantly reversed the strong suppression of ET-1 back to basal level, and had only a marginal effect in reducing the MCP-1 suppression.

* P < 0.05 (in comparison to siNeg Static). # P < 0.05 (in comparison to siNeg PS). § P < 0.05 (in comparison to siKLF2 Static).
III.D.5 AMPK Knockdown Attenuates eNOS Expressions Induced by PS Flow

In section II.D.5, I showed that AMPK acts as the upstream signaling molecule that regulates KLF2 induction by PS flow. siAMPK transfection significantly attenuated flow-induced KLF2 expression. To test whether AMPK modulation of KLF2 may affect the inflammation-associated genes under PS flow, I examined expressions of eNOS, ET-1, and MCP-1 in cells that were transfected with siAMPK and subjected to 1-hr PS flow. As shown in Fig. 14, siAMPK reduced eNOS expression to ~70% in static cells, and abolished the moderate induction (~145%) of eNOS to below basal level (~86%). In contrast, siAMPK did not affect ET-1 expression in static cells, and was not able to reverse its suppression under PS flow, as seen when using siKLF2. siAMPK also did not affect MCP-1 expression significantly in static cells, nor its suppression under PS flow. These results indicate that AMPK activation is critical in regulating the expression of eNOS, as well as KLF2, induced by PS flow. However, the suppressions of ET-1 and MCP-1 by PS flow were not affected by AMPK knockdown. Hence, the enhanced expression of eNOS, but not the suppressions of ET-1 and MCP-1, induced by PS is mediated by the AMPK-KLF2 axis.
Figure 14. siAMPK abolishes eNOS induction but does not alter ET-1 and MCP-1 suppressions by PS flow. HUVECs transfected with either siNeg or siAMPK were kept in static condition or subjected to 1-hr PS flow. The top graph represents the expression levels of eNOS and the bottom graphs represent ET-1 and MCP-1 expression levels. AMPK knockdown not only led to reduced eNOS expression in static cells, but also abolished the moderate induction of eNOS under 1-hr PS flow. However, AMPK knockdown did not alter ET-1 and MCP-1 expressions in static cells, nor their suppressions under flow.
III.E Discussion

The findings in this chapter strengthened KLF2’s role as a key transcription factor which contributes importantly in regulating the anti-inflammatory function of PS flow. First, I demonstrated that PS flow (12 ± 4 dyn/cm$^2$) kept ECs in a “non-activated”, anti-inflammatory state by regulating the expression levels of several inflammatory markers (eNOS, ET-1, and MCP-1). This result is consistent with the microarray data using steady laminar shear [Chen et al., 2001; Garcia-Cardena et al., 2001; Brooks et al., 2002]. The 1-Hz sinusoidal component of our PS flow provided the physiologically relevant condition with the pulsatility of blood flow. The sinusoidal component of the blood flow may play an important role in regulating vascular functions. Other groups [Blackman et al., 2002; Dekker et al., 2002; Desai et al., 2002] have compared the effects between steady laminar flow and pulsatile laminar flow on gene expressions. Although overall both flow patterns exert an anti-inflammatory trend, the pattern of modulation of gene expression levels differs depending on the shear magnitude and cell type used. Himburg et al. showed that the anti-inflammatory regulation under 1-Hz laminar shear stress (15 dyn/cm$^2$) was reversed at higher frequencies (2 or 3 Hz) [2007]. My results suggest that laminar shear stress in the physiological range (10~20 dyn/cm$^2$) with a pulsatile frequency corresponding to normal heart rate (1 Hz) offers athero-protective functions in the vascular endothelium by suppressing the inflammatory responses.

In addition to demonstrating KLF2 induction by PS flow, I examined the regulation of KLF2 under pro-inflammatory stimuli. Consistent with other studies using IL-1β and TNF-α [Senbanerjee et al., 2004; Lin et al., 2005], my results showed
that both the bacterial endotoxin LPS and the cytokine TNF-α significantly down-regulated KLF2 expression. These results confirmed the essential role of KLF2 in inflammatory responses. Furthermore, there is considerable evidence that KLF2 is also an important regulator of thrombogenic responses. Thus, Lin et al. demonstrated that over-expression of KLF2 significantly prolonged the blood clotting time in both basal condition and under TNF-α stimulation, while siRNA-mediated KLF2 knockdown greatly shortened the clotting time [2005]. They also showed that KLF2 knockdown led to an increased induction of the pro-coagulant TF and increased suppressions of eNOS and TM upon TNF-α stimulation. Here, I focused on examining the role KLF2 plays in maintaining ECs in an anti-inflammatory state under athero-protective PS flow. My results showed that KLF2 expression is critical for eNOS induction and ET-1 suppression, without significantly altering MCP-1 suppression under PS flow. Although MCP-1 suppression was not affected much with KLF2 knockdown, the PS anti-inflammatory trend, as evident by the eNOS induction and ET-1 suppression, was significantly impaired. Both eNOS and MCP-1 are important target genes of two pro-inflammatory transcription factors, nuclear factor-kappa-B (NF-κB) and activator protein-1 (AP-1) [Kilgore et al., 1997; Faller et al., 1999; Hamuro et al., 2002]. Both NF-κB and AP-1 are transiently responsive to laminar shear stress [Lan et al., 1994], and SenBanerjee et al. showed that KLF2 binds and activates eNOS promoter through competing with NF-κB for coactivator cyclic AMP response element-binding protein (CBP/p300) [2004]. Thus KLF2 may counteract against NF-κB and AP-1 to modulate gene expressions to exert the anti-inflammatory functions under PS flow. Comparing the modulations of eNOS, ET-1 and MCP-1 by KLF2 knockdown under flow, MCP-1
expression may be mainly controlled by other factors since the impact of siKLF2 on MCP-1 was less effective than other genes, such as eNOS and ET-1. My findings are consistent with the microarray results by Dekker et al. and Parmar et al. [2005; 2006], who showed that KLF2 expression plays a key role in regulating many other genes related to vascular tone and inflammation, including argininosuccinate synthetase (ASS), TM, Angiopoietin receptor Tie2, C-type natriuretic peptide (CNP), adrenomedullin (ADM), IL8, angiopoietin 2 (Ang-2) and others. In particular, Parmar et al. pointed out that 15.3% of all genes regulated by laminar flow were KLF2-dependent, further strengthening KLF2’s role as one of the most critical transcription factors regulating flow-modulated EC functions.

