Characterization of \textit{Athsq1}, an Atherosclerosis Modifier Locus on Mouse Chromosome 4:

Identification of \textit{Cdkn2a} as a modifier locus mediating monocyte/macrophage cell proliferation

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Abstract

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Atherosclerosis, the primary cause of heart attack, stroke, and peripheral vascular disease, is genetically complex and the genes that confer cardiovascular risk remain largely unknown. Attempts to map common atherosclerosis susceptibility loci in humans have resulted in limited success. Mouse models provide an excellent tool for dissecting genetic complexity, and testing the role of candidate genes and pathways. While recent genome-wide association studies in humans have identified multiple genetic variants associated with atherosclerosis related traits—such as circulating lipid levels and hypertension—as well as myocardial infarction and stroke, few of the causal variants or underlying mechanisms are known. The work presented in this thesis identifies genetic loci contributing to atherosclerosis susceptibility in a murine model and, based on mechanistic data obtained in mouse, strongly suggests a mechanism underlying a coronary heart disease susceptibility locus on chromosome 9p21 identified by genome-wide association studies in humans.

In this study we have used congenic mice that were previously constructed to isolate Athsq1, an atherosclerosis susceptibility locus identified in a cross between MOLF/Ei and C57BL/6J-Ldlr−/−. Atherosclerosis studies performed in congenic mice carrying the MOLF-derived susceptibility allele from chromosome 4, in the C57BL/6J-Ldlr−/− background, confirmed the existence of the Athsq1 locus and BM-derived cells were shown to play an important role. Refined mapping of the Athsq1 locus identified at least two susceptibility loci within the interval. A proximal locus was narrowed to a region containing 8-21 genes, including the region of homology with the human coronary heart disease risk interval on chromosome 9p21.
Interestingly, mRNA expression analyses in macrophages revealed markedly decreased Cdkn2a transcript expression in cells derived from congeneric mice compared to the controls. Cdkn2a is a candidate locus associated with the 9p21 locus in humans. The potential role of bone marrow–derived Cdkn2a expression in atherogenesis was tested using a bone marrow transplantation approach with Cdkn2a-deficient donor cells. Mice receiving Cdkn2a+/− deficient bone marrow exhibited accelerated atherosclerosis, increased inflammatory monocyte subsets and increased monocyte/macrophage proliferation compared to the controls. This study provides a mechanistic link between decreased Cdkn2a expression, increased monocytes/macrophages proliferation with the expansion of an inflammatory monocyte subset and increased atherosclerosis. Together, these data illustrate the utilization of mouse models as tools to elucidate causal gene(s)/loci and potential pathophysiologic mechanisms underlying genome-wide association studies-identified loci for human disease.
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Abbreviations

ABCA1-ATP binding cassette, sub-family A, member 1
Alox5-arachidonate 5- lipoxygenase
ALOX5AP-arachidonate 5- lipoxygenase-activating protein
ANOVA-analysis of variance
ANRIL-antisense non-coding RNA
apoB-apolipoprotein B
Apoe-apolipoprotein E
Athsq1-atherosclerosis susceptibility QTL1
b/b-homozygote B6-\(\text{Ldlr}^{\text{wt}}\)
BM-bone marrow
BrdU-bromodeoxyuridine
BWT-body weight
CHD-coronary artery disease
CD14-endotoxin receptor, a component of the lipopolysaccharide receptor
CD16-Fc\(\gamma\) receptor
CDK-cyclin dependent kinase
CDKN2A-cyclin-dependent kinase inhibitor 2A
CDKN2B- cyclin-dependent kinase inhibitor 2B
Chr-chromosome
CpG-cytosine and guanine nucleotides separated by a phosphate
CVD-Cardiovascular disease
DNA-deoxyribonucleic acid
ECM-extracellular matrix
EC-endothelial cell
EDTA-ethylenediaminetetraacetic acid
FH- familial hypercholesterolemia
GWAS-genome wide associate study
HDL-C-high density lipoprotein cholesterol
hr-hour
IFN-I-interferon I
IFN-γ-Interferon-gamma
INK4-inhibitors of cyclin-dependent kinase 4
iNOS-inducible nitric oxide synthase
Jun-Jun oncogene
Kb-kilobases
LD-linkage disequilibrium
LDL-low density lipoprotein
LDLR-low density lipoprotein receptor
LOD-logarithm of the odds
LPS-lipopolysaccharide
LRP6-LDL-receptor related protein 6
m/m-homozygote for the MOLF allele
MAPK-mitogen-activated protein kinase
Mb-megabase
MCP-1-monocytechemotactic protein
M-CSF-macrophage colony stimulating factor
MHC class II-major histocompatibility complex class II
MI-myocardial infarction
mmLDL-minimally modified LDL
MMP-metalloproteinase
Mtап-methylthioadenosinephosphorylase
MTAP-methylthioadenosinephosphorylase
NfкB-nuclear factor kappa-light-chain-enhancer of activated B cells
Ox40 - member of the tumor necrosis factor receptor superfamily
Ox40L-Ox40 ligand
oxLDL-oxidized LDL
p53-tumor protein 53
PCR-polymerase chain reaction
PCSK9-proproteinconvertasesubtilisin/kexin type 9
QTL-quantitative trait locus
Rb-retinoblastoma
RNA-ribonucleic acid
rtPCR-real time PCR
SD-standard deviation
SEM-standard error mean
SLE-systemic lupus erythematosus
SMC-smooth muscle cell
SNP-single nucleotide polymorphism
SORT1-sortilin 1
Sub-B,C,D-subcongenic strains B,C and D
TC-total cholesterol
TGF-β-transforming growth factor beta
TG-triglyceride
TLR-toll-like receptor
Tnfsf4-tumor necrosis factor superfamily, member 4

TNF-α-Tumor necrosis factor-alpha

TUNEL-terminal deoxynucleotidyltransferasedUTP nick end labeling

VCAM-vascular adhesion molecule

VLDL-very low density lipoprotein

wk-week

WTD-western type diet
Attestation

A. Worked performed by doctoral candidate
   a. Subcongenic strain production
   b. Aorta harvesting and atherosclerotic lesion analyses
   c. Bone marrow transplantation experiments
   d. Versican immunostaining in atherosclerotic lesions
   e. mRNA isolation and purification for microarray and gene expression data analyses
   f. Cdkn2a\(^{-}\) breeding into the B6-Ldlr\(^{-}\) background
   g. Blood collection and FACS analyses
   h. Plasma cholesterol measurements
   i. Cell proliferation assays

B. Work performed in collaboration
   a. Atherosclerotic lesion quantification performed together with Dr. Carrie Welch
   b. cDNA labeling, microarray hybridization and data analysis by Oscar Puig and colleagues at Merck Research Laboratories
   c. Splenic macrophage isolation by Yan Liu/ Dr. Norman Sharpless at The University of North Carolina
   d. Allele-specific quantitative PCR by Janakiraman Krishnamurthy / Dr. Norman Sharpless at the University of North Carolina
   e. Cdkn2a\(^{-}\) mice were obtained from Dr. Sean Morrison, University of Michigan
   f. FACS analyses performed together with post-doctoral fellow Dr. Andrew Murphy and Dr. Laurent Yvan-Charvet
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Dedication

To my family
Chapter 1. Introduction and Literature Review
A. Overview of atherosclerosis

Prevalence and risk factors

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in both men and women worldwide. Although mortality rates of CVD have declined over the past four decades in the United States, about one-third of deaths in individuals over 35 years-of-age are due to CVD complications (1, 2). It is estimated that approximately half of men and one-third of women at age 40 will manifest CVD complications (3). Atherosclerosis is the primary cause of CVD and stroke with a complex multifactorial etiology and a large genetic component underlying the disease. Despite numerous studies advancing our understanding of the etiology of atherosclerosis, the public disease burden remains high and maybe increasing (4).

Data from epidemiological studies and randomized clinical trials led to the identification of risk factors that increase the susceptibility of CVD (5). “Risk factor” is defined as any measurable characteristic of an individual that predicts the individual’s probability of developing a clinically manifest disease (Table 1-1)(6). Genetic factors, including elevated levels of atherogenic lipoproteins, play a strong role in most forms of CVD. Obesity and diabetes, two common diseases with a large genetic component, also increase the risk of developing CVD. Elevated levels of C-reactive protein, a trace plasma protein secreted in response to inflammation, are associated with CVD, suggesting that genetic factors modulating inflammation may contribute to CVD risk. Environmental factors play an important role as well. Smoking, lack of exercise and fatty diet all increase the incidence of CVD (6, 7). Patients with periodontitis (infections of tooth-supporting tissues) have higher frequency of unstable angina, suggesting that infection is involved in atherogenesis (8). However, these risk factors are not simply
additive. Thus, the complex etiology of CVD results from the combination of an unhealthy environment, genetic susceptibility and gene-environment interactions.

