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The Role of AMPK and miR-92a in the Shear Stress Regulation of KLF2

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cellular, Molecular and Developmental Biology

by

Wei Wu

December 2010

Dissertation Committee:

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2010
The Dissertation of Wei Wu is approved:

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The Role of AMPK and miR-92a in the Shear Stress Regulation of KLF2

By

Wei Wu

Doctor of Philosophy, Graduate Program in Cellular, Molecular and Developmental Biology University of California, Riverside, December 2010 Dr. John Y-J Shyy, Chairperson

The vascular endothelium is essential to maintain normal vascular homostasis and its dysfunction is a hallmark of atherosclerosis development. Upregulated by athero-protective flow, the transcription factor Kruppel-like factor 2 (KLF2) is a crucial integrator for maintaining multiple endothelial functions, including anti-inflammation, anti-thrombosis, vasodilatation, and anti-angiogenesis. To investigate the molecular mechanism by which KLF2 is regulated by different flow pattern, the regulation of KLF2 expression was examined at both transcriptional level and post-transcriptional level response to different flow pattern.

In the first part of the study, AMP-activated protein kinase (AMPK) was demonstrated to be necessary and sufficient to regulate the expression of pulsatile
shear stress (PS)-induced KLF2 and its downstream (eNOS and ET-1). In addition, the PS-induced phosphorylation of ERK5 and MEF2, which regulates the KLF2 expression, is AMPK-dependent in ECs. Furthermore, the phosphorylation levels of ERK5 and MEF2, as well as the expression of KLF2, were significantly reduced in the aorta of AMPKα2 knockout mice when compared with wild-type control mice. These findings suggest that AMPK/ERK5/MEF2 is a functional signaling for the regulation of KLF2 transcription.

MicroRNAs (miRNAs) are non-coding small RNAs that regulate gene expression at the post-transcriptional level. In the second part, the role of miRNAs, particularly miR-92a, was examined in the atheroprotective flow-regulated KLF2. First, it was demonstrated that KLF2 is regulated by miRNA by knocking down Dicer with siRNA. In silico analysis predicted that miR-92a could bind to the 3’ untranslated region (3’UTR) of KLF2. Overexpression of miR-92a precursor (pre-92a) decreased the expression of KLF2 and the KLF2-regulated endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM) at mRNA and protein levels. Subsequent studies revealed that, athero-protective laminar flow down-regulated the level of miR-92a to induce KLF2, and the level of this flow-induced KLF2 was reduced by pre-92a. Consistent with these findings, miR-92a level was lower in the endothelium of athero-protective than athero-prone areas of the mouse aorta. Furthermore, mouse carotid arteries receiving pre-92a exhibited impaired vasodilatory response to flow. Collectively, these results suggest that
athero-protective flow patterns decrease the level of miR-92a, which in turn increases KLF2 expression to maintain endothelial homeostasis.

Taken together, this study demonstrated the potency of shear stress on EC function is due to the upregulation of KLF2 at both transcriptional level and post-transcriptional level.
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List of Abbreviations

ACC: acetyl-CoA carboxylase
Ago: argonaute
AICAR: 5’-aminoimidazole-4-carboxamide ribonucleoside
AMPK: AMP-activated protein kinase
BAEC: bovine aortic endothelial cell
Ad-AMPK-CA: Adeno-AMPK-constitutively active
DMEM: Dulbecco modified Eagle medium
DMSO: dimethyl sulfoxide
DRB: 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
EC: endothelial cell
ET-1: endothelin-1
eNOS/NOS3: endothelial nitric-oxide synthase
ERK5: extracellular signal-regulated kinase 5
FISH: fluorescence in situ hybridization
HDAC: histone deacetylase
HEK293: human embryonic kidney 293
HUVEC: human umbilical vein endothelial cell
IP: immunoprecipitation
KLF2: Kruppel-like factor 2
KO: knockout

MEF2: myocyte enhancer factor-2

MEK5: MAPK/extracellular signal-regulated kinase kinases

miRISC: miRNA induced silencing complex

NO: nitric oxide

OS: oscillatory shear flow

PS: pulsatile laminar flow

TM: thrombomodulin

3’-UTR: 3’ untranslated region
Chapter 1

Introduction

1. Atherosclerosis and endothelial dysfunction

The vascular endothelium, located at the interface between the circulating blood and the vessel wall, is extensively involved in vascular homeostasis such as the balance between vasodilation and vasoconstriction, inhibition and stimulation of smooth muscle cell proliferation and migration, and thrombogenesis and fibrinolysis\(^1\). Endothelial dysfunction is an early marker for atherosclerosis\(^2\), which can be initiated by various risk factors, including elevated level of inflammatory cytokines, hypercholesterolemia, and hyperhomocysteinemia, diabetes, hypertension, disturbed flow patterns, and tobacco smoking\(^3\). These risk factors increase the endothelial permeability and recruitment of the circulating monocytes/macrophages that engulf the oxidized low-density lipoprotein (ox-LDL), fats, calcium, and fibrin within the artery wall, which causes atherogenesis\(^4\). These plaques gradually occlude the arteries, and then decrease blood flow and vascular functions. The enlarged plaque may rupture and quickly obstruct the flow of blood, which results in myocardial infarction and stroke.

Shear stress, the tangential component of the hemodynamic forces resulting from blood flow, is the most physiologically relevant stimulus that maintains endothelial cell (EC) homeostasis and prevents the development of atherosclerosis\(^5\). The transcription factor Krüppel-like factor 2 (KLF2) is a critical regulator for endothelial lineage development and multiple vascular functions\(^6,7\). The purpose of my study is
to understand the molecular mechanism by which shear stress upregulates KLF2 expression and then maintains the protective effect on ECs. In chapter 2, I describe whether AMP-activated protein kinase (AMPK) mediates shear stress-induced expression of KLF2. Experiments with the cultured ECs and mouse vessel wall demonstrated the requirement of AMPK for the activation of extracellular signal-regulated kinase 5/myocyte enhancer factor 2 (ERK5/MEF2) signaling pathway and the expression of KLF2. In chapter 3, I examined the role of miRNA, particularly miR-92a, in the atheroprotective flow-regulated KLF2. Evidence collected both in vitro and in vivo suggests that atheroprotective flow decreases the level of miR-92a, which in turn increases KLF2 expression to maintain endothelial homeostasis. Taken together, these findings indicate that the potency of shear stress on EC function is due to the regulation of KLF2 at both transcriptional and post-transcriptional levels.

2. Blood flow, shear stress, and Endothelial function

The concept that “physical exercise involving increase of cardiac output, and hence increased shear rate, might retard the development of atheroma” was first proposed 40 years ago. In the following decades, pathophysiological studies of human patients and various animal models clearly indicated that atherosclerotic lesions tend to occur in the curvatures, bifurcation, and branches of the vessels, which are termed the “atheroprone region”. In contrast, lesions develop much less in the unbranched straight parts of the arteries, known as the “atheroprotected region”. In the atheroprone region, the blood flow rate is reduced and can reverse direction during the cardiac cycle, which is called “oscillatory flow” or “disturbed flow”. In the
straight part of arteries, the rate of flow changes during the cardiac cycle, but flow is always in the forward direction and is called “laminar flow” or “pulsatile flow”\textsuperscript{13, 14}.

Shear stress, the tangential component of hemodynamic forces resulting from blood flow, is an important regulator of endothelial functions\textsuperscript{15}. ECs in the atheroprone region experience unsteady and low mean shear stress, which stimulates proatherogenic responses, such as high levels of cell turnover, inflammation, permeability, thrombosis and oxidative stress\textsuperscript{12, 13}. In contrast, ECs in the atheroprotected region experience unidirectional, high mean shear stress. Laminar shear stress maintains the quiescent phenotype of ECs and exerts vascular protective effects, such as anti-inflammation, anti-oxidation, anti-thrombosis, and promotion of vasodilation\textsuperscript{16}.

To investigate the mechanism by which shear stress modulates endothelial functions, I used the flow channel system to mimic the different flow patterns \textit{in vitro} (Fig. 1-1)\textsuperscript{17}. The laminar flow (with steady, laminar shear stress at 12 dyn/cm\textsuperscript{2}) is the most simplified flow pattern to assess the effects of atheroprotective flow in endothelial biology. To mimic more physiological conditions, pulsatile shear flow (PS) or oscillatory flow (OS) can also be generated by this system. A sinusoidal component with a frequency of 1 Hz (mimicking the pulse in the human body) can be introduced into the system by connecting a reciprocating syringe pump to the circulating system. In my study, PS or OS was applied to ECs as $12 \pm 4$ dyn/cm\textsuperscript{2} or $0 \pm 4$ dyn/cm\textsuperscript{2} respectively.
Fig. 1-1. The flow channel system used to mimic blood flow in vitro. A confluent monolayer of ECs is seeded onto the glass slide and sealed within the chamber and a gasket creates a space for flow. The flow rate is controlled by the height difference of two reservoirs. A reciprocating syringe pump is connected to the circulating system to introduce a sinusoidal component (frequency=1 Hz) onto the shear stress. (Adapted from Chien, 2007)

The impaired endothelium-dependent vasodilation, which is mediated by nitric oxide (NO), is the hallmark of endothelial dysfunction and a contributor to atherosclerosis. By using the flow channel system and animal models, many groups, including ours, have shown that laminar shear stress is an important physiological stimulus enhancing endothelial functions, in part because of the regulation of endothelial NO synthase (eNOS) and thereby NO production. At the transcriptional level, the prolonged shear stress can upregulate eNOS expression in cultured ECs and in intact arteries. At the post-translational level, shear stress can
cause eNOS phosphorylation at Ser-1177 and Ser-633 through multiple kinases, such as protein kinase A (PKA), protein kinase B (AKT) and AMPK\textsuperscript{22-25}. eNOS can also be activated by an SIRT1-induced deacetylation response to shear stress\textsuperscript{26}.

3. **KLF2, the integrator of multiple endothelial functions**

Shear stress is well known to regulate eNOS activity at the post-translation level (such as phosphorylation, acetylation). However, how shear stress regulates the expression of eNOS in the long term is unclear. Over the last several years, many studies have suggested that Kruppel-like transcription factor (KLF) is a crucial regulator of endothelial function. Notably, KLF2 is shear inducible and could bind to the eNOS promoter and regulate its transcription response to shear stress\textsuperscript{27}. In this section, I summarize the role of KLF2 in endothelial function and the regulation of KLF2 transcription.

3.1 **KLF family**

"Krüppel", the German word for “cripple”, is an important transcription factor regulating the development of *Drosophila*\textsuperscript{28, 29}. The protein is named because *Drosophila* embryos lacking the protein Krüppel have altered anterior abdominal and thoracic segments where the legs should develop from, which results in death\textsuperscript{30}. The nomenclature of the mammalian Krüppel-like factor (KLF) family is based on the homology to the DNA-binding domain of the *Drosophila* Krüppel. The first mammalian Krüppel, erythroid Krüppel-like factor (EKLF/KLF1) was identified
in red blood cells in 1993\textsuperscript{31}. It was demonstrated to play an important role in \(\beta\)-globin gene synthesis and erythrocyte development\textsuperscript{32, 33}. To date, 17 mammalian Krüppels have been identified and designated on the basis of the chronological order of discovery (ie, KLF 1-17). KLFs play key roles in regulating cellular processes in many distinct cell types, as shown by gene knockout studies\textsuperscript{34, 35}. Three members of the KLF family are expressed in ECs: KLF2, KLF4, and KLF6.

\textbf{Figure. 1-2. Schematic comparison of the members of KLF family.} The blue region is the activation domain and the purple region is the repression domain. C-terminal is the zinc finger domain. (Adapted from Jain, 2007\textsuperscript{6})

KLFs are a subclass of the zinc finger family of DNA-binding transcription factors\textsuperscript{35}. The C terminus of KLF protein contains 3 Cys2/His2 zinc finger motifs, which could bind to the conscious sequence "CACCC" or the "GT box" in the promoter region of the target genes\textsuperscript{36}. The inter-finger-space sequence contains a highly conserved 7-aa sequence, TGEKP(Y/F)X. Although KLFs have similar zinc finger domains in the C terminus, their N termini are highly diverse. Transactivation
of KLFs to the targets is mediated via their various activation or repression domains in the N terminus (Fig. 1-2).

3.2 The identification and characterization of KLF2

KLF2, a 354-aa protein, was first cloned by the Lingrel group in 1995. It was initially termed as lung Krüppel-like factor (LKLF) because it is highly expressed in lung tissues. Human KLF2 maps to chromosome 10p13.1 and has greater than 85% homology to the mouse gene. Furthermore, the critical regions of the promoter are also conserved across species, including exact identity of a 75-bp sequence. The N-terminal regulatory region contains a transcriptional activation domain (1-110 aa) and an inhibitory domain (110-267 aa). The transcriptional activation domain can interact with a coactivator (such as p300/CBP), and the complex is able to induce chromatin remodeling. The inhibitory domain interacts with the WW domain–containing E3 ubiquitin protein ligase 1 (WWP1), thus resulting in ubiquitination and proteasomal degradation of KLF2.

3.3 KLF2 animal model

In the embryo of wild-type mice, the expression of KLF2 is initially noted at embryonic day 7 (E7), then decreased at day 11, and reactivated at day 15. Thus, KLF2 expression is developmentally regulated. KLF2 knockout mice exhibit abnormal blood vessel formation because of insufficient smooth muscle cell recruitment, thus resulting in embryonic hemorrhage and death between day 12.5 and
These mice also display abnormal lung morphogenesis, which supports an essential role for this factor in lung development. The endothelial-specific KLF2 knockout mouse is also embryonic lethal because of heart failure in association with reduced vessel tone but not vascular defects. The viable hemizygous KLF2 knockout mouse (KLF2+/−) does not have an apparent endothelial phenotype. However, when crossed to apolipoprotein E (ApoE)-deficient mice, KLF2+/− mice show aggravated atherosclerosis development, possibly through increased foam-cell formation. These animal models showed that KLF2 plays an important role during development and maintains EC functions.

3.4 KLF2 regulates EC functions

In 2002, Dekker et al. first demonstrated that knockdown of KLF2 prevented flow-mediated induction of eNOS and flow-mediated reduction of endothelin-1, which is the first important evidence of KLF2 regulating flow-mediated effects in ECs. The recent transcriptome analysis showed that KLF2 governs 70% of the shear stress-regulated gene sets. Emerging evidence indicates that KLF2 is a critical integrator of multiple endothelial functions (anti-inflammation, anti-thrombosis, anti-angiogenesis, vascular tone, blood vessel development) that are resistant to atherogenesis (Fig. 1-3). For example, KLF2 inhibits NFκB-dependent vascular cell adhesion molecule 1 (VCAM-1) and E-selectin expression response to inflammatory cytokines, which in turn decreases the attachment of immune cells and rolling to ECs. KLF2 can also strongly inhibit transforming growth factor (TGF)-β
signaling by decreasing the levels of phosphorylated nuclear activating transcription factor 2 (ATF2)\textsuperscript{48}. Parmar \textit{et al.} found that induction of several vasodilatory factors that depend on KLF2 include the flow-mediated upregulation of eNOS, argininosuccinate synthetase (ASS), and C-natriuretic peptide (CNP), as well as the downregulation of endothelin-1 (ET-1) and VCAM-1\textsuperscript{7}. KLF2 has potent anti-angiogenic effects and keeps ECs quiescent by inhibiting the expression of the key VEGF receptor (VEGFR2) and then reducing EC proliferation and migration\textsuperscript{49}.

\textbf{Fig.1-3. Schematic diagram of the functions of KLF2.} (Adapted from Jain, 2010\textsuperscript{50})
3.5 The regulation of KLF2 transcription

3.5.1 Flow regulation of KLF2

As discussed above, laminar blood flow is thought to be benefit to the endothelium. The disturbed flow patterns are thought to contribute to atherogenesis in the atheroprone regions. However, the exact mechanism by which laminar flow imposes atheroprotective effects remains poorly understood.