To further elucidate the role of KLF2’s upstream regulator AMPK in the anti-inflammatory action of PS flow, I demonstrated that AMPK knockdown had a significant impact on eNOS expression in both static and flow conditions. AMPK knockdown substantially reduced eNOS expression in static cells and abolished eNOS induction in sheared cells. Previously work by Zhang et al. has shown that AMPK is a potent inducer of eNOS activity and that its activation was essential in PS flow-mediated increase in eNOS phosphorylation [2006]. Here I showed that eNOS regulation is controlled by AMPK-mediated KLF2 induction. AMPK knockdown, however, had little effect on the down-regulations of ET-1 and MCP-1 by PS flow, indicating that ET-1 and MCP-1 may not be responsive to the reduced KLF2 induction by siAMPK. Our systematic approaches show that KLF2 indeed plays a key role in regulating the anti-inflammatory function of PS flow. Through activating eNOS and suppressing ET-1 expression, KLF2 mediates vaso-dilation, inhibition of leukocyte
chemotaxis and platelet adhesion [Sun et al., 2004; Napoli et al., 2006]. As a chronic inflammatory disease, atherosclerosis progression may be prevented by controlling inflammation. Better understandings of the roles played by KLF2 as an anti-inflammatory mediator may offer potential therapeutic applications in atherosclerosis prevention and treatment.

Part of Chapter III has been submitted for publication of the material as it may appear in Circulation Research, 2008, Angela Y, Wu W, Sun W, Li YS, Shyy J, Chien S and Garcia-Cardena G. The dissertation author and Ms Wei Wu were the primary investigators and authors of this paper. All materials in this chapter were generated by the dissertation author.
Chapter IV

Role of KLF2 in the Anti-Proliferation Function of Pulsatile Laminar Flow

IV.A Abstract

The initiation of atherosclerosis starts from an inflamed endothelium to attract leukocytes/monocytes which leads to damages of ECs and increases of EC turnover. It has been shown that high laminar shear stress inhibits endothelial proliferation, prevents EC monolayer leakage and reduces monocytes accumulation. Recent studies also have identified that KLF2 plays an important role in reducing EC proliferation in the vascular system [Bhattacharya et al., 2005]. These studies prompted me to investigate the role of KLF2 in the anti-proliferative function of pulsatile laminar flow (PS). Using immunofluorescent imaging and flow cytometric analyses of BrdU incorporation, I examined the effects of KLF2 knockdown on cell proliferation rate in both static and flow conditions. My results demonstrate that KLF2 expression was crucial for PS flow-mediated endothelial quiescence; KLF2-knockdown significantly increases EC proliferation. In addition, I also examined the role of AMPK, the upstream signaling molecule for KLF2, in EC proliferation; AMPK-knockdown also reverses the cell quiescence under PS flow. These results strongly support the notion that the PS flow-regulation of EC proliferation occurs, at least in part, via the AMPK/KLF2 pathway.
IV.B Introduction

Situated at the blood-tissue interface in the vascular system, ECs play an important role in maintaining tissue integrity and preventing atherosclerosis development. An inflamed endothelium can attract leukocyte rolling and monocyte adhesion. As leukocyte infiltration progresses, damages of the endothelium caused by the immune system or other factors may result in EC apoptosis [Choy et al., 2001]. EC apoptosis subsequently leads to high cell turnover along with increased EC proliferation. In vivo, EC damage often leads to regeneration of a new endothelium, which is generated from the adjacent proliferating and migrating cells. Primary cultures of ECs from regenerated endothelium are more permeable to low-density lipoprotein (LDL) and more active in the internalization of oxidized LDL (oxLDL) than normal cells [Fournet-Bourguignon et al., 2000]. The resulting abnormal, leaky cell junctions can significantly alter vascular permeability [Weinbaum et al., 1985]. Several studies have shown that high EC turnover correlates with high permeability [Somer et al., 1971, 1972; Caplan et al., 1973]. Areas of high rate of cell proliferation with a high $[^3]H$thymidine index were shown to have an enhanced uptake of albumin labeled with the Evans blue dye. These focal areas are usually found at athero-prone locales such as branch points [Bell et al., 1974; Stemerman et al., 1986]. Studies on rat thoracic aorta also showed that LDL-permeable sites are often at vascular branches, but not along the straight part of the aorta [Herrmann et al., 1994]. These studies suggested a strong correlation between the distribution of EC proliferation and high permeability with blood flow patterns. Indeed, Levesque et al. first showed that laminar shear stress led to a dose-related reduction of cell proliferation rate [1990].
et al. [2005] showed that EC proliferation was significantly inhibited by PS flow, but enhanced by reciprocal low shear. Several other studies have served to elucidate the mechanism. Laminar shear stress activates the tumor suppressor gene p53 which subsequently activates p21\textsuperscript{cip1}, a cyclin-dependent kinases (CDKs) inhibitor [Akimoto et al., 2000; Lin et al., 2000]. The reduced CDKs activity then causes retinoblastoma (Rb) hypophosphorylation, which arrests ECs in G0/G1 phase. These findings support the hypothesis that in athero-resistant regions where flow pattern is laminar, ECs are quiescent and maintain a more intact, non-leaky phenotype. In contrast, in athero-prone regions where shear stress is low and flow recirculation occurs, ECs are “activated” and become more permeable for monocyte transmigration.

