The Role of Hemodynamics in Modulating Kaiso Expression and Activity in Atherogenesis

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ABSTRACT

Atherosclerosis is an inflammatory disease of the vasculature characterized by the accumulation of lipids beneath the endothelium leading to the formation of plaque. The generation of atherosclerosis has been shown to be a non-random process, with endothelial cells (ECs) in regions of disturbed flow, as dictated by variations in regional vascular geometry, developing an atheroprone phenotype when compared to endothelium in regions of nondisturbed, atheroprotective flow. Consequently, hemodynamics (i.e. shear stress) provide a vital mechanical signal that regulates regional susceptibility to atherosclerosis. While a great deal of research has focused on shear stress regulation of EC phenotype as it pertains to atherogenesis, large gaps remain in the understanding of protein expression and signaling pathways that mediate ECs' response to local hemodynamic environment. **We hypothesized that hemodynamic forces modulate the expression and/or activity of the transcription factor Kaiso in a manner that alters local endothelial susceptibility to atherogenesis.**

To investigate the effects of hemodynamics on Kaiso function, we utilized a novel *in vitro* system to apply physiologically determined shear stresses from a human common carotid artery (atheroprotective) or internal carotid sinus (atheroprone) to primary human endothelial cell (EC) monolayers. Through the evaluation of a luciferase-based reporter, we demonstrated that Kaiso activity is differentially regulated by hemodynamic conditions in a manner that is independent of changes in Kaiso expression or interaction with its known activity modulator p120 catenin. Having established Kaiso's dependence on hemodynamic environment, we sought to elucidate the functional role of Kaiso in

mediating transcription pathways downstream of physiological shear stress exposure. To obtain a broad characterization of Kaiso function in endothelial cells, siRNA was used to knockdown Kaiso expression in ECs exposed to atheroprotective or atheroprone hemodynamics, and changes in the transcriptome were assayed by gene microarrays. The microarrays demonstrated involvement of Kaiso in a variety of relevant signaling pathways and suggested an elevated importance of Kaiso in mediating the downstream effects of atheroprotective hemodynamics. In order to ascertain a more specific understanding of Kaiso function in response to shear stress, potential atherosclerosis-related Kaiso gene targets were identified through a bioinformatic analysis of human promoter sequences. We then investigated the response of these genes to either Kaiso knockdown or overexpression in the context of hemodynamic environment. Our findings reveal a complex and possibly bimodal activity of Kaiso that is dependent on both hemodynamic environment and loss or gain of expression.

While Kaiso demonstrated many varied functions in endothelium, our studies specifically established a novel role for Kaiso as a positive regulator of the highly anti-atherogenic KLF2/KLF4 signaling pathway downstream of exposure to atheroprotective hemodynamics. Kaiso knockdown resulted in a downregulation of eNOS, KLF2, KLF4, MEF2a, NOV, TFPI, and THBD, all of which are known mediators of anti-inflammatory and anti-thrombotic effects. This loss of atheroprotective expression correlated with increased inflammatory NFκB activity and subsequently elevated expression of the leukocyte adhesion molecules VCAM-1 and ICAM-1. To support the physiological significance of Kaiso-mediated atheroprotection, we confirmed that knockdown of Kaiso expression is sufficient to increase monocyte adhesion to EC monolayers. Taken

together, these results indicate a potentially critical role for Kaiso in conferring atheroprotection to endothelium in response protective hemodynamic exposure. This finding improves our understanding of hemodynamic modulation of athero-susceptibility and may serve to generate future therapeutic targets for the treatment and prevention of atherosclerosis.

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CHAPTER 1: SPECIFIC AIMS OF THE DISSERTATION

1.1 Specific Aim 1

To determine the effect of the hemodynamic environment on Kaiso expression, interaction with p120, and activity.

The expression of Kaiso and its binding partner, p120, were assessed as a function of the hemodynamic, shear stress environment and time in human endothelial cells (ECs). Expression of p120 was determined by Western blot, while immunoprecipitation of Kaiso allowed for subsequent quantification of both its expression and its interaction with p120, which has been shown to alter Kaiso activity. Given that expression levels alone cannot properly convey the activity of a transcription factor such as Kaiso, a reporter construct was utilized to investigate the modulation of Kaiso activity by hemodynamic environment alone. To further characterize the function of this reporter as well as allow inferences to be made linking hemodynamics to Kaiso function, similar reporter experiments were performed in ECs with exogenous manipulation of Kaiso expression. Additionally, immunohistochemistry allowed evaluation of Kaiso expression in both healthy and diseased arteries in the $ApoE^{-/-}$ mouse model of atherosclerosis.

The hypothesis of this Aim is:

Based on prior biomedical literature of downstream genes regulated by Kaiso, we hypothesize that hemodynamic forces modulate the expression and/or activity of the transcription factor Kaiso in endothelial cells.

1.2 Specific Aim 2

To define a comprehensive functional role for Kaiso in endothelium exposed to hemodynamic flow through the utilization of genome-wide expression analysis.

Given its expression in endothelium and the lack of previous studies regarding its transcriptional targets, Kaiso's functional importance as a transcription factor poses an unexplored field of study with possible implications in many important EC signaling systems, including those pertinent to atherosclerosis. To explore the genome-wide role of Kaiso in endothelium, ECs were treated with either control or Kaiso-directed siRNA then exposed to 24 hours of prone or protective hemodynamics. Total mRNA samples from these experiments were processed and analyzed via Affymetrix U133 Plus 2.0 microarrays, providing crucial information in the study of endothelial Kaiso function. Broadly, the microarrays not only discovered Kaiso regulation of many important EC signaling pathways but also highlighted flow-dependent variations in Kaiso function. Additionally, individual findings from the gene arrays provided important specific targets and pathways to investigate more extensively (Aim 3).

The hypothesis of this Aim is:

In endothelium, Kaiso plays a previously unidentified role in a variety of functional signaling pathways. Additionally, Kaiso's function within endothelial cells is flow-dependent and mediates systems with substantial influence in the process of atherogenesis.

1.3 Specific Aim 3

To investigate the biological and functional consequence associated with Kaiso-dependent regulation of inflammation in the context of atherogenesis

In addition to information obtained through genome-wide expression analysis (Aim 2), bioinformatics software was used to analyze human promoter sequences for possible Kaiso interactions and screen these potential Kaiso targets for known relevance to atherosclerosis. To examine the mechanisms by which Kaiso modulates inflammation and atherogenesis through potential gene targets and pathways, Kaiso was knocked down or overexpressed in ECs exposed to prone or protective hemodynamics, and the resulting changes in expression of target genes were examined at the mRNA and protein level. Furthermore, to evaluate downstream inflammatory consequences of Kaiso manipulation on known shear-dependent inflammatory mediators, we assessed the activities of activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Finally, to demonstrate physiologically relevant consequences of disturbances in Kaiso homeostasis, the ability of human monocytes to adhere to shear-

exposed EC monolayers was quantified with or without Kaiso knockdown. In addition, exposure to tumor necrosis factor alpha (TNFα) was used to investigate Kaiso's importance in maintaining the endothelial response to an inflammatory challenge.

The hypothesis of this Aim is:

Kaiso signaling is a key transcriptional regulator by which endothelial cells respond to hemodynamic environment, maintaining cellular homeostasis and responding to inflammatory challenges.

CHAPTER 2: BACKGROUND AND SIGNIFICANCE

2.1 Atherosclerosis is a Chronic Vascular Inflammatory Disease

The inflammatory disease atherosclerosis is characterized by the accumulation of lipids beneath vascular endothelium, which triggers an inflammatory cascade and promotes the formation of intravascular plaque [1]. This process involves secretion of a variety of inflammatory cytokines, expression of cell adhesion molecules, intimal infiltration and proliferation of smooth muscle cells, accumulation of monocytes and macrophages, the formation of foam cells from lipid engorged phagocytic cells, and even the involvement of T and B lymphocytes (reviewed in [2]).

Plaque progression can lead to arterial stenosis, which if severe enough, can result in downstream perfusion deficits and tissue ischemia. Rupture of a lesion's fibrous cap exposes the highly thrombotic plaque core, resulting in a locally occlusive thrombus formation or distal occlusion from embolization of clotting products. In the coronary arteries, stenosis from atherosclerotic lesions can lead to ischemic heart disease, whereas plaque rupture in the coronary or cerebral arterial circulation results in myocardial infarction or stroke, respectively. Given that these pathologies are some the leading causes of morbidity and mortality in the United States and the developed world, understanding, treating, and preventing atherosclerosis presents one of the most critical challenges facing modern medicine.

2.2 Role of Hemodynamics/Shear Stress in Atherosclerosis

The endothelium possesses the ability to sense and respond to hemodynamic environment through a set of signals known collectively as endothelial mechanotransduction (reviewed in [3]). This process is complex and multifactorial but involves a mechanosensory complex comprising platelet endothelial cell adhesion molecule (PECAM-1) [4-6], vascular endothelial cadherin (VE-Cadherin), and vascular endothelial growth factor receptor 2 (VEGFR2) [6], that senses mechanical shear stress and generates a wide range of downstream biological signals, including integrin activation [7, 8]. Physiologically, endothelial cells have shown an ability to respond to changes in blood flow acutely to maintain appropriate vascular tone [9-12] and chronically to remodel vessel geometry [13, 14].

The localization of atherosclerosis has been shown to be a non-random pathological process (reviewed in [15]). Early surgical studies revealed that lesions develop primarily in the following vascular beds: (I) the coronary arteries, (II) major branches of the aortic arch, (III) visceral arterial branches of the abdominal aorta, (IV) the terminal abdominal aorta and branches, or (V) a combination of the above [16]. Furthermore, lesions were shown to arise preferentially at bifurcations, areas of curvature, and other sites of geometric complexity that correlate with lower wall shear stress and non-laminar disturbed hemodynamics [17-19]. Variations in hemodynamics, due to these regional vascular geometries and the resulting hemodynamic patterns, have been shown to alter EC phenotype, shifting gene expression profiles in these cells towards either an atheroprotective or atheroprone state [20]. Studies have shown that ECs

exposed to atheroprone hemodynamic patterns display chronic inflammation, as indicated by the expression and hyperresponsiveness of NFκB subunits [20, 21], expression of cell adhesion molecules (e.g. VCAM-1, ICAM-1) [22, 23], and secretion of leukocyte recruiting cytokines (e.g. MCP-1, MIP-1 α/β, and RANTES) [24]. These mechanisms contribute to the accumulation of dendritic cells, monocytes, T cells, and other leukocytes into a lesion site [2, 23], which further drives the inflammatory response. By contrast, endothelial cells exposed to atheroprotective hemodynamics exhibit an inflammation resistant phenotype largely modulated by expression of the transcription factor Kruppel-like factor 2 (KLF2) [20, 25, 26], which serves as a mechano-activated master regulator of inflammation, thrombosis, vascular tone, and vessel development [27, 28].

Consequently, vascular hemodynamic shear stress profiles constitute a major factor in the risk of developing atherosclerosis, and recent research has begun to focus on the mechanisms by which ECs respond to their hemodynamic environment to alter regional susceptibility to atherogenesis.

2.3 Novel In Vitro Flow Model for Physiologically Relevant Hemodynamics

In vitro models of ECs under hemodynamic flow conditions have been extensively studied to further understand the role of the endothelium in atherogenesis. Throughout the last two decades, the model for studying EC function in the context of atherosclerosis has evolved to incorporate “atheroprotective-like” and “atheroprone-like” flow patterns into *in vitro* models. Using either a cone-and-plate or parallel plate chamber, steady laminar shear stress at 10-20 dynes/cm^2 is often used to create an

artificial atheroprotective flow environment; however, this method neglects the pulsatile effects of blood flow *in vivo* [29]. Attempts at recreating *in vivo* atheroprone flow patterns for use *in vitro* vary greatly in the literature. Methods have included low steady laminar flow (<5 dynes/cm^2), oscillatory flow, the formation of disturbed flow using parallel-plate and step-flow chambers, and changing the cone angle of cone-and-plate models to produce turbulence [29]. These models often make simplified assumptions of the flow within atheroprone/protective regions of the arteries. Despite their limitations, these systems have shown differential responses of ECs regarding morphology, adhesion, and permeability [30, 31]. Research has shown that cells exposed to "atheroprone-like" flow have decreased elongation and alignment, compromised junction integrity leading to increased permeability, and increased O_2^- production leading to increased monocyte adhesion [31, 32].

A novel *in vitro* model has been developed by Blackman et al. through which arterial flow patterns modeled from human circulation may be applied to ECs via a modified cone-and-plate viscometer driven by a controllable motor [30]. This model closely mimics the physiological hemodynamic shear stress forces imparted on ECs *in*

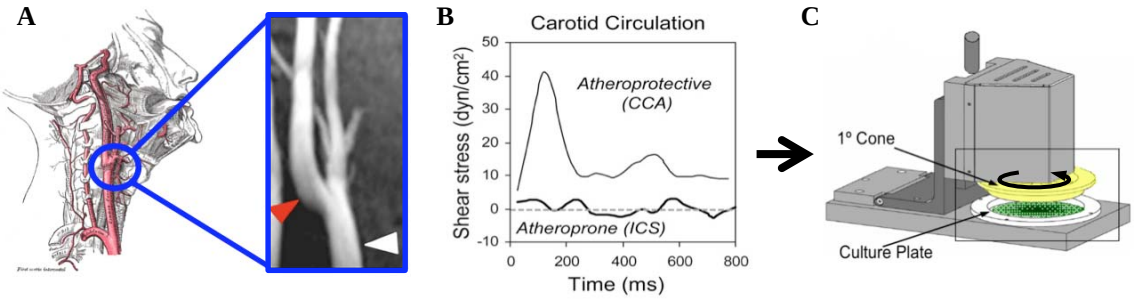

Figure 1. MRI analysis of atheroprotective and atheroprone blood flow in human carotid arteries. (A) Blood velocity data was acquired at the carotid bifurcation, which includes the internal carotid sinus (ICS) and in the common carotid artery (CCA). (B) The derived atheroprone (ICS) and atheroprotective (CCA) shear stress waveforms as a function of time. (C) Schematic of the cone-and-plate device. Modified from Gelfand et al. 2006.

vivo and overcomes many of the limitations of previous flow models. Furthermore, with advanced knowledge of the susceptibility of specific regions of the human vasculature to develop clinical atherosclerosis, Gelfand et al. utilized phase contrast magnetic resonance imaging (MRI) to measure near wall blood velocity gradients in a healthy adult male's common carotid artery (CCA) and internal carotid sinus (ICS) throughout a cardiac cycle. These measurements were then used to calculate shear stress waveforms, which serve as atheroprotective and atheroprone hemodynamic paradigms, respectively, for use in the *in vitro* model (Figure 1) [33]. Briefly, the atheroprotective waveform possesses high time-average shear stress, notable pulsatility, and a lack of flow reversal; while the atheroprone waveform has low time-average shear stress, minimal pulsatility, and periods of retrograde flow. These physiologically relevant shear stress profiles have been employed alongside mouse models of atherosclerosis to further elucidate the mechanisms by which hemodynamics modulate EC phenotype, particularly the manner by which atheroprone flow contributes to inflammation and a pro-atherogenic environment. For example, atheroprone hemodynamics induce inflammatory signaling through β-catenin/TCF activation and nuclear translocation [34] as well as fibronectin (FN) expression and subsequent integrin/NFκB activation [35]. Furthermore, prone flow is implicated in cytoskeletal reorganization and cell migration [36] and p21 activated kinase (PAK)-dependent increases in EC permeability [37]. Studies by Feaver et al. supported utilizing physiologically derived (and non-steady) flow profiles by demonstrating the ability of frequency harmonics within the complex atheroprone waveform to initiate inflammatory signaling [38]. Incorporation of smooth muscle cell (SMC)/EC co-culture into the flow model above demonstrated that atheroprone flow exposure to ECs resulted

in dedifferentiation and increased inflammation of underlying SMCs [39, 40]. Additionally, atheroprone hemodynamics have also been shown to induce compensatory mechanisms such as mitigation of vascular cell adhesion molecule (VCAM-1) expression through interleukin 8 (IL-8) secretion [41] and pro-survival GRP78 (glucose regulated protein, 78kDa) upregulation in response to endoplasmic reticulum stress [42].

While the mechanisms by which atheroprone hemodynamics induce an atherogenic environment are becoming increasingly understood, less evidence exists as to how protective flow conveys an athero-resistant phenotype, a question that serves as major motivation for the studies in this dissertation.

2.4 Kruppel-Like Factors as Hemodynamic-Dependent Mediators of EC Atheroprotection

Unlike areas of bifurcation and curvature, long and relatively straight portions of vasculature are typically spared from atherosclerotic lesion development. In these regions (e.g. the CCA, as discussed above), blood flow is characteristically higher shear stress, unidirectional, laminar, and pulsatile. In one of the early *in vitro* applications of "atheroprotective-like" steady laminar sheer stress to cultured ECs, results indicate an upregulation of manganese superoxide dismutase, cyclooxygenase-2, and endothelial cell nitric oxide synthase (eNOS), three genes with promising anti-atherosclerotic potential [43]. Later studies involving the application of various forms of protective shear stress to ECs reveal significant induction of the transcription factor KLF2 [20, 25, 26], which was the first EC transcription factor shown to be under hemodynamic control. Given its robust

upregulation by shear stress, the mechanisms by which KLF2 could convey EC protection became a topic of great interest. KLF2 is expressed selectively in atheroprotected regions of human vasculature [25], and *in vitro* it drives expression and activity of eNOS, while inhibiting VCAM-1 and E-selectin expression, resulting in diminished leukocyte attachment [26]. Having firmly established the potential of KLF2 as a shear-stress induced mediator of atheroprotection, researchers manipulated KLF2 expression and utilized genome-wide microarray expression analysis to investigate the extent of KLF2 signaling. Their findings support KLF2 as a crucial master regulator of EC response to atheroprotective hemodynamics with major implications in cell migration, vascular tone, inflammation, thrombosis, and vessel development [28, 44]. Specifically, possible KLF2-mediated mechanisms for atheroprotection include KLF2-driven expression of eNOS and thrombomodulin (THBD), while suppressing the pro-coagulation proteins plasminogen activator inhibitor-1 (PAI-1/Serpine1) and tissue factor (TF/F3) as well as multiple inflammatory cytokines [28, 44], while thwarting the activation of ECs by the major inflammatory mediator interleukin 1 beta (IL-1β) [28].