Using a gene profiling approach, the Horrevoets group first demonstrated that KLF2 is induced by prolonged laminar flow in cultured ECs\(^4\)\(^6\). Furthermore, this group showed with \textit{in situ} hybridization that KLF2 is highly expressed in the linear segments of the vessel and is decreased at branch points, which are the more atheroprone regions of the vasculature. Parmar \textit{et al.} demonstrated the flow-dependent expression of endothelial KLF2 \textit{in vivo} using the \textit{sih} zebrafish mutant, which lacks blood flow\(^7\). \textit{In vitro} and \textit{in vivo} experiments with vascular ECs indicate that KLF2 exhibits sustained induction under laminar shear stress. Furthermore, Wang \textit{et al.} reported that pulsatile flow induced sustained expression of KLF2, but oscillatory flow caused prolonged suppression after a transient induction in ECs\(^5\)\(^1\).

3.5.2 MEF2, an important transcription factor of KLF2

Several groups used promoter analysis to elucidate the mechanism of flow-mediated induction of KLF2. In 2004, Huddleson \textit{et al.} identified a 62-bp shear stress-responsive region in the KLF2 promoter that contains a 30-bp (-138 to
-108 bp) tripartite palindrome motif. Deletion analysis revealed that with lack of this region, KLF2 expression would not be upregulated by shear stress. The authors further found a consensus myocyte enhancing factor 2 (MEF2)-binding site (ctaaatttag) in this region. Chromatin immunoprecipitation (ChIP) assays revealed that both MEF2A and MEF2C bind to this region of the KLF2 promoter in ECs.

MEF2 factors are members of the MADS box (MCM1, Agamous, Deficiens, Serum response factor) family of transcription factors that bind to A/T-rich sequences. MEF2 was initially identified as a transcription factor that activates the expression of skeletal and cardiac muscle structural genes. Recently, MEF2 was found to be required for EC proliferation and viability and involved in the pathogenic mechanisms associated with endothelial disorders. For example, mutations in the human \textit{mef2a} gene have been observed in patients with inherited coronary artery disease. \textit{mef2c}-knockout mouse is lethal, with profound cardiac and vascular defects due to the impaired endothelial integrity and permeability.

3.5.3 The regulation of MEF2 transactivation

The identification of MEF2 factors as regulators of KLF2 expression provided an immediate link to flow. ERK5 (also known as big mitogen-activated protein kinase 1) was identified as a highly flow-induced factor by the Berk group, in 1999. MEF2 is one of the best-characterized targets of ERK5. ERK5 is capable of greatly enhancing the transactivation activity of MEF2C by phosphorylating MEF2C at Ser 387 and Thr 300 and MEF2A at Ser 355, Thr 312 and
Thr 319 both *in vitro* and *in vivo*. Yeast 2-hybrid screening and co-immunoprecipitation also showed that ERK5 and MEF2C interact with each other.

The connection between KLF2 and this pathway was confirmed by consequent work. Loss-of-function studies performed by the Winoto group showed that ERK5 is essential for embryonic KLF2 expression and that ERK5 drives KLF2 transcription by activating MEF2 transcription factors. Consistent with this observation, Parmar *et al.* showed that overexpression of a dominant-negative MEF2 or mutant MEK5 (an upstream activator of ERK5) prevented flow-mediated induction of KLF2 expression in endothelial cells. These results indicated that the mechanisms linking shear stress and KLF2 expression involve activation of a MEK5/ERK5/MEF2 pathway.

### 3.5.4 Other transcription factors

In addition to MEF2, several transcription factors (TFs) that bind to the KLF2 promoter by using DNA affinity chromatography and mass spectrometry were identified. These factors include p300/CBP-associated factor (PCAF), heterogeneous nuclear ribonucleoprotein D (hnRNP D), and nucleolin. Furthermore, these proteins were required for the shear induction of KLF2 by chromatin immunoprecipitation and gel-shift analysis. Shear stress regulates the binding of this complex through a phosphoinositide-3 kinase (PI3K)-dependent/Akt-independent pathway.
3.6 Other stimuli regulates KLF2 expression

Considering its beneficial effects to the endothelium, KLF2 is an ideal target for drug development for cardiovascular disease. Recently, several medicines and chemicals have been shown to improve EC function by regulating KLF2 expression.

3.6.1 Statins

Statins act to reduce plasma cholesterol levels by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and constitute the most widely prescribed class of drugs for reducing morbidity and mortality associated with cardiovascular disease. Interestingly, many of the benefits of statins may extend beyond their lipid-lowering effects\textsuperscript{66-68}. The pleiotropic effects include the improvement of endothelial function, inhibition of vascular inflammation and oxidation, and stabilization of atherosclerotic plaques. At the cellular level, similar to shear stress, statins have been shown to induce eNOS phosphorylation and also upregulate eNOS gene expression in endothelium\textsuperscript{69}.

Because of much overlap between the beneficial cellular effects of statins and shear stress, several groups studied whether KLF2 mediates statin-induced effect in ECs. Indeed, multiple statins could induce KLF2 expression\textsuperscript{70-72}, which suggested that statins induce KLF2 expression by inhibiting a Rho pathway. Promoter deletion and mutational analysis identified that the MEF2-binding site of KLF2 promoter is
also required for statin-mediated KLF2 induction. Furthermore, KLF2-knockdown experiments demonstrated that KLF2 is required for statin-mediated induction of eNOS and TM mRNA and protein levels. These data strongly implicate KLF2 as a novel nuclear mediator of statin effects in ECs.

3.6.2 Resveratrol

Resveratrol (3,5,4′-trihydroxystilbene), a natural polyphenol and phytoalexin, has a wide range of beneficial effects on the cardiovascular system. The NAD+-dependent deacetylase Sirtuin 1 (SIRT1), activated by resveratrol, mediates the effect of anti-inflammatory, vasodilatory, and anti-thrombotic properties to the vasculature. Resveratrol and SIRT1 activators improved endothelial dysfunction and reduced vascular inflammation in mice. Previous studies have demonstrated that endothelial-specific overexpression of SIRT1 improves endothelial function and decreases atherosclerosis in ApoE-null mice. Recently, the Garcia group demonstrated that resveratrol could induce the expression of KLF2 in cultured HUVECs. This induction depended on SIRT1 and sequentially the MEK5/MEF2 pathway but not ERK5.

3.6.3 Angiopoietin-1

Angiopoietin-1 (Ang1) is a ligand for the endothelium-specific receptor tyrosine kinase Tie-2. Ang1/Tie2 signaling promotes vascular formation during development and physiological and pathological angiogenesis but maintains the
quiescence of mature blood vessels in normal adult vasculature by enhancing vascular integrity and endothelial survival\cite{80}. In the presence of cell–cell contact, Ang1-induced KLF2 expression was demonstrated by microarray analysis\cite{81}. The following study showed that Ang1/Tie2 signal stimulates transcriptional activity of MEF2 through a PI3K/AKT pathway to induce KLF2 expression, thereby contributing to vascular quiescence\cite{82}.

![Fig. 1-4. Schematic diagram of the transcriptional regulation of KLF2 in endothelial cells. (Adapted from Jain, 2010\textsuperscript{50})](image)

3.6.4 Cytokine-mediated Inhibition

An important observation is that many proinflammatory stimuli inhibit KLF2 expression in ECs. Kumar et al. determined that TNF\textsubscript{α} inhibition of KLF2 is mediated by both the NF-\textsubscript{κ}B and histone deacetylase (HDAC) pathways\cite{83}. A combination of promoter deletion and mutational analysis, coimmunoprecipitation and
chromatin immunoprecipitation assays revealed that HDAC4/5 and p65 (a component of NF-κB) can form a tri-mer complex with MEF2 factors on the KLF2 promoter and can inhibit the ability of MEF2 to induce KLF2 expression. This work suggests that endothelial activation by inflammatory cytokines is potentially mediated by reduction in KLF2 activity, thus leading to unopposed NF-κB activity and its subsequent deleterious effects.

4. AMPK, an energy sensor in vascular biology

AMPK has long been recognized as a metabolic switch that senses and regulates the cellular energy homeostasis. Mammalian AMPK is a trimeric enzyme composing a catalytic α subunit and regulatory β and γ subunits. AMPK activation requires the phosphorylation of Thr172 at the catalytic α-subunit by an upstream AMPK kinase. Under several physiological and pathological conditions, such as exercise, hypoxia, and nutrient depletion, an increased AMP-to-ATP ratio rapidly activates AMPK by LKB1 (encoded by the Peutz-Jegher tumor suppressor gene). AMPK can also be activated by CaMKK with changes in the intracellular level of Ca^{2+}, which is independent of elevated AMP/ATP ratio. Such an activation “switches on” ATP-generating catabolic processes such as glycolysis and fatty acid oxidation but “switches off” ATP-consuming anabolic pathways, such as fatty acid and protein synthesis, to restore the energy balance in the cell. Recent studies indicated that AMPK, by activating eNOS and leading to the NO production, may also play an important role in vascular biology. Zhang et al. demonstrated for the
first time that shear stress activates AMPK in ECs, then induced not only eNOS phosphorylation at Ser 1179 but also eNOS expression with increased NO release. A more recent study in our lab showed that AMPK also can phosphorylate eNOS at Ser-633 constantly in response to laminar shear stress. Furthermore, several widely used pharmacological agents, including statins and metformin, have been shown to activate AMPK-eNOS signaling in ECs. Considering the critical role of AMPK and KLF2 in EC function, I hypothesize that AMPK may mediate shear stress-induced KLF2/eNOS expression. This hypothesis was tested in the first project of this dissertation. The experimental approaches and results are presented in Chapter 2.

5. Flow regulates KLF2 at the post-transcriptional level via miRNAs

Although both atheroprotective flow and statins could induce KLF2 transcription to a similar level, flow is much more potent to enhance the levels of the KLF2 downstream targets eNOS and TM. One of the explanations is that shear stress, but not statins, increases the stability of KLF2 mRNA, thus resulting in increased KLF2 protein expression and concomitant strong induction of KLF2 downstream targets. The molecular basis of such an increased stability of KLF2 mRNA remains elusive.

Recently, microRNAs (miRNAs) have emerged as important regulators at the posttranscriptional level. MiRNAs are short (20–23 nt), endogenous, single-stranded RNA molecules that regulate gene expression by degradation of mRNA and
repression of translation\textsuperscript{94}. In humans, miRNAs are predicted to control the activity of more than 60% of all protein-coding genes and affect many cellular pathways, including development, cancer and cardiovascular disease\textsuperscript{95}. In this section, I summarize the miRNA biogenesis, the repression mechanism of miRNAs on targets, and the function of argonaute (Ago) proteins in the miRNA-induced silencing complex (miRISC). Specifically, I discuss the regulation and function of the miR-17\textasciitilde92 cluster.

5.1 miRNA Biogenesis

According to the genomic organization and the regulation of transcription, miRNAs are separated into intronic and intergenic miRNAs\textsuperscript{96}. Intronic miRNAs are in the intron of encoding genes, which are expressed with their host genes, spliced out of the transcript and further processed into mature miRNAs\textsuperscript{97}. Intergenic miRNAs are in the non-coding or the intron region of a given gene but in the opposite orientation to the gene transcript\textsuperscript{96}. These miRNAs have their own promoter region and are transcribed independently. In animals, miRNAs are frequently transcribed together as polycistronic primary transcripts that are processed into multiple individual mature miRNAs\textsuperscript{98}. The genomic organization of these miRNA clusters is often highly conserved, which suggests an important role for coordinated regulation and function.
Most mammalian miRNAs are transcribed by RNA polymerase II (pol II), which generate a primary miRNA (pri-miRNA) transcript that contains one or more hairpin structures, each composed of a stem and a terminal loop\(^99,^{100}\). In the nucleus, the pri-miRNA is cleaved into a 70-nt hairpin-structured precursor (pre-miRNA) by Drasha (an RNase III enzyme) and its cofactor DGCR8\(^{101}\). Pre-miRNAs, which fold into stem-loop structures, is recognized by exportin 5 (Exp5) (the nuclear export factor) and then trafficked from the nucleus to the cytoplasm\(^{102}\). In the cytoplasm, pre-miRNA hairpin is recognized by Dicer, another RNase III-like endonuclease, and cut the loop end off, thus creating an 18- to 24-nt RNA duplex\(^{103}\). Subsequently, the miRNA duplex is incorporated into the miRNA-induced silencing complex (miRISC), the final effector\(^{104}\). Once loaded, one strand of the duplex, which is relatively thermodynamically stable, remains as a mature miRNA (the guide strand or miRNA), whereas the other strand (the passenger strand or miRNA*) is degraded. Typically, the miRNA strand, whose 5’ end is less stably base-paired, will be more frequently chosen as the guide.

### 5.2 Principles of target reorganization in animals

Once the miRISC is assembled, the miRNA guides the complex to its target by base-pairing with the target mRNA. In plants, miRNAs bind to a generally perfectly complementary site in either the coding or 3’-untranslated regions (UTRs) of the target mRNA\(^{104}\). However, most animal miRNAs bind to multiple, partially complementary sites in the 3’-UTR of targets\(^{105}\). The most stringent requirement is a
perfect match of the nucleotides 2–8 in the 5’ end of the miRNA, which represent the “seed sequence”. In addition, an A residue across position 1 of the miRNA and an A or a U across position 9 improve miRNA activity, although they do not need to base-pair with miRNA nucleotides. Complementarity of the miRNA 3’ half is quite relaxed, though it stabilizes the interaction. Generally, miRNA–mRNA duplexes contain mismatches and bulges in the central region, which prevent endonucleolytic cleavage of mRNA by an RNAi mechanism. Furthermore, an AU-rich neighbourhood, the position at each end of 3’ UTR and longer 3’ UTRs of mRNA increase the chance of miRNA binding. These factors can make the 3’ UTR regions less structured and hence more accessible to miRISC recognition. In addition, multiple sites for the same or different miRNAs are generally required for effective repression. When miRNAs are tethered by binding to the closed sites (within 10–40 nt), they tend to act cooperatively. Therefore, their effect exceeds that expected from the independent contributions of two single sites.

5.3 Repression mechanism

The molecular mechanism underlying miRNA-mediated silencing is still not entirely clear. A large number of studies showed that miRNAs inhibit translation or destabilize mRNA by deadenylation and degradation. In the following section, I introduce these repression mechanisms.
5.3.1 mRNA cleavage mediated by Ago2-associated RISC

Both the siRNAs and miRNAs, when perfectly base-paired to their target mRNA, could direct cleavage of the target mRNA. This cleavage is a result of the “Slicer” activity in the RISC. Argonate protein, which contains the signature PAZ and PIWI domains, is the most important and best-characterized components of miRISC. Structural studies revealed that miRNAs can be limited between the PAZ and PIWI domains, thus positioning the target mRNA closed to the catalytic center.

Mammals contain 4 AGO proteins, Ago1 to Ago4. Although all human Ago proteins bind both miRNAs and siRNAs, only Ago2-containing complexes can cleave mRNA targets. The mammalian miR-196 has a near-perfect complementary sequence in the 3’ UTR of the Hoxb8 mRNA and leads to direct mRNA cleavage and degradation of the target mRNA. All the other mammalian miRNAs are believed to base-pair to their targets at partially complementary sites and inhibit protein accumulation.

5.3.2 Repression of translation

At the beginning of the mammalian miRNA study, many reports showed that most of miRNAs are partially complementary to their multiple target mRNAs, thus resulting in the inhibition of protein accumulation without strongly affecting mRNAs. The repression of translation is the predominant effect of miRNA silencing.

miRNAs could repress the initiation of translation by recruiting proteins that interfere with the translational initiation factor eIF4E–eIF4G interaction or bind
directly to the m7GpppN cap, which inhibits the assembly of the 40S initiation complex and the joining of the 60S subunit\textsuperscript{107, 108}. In addition, miRNAs also could decelerate translation elongation or induce the ribosome drop off to terminate the translation early. Some studies also showed that the synthesized protein could be rapidly degraded by proteases recruited by miRISC\textsuperscript{111}.