Table 1-1  Genetic and environmental risk factors affecting atherosclerosis and CVD

<table>
<thead>
<tr>
<th>Genetic factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elevated levels of LDL/VLDL</td>
</tr>
<tr>
<td>Decreased levels of HDL</td>
</tr>
<tr>
<td>Elevated levels of Lipoprotein (a)</td>
</tr>
<tr>
<td>Elevated levels of homocysteine</td>
</tr>
<tr>
<td>Family history</td>
</tr>
<tr>
<td>Diabetes and obesity</td>
</tr>
<tr>
<td>Elevated levels of fibrinogen, plasminogen activator inhibitor type 1 and platelet reactivity</td>
</tr>
<tr>
<td>Gender (male)</td>
</tr>
<tr>
<td>Elevated inflammatory signals-C reactive protein</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Environmental factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
</tr>
<tr>
<td>Smoking</td>
</tr>
<tr>
<td>Exercise</td>
</tr>
<tr>
<td>Infection</td>
</tr>
<tr>
<td>Fetal environment</td>
</tr>
</tbody>
</table>

Adapted from (9, 10)

**Development and progression**

The normal artery consists of four major morphologically distinct concentric layers. The endothelial cell monolayer acts as a barrier between the blood and underlying tissue. The subendothelial space, intima, is a very thin layer of extracellular connective tissue, mainly comprised of proteoglycans and collagens. The peripheral side of it is surrounded by an internal elastic lamina. The middle layer, the media, consists mainly of smooth muscle cells that act to contract the vessel. The outer layer, adventitia, is composed of connective tissue with fibroblasts and smooth muscle cells.
Lesion initiation

Numerous pathological and molecular studies have shed light on the morphological and cellular changes that occur during atherogenesis (reviewed in (9, 11)). The endothelium, connected by intercellular tight junctions, acts as a selectively permeable barrier between blood and tissues(12). It regulates vascular tone, vascular remodeling, inflammation and thrombosis. Fluid shear stress is an important physical force acting on endothelial cells (ECs). Sites of low shear stress, high turbulent flow are the preferential vascular bed for the initiation of atherosclerosis (13). Cells in regions of arterial branching or curvature, where flow is disturbed, show increased permeability to macromolecules such as LDL and are preferential sites for lesion formation(12).

Atherosclerosis studies in genetically altered mice showed that leukocyte adhesion molecules play a role in the initiation of atherosclerosis(14)(Figure 1-1). Monocyte rolling on endothelial cells requires adhesion molecules of the selectin family. Apoe<sup>−/−</sup> mice deficient in P-selectin and E-selectin showed a reduction of atherosclerotic lesion formation(15). Ldlr<sup>−/−</sup> mice expressing a truncated form of vascular cell adhesion molecule-1 (VCAM-1) showed decreased early lesion formation (16).

One of the key initiating events in atherosclerosis is LDL accumulation in the subendothelial matrix. LDL diffuses passively through endothelial cell junctions, and its retention in the vessel wall involves interactions between the LDL constituent apolipoprotein B (ApoB) and matrix proteoglycans(Figure 1-1). Other ApoB-containing lipoproteins, namely lipoprotein(a) and remnants, can also accumulate in the intima and promote atherosclerosis(17). The trapped LDL undergoes modification. One of the modifications most significant for early lesion formation is lipid oxidation. Enzymes
involved in such modification include 12, 15-lipoxygenase (15-LO), inducible nitric oxide synthase (iNOS), myeloperoxidase (MPO), and various lipases (18). Modified LDL is taken up in an unregulated manner by scavenger receptors, SR-A and CD36, on macrophages to form lipid-engulfed “foam” cells (19, 20). Foam cells collect to form “fatty streaks”, the earliest lesion of the atherogenic process.

High-density lipoprotein (HDL) exhibits multiple protective effects against atherosclerosis. HDL promotes reverse cholesterol transport via cholesterol efflux mechanisms involving ATP-binding cassette transporters (21, 22). In addition, HDL plays a protective role by inhibiting lipoprotein oxidation. The antioxidant properties of HDL are due in part to serum paraoxonase (PON1), an esterase carried on HDL that can degrade certain biologically active oxidized phospholipids (23). HDL also inhibits endothelial inflammation, promotes endothelial nitric oxide production, and inhibits platelet aggregation and coagulation (24). More recently, HDL has been shown to decrease leukocytosis by inhibiting the proliferation of hematopoietic stem and multipotent progenitor cells and ultimately reverse accelerated atherosclerosis phenotypes (25). These data suggest that increasing circulating HDL levels may be a beneficial therapeutic mechanism for the treatment/prevention of atherosclerosis.
Figure 1-1  Initiation of atherosclerosis

LDL is subjected to oxidative modifications in the subendothelial space. Minimally-modified LDL (mmLDL) is oxidized extensively to form “oxidized LDL” (oxLDL) by 15-lipoxygenase (15-LO) and inducible nitric oxide synthases (iNOS). Cell adhesion molecules expressed on endothelial cells recruit monocytes. Adherent monocytes transmigrate between endothelial cells and differentiate into macrophages promoted by M-CSF. oxLDL engulfed by macrophages via scavenger receptor CD36 or SR-A is subjected to esterification by acetyl-coenzyme A acetyltransferase (ACAT), leading to foam cell formation. ApoE secreted by macrophages facilitates cholesterol efflux to HDL or exportation of lipids via ABCA1 to HDL. Adapted from (11).

Leukocyte recruitment:

The recruitment of monocytes and lymphocytes to the artery wall is triggered by oxLDL, which stimulates ECs to produce numerous proinflammatory molecules including adhesion molecules and growth factors, such as the macrophage colony-stimulating factor (M-CSF). Adhesion of monocytes and T cells to the endothelium is
mediated by VCAM-1 via interaction with integrin VLA-4 and adhesion molecules from the selectin family, E-selectin and P-selectin(26). Mice deficient in monocytechemotactic protein-1 (MCP-1) or its receptor, CCR2, have significantly reduced atherosclerotic lesions(27, 28), suggesting that MCP-1/CCR2 interaction plays a role in monocyte recruitment in atherosclerosis.

Lesion progression and thrombosis:

As atherosclerosis progresses, accumulation of cholesterol ester, SMC migration and proliferation, and SMC-derived extracellular matrix (ECM) deposition occurs within plaques and leads to fibrous-fatty plaque formation. Cytokines and growth factors secreted by macrophages and T cells are important for SMC migration/proliferation and ECM production. Activation of lesional macrophages characterized by expression of MHC class II molecules and inflammatory cytokine production, such as TNF-α, IL-6, and MCP-1. Concomitantly, T cells express both Th1 (IFNγ) and Th2 cytokines (IL-4, IL-10)(Figure 1-2) (29). As atherosclerotic lesions develop into advanced plaques, there can be ischemic symptoms as a result of progressive narrowing of the vessel lumen. On the other hand, acute cardiovascular events such as myocardial infarction (MI) and stroke could happen as a result of plaque rupture and thrombosis (30, 31). Plaque rupture usually occurs at the shoulder regions of plaques exhibiting thin fibrous caps and large necrotic cores(30). The thickness of the fibrous cap reflects matrix production (secreted by SMCs) and degradation (by secreted metalloproteinases, MMPs). Plaque rupture exposes intimal lipids and tissue factor to blood components, initiating a cascade of coagulation events, platelet adherence, and thrombosis (Figure 1-2). The thrombogenicity of the lesion core likely depends on the presence of tissue factor, a key
protein in the initiation of the coagulation cascade. Neovascularization also affects the stability of atherosclerotic lesions, as it is prevalent in human advanced lesions.

Figure 1-2  Lesion progression and thrombosis
Interaction of macrophage foam cells and T cells creates a chronic inflammatory state. Cytokines produced by macrophages and lymphocytes are either pro-atherogenic (IFN-γ, CD40) or anti-atherogenic (IL-4, IL-10). Smooth muscle cells migrate from the medial wall to proliferate in the subendothelial space and secrete extracellular matrix to form a fibrous cap. As the plaque develops into a complex lesion, macrophages undergo apoptosis and necrosis to form a necrotic core. Matrix metalloproteinases secreted by macrophages lead to the thinning of the fibrous cap, resulting in a vulnerable plaque prone for plaque rupture. Plaque destabilization exposes blood components to tissue factor, initiating coagulation and the recruitment of platelets and thrombus formation. Adapted from (11).

Monocytes and macrophages in atherosclerosis

The role of macrophages is central to plaque development. Human monocytes were initially identified by high cell surface expression of CD14 (a component of the lipopolysaccharidereceptor) and CD16 (Fcγ receptor). Recently Cros et al. classified three distinct human monocyte subsets; CD14⁺CD16⁻accounting for 85% of blood monocytes, and CD14⁺CD16⁺ and CD14⁺CD16⁺ which comprise the remainder of monocytes in healthy individuals (32).
Accumulating evidence suggests that monocytosis is an independent risk factor for CHD (33, 34). In addition, studies have revealed the importance of different monocyte subsets in association with CHD related complications. The predominant CD14⁺CD16⁻ subset in patients with stable CHD has been correlated with risk factors and family history (35). CD14⁺CD16⁻ cell counts correlate negatively with the serum concentrations of HDL and positively with levels of low density lipoprotein and lipoprotein(a) (36). However, after MI, it has been shown that CD14⁺CD16⁻ monocyte counts exhibit an inverse correlation with left ventricular recovery (37). Interestingly, increased levels of the CD14⁺CD16⁻ subset has been associated with preferential uptake of oxLDL (38), increased serum TNF-α levels (39) and increased cardiovascular events (39, 40). Further studies with careful classifications are required to define the relative role of monocyte subsets in atherogenesis.