Further analysis revealed mechanisms by which KLF2 upregulates eNOS and THBD expression and their resulting atheroprotective activities. Not only does KLF2 directly induce eNOS expression through direct promoter binding and recruitment of cyclic AMP response element-binding protein (CBP/p300) as a cofactor [26], but KLF2 has other indirect mechanisms for increasing eNOS effectiveness as well. KLF2 expression has been shown to inhibit the eNOS modulator Caveolin-1, while inducing argininosuccinate synthase, which is a limiting enzyme in eNOS substrate bioavailability [27, 44-47]. The resulting increase in eNOS expression and activity has the potential for

multiple atheroprotective benefits. Increased eNOS production of nitric oxide (NO) has been shown to critically regulate physiological vasomotor tone [48-50], as well as convey anti-inflammatory, anti-thrombotic, anti-platelet, anti-proliferative, and anti-migratory effects in vessels [27, 51, 52]. Dysregulation of eNOS and NO production has been shown to result in vascular endothelial dysfunction and play a major role in atherosclerosis [53-55]. Similarly, KLF2 strongly induces THBD expression and activity through direct interaction with its promoter [56]. THBD then has potent anti-inflammatory and anti-thrombotic effects by serving as a cofactor for thrombin-dependent generation of activated protein C [57, 58]. Consequently, flow-dependent, KLF2-mediated upregulation of eNOS and THBD may serve as critical atheroprotective events.

Additional studies have revealed that the ability of KLF2 to inhibit expression of inflammatory mediators is at least partially the consequence of reducing activation of NFκB and AP-1 transcription factor complexes [59-61], both well-known mediators of pro-inflammatory expression patterns. Inflammatory NFκB activity is further diminished by shear stress/KLF2-dependent expression of nephroblastoma overexpressed protein (NOV/CCN3) [62]. NOV was initially discovered as a protein upregulated in nephroblastoma tumors of chickens [63, 64] that may have cell proliferatory implications in multiple cancers [65-68]. Though expressed in diverse human tissues with ambiguous interactions in many potential signaling pathways (reviewed in [69]), NOV is found in vasculature [70-72] and dynamically regulated by vascular injury [73], suggesting a role in vessel inflammatory response. Furthermore, NOV is directly regulated by KLF2 binding to its promoter, and its expression is induced by both protective laminar shear

stress and statin treatment [62]. Although the mechanism is uncertain, NOV expression correlates with a loss of NFκB activation, inhibition of VCAM-1 expression, and a subsequent reduction in monocyte adhesion [62]. The recently defined KLF2 dependence and anti-inflammatory function of NOV present an intriguing but incompletely described mechanism for hemodynamic-dependent inhibition of atherogenesis that warrants further investigation.

Although significantly less understood, kruppel-like factor 4 (KLF4) is also induced by shear stress [74, 75] and similarly induces eNOS and THBD expression, while inhibiting TF, VCAM-1, and inflammatory cell adhesion [75]. However, unlike KLF2, KLF4 is also stimulated by exposure of ECs to challenges from inflammatory cytokines, suggesting it may serve a compensatory role should KLF2 become attenuated [27, 75].

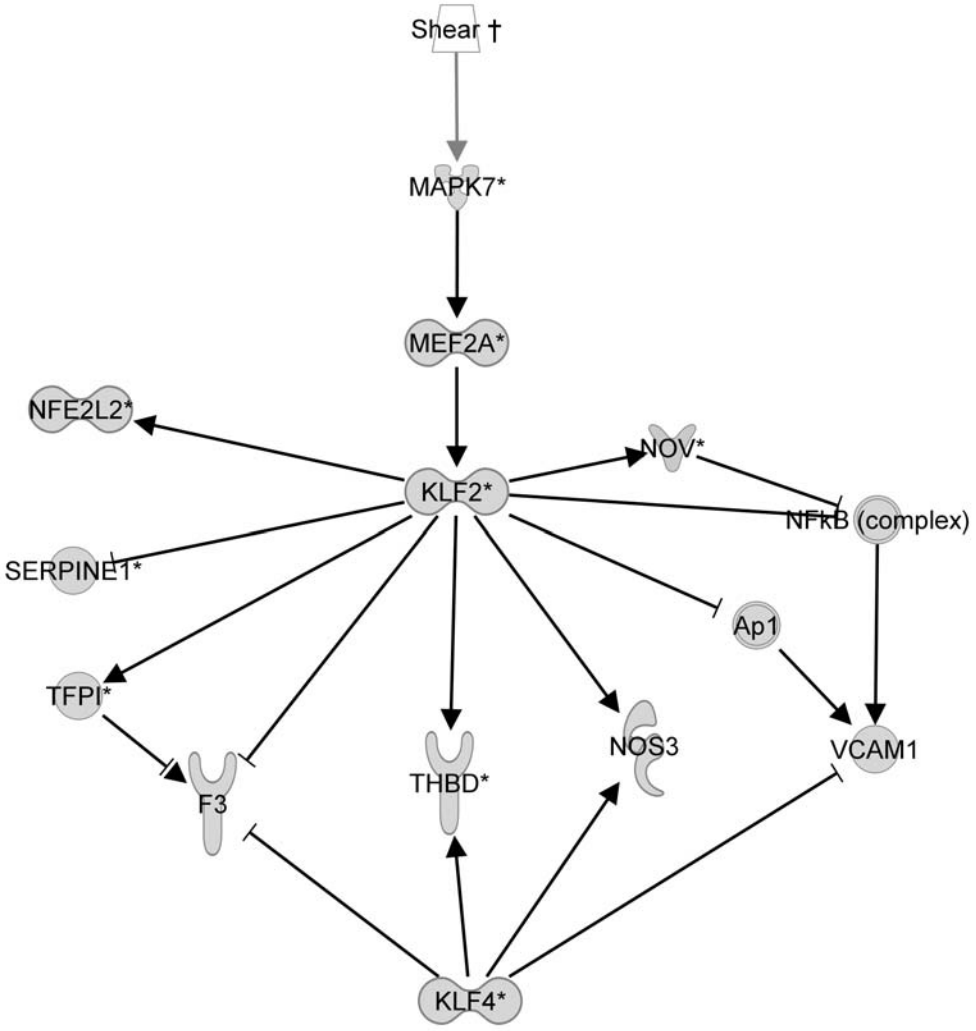

Figure 2. KLF2/4 are implicated in a variety of atheroprotective EC signaling pathways downstream of exposure to flow. Protein interactions pertinent to the studies within this dissertation are highlighted above. This figure was generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com)

2.5 Investigation of p120 Leads to Discovery of the Novel Transcription Factor Kaiso

Over the previous decade, the multifunctional protein p120-catenin (p120) has become the subject of an increasing amount of research, principally in the field of cancer. p120 is the prototypical member of a subfamily of armadillo-related proteins, which

includes p120, p0071, δ-catenin/NPRAP, ARVCF, and plakophilins 1/2/3 [76]. Additionally, p120 shares similarities with the highly studied protein, β-catenin, which has major functions maintaining the stability and integrity of cell-cell junctions [77] and, upon initiation by Wnt/wingless or other signals, translocates to the nucleus where it activates TCF transcription factors and regulates transcription. Unlike the well-characterized pathways for Wnt/β-catenin/TCF signaling, the knowledge of p120's role in cell biology is only beginning to be understood. While a large fraction of the previous p120 studies have focused on potential links to cancer and tumor progression, several of its functions and properties appear critical to the study of EC biology and atherogenesis. p120 has been shown to localize to three distinct subcellular pools: cell-cell junctions, cytoplasm, and nucleus (Figure 3), with varying functions associated with each of these locations. Within cell-cell junctions, p120's association with the juxtamembrane domain of VE-Cadherin has been shown to prevent endocytosis and turnover of the cadherin. Conversely, loss of p120 leads to greatly increased cadherin entry into lysosomal pathways with a loss of adherens junction stability [78-80] and a corresponding increase in permeability [81]. In the cytoplasm, p120 has been demonstrated to act as an inhibitor of Rho kinase through interactions with p190RhoGAP [82]. Additionally, p120 has been shown to associate with microtubule networks [83], stabilizing their dynamics, reducing catastrophe rates [84], and anchoring their minus ends at adherens junctions to stabilize cell-cell contacts [85]. Finally, like β-catenin an increasing amount of research has begun to focus on p120's ability to translocate to the nucleus, where it has the potential to regulate gene transcription. Yeast two-hybrid assays using p120 as bait unveiled interactions with a novel pox virus, zinc finger (POZ-ZF) transcription factor, which was

given the name Kaiso [86]. However unlike β-catenin, the cellular events that result in p120 translocation to the nucleus and the consequences of p120/Kaiso interaction on transcriptional events remain poorly understood and may play a novel role in atherogenesis.

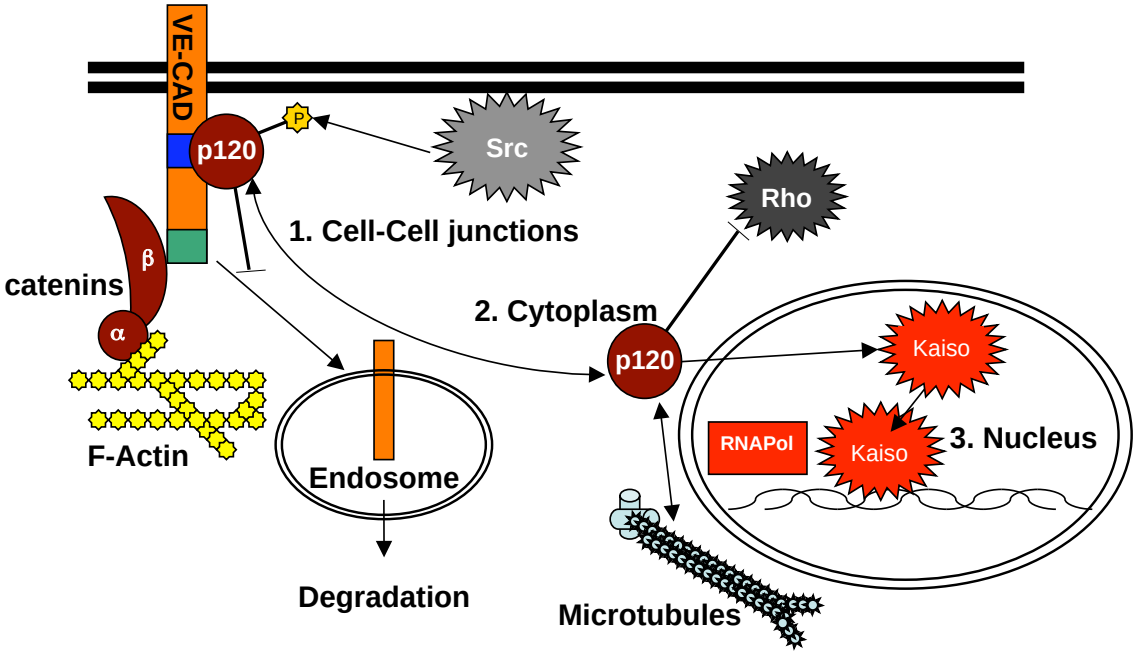

Figure 3. Subcellular localization of p120. p120 has been shown to localize to three distinct subcellular pools and possesses specific functions associated with each pool.

2.6 Kaiso-Mediated Regulation of Transcription as a Complex Potential Factor in Athero-Susceptibility

Kaiso was originally characterized as a transcriptional repressor that binds DNA bi-modally at methylated CpG dinucleotides and at the sequence-specific Kaiso binding site (KBS) TCCTGCNA [87, 88]. These findings led to the detection of nuclear localization of p120 [89, 90]. Given that separate studies had shown p120's interaction

with the transcription factor Kaiso and its ability to translocate to the nucleus, more recent studies have sought to elucidate the impact of p120 and Kaiso on gene transcription. *In vitro* studies involving the use of an artificial 4xKBS-luciferase reporter in HeLa cells indicated that nuclear localization of p120 is sufficient to reverse Kaiso-mediated transcriptional repression [91]. This study was further corroborated by evidence that Kaiso repression of several target genes, including the known Wnt/TCF/β-catenin targets *matrilysin* (in HeLa, HT-29, and HEK 293) and *siamois* (in *Xenopus laevis)*, can be alleviated by p120 expression [92, 93]. Other than some evidence of shared targets with TCF/β-catenin, the genes that are regulated by Kaiso remain mostly unknown and may provide a mechanism for determining susceptibility of tissues to atherosclerosis.

While early studies showed Kaiso negatively regulating transcription of target genes unless inhibited by association with p120, more recent investigations have indicated much greater complexity than initially thought. Research in bovine pulmonary artery and human dermal microvascular endothelial cells supports Kaiso as a transcriptional repressor but differs from previous studies by demonstrating that p120 association further contributes to Kaiso's repressive activities [94]. Interestingly, using *Xenopus* embryos and 293T cells, Iioka et al. demonstrated Kaiso's dose-dependent ability to either induce or repress canonical Wnt/β-catenin signaling. In these experiments, loss-of-function or mild overexpression experiments indicated Kaiso's positive effects, while moderate-to-high overexpression of Kaiso revealed repression of the same signals [95]. Similarly, in recent studies an additional model for Kaiso's regulatory abilities has been suggested. After demonstrating that Kaiso binds TCF3/4 and that this interaction mutually excludes either protein from binding to DNA and altering

transcription, it has been proposed that some of Kaiso's major repressive abilities derive from the ability to sequester TCF and block β-catenin-dependent activation of transcription [96]. However, it has also been shown that Wnt ligand binding results in CK1-dependent phosphorylation of p120, releasing it from cadherins and increasing its interaction with Kaiso. This interaction then relieves Kaiso's inhibition of TCF/β-catenin complexes and restores transcription of downstream targets [97, 98]. Previous work in our lab has demonstrated that exposure of ECs to atheroprone flow increases nuclear accumulation of β-catenin, resulting in TCF activation and β-catenin/TCF-dependent expression of fibronectin [34]. However, it is not known what role p120/Kaiso response to flow may have in this process, either through direct β-catenin/TCF interaction or through independent pathways. Although the quantity of Kaiso literature has increased drastically in previous years, these complex and often conflicting reports portray an incompletely understood protein with a potentially significant role in vascular physiology more broadly and atherogenesis more specifically.

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell Culture

Primary human umbilical vein endothelial cells (HUVEC) for *in vitro* studies were commercially obtained (Lonza, Vec Technologies, Cell Applications Inc.) or isolated and expanded from fresh umbilical vein specimens as previously described [30]. Umbilical cord specimens collected up to eight hours after childbirth were obtained from the Labor and Delivery Unit at the University of Virginia Health System (UVA Human Investigation Committee Approval #10486) or Martha Jefferson Hospital System (IRB Approval #0179). These cells were grown and passaged in Medium 199 (M199, Lonza) supplemented with 10% fetal bovine serum (FBS, Hyclone), 2 mM L-glutamine (Gibco), 100 U/mL Penicillin (Gibco), 100 μg/mL Streptomycin (Gibco), 10 μg/mL heparin, and 5 μg/mL endothelial cell growth supplement (ECGS, Biomedical Technologies). HUVECs up to passage 3 were plated in the same media at a density of 80,000 cells/cm^2 on gelatin-treated plastic for at least 18hrs prior to experimentation.

3.2 In Vitro Application of Hemodynamic Shear Stress

Confluent EC monolayers grown on gelatin coated 10cm dishes (Nunc) were washed with Dulbecco's Phosphate Buffered Saline (DPBS, Hyclone) containing calcium and magnesium, then covered in a flow-specific modified formulation of cell culture medium that contains reduced serum (2% FBS) and incorporates 4% dextran (Sigma) to

increase media viscosity to 4 cP, approximately the viscosity of human blood. The modified cone-and-plate described in chapter 2.3 was then used to apply atheroprotective (CCA) or atheroprone (ICS) hemodynamic waveforms for a predetermined period of time. During this period, the device was enclosed, allowing the environment to be maintained at a physiological 37 degrees Celsius and 5% CO_2 concentration (for pH). Flow media was exchanged continuously at a rate of approximately 3.6 mL/hour. At the conclusion of the experiment, monolayers were visually observed under light microscope, washed in DPBS, and then treated as appropriate to generate samples for various analyses.

3.3 siRNA Knockdown and Lentiviral Overexpression of Kaiso

To knockdown endogenous Kaiso expression, ECs were plated without antibiotics at 60% confluence in 10 cm gelatin-coated dishes and allowed to adhere for at least 2 hours, at which point the cells were washed in DPBS. Cells were then transfected with 0.87 pmol of ON-TARGETplus non-targeting control pool (D-001810, Thermo Scientific) or human Kaiso SMARTpool (ZBTB33, L-019982, Thermo Scientific) in 52 μL of Oligofectamine (Invitrogen) and 6.9 mL of OptiMEM-1 (Invitrogen) for 5 hours. After 5 hours, the dishes were flooded with 8 mL of cell culture medium minus antibiotics. 24 hours post-transfection this mixture was aspirated off and normal cell culture medium was applied. Cells were used for experimentation 48 hours after initial siRNA transfection.

To overexpress Kaiso in primary EC culture, a lentiviral Kaiso expression vector was constructed. A generously gifted a mammalian pCS2+myc-hKaiso expression plasmid from Dr. Juliet Daniel (McMaster University, [86, 99]) served as a template for hKaiso cloning into the *BamH1* site of pBOB-GFP. A stop codon was included upstream of the GFP to prevent its expression. Unmodified pBOB-GFP was used as an empty control. The hKaiso or GFP-control was transfected using Effectene (Qiagen) into Lenti-X 293T (Clontech) cells along with a fourth generation Lenti-X HTX Packaging System (Clontech) according to manufacturer's protocols. 24 hours post-transfection, media was aspirated off and replaced with EC cell culture medium without antibiotics. 48 hours post-transfection, viral-laden supernatant was filtered and aliquoted for future use. For transduction of ECs, cells were plated for at least 1 hour and the media was removed and replaced with lentiviral-laden media and 9 μg/mL polybrene (Roche) at an estimated MOI of 50. After 24 hours of virus exposure, media was replaced with fresh EC cell culture medium. Cells were utilized for experimentation 72 hours after the initiation of transduction.

3.4 Western Blotting and Immunoprecipitation

Protein was isolated by direct lysis of cells in the culture dish with MAPK sample buffer and 50 mM DTT. After SDS-PAGE, proteins were transferred to nitrocellulose membrane using an iBlot system (Invitrogen), and blocked 1 hour at room temperature using Odyssey Blocking Buffer (LI-COR). Primary antibody incubation (Table 1) occurred overnight at 4 decrees Celsius, followed by washing and application of

appropriate IRDye secondary antibodies (LI-COR) according to manufacturer's protocols. Quantification of protein intensity was performed using the LI-COR Odyssey system, using normalization to housekeeping α-tubulin where appropriate.