**5.3.3 Compartmentalization of miRNA repression**

P-bodies are now known to be temporary sites of storage for repressed mRNAs in yeast and mammals\textsuperscript{112, 113}. AGO proteins, miRNAs and mRNAs repressed by miRNAs are all enriched in P-bodies, which suggests that P-bodies function in miRNA repression and in the fate of repressed mRNAs. Within the P-bodies, miRNA targets are sequestered from translational machinery and are subjected to mRNA degradation. This repression is reversible depending on different environment and stimuli. For example, the endogenous cationic amino acid transporter 1 (CAT-1) mRNA, a target of *miR-122*, localizes to P-bodies when translation is repressed but not when it is reversed by stress\textsuperscript{114}.

**5.3.4 mRNA deadenylation and decay**

Although initial studies suggested that the levels of mRNAs remain mostly unchanged with the miRNA effect, more recent work has demonstrated that miRNAs can induce pronounced target mRNA degradation\textsuperscript{115}. Likewise, microarray studies of mRNA levels in cells and tissues with altered miRNA levels revealed marked
changes in the abundance of dozens of validated or predicted miRNA targets, consistent with a role for miRNAs in mRNA destabilization\textsuperscript{105}.

**Fig. 1-5. The mechanism of miRNA-mediated translational repression in animal cells.** The miRISC–mRNA interaction can lead to several modes of direct translational repression. (a) Initiation block: The recruitment of 40S and/or 60S ribosomes near the 5′ cap of mRNA is inhibited. (b) Ribosomal drop-off: The 40S/60S ribosomes are dissociated from mRNA. (c) Stalled elongation: The 40S/60S ribosomes are prohibited from joining during the elongation process. (d) The indirect translational repression by mRNA degradation and sequestration in P-bodies. (Adapted from Sun, 2010\textsuperscript{116})

In eukaryotes, mRNA degradation has two pathways, each of which is initiated by a gradual shortening of the mRNA poly(A) tail. The mRNA body can then be degraded by progressive 3′→5′ decay, which is catalyzed by the exosome or by the removal of the 5′ cap followed by 5′→3′ degradation, which is catalyzed by the exonuclease\textsuperscript{117}. These decay machinery components are enriched in P-bodies, where miRISC is stored. For example, the P-body protein GW182, which is a key factor
that marks mRNAs for decay, could interact with Ago1 in mammals and worms. GW182 depletion leads to an upregulation of many mRNA targets that are also upregulated by Ago1 depletion. Depletion of the components of the CCR4–NOT deadenylating complex prevents the decay-promoting activity of GW182, which suggests that GW182 recruits CCR4–NOT to repressed mRNAs.

5.3.5 Translation repression, mRNA destabilization, both?

The exact molecular basis underlying miRNA-mediated gene silencing is not entirely clear. Many examples showed endogenous mammalian mRNAs that are regulated by miRNAs at the level of translation without change in target mRNA levels. Many examples show that the target mRNA levels were altered. In this context, translational repression may be the primary event, with any reduction in mRNA levels a consequence of translational repression and subsequent relocalization to P-Bodies. In one of the most recent studies, ribosome profiling was used to measure the overall miRNA effects on protein production and compare these to simultaneously measured effects on mRNA levels. The lowered mRNA levels account for most (≥84%) of the decreased protein production. These results indicated that destabilization of target mRNAs is the predominant reason for reduced protein output.

Whether Ago1 or Ago2 is more important for miRNA function in mammals is still unclear. Among the Ago family members, Ago1 mediates the miRNA-induced translational inhibition and deadenylation and 5’→3’ decay. Neither of the cases
requires a perfect match between miRNAs and the 3’ UTR of the targeted mRNAs. However, Ago2 modulates mRNA degradation, a process that requires a near-perfect complementary sequence in the 3’ UTR of the target.\textsuperscript{117, 118} Recently, a chaperone model suggested that Ago1 facilitates the association of the guide strand of the miRNA with the target mRNA and then Ago2 is recruited to the miRNA–mRNA complex.\textsuperscript{120} Even with no perfect match between the miRNA and the target mRNA, Ago2 still could incorporate to miRISC and degrade mRNA. In the chapter 3, I describe the function of Ago1 and Ago2 in regulation.

6. miRNA and vascular biology

6.1 The role of miRNAs in endothelial cells

The importance of miRNAs in endothelial function was first revealed by disrupting the function of Dicer, a key enzyme for miRNA biogenesis\textsuperscript{121, 122}. Dicer1-null embryonic stem (ES) cells are unviable, and the homozygous Dicer knockout mouse is early embryonic lethal\textsuperscript{123, 124}. Dicer hypomorphic mice have defects in vascular remodeling during development or ovary angiogenesis\textsuperscript{125}. EC-specific deletion of Dicer in mice provided direct \textit{in vivo} evidence that endothelial miRNAs are required for postnatal angiogenesis in response to angiogenic stimuli, including VEGF, tumors, limb ischemia, and wound healing\textsuperscript{121}. Knockdown of Dicer \textit{in vitro} in human ECs results in decreased angiogenesis and EC proliferation and impaired morphogenesis\textsuperscript{121}. These situations are due to upregulation of
thrombospondin-1 (Tsp-1), an inhibitor of angiogenesis, as well as other key regulators of endothelial function in ECs\textsuperscript{125}. Tsp-1 is a predicted target of the let-7 family and miR-17-92 cluster. The transfection of the miR-17-92 cluster rescued the Tsp-1 expression and EC proliferation.

miRNA profiling revealed about 40 miRNAs that are highly expressed in human umbilical vein ECs (HUVECs), including miR-221/222, miR-21, the let-7 family, the miR-17-92 cluster and miR-126\textsuperscript{121, 122, 126}. These miRNAs provide a new layer of information on the regulation of multiple EC functions (see Table 1-1). For example, miR-126 is the only EC-specific expressed miRNA and the first vascular miRNA to be knocked out in mice\textsuperscript{127}. The deletion of miR-126 caused leaky vessels and partial embryonic lethality because of loss of vascular integrity and defective angiogenesis\textsuperscript{128}. MiR-126 null mice also showed reduced survival and defective neovascularization after myocardial infarction. The proangiogenic function of miR-126 is mediated by promoting mitogen-activated protein kinase (MAPK) and PI3K signaling in response to VEGF, through targeting negative regulators of these signaling pathways, including Spred-1 and PI3K regulatory subunit 2 (PI3KR2)\textsuperscript{129}. Besides Spred-1 and PI3KR2, miR-126 also targets vascular cell adhesion protein 1 (VCAM-1), thereby regulating the adhesion of leukocytes to the endothelium, which suggests a role of miR-126 in vascular inflammation\textsuperscript{128}.
Table 1-1. The miRNAs and vascular function

<table>
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<tr>
<th>Vascular function</th>
<th>miRNAs</th>
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<td>Plasma cholesterol</td>
<td>miR-122</td>
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6.2 Shear stress and miRNAs

The endothelial function response to shear stress is regulated by transcription and signaling networks and also miRNAs. Recently, shear-regulated miRNA profiles of ECs were reported by 3 different groups. Wang et al. compared the miRNA profile of HUVECs under 24-hr PS to that of the static condition. Eight miRNAs were upregulated (including the miR-23b cluster) and 13 miRNAs were downregulated (including miR-17–92, miR-16, and miR-221 clusters) in ECs in response to PS. Specifically, miR-23b mediated shear-induced cell arrest by regulating the expression.
level of E2F1 and retinoblastoma (Rb) hypophosphorylation. Qin et al. reported that 35 miRNAs were significantly upregulated and 26 miRNAs were significantly downregulated in 12-hr laminar-flow–treated HUVECs as compared with static control cells. Specifically, the upregulated miR-19a regulated the inhibition of EC proliferation by targeting cyclin D response to steady laminar flow. In Weber et al.’s study, 13 miRNAs were upregulated in HUVECs exposed to prolonged unidirectional shear stress (USS, 24 h, 15 dyn/cm²). The miRNA with the greatest change was miR-21, which directly targets PTEN. HUVECs overexpressing miR-21 showed decreased apoptosis and increased eNOS phosphorylation and nitric oxide (NO*) production.

Recently, Fang et al. reported differential endothelial miRNA expression in vivo at athero-susceptible and -protected regions of the aorta and renal arteries in normal adult swine. The authors identified 7 downregulated and 27 upregulated miRNAs in athero-susceptible aortic arch (AA) endothelia as compared with athero-protected dorsal descending thoracic aorta (DT). miR-10a, the miRNA most significantly downregulated by athero-susceptible flow, despressed its targets MAP3K7 and βTRC and then upregulated IκB/NF-κB–mediated inflammation in this area. This study is the first to link miRNA regulation in arterial endothelium with endothelial athero-susceptibility or -protection signatures in vivo. Interestingly, the profile in this study also showed miR-92a is upregulated by athero-susceptible flow. This result is consistent with my second project described in Chapter 3.
7. miR-17-92 cluster

The miR-17-92 cluster, the first set of miRNAs, are commonly overexpressed in human tumors and have been implicated in the control of tumor growth, survival and angiogenesis. Recently, some studies indicated that this cluster also plays an important role in the regulation of endothelial function. In the following section, I summarize the genome organization, regulation and function of this cluster.

7.1 Genome Organization of the miR-17-92 cluster

The miR-17-92 cluster is a polycistronic miRNA gene within an 800-bp region located in the third intron of a primary transcript (C13orf25) on human chromosome 13\textsuperscript{133}. This cluster encodes 7 individual mature miRNAs (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b and miR-92a). Both the sequences of these mature miRNAs and their organization are highly conserved in all vertebrates. The mammalian genome has two miR-17-92 cluster paralogs because of ancient gene duplications. The miR-106b-25 cluster is located on human chromosome 7 and the miR-106a-363 cluster on the X chromosome. Each cluster contains homologous miRNAs to a subset of miR-17-92 components. According to their seed sequence, these mature miRNAs can be categorized into 4 miRNA families: the miR-17 family (miR-17, miR-20a/b, miR-106a/b, and miR-93); miR-18 family (miR-18a/b); miR-19 family (miR-19a/b); and miR-25 family (miR-25, miR-92a and miR-363)\textsuperscript{134}. 
The miR-17-92 cluster was first identified as a potential oncogene because of its genomic amplification and elevated expression in B-cell lymphoma cell lines\textsuperscript{151}. The following studies showed the enhanced expression of the miR-17-92 cluster in multiple hematopoietic malignancies and solid tumors\textsuperscript{152}. In addition to being expressed in cancer cells and tissue, miR-17-92 is upregulated in ischemic muscle or the heart and induced by pro-inflammatory cytokines such as interleukin 6 (IL-6) in ECs\textsuperscript{153}.\textsuperscript{150}
Expression profile studies, miRNA functional screens, and computational analyses demonstrated that the miR-17-92 cluster is a key component of the oncogenic and tumor suppressor network. The first evidence came from O'Donnell, who demonstrated that c-myc stimulates the transcription of the cluster in human B-cells and chronic myeloid leukemia cells\textsuperscript{154,155}. The oncogenes c-myc and n-myc are well known to regulate cell proliferation, growth and apoptosis, which is consistent with the function of miR-17-92 in promoting proliferation. In addition to myc, the E2F family, AML1 and Cyclin D1 are targeted by miR-17 and miR-20a and in turn can bind to the promoter region of miR-17-92, thereby establishing a negative feedback loop (Fig. 1-7)\textsuperscript{147,156,157}. In contrast, a recent study indicated that p53 could inhibit the expression of the miR-17-92 cluster and then promote apoptosis in a colon cancer cell line under hypoxia\textsuperscript{158}. p53 blocks the initiation of transcription because its binding site is overlapped with a TATA-box within the miR-17-92 promoter. Moreover, STAT3 could bind to the promoter region of the miR-17-92 cluster and then miR-17-5p and miR-20 target on bone morphogenetic protein receptor type II (BMPR2), which promoted cell survival in HPAEC under pulmonary hypertension or stimulated the pro-inflammatory cytokine IL-6\textsuperscript{153}. 
7.3 MiR-92a and EC function

Although miR-17-92 is frequently overexpressed in different types of tumors, no evidence exists of the direct effect of miR-92a on cell survival in the context of cancer. Moreover, crucial studies showing that the miR-17-92 cluster with a truncated cluster lacking miR-92a suggested that miR-92a does not necessarily augment lymphoma growth\textsuperscript{133}. In ECs, overexpression of miR-92a does not change EC apoptosis and proliferation\textsuperscript{160}.
To date, only one group reported miR-92a function in cardiovascular system\textsuperscript{160}. Bonauer \textit{et al.} demonstrated that miR-92a negatively regulates angiogenesis of ECs \textit{in vitro} and \textit{in vivo} by loss- and gain-of-function experiments. Since miR-92a was significantly upregulated after induction of ischemia in animal models \textit{in vivo}, the authors used antigomir, the modified antasense oligo of miR-92a, to determine the therapeutic effect of miR-92a inhibition. Indeed, systemic inhibition of miR-92a improved the functional recovery in models of hind-limb ischemia and acute myocardial infarction. By using mRNA expression array, the authors found the changed expression of several genes related to endothelial function. Specifically, the intergrin subunit 5 (ITGA5) was identified as a direct target of miR-92a in ECs; miR-92a is a crucial regulator of EC functions and vessel growth by mediation of cell–matrix interaction, anti-apoptotic signaling and cell migration. Interestingly, miR-92a overexpression in HUVECs suppressed the expression of several KLF2-regulated genes such as eNOS and TM\textsuperscript{160}. The lack of miR-92a binding sites in the 3’UTR of these genes suggests the involvement of a mechanism other than direct targeting of miR-92a. In chapter 3, the role of miR-92a in shear-regulated EC function is described.
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Chapter 2

Flow Activation of AMP-activated Protein Kinase Leading to The
Expression of Krüppel-like Factor 2

2.1 Abstract

Objective: Vascular endothelial cells (ECs) confer atheroprotection at locations of the arterial tree where pulsatile laminar flow (PS) exists with a high shear stress and a large net forward direction. We investigated whether the PS-induced expression of the transcription factor Krüppel-Like Factor 2 (KLF2) in cultured ECs and mouse vessel wall is regulated by AMP-activated protein kinase (AMPK).

Methods and results: the AMPK inhibition by siRNA had a significant blocking effect on the PS-induced KLF2 expression. The induction of KLF2 by PS led to augmentation of eNOS and suppression of ET-1, which can be reversed by KLF2 siRNA. In addition, PS induced the phosphorylation of ERK5 and MEF2, which increased the KLF2 expression. These mechanotransduction events were abrogated by the blockade of AMPK. Furthermore, the phosphorylation levels of ERK5 and MEF2, as well as the expression of KLF2, were significantly reduced in the aorta of AMPKα2 knockout mice when compared with wild-type control mice.
**Conclusion:** The flow-mediated AMPK activation is a novel KLF2 regulatory pathway in vascular endothelium that acts via ERK5/MEF2.

### 2.2 Introduction

Vascular endothelial cells (ECs) in the arterial tree are subjected to shear stress resulting from blood flow. ECs exposed to laminar flow patterns are spared from early lesions of atherosclerosis. In contrast, ECs at the arterial bifurcations and curvatures, where disturbed flow patterns exist, are susceptible to the development of atherosclerotic lesions. There is ample evidence indicating that the different patterns of shear stress associated with laminar vs. disturbed flows play a significant role in regulating endothelial phenotype and vascular homeostasis.

Krüppel-like factor 2 (KLF2) is a transcription factor whose expression is flow-dependent *in vitro* and *in vivo* and has been shown to regulate various EC functions related to inflammation, thrombosis, proliferation, and vascular tone\(^1-^3\). KLF2 belongs to the family of KLF zinc-finger transcription factors that are important regulators of cell differentiation and development\(^4\). Using microarray analysis, Dekker et al. demonstrated that KLF2 is upregulated by prolonged laminar shear stress and that it is expressed in ECs of atherosclerosis-resistant regions of the human aorta\(^1\).
Subsequent work by J. Lingrel and colleagues demonstrated that laminar shear stress induces transcriptional activation of KLF2 in ECs. Our previous studies showed that KLF2 was induced by the atheroprotective flow, but suppressed by atheroprone flow. KLF2 has been implicated in mediating the anti-inflammatory effects of flow, presumably by inhibiting pro-inflammatory transcription factors, such as ATF2, AP-1, and NFκB, which in turn regulate gene expression.