In mice, two subpopulations of circulating monocytes can be distinguished by the expression of Ly6C antigen (41). An “inflammatory” subset, Ly6C<sup>hi</sup> (CX<sub>3</sub>CR<sub>1</sub><sup>lo</sup>CCR2<sup>+</sup>Ly6C<sup>hi</sup>), are released from bone marrow into the blood via CCR2 (42-44) and preferentially recruited to inflamed tissues (41). A “resident” subset, Ly6C<sup>lo</sup> (CX<sub>3</sub>CR<sub>1</sub><sup>hi</sup>CCR2<sup>−</sup>Ly6C<sup>lo</sup>), have high expression of cell surface receptor CX<sub>3</sub>CR1 (CX<sub>3</sub>CL1-receptor, mediates monocyte adhesion and survival (45)) and exhibits CX3CR1-dependent crawling on the endothelium of non-inflamed tissues (46).

Monocyte subsets are functionally heterogeneous. During MI, Ly6C<sup>hi</sup> cells are mobilized from the spleen reservoirs (47) and scavenge necrotic debris in the damaged tissue to produce TNF-α and proteolytic enzymes (48). In response to bacterial, viral or parasitic infection, Ly6C<sup>hi</sup> cells produce TNF-α, iNOS, IL-12, and type 1 interferon to
ward off infection (49, 50). During muscle injury, Ly6C<sup>hi</sup> expression decreases concomitant to increased expression of mature macrophage markers F4/80 and TGF-β (51). In contrast, Ly6C<sup>lo</sup> cells contribute to tissue healing in the infarcted myocardium rather than inflammation, promoting myofibroblast accumulation, angiogenesis, and deposition of collagen(48). They have also been shown to give rise to macrophage populations in the lung (52) and at the site of infection (peritoneum) (46). However, the exact function of Ly6C<sup>lo</sup> in atherogenesis remains to be determined. During atherosclerosis, Ly6C<sup>hi</sup> monocytes infiltrate into the lesions and differentiate into macrophages(53), this depends on CCR2, CCR5 and CX<sub>3</sub>CR1(54). On the other hand, CCR2<sup>-</sup>Ly6C<sup>lo</sup> monocytes are partially dependent upon CCR5. This suggests that Ly6C<sup>hi</sup> monocytes could be a potential therapeutic target by antagonizing CX<sub>3</sub>CR1 in ameliorating CCR2<sup>+</sup> monocyte recruitment to plaques without impairing other CCR2-dependent responses to inflammation.
Figure 1-3  Blood monocytes in atherosclerosis
During atherosclerotic lesion development, inflammatory monocytes (Gr1+/Ly6C\textsuperscript{hi}) are recruited into the intima and differentiate into macrophages. Patrolling monocytes (Gr1⁻/Ly6C\textsuperscript{lo}) patrol the endothelium scavenging lipid derivatives, dead and/or dying cells. Little is known about the influx and potential role of Gr1⁻/Ly6C\textsuperscript{lo} in atherosclerosis progression. Adapted from (26).

B. Genetics and atherosclerosis in humans

Genetic factors play a significant role in atherosclerosis, with an estimated heritability of over 50%(55). Twin and family studies have established that CHD aggregates in families(6). Family history of early onset CHD has long been considered a risk factor for disease(56). The most rigorous analysis of the inheritance of MI risk to date, the Framingham Offspring Study(57), found that individuals with at least one parent with premature CHD (defined as age of onset <55 years in men, <65 years in women) experienced a significant increase in risk of suffering a cardiovascular event compared
to individuals with no such family history. The investigators observed an odds ratio of 2.0 and 1.7 in men and women, respectively, after adjustment for other traditional cardiovascular risk factors (57). Furthermore, sibling history of MI was observed to be a greater risk factor than parental history of early-onset CHD (58). These data established family history as a powerful risk predictor for CHD.

Genes associated with a disease or trait can be studied in two ways. The first strategy, termed “forward genetics”, involves the testing of a hypothesis associated with a known candidate gene. This can be done by genetic modification of a known gene in rodent models, or testing for association of polymorphisms (variants that cause protein or functional alteration) in candidate genes in humans. The other method, sometimes termed, “reverse genetics”, involves linkage or association of a particular region of the genome with disease—without any prior knowledge about the underlying gene. This later approach involves genome-wide linkage and association studies (reviewed in (59)).

**Human Mendelian disorders**

Mendelian disorders are single-gene traits that are transmitted in an autosomal dominant, recessive or X-linked manner (60). Familial hypercholesterolemia (FH) is a classic example of a Mendelian disorder associated with CAD (6). This disease is due to mutations in the LDL receptor gene (LDLR). Individuals with homozygous FH have extremely high LDL cholesterol levels and often develop premature coronary artery disease. Loss-of-function mutations in LDLR were identified in fibroblasts derived from FH patients, and found to be transmitted within families in an autosomal dominant manner (61). Similarly, familial defective ApoB100 (FDB) results in hypercholesterolemia because the abnormal LDL binds poorly to LDL receptors (62). Mutations in the proprotein convertase subtilisin/kexin type 9 gene (PCSK9) have also been associated
with autosomal dominant hypercholesterolemia (63). Tangier disease, resulting in extremely low HDL cholesterol levels, is an autosomal recessive disorder. In Tangiers, mutations in the ATP-binding cassette A1 gene (ABCA1) result in ineffective export of cholesterol from peripheral tissues to apolipoprotein A1, the major protein component of circulating HDL particles (64, 65). The identification of specific genes underlying Mendelian disorders have unraveled mechanisms for lipid metabolism and provided the opportunity to develop therapeutic targets for dyslipidemia, such as cholesterol-lowering drugs statins by promoting LDL catabolism.

**Linkage and association studies**

The success to new gene discovery starts with the establishment of genome-wide evidence for linkage or association in a single study, or consistent suggestive evidence in several independent studies(66). Linkage studies are performed by testing numerous genetic markers distributed throughout the genome to test for coinheritance of the alleles and the trait, and the output is given as a likelihood ratio statistic or a log of odds ratio (LOD) score (67). A LOD score >3 is considered significant evidence in humans and LOD scores of >2.8 and 4.3 are considered suggestive and significant evidence respectively for linkage in mice.

Until recently, linkage analysis was widely used in studies aimed at identifying new genes for CVD. Helgadottir et al.(68) performed linkage analysis using Icelandic families (713 individuals) with Mland 1,068 microsatellite markers distributed throughout the genome. A linkage signal (LOD 2.86) was observed on chromosome (chr) 13. This interval was narrowed using an additional 120 microsatellite markers in 802 MI cases and 837 controls. A 4-SNP haplotype spanning the ALOX5AP gene (encoding arachidonate 5-lipoxygenase-activating protein) was associated with risk of
Another linkage study, involving a four-generation family with early-onset coronary disease transmitting in an autosomal dominant pattern, reported a linkage signal on chr 12p13(69). The linkage interval was narrowed to a 750-kb region harboring six genes. A missense mutation in LRP6 (*LDL receptor-related protein 6*) was found to segregate with family members having early onset CAD. This variant was not observed in 2000 controls. The mutation was further associated with high LDL, high triglycerides, hypertension, diabetes, and low bone density. Linkage analyses have been most successful in identifying rare causal variants with modest to large effects. However, such loci explain only a small percentage of the total population variance in risk for disease.

**Genome wide association studies (GWASs) and CVD**

The completion of the human genome sequence (70, 71), identification of millions of SNPs subsequently cataloged in public databases (72), and the initiation of the International Hap Map Project (73) have enabled us to further identify genes underlying common diseases. GWASs represent an unbiased genetic approach designed to detect common DNA variants (minor allele frequency >5%) associated with traits or diseases. These variants typically have modest to small effects on disease susceptibility. Using this approach, a dense set of SNPs are genotyped across the whole genome to search for genetic variants contributing to disease susceptibility or variation in levels of risk factors for disease-related heritable quantitative traits (74).

Over the past few years, GWASs have identified numerous robust associations between SNPs on specific chromosomal loci and complex diseases such as type 2 diabetes (75, 76), cancer (77, 78), Crohn’s disease (79, 80), rheumatoid arthritis (81, 82) and CHD (83, 84). Much success has been achieved for CHD-related traits including hypertension and plasma lipid levels (83, 85). More than a dozen loci associated with
systolic blood pressure, diastolic blood pressure or hypertension have been identified (86, 87). Similarly, 95 loci associated with plasma lipid levels have been identified to date (88-90). A notable finding of these studies is that some of the same genes that cause Mendelian lipid disorders also underlie variation of lipid levels in the general population.