For Kaiso immunoprecipitation, samples were lysed in 1% Triton lysis buffer with 1:100 protease inhibitor cocktail (P8340, Sigma). 800 μg of protein was incubated overnight at 4 degrees Celsius with 20 μg of monoclonal Kaiso antibody (6F, Invitrogen) and 1.5 mg of Dynabeads Protein G (Invitrogen). Beads were washed and the precipitated protein was eluted with 20 μL of MAPK sample buffer and 50 mM DTT. Samples were then Western blotted as described above.

Protein	**Host**	**Clone**	**Manufacturer**	**Dilution**
αTubulin	mouse	DM1A	Sigma	1/750
αTubulin	chicken	pAb	Sigma	1/1000
eNOS	mouse	3	BD Transduction	1/1000
FN	mouse	10	BD Transduction	1/1000
ICAM-1	goat	BBA17	R&D	1/1000
Kaiso	rabbit	pAb	Albert Reynolds	1/1000
p120	mouse	15D2	Santa Cruz	1/200
VCAM-1	rabbit	H-276	Santa Cruz	1/750

Table 1. Primary antibodies used in Western blotting.

3.5 mRNA Isolation and Real Time RT-PCR Quantification

Upon completion of hemodynamic experiments, cells were observed by light microscope and washed in cold DPBS. RNA was then isolated from samples via PureLink RNA mini kit (Life Technologies), and served as a template for constructing cDNA with a iScript cDNA Synthesis kit (Bio-Rad). Genes of interest (Table 2) were then assayed in duplicate using real time reverse transcriptase polymerase chain reaction

(RT-PCR) and FastStart Universal SYBR Green Master (Roche) for visualization. Housekeeping gene β-2-microglobulin (B2M) was used for normalization.

Gene	Sense Primer	Anti-sense Primer
B2M	AGCATTCGGGCCGAGATGTCT	CTGCTGGATGACGTGAGTAAACCT
E-Selectin	AATCCCAGTTTGTGAAGCTTTCCA	GCCAGAAGCACTAGGAAGACAATT
eNOS	CTCCATTAAGAGGAGCGGCTC	CTAAGCTGGTAGGTGCCTGTG
FN	AACGATCAGGACACAAGGAC	CCTCTCACACTTCCACTCTC
ICAM-1	TCGCTATGGCTCCCAGCAGC	TTCCGGTTGTTCCCAGGCAGG
Kaiso	ACACCACTTTCTACACCACC	TTATTTCCTCCTCCTCCTCTTC
KLF2	GCTGAGTGAACCCATCCTGCC	CGCTGTTGAGGTCGTCGTCG
KLF4	GGCCAGAATTGGACCCGGTGTAC	GCTGCCTTTGCTGACGCTGATGA
MEF2a	GAACAAGAAGGAACACAGAGG	TGTAGGACAAAGCATTGGGG
NFκB	ATCATCCACCTTCATTCTCAAC	CAAATCCTCCACCACATCTTC
NOV	CAGCAACCAGACTGGCATC	GAATTTGCAGCTTGGCTGA
PAI-1 (Serpine1)	GAGGTGCCTCTCTCTGCCCTCACCAACATT	AGCCTGAAACTGTCTGAACATGTCG
TF/F3	CTGGAGACAAACCTCGGACAGCCA	CGGGCTGTCTGTACTCTTCCGG
TFPI	GATGGTCCGAATGGTTTCCAGGTGG	TGGGCGGCATTTCCCAATGACT
THBD	CCTCCATGCATCTCATAGCA	CGGGTTGTGTGTCTGTTCAC
VCAM-1	GTTTGTCAGGCTAAGTTACATATTGATGA	GGGCAACATTGACATAAAGTGTTT

Table 2. Primers used in real time RT-PCR.

3.6 Luciferase Reporter Assays of Transcription Factor Activity Levels

Adenoviral luciferase reporter vectors Ad-NFκB-Luc and Ad-AP1-Luc were commercially obtained (1740 and 1670, Vector Biolabs). To obtain similar adenoviral-based reporters of Kaiso activity, 4xKBS-Luc was cloned out of a pGL3-4xKBS-Luc mammalian expression plasmid (gift from Dr. Juliet Daniel, [91, 93, 94]) and commercially packaged into a custom Ad-4xKBS-Luc vector (Vector Biolabs). 24 hours prior to hemodynamic exposure, ECs were infected with adenovirus at 50 MOI. At the completion of the experiment, ECs were observed by light microscopy, scraped in DPBS, pelleted by centrifugation, and lysed in 100 μL of Passive Lysis Buffer (Promega) for 30

minutes at 4 degrees Celsius. Luciferase Assay Reagent substrate (Promega) was added and luminescence was quantified according to manufacturer's protocols. For each sample, resulting relative luminescence units (RLUs) were normalized to total protein concentration, which was quantified by Coomassie Plus Reagent (Thermo Scientific).

3.7 Monocyte Adhesion Assay

THP-1 monocytes were washed twice in DPBS, pelleted, and resuspended in serum free RPMI (Gibco) at a concentration of $1x10^6$ cells/mL. The cells were then labeled with 1 μg/mL Calcein AM (Life Technologies) for 15 minutes at 37 degrees Celsius. THP-1 cells were then pelleted, washed in M199, pelleted again, and resuspended in serum free M199 at a concentration of $1x10^6$ cells/mL. Upon completion of 24 hours of hemodynamic exposure with or without exposure to 0.1 ng/μL TNFα for the final 6 hours of flow, EC monolayers in 10 cm dishes were washed in serum free M199 and 3 million labeled THP-1 cells were applied in 8 mL of serum free M199 for 30 minues at 37 degrees Celsius. Samples were then washed twice in DPBS, fixed with 4% paraformaldehyde (PFA) at room temperature for 15 minutes, and washed another four times in DPBS. Monolayers were dried and glass coverslips were mounted with ProLong Gold Antifade Reagent with DAPI (Life Technologies). At least 7 representative images for each sample were obtained at 20x magnification with a fluorescence microscope operated in the 488 nm channel. The number of adherent monocytes per 20x field was quantified by particle analysis in ImageJ (NIH).

3.8 Gene Expression Microarray Analysis

siKaiso or siControl treated ECs were exposed to either atheroprotective or atheroprone flow, as described above, for 24 hours, after which the cells were observed by light microscopy, washed with cold DPBS, and then RNA was isolated from samples via PureLink RNA mini kit (Life Technologies). Total RNA samples in triplicate from each of these four treatment groups were commercially processed and analyzed on GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix) by Expression Analysis (Durham, NC). siKaiso treated ECs were compared to siControl for atheroprotective hemodynamics, atheroprone hemodynamics, and globally. Correcting for variation between individual microarray results, a statistical analysis of fold changes, average expressions, and false discovery rates was provided by Dr. Aaron Mackey (University of Virginia Center for Public Health Genomics), utilizing R Project for Statistical Computing software (www.r-project.org). Heatmaps were generated by utilizing the gplots programming tool in R version 2.11.0 [100]. Additionally, the signaling pathways, networks, functions, biological ramifications, etc. of Kaiso-dependent genes were visualized and analyzed through the use of IPA (Ingenuity® Systems, www.ingenuity.com). Functional clustering of findings by gene ontology terms was completed in the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 [101, 102]. Venn diagrams were generated for viewing relationships between different comparison groups through use of the publicly available VENNY program [103].

3.9 Kaiso Immunohistochemistry in ApoE$^{-/-}$ Mice

The well-established mouse model for studying atherosclerosis, a genetic knockout of apolipoprotein E (ApoE$^{-/-}$), has been shown to develop atherosclerotic plaques similar to those found in humans but at an accelerated rate [104, 105]. To establish Kaiso expression in healthy and diseased regions of vasculature *in vivo*, ApoE$^{-/-}$ mice were fed a pro-atherogenic Western diet for 8 or 20 weeks (in accordance with University of Virginia ACUC protocol #3597) and euthanized. The aortic arch was dissected out, fixed in paraformaldehyde, paraffin embedded, and then prepared in cross-sections. Kaiso was immunofluorescently labeled by primary rabbit polyclonal Kaiso antibody (gifted from Dr. Albert Reynolds) and fluorophore-tagged anti-rabbit secondary before confocal microscopy imaging. TOTO-3 served as a nuclear marker.

3.10 Statistical Analysis

All data is presented as means with associated standard error. Statistical significance (p-value < 0.05) was determined by 1- or 2-tailed Student's t-test or ANOVA, as appropriate. In instances of comparing fold changes of treatment groups relative to control, p-values were obtained by a one-sample t-test to determine if the fold change is significantly different than a value of 1 (no change).

CHAPTER 4: THE EFFECT OF HEMODYNAMIC ENVIRONMENT ON KAISO EXPRESSION, INTERACTION WITH p120, AND ACTIVITY

4.1 Methodology

In order to create a time–course of p120 and Kaiso expression and interaction levels, EC monolayers were plated to confluence and exposed to atheroprotective or atheroprone hemodynamics for 6, 16, 24, or 48 hours of flow. p120 is abundant in ECs and was easily resolved by SDS-PAGE and Western blotting of whole cell lysates. In order to quantify Kaiso expression and its relative interaction with p120, Kaiso was immunoprecipitated by antibody-covered magnetic beads, and the resulting Kaiso complexes were eluted and separated by SDS-PAGE and quantified by Western blotting for both Kaiso and Kaiso-bound p120.

To determine the effect of hemodynamic environment on Kaiso activity, ECs were infected with Ad-4xKBS-Luc reporter and exposed to atheroprotective or atheroprone flow for 6, 16, or 24 hours. Luciferase expression was quantified and normalized to luciferase expression at the initiation of flow (T_0).

To characterize the effects of Kaiso expression on Ad-4xKBS-Luc reporter activity, Kaiso was knocked down by siRNA or overexpressed by a lentiviral vector and exposed to 24 hours of either atheroprotective or atheroprone flow. Control siRNA or empty lentivirus was used as negative controls for comparison.

Finally, to confirm the expression of endothelial Kaiso *in vivo*, ApoE$^{-/-}$ mice on 8 or 20 weeks of Western diet served as models of early and intermediate/advanced

atherosclerosis, respectively. Fluorescently labeled cross sections of ascending aorta were observed by confocal microscopy to confirm Kaiso localization within the mouse vasculature.

4.2 Results

p120 expression is differentially regulated by flow, but Kaiso expression is not

Western blot analysis of whole cell lysate indicated that total expression of p120 was significantly higher in EC monolayers exposed to atheroprotective hemodynamics when compared to atheroprone flow (Figure 4A). While this result was only significant at 24 hours (P=0.03), there appears to be a trend of acute (6h, P=0.15) and prolonged (48h, P=0.28) activation of p120 expression in protective flow relative to prone.

On the other hand, immunoprecipitation and quantification of Kaiso revealed that the protein was not differentially expressed between the two hemodynamic profiles at any time point examined. Furthermore, the time-course revealed nearly identical Kaiso expression trends in ECs exposed to flow, with initiation of either hemodynamic waveform resulting in an acute (6-24 hours) but nonsignificant induction of Kaiso expression that rebounded fully by 48 hours.

p120 interaction with Kaiso is not regulated by hemodynamics

Co-immunoprecipitation of Kaiso complexes revealed that, like total Kaiso expression levels, Kaiso's interaction with p120 is not hemodynamically regulated. As shown previously by Western blot [106], p120 isoforms 1 and 2 migrate in an

approximately 120kD band, while isoforms 3 and 4 resolve near 100kD and 70kD, respectively (Figure 4B). Each p120 band was analyzed individually, as were various combinations of the 3 bands. At each time point, neither single bands, combinations of 2 bands, or total p120 displayed p120/Kaiso interaction that was differentially regulated between protective and prone hemodynamic profiles. The lowest molecular weight p120, isoform 4, displayed highly variable and unpredictable binding to Kaiso, skewing total p120 quantification. Consequently, the data is best represented by separating total p120 co-immunoprecipitation into Kaiso interaction with p120 isoforms 1-3 or isoform 4 alone (Figure 4C).

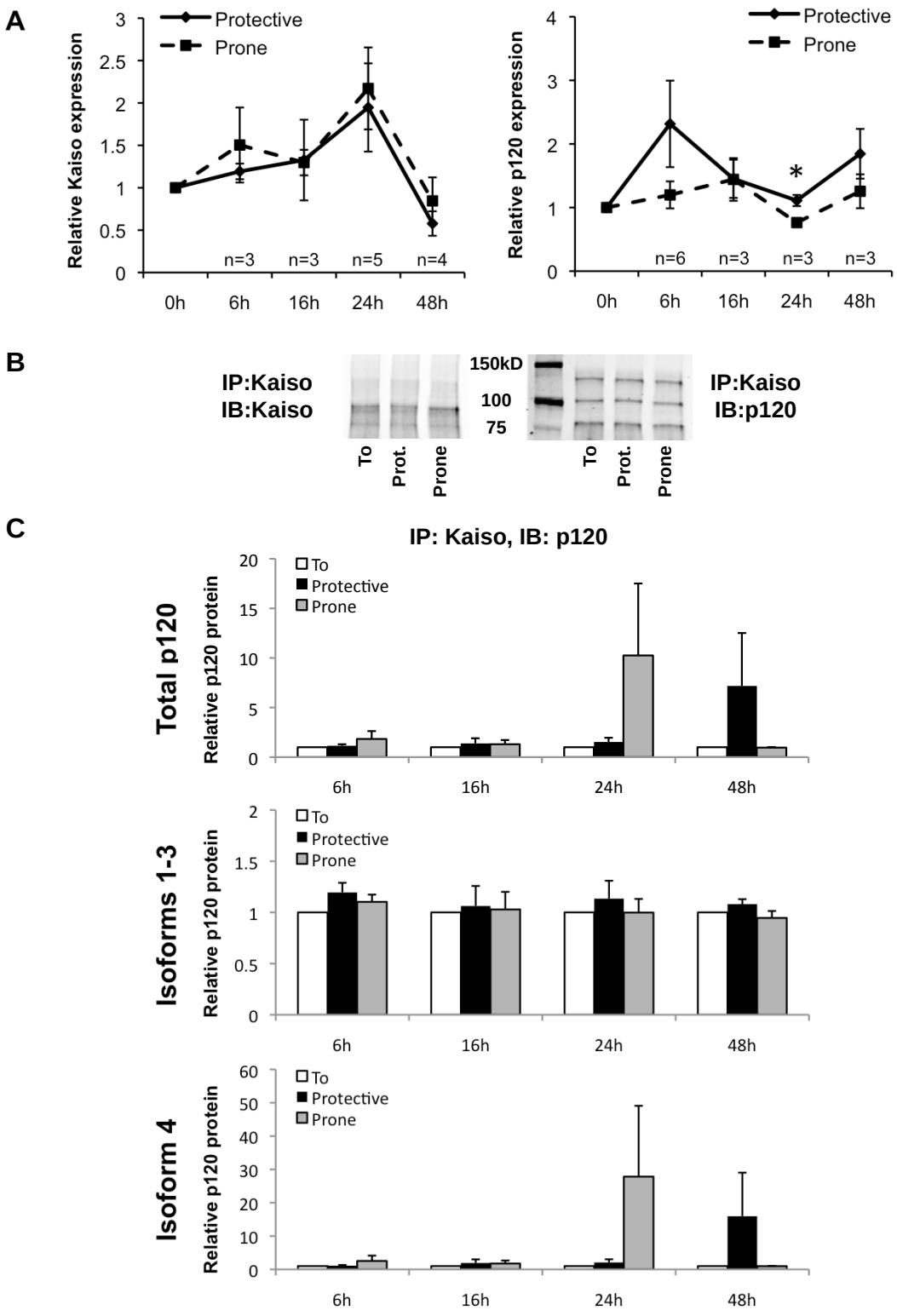

Figure 4. Regulation of p120 and Kaiso expression and interaction by hemodynamics. (A) Protein expression levels of p120 and Kaiso over time in ECs exposed to hemodynamic flow. (B) Representative blot demonstrating the co-immunoprecipitation of p120 and Kaiso at 16h. (C) Quantification of p120 interaction with Kaiso in ECs under flow by co-immunoprecipitation, including a separation of p120 isoforms 1-3 (combined) from highly variable isoform 4. *$p<0.05$

Kaiso activity is differentially regulated by hemodynamic environment

Our hypothesis postulates that hemodynamic environment modulates Kaiso expression and/or activity, but our evidence suggests that hemodynamics do not differentially regulate Kaiso expression or its interaction with known activity modulator p120. It was therefore important to directly determine the shear stress dependent regulation of Kaiso activity.

ECs infected with Ad-4xKBS-Luc and exposed to either protective or prone hemodynamics revealed that luciferase expression under the control of Kaiso binding was significantly higher in atheroprone conditions than atheroprotective at 16 hours (1.51 fold, $p = 0.041$) and 24 hours (1.69 fold, $p = 0.042$) (Figure 5A). Given the uncertainty of Kaiso's transcriptional regulatory mechanisms, this result may be interpreted as increased inhibitory activity in atheroprotective flow or increased positive activity in atheroprone conditions (see 4.3 Discussion). Consequently, to characterize the response of this reporter to Kaiso in our novel *in vitro* endothelial cell hemodynamic system, additional 24 hour experiments were conducted, incorporating manipulation of Kaiso expression by siRNA-mediated knockdown (Figure 5B) or lentiviral-mediated overexpression (Figure 5C). First, negative controls from both experiments confirmed the increase in reporter activity in atheroprone conditions relative to atheroprotective. Unexpectedly, regardless of the hemodynamic condition, both knockdown (mean expression = 0.51 fold) and overexpression (mean expression = 4.38 fold) of Kaiso resulted in an increase in reporter activity. This is a novel finding in our hemodynamic model that is in stark contrast to many of the previously reported findings using a similar reporter construct that define Kaiso as purely repressive [91, 93, 94].