Similar to the anti-proliferative characteristics of athero-protective laminar flow, the transcription factor KLF2 has also been shown to regulate cell proliferation in several cell types. In T-lymphocytes, KLF2 expression is both necessary and sufficient to program cell quiescence characterized by decreased proliferation, reduced cell size and protein synthesis [Kuo et al., 1997]. Buckley et al. showed that KLF2-deficient T-cells exhibited increased proliferation, and that KLF2 exerted anti-proliferative effects via suppression of the proto-oncogene c-Myc [2001]. In addition, in ovarian cancer cell lines, KLF2 reintroduction decreased cell growth by negatively regulating the transcription of a G2/M phase-transitioning tyrosine kinase, WEE1 [Wang et al., 2005]. Most importantly, Bhattacharya et al. showed that in vascular ECs, adenoviral-mediated KLF2 over-expression inhibited VEGF-A induced angiogenesis \textit{in vivo} and retarded cell proliferation \textit{in vitro} [2005]. KLF2 over-expression also abrogated VEGF-A-mediated inductions of VCAM-1 and TF. In
summary, these studies strongly suggest that KLF2 possesses a strong inhibitory effect on cell growth and proliferation, and keeps cells in a “non-activated” state.

Studies carried out in the previous two chapters have shown that the AMPK-KLF2 axis plays a key role in regulating the anti-inflammatory function of PS flow. The aim of the current chapter is to elucidate KLF2’s role in the anti-proliferative function of laminar shear stress. I first demonstrated that our in vitro PS flow system leads to cell quiescence in cultured HUVECs. By using small-interference RNA, the effects of KLF2 and AMPK knockdown on cell proliferation were then examined under long-term PS flow. Immunofluorescent imaging for BrdU-Labeling Index was used to quantify cell proliferation, and flow cytometry was employed to analyze cell cycle progression. My hypothesis is that KLF2 expression is essential for cell quiescence under athero-protective PS flow. The goal was to contribute to the understanding of the role KLF2 serves as a crucial player in atherosclerosis prevention, by decreasing cell turnover rate in the endothelium, maintaining vascular integrity, and reducing macromolecular permeability.
IV.C Materials and Methods

IV.C.1 Cell Culture and Pulsatile Laminar Flow Experiment

Human umbilical vein endothelial cells (HUVECs) between passages 2 to 6 were used for all experiments under the same culture condition as described in section II.C.1. The procedures for PS flow experiment were described in section II.C.2.

IV.C.2 RNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction

Total RNA was isolated using the procedure described in section II.C.3. cDNA synthesis and real-time quantitative PCR (qPCR) were carried out using the same conditions as in section III.C.2. The human primers sequences are shown in Table 4.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| GAPDH | Forward: 5’- ATGACATCAAGAAGGTGGTG -3’  
Reverse: 5’- CATACCAGAAATGAGCTTG -3’ |
| KLF2 | Forward: 5’- AGACCTACACCAAGAGTTGCACTG -3’  
Reverse: 5’- CATGTGCCGTGTCCATGTCAGC -3’ |
| AMPK | Forward: 5’- GAATGAAGGCTGGATGAAA -3’  
Reverse: 5’- TTCTGGGAGCAGCATAGTTGG -3’ |

Table 3. Primers for qPCR.

IV.C.3 Cell Proliferation Assay

Bromodeoxyuridine (BrdU) incorporation assay was used to assess cell proliferation. BrdU, an analog of the DNA precursor thymidine, is incorporated into newly synthesized DNA by cells entering and progressing through the S-phase (DNA synthesis) of cell cycle. Thus, BrdU incorporation assay serves as an analytical method to differentiate cells undergoing active DNA synthesis during proliferation from non-proliferating cells. BrdU (BD Pharmingen, San Diego, CA) was added to the
ECs 2 hr prior to the end of a 24-hr PS flow experiment and the parallel static control to achieve a final concentration of 10 µM BrdU in the media. For immunofluorescent imaging analysis, at the end of the 2-hr BrdU incubation period, 4% paraformaldehyde/PBS was used for fixation of cells, followed by permeabilization using 0.5% Triton X-100, treatment of 0.5 mg/mL NaBH₄, 2N HCl at 37°C, and 0.1M sodium tetraborate. After incubation of anti-BrdU conjugated with Alexa Fluor 488 (FITC-conjugated anti-BrdU antibody, BD Pharmingen) and Propidium Iodine (PI from Sigma), fluorescent images of cells were taken using a Nikon Microphot-FX upright fluorescence/phase contrast/DIC microscope with a CCD camera. Cell proliferation rate was calculated as the BrdU Labeling Index (BLI), which was the ratio between BrdU-stained cells (green fluorescent signals) and PI-stained cells (red fluorescent signals). BrdU-stained cells represent cells undergoing cell division, while PI staining shows the total number of cells.

In addition to the immunofluorescent imaging analysis, a two-color flow cytometric analysis method was adopted to produce detailed quantitative data. With the BrdU flow kit purchased from BD Pharmingen (San Diego, CA), ECs undergoing 2-hr BrdU incorporation were fixed and processed according to the manufacturer’s protocol. After DNAse treatment at 37°C, cells were stained with anti-BrdU conjugated with Alexa 488 and 7-amino-actinomycin D (7-AAD), a DNA content staining agent. Cell samples were then analyzed on a Becton Dickinson FACScan flow cytometer with the use of BD CellQuest software. Distinctive cell populations were identified to be in different phases of the cell cycle, including G0/G1 phase, G2 +
M phase, and S phase. Quantified cell numbers in each population were generated using a CellQuest analysis tool.