Functioning as a “fuel gauge” in multiple organ systems, AMPK is also important in the vessel wall. Nagata et al. showed that hypoxia activates AMPK in human umbilical vein ECs (HUVECs), as indicated by increased phosphorylation at Thr-172 of the AMPKα subunit. The suppression of AMPK signaling by a dominant-negative mutant of AMPK (AMPK-DN) inhibited vascular endothelial growth factor (VEGF)-enhanced EC migration and hypoxia-induced differentiation into tube-like structures. Notably, AMPK phosphorylates eNOS at Ser-1177/1179 and thereby augments the eNOS-derived NO bioavailability. We have previously demonstrated that shear stress regulates AMPK in ECs and that this may account for the NO bioavailability and cell cycle arrested in G0/G1 under steady laminar flow.
Based on the findings that both AMPK and KLF2 are regulated by atheroprotective flow and both have similar effects on EC biology, here we investigate whether AMPK activation is functionally linked to KLF2 expression in the vascular endothelium.

2.3 Materials and Methods

2.3.1 Cell culture and reagents

Human umbilical cord venous endothelial cells (HUVECs) were isolated from human umbilical cord veins with collagenase as previously described. The cells were cultured on 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% CO₂-95% air incubator at 37°C. Cells within passages 2-6 were used in the experiments.

Antibodies used in this study were purchased from the following commercial sources: anti-phospho-AMPKa Thr-172, anti-AMPKa, anti-phospho-ACC Ser-79, and anti-ACC (Cell Signaling Technology, Beverly, MA); anti-phospho-MEF2 Thr-312 (Abcam, Cambridge, MA), anti-phospho-ERK5 Thr-218/Tyr-220 (Millipore,
Billerica, MA) anti-β actin (Sigma, St. Louis, MO): anti-α tubulin (Sigma, St. Louis, MO). The anti-KLF2 antibody was obtained by immunization of the N-terminal region (aa 1-245) of human KLF2 fused to Fc. Serum was tested for KLF2 reactivity, and hybridoma supernatants were tested by ELISA assays. The specificity of antibody was tested by immunoblotting lysates collected from HEK293 cells transfected with cDNAs encoding KLF2, KLF4, KLF6, and KLF13. Immunoreactivity was detected only in cells transfected with KLF2. Compound C was from Calbiochem (La Jolla, CA). The siRNA targeting KLF2 sequence [nucleotides 1482-1502 (AATTTGTACTGTCTGCGGCAT) of the cDNA (GenBank Accession No. NM_016270)] was custom synthesized by Ambion (Austin, TX). The siRNA targeting AMPK-α1 and AMPK-α2 catalytic-subunits (Hs_PRKAA1_5_HP, Hs_PRKAA2_6 HP Validated siRNA) were purchased from QIAGEN (Valencia, CA). A negative control siRNA (Silencer Negative Control #1) and a positive control siRNA (Silencer FAM labeled GAPDH siRNA) for monitoring siRNA delivery efficiency were purchased from Ambion.

2.3.2 Flow experiments

A circulating flow system, previously described, was used to impose shear stress on a confluent monolayer of HUVECs seeded on a collagen I-coated glass slide
A reciprocating syringe pump was connected to the circulating system to introduce a sinusoidal component (frequency = 1 Hz) onto the shear stress. 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 shear flow (PS) was applied to cells with a shear stress of 12 ± 4 dyn/cm².

2.3.3 RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated with the use of Trizol reagent (Invitrogen). Reverse transcription was carried out with 3 μg of total RNA by the Superscript II reverse transcriptase (Invitrogen). The synthesized cDNA was used to perform real-time quantitative PCR (qPCR) with the iQ SYBR Green supermix (Bio-Rad, Hercules, CA) on the iCycler real-time PCR detection system (Bio-Rad). The sequences of primer sets were: KLF2, AGACCTACACCAAGAGTTCGCATC and CATGTGCCGTTTTCATGTGCAGC; eNOS, TGGTACATGAGCACTGAGATCG and CCACGTTGATTTCCACTGCTG; AMPK, GAATGGAAGGCTGGATGAAA and TTCTGGTGCGCATAGTTGG; endothelin-1 (ET-1), TCCTCTGCTGCTTACTGCTG and CAGAAAATCCACCCTTGTT; GAPDH, ATGACATCAAGAAGGTGGTG and CATACCAGGAAATGAGCGT.
2.3.4 Protein isolation and immunoblotting

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 100 μg/mL phenylmethanesulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na₃VO₄), 2 mM sodium fluoride (NaF), and 150 KIU/mL aprotinin. Proteins for in vivo experiments were isolated from the aorta harvested from control (C57BL/6J) or AMPKα2-knockout mice. Equal amounts of denatured protein lysates were separated on SDS polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After 1-hr 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), followed by ECL detection (GE Healthcare, Piscataway, NJ). The density of the protein bands was quantified by using the ImageJ software (National Institutes of Health).

2.3.5 siRNA transfection

HUVECs were transfected with siRNA using the siPORT NeoFX transfection reagent (Ambion). The cells were incubated with the siRNA-transfection reagent complex in 2 mL of culture media on a glass slide (for flow experiments) for 12 hr.
Fresh culture media were then added and replaced again at 24 hr. Twenty-four or 48 hr post-transfection, the cells were used in PS experiments.

2.3.6 Animal Experiments

All experiments were performed according to institutional protocols (University of California) using 8-week-old male mice. After euthanization, the abdominal aorta was harvested from C57BL6J wild-type mice and AMPKα2−/− mice (a gift from Dr. B. Viollet in Institute Cochin, Université Paris 5). Aortas were then homogenized, and two aortic extracts were pooled to yield one sample, which was then analyzed for protein expression and phosphorylation by immunoblotting. Three independent experiments were performed (six animals total) for each study.

MEF2 en face staining was performed according to protocols previously published. The aortas used were isolated from 10-week-old male mice. The primary antibody was rabbit anti-phospho-MEF2 antibody (Abcam, Cambridge, MA) and the second antibody is Alexa 647-labeled goat anti-rabbit IgG (Invitrogen, San Diego, CA). Rabbit IgG was used as negative control. The quantitative analysis was performed using LCS Lite software (version 2.0).
2.3.7 Statistical analysis

All data were analyzed by Student’s *t* test (for paired testing between two groups used only once) or two-way ANOVA (for testing of multiple groups). Results are expressed as mean±SD from at least three independent experiments. *P* values < 0.05 were considered to be statistically significant.

2.4 Results

2.4.1 Pulsatile shear flow activates AMPK and induces KLF2 expression in ECs

We have previously shown that a laminar steady flow activates AMPK in bovine aortic endothelial cells (BAECs)\(^{15,16}\). Here, we investigate the effect of PS in regulating AMPK activities in HUVECs. As shown in Fig. 2-1A, PS with a shear stress of 12 ± 4 dyn/cm\(^2\) and a frequency of pulsatility at 1 Hz increased the phosphorylation of AMPK Thr172 and its target ACC Ser79 as early as 5 min. The increased phosphorylation of AMPK and ACC peaked at 1 hr and remained elevated for at least 4 hr. The elevation could lasted for 16 hr (data not shown). Thus, in contrast to the transient AMPK activation under steady flow in BAECs\(^{15,16}\), the phosphorylation of AMPK under PS was sustained in HUVECs (Fig 1A). Under the same experimental conditions, PS also caused the induction of the KLF2 gene at both mRNA and protein levels, as determined by real-time qPCR and Western blot analysis,
respectively. As seen in Figure 1B, consistent with the time course of PS-mediated AMPK activation, the increase in KLF2 mRNA began 1 hr after the initiation of PS and remained at a high level for more than 4 hr. Concomitantly, there was an increase in KLF2 protein expression 4 hr after the initiation of PS (Figs. 1C).

2.4.2 AMPK regulates KLF2 expression in response to PS

Since both AMPK and KLF2 play important roles in flow-regulated eNOS activities/expression, we investigated whether AMPK activation is critical for the induction of KLF2 under PS. We inhibited AMPK activity by treating ECs with Compound C (an AMPK antagonist) or knocked down AMPK expression using siRNA. The levels of KLF2 expression in control and treated cells exposed to PS or maintained under static (no flow) conditions were then assessed. As shown in Fig. 2-2A, Compound C reduced basal expression of KLF2 in static cells, and abolished the shear-induction of KLF2 expression at 4 hr. The negative regulation of KLF2 by Compound C was concentration-dependent (supplementary Fig. 2-S1). Similar to the effect of Compound C, siRNA knockdown of either AMPKα1 or AMPKα2 significantly reduced the 4-hr shear-induction of KLF2, with a marginal decrease in the basal expression of KLF2 (Fig. 2-2B,C). These results confirmed that AMPK activation is critical for the induction of endothelial KLF2 in response to PS.
2.4.3 KLF2 regulates the eNOS and ET-1 expression in response to PS

In order to further delineate the effects of PS-induced KLF2 on its downstream targets, we first determined the optimal siRNA concentration, which was able to knockdown KLF2 expression in HUVECs with the efficiency of ~80% suppression (supplementary Fig. 2-S2). We next examined the expression of two known targets of KLF2, eNOS and ET-1. As seen in Fig. 2-2D, silencing of KLF2 expression led to a reduced level of eNOS in static cells and also a significant decrease in eNOS induction in ECs exposed to PS. Knockdown of KLF2 also resulted in the abolishment of PS-suppression of ET-1, which remained at basal level in the presence of PS (Fig. 2-2E). These results suggest that KLF2 acts as a key transcription factor mediating the PS-dependent expression of eNOS and ET-1, two critical genes for the maintenance of vascular homeostasis and vasomotor tone.

2.4.4 AMPK-KLF2 signaling in cultured ECs

To further elucidate the mechanism of KLF2 upregulation by AMPK, HUVECs were treated with AICAR (an AMPK agonist), or infected with Ad-AMPK-CA expressing a constitutively active form of AMPK. As shown in Fig. 2-3A, KLF2 expression increased significantly in ECs treated with AICAR and those infected with Ad-AMPK-CA as indicated by real-time Taqman PCR.
Since the MEK5/ERK5/MEF2 pathway regulates the flow-induced KLF2, and the phosphorylation of MEF2 at Thr312 by ERK5 increases the rate of MEF2-mediated transcription\(^3\), \(^20\), we tested whether AICAR-induction of KLF2 mRNA is mediated by MEK5 and MEF2. As seen in Fig. 2-3B, C, the increase in KLF2 in ECs treated with AICAR was abrogated by infection with Ad-MEK5-DN (the dominant negative mutant of the ERK5-upstream kinase MEK) or Ad-MEF2-DN expressing a dominant negative mutant of MEF2.

We then explored whether AMPK activation can increase the phosphorylation of MEF2 through the MEK5/ERK5 pathway. The AICAR-induced phosphorylations of AMPK Thr172, ERK5 Thr218, and MEF2 Thr312 were markedly decreased in ECs in which AMPK\(\alpha_1\) was knocked down in comparison to those in cells treated with control siRNA (Fig. 2-4A). The knockdown of AMPK\(\alpha_2\) also resulted in similar reductions (Fig. 2-4B). In consistent with these results, the AICAR-increased ERK5 phosphorylation was inhibited by Compound C (Fig. 2-S3A) and AD-MEK5-DN (Fig. 2-S3B). More importantly, Compound C treatment was able to inhibit the PS flow-increased AMPK activity and ERK5 and MEF2 phosphorylation (Fig. 2-4C). These results established that the activation of KLF2 by AMPK is mediated through ERK5 and MEF2 in cultured ECs.
2.4.5 AMPK-KLF2 signaling *in vivo*

To examine the role of the AMPK-KLF2 pathway *in vivo*, KLF2 expression was studied in AMPK knockout mice. AMPK α2 seems to play a more important role than AMPK α1 in fuel sensing and downstream signaling *in vivo* \(^{19,21}\). Although we showed that both AMPK isoforms (α1 and α2) have similar effects on KLF2 expression in cultured endothelial cells, we studied the AMPK-KLF2 signaling in α2\(^{-/-}\) mice due to *in vivo* results of α2\(^{-/-}\) mice studies in fuel sensing and downstream signaling are more robust \(^{19,21}\), as well as the availability of the α2\(^{-/-}\) mice. To this end, aortas were harvested from C57BL6 wild-type and AMPKα2\(^{-/-}\) mice, protein lysates were prepared and use for immunoblotting analysis to assess the expression levels of KLF2 and its downstream target gene eNOS and the phosphorylation state of its upstream regulators AMPK, ERK5, and MEF2. As shown in Fig. 2-5A, aortas from AMPKα2\(^{-/-}\) mice exhibited reduced expressions of KLF2 and eNOS when compared to aortas from wild-type control mice. Moreover, aortas from AMPKα2\(^{-/-}\) mice displayed a reduced phosphorylation level of AMPK, which was accompanied by decreases in ERK5 and MEF2 phosphorylations (Fig 5B). p-MEF2 *en face* immunostaining confirmed that the level of MEF2 phosphorylation in the thoracic endothelium of AMPKα2\(^{-/-}\) mice was lower than that in the wild-type controls (Fig
Collectively, these results indicate that AMPK is a regulator of KLF2 expression in the vascular wall *in vivo*, and support our *in vitro* mechanistic studies.

### 2.5 Discussion

In order to elucidate the roles of wall shear stress in regulating KLF2 expression and modulating vascular homeostasis, we investigated additional mechanotransduction mechanisms by which shear stress upregulates KLF2 expression in vascular ECs. Data collected from both *in vitro* and *in vivo* experiments revealed that AMPK is an upstream kinase regulating the PS-induced endothelial KLF2 expression. Our results also link the AMPK regulation to the ERK5/MEF2/KLF2 pathway, thus providing a detailed mechanistic explanation of KLF2 induction by PS via AMPK, ERK5, and MEF2 signaling.

AMPK, functioning as a cellular energy sensor, plays important roles in vascular biology. Many stimuli such as hypoxia, estrogen, shear stress, adiponectin and statins can act on the vascular EC to activate AMPK, which in turn phosphorylates eNOS Ser1177 to enhance the NO bioavailability in ECs. KLF2, upregulated by the atheroprotective laminar shear stress, causes elevation of eNOS expression and integration of the flow-mediated endothelial atheroprotective phenotype. The mechanism underlying the shear stress-induced KLF2 has been
investigated by several groups. Lingrel et al. showed the importance of a phosphoinositide-3-kinase (PI3K)-dependent/Akt-independent pathway in the activation of KLF2 by shear stress and the involvement of nucleolin\textsuperscript{5,25}. Parmar et al. showed that the flow-dependent activation of KLF2 is mediated via a MEK5/ERK5/MEF2 signaling pathway, suggesting the involvement of MAPK family in the regulation of KLF2\textsuperscript{3}. Van Thienen et al. demonstrated that shear stress sustain KLF2 expression through mRNA stabilization via PI3K dependent pathway\textsuperscript{30}. However, Lingrel’s group demonstrated that KLF2 promoter can be transcriptional activated by shear stress, and the shear responsive element is located at position of -195 to -95 bp in front of the transcription start site\textsuperscript{5}. These results indicate the both mRNA stabilization and transcription are important for the shear-regulation of KLF2 expression. Here, we identify the AMPK as a key upstream regulator for ERK5 signaling that leads to KLF2 expression under shear. In RAW 264.7 cells, the PI3K inhibitor Wortmannin has been shown to abolish the nicotine-induced AMPK phosphorylation \textsuperscript{26}, indicating the potential interaction between PI3K and AMPK. However, in our hands, blocking PI3K with Wortmannin or LY2943002 had little effect on shear-induced AMPK activation and KLF2 expression (data not shown). Further investigation will be needed to understand the role of PI3K in shear-regulated
AMPK-KLF2 pathway. It has been shown in ECs that AMPK is activated by two kinases, Peutz-Jeghers syndrome kinase LKB1 and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase kinase (CaMKK)\textsuperscript{27,28}. Our earlier work\textsuperscript{15} identified LKB1 as the upstream kinase causing AMPK Thr172 phosphorylation under steady shear, and we postulate here that PS activates AMPK via the same pathway. The mechanisms by which shear stress modulates the KLF2 expression, however, are complex and involve multiple signaling pathways.