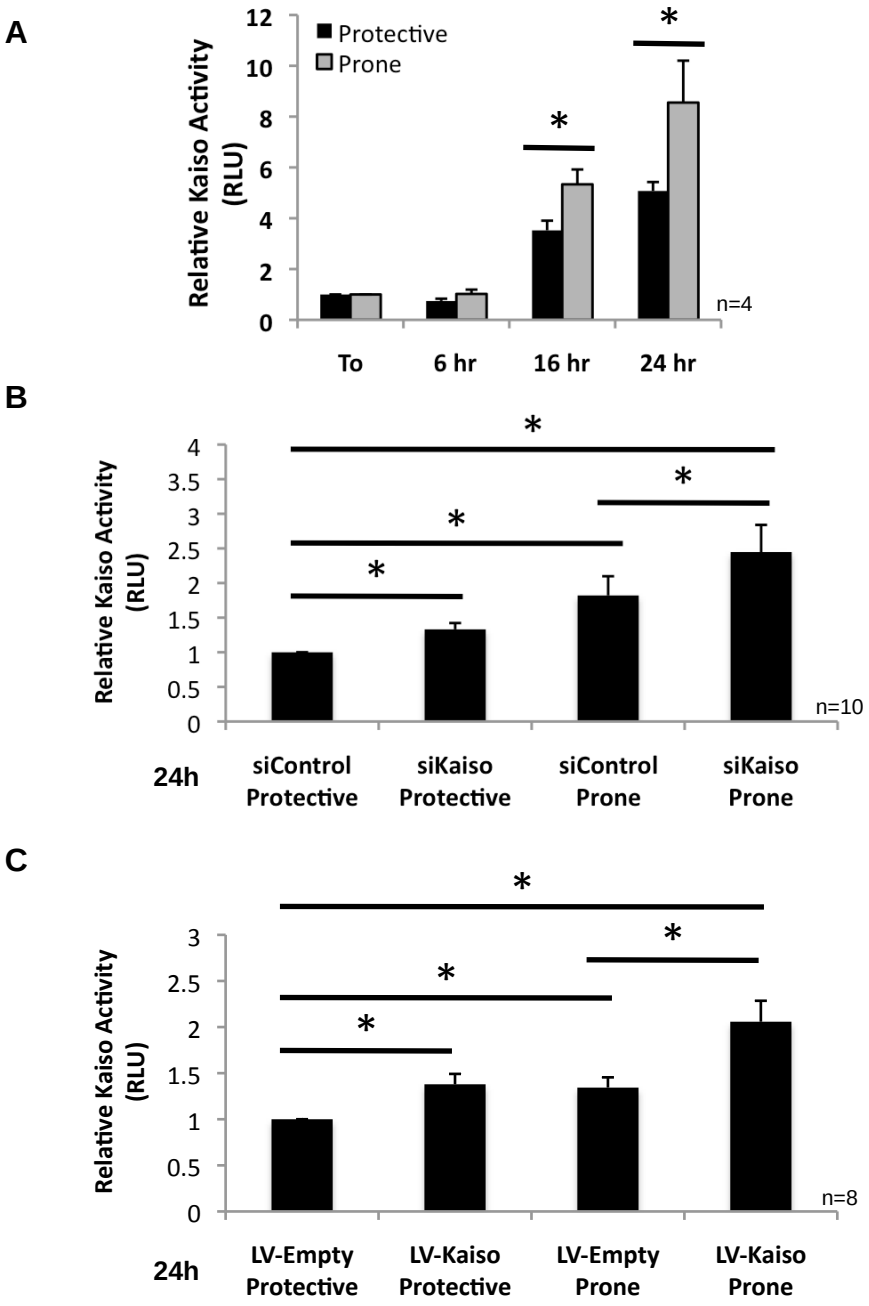

Figure 5. Regulation of a Kaiso activity reporter by hemodynamics and manipulation of Kaiso expression. (A) ECs were infected with Ad-4xKBS-Luc and exposed to protective or prone flow for varying degrees of time, revealing higher luciferase expression in atheroprone flow. For 24 hours of either protective or prone flow, Kaiso was modulated by (B) siRNA knockdown or (C) lentiviral overexpression and the effects on Kaiso activity were quantified. Both overexpression and knockdown of Kaiso result in increased Kaiso reporter activity. *$p<0.05$

Kaiso is expressed in the endothelium and underlying structures in the large arteries of ApoE$^{-/-}$ mice

As expected, ApoE$^{-/-}$ mice fed a Western diet for 8 or 20 weeks generated examples of early or intermediate/advanced atherosclerotic disease, respectively, in the ascending aorta. Confocal microscopy of Kaiso-labeled cross sections confirmed that Kaiso was expressed in the endothelium and underlying tissues of healthy vessel wall (Figure 6 A, B, C) as well as within both early (Figure 6 A, C) and more advanced (Figure 6 B, D) lesions. Higher magnification images overlaying Kaiso expression with the nuclear marker TOTO-3 highlight the nuclear/peri-nuclear localization of Kaiso in endothelial cells and suggest a more diffuse staining pattern in the underlying vessel wall or lesion (Figure 6 C, D).

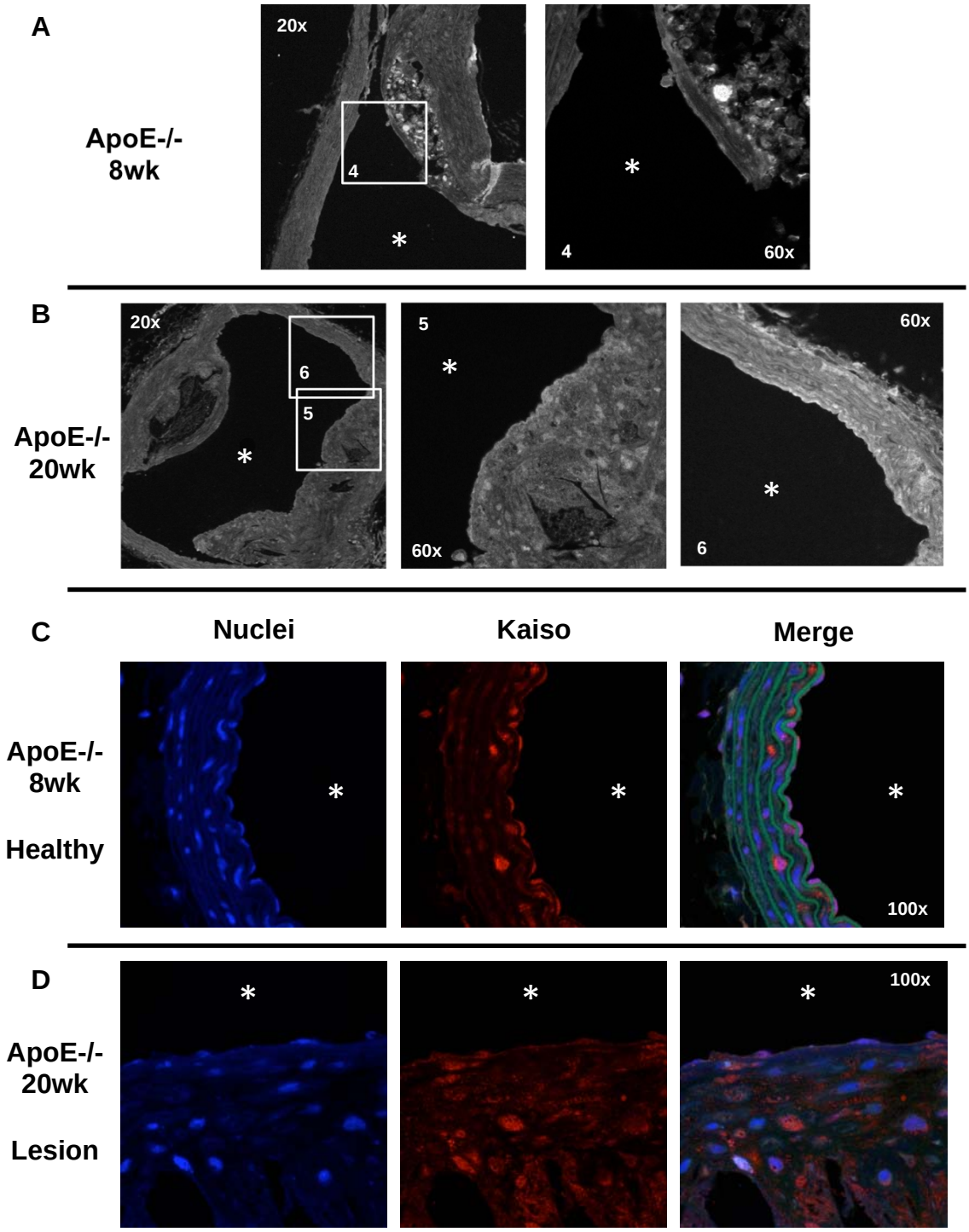

Figure 6. Representative confocal images of Kaiso expression in the ascending aorta of an ApoE$^{-/-}$ mouse. In mice fed (A) 8 or (B) 20 weeks of Western diet to stimulate lesion development. Kaiso is expressed in both healthy and diseased tissue at both time points. Higher magnification (100x) images of Kaiso expression in a (C) healthy vessel wall from an 8 week ApoE$^{-/-}$ mouse or (D) advanced lesion of a 20 week ApoE$^{-/-}$ mouse, overlayed with a nuclear stain. Kaiso demonstrates both (peri-)nuclear localization and more diffuse patterns. * represents the location of the vessel lumen.

4.3 Discussion

With increasing literature establishing p120's importance in maintaining endothelial monolayer integrity and stability, which are vital in preventing atherogenesis, understanding p120's other possible functions in endothelial cells is a priority. One of p120's least understood roles remains its interaction with the transcription factor Kaiso. While the fundamental knowledge of Kaiso is still growing, it remains a relatively newly discovered and largely mysterious protein, particularly in the field of hemodynamic regulation of EC biology. In particular, the cellular events that control Kaiso activity, the genes that are transcriptionally regulated as a result, and the downstream physiological consequences of this signaling have remained highly elusive. Given the emerging importance of p120 to EC biology and the expression of Kaiso in human and mouse endothelium with unknown significance, we sought to establish the regulation of Kaiso expression and activity by hemodynamic environment, as this has been shown to regulate countless signaling pathways involved in atherosclerosis.

Although Kaiso was initially considered a negative regulator of gene transcription whose inhibitory abilities were relieved by p120 binding [91-93], increasing evidence has begun to display a much greater complexity to Kaiso activity than previously thought [94-98]. Our research adds to this increasing view of Kaiso complexity. For example, despite no significant difference in Kaiso expression or interaction with p120, we demonstrated that Kaiso reporter activity is increased under atheroprone hemodynamic conditions compared to protective. Since expression studies alone provide limited information about transcription factor activity levels, this result revealed that

hemodynamic modulation of Kaiso activity is independent of both total expression levels and p120 interaction. This raises multiple questions. First, what are the mechanisms by which hemodynamics modulate Kaiso activity if not through expression or interaction with p120? Possible explanations for this include shear stress-mediated regulation of Kaiso shuttling into and out of the nucleus, post-translational modifications to Kaiso that influence activity, and binding of Kaiso to currently unknown protein-binding partners. Given that Kaiso has been shown to physically interact with both p120 and β-catenin/TCF, it is highly conceivable that other undiscovered protein interactions exist that might be regulated by hemodynamics and have an influence on activity. Second, the reporter findings raise a question about how increased luciferase expression under the control of Kaiso binding sequences could be interpreted. Does higher reporter activity in atheroprone conditions indicate an increase in Kaiso's ability to positively regulate transcription in prone flow, an increase of Kaiso-mediated repression in protective flow, or some combination of the two? Iioka et al. have demonstrated the possibility that Kaiso can act as a bimodal modulator of transcription [95], so none of those possibilities can be excluded.

To determine whether Kaiso was acting as a positive or negative regulator of transcription in our hemodynamic reporter system, we performed a set of 24 hour Kaiso reporter experiments under protective or prone flow in which we knocked down or overexpressed Kaiso. Surprisingly, regardless of the flow paradigm, both Kaiso knockdown and overexpression resulted in higher reporter activity. While this confounding result does not solidly define Kaiso as solely a positive or negative regulator of transcription in ECs, it further suggests that simpler preconceived notions of Kaiso

function may not be applicable in our system and that this incompletely understood transcription factor may behave in ways not previously considered. Furthermore, these results might be explained by dose-dependency of Kaiso expression levels. In the Iioka et al. study, loss-of-function and moderate gain-of-function demonstrated the ability of Kaiso to activate downstream signaling pathways, while high levels of overexpression resulted in inhibitory effects. Our findings may coincide with this and other increasing evidence of Kaiso complexity. It is also possible that the simplified structure of the reporter system neglects to account for other potential Kaiso functions that occur endogenously. While control of this reporter is only moderated by consensus KBS binding sites immediately upstream of the active promoter, Kaiso has demonstrated ability to bind methylated CpG dinucleotides as well, which is a possibility neglected by this system. Additionally, in Kaiso regulation of endogenous genes, there could other unknown binding elements upstream or downstream that interact with Kaiso other transcription factors and further modulate transcription in unanticipated ways. Finally, Kaiso may be able to modulate transcription of endogenous target genes through trans-regulatory methods, by controlling the expression and/or activity of another transcription factor that then binds cis-elements in the promoter of the target gene. This possibility cannot be accounted for in the simplified promoter of the Kaiso activity reporter used in these experiments. Regardless, the results suggest that Kaiso expression and activity is tightly controlled by EC environment and that artificial manipulation of Kaiso expression may upset cell equilibrium and result in unpredicted effects.

In summary, hemodynamic environment was shown to regulate Kaiso activity despite no changes in its expression or interaction with p120. Attempts to directly

determine Kaiso's effect on transcription of its reporter construct suggest that Kaiso has complex activity and perhaps cannot be simply defined as either an inhibitor or activator of transcription but may instead have elements of both of these activities. Combined with the lack of knowledge regarding downstream targets, evidence of hemodynamic regulation of Kaiso activity supports the hypothesis that Kaiso may play a novel role in EC response to hemodynamics with major implications in atherosclerosis.

CHAPTER 5: DEFINING A COMPREHENSIVE FUNCTIONAL ROLE FOR KAISO IN ENDOTHELIUM EXPOSED TO FLOW THROUGH THE UTILIZATION OF GENOME-WIDE EXPRESSION ANALYSIS

5.1 Methodology

In order to evaluate the genome-wide functional contributions of Kaiso, ECs were treated with control or Kaiso-directed siRNA and exposed to atheroprotective or atheroprone hemodynamics for 24 hours. Affymetrix U133 2.0 Plus microarrays were utilized to quantify relative changes in the expression of a comprehensive library of human transcripts. For experiments performed in triplicate, expression comparisons were made between siControl and siKaiso treatment groups for each hemodynamic condition as well as globally. IPA (Ingenuity® Systems, www.ingenuity.com) and DAVID Bioinformatics [101, 102] were used to explore relationships, signaling pathways, and functional enrichments within the Kaiso-dependent genes revealed by the microarray analysis.

5.2 Results and Discussion

Quantifying of the hemodynamic trends of Kaiso-dependent genes

A quantification of genes whose expression was Kaiso-dependent, as determined at false discovery rates (FDRs) less than 0.20, 0.10, or 0.05, is summarized in Table 3. Briefly, statistical combination of findings from both hemodynamic conditions (global

siKaiso v siControl	FDR < 0.20		FDR<0.10		FDR<0.05	
	# Genes Up	# Genes Down	# Genes Up	# Genes Down	# Genes Up	# Genes Down
Global	926	984	166	177	20	22
Protective	363	442	80	81	11	7
Prone	217	144	8	7	3	2

Table 3. Kaiso knockdown resulted in similar numbers of up- and downregulated genes and preferentially affects atheroprotective transcription. Genes up- or downregulated by Kaiso siRNA treatment were quantified by individual hemodynamic conditions or globally at a range of false discovery rates (FDR).

comparison) at an FDR<0.20 demonstrated a total of 1910 transcripts that were differentially expressed as a result of Kaiso knockdown. Quantifying the results by individual hemodynamic condition reveals that considerably more genes were affected by loss of Kaiso under atheroprotective conditions relative to atheroprone, a trend that was not dependent on the FDR. This result suggests that Kaiso may play a larger or more important role in mediating the downstream consequences of EC exposure to atheroprotective hemodynamics. Interestingly, at all FDRs, loss of Kaiso expression resulted in nearly equal numbers of upregulated and downregulated genes for each hemodynamic condition and globally. It is possible that Kaiso could possess solely repressive abilities and by inhibiting other repressive transcription factor pathways indirectly result in positive expression outcomes; however, this finding could also suggest that Kaiso demonstrates bimodal transcriptional regulatory abilities. Unfortunately, this analysis lacks the capability to differentiate between direct and indirect influences of Kaiso knockdown.

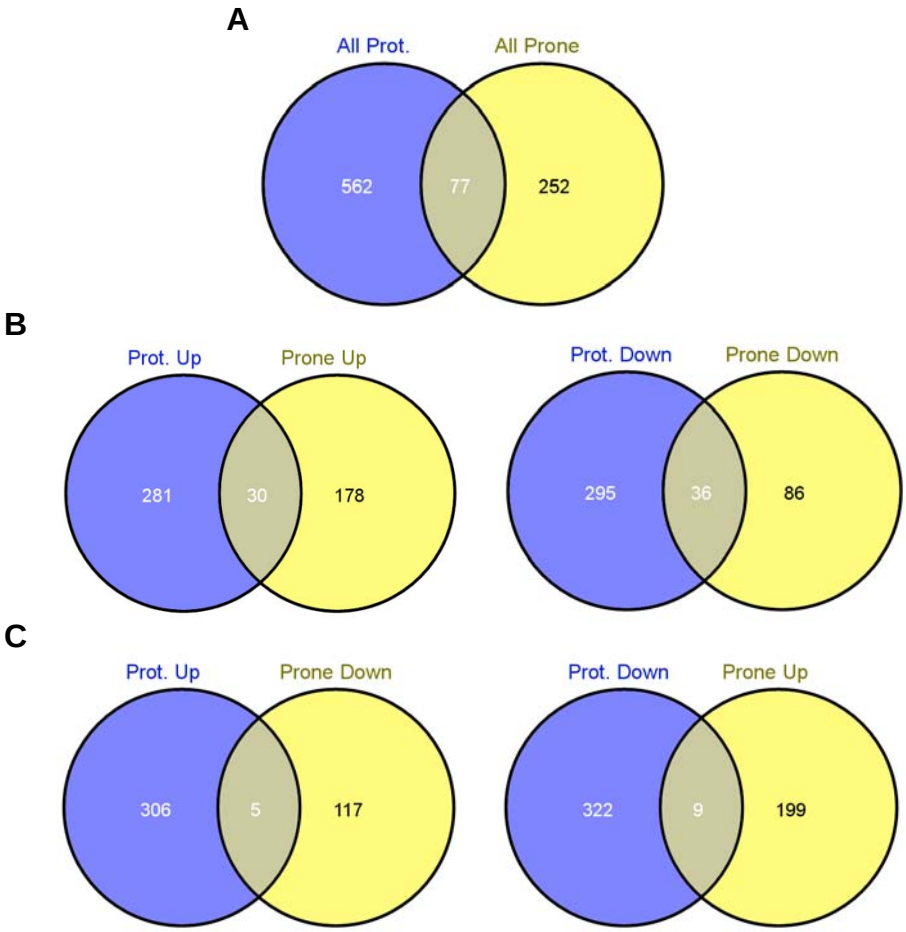

Figure 7. Kaiso siRNA treatment resulted in both flow-dependent and flow-independent changes in downstream transcripts. For an FDR<0.20, the number of unique and shared findings is displayed for (A) all upregulated and downregulated genes. (B) Separating results into upregulated or downregulated genes demonstrates flow-dependence. (C) Genes exhibiting an upregulation under one hemodynamic condition but downregulation under the other are identified.