The most highly replicated locus associated with CHD and MI identified by GWASs is a locus on chr 9p21 (91-94). This locus has been confirmed in multiple European groups (94-101), as well as other ethnic groups including Chinese (102), Japanese, Korean (101) and East Indian populations (103). The frequency of the risk allele is almost 50%, with a 20%-30% increased risk for CHD per copy (84). The high frequency of the risk allele confers a substantial impact at the population level. The locus is not associated with traditional cardiovascular risk factors such as blood pressure, cholesterol level, body mass index or smoking (94, 95), suggesting novel pathways influencing CHD and/or MI risk. Several studies have reported conflicting results about associations of 9p21 with expression of nearby genes. Individuals carrying the risk allele have been shown to exhibit decreased expression of p16\textsuperscript{INK4a}, p19\textsuperscript{ARF}, and p15\textsuperscript{INK4b} - inhibitors of cellular proliferation encoded by the nearby CDKN2A/B locus – as well as proximal transcripts of a non-coding mRNA ANRIL, in some studies (104-106) but not others (107, 108). The inconsistency of these data may be due to relatively small sample sizes or tissue specific regulation of CDKN2A, CDKN2B and MTAP genes.

Studies have shown that risk variants of 9p21 do not affect early CHD markers, such as carotid intima-media thickness and brachial flow-mediated dilatation (109). On the other hand, Horne et al. found no association of 9p21 with either the severity or extent of CHD assessed angiographically, thus leading to their conclusion that 9p21 locus plays a
role at an early stage of CHD (110). It remains to be determined at which stage this locus comes into action.

There are several findings of chr 9p21 that show the genetic complexity of a human disease locus. Firstly, the locus underscores the importance of non-traditional risk factors in determining disease susceptibility. Only 2 out of 8 chromosomal regions affecting MI or CHD (in replicated studies) (84, 111-113) are associated with traditional risk factors, thus suggesting novel mechanisms underlying disease. A robust association was found with abdominal aortic aneurysm and intracranial aneurysm (98, 114), suggesting that the underlying gene affects local vessel wall integrity. Secondly, two loci that are associated with type 2 diabetes (T2D) map to a similar region of chr 9p21 (82). However, the associated SNPs flank the CHD risk locus (with no overlap), implicating that the causal genes and/or regulatory loci for CHD and T2D are distinct. Thirdly, the associated SNPs map to a region with no known protein coding genes (Figure 1-4). The linkage disequilibrium (LD) block, ~58 Kb, lies more than 100 Kb away from the nearest protein coding genes, CDKN2A (encoding p16\textsuperscript{INK4a}, p14\textsuperscript{ARF}), CDKN2B (p15\textsuperscript{INK4b}) and the MTAP gene (methylthiosadenosine phosphorylase). This suggests the possibility that the causative variants are non-coding RNAs or regulatory elements affecting expression of one or more flanking genes. Despite these discoveries, the causative the link between 9p21 variants and CAD remains to be understood.
Figure 1-4 Genomic organization of the CDKN2A/B locus near the 9p21 CHD locus.

(A) The exon-intron structure of annotated genes is shown in different colors for distinction. p16\textsuperscript{INKA} and p14\textsuperscript{ARF} share exons 2 and 3 but have different exon1, resulting in two different reading frames encoding two distinct proteins. The ANRIL gene overlaps the two exons of p15\textsuperscript{INK4b} and is transcribed in opposite direction from the p15\textsuperscript{INK4b}, p16\textsuperscript{INK4a}, p14\textsuperscript{ARF} (mouse: p19\textsuperscript{ARF}) genecluster.

(B) The linkage disequilibrium (LD) blocks on chr 9p21.3. Regions of LD are shaded on a continuous scale, with red and white the strongest and least association respectively. Haplotype blocks are framed by triangles. Figure adapted and modified from (115).

C. **Mouse genetics and atherosclerosis**

**Atherosclerotic mouse models**

Two strains of genetically modified mice have been used for studying atherosclerosis: Apoe\textsuperscript{-/-} and Ldlr\textsuperscript{-/-}. Apoe\textsuperscript{-/-} mice lack apolipoprotein E, a key component in cholesterol metabolism. Apoe\textsuperscript{-/-} mice develop spontaneous hypercholesterolemia and atherosclerotic lesions with standard chow feeding, and high-fat diet exacerbates the disease (116).
Low-density lipoprotein receptor (Ldlr)-deficient mice develop atherosclerosis only when fed a high-cholesterol diet (67). Crossbreeding of these mice with animals carrying deletion/overexpression constructs or tissue-specific promoters allows for testing the role of candidate genes and tissues in atherosclerosis development. Moreover, the use of bone marrow (BM) transplantation offers insights to the role of BM or non-BM derived cells in the pathogenesis of the disease.

**Complex trait and disease locus mapping strategies**

The efficiency of mapping loci for complex traits in the mouse ultimately depends on good genetic mapping resolution. Interval-specific congenics provide one approach to QTL fine mapping in rodents. These mice are created by repeated backcrossing of mice carrying a specific disease susceptibility interval from a donor strain into a recipient background strain. Using marker-assisted selection, a particular genomic region of interest can be selectively introgressed from the donor strain into the recipient strain (117). Subsequent intercrossing is then performed to generate segment homozygotes. Isolating the region of interest in a uniform genetic background enables the standardization of genetic background effects and allows characterization of the phenotype in multiple genetically identical individuals (reviewed in (118-120)). Thus, interval-specific congenics provide a valuable tool to identify genes underlying complex traits/diseases of interest.

There are several practical considerations when narrowing the susceptible interval and identifying the underlying genes. Regions that are identical by descent between two parental strains are highly unlikely to contain causal genetic variants underlying QTLs and can be excluded as candidate regions. Wild-derived mouse strains provide great genetic variation compared to standard laboratory inbred strains. However, a high
degree of polymorphism complicates the distinguishing of causal and non-causal SNPs (118).

Comparative mapping of mouse and human atherosclerosis-related QTLs reveals that many mouse QTLs found in both Apoe<sup>−/−</sup> and Ldlr<sup>−/−</sup> crosses are concordant with the mapped locations of human QTLs, and vice versa (121). Comparative mapping can facilitate the identification of genes underlying QTLs by excluding non-concordant regions of the mapped intervals. This is based on the assumption that orthologous genes underlie QTLs in both species, thus narrowing down the regions of interest. For atherosclerosis, 13 of 21 (62%) of mouse QTLs are concordant with human QTLs, and 17 of 27 (63%) of human QTLs are concordant with mouse QTLs (122). This suggests that more than half of the disease loci underlying atherosclerosis QTLs are conserved between mouse and human. Using this approach may facilitate the pinpointing of loci /genes that are relevant to human diseases.

**Genes identified from QTL studies**

The identification of causal genes underlying atherosclerosis QTLs in model organisms has been modestly successful. Two examples for mouse atherosclerosis QTLs are 1) arachidonate 5-lipoxygenase (Alox5) underlying QTL Artles (for arterial lesions) on chr 6; and 2) tumor necrosis factor, superfamily 4 (Tnfsf4, a.k.a. Ox40l) for QTL Ath1 (for atherosclerosis susceptibility 1) on chr 1. 5-LO is the rate-limiting enzyme in leukotriene synthesis and is expressed primarily in leukocytes, including monocytes and macrophages. Leukotrienes are potent pro-inflammatory lipid mediators and it has been suggested that 5-LO could play a role in atherosclerosis by regulating these pathways. The original linkage peak for Artles was found in an F2 population derived from a cross between C57BL/6J and CAST/Ei (123). Two conserved 5-LO amino acid
changes were associated with reduced 5-LO expression in the congenic C57BL/6J mice carrying the CAST atherosclerosis Alox5 allele (124). The creation of reciprocal subcongenic lines showed that two other loci within the same QTL, named Ath37 and Ath38, play significant roles in the effect of this QTL (125). Genetic linkage and association studies in Icelandic families subsequently revealed genetic variants in ALOX5AP that confer risk for MI and stroke, likely through increased production of leukotrienes in neutrophils (68). The associated SNPs in the ALOX5AP were further confirmed in Scottish, Japanese and European descent in the US (68, 126-128). This provides an example of identifying genes underlying human disease using mouse QTL studies.