**IV.C.4 siRNA Transfection and Adenoviral Infection**

HUVECs were reverse transfected with siNeg, siKLF2, or siAMPK using Ambion siPORT NeoFX transfection reagent using the procedure as described in section III.C.4. 32 hr post transfection, cells were either kept in static or subjected to 24-hr PS flow. After 2-hr BrdU incorporation (22nd~24th hr), cells were collected for proliferation assay analysis as described in section IV.C.3. For adenoviral infection experiment, an adenovirus over-expressing mouse KLF2 (AdKLF2) was kindly provided by Dr. Mukesh Jain at Case Western University. For the control, a GFP-expressing adenovirus (AdGFP) was used. Appropriate amounts of viral supernatant were used to infect sub-confluent HUVECs, and cell proliferation assay was performed one day after infection.

**IV.C.5 Statistical Analysis**

Data for all figures were analyzed by paired Student’s t-test. Results were expressed as mean ± SD from at least 3 independent experiments. *P values < 0.05 were considered to be statistically significant.
IV.D Results

IV.D.1 Long-term Pulsatile Laminar Flow Keeps Vascular Endothelium in a Quiescent State

Since Levesque et al. [1990] first demonstrated that laminar steady shear stress and pulsatile shear stress in the physiological range (5-13 dyn/cm$^2$) caused a significant reduction of cell growth rate in cultured bovine aortic endothelial cells (BAECs), several studies have shown similar observations in the same or different cell systems [Akimoto et al., 2000; Lin et al., 2000; Kadohama et al., 2007; Yao et al., 2007]. I have tested our in vitro PS flow system by subjecting HUVECs to 24-hr PS flow and examined cell proliferation rate using the BrdU incorporation assay as described in section IV.C.3. Using both immunofluorescent imaging and flow cytometric analyses, I observed that 24-hr PS flow led to a significant reduction in cell proliferation rate in comparison to that in static condition. As shown in Fig. 15A, fluorescent images showed that the number of BrdU-positive cells (green/yellow signals) was substantially reduced under 24-hr PS flow; with the BrdU Labeling Indices (BLI) of 21.5 ± 2.03 % at static condition, versus 2.45 ± 1.02 % at PS condition.

In addition to immunofluorescent imaging, I used flow cytometric method to obtain detailed quantitative results for cell cycle analysis. As shown in Fig. 14B, the upper left plot described the light scattering properties of the cell as it passes through the flow cytometry laser. The forward scatter channel (FSC) detects low angle, scattered light, and is proportional to the cell size. The side scatter channel (SSC) detects high angle, scattered light, which is proportional to cell granularity and internal
complexity. When FSC is plotted vs. SSC, different cell populations can be discriminated based on cell size and granularity. The FSC vs. SSC plot (upper left plot of Fig.15B) showed that the HUVECs used in the present experiments had a very uniform size, with little change in the granularity. The upper right plot (Fig. 15B) was 10,000-cell count (to give statistical significance) in the direction of FL1 and FL3 channels. FL1 channel (emission at 518 nm) detects FITC anti-BrdU signals, while FL3 channel (emission at 647 nm) detects 7-AAD signals. Using the BD CellQuest software, I applied “Region Gates Calculation” to identify cell populations that had been stained for different levels of incorporated BrdU and total DNA content (7-AAD). R5 included cells with low levels of BrdU incorporation and total DNA content, and were depicted as in the G0/G1 phase. R6 were cells with low level of BrdU incorporation and with higher total DNA content, indicating they were in the G2 + M with newly synthesized DNA. Lastly, R7 were cells with high level of BrdU incorporation and moderate amount of total DNA content, indicating the active DNA synthesis in S phase. The bottom left plot of Fig.15B demonstrated cell count against FL1 channel, representing the two populations of BrdU-negative cells (low BrdU signal) vs. BrdU-positive cells (high BrdU signal). The bottom right plot demonstrated cell count against FL3 channel, representing cells in the range of total DNA content. These plots provided information for quantitative cell cycle analyses.

Fig. 15B clearly illustrated the effect of 24-hr PS flow on EC cell cycle. PS flow significantly increased the number of cells in quiescent G0/G1 phase, while decreasing the number of cells in the actively proliferating S phase. The number of cells in G2 + M phase did not change significantly under flow. Comparing the cell count vs. FL1
channel plots between static and 24-hr PS flow, there was a dramatic leftward shift of cell population into the BrdU-negative region under 24-hr PS. Fig. 15C shows the statistic analysis of the results. PS significantly increased the %cells in G0/G1 phase (71.48 ± 5.95 %) in comparison to static condition (57.23 ± 8.14 %). Furthermore, PS also significantly reduced the cells in S-phase (1.52 ± 1.10 %) in comparison to static cells (18.81 ± 6.83 %). There is no significant difference in cells at G2 + M phase between PS cells (23.96 ± 5.11 %) and static cells (26.99 ± 5.98 %).

The flow cytometric results were consistent with those from immunofluorescent imaging analysis. Here I show that long-term PS flow kept the endothelium in a quiescent state through the inhibition of active DNA synthesis, thus preventing cells from entering S-phase. These findings provide the foundations for performing our studies on the role of KLF2 in the anti-proliferative function of PS flow.
A.