While Table 3 demonstrates the raw sums of Kaiso-dependent genes for various treatment conditions, Venn diagrams present the relationships among comparison groups and highlight unique and shared results (Figure 7). While this visualization confirms that both upregulation and downregulation of gene transcripts were more prevalent under protective hemodyamics compared to prone, it additionally highlights subsets of genes that are either hemodynamically dependent or independent. While a smaller subset of genes demonstrated the same expression trend regardless of the hemodynamic

environment, the majority of findings are unique to either protective or prone hemodynamics, and may be considered both Kaiso- and hemodynamically dependent. While these unique transcripts may comprise genes that are predominantly expressed under only one hemodynamic condition, and thus Kaiso knockdown only generated a measurable response in the proper environment, they may also demonstrate hemodynamic-dependent differences in Kaiso function. The possibility of hemodynamic "switching" of Kaiso function is further validated by a small subset of genes whose Kaiso-dependent upregulation in one condition was contrasted by downregulation in the other hemodynamic context (Figure 7C). The microarray analysis revealed 14 such genes that had conflicting responses to Kaiso knockdown in the two hemodynamic conditions. Unfortunately, these genes do not demonstrate any functional clustering by gene ontology terms, likely due to the small sample size. Taken together, the hemodynamic dependence of Kaiso transcriptional regulatory ability demonstrates the importance of investigating both Kaiso function and EC transcriptional patterns within the proper context of physiological flow.

Analysis of EC Kaiso function in atheroprotective and atheroprone hemodynamic conditions

Figure 8 graphically displays $\log_2$ fold changes in gene expression resulting from Kaiso knockdown under either hemodynamic condition and globally in a heat map format. A broad FDR<0.20 and a more stringent FDR<0.05 are shown simultaneously to give both a highly inclusive and a more specific qualitative analysis, respectively. Like

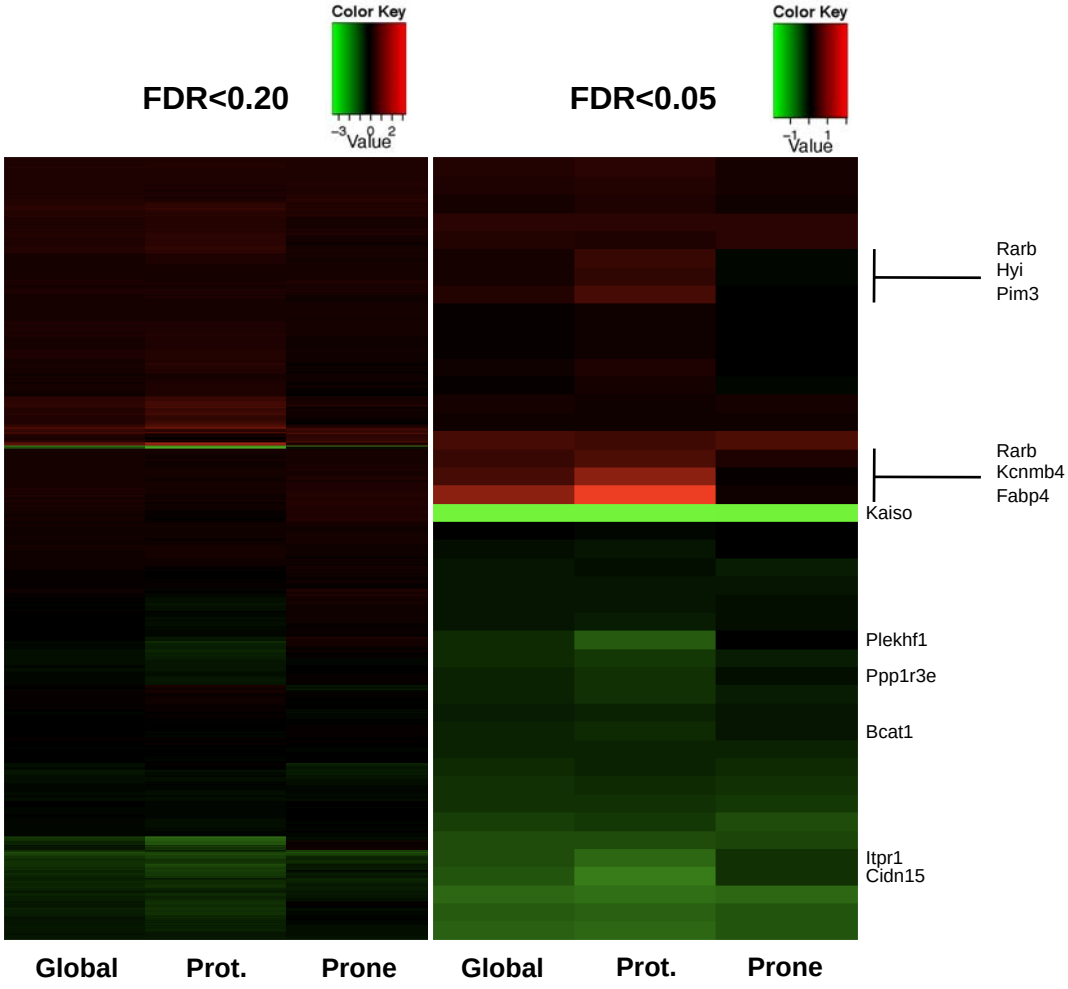

Figure 8. The quantity and magnitude of changes in Kaiso-dependent gene expression was elevated under atheroprotective hemodynamics relative to atheroprone. Log_2 fold changes in gene expression as a result of Kaiso knockdown are colorimetrically represented at both broad (<0.20) and stringent (<0.05) FDR. Genes notably upregulated (red) or downregulated (green) under atheroprotective but not atheroprone hemodynamics are annotated on the right.

the quantitative trends discussed above, this analysis of fold changes suggests the increased role for Kaiso-dependent regulation of transcription under atheroprotective hemodynamic conditions. In general, the heat map displays visual confirmation that both the quantity of genes differentially expressed and the magnitude of their fold changes are elevated in protective hemodynamics relative to prone. Similarly, within a single hemodynamic condition, the up- and downregulation of genes is not only similar in quantity, but also roughly equivalent in terms of magnitude. This qualitative evaluation corroborates the implication that loss of Kaiso similarly affects both upregulation and downregulation of downstream targets, with greater predilection for atheroprotective hemodynamics. In the case of the more strict FDR<0.05 heat map, it was possible to identify individual genes with highly contrasting findings between hemodynamic conditions, which are annotated on the right side of Figure 8. While no unifying functional clustering can be found for this small subset, these genes are involved in a wide variety of cellular functions including growth/differentiation/proliferation/survival, calcium release and signaling, and metabolic pathways.

Given the indications that Kaiso's role in EC biology varies between hemodynamic conditions, we evaluated the signaling pathways and gene ontology (GO) clustering of Kaiso-dependent genes for each hemodynamic environment separately. The top networks and functions of Kaiso-dependent genes, as determined by pathway analysis, are summarized in Table 4. Additionally, clustering datasets based on GO terms identified cellular functions that are overrepresented in Kaiso-dependent genes compared to the human genome. The statistically significant enriched GO terms are catalogued in

Atheroprotective siKaiso v siControl	
Top Networks	**Relevant Hubs**
Cell Signaling, Post-Translational Modification, Hereditary Disorder	NFkB
Hereditary Disorder, Renal and Urological Disease, Developmental Disorder	ERK1/2
Cell Morphology, Cellular Assembly and Organization, Cellular Function and Maintenance	PI3K
Dermatological Diseases and Conditions, Inflammatory Disease, Inflammatory Response	VEGF
Embryonic Development, Organismal Development, Tissue Development	FAK, RNApolyII, caspase
Top Molecular and Cellular Functions	
Cellular Movement	Protein Degradation
Cellular Growth and Proliferation	Protein Synthesis
Post-Translational Modification	

Atheroprone siKaiso v siControl	
Top Networks	**Relevant Hubs**
Cancer, DNA Replication, Recombination, and Repair, Cell Cycle	NFkB
Cell Cycle, Cellular Function and Maintenance, Carbohydrate Metabolism	ERK1/2, AP-1
Connective Tissue Disorders, Dental Disease, Developmental Disorder	MDM2, BRCA1, Cyclin A
Cellular Assembly and Organization, Cellular Dev., Cellular Function and Maintenance	Jnk
Cancer, Developmental Disorder, Immunological Disease	Akt, NFAT
Top Molecular and Cellular Functions	
Cell Morphology	Cell Death and Survival
Cellular Development	Carbohydrate Metabolism
Cell Cycle	

Table 4. The top Kaiso-dependent signaling networks and molecular and cellular functions as determined by IPA (Ingenuity® Systems, www.ingenuity.com). Genes identified under atheroprotective (top) and atheroprone (bottom) hemodynamics were analyzed separately.

Atheroprotective siKaiso v siControl			
Gene ontology term	**# of Genes**	**Fold Enrichment**	**P-value**
Actin binding (GO:0003779)	27	2.94	2.70E-05
Intracellular non-membrane-bounded organelle (GO:0043232)	107	2.93	8.30E-04
Actin filament-based process (GO:0030029)	19	2.65	9.90E-04
Mitotic cell cycle (GO:0000278)	25	2.26	1.20E-03
Zinc ion binding (GO:0008270)	104	2.16	4.40E-04
Notch signaling pathway (GO:0007219)	7	2.04	6.80E-03
Lysosome (GO:0005764)	14	1.88	1.40E-02
RNA binding (GO:0003723)	35	1.79	2.00E-02
Protein catabolic process (GO:0030163)	31	1.67	2.20E-02
Heart morphogenesis (GO:0003007)	7	1.64	3.20E-02
Actomyosin structure organization (GO:0031032)	4	1.61	6.20E-02
Regulation of transcription (GO:0045449)	100	1.61	5.40E-02
Apoptosis (GO:0006915)	32	1.57	8.60E-03
Ubiquitin-protein ligase activity (GO:0004842)	11	1.55	2.30E-02
Protein localization (GO:0008104)	40	1.48	3.40E-02
Microtubule cytoskeleton (GO:0015630)	26	1.48	3.30E-02
Negative regulation of cell adhesion (GO:0007162)	7	1.45	2.60E-03
Nuclear lumen (GO:0031981)	58	1.43	3.20E-02
Regulation of cell migration (GO:0030334)	13	1.43	9.70E-03
Sphingoid metabolic process (GO:0046519)	6	1.41	1.50E-02
Cytoplasmic membrane-bounded vesicle (GO:0016023)	28	1.34	1.20E-02
Atheroprone siKaiso v siControl			
Gene ontology term	**# of Genes**	**Fold Enrichment**	**P-value**
Intracellular organelle lumen (GO:0070013)	53	3.86	2.40E-06
Intracellular non-membrane-bounded organelle (GO:0043232)	63	2.85	1.30E-04
Cellular macromolecule catabolic process (GO:0044265)	26	2.21	1.40E-03
RNA binding (GO:0003723)	25	2.18	2.50E-03
Golgi apparatus (GO:0005794)	24	2.05	8.00E-03
Mitotic cell cycle (GO:0000278)	16	2.01	3.00E-03
Chromosome (GO:0005694)	15	1.88	1.20E-02
DNA repair (GO:0006281)	11	1.83	3.40E-02
Nucleotide binding (GO:0000166)	60	1.56	1.20E-03
Mitochondrion (GO: 0005739)	33	1.54	2.70E-04
Zinc ion binding (GO:0008270)	50	1.3	1.10E-01

Table 5. Representative GO terms from significant GO term clusters of Kaiso-dependent genes. The number of genes associated with the GO term and the extent to which the GO term is overrepresented in dataset compared to the human genome, and the statistical significance are shown for the atheroprotective (top) and atheroprone (bottom) datasets.

Table 5. Clearly, both pathway analysis and gene ontology clustering demonstrate that genes downstream of Kaiso regulation are involved in a wide variety of critical EC processes. While there is some degree of overlap between Kaiso function in atheroprotective and atheroprone conditions, this hemodynamically separated analysis helps to characterize different roles for Kaiso in the context of hemodynamic environment. Notably, the networks and GO terms implicated under atheroprotective conditions suggest heavy Kaiso involvement in regulating cellular movement, cytoskeletal organization, transcription, protein synthesis, and post-translational modification. Conversely, in atheroprone hemodynamics Kaiso demonstrates a bias for regulating DNA repair, mitosis, and cell cycling with increased relevance in cancer biology. While the networks revealed by pathway analysis differed depending on hemodynamic environment, the top two networks for either hemodynamic condition centered around hubs of NFκB and ERK1/2 activity. Each of these highly mechano-sensitive proteins is known to play major roles in EC response to shear stress exposure, further implicating Kaiso's importance in mediating responses to hemodynamic environment and suggesting that even hemodynamically divergent Kaiso signaling pathways converge on critical proteins downstream of shear stress exposure.

The induction of NOV by atheroprotective hemodynamics is highly Kaiso-dependent

As discussed in section 2.4, previous literature demonstrates that NOV expression is under the direct control of KLF2, is upregulated in ECs by exposure to laminar shear stress, and serves to inhibit activation of inflammatory NFκB signaling [62]. Interestingly, our microarray comparison of siControl atheroprotective flow to siControl

atheroprone flow revealed that out of the 7541 transcripts differentially regulated by hemodynamics at an FDR<0.20, NOV was the fourth most positively induced gene by atheroprotective flow ($log_2FC=5.195$, FDR=0.00146). However, comparing siKaiso to siControl in atheroprotective conditions where it was highly upregulated, NOV was the single most Kaiso-dependent gene, with drastically reduced expression levels upon loss of Kaiso (log2FC=-3.555, FDR=0.0921). Together, these results suggest NOV is a critical mediator of anti-inflammatory effects in response to atheroprotective hemodynamic exposure, but this induction is highly Kaiso-dependent. This data supports our hypothesis that Kaiso functions to alter EC athero-susceptibility and, along with pathway analysis implicating Kaiso upstream of NFκB signaling, represents an incredibly promising signaling pathway to pursue experimentally.

5.3 Summary

Our genome-wide microarray analysis provided insight into Kaiso's previously unexplored role in mediating downstream EC signaling pathways in response to hemodynamic environment. We demonstrated that while loss of Kaiso resulted in a similar proportion of up- and downregulated genes, substantially more genes were affected under atheroprotective conditions, highlighting the potential importance of Kaiso in mediating signaling pathways downstream of protective hemodynamics. We also showed that while there was overlap between Kaiso-dependent genes and functional pathways implicated in response to different hemodynamic exposures, many gene findings and their resultant functional consequences were unique to either

atheroprotective or atheroprone environment. This flow-dependence was particularly highlighted by a small subset of genes that was upregulated by Kaiso knockdown in one hemodynamic condition and downregulated in the other. Finally, we established that the prominent induction of NOV by atheroprotective hemodynamics was highly Kaiso-dependent. Given the known inhibition of NFκB activation by NOV [62] and the implication of Kaiso upstream of NFκB signaling by our pathway analysis, this discovery constituted a potential role for Kaiso in atheroprotection that was worthy of further specific investigation.

CHAPTER 6: INVESTIGATING THE MECHANISMS BY WHICH KAISO MODULATES INFLAMMATION AND ATHEROGENESIS THROUGH REGULATION OF GENE TRANSCRIPTION

6.1 Introduction / Methodology

As previously mentioned, little is known about Kaiso's gene targets. To generate an intitial list of candidate genes, Genomatix Software Suite (Genomatix Software Inc., Ann Arbor) was used to mine all human promoter sequences for instances of the putative Kaiso Binding Sequence (KBS). IPA (Ingenuity® Systems, www.ingenuity.com) was then utilized to cross-reference this list of potential Kaiso target genes with databases of genes previously implicated in atherosclerosis. As a result, genes known to be involved in the process of atherosclerosis and containing at least one instance of the KBS were isolated for future experimental screening. Due to Kaiso's demonstrated ability to bind both methylated CpG nucleotides and TCF/β-catenin, additional genes of interest were added for experimental analysis as appropriate.

To investigate target genes for Kaiso dependence, Kaiso was knocked down by siRNA or lentivirally overexpressed in ECs exposed to 24 hours of either atheroprotective or atheroprone hemodynamics, and expression of candidate genes was determined by real time RT-PCR and/or Western blotting. In order to examine consequences of Kaiso manipulation on downstream inflammatory transcription factors, identical hemodynamic experiments were performed on ECs transduced with Ad-NFκB-Luc or Ad-AP1-Luc activity reporters.

Additionally, to determine if loss of Kaiso increases inflammatory cell adherence in ECs exposed to non-inflammatory atheroprotective flow, EC monolayers were treated with Kaiso siRNA and exposed to protective flow for 24 hours, incubated with labeled THP-1 monocytes, fixed, and imaged. Monocyte adhesion studies were completed with or without an inflammatory challenge of 0.1 ng/μL TNFα for the final 6 hours of hemodynamic exposure.

6.2 Results

Many potential Kaiso gene targets have known implications in atherosclerosis and have previously demonstrated regulation by hemodynamic environment

Our bioinformatics analysis with Genomatix Suite revealed 8,599 instances of the KBS in human promoters. While this represents an unmanageable number of genes to screen experimentally, cross-referencing with IPA revealed only 45 genes that had been previously implicated in atherosclerosis. A subset of these genes, including many that have been previously demonstrated to be regulated by flow, is shown in Table 6. Additionally, due to known responses to shear stress and relationships with other genes containing KBS(s), the following genes were included for experimental evaluation: KLF2, E-Selectin, NOV, TFPI, and VCAM-1. This bioinformatic analysis not only supported the hypothesis that Kaiso-dependent regulation of transcription is potentially critical in mediating atherogenesis but also provided candidate genes to investigate individually.