Another example is the identification of Tnfsf4 by Wang et al. (129). The Ath1 locus was originally mapped to a 0.66 cM interval on chr 1 in a cross between C57BL/6J and C3H/HeJ (130, 131). Tnfsf4, coding for Ox40 ligand (Ox40L), is expressed in the aorta and the level of its expression was significantly higher in the atherosclerosis susceptible C57BL/6J strain than in the resistant C3H/HeJ strain. The Ox40L-Ox40 pathway is known to control lymphocyte proliferation and survival. Mice overexpressing Tnfsf4 manifest more atherosclerosis than wild-type mice. Deficiency in Tnfsf4 expression, or inhibition of the Ox40L-Ox40 pathway, protects from atherosclerosis (132). In addition, human association studies showed that polymorphisms in TNFSF4 were more frequent in individuals with MI (129) and stroke (133) than unaffected controls. These data support a role for Tnfsf4 in determining disease susceptibility in mouse and human. Therefore, the Ox40L-Ox40 pathway may be targeted for treating human atherosclerosis disease.
More recently, integrating genetic and gene expression data has been successful in mapping genes underlying complex traits. Transcripts influenced by loci nearby to the structural genes are referred to as *cis*-acting QTLs; others influenced by loci mapping to a completely different genomic location are referred to as *trans*-acting QTLs. When *cis*-QTLs co-localize with QTLs for a clinical trait, they can facilitate the identification of the causal genes. Successful examples include identifying candidate genes underlying behavioral phenotypes using brain tissues from recombinant inbred lines (134). Another example includes the lipoxygenase gene, *Alox15*, that was found to be an important regulator of bone mass using a differential gene expression approach comparing congenic vs non-congenic mice. The role of *Alox5* was further proven using knockout mice and pharmacological inhibitors targeting the encoded enzyme and showing improve Subsequent intercrossing is then performed to generate segment homozygotes. Isolating the region of interest in a uniform genetic background enables d bone density and strength (135). These studies have demonstrated the great potential for systems biology approaches, allowing the identification of networks of coregulated genes and pathways (136, 137).

The utility of genes/loci identified by human GWASs requires the elucidation of functional mechanisms underlying disease. Recently, the *CELSR2-PSRC1-SORT1* locus was identified by GWAS to be associated with LDL levels and MI. Using genetically altered mice, it was demonstrated that a non-coding polymorphism creates a transcription binding site that alters *Sort1* expression, thus altering plasma LDL and VLDL by modulating hepatic VLDL secretion (138, 139). Therefore, genetically-altered mouse models provide great tools to understand the functional mechanisms of DNA variants identified by GWASs.
Table 1-2  Examples of candidate genes identified for complex traits in genetic studies using rodent models

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Phenotype</th>
<th>Variation</th>
<th>Gene identification method</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alox15</td>
<td>Arachidonate12/15</td>
<td>Abnormal bone mass</td>
<td>4%</td>
<td>Expression differences, analysis of lipoxygenase knockout alleles, pharmacological inhibition</td>
<td>(135)</td>
</tr>
<tr>
<td>Rgs2</td>
<td>Regulator of G-protein signaling 2</td>
<td>Fear-related behavior</td>
<td>5%</td>
<td>Outbred mice, QTL–knockout interaction</td>
<td>(140)</td>
</tr>
<tr>
<td>Cyp11b1</td>
<td>Cytochrome P450, subfamily type1</td>
<td>Abnormal blood pressure</td>
<td>5%</td>
<td>Congenics, sequence variants</td>
<td>(141)</td>
</tr>
<tr>
<td>Lipc</td>
<td>Hepatic lipase</td>
<td>Obesity</td>
<td>7%</td>
<td>Multiple crosses, knockout interaction</td>
<td>(142)</td>
</tr>
<tr>
<td>C5</td>
<td>Complement component 5</td>
<td>Experimental allergic asthma</td>
<td>8%</td>
<td>Expression differences, loss-of-function mutation</td>
<td>(143)</td>
</tr>
<tr>
<td>Ace2</td>
<td>Angiotensin 1</td>
<td>Abnormal blood pressure</td>
<td>12%</td>
<td>Knockout phenotype</td>
<td>(144)</td>
</tr>
<tr>
<td>Apoa2</td>
<td>Apolipoprotein A2</td>
<td>HDL cholesterol</td>
<td>23%</td>
<td>Multiple-strain haplotypes, coding sequence variants</td>
<td>(145)</td>
</tr>
<tr>
<td>Mpdz</td>
<td>Multiple PDZ domain protein</td>
<td>Pentobarbital drug withdrawal with seizures</td>
<td>25%</td>
<td>Genotype-dependent differences in coding sequence and expression</td>
<td>(146)</td>
</tr>
<tr>
<td>Ncf1</td>
<td>Neutrophilic cytosolic factor 1</td>
<td>Severe arthritis</td>
<td>25%</td>
<td>Functional sequence change, pharmacological treatment</td>
<td>(147)</td>
</tr>
<tr>
<td>Kcnj10</td>
<td>Potassium inwardly-rectifying channel, subfamily J, member 10</td>
<td>Susceptibility to seizure</td>
<td>25%</td>
<td>Coding sequence variants, multiple-strain haplotypes</td>
<td>(148)</td>
</tr>
<tr>
<td>Ptp replacement (Sc1</td>
<td>Protein tyrosine phosphatase receptor type, J polypeptide</td>
<td>Susceptibility to colon cancer in human cancer</td>
<td>29%</td>
<td>Recombinant haplotypes, sequence differences, loss of heterozygosity</td>
<td>(149)</td>
</tr>
<tr>
<td>Tas1r3</td>
<td>Taste receptor, type 1, member 4</td>
<td>Saccharin response</td>
<td>30%</td>
<td>Multiple-strain haplotypes, coding sequence variants</td>
<td>(150)</td>
</tr>
<tr>
<td>Nppa</td>
<td>Natriuretic peptide</td>
<td>Cardiac</td>
<td>44%</td>
<td>Promoter variants</td>
<td>(151)</td>
</tr>
<tr>
<td>precursor A</td>
<td>hypertrophy</td>
<td>increase gene expression</td>
<td></td>
<td></td>
<td></td>
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<td>-------------</td>
<td>-------------------------------------------------</td>
<td>--------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cd36</strong></td>
<td>Fatty acid translocase</td>
<td>Abnormal fatty acid metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fatty acid translocase</td>
<td>50%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Expression differences, loss-of-function</td>
<td>mutation, transgene complementation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mutation, transgene complementation (152)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pla2g2a</strong></td>
<td>Phospholipase A2, group IIA (platelets, synovial fluid)</td>
<td>Modifier of tumors in intestinal cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td>Frameshift loss of function and transgene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>complementation (cosmid)</td>
<td>(153)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mtap1a</strong></td>
<td>Microtubule-associated protein 1a</td>
<td>Modifier of hearing loss</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>57%</td>
<td>Coding sequence variants, transgene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>coding sequence complementation (cosmid)</td>
<td>(154)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Adapted from (120)

D. **Aim of the thesis**

The aim of this thesis is to identify an atherosclerosis susceptibility gene underlying the *Athsq1* locus on mouse chr 4. Chapter two focuses on fine mapping of the chr 4 locus and identification of the cell types that are important determinants of the disease phenotype. In chapter three, gene expression analyses, functional analyses, and a gene-targeted mouse model are used to implicate the *Cdkn2a* locus (encoding *p16*<sup>INK4a</sup> and *p19*<sup>ARF</sup>, cell cycle inhibitor proteins) as a causal susceptibility locus.
Chapter 2. Fine Mapping of the *Athsq1* Locus
Abstract

Objective: Cardiovascular disease is complex, involving both genetic and environmental factors. Much effort has focused on identifying underlying genetic factors but with modest success. A mouse genetics approach provides tools to dissect genetic complexity and provide uniform models for mechanistic studies.

Methods and results: Atherosclerosis susceptibility QTL1 (Athsq1) was previously identified on chromosome (chr) 4 in a cross between MOLF/Ei and C57BL/6J-Ldlr−/−. Congenic mice carrying the MOLF-derived susceptibility allele in the C57BL/6J-Ldlr−/− genetic background confirmed the existence of the Athsq1 locus and revealed an association with increased accumulation of versican, presumably a proatherogenic matrix component abundant in human lesions. Here we provide refined mapping of the Athsq1 locus by creating a series of subcongenic strains. A distal locus in the Athsq1 interval shows proatherogenic activity after 6-wk western type diet (WTD) feeding, while an interval located near the proximal end of the Athsq1 locus is capable of conferring atherosclerosis later during the development of the plaque (9-wk WTD feeding). The proximal interval was further narrowed to a genomic region containing 8-21 genes, including the region of homology with a human CHD risk interval on chr 9p21. Bone marrow (BM) transplantation studies were performed to identify cell types that are responsible for the atherosclerosis phenotype. B6-Ldlr−/− mice receiving BM derived from the Athsq1 congenic exhibited increased atherosclerotic lesion area and versican accumulation compared to the controls. A reciprocal BM transplantation study confirmed that smooth muscle cells (SMCs) and endothelial cells (ECs) are not involved in the accelerated atherosclerosis of the 17-Mb congenics. These data provide evidence that
the accelerated atherosclerotic phenotype is mediated by BM-derived cells but not resident cells of the vascular wall.