Figure 15. (A) 24-hr PS flow significantly reduces cell proliferation in HUVECs (fluorescent imaging). Cells were subjected to 24-hr PS flow, with BrdU added into the media during the last 2 hr of the 24-hr period. Cells were fixed and stained with anti-BrdU and PI. The upper panels are immunofluorescent images taken using an upright fluorescence microscope with a CCD camera. The number of proliferating cells (green/yellow signals) was significantly reduced under 24-hr PS flow in comparison to the static condition. The lower panel presents the calculated BLI: static (21.5 ± 2.03 %) vs. 24-hr PS (2.45 ± 1.02 %).
Figure 15. (B) Flow cytometric analyses for cells in static condition vs. 24-hr PS flow. Flow cytometric analysis for the static sample. R5, R6, and R7 region gates denote cells in G0/G1, G2 + M, and S-phase, respectively. Consistent with the results from fluorescent imaging, in static condition there were about 16% cells in S-phase (BrdU positive) actively proliferating (lower table).
Figure 15. (B, cont’d) Comparing to the static sample, 24-hr PS flow dramatically reduced the number of cells in S-phase (BrdU positive) from 16% (static) to 2.5% (lower table). The cell count vs. FL1 plots (lower left panel) show that there was a leftward shift of cell population to BrdU-negative cells in 24-hr PS flow sample (previous page).
Figure 15. (C) Flow cytometric cell cycle analysis shows that cells subjected to 24-hr PS flow mainly reside in G0/G1 arrest and very few cells are in active DNA-synthesizing S-phase. EC cell cycle analysis was performed using data derived from flow cytometry. Under 24-hr PS flow, there was a significant increase of cell number in G0/G1 phase and a significant decrease of cell number in S-phase. The number of cells in G2 + M phase was not significantly changed. These results confirmed that PS flow exerted anti-proliferative action in vascular endothelium.

* P < 0.05 (in comparison to Static: phase-to-phase matching).
IV.D.2 Knocking Down KLF2 Inhibits the Anti-Proliferative Function of PS Flow: Immunofluorescent Analysis

Following the demonstration that 24-hr PS flow exerts anti-proliferative effect on cultured HUVEC, I proceeded to investigate the role of KLF2 in the flow-mediated endothelial quiescence. It has been shown that KLF2 inhibits cell growth and proliferation in several cell systems, including T-cells and vascular endothelial cells [Wu et al., 2005; Bhattacharya et al., 2005]. I hypothesized that KLF2 may be the key regulator for endothelial quiescence under pulsatile laminar shear stress.

Cells reverse transfected with siNeg (control) or siKLF2 were either kept in static condition or subjected to 24-hr PS flow. BrdU incorporation described in section IV.C.3 was performed to assess the effect of KLF2 knockdown on cell proliferation under PS flow. From immunofluorescent images (Fig. 16), cells transfected with siNeg exhibited similar proliferation rates as those with no transfection (in section IV.D.1). Cell proliferation was reduced from 20% in siNeg static cells to 2% in siNeg PS-sheared cells (Fig. 16), consistent with the effects of PS on cells without siRNA transfection (Fig. 15A). These results suggested that the siRNA transfection did not interfere with basal cell proliferation. siKLF2 transfection increased the cell proliferation slightly to 25% in static cells, but significantly inhibited the strong anti-proliferative effect of PS flow, bringing cell proliferation rate up to 11% in comparison to 2% in siNeg PS-sheared cells. These results indicated that KLF2 knockdown diminished the quiescent phenotype induced by flow, suggesting that KLF2 expression is essential in mediating the anti-proliferative effect of PS flow.
Figure 16. siKLF2 significantly reverses the anti-proliferative effect of 24-hr PS flow (fluorescent imaging). 24-hr PS flow led to a strong reduction of cell growth/proliferation in siNeg-transfected cells (upper panels). siKLF2 transfection increased the basal proliferation rate in static cells, and significantly inhibited the PS-reduction of cell proliferation from 2% to 11% (lower panels).

* P < 0.05 (in comparison to siNeg Static).  # P < 0.05 (in comparison to siNeg PS).  § P < 0.05 (in comparison to siKLF2 Static).
IV.D.3 KLF2 Expression is Crucial for Keeping Vascular ECs in an Anti-Proliferative State: Flow Cytometric Analysis

Immunofluorescent imaging analysis from the previous section provided the evidence that KLF2 expression is critical for endothelial quiescence under PS flow. To verify my hypothesis with a detailed quantitative approach, I performed flow cytometric analysis to examine the effect of KLF2 knockdown on the anti-proliferative function of 24-hr PS flow. Fig. 17A presents the region gate analyses for the four experimental groups: siNeg static, siNeg flow, siKLF2 static, and siKLF2 flow. PS flow significantly increased the number of cells in G0/G1 phase (69.5%) and decreased the number of cells in S-phase (3.5%), as shown in the siNeg flow sample. siKLF2 transfection did not alter basal cell proliferation rate in static condition; under PS flow, however, the percentages of cells decreased in the G0/G1 quiescent phase (60.0%) and increased in the S-phase (8.2%). The quantitative bar graphs in Fig. 17B summarized results from 3 independent experiments, and clearly illustrate that KLF2 knockdown attenuated the anti-proliferative action of PS flow, and shifted cell populations to a less quiescent phenotype comparing to siNeg-transfected cells under flow. These data confirm that KLF2 acts as a key molecule for maintaining endothelial quiescence and regulating cell cycle under long-term PS flow.
Figure 17. (A) Flow cytometric analysis confirms the reversal of the antiproliferative effect in sheared cells due to siKLF2. Cell count “region gate” analyses showed that siKLF2 transfection significantly decreased the number of cells in G0/G1 phase (comparing R5 gates in siNeg flow and siKLF2 flow), and increased the number of cells in S-phase under PS flow (R7 gates in siNeg flow and siKLF2 flow).

* P < 0.05 (in comparison to siNeg Static). # P < 0.05 (in comparison to siNeg PS). § P < 0.05 (in comparison to siKLF2 Static).