Since both AMPK and KLF2 are regulated by shear stress and able to augment nitric oxide production, the present study focused on elucidating the role of the AMPK pathway in regulating the shear-induction of KLF2 expression. In contrast to the previous observation that steady laminar shear stress led to a transient activation (5-30 min) of AMPK in cultured BAECs\textsuperscript{16}, we found that PS (12 ± 4 dyn/cm\textsuperscript{2} laminar shear stress with 1 Hz pulsatility) caused a 4-hr sustained activation of AMPK in cultured HUVECs, and such a sustained AMPK activation can last up to 16hr (data not shown). Our studies of steady laminar flow in HUVECs also demonstrated a sustained AMPK phosphorylation up to 16 hr (data not shown). The different of shear-induced AMPK activation is likely due to the variations of endothelial cell sources, bovine vs. human. In the current study, we identified the necessity of AMPK
for the shear-induced phosphorylation of ERK5 and MEF2, and expression of KLF2 and its downstream target genes. We also demonstrated that activation of AMPK (by AICAR) is sufficient for the induction of KLF2 expression, as well as the phosphorylation of its upstream signaling molecules ERK5 and MEF2. These results indicate an important role of AMPK in modulating the shear-activation of the ERK2/MEF2/KLF2 pathway, and establish a novel link between the AMPK and the ERK5 signaling pathways.

To further validate this newly identified relationship between AMPK and ERK5/MEF2/KLF2 pathway, in vivo experiments were performed. We investigated the expressions of KLF2 and the activity levels of ERK5 and MEF2 in AMPK α2 knockout (AMPKα2/-) mice. While AMPK α1 is more abundant in ECs, α2 also plays an important role in EC functions, e.g. migration and tube formation. Although our in vitro studies mainly addressed AMPKα1, it appears that AMPKα2 plays a similar role (see Figs. 2C and 3E). Since comparison of AMPK α1/- versus α2/- mice demonstrated that α2/- plays more important roles in fuel sensing and downstream signaling in vivo and due to the availability of the α2/- mice, we studied the AMPK-KLF2 signaling in the vessels of α2/- mice. Our results demonstrated that KLF2 expression was significantly reduced and the
phosphorylations of ERK5, MEF2, and eNOS were greatly attenuated in the aortas of AMPKα2-/- mice. Due to the EC-specificity of KLF2, we are confident that the reduction aortic KLF2 expression in α2-/- mice occurs in endothelium. To further demonstrate vascular location of the reduction of MEF2 activation, we performed en face staining of phospho-MEF2 to confirm the reduction in aortic endothelium.

In summary, this study suggested AMPK is an upstream signaling molecule of the ERK5/MEF2/KLF2 pathway, and documented the requirement of AMPK for the activation of ERK5/MEF2 signaling pathway and the expression of KLF2 in the mouse vasculature. Our findings established that the PS-activation of the AMPK/ERK5/MEF2/KLF2 pathway plays an important role in the regulation of vascular homeostasis.
References


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Fig. 2-1. PS activates AMPK and induces KLF2 in cultured ECs. Confluent HUVECs were exposed to PS for the indicated times. Static controls are represented as “time 0”. The mean shear stress was 12 dyn/cm² (represented by dash line) with ± 4 dyn/cm² oscillation at 1 Hz. (A, C) ECs were lysed, the lysates were separated by SDS-PAGE, and the membranes were probed with antibodies as indicated. (B) RNA samples were isolated, and the levels of KLF2 mRNA were determined and quantified by real-time PCR, with the results normalized by GAPDH. The quantitative graphs below show the ratios of phosphorylated AMPK or ACC to total AMPK or ACC, respectively, in (A), KLF2 mRNA to GAPDH mRNA in (B), and KLF2 to α-tubulin in (C) at various time points. The shear results were normalized by that of the corresponding static control. The data represent mean ± SD from 3 independent experiments. * indicates p<0.05 between the two groups being compared.
Fig. 2-2. AMPK regulates the PS-induced KLF2 and its target genes. HUVECs were treated with Compound C (15 µM) for 30 min (A), transfected with AMPKα1 siRNA (B), AMPKα2 siRNA (C), or KLF2 siRNA (D, E) for 48 hr. Control siRNA were used in the parallel experiments. The ECs were then subjected to PS for 4 hr. RNA samples were isolated and the levels of KLF2 (A-C), eNOS (D), and ET-1 (E) mRNA were quantified by real-time qPCR. The results were normalized with GAPDH. The data represent mean ± SD from 3 separate experiments. * indicates $p<0.05$ between the two groups being compared.
Fig. 2-3. AMPK activates KLF2 via the ERK5-MEF2 pathway in cultured ECs. (A) KLF2 and GAPDH transcripts were analyzed by quantitative RT-PCR in HUVECs treated with 1 mM AICAR for 8 hr (left panel), and with Ad-null or Ad-AMPK-CA for 24 hr (right panel). (B,C) HUVECs were infected with Ad-GFP together with Ad-MEK5-DN or Ad-MEF2-DN followed by AICAR stimulation. Total RNA was extracted and the levels of KLF2 and GAPDH mRNA analyzed by quantitative RT-PCR. The data represent mean ± SD from 3 separate experiments.
**Fig. 2-4. AMPK upregulates ERK5-MEF2 in ECs.** AMPKα1 (A) or AMPKα2 (B) was knocked down by siRNA as described in Fig. 2-2. The cells were then treated with 1 mM AICAR or vehicle control 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). The bar graphs below are the statistic analysis of the ratio of p-ERK5 or p-MEF2 to that of total ERK5 and MEF2 respectively. The data represent mean ± SD from 3 separate experiments. * indicates \( p < 0.05 \) between the two groups being compared. (C) HUVECs were treated with Compound C (15 µM) for 30 min and then subjected to PS flow for 0.5 and 1.5 hrs. Cell lysates were analyzed by Western blotting with anti-p-ACC(Ser79), anti-p-ERK5(Thr218) and p-MEF2(Thr312). The data represent mean ± SD from 3 separate experiments. * indicates \( p < 0.05 \) between the two groups being compared.
Fig. 2-5. AMPK mediates KLF2 expression via the ERK5-MEF2 pathway in mouse models. Expression of KLF2 and phosphorylations of ERK5 and MEF2 are higher in the aorta of wild-type mice than those in AMPKα2−/− mice. Two aortic extracts from the same line were pooled and subjected to immunoblotting to determine (A) the levels of KLF2, eNOS, and α-tubulin expression, and (B) the phosphorylation of AMPK, ERK5, and MEF2; (C) en face immunostaining of phospho-MEF2 in thoracic endothelium. Fig. 2-5C: The thoracic aortas from C57BL6 and AMPKα2−/− mice were immunostained with anti-phospho-MEF2 followed by Alexa 647-conjugated secondary antibody. Nuclei were counterstained with DAPI. The immunostained images of anti-phospho-MEF2 and DAPI were obtained by confocal microscopy. Shown are representative images from 4 animals in each group. * indicates p<0.05 between the wild-type and AMPKα2−/− samples.
Fig. 2-S1. Inhibition of KLF expression by Compound C is concentration dependent. (A) Compound C at 20, 15, or 10 mM was added to cultured HUVECs for 30 min before cells were subjected to 1-hr PS flow. A corresponding concentration of Compound C was included in the circulating media during the flow. The inclusion of Compound C completely inhibited PS flow activation of p-ACC. The high concentration (20 and 15 mM) of Compound C also suppressed the basal level expression of KLF2 (B) both in static ECs and in those under 1-hr PS flow. However, 10 µM of Compound C kept KLF2 expression at basal level in static condition, but significantly reduced the level of KLF2 induction under 1-hr 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).
Fig. 2-S2. Optimization for KLF2 knockdown. Different amounts of KLF2 siRNA at 100 to 800 pmoles/slide were used to transfect HUVEC for 48hr. The RNA samples were isolated and the expression of KLF2 was quantified by real-time qPCR, and normalization to that of GAPDH. The optimal knock-down efficiency (~80%) was found to be at 400 pmoles/slide, which is equivalent to 40 nM.
Fig. 2-S3. AICAR-induced ERK5 phosphorylation is compound C sensitive and MEK5 dependent. (A) AMPK was blocked by Compound C (CompdC) to test the role of AMPK in the downstream signaling events. The cells were then treated with 1 mM AICAR or vehicle control for 1 hr before being lysed. (B) Cells were infected with Ad-GFP or Ad-MEK5-DN for 24 hr before treatment with 1 mM AICAR for 1 hr. Cell lysates were analyzed by Western blotting with anti-p-AMPK(Thr172), or anti-p-ERK5(Thr218). The same blots were stripped and re-blotted with anti-a-tubulin to demonstrate the equal loading of proteins for the lanes.
Chapter 3

Flow-regulation of Krüppel-like Factor 2 Is Mediated by MicroRNA-92a

3.1 Abstract

Background—Upregulated by atheroprotective flow, the transcription factor Krüppel-like factor 2 (KLF2) is crucial for maintaining endothelial function. MicroRNAs (miRNAs) are non-coding small RNAs that regulate gene expression at the post-transcriptional level. We examined the role of miRNAs, particularly miR-92a, in the atheroprotective flow-regulated KLF2.

Methods and Results—Dicer knockdown increased the level of KLF2 mRNA in human umbilical vein endothelial cells (HUVECs), suggesting that KLF2 is regulated by miRNA. In silico analysis predicted that miR-92a could bind to the 3’ untranslated region (3’UTR) of KLF2. Overexpression of miR-92a precursor (pre-92a) decreased the expression of KLF2 and the KLF2-regulated endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM) at mRNA and protein levels. A complementary finding is that a miR-92a inhibitor (anti-92a) increased the mRNA and protein expression of KLF2, eNOS, and TM. Subsequent studies revealed that, atheroprotective laminar flow downregulated the level of miR-92a to induce KLF2, and the level of this flow-induced KLF2 was reduced by pre-92a. Furthermore, miR-92a level was lower in HUVECs exposed to the atheroprotective pulsatile shear flow (PS) than under atheroprone oscillatory shear flow.
Anti-Ago1/2 immunoprecipitation coupled with RT-PCR revealed that PS decreased the functional targeting of miR-92a/KLF2 mRNA in HUVECs. Consistent with these findings, miR-92a level was lower in the endothelium of atheroprotective than atheroprone areas of the mouse aorta. Furthermore, mouse carotid arteries receiving pre-92a exhibited impaired vasodilatory response to flow.

**Conclusions**—Atheroprotective flow patterns decrease the level of miR-92a, which in turn increases KLF2 expression to maintain endothelial homeostasis.

### 3.2 Introduction

The vascular endothelium, located at the interface between the circulating blood and the vessel wall, is exposed to shear stress resulting from blood flow. The endothelium in straight parts of the artery tree is subjected to pulsatile shear stress with a significant forward direction, which is an important physiological stimulus enhancing vessel compliance and conferring anti-thrombotic, -adhesive, and -inflammatory effects. In contrast, disturbed flow patterns at the arterial bifurcations and curvatures can cause endothelial dysfunction, which initiates atherosclerosis\(^1\text{-}^4\).

The transcription factor Krüppel-like factor 2 (KLF2) is a critical integrator for endothelial lineage development and vascular functions (reviewed in references 5-7). KLF2 can be highly induced by shear stress with a significant forward direction (whether steady laminar flow or pulsatile flow), as well as HMG-CoA reductase inhibitors (i.e., statins). Previous studies have shown that KLF2 can be induced by shear stress at both transcriptional and post-transcriptional levels. The induction of KLF2 mRNA by laminar
shear stress has been suggested to be mediated through the MEK5/ERK5/MEF2 pathway, which is AMPK dependent\textsuperscript{8-13}. Shear stress increases the stability of KLF2 mRNA, with attendant elevation of KLF2 protein\textsuperscript{14}. The molecular basis of such an increased stability of KLF2 mRNA has not been established.

MicroRNAs (miRNAs) are non-coding small RNAs that regulate gene expression at the post-transcriptional level\textsuperscript{15-17}. Ranging from 18 to 24 nt (22 nt in general), miRNAs bind to the 3’ untranslated region (3’UTR) of their target mRNAs, with ensuing suppression of protein translation or enhancement of mRNA degradation. Analysis of miRNAs expressed in arterial walls and cultured vascular endothelial cells (ECs) has shown that approximately 40 miRNAs are highly expressed in ECs\textsuperscript{18-20}. These miRNAs play important roles in regulating blood vessel development, wound healing, redox signaling, inflammatory responses, and angiogenesis (reviewed by reference 21). Using miRNA microarrays, we and others have shown that laminar shear stress upregulates a set of miRNAs in ECs \textit{in vitro} and \textit{in vivo}\textsuperscript{22-25}. Functionally, miR-21 increases NO bioavailability and reduces EC apoptosis\textsuperscript{22}, miR-19a and miR-23b regulate EC proliferation\textsuperscript{23, 24}, and miR-10a is anti-inflammatory\textsuperscript{25}. Several recent reports demonstrated that the miR-17~92 cluster regulates cardiac development, EC proliferation, and angiogenesis (reviewed by reference 26). In this cluster, miR-92a was the first miRNA identified to regulate angiogenesis. Loss- and gain-of-function experiments showed that miR-92a inhibited angiogenesis \textit{in vitro} and \textit{in vivo}\textsuperscript{27}. Importantly, miR-92a overexpression in human umbilical vein ECs (HUVECs) suppressed the expression of several KLF2-regulated genes such as endothelial nitric oxide synthase (eNOS) and
thrombomodulin (TM)\textsuperscript{27}. The lack of miR-92a binding sites in the 3’UTR of these genes suggests that a mechanism other than direct targeting of miR-92a is involved.

Here, we report that atheroprotective flow causes downregulation of miR-92a in ECs, which in turn elevates the KLF2 mRNA. The functional consequences of the flow-regulated miR-92a/KLF2, including the augmentation of eNOS and TM levels and increase of NO bioavailability, can be mimicked by inhibition of miR-92a. These findings have led to a new paradigm of mechanotransduction that involves the shear modulation of miRNAs, KLF2 expression, and vascular homeostasis.

3.3 Materials and Methods

3.3.1 Cell culture and reagents

HUVECs were cultured as described in chapter 2. BAECs and human embryonic kidney 293 (HEK293) cells were cultured in DMEM containing 10% FBS. All cell cultures were kept in a humidified 5% CO\textsubscript{2}-95% air incubator at 37°C.

Western blot analysis was performed with the use of antibodies against eNOS (Cell Signaling, Beverly, MA), TM (ABcam, Cambridge, MA), KLF2, histone H1 (Santa Cruz Biotechnology, Santa Cruz, CA), and α-tubulin (Sigma, St Louis, MO). The anti-KLF2 antibody was obtained by immunizing rabbits with 2 separate synthetic peptides (CALSEPIPSFST-amide and Ac-ALSEPIPSFST-Ahx-C-amide) corresponding to human KLF2. The antiserum was purified by affinity column and tested by ELISA.
3.3.2 miRNA and mRNA Real-time quantitative reverse-transcription polymerase chain reaction analysis

Quantitative Real-time PCR (qRT-PCR) was performed to measure the level of miR-92a with the TaqMan miRNA reverse transcription kit and the TaqMan miRNA assay kit according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA) with IQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). U6 small nucleolar RNA was used as the housekeeping small RNA reference gene. For the quantification of KLF2, eNOS, and TM mRNA, qRT-PCR was performed with the iQ SYBR Green supermix (Bio-Rad). The sequences of primer sets were: KLF2, AGACCTACACCAAGAGTTCTCGATC and CATGTGCCGTTTCATGTGCAGC; eNOS, TGGTACATGAGCACTGAGATCG and CCACGTTGATTTCCACTGCTG; GAPDH, ATGACATCAAGAAGGTGGTG and CATACCAGGAATGAGCCTTG. Expression levels of miRNA and mRNA were quantified employing the 2 (-ΔΔCT) relative quantification method.