**Conclusions:** This study confirms that there are at least two loci in the MOLF-derived Athsq1 locus that have proatherogenic activity with the smallest locus containing 8-21 genes. In addition, we found evidence that BM-derived cells are causative for the accelerated atherosclerosis phenotype observed in the congenics.
**Introduction**

CHD is highly prevalent in the population and remains an important public health challenge. Atherosclerosis is the main cause of CHD and is a complex disease resulting from genetic, environmental and gene-environment interactions. Attempts to identify the genetic components have been hampered by clinical and genetic heterogeneity, polygenic inheritance and environmental factors. Intense effort has been made to dissect the genetic component of atherosclerosis using traditional genetic approaches but with modest success (155). Animal models can facilitate the discovery of genetic factors and molecular pathways underlying human disease.

Quantitative trait loci (QTLs) were initially mapped in mouse using recombinant inbred lines (32, 130, 156). Later studies reported the use of congenic strains to identify QTLs (40, 123, 157-159). To date, more than thirty different mouse atherosclerosis loci have been identified (121). However, very few of the underlying genes have been identified and tested for a role in human disease. Apoa2 was the first gene identified by mouse genetic mapping (160, 161), with increased levels of apoA-II associated with metabolic disorders in humans (59). Similarly, Alox5 and Tnfsf4 were identified by mouse genetic mapping and polymorphisms in these genes have been found to be associated with MI and stroke in humans (68, 124, 129). Thus, mouse genetic mapping provides a great tool to identify underlying genes for atherosclerosis-related traits and ultimately discover mechanisms for human disease.

We previously reported the localization of atherosclerosis susceptibility QTL 1, Athsq1, to chr 4 using a cross between strains MOLF/Ei and C57BL/6J-Ldlr<sup>+</sup>(157). The Athsq1 locus was confirmed with the creation of congenic strains (162). Congenic mice carrying a 54-Mb atherosclerosis susceptibility locus derived from the MOLF strain was introgressed onto the C57BL/6J-Ldlr<sup>+</sup> genetic background. Homozygous congenic mice
exhibited up to 4.5-fold greater lesion area compared to noncongenic littermates \((p<0.0001)\). This locus affects lesion susceptibility in a gene dosage dependent manner and acts independently of plasma lipid levels. Lesions from the congeneric mice exhibited more advanced plaque characteristics including thick fibrous caps and greater necrotic core areas compared to controls. These mice also exhibited prominent accumulation of a proatherogenic matrix protein, versican, located at the intima-media interface beneath plaques. However, the large interval (54 Mb) contained numerous genes (~650 genes) making candidate gene analysis impossible at this point.

Here, we report refined mapping of the \(Athsq1\) locus to narrow the list of candidate causal genes by generating a series of subcogenic strains. Using different durations of WTD feeding, we identified at least two loci in the \(Athsq1\) interval. One of these loci was narrowed down to a 5.4-9 Mb interval that contains the region of homology to a human CHD locus on 9p21 (91-94). BM transplantation studies revealed that the accelerated atherosclerosis and versican accumulation in the congenics are mediated by BM-derived cells, not SMCs or ECs.

Material and Methods

Mice. MOLF/Ei (MOLF) and B6.129S7-\(Ldlr^{tm1Her}\) (B6-\(Ldlr^{-/-}\)) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.MOLF-\(Athsq1\)congenic mice were generated by introgression of a 54 Mb MOLF donor interval into the B6-\(Ldlr^{-/-}\) background, followed by intercrossing to produce homozygotes as described (162). Subcongenic mice (17, 11, 4 Mb and Sub-B and -C strains) were derived from the 54 Mb strain. Recombinants were identified by marker-assisted selection (163), using
microsatellite markers spanning the 54-Mb interval inherited from the MOLF parental strain. Mice carrying similar (ie. closely-located) breakpoints were intercrossed at N13-N17 generations. A 51 Mb congenic strain carrying a nearby breakpoint relative to the 54 Mb interval was also used for BM transplantation studies and versican staining. An atherosclerosis study was performed on 51 and 54 Mb congenics, which were derived from the N6 and N14 generation respectively. There is a clear lesion susceptibility phenotype present in both the 51- and 54-Mb strains (Figure 2-1) (the difference may be due to experiment-experiment variation). These data suggest that the lesion phenotype was still present in a more uniform (N14) background and not dependent on the presence of other donor regions present at the N6 generation. Subcongenic mice (males and females) were fed Western-type diet (WTD) (TD88137; Harlan Teklad) for 6- or 9-weeks before sacrifice as indicated. All procedures were in accordance with Institutional guidelines.

Figure 2-1  A clear lesion susceptibility phenotype present in both 51 and 54 Mb congenic strains.
Atherosclerotic lesion area in (A) 51 Mb congenic mice (220 577 ± 153 238 µm²/section) vs the controls (52 526 ± 17 512 µm²/section) after a 6wk WTD compared to (B) 54 Mb congenic mice (22 3726 ± 38 938 µm²/section) vs the controls (111 543 ± 69 664 µm²/section) after 7 wk WTD. These data suggest that the lesion phenotype was still present in a more uniform (N14) background and not dependent on the presence of other donor regions present at the N6 generation. Shown is mean ± SD.

DNA Extraction and LdlrGenotyping. DNA was extracted from tail tips by an alkaline lysis protocol (164). Briefly, the tail tips were incubated in 50mM NaOH for 1hr at 95°C, vortexed and neutralized in 1 M Tris (pH 8). Cellular debris was pelleted by centrifugation and the supernatant was used for genotyping. Ldlr alleles were typed by polymerase chain reaction (PCR) with the primer information obtained from http://jaxmice.jax.org/protocolsdb/f?p=116:2:2955415530757048::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:6048,007068 (157,162). Custom-made oligonucleotides were synthesized commercially (Invitrogen). Microsatellite markers were typed by PCR using the following protocol: 30 seconds denaturing at 95 ºC, 30 seconds annealing at 60 ºC, 2 minutes extension at 72 ºC for a total of 35 cycles, and one final extension at 72 ºC for 10 minutes.

Atherosclerotic Lesion Measurements. Serial sections were prepared from the aortic root as described (157,162). Anesthetized mice were killed by cervical dislocation. Mouse hearts were perfused using 10 ml of PBS (Invitrogen), and the aortic roots were dissected and fixed in 10% formalin (Fisher). 6-µm sections from the proximal aortas were serially paraffin-sectioned and stained with hematoxylin and eosin (H&E) (Sigma). Lesion areas were quantified by morphometric analysis using every tenth section, for a total of six sections per mouse. Morphometric analysis was performed by outlining lesion areas delineated by H&E staining using ImagePro 3.0 Plus (Media Cybernetics). Mean
aortic lesion area was obtained by averaging lesion areas from six sections from the same mouse.

**Immunohistochemistry.** Aortic tissues of 6-μm paraffin-embedded sections mounted on Super Frost slides were deparaffinized, rehydrated, and stained for versican using a rabbit polyclonal antibody against the β-gag domain (Chemicon International) and detected by an indirect avidin–biotin–peroxidase method. Briefly, sections were sequentially incubated and washed in phosphate-buffered saline (PBS). A polyclonal antibody specific for versican was diluted to 6-8 µg/ml in PBS, followed by hybridization with biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex. Sections stained without primary antibody were used as negative controls.

**BM Transplantation.** BM transplantation was performed as previously described (165). Recipient mice were given acidified water (pH 4.5) containing 100 mg/liter neomycin (Sigma) and 10 mg/liter polymyxin B sulfate (Sigma) one week before and two weeks after BM transplantation. Eight-week-old male or female B6- \( Ldlr^{-/-} \) mice were lethally irradiated with 1000 rads (10 Gy) from a cesium gamma source. BM was collected from femurs and tibias of donors, by flushing with sterile RPMI 1640 media containing 2% FBS, 5 U/ml heparin, 50 U/ml penicillin, and 50 µg/ml streptomycin. Each recipient mouse was injected with 4–5 × 10^6 BM cells through the tail vein. Reconstitution of the BM with donor cells was checked at 6 weeks postinjection using microsatellite marker \( D4Mit185 \), located within the 17-Mb donor interval. Mice were then fed WTD for 11 weeks, euthanized, and the aortic roots dissected and sectioned as above.

In the first BM transplantation study, irradiated recipient B6-\( Ldlr^{-/-} \) mice were injected with BM derived from 51-Mb congenics or non-congenic controls. In the second study,
recipient B6-\(Ldlr^{-+}\) or 17-Mb congenic mice were each injected with BM donor cells from B6-\(Ldlr^{-+}\) mice.

**Statistical Analysis.** ANOVA and t-test were performed with or without square root or log transformation, as indicated, using STATVIEW 5.0 (Abacus Concepts, Inc). Both males and females were included in the lesion analyses using genotype and sex as factors in the ANOVA. The threshold for significance was set at \(p = 0.05\). Data shown are mean ± SD.