<table>
<thead>
<tr>
<th>Cell Number (%)</th>
<th>siNeg Static</th>
<th>siNeg PS Flow</th>
</tr>
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<tr>
<td>G0/G1 phase</td>
<td>55.3 ± 6.12</td>
<td>69.7 ± 3.41 *</td>
</tr>
<tr>
<td>S-phase</td>
<td>22 ± 9.3</td>
<td>1.77 ± 1.45 *</td>
</tr>
<tr>
<td>G2 + M phase</td>
<td>22.7 ± 3.20</td>
<td>28.6 ± 3.88</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell Number (%)</th>
<th>siKLF2 Static</th>
<th>siKLF2 PS Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1 phase</td>
<td>53.1 ± 2.56</td>
<td>61.6 ± 5.47 * # §</td>
</tr>
<tr>
<td>S-phase</td>
<td>22.9 ± 6.86</td>
<td>6.89 ± 1.93 * # §</td>
</tr>
<tr>
<td>G2 + M phase</td>
<td>24.0 ± 4.31</td>
<td>31.49 ± 4.58 * §</td>
</tr>
</tbody>
</table>
Flow cytometric cell cycle analysis shows the differences in cell populations between siNeg-transfected vs. siKLF2-transfected cells under PS flow. In siNeg-transfected cells, PS flow significantly reduced cell growth, increasing cell number in G0/G1 phase while decreasing cell number in S-phase. In static cells, siKLF2 transfection did not significantly alter cell proliferation rate in comparison with siNeg. In PS-sheared cells, siKLF2 transfection significantly reduced the number of cells in G0/G1 phase and increased the number of cells in S-phase, when compared to the corresponding data for siNeg transfection.

* P < 0.05 (in comparison to siNeg Static).  # P < 0.05 (in comparison to siNeg PS).  § P < 0.05 (in comparison to siKLF2 Static).
IV.D.4 KLF2 Over-expression Leads to a Less-Proliferative EC Phenotype

We have identified KLF2 as a key regulator of endothelial quiescence under long-term PS flow. The results from the previous section suggested that siKLF2 did not significantly alter the basal cell proliferation rate in static cells. Instead of knocking down KLF2 expression, here we investigated the effect of KLF2 over-expression on the basal cell proliferation rate in static cells. As demonstrated by Bhattacharya et al. [2005] using [³H]thymidine uptake assay, over-expression of KLF2 slightly decreased cell proliferation rate in control cells and strongly reduced VEGF-A induced cell proliferation. In the current study, we infected sub-confluent HUVECs with adenoviral vectors of Ad-GFP (control) and Ad-KLF2. BrdU incorporation assays were performed one-day post infection. Fluorescent images (Fig. 18) showed that Ad-KLF2 infection decreased the basal cell proliferation from 17.9 % (as in Ad-GFP cells) to 8.5 %. Our results demonstrated that KLF2 over-expression led to a reduction in endothelial proliferation.
Figure 18. KLF2 over-expression leads to a less proliferative EC phenotype. Fluorescent images were taken using an upright fluorescence microscope with a CCD camera. Since both Ad-GFP and Ad-KLF2 (tagged with GFP) expressed green fluorescent signals and that infection efficiency was almost 100% (quantified previously), we used the FITC channel for recording total cell numbers and switched the FITC signals to red color. An anti-BrdU conjugated with Alexa Fluro 546 was used for BrdU staining, which was then recorded using the TRITC channel. The signals were switched to green color. Thus, the merged images presented BrdU Labeling Index as the number of green cells/red cells. The bottom bar graph showed the quantified cell proliferation rates of Ad-GFP and Ad-KLF2. Over-expression of KLF2 decreased the basal cell proliferation rate to about half (1/2).
IV.D.5 AMPK Knockdown Reverses EC Cell Cycle Regulation Under PS Flow:

Flow Cytometric Analysis

In section II we identified AMPK as the upstream regulator of KLF2 induction under PS flow. As demonstrated that KLF2 plays a critical role in mediating the anti-proliferative effect of 24-hr PS flow, we investigated the role of AMPK in modulating flow-regulated cell quiescence.

HUVECs were reverse transfected with either siNeg or siAMPK, and then kept in static condition or subjected to 24-hr PS flow. Cells were incubated with BrdU during the last 2 hr of flow, and then fixed and prepared for flow cytometric cell cycle analysis. As shown in Fig. 19A (upper panels), PS flow significantly inhibited DNA synthesis in S-phase (0.9 %) compared to that in static cells (17.9 %). siAMPK transfection slightly increased the number of cells in the G2 + M mitotic phase (28.7 %) compared to that in siNeg cells (24.5 %) with no significant difference in the S phase (18.9% vs. 17.9%). Most interestingly, although under flow siAMPK did not substantially affect the number of cells in S-phase (2.0 %), a significant cell population was shifted from quiescent G0/G1 phase (56.4 %) to mitotic G2 + M phase (41.6 %), in comparison to siNeg cells (G0/G1: 69.6 %; G2 + M: 29.5 %). Fig. 19B presented the quantitative data summarizing the effect of AMPK knockdown in attenuating the anti-proliferative effect of PS flow, leading to a majority of mitotic cells clustering in the G2 + M phase. This trend is similar to what Guo et al. observed [2007] using an adenovirus expressing dominant negative mutant of AMPK and examining its effect on bovine aortic endothelial cell proliferation under steady laminar flow. These results suggested that KLF2 and its upstream signaling molecule
AMPK act as the key mediator for the anti-proliferative function of athero-protective PS flow.
A.