3.3.3 Knockdown and overexpression of miR-92a and KLF2

miR-92a was knocked down by using anti-92a (Ambion Inc, Austin, TX). The overexpression of miR-92a was achieved by transfecting cells with miR-92a precursor (pre-92a) (Ambion). Dicer1 was knocked down by using small interfering RNA (siRNA) obtained from Qiagen (Valencia, CA). For KLF2 overexpression in ECs, 0.4 g pcDNA-CMV-KLF2 was used to transfect 10^6 cells. The transfections were carried out by use of lipofectamine 2000 (Invitrogen, Carlsbad, CA).
3.3.4 Shear stress experiments

A parallel-plate flow system was used to impose shear stress on ECs cultured in flow channels as described in chapter 2. Laminar flow, pulsatile shear flow (PS), and oscillatory shear flow (OS) were applied to ECs with shear stresses of 12 dyn/cm², 12 ± 4 dyn/cm², and 0 ± 4 dyn/cm² (frequency = 1 Hz), respectively.

3.3.5 Plasmid construction and luciferase assay

The full length of human KLF2 3’-UTR sequence was amplified and inserted to the downstream of the luciferase reporter gene of the pMIR-REPORT vector (between Hind III and Spe I site) (Ambion). The FLAG-KLF2 plasmid was constructed by fusing a CMV-driven FLAG tag with KLF2 cDNA (including 3’UTR). The primer set: Forward (EcoRI): cgggaatctATGGCGCTGAGTGAACCC and Reverse (BamHI): cgcggtacctAACCAACCCAGCAAAATC. Luc-KLF2 (Luc-mut) and FLAG-KLF2 (mut) with a mutated miR-92a binding site were created by using QuickChange site-directed mutagenesis (Stratagene, La Jolla, CA). The sequences of the mutagenic oligonucleotides were: GCCACTTTAATTATTCGGGCCATTATTGGAAAAACAAAAACTCG (sense) and CGAGTTTTGTATTTTCAAAATGCGCAAAATTTAAGTGCG (anti-sense). The deletion of miR-92 binding site in pMIR-KLF2-3’UTR was constructed by two-step PCR. The sequences of the primer sets were: GCCACTTTAATGCACACAAAACACTCGTCAAGG, CCTTGACGAGTTTTTGGGTGCATTAAGTGCG. The miR-92a reporter (Luc-92a) contained a luciferase reporter and 2 copies of sequences complementary to miR-92a (Luc-2xmiR92a). The reporter constructs were co-transfected with pre-92a or anti-92a
(20 nM) into HEK293 cells or BAECs by use of lipofectamine 2000 (Invitrogen). Luciferase expression was measured by luciferase reporter and β-galactosidase enzyme assays (Promega, Madison, WI).

3.3.6 NO bioavailability

HUVECs were transfected with anti-92a or BAECs were co-transfected with pre-92a and pcDNA-CMV-KLF2 for 48 hr and then the medium was replaced by phenol-free and serum-free DMEM. After 8 hrs, the medium samples were removed for immediate determination of total nitrate/nitrite concentration with nitrate/nitrite fluorescence assay kit (Cayman Chemicals).

3.3.7 In situ hybridization

To detect miR-92a expression, the aortic arch and thoracic aorta segment were processed and stained by modified protocol29. In brief, the mice were perfused with 10 ml of 4% paraformaldehyde (PFA) after the sacrifice. Aortas were collected and immersed in 4% PFA for 24 h at 4°C, then in 0.5 M sucrose at 4°C for 48 h. For inactivation of enzymes in tissues, samples were acetylated by incubating for 30 min in a 1 ml solution of freshly prepared acetylation solution, containing 0.06N HCl, 1.2% (v/v) triethanolamine and 0.6% (v/v) acetic anhydride. the samples were pre-hybridized with 500 μl of hybridization buffer containing 50% formamide, 5x SSC, 1x Denhardt’s solution, 0.3M NaCl, 20mM Tris-HCl, pH8.0, 5mM EDTA, 10mM NaPO4, pH8.0, 10% Dextran Sulfate, 500 μg/ml yeast tRNA (Sigma), at 57°C for 0.5 h. For hybridization, 5 pmol of FITC-labeled LNA probe diluted in 100 μl of hybridization buffer were applied.
The samples were incubated in a sealed humidified chamber for 4 h at a temperature 20°C below the TM of the LNA oligonucleotide probe. The samples were washed twice in 1 ml of a solution containing 50% formamide, 1x SSC, and 0.1% Tween-20 for 30 min at the same temperature as probe hybridization twice. Finally, samples were washed in 1.5 ml 0.2x SSC for 15 min and once in 1 ml of 0.1% Tween in PBS at room temperature. Then the segments were incubated in anti-FITC-HRP (PerkinElmer Life Sciences) diluted in blocking solution overnight at 4°C. The samples were incubated with 100 μl of TSA Plus FITC System working solution for 10 min at 25°C in the dark according to the manufacturer’s protocol (PerkinElmer Life Sciences). The samples were then washed three times in TNT buffer. Aortas were opened and mounted on slides with mounting medium containing DAPI (Invitrogen, CA) and samples processed for microscopy.

3.3.8 Local oligo delivery

Chemically modified oligonucleotides (antagomir) comprising a sequence complementary to the mature miR-92a (anti-miR92a) were used to inhibit miR-92a activity. All experiments were performed according to institutional protocols (University of California) using 8-week-old male mice. To deliver antagomir-92 and precursor miR-92 into vascular tissue and to avoid any potential systemic side effects, an established local oligo delivery model via F-127 pluronic gel was used as previously described.\(^3\) Briefly, 15 μg of these oligonucleotides (Dharmacon, Lafayette, CO) were mixed with 50 μl 25% F-127 pluronic gel (Sigma) at 4°C. Immediately after anesthesia and incision, carotids were locally applied by the gel mixture. Pluronic poloxamer gel is liquid at 4°C.
but rapidly solidifies at 37°C when in contact with mouse tissues. It is hydrophilic and degrades rapidly in an aqueous environment. After gelification, the skin was reapproximated by a reabsorbable suture.

3.3.9 Vasodilation

The carotid arteries were isolated immediately after sacrifice and were mounted on two glass cannulae in a perfusion myograph chamber which is connected to the SoftEdge Acquisition Subsystem. The vessel chamber was superfused with warmed PSS containing 130mM NaCl, 10mM HEPES, 6mM glucose, 4mM KCl, 4mM NaHCO₃, 1.8 mM CaCl₂, 1.18 mM KH₂PO₄, 1.2mM MgSO₄, and 0.025mM EDTA, pH7.4. Carotid arteries were imaged by a video camera attached to a microscope. A video dimension analyzer (Living System) measured external diameter and data was collected via BioPac MP100 hardware and Biopac AcqKnowledge software (BioPac, Goleta, CA). The arteries were maintained at an intraluminal pressure of 100mmHg for the duration of the experiment. The vessels were equilibrated for 30 min before extraluminal administration of 1 μM phenylephrine. After the maximal constriction, flow rate was increased to 400 μl/min, which corresponded to a shear stress of 12dyn/cm². The diameter changes induced by flow will be recorded.

3.3.10 Computational analysis for KLF2-regulated miRNAs

The transcriptional start sites (TSSs) of the selected miRNAs were obtained from mirStart database (http://mirstart.mbc.nctu.edu.tw/), which contains the predicted promoters of human miRNAs. The miRNA promoters were identified by the supports of
several experimental datasets derived from TSS-relevant experiments, including CAGE tags, TSS Seq tags and ChIP-seq of H3K4me3 enrichment.

JASPAR\textsuperscript{31} was utilized to identify the potential binding sites of KLF2 within the promoter regions (flanking -3000\textendash +500 according to TSS) of the reported shear-regulated miRNAs. The position weighted matrix (PWM) of KLF4 was used to identify KLF2-regulated miRNAs since the binding motifs of KLF2 and KLF4 are highly similar.

3.3.11 Statistical analysis

All data were analyzed by Student’s \textit{t} test (for paired testing between two groups used only once). Results are expressed as mean\textpm SD from at least three independent experiments. \( P \) values < 0.05 were considered to be statistically significant.

3.4 Results

3.4.1 miRNAs are involved in the regulation of KLF2 expression.

To investigate whether the shear stress-induction of KLF2 can be mediated at the post-transcriptional level, HUVECs were pre-sheared with laminar flow for 6 hr and then treated with 5,6-dichloro-1-\( \beta \)-D-ribobenzimidazole (DRB) to terminate transcription so that KLF2 mRNA stability could be monitored. As shown in Fig. 3-1A, the degradation rate of KLF2 mRNA in HUVECs exposed to laminar flow was much slower than that under static controls. To explore whether miRNAs are involved in the regulation of KLF2, we knocked down Dicer to block the miRNA biogenesis and found that the levels of KLF2 mRNA and protein increased (Fig. 3-1B,C). The levels of eNOS and TM,
which are downstream targets of KLF2, also increased in ECs with Dicer knockdown (Fig. 3-1C).

By using miRanda, microCosm, and TargetScan, we explored the putative miRNA binding sequences at the 3’UTR of KLF2 mRNA. Twenty miRNA binding sites with 45 miRNAs were predicted (Fig. 3-1D). miRanda, microCosm, and TargetScan predicted, in common, that the segment between 228 to 249 nt contains binding sequences for 8 miRNAs (i.e., miR-25, -32, -92a, -92b, -363, -367, -29a, and -29b). Analysis of the secondary structure of this segment by RNAcofold (http://www.tbi.univie.ac.at) revealed that the miRNA binding locus was located in “unstable” regions with multi-branching loops, characteristic of a miRNA target site. Among these putative 8 miRNAs, miR-29 and miR-92a are highly expressed in ECs\(^{18-20}\). Interestingly, a nucleotide sequence, GGUGCAAAUA, complementary to the seed region of miR-92a, is highly conserved among the human, chimpanzee, mouse, and rat KLF2 3’UTR (Fig. 3-1E).

### 3.4.2 miR-92a targets KLF2 mRNA

To explore whether miR-92a targets KLF2 mRNA, HUVECs were transfected with pre-92a. qRT-PCR was performed and confirmed the increased expression of miR-92a and decreased level of KLF2 mRNA in pre-92a-transfected cells, as compared with the control RNA-transfected HUVECs (Fig. 3-2A). Furthermore, the mRNA level of eNOS and TM decreased by ~50%. In complementary experiments, HUVECs transfected with anti-92a exhibited higher levels of KLF2, eNOS, and TM mRNAs (Fig. 3-2B); and protein (Fig. 3-2C).
To demonstrate the direct targeting of KLF2 3’UTR by miR-92a, we created a CMV-driven expression plasmid encoding the wild-type KLF2 3’UTR fused with a FLAG tag [FLAG-KLF2(WT)]. We also fused FLAG to a mutated KLF2 3’UTR in which the miR-92a binding site was altered [FLAG-KLF2(mut)] or deleted [FLAG-KLF2(Δ)]. Together with pre-92a or control RNA, these KLF2 expression plasmids were transfected into HEK293 cells, which have a high transfection efficiency and a low level of endogenous KLF2. As shown in Fig. 3-2D, pre-92a, but not control RNA, decreased the level of FLAG-KLF2(WT) fusion protein. In parallel experiments, the expression of FLAG-KLF2(mut) or FLAG-KLF2(Δ) was unaffected by the co-transfected pre-92a.

We also created reporter constructs containing luciferase fused to the wild-type KLF2 3’UTR [Luc-KLF2(WT)] or the mutated KLF2 3’UTR [Luc-KLF2(mut)]. HEK293 cells were co-transfected with Luc-KLF2(WT) or Luc-KLF2(mut) together with pre-92a or control RNA. As shown in Fig. 3-2E, the transfected pre-92a decreased the luciferase activity of Luc-KLF2(WT), as compared with cells co-transfected with control RNA but was unable to decrease the luciferase activity of Luc-KLF2(mut). Together, the data from Fig. 3-2 suggest that the 3’UTR of KLF2 mRNA contains a functional miR-92a target site. The interaction of miR-92a with KLF2 mRNA through this site causes the degradation of KLF2 mRNA and/or decreased translation of KLF2.

3.4.3 Shear stress-regulation of KLF2 is mediated by miR-92a

Because shear stress upregulates, while miR-92a downregulates, KLF2 mRNA in ECs, we examined the effect of laminar flow on miR-92a. As shown in Fig. 3-3A, the level of miR-92a in HUVECs decreased after exposure to laminar flow for 8 hr, and this
decrease lasted for at least 16 hr. To explore whether miR-92a is involved in the shear stress-regulated KLF2, HUVECs were transfected with pre-92a and then exposed to laminar flow. As shown in Fig. 3-3B, C, pre-92a transfection attenuated shear stress induction of KLF2 at both mRNA and protein levels. Furthermore, the shear stress-induction of eNOS and TM was downregulated by pre-92a in a similar manner (Fig. 3-3D-F).

3.4.4 Differential regulation of miR-92a by PS versus OS

Because of the atheroprotective versus atheroprone natures associated with PS and OS, we compared the miR-92a levels in HUVECs subjected to PS and OS. Both qRT-PCR and miRNA microarray showed that the expression of miR-92a was attenuated in ECs exposed to PS as compared with OS (Fig. 3-4A, B). To further test the flow regulation of miR-92a/KLF2, we created a reporter construct in which luciferase was fused to 2 copies of the miR-92a binding site found in the KLF2 3’UTR (Luc-2xmiR92a). As shown in Fig. 3-4C, PS caused a 2-fold increase in luciferase activity, as compared with static controls. In contrast, OS significantly reduced the luciferase activity, as compared with static controls (Fig. 3-4D). Furthermore, PS increased whereas OS slightly decreased the luciferase activity of Luc-KLF2(WT) (Fig. 3-4E, F).

Because miRNA targeting mRNAs depends on the association of the miRNA/mRNA complex with Ago proteins to form miRNA-induced silencing complex (miRISC), we investigated the association of miR-92a and KLF2 mRNA with Ago1 and Ago2 in HUVECs under PS. As shown in Fig. 3-5A and C, the levels of miR-92a and KLF2 mRNA associated with Ago1 or Ago2 immunoprecipitated from HUVECs
subjected to PS were lower than that in static controls. Similar experiments were performed with HUVECs exposed to OS. Contrary to PS, OS increased the miRISC-associated miR-92a and KLF2 mRNA (Fig. 3-5B, D). The expression of neither Ago1 nor Ago2 was affected by the applied PS and OS (Fig. 3-5E).

To determine whether miR-92a was differentially regulated by different flow patterns in vivo, we performed FISH on the aortic arch and thoracic aorta of C57BL6 mice. The endothelium experiences atheroprotective flow in the thoracic part and atheroprone flow in the aortic arch. As shown in Fig. 3-5F, the level of miR-92a in endothelium of the inner curvature of the aortic arch, where flow patterns are disturbed, was significantly higher than that in the thoracic aorta, which experiences undisturbed PS.

3.4.5 miR-92a regulates NO bioavailability

A functional consequence of KLF2-regulated eNOS is the increase in NO bioavailability in ECs. Hence, we examined whether NO production is affected by miR-92a. As shown in Fig. 3-6A, anti-92a overexpression in HUVECs enhanced NO production. To show that miR-92a targeting KLF2 mRNA is involved in the enhanced NO bioavailability, we co-transfected BAECs with FLAG-KLF2(WT) and pre-92a. KLF2 overexpression indeed increased NO production, which was largely abolished by the co-transfected pre-92a (Fig. 3-6B).