	Gene	# of Kaiso Binding Sites (KBS)	Pro / Anti-Inflammatory	Regulated by Hemodynamics in Literature
Lipid Regulation	ABCA1	2	-	Yes
	ACAT2	1	-	-
	APOA5	1	-	-
	APOC1	2	-	-
	APOC3	1	-	-
	CD36	4	-	Yes
	HMGCR	2	-	-
	IGF1	1	-	-
	LDLR	1	-	Yes
	LRP1	1	-	-
	LRP5	2	-	-
Transcription Factor	FOS	1	-	Yes
	KLF2	0	Anti	Yes
	KLF4	1	Anti	Yes
	KLF6	2	-	-
	LXRA	1	-	Yes
	MEF2A	1	Anti	Yes
	NFKB1	1	Pro	Yes
Miscellaneous Mediators of Inflammation	ALOX15	1	Pro	-
	CCR2	1	Pro	-
	CD40	1	Pro	-
	E-selectin	0	Pro	Yes
	eNOS	1	Anti	Yes
	FASLG	1	Pro	Yes
	FN	2	Pro	Yes
	ICAM-1	1	Pro	Yes
	IFNGR1	2	Pro	-
	IL1R1	3	Pro	-
	IL6R	1	Pro	-
	MYD88	1	Pro	-
	NOV	0	Anti	Yes
	PAI-1	2	Pro	Yes
	TF/F3	1	Pro	Yes
	TFPI	0	Anti	Yes
	THBD	1	Anti	Yes
	TLR4	1	Pro	Yes
	VCAM-1	0	Pro	Yes

Table 6. A subset of atherosclerosis related genes that are potentially regulated by Kaiso (from bioinformatics analysis). The number of Kaiso binding sites revealed by promoter analysis, the generalized inflammatory properties, and regulation by hemodynamics are indicated. Genes that were experimentally investigated at the mRNA and/or protein level are shaded gray.

Endothelial response to Kaiso is dependent on hemodynamic environment

By manipulating Kaiso expression in ECs exposed to hemodynamic flow, we found that the response of Kaiso target genes and/or their downstream signaling partners was highly dependent on whether cells were exposed to atheroprotective or atheroprone hemodynamics. For the expression or activity of any downstream target, we would expect that manipulating Kaiso expression unidirectionally would have an effect in either atheroprotective flow, atheroprone flow, or the same effect in both. While this was the case for the target genes observed in Kaiso overexpression experiments, knockdown of Kaiso by siRNA resulted in unpredicted findings (results summarized in Table 7). As discussed in Chapter 2.4, KLF2 and KLF4 are known to be crucial mediators of anti-atherogenic signaling pathways downstream of EC exposure to protective shear stresses. However, in our atheroprotective flow conditions, siKaiso resulted in the downregulation of KLF2 and KLF4 and a subsequent increase in NFκB activity. Conversely, the exact same siRNA treatment under atheroprone conditions resulted in the opposite response, KLF2 and KLF4 upregulation and a loss of NFκB activity (Figure 9). As anticipated for a given hemodynamic environment, KLF2 and KLF4 responded similarly and their expression was inversely related to NFκB activity, but contrasting findings between the two flow paradigms was unexpected. These results confirm the importance of studying EC signaling mechanisms in physiologically relevant contexts and suggest that in endothelium Kaiso may function differently depending on the hemodynamic environment of the cell.

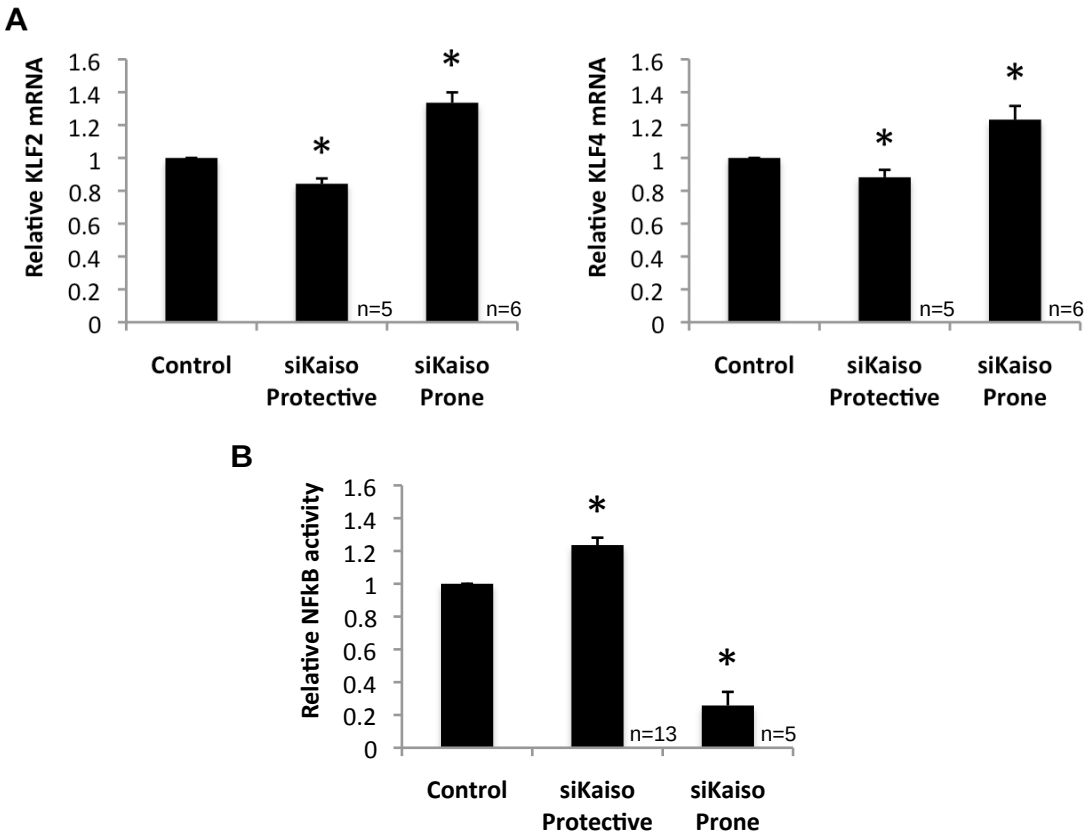

Figure 9. siRNA-mediated knockdown of Kaiso expression has contradictory consequences that are dependent on the hemodynamic environment of the experiment, as demonstrated by the effect of siKaiso on (A) KLF2 and KLF4 expression and (B) downstream NFκB activity. * $p<0.05$

Several Kaiso downstream targets are expressed unpredictably when comparing Kaiso knockdown to overexpression

Similarly, when examining the response of a potential gene targets to Kaiso manipulation in one hemodynamic condition (i.e. either atherprotective or atheroprone), one would expect that if Kaiso knockdown and overexpression both result in an effect that these findings would be in opposition. For example, if Kaiso knockdown resulted in downregulation of a target gene in one hemodynamic environment, we would have anticipated that in the same flow environment Kaiso overexpression would result in upregulation of the target or have no significant effect. While this was the case for many

of the genes examined, we discovered many unpredicted instances where knockdown and overexpression of Kaiso had similar findings (results summarized in Table 7). In atheroprotective flow, both knockdown and overexpression of Kaiso resulted in downregulation of KLF4, NOV, and THBD. Likewise in atheroprone flow, siKaiso and LV-Kaiso treament both resulted in a loss of TFPI transcript (Figure 10). These findings are similar to the Ad-4xKBS-Luc reporter characterization (Figure 5 B,C) in that knocking down and overexpressing Kaiso had similar consequences; however, in the observation of these potential target genes, either Kaiso manipulation resulted in reduced transcriptional output. The Kaiso-dependence of KLF4 transcription was particularly intriguing. As discussed above, in atheroprotective flow both Kaiso overexpression and knockdown resulted in a loss of KLF4 mRNA; however, under atheroprone flow conditions siKaiso resulted in an upregulation of KLF4, while overexpression had the expected opposite effect (Figure 10B). As a result, in the evaluation of a single potential Kaiso target, manipulation of Kaiso expression had both anticipated and unexpected findings, depending on the hemodynamic environment.

In combination with the uncertainty in literature, the confounding Kaiso reporter studies, and the dependence of Kaiso function on hemodynamic environment, the intriguing results of Kaiso manipulation further confirm that its signaling is a complex system. Based on these findings we hypothesize that Kaiso may serve as a tightly controlled fulcrum on which the delicate balance of EC signaling downstream of hemodynamic exposure rests. Therefore, disturbances in hemodynamic environment or Kaiso expression may disrupt this balance with unpredictable and far-reaching consequences in both Kaiso signaling and EC biology as a whole.

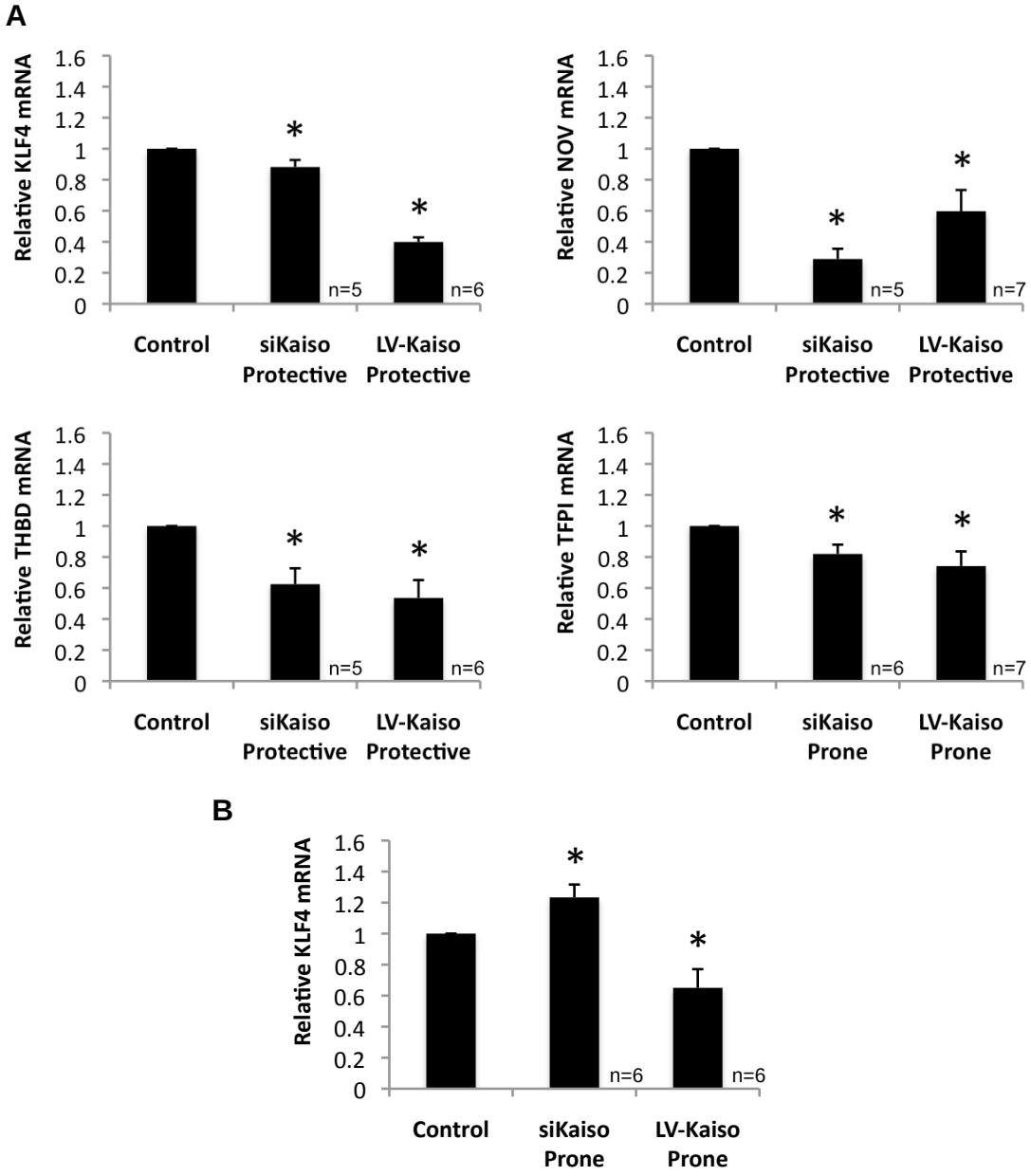

Figure 10. Knockdown (siKaiso) and overexpression (LV-Kaiso) of Kaiso can mediate similar effects on downstream genes, (A) as seen in downregulation of KLF4, NOV, and THBD under atheroprotective hemodynamics and TFPI in atheroprone hemodynamics. (B) Unlike atheroprotective conditions, in atheroprone flow, the knockdown and overexpression of Kaiso have opposite effects on KLF4 transcription. * $p<0.05$

Kaiso acts as an equilibrium setpoint that maintains EC inflammatory status, while perturbations in Kaiso expression generate disarray

As we have demonstrated, Kaiso function clearly demonstrates dependence on both hemodynamic environment as well as experimental conditions. With this in mind, we began the process of defining the role of Kaiso signaling in response to EC exposure to hemodynamic shear stress. Through the four experimental conditions of knocking down or overexpressing Kaiso in either atheroprotective or atheroprone hemodynamic conditions, we demonstrated Kaiso-dependent responses in many potential target genes including anti-atherogenic (eNOS, KLF2, KLF4, MEF2a, NOV, TFPI, and THBD) and pro-atherogenic (FN, ICAM-1, NFkB, PAI-1, TF, VCAM-1) mediators (results summarized in Table 7). When viewed as a whole across the four experimental groups, several conclusions can be reached. First, as discussed above, many paired experiments have contradictory findings that indicate the complex regulation of Kaiso and its uncertain functional mechanisms. Second, both knockdown and overexpression of Kaiso resulted in significant downregulation of target genes, but some upregulation was seen as well, suggesting that Kaiso may possess bimodal mechanisms of regulating transcription and these mechanisms are dependent on environmental conditions. Third, Kaiso manipulation caused disarray in the balance of pro- and anti-atherogenic signaling proteins investigated, often affecting proteins on both sides of the balance in ways that made pattern observation difficult. For instance, in atheroprone conditions knocking down Kaiso resulted in a decoupling of the KLF2 – NFκB – VCAM-1 signaling pathway. While an upregulation of both KLF2 and KLF4 led to the expected decrease in inflammatory NFκB activity, expression of VCAM-1 protein actually increased (Figure

11). With the exception of the experimental group involving siKaiso treatment under atheroprotective flow, the various up- and downregulation of Kaiso target genes did not reveal a clear net change in athero-susceptibility or inflammatory status. All of these findings continue to implicate Kaiso as a finely controlled and flow-dependent setpoint for maintaining EC equilibrium.

A

		mRNA			
		siKaiso		LV-Kaiso	
		Protective	Prone	Protective	Prone
Anti-Atherogenic	eNOS	Down[1]	-	-	Up
	KLF2	Down	Up	-	-
	KLF4	Down	Up	Down	Down
	MEF2a	Down	-	-	Down
	NOV	Down	-	Down	Down
	TFPI	Down	Down	-	Down
	THBD	Down	-	Down	-
Pro-Atherogenic	E-selectin	-	-	NA	NA
	FN	-	-	Down	Down
	ICAM-1	-	Up	NA	NA
	NFkB	-	-	Down	Down
	PAI-1	-	-	Down	Down
	TF	Up	-	Down	Down
	VCAM-1	Down	-	-	Up

B

		Downstream Physiological Consequences			
		siKaiso		LV-Kaiso	
		Protective	Prone	Protective	Prone
Pro-Athero	NFkB activity	Up	Down	-	-
	AP-1 activity	-	-	NA	NA
	FN protein	-	-	Down	Down
	ICAM-1 protein	Up	Up	NA	NA
	VCAM-1 protein	Up	Up	-	Up

Legend	
Up	$p < 0.05$
Up	$0.1 > p > 0.05$
Down	$0.1 > p > 0.05$
Down	$p < 0.05$

Table 7. Summary of findings when Kaiso expression is manipulated in ECs exposed to hemodynamic flow. Manipulating Kaiso expression results in various changes in (A) expression of potential target gene mRNAs with (B) corresponding downstream physiological consequences. Both trending and statistically significant findings are presented as outlined in the legend. [1]represents the confirmation of an mRNA trend with statistically significant protein data.

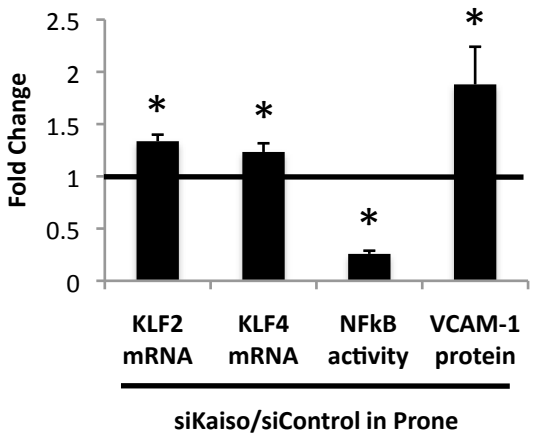

Figure 11. siKaiso treatment in atheroprone flow decouples NFkB/VCAM-1 signaling. Kaiso knockdown in ECs under atheroprone flow conditions resulted in an increase in protective KLF2/4 expression and a corresponding loss of NFkB activity; however, despite this highly anti-inflammatory response, VCAM-1 expression still increased. n=6,6,5,6 respectively. * $p<0.05$

Kaiso siRNA experiments demonstrate the Kaiso-dependence of atheroprotective shear stress induction of the KLF2/4 signaling pathway

While many of the consequences of manipulating Kaiso expression in ECs under hemodynamic conditions revealed confounding results, treatment of ECs with Kaiso-directed siRNA revealed a clear role for Kaiso in driving shear stress-dependent anti-inflammatory pathways in response to atheroprotective hemodynamics. Given that our gene microarray data supported the importance of Kaiso-mediated signaling in atheroprotective conditions, we focused our efforts on characterizing the role of Kaiso in mediating downstream effects of atheroprotective hemodynamics and the functional consequences of disrupting Kaiso signaling in protective flow.

As expected, mRNA levels of the anti-atherogenic proteins MEF2a, KLF2, KLF4, eNOS, NOV, and THBD were significantly lower in ECs exposed to atheroprone hemodynamics when compared to atheroprotective (Figure 12A). However, siRNA-

mediated knockdown of Kaiso under protective hemodynamics significantly reduces the transcription of these genes as well (Figure 12B), causing ECs under atheroprotective flow to be more phenotypically comparable to atheroprone. While the downregulation of eNOS mRNA was just shy of statistical significance, analysis of protein expression confirmed that siKaiso treatment resulted in significant loss of eNOS (Figure 12C). These results indicate a role for Kaiso as a positive regulator of anti-atherogenic pathways downstream of atheroprotective shear stress exposure.