**Results**

**Fine mapping of the \(Athsq1\) locus reveals at least two atherosclerosis susceptibility loci within the 54 Mb interval.** In order to refine the localization of the susceptibility gene(s) residing in the \(Athsq1\) locus, subcongenic strains were created. \(Athsq1\) full congenics were backcrossed to B6-\(Ldlr^{-+}\) mice. Chromosomal recombinations were identified using microsatellite markers polymorphic between MOLF and B6. Heterozygotes carrying similar (i.e. closely located) breakpoints were intercrossed to produce homozygotes. In total, six homozygous subcongenic strains were created (Figure 2-2, A). Strains carrying a 17-, 11- or 4-Mb proximal region of the MOLF-derived interval, as well as a strain carrying just the distal end of the 17-Mb interval (Sub-C) were identified. In addition, a Sub-B strain was identified carrying a distal end of the 54-Mb full interval (Figure 2-2, A).

Following 6-wk WTD feeding, mean lesion area of 17-Mb congenic mice did not differ significantly compared to the control strain (b/b) (Figure 2-2, B). Consistent with this observation, mice carrying the 11- or 4-Mb and Sub-C intervals, which cover the proximal and distal ends of the 17-Mb region, did not show significant differences in
lesion area compared to the b/b controls. However, the Sub-B congenic strain, which carries the distal end of the 54 Mb interval, exhibited significantly larger mean lesion area than the controls, indicating the existence of one or more susceptible alleles (Figure 2-2B). Thus, at the 6-wk feeding timepoint, a distal locus within the 54-Mb interval confers susceptibility for atherosclerosis.

Analysis of atherosclerotic lesion development was then determined in a subset of congenic mice following 9-wk WTD feeding. Mice carrying the 17- or 11-Mb subinterval, but not the 4-Mb interval, exhibited approximately 2-fold greater mean lesion area than non-congenic controls (p<0.0001) (Figure 2-2, C). These data indicate that there is a locus at the proximal end of the 54 Mb interval, excluding the 4-Mb region, that is detectable only after a longer (9-wk vs. 6-wk) WTD feeding period.

Together, these data suggest that there are at least two different loci contributing to atherosclerosis susceptibility in the Athsq 1 congenic mouse, possibly acting at different stages of atherogenesis.

To determine whether versican accumulation was associated with the proximal locus, immunostaining was performed in lesions derived from 17-Mb congenic or non-congenic control mice. Using an antibody directed against the β-gag domain of versican, 17-Mb congenic mice showed a prominent accumulation of versican compared to controls (Figure 2-2, D). These data suggest that versican plays a role in the development of accelerated plaque formation observed in the congenics.
Figure 2-2  Refined mapping of the \textit{Athsq1} interval reveals two distinct loci detectable after different periods of WTD feeding.

(A) Physical map used for the construction of subcongenic strains on mouse chr 4. A series of subcongenic strains derived from the 54-Mb full congenics was created. The extent of MOLF donor intervals (white boxes) introgressed into the B6-Ldlr\textsuperscript{-/-} background (black boxes) is indicated along the top in Mb; b/b, homozygosity for B6 alleles; m/m, homozygosity for MOLF alleles. (B) Mean lesion areas for each subcongenic strain after a 6-wk WTD feeding period. The increased atherosclerotic lesion area observed in the Sub-B congenic strain suggests a locus at the distal end of the 54 Mb interval acting at
an early stage of atherogenesis. (C) After 9-wk WTD feeding, 17-Mb and 11-Mb subcongenic strains revealed increased susceptibility for atherosclerosis, suggesting an additional locus located in the proximal region of the 54 Mb interval. A 4-Mb interval at the proximal tip of the full interval does not confer increased susceptibility for atherosclerosis. ANOVA was performed with square root transformation. Horizontal bars indicate mean values for males (blue) and females (red) for each genotypic group. NS, not significant. D) Prominent versican accumulation in the 17-Mb congenics compared to the controls after 9-wk WTD feeding.

Conserved chromosomal homology between the *Athsq1* proximal locus and human chr 9p21 including a widely-replicated CHD locus. The endpoints of the proximal susceptible interval were defined by microsatellite marker genotyping. As shown in Figure 2-3, the minimal interval is defined by markers *D4Mit27* and *D4Mit350* (88.7 - 94.1 Mb). The maximal interval is defined by markers *D4Mit349* and *D4Mit154* (87.4 - 96.4 Mb) (Figure 2-3, B). The interval contains 8-21 known protein coding genes/gene families (Figure 2-3, A). Comparative analysis of mouse and human homologies revealed that the proximal *Athsq1* interval overlaps the region of homology with the human CHD locus on chr 9p21 (Figure 2-3, C). The closest protein coding genes transcribed from the *CDKN2A/B* locus are conserved in mouse and human. Thus, *Cdkn2a* and *Cdkn2b* are strong candidate genes for atherosclerosis and warrant further testing.
Figure 2-3  The proximal *Athsq1* locus harbors 8-21 genes and contains the region of homology with a human CHD risk interval on chr 9p21

(A) List of genes residing in the *Athsq1* proximal interval (taken from the Mouse Genome Informatics Database, MGD). (B) Schematic of the 5.4 – 9 Mb interval defined by microsatellite markers. The white rectangle denotes the minimally mapped susceptibility locus derived from MOLF/Ei. Recombination breakpoints occur between *D4Mit349/D4Mit27* (proximal end) and *D4Mit350/D4Mit154* (distal end). The black rectangles denote alleles derived from B6. (C) Magnification of the area containing the region of homology with a human CHD risk interval on 9p21 (shown in red). The orientation of flanking genes is shown with blue boxes.

**Accelerated atherosclerosis in the *Athsq1*congenic mouse is mediated, at least in part, by BM-derived cells.** BM transplantation studies are frequently performed to identify the role of BM–derived cells vs resident cells of the vascular wall in mouse
models of atherosclerosis (168). In order to identify the cell type(s) responsible for the pro-atherogenic phenotype of the Athsq1congenic mice, we performed a series of BM transplantation experiments. To test the role of BM-derived cells, lethally-irradiated B6-Ldlr<sup>−/−</sup> recipient mice were injected with donor BM derived from the congenic or noncongenic mice (both on the B6-Ldlr<sup>−/−</sup> background). After recovery/repopulation of the BM-derived cells with donor cells, mice were fed 11-wk WTD and atherosclerotic lesions were analyzed from the proximal aorta. Lesion area was significantly increased in mice receiving congenic (51 Mb)- derived BM compared to mice receiving noncongenic (b/b) BM (p=0.0016; Figure 2-4,A). Greater accumulation of versican in mice receiving congenic-derived BM was also observed (Figure 2-4,B-C): a 2.5-fold increase in versican-positive medial area (Figure 2-4,D) in mice receiving congenic BM compared to the controls. These results indicated that BM-derived cells play an important role in the development of lesion susceptibility in the B6-Ldlr<sup>−/−</sup> model.

![Figure 2-4](image_url)

Figure 2-4  Increased lesion area and versican accumulation in B6-Ldlr<sup>−/−</sup> mice transplanted with Athsq1congenic BM compared to noncongenic controls.
(A) Atherosclerotic lesion area after 11-wk WTD feeding of B6-\(Ldlr^{\overline{c}}\) mice receiving b/b or 51-Mb BM. Horizontal bars represent group means. (B, C) Representative cross-sections including atherosclerotic lesions derived from BM-transplanted mice immunostained with versican (red). Pl, plaque; M, media. (D) Quantification of versican-positive medial area by morphometric analysis shown in (B, C). Data analyzed by \(t\)-test with square root transformation. Female B6-\(Ldlr^{\overline{c}}\) mice were used as recipients.

A reciprocal BM transplantation study was performed to determine whether vascular wall cells, including SMCs or ECs, contributed to the pro-atherogenic phenotype conferred by the proximal locus. BM derived from B6-\(Ldlr^{\overline{c}}\) controls was injected into lethally-irradiated 17-Mb congenic and non-congenic controls. Following repopulation of the BM, mice were fed 11-wk WTD and analyzed for atherosclerotic lesion development. No differences in mean lesion areas were observed for males or females, or both sexes combined (Figure 2-5). Importantly, these data indicate that vascular wall-derived cells, ECs and/or SMCs, are not involved in the accelerated atherosclerosis phenotype associated with this locus. Thus, we focused further mechanistic studies on the role of BM-derived monocyte/macrophages.
Discussion

Generation of subcongenic strains derived from the full congenic Athsq1 strain revealed at least two loci in the Athsq1 interval: 1) a distal locus acting early in lesion development (6-wk WTD feeding) and 2) a proximal locus acting at a later stage (9wk-WTD feeding). Moreover, BM transplantation studies revealed that BM-derived cells are responsible for the accelerated atherosclerosis phenotype seen in the congenics.