To determine the role of miR-92a in regulating vascular functions in vivo, we delivered pre-92a into the mouse carotid artery by a local oligo delivery system. Carotid arteries were used because the associated flow conditions have been defined and gene expression in these vessels can be manipulated by pluronic gel-based gene delivery. As
shown in Fig. 3-6C, the expressions of KLF2 and eNOS were significantly lower in vessels with pre-92a treatment. The role of miR-92a in the flow-mediated vasodilation was then investigated. As shown in Fig. 3-6D, the flow-mediated vasodilation was suppressed in vessels with overexpressed miR-92a. Importantly, L-NAME administration inhibited the flow-mediated vasodilation in vessels receiving control RNA, but had little effect on vessels overexpressing pre-92a. These results indicate that miR-92a is a critical molecule that regulates the eNOS-derived NO bioavailability in vivo.

3.5 Discussion

Shear stress with a forward direction has been shown to upregulate KLF2 in ECs5-7, which in turn modulates the expression of ~70% of the genes that are responsive to shear stress33. Early studies demonstrated that shear stress enhances KLF2 transcription via the binding of several transcription factors (TFs) to the promoter of the KLF2 gene (see Table 4-1). Post-translational modifications (e.g., phosphorylation and acetylation/deacetylation) of these TFs play important roles in changing the transcriptional activity of KLF213,34. Results from the present study show that shear stress also regulates KLF2 expression at the post-transcriptional level, which is mediated by the decreased level of miR-92a. Thus, the expression of KLF2 can be modulated at multiple levels, including transcription, post-transcriptional via miR-92a, and post-translational modifications.

miR-92a belongs to the miR-17~92 cluster. Also known as oncomiR-1, the miR-17~92 cluster is located in the third intron of the C13orf25 locus at 13q31-q32. Overexpression of miR-92a in ECs blocks angiogenesis in vitro and in vivo, which
suggests that miR-92a is a negative regulator of some endothelial functions. It has since been found that other miRNAs of the miR-17–92 cluster, such as miR-17, -18a, -19a, and -20a, are also anti-angiogenic. Sharing the same promoter, various miRNAs within the miR-17–92 cluster would be suppressed by PS and/or induced by OS in a similar manner. Indeed, the expression of miR-17, -18a, -19b, and -20a in HUVECs was downregulated by PS but upregulated by OS (Fig. 3-7). However, some miRNAs within the miR-17–92 cluster may not target KLF2 mRNA, since only the seed sequence of miR-92a is complementary to the KLF2 3’UTR.

The expression of the miR-17–92 cluster can be modulated by several transcription factors or signaling molecules. Overexpression of c-Myc, cyclin D1, and E2F increased the expression of the miR-17–92 cluster in cancer cells. ChIP assay has shown the binding of c-Myc, E2F, and cyclin D1 to the upstream promoter region of the miR-17–92 cluster. In addition, a stat3 binding site is present in this promoter. Given the positive effect of c-Myc, cyclin D1, E2F, and stat3 on the induction of the miR-17–92 cluster, one would assume that OS upregulates and that PS downregulates these proteins. Indeed, prolonged laminar flow has been shown to suppress the expression of these proteins. Since miR-92a targets the KLF2 3’UTR, the PS-downregulation of miR-92a should lead to an elevation of KLF2 expression at the post-transcriptional level. Consequently, the “de-suppressed” KLF2 transactivates its target genes (e.g., eNOS, and TM), which are otherwise suppressed under static or OS conditions. In addition to the regulation of NO and its consequent vasodilation, this mechanism involving miR-92a targeting KLF2 may also regulate other KLF2-dependent
genes such as von Willebrand factor, FLK1, and Tie-2, which are critical for EC lineage development and vascular functions\textsuperscript{27, 43, 44}.

The results presented in Figs. 2 and 3 show that miR-92a regulates KLF2 at both mRNA and protein levels. When assembled into miRISC, the mature miRNAs can cause the degradation of their target mRNAs or interfere with the translational process\textsuperscript{15-17}. Among the Ago family members, Ago1 mediates the miRNA-induced translational inhibition\textsuperscript{16, 45}. Recent studies have shown that the Ago1-involved miRISC could also destabilize target mRNAs by deadenylation and 5'→3' decay after decapping\textsuperscript{16, 45}. Neither of the mechanisms requires a perfect match between miRNAs and the 3'UTR of the targeted mRNAs. However, mRNA degradation modulated by Ago2 requires a near-perfect complementary sequence in the 3'UTR of the target\textsuperscript{46, 47}. The miR-92a/KLF2 mRNA targeting lacks a perfect match, since only the seed region of miR-92a (8 nt) is complementary to the KLF2 3'UTR. The RNA chaperone model\textsuperscript{48} suggests that Ago1 may facilitate the association of the guide strand of miR-92a with the KLF2 mRNA, and then Ago2 is recruited to the miRISC complex. This model explains the increased association of miR-92a and KLF2 mRNA with both Ago1 and Ago2 in ECs under OS (Fig. 3-5B,D).
References


Figure 3-1. miRNAs are involved in the regulation of KLF2 mRNA. (A) HUVECs were subjected to laminar flow (12 dyn/cm²) for 6 hr. After the addition of 5,6-dichloro-1-ß-D-ribobenzimidazole (DRB; 2 μg/ml), then the cells were continuously exposed to laminar flow or static conditions for additional 2 and 4 hr. The levels of KLF2 and GAPDH mRNAs were measured by qRT-PCR, and the KLF2/GAPDH mRNA ratio is plotted as a percentage of that in the untreated static cells. (B,C) HUVECs were transfected with 20 nM Dicer siRNA or control RNA for 48 hr. RT-PCR (B) and Western blot analysis (C) were performed to detect the mRNA levels of KLF2 and eNOS and protein levels of KLF2, eNOS, and TM, respectively. Histone H1 served as the internal control of nuclear extracts. The bar graphs are mean ± SD from 3 independent experiments. * p<0.05 between Dicer siRNA and control RNA. (D) The miRNA binding sites in the KLF2 3’UTR are predicted by bioinformatics algorithms. (E) The seed region of miR-92a and its target sequences at the KLF2 3’UTR of several mammalian species: Homo sapiens, Pan troglodytes, Mus Musculus, and Rattus Norvegicus.
Fig. 3-2. miR-92a targets KLF2 mRNA and decreases KLF2 translation. HUVECs were transfected with 20 nM miR-92a precursor (pre-92a) or control RNA. At 48 hr, the mRNA levels of miR-92a and KLF2, eNOS, and TM as ratios to GAPDH were assessed by qRT-PCR. In (B) and (C), HUVECs were transfected with 20 nM miR-92a inhibitor (anti-92a) or control RNA. The mRNA levels of KLF2, eNOS, and TM as ratios to GAPDH were assessed by qRT-PCR. The protein levels of KLF2, Histone H1 and eNOS were determined by Western blotting. (D) HEK293 cells were transfected with the wild-type FLAG-KLF2 (WT), FLAG-KLF2 (mut) (miR-92a binding site mutation), or FLAG-KLF2 (Δ) (miR-92a binding site deletion) together with 20 nM pre-92a or control RNA for 48 hr. The cells were then lysed, and the level of exogenously expressed FLAG-KLF2 fusion proteins was detected by Western blot analysis with anti-FLAG. Shown in the bottom panel is the densitometry analysis of the protein amount normalized to that in the control RNA-transfected cells. (E) HEK293 cells were transfected with the wild-type Luc-KLF2-3’UTR(WT) or Luc-KLF2-3’UTR(mut) together with 20 nM pre-92a or control RNA and 0.1 μg CMV-β-gal. The luciferase activity was normalized to that of β-gal. The data represent mean ± SD from 3 independent experiments. * p<0.05 between the 2 groups being compared.
Fig. 3-3. Shear stress-induction of KLF2 is mediated through miR-92a. (A) HUVECs were exposed to laminar flow for 4, 8 or 16 hr. RT-PCR was performed to detect the level of miR-92a, which was normalized to that of U6 RNA. * p<0.05 compared with static controls (time 0). (B-F) HUVECs were transfected with 20 nM control RNA or pre-92a for 48 hr and then exposed to laminar flow for 8 hr. (B) KLF2 mRNA level was detected by qRT-PCR and (C) protein level was assessed by Western blot analysis. (D) eNOS and (E) TM mRNA levels were detected by qRT-PCR and (F) protein level was assessed by Western blot analysis, and the results of statistical analyses are shown in the right. The data represent mean ± SD from 3 independent experiments. * p<0.05 between ECs transfected with pre-92a and control RNA.
Figure 3-4.

Fig. 3-4. PS downregulates, but OS upregulates, miR-92a expression in ECs. (A) HUVECs were exposed to PS (12±4 dyn/cm²) or OS (0±4 dyn/cm²) for 8 hr. qRT-PCR was performed to detect the level of miR-92a, which was normalized to that of U6 RNA. (B) The expression of miR-92a in ECs exposed to PS or OS flow assessed by miRNA microarray. (C,D) BAECs were transfected with Luc-2×miR92 reporter or control plasmid for 24 hr and then exposed to PS or OS flow for 12 hr. The luciferase activity was measured and normalized to β-gal activity. (E, F) BAECs were transfected with Luc-KLF2(WT) or Luc-KLF2(mut) for 24 hr and then exposed to PS or OS flow for 12 hr. The luciferase activity was measured and normalized to β-gal activity. The data represent mean ± SD from 3 independent experiments. * p<0.05 between the 2 groups being compared.
Figure 3-5.

Fig. 3-5. miRISC regulates miR-92a. HUVECs were exposed to PS (A,C) or OS (B,D) for 8 hr. The Ago1- or Ago2-associated miRNAs and mRNAs were enriched by IP with the use of anti-Ago1 (A,B) or anti-Ago2 (C,D). mRNA levels of miR-92a and KLF2 were detected by qRT-PCR and normalized to those of Ago1 or Ago2 protein. The data represent mean ± SD from 3 independent experiments. * p<0.05 for PS or OS vs. static controls. (E) Protein levels of Ago1 and Ago2 assessed by Western blot analysis with anti-Ago1 and anti-Ago2 and normalized to that of α-tubulin. (F) Levels of miR-92a in endothelium of the thoracic aorta and aortic arch of C57BL6 mice were assessed by in situ hybridization with LNA miR-92a probe (green). Nuclei were counterstained with DAPI (blue). Images are representative of 4 animals.
Fig. 3-6. miR-92a regulates endothelial function in vitro and ex vivo. HUVECs were transfected with control or anti-92a RNA (A) or BAECs were co-transfected with pre-92a and CMV-KLF2 (B). After 48 hr, the NO bioavailability was detected by fluorometric assay and expressed as NOx. The data represent mean ± SD from 3 independent experiments. (C) pre-92a or control RNA was administered to the carotid arteries of C57BL6 mice by pluronic gel F-127. Five days later, the arteries were isolated. The expression levels of miR-92a, KLF2 and eNOS in the isolated vessels (n=6) were assessed by qRT-PCR. miR-92a level was normalized to that of U6 RNA, whereas KLF2 and eNOS levels were normalized to that of GAPDH. (D) The flow-induced vasodilation ex vivo was measured by use of the SoftEdge Acquisition Subsystem in the presence or absence of L-NAME (1 μM). The flow-induced diameter changes in various experimental groups were compared with those of vessels receiving control RNA set as 1. The bars represent mean±SD from the indicated number of vessel specimens, with control vessels set as 1.
Fig. 3-7. The expression of miR-17-92 cluster under the different flow pattern. HUVECs were exposed to a PS (12 ± 4 dyn/cm²) or OS (0 ± 4 dyn/cm²) for 24 hr and then lysed. The amount of miR-92a were assessed by miRNA microarray. The level in ECs exposed to OS, averaged from 3 experiments was normalized to that in cells exposed to PS.
Chapter 4

Conclusion and perspectives

1. Introduction

KLF2 is an important integrator of multiple endothelial functions. The study of the mechanism by which atheroprotective flow upregulates KLF2 expression and maintains ECs homeostasis can increase our understanding of atherosclerosis and help develop novel strategies for its prevention and treatment. In this study, I explored the regulation of KLF2 expression at both the transcriptional level and the post-transcriptional level in response to shear stress. The major findings are as follows: (1) AMPK mediates shear stress-upregulated KLF2 transcription in ECs and \textit{in vivo} by activation of the MEK5/ERK5/MEF2 pathway; and (2) Atheroprotective flow patterns (laminar flow and pulsatile flow) decrease the level of miR-92a, which in turn increases KLF2 expression at the post-transcription level. These two mechanisms synergistically function to maintain endothelial homeostasis. Therefore, my study summarized in this dissertation provide a novel molecular mechanism by which different flow patterns can regulate KLF2 expression and modulate vascular function.

2. Shear stress regulates KLF2 expression at the transcriptional level

KLF2 is highly expressed in the endothelium of the atheroprotective regions of the vessel and reduced in that of the atheroprone region, such as the branch points or bifurcations\textsuperscript{1}. Because both AMPK and KLF2 are regulated by shear stress and can
augment nitric oxide production, I describe my study of the role of the AMPK in regulating the shear stress-induction of KLF2 expression in chapter 2.

In my study, an AMPK inhibitor (Compound C) or siRNA to target AMPK were used to block AMPK function, and an AMPK activator (AICAR) and an adenovirus expressing a constitutively active form an AMPK were used to mimic a gain of function. Indeed, the inhibition of AMPK had a significant blocking effect on the shear stress-induced KLF2 expression, and the activation of AMPK is sufficient for the induction of KLF2 expression.

Furthermore, I explored the mechanism underlying AMPK regulation of shear stress-induced KLF2 expression. MEF2 is an important transcription factor for KLF2 in ECs; this finding was demonstrated by the identification of the MEF2 binding site in the shear stress response region of the KLF2 promoter. Post-translational modifications such as phosphorylation provide a mechanism to regulate the transactivation of MEF2. Previous studies have shown that ERK5 (BMK1) is capable of greatly enhancing the transactivation of MEF2C by phosphorylating MEF2C at Thr 300 and MEF2A at Thr 312. Parmar et al. showed that the flow-dependent activation of KLF2 is mediated via the MEK5/ERK5/MEF2 signaling pathway, which suggests the involvement of the MAPK family in the regulation of KLF2. In chapter 2, I further identified that AMPK is necessary to activate MEK5/ERK5/MEF2 signaling by use of AMPK siRNA to knockdown AMPK, which in turn induced KLF2 expression in response to shear stress. I also demonstrated that
activation of AMPK by AICAR is also sufficient for the induction of the phosphorylation of ERK5 and MEF2, which was subsequently confirmed *in vivo* with AMPKα2-/- mice. The phosphorylation of ERK5, MEF2, and eNOS was greatly attenuated and KLF2 expression was significantly reduced in the aortas of AMPKα2-/- mice. To further demonstrate the vascular location of the reduction of MEF2 activation, I performed *en face* staining of phospho-MEF2 to confirm the reduction in aortic endothelium of AMPK α2-/- mice. These results indicate that AMPK plays a critical role in modulating the shear stress activation of the ERK2/MEF2/KLF2 pathway and establishes a novel link between AMPK and ERK5/MEF2 signaling pathway.

In summary, my study suggested that AMPK is an upstream signaling molecule of the ERK5/MEF2/KLF2 pathway and is required for subsequent expression of KLF2 in ECs and in mouse vasculature. These findings established that the PS activation of the AMPK/ERK5/MEF2/KLF2 pathway plays an important role in the regulation of vascular homeostasis.

**Remaining questions**

1) Table 4-1 lists the TFs, including MEF2, that can bind to the KLF2 promoter region. Among these TFs, none are endothelial-specific, but the binding of these proteins to the KLF2 promoter is regulated by shear stress in ECs, which is probably due to the post-translational modification. For example, the binding of nucleolin to the KLF2 promoter is flow specific and PI3K dependent in ECs. Nucleolin could be
highly phosphorylated at specific residues, which induces a conformational change leading to its active form\(^5\). Co-IP experiments showed that nucleolin can interact with the p85 regulatory subunit of PI3K, but the phosphorylation site has not been identified yet\(^4\). However, by use of our system, blocking PI3K with Wortmannin or LY2943002 had little effect on shear-induced AMPK activation and KLF2 expression, which indicates that the AMPK-KLF2 pathway is independent to PI3K. Therefore, nucleolin may be phosphorylated by response of another kinase such as AMPK or PKA to shear stress. Further investigation will be needed to understand how shear stress regulates the transactivation of TFs on KLF2 expression.