<table>
<thead>
<tr>
<th>Cell Number (%)</th>
<th>siNeg Static</th>
<th>siNeg PS Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0/G1 phase</td>
<td>57.61 ± 9.99</td>
<td>69.55 ± 7.86 *</td>
</tr>
<tr>
<td>S-phase</td>
<td>17.88 ± 3.56</td>
<td>0.93 ± 0.26 *</td>
</tr>
<tr>
<td>G2 + M phase</td>
<td>24.51 ± 6.44</td>
<td>29.52 ± 8.07</td>
</tr>
<tr>
<td>siAMPK Static</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G0/G1 phase</td>
<td>52.37 ± 8.81 *</td>
<td>56.41 ± 9.98 * §</td>
</tr>
<tr>
<td>S-phase</td>
<td>18.89 ± 4.22</td>
<td>2 ± 1.47 * §</td>
</tr>
<tr>
<td>G2 + M phase</td>
<td>28.73 ± 4.81 *</td>
<td>41.59 ± 11.26 * §</td>
</tr>
</tbody>
</table>

Figure 19. (A) Flow cytometric analysis shows that siAMPK increases the number of cells in G2 + M phase without significantly altering the number of cells in S-phase under PS flow. Cell count “region gate” analyses showed that under PS flow, siAMPK transfection shifted cell populations to the mitotic G2 + M phase (R6) from the quiescent G0/G1 phase (R5) compared to siNeg. Although this effect was on different phases of the cell cycle from that of siKLF2, which shifted cells to S-phase, siAMPK also inhibited the anti-proliferative characteristics of flow.  * P < 0.05 (in comparison to siNeg Static).  # P < 0.05 (in comparison to siNeg PS).  § P < 0.05 (in comparison to siAMPK Static).
B.

Figure 19. (B) Flow cytometric cell cycle analysis comparison between siNeg-transfected and siAMPK-transfected cells under PS flow. In siNeg-transfected cells, PS flow significantly reduced cell growth, consistent with what was shown in Fig.13B. In static cells, siAMPK transfection slightly increased cell proliferation rate by reducing cell number in G0/G1 phase and increasing cell number in G2 + M phase. In PS-sheared cells, siAMPK did not significantly alter cell number in S-phase, but it did shift cell population from G0/G1 phase to G2 + M phase. These results demonstrated that AMPK expression also plays a crucial role in PS flow-mediated cell quiescence.

* P < 0.05 (in comparison to siNeg Static).  # P < 0.05 (in comparison to siNeg PS).  § P < 0.05 (in comparison to siAMPK Static).
IV.E Discussion

In this chapter I showed that KLF2 expression is essential in regulating the anti-proliferative function of PS flow. Long-term PS flow (12 ± 4 dyn/cm$^2$) led to a significantly reduction of cell proliferation, evident by both immunofluorescent imaging and flow cytometric analyses (Fig. 15). Thus, PS flow exerts an anti-proliferative effect similar to that of steady laminar flow [Lin et al., 2000]. There are some mixed reports in the literature regarding whether pulsatile or steady flow exhibits a stronger growth-arrest effect. Levesque et al. [1990] showed that pulsatile flow produced an exaggeration of the effect observed in response to steady flow; while Kadohama et al. [2007] showed that steady flow had a stronger effect in suppressing cell proliferation. The discrepancy could be due to differences in cell culture conditions such as the extracellular matrix used, etc. The results of cell proliferation analysis suggested that EC monolayer subjected to the PS flow patterns along the straight part of the arteries may provide a more selective barrier in limiting macromolecule permeability due to the PS-regulated low turnover rate. Such EC phenotype may contribute substantively in maintaining vascular homeostasis.

Transcription factors often play important roles in development through regulation of cell growth and tissue differentiation. KLF2 has been shown to regulate cell quiescence in many cell types. In the current study, my results showed that KLF2 expression is necessary for the anti-proliferative effect in cultured HUVECs under PS flow. Figs. 16 and 17 demonstrated that in the presence of PS flow, knocking down KLF2 expression significantly increased cell proliferation compared to that of control transfection. Although the PS-induced EC growth arrest was not completely abolished,
possibly due to the fact that siKLF2 did not give 100% KLF2 knockdown (see siKLF2 Static vs. siKLF2 PS), I successfully demonstrated the importance of KLF2 in PS flow-mediated anti-proliferation. To further investigate KLF2’s role in EC proliferation, I showed that KLF2 over-expression was sufficient to program cell quiescence (Fig. 18). Bhattacharya et al. [2005] demonstrated that KLF2 over-expression caused a slight reduction of cell proliferation rate with a reduced thymidine uptake (~10%, with no statistically significance shown) in untreated cells, while significantly inhibited the increase in cell proliferation induced by VEGF-A. My experiment showed a stronger cell growth arrest effect by KLF2 in untreated cells than in the study by Bhattacharya et al.; this could be due to differences in adenoviral infection procedures and different cell assays (BrdU incorporation vs. thymidine uptake). Lastly, by knocking down KLF2’s upstream regulator AMPK, I found that AMPK also contributes to maintaining EC quiescence under PS flow. Similar to the results shown by Guo et al. using steady flow [2007], under PS flow AMPK knockdown reduced the number of cells in G0/G1 phase and shifted the cell population to G2 + M phase, with little effects on the number of cells in S-phase (Fig. 19B). The composite results I obtained in this chapter suggest that AMPK/KLF2 axis plays a role in the anti-proliferative function of PS flow.