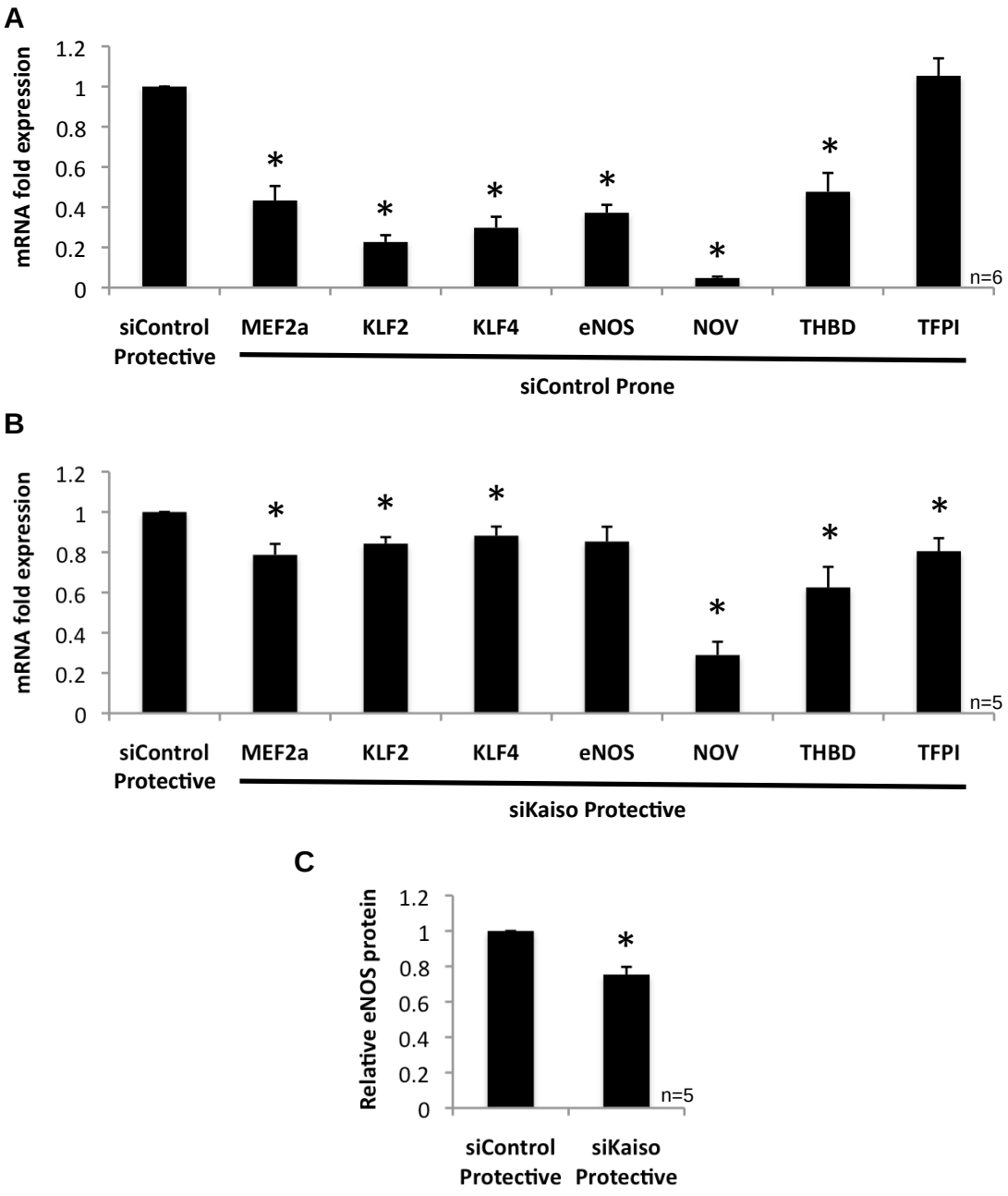

Figure 12. Kaiso positively regulates anti-atherogenic genes under atheroprotective hemodynamics. (A) Known anti-atherogenic genes have reduced mRNA expression under atheroprone flow conditions; however, (B) in atheroprotective hemodynamics knockdown of Kaiso resulted in a decrease of these transcripts as well. (C) While eNOS mRNA only trended down, siKaiso resulted in signicant loss at the protein level. * $p<0.05$

Under atheroprotective hemodynamics, loss of Kaiso-dependent signaling results in physiologically relevant increases in inflammation

While the reduction of known anti-atherogenic and anti-inflammatory genes by loss of Kaiso is remarkable, we sought to demonstrate the physiological significance of Kaiso loss by confirming a corresponding increase in downstream inflammatory pathways. We first utilized luciferase-based reporters to evaluate the activity levels of NFκB and AP-1, both of which are known to be shear stress-dependent master regulators of inflammation opposed by KLF2 signaling (Figure 13A). As hypothesized, siKaiso treatment of ECs under protective hemodynamics resulted in an increase in NFκB activity. Interestingly, AP-1 did not demonstrate any dependence on Kaiso, suggesting that the effects of Kaiso signaling bypass this important transcription factor family. Additionally, we investigated protein expression levels of the inflammatory cell adhesion molecules VCAM-1 and ICAM-1, which are positively regulated by NFκB activation (Figure 13B). As expected due to the increase in NFκB activity, protein levels of both cell adhesion molecules were increased by siRNA-mediated Kaiso knockdown. Taken together, these results indicate that Kaiso-mediated activation of anti-atherogenic genes inhibits activation of NFκB and expression of cell adhesion molecules, two critical factors in the development of atherosclerosis.

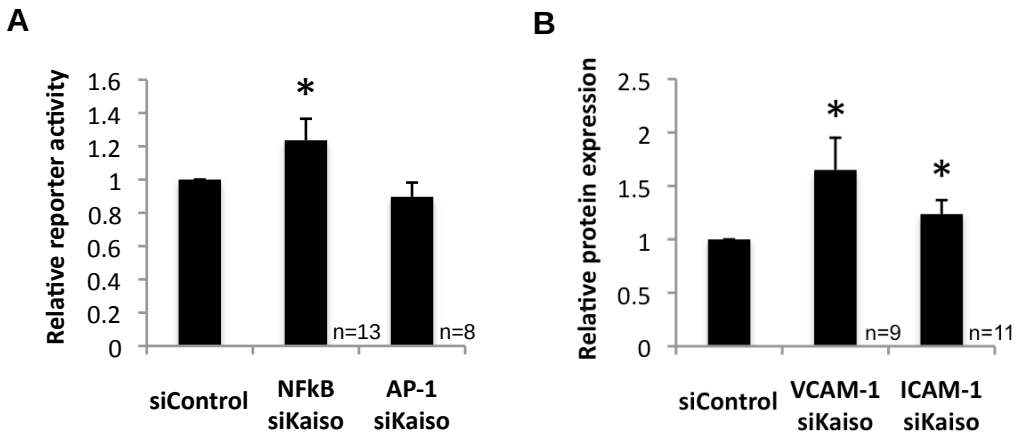

Figure 13. Under atheroprotective hemodynamics, loss of Kaiso results in an increase in physiologically relevant measures of endothelial inflammation. Kaiso knockdown by siRNA results in (A) increased NFkB activation and a (B) corresponding upregulation of cell adhesion molecule expression. * $p<0.05$

Kaiso knockdown increases monocyte adhesion to ECs exposed to atheroprotective hemodynamics

We have demonstrated that Kaiso knockdown results in a decrease in the anti-inflammatory pathways that are typically activated by endothelial exposure to protective shear stresses. This loss of protection was linked to a downstream upregulation of NFκB activity and the expression of VCAM-1 and ICAM-1. These cell surface molecules are critical components of leukocyte adhesion and extravasation at the site of developing atherosclerotic lesions. As such, we sought evaluate whether Kaiso knockdown would increase monocyte adhesion to EC monolayers exposed to typically monocyte-resistant atheroprotective hemodynamics. Consequently, we found that siKaiso treatment was sufficient to augment monocyte adhesion in protective hemodynamics, both with and without the presence of an additional inflammatory challenge via 0.1 ng/μL TNFα (Figure 14). While many of the other Kaiso-dependent findings represent novel Kaiso functions in endothelial signaling, these results highlight a major biological

consequence of Kaiso's role in mediating atheroprotection downstream of hemodynamics.

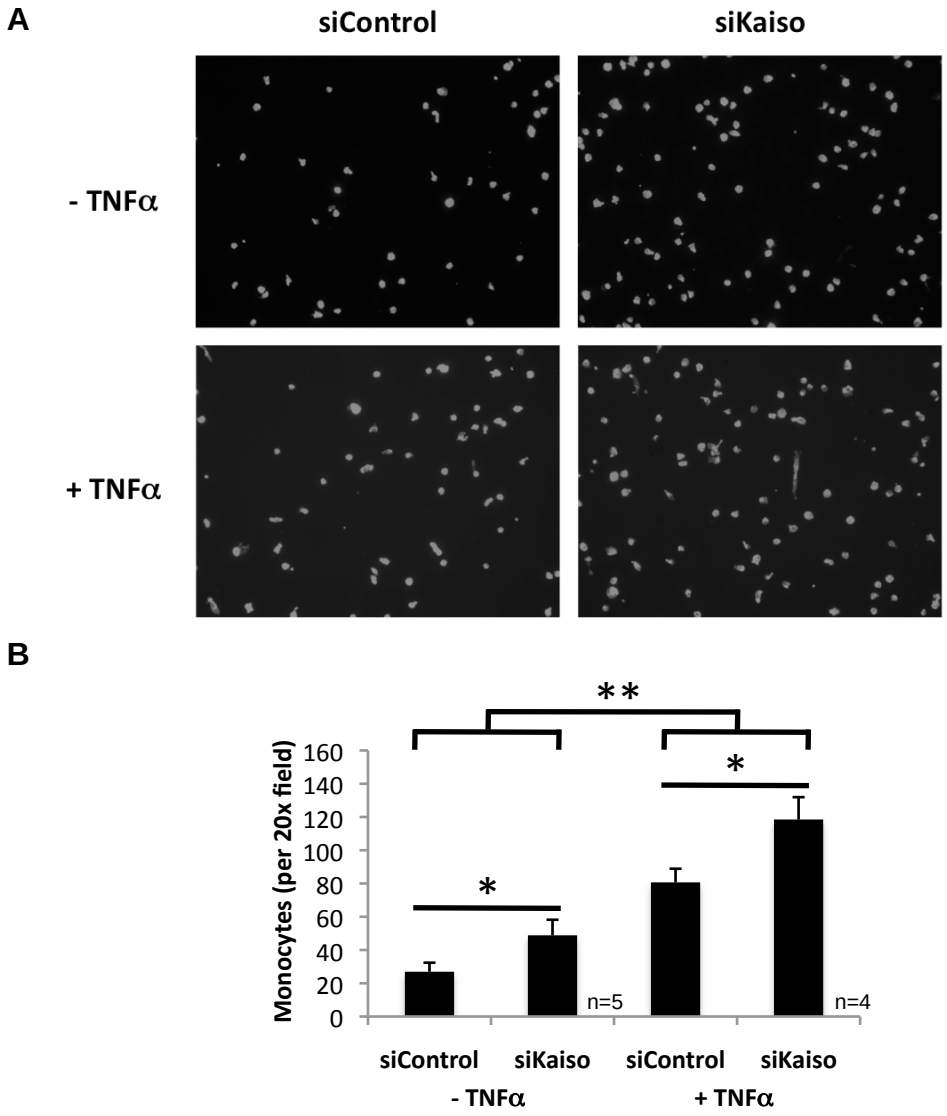

Figure 14. In EC monolayers exposed to atheroprotective hemodynamics, Kaiso knockdown was sufficient to increase monocyte adhesion. (A) Representative 20x images of adhered monocytes on EC monolayers preconditioned with 24 hours of protective hemodynamics. (B) With and without an inflammatory TNFa challenge, loss of Kaiso results in increased adhesion. *p<0.05, **p<0.001

6.3 Discussion

Since its discovery as a transcription factor binding partner of p120 in 1999 [86], an increasing amount of studies sought to elucidate the functional significance of Kaiso. Although initially described as a transcriptional inhibitor whose repressive abilities can be overcome by p120 binding [91-93], later studies challenged this initial understanding and suggested that Kaiso-mediated regulation of transcription may be much more complex and situational. Iioka et al. was the first to describe potential dose-dependent bimodal activity of Kaiso [95], and many additional studies established a link between Kaiso and β-catenin/TCF signaling [92, 95-98, 107], further adding to the evolving view of Kaiso's role in cell biology. While our studies add questions to the increasingly nebulous view of Kaiso, we believe they also give clarity to a novel role for Kaiso in maintaining endothelial cell homeostasis and driving the anti-atherogenic responses downstream of atheroprotective hemodynamic exposure.

First, because very few targets of Kaiso-mediated transcriptional regulation were defined by previous literature, we used bioinformatics to generate a practical list of potential Kaiso-regulated genes in atherogenesis and sought to systematically determine the effects of manipulating Kaiso on their expression, all within the context of physiologically relevant hemodynamics. Interestingly, when observing the expression patterns of target genes created by altering Kaiso availability, we discovered that Kaiso's function is highly dependent on not only the hemodynamic environment applied but also whether Kaiso was overexpressed or knocked down. In several instances target genes exhibited a similar response to either siKaiso or LV-Kaiso treatment. While this finding

is difficult to explain mechanistically, it parallels Iioka et al.'s description of Kaiso's bimodal function [95] as well as our characterization of the Ad-4xKBS-Luc reporter system under flow. Likewise, knocking down Kaiso expression by siRNA elicited conflicting responses of some target genes depending on the exposure of either atheroprotective or atheroprone hemodynamics, a finding also supported by our microarray analysis. Interestingly, the specific loss of Kaiso studies demonstrated that Kaiso positively drives KLF2 and KLF4 expression in atheroprotective conditions but inhibits their expression under atheroprone. Such findings suggest that Kaiso may act as a shear-stress dependent switch whose functionality varies depending on the hemodynamic environment. One possible explanation for this finding could be that hemodynamics differentially regulate Kaiso interaction with unknown binding partners, which are then able to bimodally modulate Kaiso's inherent transcriptional regulatory abilities. Additionally, under atheroprone hemodynamics, siKaiso treatment even resulted in the decoupling of NFκB activity and VCAM-1 expression. For some of the experimental conditions evaluated, both pro- and anti-inflammatory pathways seemed to be affected randomly by Kaiso manipulation, with difficult to interpret consequences. Taken together, we believe that these findings suggest that Kaiso activity is bimodal and is finely controlled by endothelial cells in response to their hemodynamic environment, serving a role in maintaining inflammatory homeostasis. As a result, perturbations in Kaiso signaling result in instability and disequilibrium of normally predictable inflammatory pathways.

While many of the consequences of altering Kaiso expression were highly complex and difficult to predict and explain, one trend became apparent. siKaiso

treatment in ECs clearly demonstrated a novel role for Kaiso in positively regulating the highly anti-atherogenic KLF2/KLF4 pathway in response to atheroprotective flow. Endothelial cells exposed to atheroprotective shear stress utilize KLF2/KLF4 activation to increase production of many anti-inflammatory and anti-thrombotic proteins including TFPI, THBD, eNOS/NOS3, and NOV. In turn, these proteins inhibit NFκB activation and a variety of downstream inflammatory consequences [27]. As expected, genes in KLF2/KLF4 pathway were much more highly expressed under atheroprotective flow, but interestingly, this upregulation was Kaiso-dependent. As a result, reduction in Kaiso expression resulted in a downregulation of many of these anti-atherogenic genes despite exposure to atheroprotective hemodynamics. Although these findings alone implicate Kaiso as novel mechanism by which atheroprotective shear stress confers anti-atherogenic benefits, we sought to confirm that loss of Kaiso was sufficient to increase physiologically relevant measures of inflammation. We established that Kaiso knockdown triggers increases in NFκB activity and subsequent cell adhesion molecule (VCAM-1 and ICAM-1) expression. Interestingly, AP-1 activity was unchanged, suggesting that Kaiso-dependent KLF2 activity favors repression of NFκB activation and either spares AP-1 or that other compensatory mechanisms keep AP-1 activity minimized. As a final demonstration of Kaiso's physiological importance, we show that knockdown under protective flow not only increases expression of leukocyte cell adhesion molecules but also enhances monocyte adhesion. Given the importance of monocyte extravasation in the progression of an atherosclerotic lesion, this finding further solidifies the atheroprotective role of Kaiso in endothelium. The Kaiso

upregulation of atheroprotective pathways and resulting reduction in indicators of inflammation are summarized in Figure 15.

Though we have defined a role for Kaiso in mediating the downstream effects of atheroprotective hemodynamics, these studies do not indicate the mechanism by which Kaiso regulates the atheroprotective signaling pathway that we have outlined. Given the wide range of genes that were identified as Kaiso-dependent by the microarray analysis in addition to the conflicting and confusing findings when evaluating individual genes experimentally, it is possible that Kaiso is operating at multiple levels and through many additional transcription factors. We have demonstrated that Kaiso manipulation regulates the mRNA of the transcription factors MEF2a and KLF2, and others have shown a high degree of overlap with β-catenin/TCF transcription factor signaling. Each of these transcription factors then has many gene targets of its own, and the resulting complexity makes identifying specific points of Kaiso interaction difficult. Intriguingly, both the gene microarray and individual real time RT-PCR results revealed NOV as one of the genes most highly regulated by Kaiso. While NOV has been shown to inhibit NFκB activation [62], the mechanism for this is currently unknown. Furthermore, NOV has a variety of protein interactions in prominent signaling pathways with unknown consequences. An amino-truncated form of the protein has the potential to translocate to the nucleus where its impact on transcriptional regulation is completely undetermined (reviewed in [69]). Since NOV is known to be under the direct transcriptional regulation of KLF2, some of Kaiso's effects were demonstrated to be upstream of NOV. However, it is conceivable that one of NOV's unknown functions is to generate positive feedback on MEF2a, KLF2, and/or KLF4 expression to maintain the entire anti-inflammatory

pathway. Even if our findings cannot be fully explained solely by the potential direct control of NOV expression, it is possible that Kaiso directly or indirectly, through other transcription factor intermediaries, regulates multiple genes within this signaling pathway. If these transcription factor intermediaries have their own responses to hemodynamic exposure that are independent of Kaiso, some of the confounding results of the other experimental conditions could be explained. While the mechanisms remain elusive, we believe that our findings define an important and highly novel role for Kaiso in mediating atheroprotection in endothelial cells exposed to hemodynamics.