2) In addition to phosphorylation, the transactivation of the MEF2 family has also been shown to be regulated by the recruitment of cofactors\(^6\). Wang \textit{et al.} demonstrated a novel role for histone deacetylase 5 (HDAC5) in flow-mediated KLF2 expression by regulating MEF2A activity\(^7\). The authors showed that shear stress induced HDAC5 phosphorylation and nuclear export in endothelial cells via a calcium/calmodulin-dependent pathway, thus leading to dissociation of HDAC5 and MEF2 and enhancing MEF2 activity. The activated MEF2 subsequently increased the expression of KLF2 and eNOS. Moreover, the authors identified that S259 and S498 of HDAC5 are critical phosphorylation sites for this regulation, but they did not identify the upstream kinase of HDAC5. Previous studies, including ours, showed that CaMKK is a Ca-dependent kinase and is an upstream kinase of AMPK\(^8\). These results suggest that AMPK may play a role in the phosphorylation of HDAC5 in
response to shear stress. *In vitro* kinase assays could be performed to examine whether AMPK phosphorylates HDAC5 directly. The blockage of AMPK by siRNA could be used to examine whether AMPK mediates shear stress-induced HDAC5 translocation to cytoplasm.

3. **Shear Stress regulates KLF2 expression at the post-transcriptional level**

Shear stress (improved by exercise training) is more potent than statins (pharmaceutical) in increasing the levels of the KLF2 downstream targets (eNOS and TM), despite being able to induce KLF2 transcription to a similar level. This result is due in part to the role of shear stress in promoting KLF2 stabilization. Results in chapter 3 show that shear stress regulates KLF2 expression at the post-transcriptional level, which is mediated by the decreased level of miR-92a.

In chapter 3, using RT-PCR, Luciferase reporter assays, and Fluorescence *in situ* Hybridization (FISH), I demonstrated that atheroprotective flow (LS and PS) downregulates miR-92a expression and atheroprone flow (OS) upregulates miR-92a expression in ECs (Fig.3-3,4). Consistent with my result, a recent report also demonstrated by microarray analysis that miR-92a expression is increased in HUVECs under PS compared to the static condition⁹. Moreover, miR-92a expression was found elevated in the endothelium of the athero-susceptible aortic arch (AA) as compared with that of the atheroprotective dorsal descending thoracic aorta (DT) in swine¹⁰.
However, the mechanism by which the different flow patterns regulate the expression of miR-17-92 cluster remains unknown. Oxidative stress, which is relatively higher in the atheropron region under disturbed flow, is a major determinant of endothelial adhesiveness for monocytes and lesion formation\textsuperscript{11}. A recent study in our lab showed that both H\textsubscript{2}O\textsubscript{2} and angiotensin II could increase the expression of miR-92a. Similarly, IL-6, an oxidative stress inducing cytokine, is related to pulmonary arterial hypertension, can upregulate the expression of the miR-17-92 cluster in HPAECs\textsuperscript{12}. These results indicate that oxidative stress probably plays an important role in the regulation of the miR-92a in response to different flow patterns in ECs.

The previous studies revealed that c-Myc, E2F, STAT3 and cyclin D1, which are related to the regulation of cell cycle, could bind to the miR-17-92 promoter and upregulate its transcription\textsuperscript{13, 14}. In contrast, p53 inhibits the transcription by competing with the transcription machinery to bind to the same region of the miR-17-92 promoter (TATA box)\textsuperscript{15}. In addition to these TFs, 37 potential TFs binding sites were revealed in the promoter region (-5000 to +500 bp) of the miR-17-92 cluster by \textit{in silico} analysis. Several of these TFs may be regulated by laminar flow (listed in Table 4-2), such as activating transcription factor 2 (ATF2) and PPARG. The nuclear activation of ATF2, which can induce the expression of pro-inflammatory genes, is inhibited by laminar flow\textsuperscript{16}. Moreover, phosphorylated ATF2 protein level is elevated in endothelial cells overlying early atherosclerotic plaques as compared
with healthy endothelium\textsuperscript{16}. Considering the expression and function of ATF2, these findings suggest that ATF2 probably mediates flow-regulated KLF2 expression.

**The function of miR-92a in ECs**

MiR-17-92 was considered an oncomiR because of its overexpression in several types of lymphoma and solid tumors\textsuperscript{17, 18}. In addition to the cluster’s role in tumorigenesis, it is also a regulator of hematopoiesis and immune function\textsuperscript{19, 20}. Even though miR-92a is highly expressed in ECs, little is known about the function of miR-92a in ECs. Gain-of-function experiments revealed that miR-92a does not influence apoptosis and proliferation of ECs. Overexpression of miR-92a in ECs blocks angiogenesis *in vitro* and *in vivo*, which suggests that miR-92a is a negative regulator of endothelial functions\textsuperscript{21}. In chapter 3, I demonstrated that miR-92a can directly bind to the KLF2 3’ UTR and decrease shear-induced KLF2 and its targets (eNOS and TM) mRNA and protein levels. A functional consequence of this effect is the increase in NO bioavailability in ECs. The enhanced overexpression of miR-92a alleviated flow-induced vasodilation in mouse carotid artery. The result of L-NAME administration, which blocked endothelial-dependent vasodilation, indicated that miR-92a not only decreased endothelial-dependent but also endothelial-independent vasodilation. The impaired vasodilatory ability is an indicator of EC dysfunction.
miRNAs regulate KLF2

Within the KLF2 3’ UTR are 20 predicated putative miRNA binding sites that can be regulated by 45 potential miRNAs. In addition to miR-92a, several other miRNAs are downregulated by PS and upregulated by OS; examples are miR-25, 30, 99 and 106. Further experiments could validate the regulatory role of these miRNAs on KLF2 expression and endothelial function. MiR-92a and these other miRNAs might act in synergy to regulate the KLF2 and other targets in response to differential flow pattern. These findings would provide information on a novel and fine regulatory layer of endothelial function.

miRNA subcellular localization

Much evidence indicates that the components of the miRISC may not localize in the cytosol, but occurs in different cellular organelles or structures\(^2^1\). Both Dicer, the key enzyme for miRNA biogenesis, and Ago, the key component of miRISC, have been reported to be associated with the endoplasmic reticulum (ER) and Golgi membranes, which could facilitate the local biogenesis or action of miRNAs in their vicinity\(^2^2^\)\(^-^2^4\). miRISC can also accumulate in discrete organelles known as P-bodies, which contain the enriched proteins involved in mRNA deadenylation, decapping, and degradation. In my study, FISH imaging showed that miR-92a mainly distributes in the cytosol close to the nucleus (Fig. 3-5). The possible explanation is that miR-92a mediates the binding of miRISC to ER, which directly connects to the nuclear envelope. Such a unique association may facilitate miR-92a targeting KLF2.
4. **Shear stress regulates KLF2 expression at the post-translational level**

To date, very little is known about the role of posttranslational modification of KLF2 and the role in mediating its functions. The Lingrel group identified that WW domain-containing E3 ubiquitin protein ligase 1 (WWP1) can interact with the inhibitory domain (110-267 aa) of KLF2, thus leading to the ubiquitination and proteasomal degradation of KLF2. K121 is the critical site for ubiquitin conjugation. *In silico* analysis revealed that this site and K148 are also the predicted sites (ψ-K-X-E) for SUMOylation, which probably modulates KLF2 function. KLF2 can interact with the transcriptional coactivator cyclic AMP response element–binding protein (CBP/p300), inducing eNOS and inhibiting E-selectin and VCAM-1. p300 and CBP each contain a protein or histone acetyltransferase (PAT/HAT) domain that can modulate the binding TFs and regulate their transactivation. Interestingly, K121 and K148 are also the potential acetylation sites for KLF2. In addition, the transactivation of KLF2 can be modulated by the recruitment of cofactors. For example, ERG, an endothelial-specific TF of ETS family, forms a physical complex with KLF2 and synergistically activates transcription of *Flk1* during embryonic vascular development. These experimental results and prediction indicate that post-translational modification is important for the shear stress-regulated KLF2 function.
5. KLF2 regulates the expression of miRNAs

KLF2, functioning as a TF, regulates the expression of both genes and miRNAs. The first evidence of regulating miRNA is that KLF2 induces the expression of miR-126, an endothelial-specific miRNA, which results in activating Vegf signaling and guiding angiogenesis during zebrafish development\textsuperscript{28}. Recently, Harris \textit{et al.} showed that KLF2 binds to the promoter region of miR-126 and directly regulates the transcription of miR-126\textsuperscript{29}. In addition to miR-126, a set of shear stress-upregulated miRNAs that have the KLF2 binding site in their promoter region were revealed by bioinformatics analysis (Table 4-3). These miRNAs are probably induced by shear stress through KLF2. The targets of KLF2 would be further identified by ChIP-Seq on a genome-wide scale.

6. Summary

From knowledge gained from the current study and that in the literature, Fig. 4-1 summarizes a network of molecular events leading to the induction of KLF2 in ECs by atheroprotective flow. AMPK/MEF2 signaling and several TFs modulated by the imposed flow are directly involved in the induction of \textit{klf2} gene (Table 4-1), whereas other TFs may downregulate the miR-17–92 cluster (Table 4-2). Thus, KLF2 is regulated at both transcriptional and post-transcriptional levels. The upregulated KLF2, functioning as a TF, transactivates a panel of genes related to EC lineage, as well as miRNAs\textsuperscript{28}. Recently, Harris \textit{et al.} showed that KLF2 regulates the transcription of miR-126 directly\textsuperscript{29}. In addition to miR-126, bioinformatics
analysis revealed a set of shear stress-upregulated miRNAs that have the KLF2-binding site in their promoter region (Table 4-3). Moreover, the downregulation of miR-92a leads to augmented IGTA5 and upregulation of miR-126 results in attenuated SpredI. Collectively, these miRNA-regulated events and KLF2 targets (e.g., eNOS, TM) maintain the EC lineage\textsuperscript{24, 26}. These results provide an integrated paradigm of how miRNAs and TFs enhance EC functions under atheroprotective flow.

Because AMPK can regulate various signaling pathways, including KLF2, activation of AMPK has become an emerging target for the prevention and treatment of atherosclerosis. Because of its negative regulation of angiogenesis and vasodilation, miR-92a may also be an effective target for therapeutic agents for cardiovascular diseases. miRNA could be readily inhibited \textit{in vivo} by use of the stable modified antagomiR, and this agent could be efficiently delivered into various tissues in animal models\textsuperscript{30-32}. If miRNA-based therapeutics indeed becomes a reality, miR-92a will be one of the miRNA candidates to be considered.
Fig. 4-1. Shear stress regulation of KLF2. The diagram shows the regulatory circuitry of the responses of transcription factors and miRNAs to atheroprotective shear flow. The circled Roman numerals refer to the Table numbers. Shear stress with a forward direction regulates the expression of KLF2 through AMPK/MEF2 signaling and other TFs (Table I) and the expression of miR-92a through TFs (Table II). Serving as a transcription factor, KLF2 transactivates the expression of downstream genes such as eNOS and TM. In addition, KLF2 may bind to the promoter region of some miRNAs, including miR-126, to upregulate their transcription directly (Table III). In turn, the network of KLF2 and miRNAs regulates the expression of factors that control anti-inflammatory, -thrombotic, -proliferative, -angiogenic, -oxidant, and -fibrotic effects to enhance EC lineage development and maintain vascular functions. The methods for computational analysis are described in the supplements.
## Table 4-1. Transcription factors regulating KLF2

<table>
<thead>
<tr>
<th>TF</th>
<th>Regulation mode</th>
<th>Shear stress regulated</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF2A</td>
<td>Activated</td>
<td>yes</td>
<td>3, 33, 34</td>
</tr>
<tr>
<td>MEF2C</td>
<td>Activated</td>
<td>yes</td>
<td>3, 33, 34</td>
</tr>
<tr>
<td>BRG1</td>
<td>Unspecified</td>
<td>unknown</td>
<td>35</td>
</tr>
<tr>
<td>p300</td>
<td>Activated</td>
<td>yes</td>
<td>26, 36-38</td>
</tr>
<tr>
<td>PCAF</td>
<td>Activated</td>
<td>Yes</td>
<td>36, 38</td>
</tr>
<tr>
<td>hnRNP D</td>
<td>Activated</td>
<td>Yes</td>
<td>36, 38</td>
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<tr>
<td>hnRNP U</td>
<td>Activated</td>
<td>Yes</td>
<td>36, 38</td>
</tr>
<tr>
<td>Nucleolin</td>
<td>Activated</td>
<td>Yes</td>
<td>4</td>
</tr>
<tr>
<td>SP1</td>
<td>Activated</td>
<td>yes</td>
<td>39</td>
</tr>
<tr>
<td>Oct-3/4</td>
<td>Activated</td>
<td>unknown</td>
<td>40-43</td>
</tr>
<tr>
<td>SOX2</td>
<td>Activated</td>
<td>unknown</td>
<td>40, 41, 44</td>
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### Table 4-2. Transcription factors regulating the miR-17~92 cluster

<table>
<thead>
<tr>
<th>TF</th>
<th>Regulation mode</th>
<th>Shear stress</th>
<th>References</th>
</tr>
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<tr>
<td>c-Myc</td>
<td>Activated</td>
<td>Inhibited</td>
<td>45, 46</td>
</tr>
<tr>
<td>E2F</td>
<td>Activated</td>
<td>Inhibited</td>
<td>47, 48</td>
</tr>
<tr>
<td>STAT3</td>
<td>Activated</td>
<td>Inhibited</td>
<td>12, 49</td>
</tr>
<tr>
<td>cyclin D1</td>
<td>Activated</td>
<td>Inhibited</td>
<td>50, 51</td>
</tr>
<tr>
<td>P53</td>
<td>Inhibited</td>
<td>Activated</td>
<td>15, 52</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Inhibited</td>
<td>Inhibited</td>
<td>19</td>
</tr>
<tr>
<td>ATF2</td>
<td>predicted</td>
<td>inhibited</td>
<td>16</td>
</tr>
<tr>
<td>CREB</td>
<td>predicted</td>
<td>Activated</td>
<td>53</td>
</tr>
<tr>
<td>PPARG</td>
<td>predicted</td>
<td>Inhibited</td>
<td>54</td>
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<tr>
<td>SP1</td>
<td>predicted</td>
<td>Activated</td>
<td>55</td>
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Table 4-3. KLF2-targeted miRNAs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Host gene</th>
<th>Function</th>
<th>Validated</th>
<th>Reference</th>
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<tbody>
<tr>
<td>hsa-miR-126</td>
<td>Egfl7</td>
<td>Angiogenesis</td>
<td>Yes</td>
<td>9, 29</td>
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<tr>
<td></td>
<td></td>
<td>Anti-inflammation</td>
<td></td>
<td></td>
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<tr>
<td>hsa-miR-30a</td>
<td>C6orf155</td>
<td>angiogenesis</td>
<td>predicted</td>
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<tr>
<td>hsa-miR-483-5p</td>
<td>IGF2</td>
<td>unknown</td>
<td>predicted</td>
<td>51</td>
</tr>
<tr>
<td>hsa-miR-101-1</td>
<td>Intergenic</td>
<td>Cell growth</td>
<td>predicted</td>
<td>57, 58</td>
</tr>
<tr>
<td>hsa-miR-181d</td>
<td>Intergenic</td>
<td>differentiation</td>
<td>predicted</td>
<td>57, 59</td>
</tr>
<tr>
<td>hsa-miR-15a</td>
<td>DLEU2</td>
<td>apoptosis</td>
<td>predicted</td>
<td>57, 60</td>
</tr>
<tr>
<td>hsa-miR-148a</td>
<td>Intergenic</td>
<td>Cell survival</td>
<td>predicted</td>
<td>57, 61</td>
</tr>
<tr>
<td>hsa-miR-365-1</td>
<td>Intergenic</td>
<td>unknown</td>
<td>predicted</td>
<td>57</td>
</tr>
</tbody>
</table>
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