Other studies have investigated the mechanisms by which KLF2 leads to cell growth arrest. In T-cells, KLF2 mediates cell quiescence via down-regulating c-Myc [Buckley et al., 2001]; in ovarian cancer epithelial cells, KLF2 inhibits cell proliferation via suppressing WEE1[Wang et al., 2005]; in leukemia cells, KLF2 retards cell growth by up-regulating p21 [Wu et al., 2004]. I have now identified
KLF2 as holding a key role in the anti-proliferative function of PS flow, and it would be interesting to study further the molecular mechanisms by which KLF2 mediates anti-proliferation in vascular endothelium in the future. In addition, several studies have suggested KLF2 possesses anti-apoptotic characteristics in different cells types. Lin et al. showed that ectopic expression of KLF2 protected tumor necrosis factor receptor-associated factor 2 (TRAF2)-deficient mouse fibroblasts from TNF-induced apoptosis [2003]. In vascular ECs, KLF2 knockdown increased cell sensitivity to oxLDL-induced apoptosis [Wang et al., 2006]. These studies suggest that KLF2 not only regulates cell proliferation but may also regulate cell death. Therefore, KLF2 may be the key player in controlling EC turnover, cell junction integrity, and EC monolayer permeability. Understanding KLF2’s role in endothelial proliferation and apoptosis may raise the possibility of using KLF2 as a therapeutic target to regulate EC homeostasis for atherosclerosis prevention/treatment.
Chapter V

Summary and Conclusions

In the cardiovascular system, it has been widely recognized that atherosclerotic lesions are preferentially located at vessel bifurcations with significant curvatures and branches, while straight part of the arteries are spared from atheroma formation. The “site-specificity” of atherosclerosis suggests some localized factor such as blood flow pattern plays a key role in the pathological progression of the disease, in addition to systemic risk factors such as high cholesterol and lipid levels. ECs in the straight vessels are subjected to laminar flow with high fluid shear stress. The correlations between laminar flow and athero-resistant EC phenotype have received enormous attention and interests for the better understanding of the athero-protective effects of this unique hemodynamic stimulus. It has been shown that laminar flow modulates diverse endothelial functions such as survival, proliferation, migration, etc., through regulation of various signaling molecules, gene expressions, and protein activities. In particular, KLF2 has recently been identified as a shear-induced transcription factor that possesses anti-thrombotic and anti-inflammatory properties [Dekker et al., 2002]. Despite of the efforts by many investigators, the molecular mechanism by which KLF2 is regulated by laminar flow and its role in the athero-protective functions of flow are not completely understood.

The purposes of this dissertation are to elucidate the upstream signaling pathway that regulates KLF2 induction by laminar flow with a physiological-relevant “1-Hz” pulsatile component, and to study KLF2’s role in the anti-inflammatory and anti-
proliferative responses in ECs under flow. An in vitro pulsatile laminar flow (PS) system was set up to impose laminar shear stress of $12 \pm 4$ dyn/cm$^2$ on cultured human endothelial cells HUVECs. In Chapter II, I was able to show that KLF2 is not only induced at the mRNA level by PS flow, but is also activated at the protein level. AMPK, a protein kinase sensitive to cellular ratio of AMP/ATP, was also activated by PS flow through phosphorylation on its Thr172 site. Blocking AMPK activity led to reduced induction of KLF2 by PS flow, while constitutively active AMPK induced KLF2 expression in static cells. These results demonstrated that AMPK acts as the upstream signaling molecule that regulates KLF2 expression under flow. In addition, my collaborative work showed that AMPK is linked to the previously known ERK5/MEF2 pathway that regulates KLF2 induction by flow [Parmar et al., 2006]. Our results from both in vitro and in vivo studies provide foundations for a complete mechanistic explanation of PS flow-induced KLF2 expression via AMPK/ERK5/MEF2 pathway.

In Chapter III, I focused on elucidating the role of KLF2 in the anti-inflammatory response in ECs exposed to PS flow. Similar to steady laminar flow [Brooks et al., 2004], PS flow kept ECs in an anti-inflammatory state by mediating several inflammation-related gene expressions. KLF2 and eNOS were both activated while ET-1 and MCP-1 were both suppressed by PS flow. Knocking down KLF2 expression using siRNA led to a reduced eNOS induction, a restored ET-1 expression back to basal level, and had marginal effect on MCP-1 suppression under PS flow. Interestingly, knocking down KLF2’s upstream regulator AMPK using siRNA reduced only eNOS induction, without affecting ET-1 and MCP-1 suppression under
flow. Although KLF2 may exert different degrees of control over these inflammation-related genes, my results demonstrate that its expression plays a crucial role in ensuring the anti-inflammatory response in ECs subjected to PS flow.

In Chapter IV, I studied the role of KLF2 in the anti-proliferative response in ECs exposed to PS flow. PS flow has been shown to induce EC growth arrest [Li et al., 2005]. Lower endothelial turnover due to reduced cell death and cell proliferation correlates with a lower endothelial permeability for leukocyte migration or macromolecule transport [Weinbaum et al., 1985]. Using BrdU-incorporation assay along with both immunofluorescent imaging and flow cytometric analyses, I showed that PS flow kept majority of the cells in quiescent G0/G1 phase, with very few in active DNA-synthesizing S-phase. siRNA-mediated KLF2 knockdown reversed the prominent anti-proliferative effect of PS flow and increased the number of cells in S-phase. Notably, AMPK knockdown using siRNA did not significantly increase the number of cells in S-phase, but increased that in mitotic G2 + M phase under PS flow. These results suggest that elevated KLF2 expression under PS flow is responsible for maintaining the anti-proliferative EC phenotype.

The findings of this dissertation include identification of a novel signaling molecule AMPK through which PS flow induces KLF2 expression, and elucidation of the critical role KLF2 plays in regulating anti-inflammatory and anti-proliferative responses in ECs subjected to PS flow. The results of these studies present a regulatory pathway involving AMPK, ERK5 and MEF2, which sit upstream of KLF2 upon flow stimulation. These findings contribute significantly to the better understanding of the endothelial response to hemodynamic flow. By inducing KLF2
expression, laminar flow keeps the endothelium in an anti-inflammatory and anti-proliferative state, and hence prevents cells from developing into an atherogenic phenotype. In conclusion, KLF2 holds a critical role in maintaining healthy endothelium and may provide potential therapeutic application in treating cardiovascular disease.

Figure 20. Summary diagram for the thesis.
REFERENCES