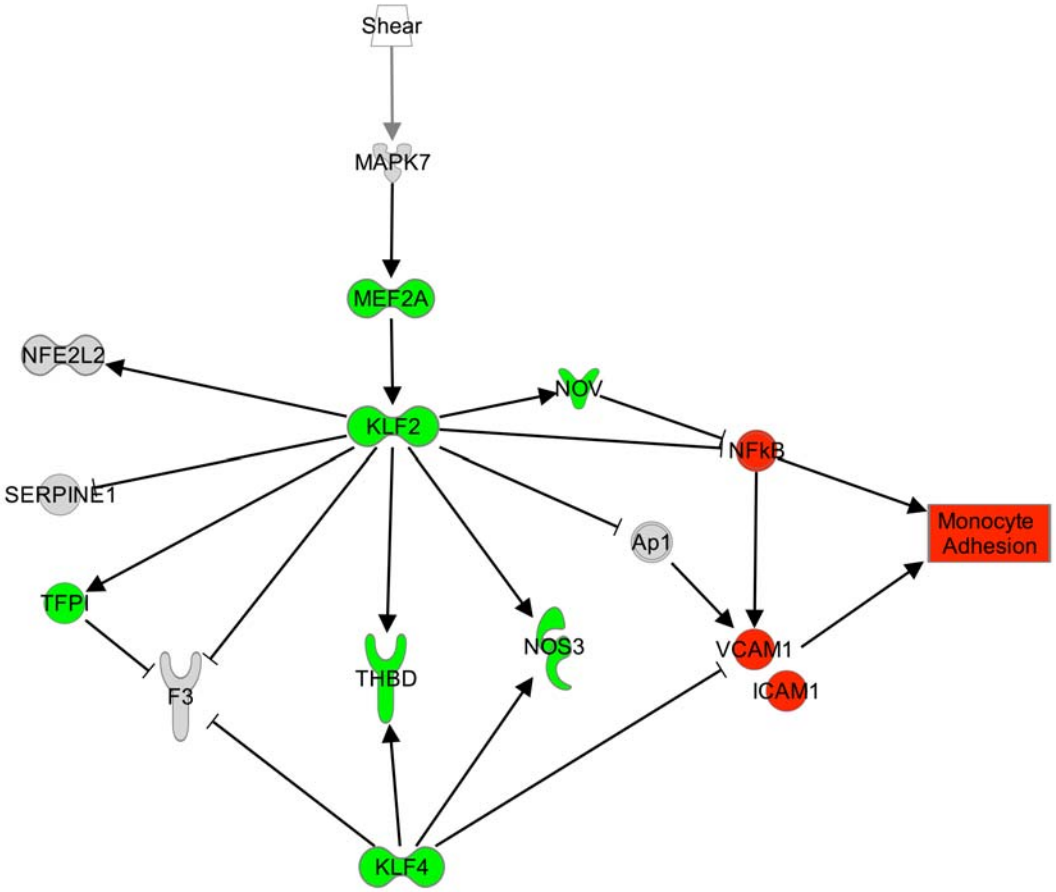

Figure 15. Knockdown of Kaiso under atheroprotective flow caused a downregulation of anti-atherogenic KLF2/KLF4 signaling. Protective genes that were downregulated after Kaiso loss are indicated in green, while corresponding increases in downstream inflammatory processes are highlighted in red. This figure was generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com)

CHAPTER 7: SUMMARY, CLOSING REMARKS, AND FUTURE DIRECTIONS

7.1 Summary

In this dissertation we hypothesized that hemodynamics modulate the activity of the transcription factor Kaiso in a manner that alters local endothelial susceptibility to atherogenesis. The following is a summation of the major findings of this study:

- Kaiso activity is regulated by hemodynamic exposure, as evidenced by increased Ad-4xKBS-Luc reporter expression under atheroprone flow.
    - The modulation of Kaiso activity is not attributable to changes in total expression, which is not differentially regulated by hemodynamics.
    - p120 expression is elevated in endothelial cells exposed to atheroprotective hemodynamics, but the magnitude of its physical interaction with Kaiso is unchanged and does not account for flow-dependence of Kaiso activity.
    - Under both protective and prone hemodynamics, characterization of the Ad-4xKBS-Luc reporter reveals that Kaiso knockdown and overexpression both result in an increase in reporter activity.
- Kaiso is expressed in the endothelium and underlying structures of healthy vessel wall, early atherosclerotic lesions, and advanced atherosclerotic lesions in $ApoE^{-/-}$ mice, a major scientific model of atherogenesis.
- Genome-wide analysis of Kaiso function demonstrates many notable trends

  - While loss of Kaiso results in a similar proportion of upregulated and downregulated genes, substantially more genes are affected under atheroprotective conditions.
  - Pathway analysis and gene ontology clustering of Kaiso-dependent genes for each hemodynamic condition implicates Kaiso in a wide range of EC functions. While there were similarities between conditions, these evaluations suggest possible divergence in Kaiso function dependent on hemodynamic environment.
  - Atheroprotective induction of NOV expression is highly Kaiso-dependent.
- Bioinformatics analysis of human promoter sequences was used to generate a list of possible Kaiso gene targets, which were then screened to isolate the potential targets that have been previously implicated in atherosclerosis.
- Under hemodynamic conditions, manipulation of Kaiso expression levels demonstrates complexity in Kaiso's regulation of downstream genes
  - The effect of Kaiso manipulation on target gene expression is dependent on the hemodynamic exposure.
    - In protective hemodynamics, Kaiso knockdown causes a downregulation of KLF2 and KLF4, while the same treatment results in upregulation under atheroprone hemodynamics.
    - In many other cases, changes in target gene expression under a given Kaiso treatment are unique to one hemodynamic condition, with the other condition demonstrating no significant change.

- Analysis of Kaiso's functionality is dependent on the experimental method utilized to manipulate Kaiso expression.
  - For a single hemodynamic environment, Kaiso overexpression or knockdown demonstrates similar responses in the expression of several downstream genes

- Kaiso positively regulates a variety of anti-atherogenic genes in the KLF2/KLF4 signaling pathway in response to atheroprotective hemodynamics.
  - Loss of Kaiso results in a reduced induction of anti-inflammatory, anti-thrombotic genes (MEF2a, KLF2, KLF4, eNOS, NOV, TFPI, and THBD) by atheroprotective shear stress.
  - Downregulation of anti-atherogenic genes as a result of Kaiso knockdown associates with a corresponding increase in NFκB activity and VCAM-1/ICAM-1 expression.
  - Loss of Kaiso is sufficient to enhance monocyte adhesion to endothelial monolayers exposed to atheroprotective hemodynamics, with and without the presence of an additional inflammatory cytokine challenge.

7.2 Closing Remarks

Atherosclerosis is one of the most prevalent and widely recognized pathological conditions in the Western hemisphere. Diagnosis and treatment of this inflammatory vascular disease utilizes a large fraction of medical resources and represents a major portion of the healthcare costs. Furthermore, despite increased awareness, medication options, and surgical treatments, the morbidity and mortality associated with this disease remains very high.

While the molecular events that lead to the development of atherosclerotic lesions have been the subject of an incredible amount of research for decades, large gaps remain in the understanding of this complex process. Improving our knowledge of the fundamental biology behind atherosclerotic lesion development presents a great opportunity to lay the foundation for future advances in both disease prevention and medical intervention. One area of increasing research in recent decades has been the concept of endothelial cell mechanotransduction, the method by which the cells lining blood vessels sense mechanical signals such as blood pressure and shear stress and respond with downstream signaling to regulate the cell's response to its environment. Increasing literature has noted that the predictable locations of lesions within human vasculature correlate with particular vessel geometries that impart disturbed shear stresses on the endothelium. Researchers then hypothesized that the hemodynamic environment of endothethium can predispose vessel wall to lesion development and sought to define the mechanisms by which cells sense and respond to this environment. While many experimental models have been utilized to evaluate the response of endothelial cells to

their hemodynamic environment, the modified cone-and-plate system employed in this dissertation has the advantageous ability to apply physiologically-determined hemodynamic patterns *in vitro*. We believe that this novel system presents a unique means with which to investigate signaling events involved in the process of endothelial cell dysfunction and atherogenesis.

We hypothesized that Kaiso, a poorly understood transcription factor with largely unknown functions in gene regulation, was regulated by hemodynamics, and that its activity has consequences in athero-susceptibility. We confirmed that Kaiso was not only expressed in both human and mouse endothelium, but that its activity is differentially regulated by hemodynamic environment. We further demonstrated that this shear stress-dependent regulation is not attributable to changes in either total expression or by binding to its known modulator p120.

Having supported our initial hypothesis that Kaiso is implicated in endothelial cell response to hemodynamic environment, we sought to systematically determine the functional impact of Kaiso by manipulating its expression under flow and examining the downstream effects in gene transcription. This we accomplished at the whole-genome level as well as within single signaling networks. Broadly defined, Kaiso is a highly complex and often unpredictable mediator of equilibrium in endothelial cells. At the genome level, Kaiso is equally implicated in positive and negative regulation of downstream genes and is involved in a wide range of cellular activities. Furthermore, the quantity of Kaiso-dependent genes is notably higher in cells exposed to atheroprotective flow, suggesting the particular importance of Kaiso in mediating signals downstream of protective shear stress. In the more specific approach of evaluating individual potential

target genes, the response to manipulation of Kaiso expression was dependent on both the hemodynamic environment and whether the experiment was testing a loss or overexpression of Kaiso. Perturbations in Kaiso expression resulted in expression patterns of both pro- and anti-inflammatory pathways that were often contradicting and indicated a dysregulation of traditionally defined signaling pathways. We believe this evidence suggests that Kaiso may serve as a central signaling node regulating the delicate balance between pro- and anti-atherogenic signaling in ECs exposed to shear stress.

Though consideration of the data as a whole suggests that Kaiso is a tightly controlled regulator of endothelial equilibrium, focusing on the loss of Kaiso experiments under atheroprotective hemodynamics demonstrated a highly novel role for Kaiso as a positive regulator of the anti-atherogenic KLF2/KLF4 signaling pathway. Consequently, Kaiso knockdown not only reduced expression of a variety of anti-inflammatory/anti-thrombotic genes but also increased multiple measures of inflammation, ultimately leading to enhanced adhesion of monocytes. This final finding confirms the physiological relevance of Kaiso signaling in atheroprotection and serves as validation of our hypothesis.

7.3 Future Directions

Within the field of endothelial cell biology and the development of atherosclerosis, the concept and consequences of mechanotransduction represent one of the more recent and promising arenas of study. Endothelial cells have been shown to respond to their shear stress environment through a variety of signaling mechanisms that alter their susceptibility to atherogenesis. With this in mind we hypothesized that the transcription factor Kaiso is a mechanism by which ECs respond to their hemodynamic environment with implications in atherosclerosis and inflammation. Through a systematical evaluation of Kaiso's functions in hemodynamic environment, we established a role for this protein as both a general mediator of endothelial cell equilibrium and, specifically, a positive regulator of the anti-atherogenic KLF2/KLF4 pathway, which is activated by atheroprotective flow. Nonetheless, throughout the course of this work, we revealed a great deal of complexity in Kaiso signaling and generated several questions about Kaiso function that remain incompletely understood.

In vivo verification and validation of Kaiso's importance in atherogenesis

The expression of Kaiso in human endothelial cells has been confirmed *in vitro*, and observations from the $ApoE^{-/-}$ mouse model displayed Kaiso localization in both diseased and lesion free regions of mouse vasculature. Additional studies could be undertaken to more fully establish the expression pattern of Kaiso in the actual development of atherosclerosis. To accomplish this, vascular tissue from both mouse models and actual human large vessels could be isolated and imaged for Kaiso expression

in both cross sections and *en face* preparations. By determining the expression levels and nuclear accumulation of Kaiso in atheroprotected regions such as the common carotid artery and comparing this to Kaiso abundance in atheroprone areas such as the internal carotid sinus during non-diseased, early lesion, and advanced lesion states, one could increase the understanding of Kaiso's function in the actual disease process. Given our demonstration of Kaiso as a positive driving force in the atheroprotective anti-atherogenic pathway, we anticipate that Kaiso expression and/or nuclear localization would be increased within atheroprotected vasculature. However, our reporter data indicated that differences in Kaiso activity were not the result of increased expression. Additionally, while both the genome-wide analysis and individual gene target studies indicated an important role for Kaiso in atheroprotected endothelium, they also suggested an incompletely understood function for Kaiso under atheroprone hemodynamics. Consequently, a more sophisticated analysis of Kaiso expression in human and mouse tissue could verify the elevated importance of Kaiso in response to atheroprotective flow or imply equally important but less understood functions in atheroprone conditions.

Evaluating the dose- and hemodynamic-dependency of Kaiso functions

As discussed, interpretation of Kaiso function was dependent on the use of either knockdown or overexpression techniques, and in either case depended on the hemodynamic environment. Like the Ad-4xKBS-Luc reporter characterization, the evaluation of individual gene targets demonstrated that loss or gain of Kaiso expression can have similar effects in certain situations. While there is some support for this finding in literature, the mechanism by which this occurs is not understood. Iioka et al.

demonstrated that Kaiso loss of function and mild gain of function can have similar upregulatory signaling effects, while higher overexpression of Kaiso became highly repressive [95]. Because in our studies it was advantageous to ensure highly repeatable experimental conditions in order to determine the effect of hemodynamics on Kaiso signaling, it remains to be seen if Kaiso dose-dependency affects the downstream response of target genes in our system. Consequently, future reporter and gene studies could incorporate variable amounts of siRNA or LV-Kaiso in order to generate a broad range of Kaiso expression levels in which downstream results may be monitored. Additionally, we displayed evidence that Kaiso's role varies notably between hemodynamic environments, and in some cases Kaiso appears to have opposing functions depending on protective or prone shear stress exposure. Such studies suggest that Kaiso may behave as a molecular "switch", behaving in one manner under atheroprotective hemodynamics but undergoing some functional change in atheroprone conditions. While the mechanisms for both dose-dependent variability and hemodynamic switching are not known, these findings could be explained by changes in Kaiso's binding partners. Although Kaiso is known to interact with p120 and β-catenin/TCF, it is reasonable to assume that not all of its binding partners have been discovered or evaluated, and these unknown binding partners could have major implications in the regulation of Kaiso function. To test this hypothesis, ECs could undergo dose-dependent manipulation of Kaiso expression preceding exposure to either protective or prone hemodynamics. Following shear stress exposure, Kaiso complexes isolated by co-immunoprecipitation could be separated by SDS-PAGE, stained for total protein, and the identity of any unknown bands ascertained by mass spectrometry techniques.

Determining the mechanism by which Kaiso activates the KLF2/KLF4 pathway

The principal finding of this dissertation is that shear stress mediated upregulation of the atheroprotective KLF2/KLF4 pathway is Kaiso-dependent. We not only provided evidence of Kaiso's positive regulation of this pathway, but also demonstrated physiologically relevant increases in inflammation upon loss of Kaiso. Unfortunately, the mechanism by which Kaiso exerts control over this pathway remains elusive. Because our studies display possible bimodal Kaiso activity, we are unable to conclude if Kaiso's activation of this pathway is through direct positive regulation of pathway proteins or through repression of proteins responsible for inhibiting pathway activation. In the case of direct positive regulation of pathway genes, chromatin immunoprecipitation (ChIP) could be utilized to demonstrate increased binding to the promoters of genes within this pathway under atheroprotective conditions. Similarly, this technique could be used to probe for Kaiso interaction with the promoters of the known inhibitors of each gene in the KLF2/KLF4 signaling pathway. In a much more general approach to this question, ChIP-sequencing could be used to determine what promoters are being regulated by Kaiso at the genome-wide level and how this regulation changes with respect to hemodynamic environment. Because of the broad nature of this method, the results are less likely to provide evidence of the direct or indirect regulation of this particular pathway by Kaiso but would likely increase the fundamental understanding of Kaiso's role in hemodynamic regulation of endothelial cell biology.

Further investigation of the Kaiso-dependent regulation of NOV and its consequences in atheroprotection

NOV is one of most intriguing genes in the KLF2 signaling pathway that warrants additional future investigation. Only as recently as 2010 was NOV demonstrated to be a KLF2-dependent mechanism by which protective shear stress inhibits activation of inflammatory NFκB signaling. In our gene microarray studies, NOV was found to be one of the 10 most upregulated genes in atheroprotective flow, suggesting a high level of importance in atheroprotection. While expressed highly in atheroprotective conditions, NOV was the single most downregulated gene by Kaiso knockdown under protective hemodynamics, identifying a strong dependence on the presence of Kaiso. Similarly, our real time RT-PCR findings confirmed that under atheroprotective hemodynamics NOV was the most Kaiso-dependent gene of all the potential targets evaluated. Unfortunately, the multiple functions and mechanisms of this protein are largely unknown. Future investigation of NOV should include the methods by which Kaiso regulates NOV expression, the mechanism by which NOV inhibits NFκB activation, and the ability of NOV to regulate the transcription of other genes relevant to atherogenesis (including potential feedback on the KLF2 pathway).

Defining a role for Kaiso in atheroprone hemodynamic signaling

Through genome-wide microarray analysis and evaluation of the expression of individual target genes with known roles in atherosclerosis, we were able to define a clear role for Kaiso in driving anti-atherogenic signaling in response to atheroprotective hemodynamic environment. However, as discussed throughout this dissertation,

interpretation of Kaiso's function in endothelial cells exposed to atheroprone conditions remains much more imprecise due somewhat conflicting RT-PCR findings demonstrated in response to Kaiso manipulation. Future work could attempt to clarify Kaiso's role downstream of atheroprone shear stress exposure. First, as discussed above, probing the possibility of Kaiso "switching" based on hemodynamic environment could be highly informative. Additionally, while we interpreted the variable findings in potential Kaiso target genes under prone flow as a requirement for Kaiso expression in order to maintain inflammatory equilibrium, expansion of the list of genes examined could result in a more sophisticated view of Kaiso. It is possible that examining the proper genes could even result in defining a major signaling pathway for Kaiso in atheroprone hemodynamics, as was accomplished in protective flow. While the genes screened in this dissertation were rationally selected by a bioinformatic analysis of promoter sequences and involvement in atherosclerosis, additional potential Kaiso-dependent pathways could be informed by our microarray analysis. Furthermore, while the genes we initially evaluated focused on inflammation, future pathway examination could be extended to include lipid metabolism, thrombotic pathways, proliferation and survival, cell motility, or a variety of other mechanisms critical to endothelial cell biology.

CHAPTER 8: REFERENCES

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