Gene(s) that are located in the distal locus likely play a role in initiation of atherogenesis including activation of the endothelium, leukocyte recruitment, monocyte migration into the intima, or foam cell formation. The gene(s) located in the proximal locus likely act at later events such as inflammatory cell proliferation (reviewed in (9)). To accurately define what stage the loci act during atherosclerosis development, the atherosclerotic lesions should be categorized carefully according to the morphologic features, for example, lesion size, necrotic core area and lesional cap thickness etc. This would assist in clearly defining the phases of genes/loci that come into action during atherosclerosis development.

Comparative mapping of mouse and human chromosomes revealed that the narrowed proximal locus contains the region of homology with the human CHD locus on 9p21 (91-94). Moreover, fine mapping of the locus narrowed the list of candidate genes to 8-21 genes, including the region of homology with 9p21. The associated locus
overlaps a long non-coding RNA, ANRIL, with the closest protein coding genes transcribed from the CDKN2A/B locus. These protein coding genes are involved in cell cycle regulation and are key tumor suppressor genes (169, 170). The proteins encoded by CDKN2A/B function as inhibitors of cell proliferation (Fig 1.) The binding of \( p15^{INK4b} \) (encoded by CDKN2B) and \( p16^{INK4a} \) (encoded by CDKN2A) to cyclin dependent kinase 4/6 (CDK4/6) blocks the assembly of catalytically active cyclin D–CDK complexes, inhibiting CDK4/6-mediated phosphorylation of retinoblastoma (Rb), leading to G1 cell cycle arrest (169, 171).

The tumor suppressor activity of \( p14^{ARF} \) (mouse p19\(^{ARF}\), encoded by CDKN2A) is largely ascribed to its ability to regulate p53 in response to aberrant growth or oncogenic stress, thus leading to apoptosis when \( p14^{ARF} \) is present (169, 171). It has been hypothesized that regulatory elements underlie the associated CHD locus, regulating the expression of CDKN2A/B genes (104, 105, 108, 166, 172). Thus, the accelerated atherosclerosis observed in the congenics might be due to increased proliferation of vascular cells, including ECs, SMCs or macrophages. Since the BM transplantation study revealed that BM-derived cells, but not resident vascular wall cells, are critical for atherogenesis in the congenics, we hypothesized that the accelerated phenotype observed in the congenics is due to increased proliferation of BM-derived cells.

Atherogenesis is initiated by lipoprotein retention and modification, ECM deposition, and inflammatory cell recruitment. Versican, a presumed proatherogenic ECM protein was examined to confirm the previous findings that versican accumulation segregates with increased atherosclerosis. Analysis of ECM composition in the 17-Mb congenic mice and BM-transplanted animals receiving 51-Mb congenic BM revealed prominent accumulation of versican. In atherosclerosis, versican is prominent in the ECM of early
intimal thickenings as well as advanced lesions (173). Versican binds LDL particles with high affinity and increases LDL atherogenicity (174). Due to the structure of versican, it has been speculated that accumulation of versican in the vessel wall may promote both extracellular lipoprotein retention and intracellular lipid uptake leading to foam cell formation (175). Versican-hyaluronan complexes promote macrophage adhesion through cell surface receptors and cell adhesion molecules. This would presumably lead to the deposition of a proatherogenic matrix in the Athsq1 congenics.

The distal end of the Athsq1 locus, defined by the Sub-B strain, contains a 34.1 Mb interval harboring ~520 annotated genes. The large number of genes prohibits any meaningful discussion of potential candidate genes. Since the Athsq1 locus (54-Mb) is a large interval, we cannot exclude the possibility that there are genes having opposing effects. Among the genes residing in the narrowed 5.4-9 Mb interval interval, type I-interferon (IFN-I) family is worthy of note. Elevated levels of IFN-I (including IFN-α and -β) (176) have been observed in patients with systemic lupus erythematosus (SLE). High levels of IFN-I results in endothelial progenitor cell depletion and dysfunction thus leading to increased cardiovascular risk in SLE patients (177). Goossens et al (178) have shown that IFN-I induces chemotactic factors and enhances macrophage adhesion in vitro. IFN-β promotes macrophage, but not neutrophils or T-cell content, in atherosclerotic carotid arteries of Apoe<sup>−/−</sup> mice. BM lacking IFN-I reduces atherosclerosis development (178). These studies have provided evidence that IFN might be a potential candidate gene. Further analysis, including sequencing, expression, and functional studies of the gene will be required to determine if genes of the IFN-I family located in the 5.4-9 Mb interval is another culprit gene.

The finding of BM-derived cells plays a role in atherogenesis in the Athsq1
congenics is intriguing. BM cells are precursors of peripheral blood mononuclear cells (PBMCs). PBMCs play a critical role in inflammation during atherogenesis and constitute a major cellular component of atherosclerotic lesions. Using PBMCs from patients with severe carotid atherosclerosis, gene expression analyses revealed a number of regulatory genes and transcription factors that were altered in patients compared with controls without measurable disease (179). Genomic signatures derived from BM progenitor cells differentiate between vascular damage and restoration in mice and were homologous to those seen in humans (180). These studies validate genomic gene sets discovered in the mouse and provide potential applications to human disorders. Thus, gene expression profiling using PBMCs should provide further insights into the underlying mechanism for accelerated atherosclerosis observed in congenic and different subcongenic strains.
Chapter 3.  *Cdkn2a* is an Atherosclerosis Modifier Locus That Regulates Monocyte/Macrophage Proliferation
Abstract

Objective: GWAS studies have been fruitful in identifying genetic variant loci underlying complex traits. The most highly replicated locus for CHD is on chr 9p21.3. However, the functional link between genetic variants and disease remains to be understood.

Methods and Results: We have localized a murine atherosclerosis susceptibility locus to a region of chr 4 containing 8-21 genes/gene families, including Cdkn2a/b. In the current study, mRNA expression analyses in macrophages showed markedly decreased Cdkn2a (cyclin-dependent kinase inhibitor 2a) transcript expression, including both p16\textsuperscript{INK4a} and p19\textsuperscript{ARF}, but not Cdkn2b (p15\textsuperscript{INK4b}), in cells from susceptible congenic mice compared to controls. Interestingly, the congenic mice also exhibited increased circulating levels of Ly6C\textsuperscript{hi} inflammatory monocytes compared to controls. To directly test the role of BM-derived Cdkn2a transcripts in atherogenesis and inflammatory cell proliferation, we performed a BM transplantation study utilizing Cdkn2a\textsuperscript{+/−} cells in the Ldlr\textsuperscript{−/−} mouse model. Cdkn2a\textsuperscript{+/−} recipients exhibited accelerated atherosclerosis, increased inflammatory monocyte subsets and increased monocyte/macrophage proliferation compared to controls.

Conclusions: We have used an unbiased mouse genetics approach to identify the Cdkn2a locus as a modifier of atherosclerosis susceptibility through altered expression in monocyte lineage cells. The data provide a novel mechanistic link between decreased expression of Cdkn2a transcripts, increased monocyte/macrophage proliferation, expansion of the Ly6C\textsuperscript{hi} inflammatory monocytes and accelerated atherosclerosis.
Introduction

GWASs have identified numerous genetic variants associated with complex traits (75-84). Some of the susceptibility variants lie outside of coding regions, and are assumed to influence transcript regulation rather than gene function. Recent studies in animal models (139, 166, 167, 172, 181, 182) have shed light on the genetic variants identified by GWASs, stressing the importance of model organisms.

The most highly replicated risk locus for CHD (including MI, stroke and aortic aneurysm) is on chr 9p21.3, accounting for a substantial part of atherosclerotic risk in the general population(91-93). The association at 9p21 appears to be the strongest common (minor allele frequency > 10%) genetic determinant in the human genome, yet the association is independent of traditional risk factors for atherothrombotic disease, such as plasma cholesterol levels and hypertension. The SNPs most strongly associated with disease risk map to a 58-kb region that includes a long, non-coding RNA (ANRIL) but is devoid of known coding genes (Figure 1-4). Individuals carrying the risk allele have been shown to exhibit decreased expression of p16\textsuperscript{INK4a}, p19\textsuperscript{ARF}, and p15\textsuperscript{INK4b} - inhibitors of cellular proliferation encoded by the nearby CDKN2A/B locus – as well as proximal transcripts of ANRIL, in some studies (104-106) but not others (107, 108). Targeted deletion of the homologous region in mice resulted in markedly decreased expression of all three transcripts encoded by the Cdkn2a/b locus, and increased proliferation of vSMCs \textit{in vitro}, but effects on atherosclerosis were not reported (166). Thus, the role of these transcripts and the pathogenic mechanism leading to increased atherosclerotic risk remains unclear.

We previously performed refined genetic mapping of a murine atherosclerosis susceptibility locus and confirmed that the susceptibility interval contains the region of
homology with the human 9p21 CVD locus. In the current study, gene expression studies revealed decreased mRNA levels of \( p16^{\text{INK4a}} \) and \( p19^{\text{ARF}} \), but not \( p15^{\text{INK6}} \), in macrophages derived from susceptible congenic mice compared to controls. As we had previously shown that BM-derived cells were capable of conferring the pro-atherogenic phenotype of the congenics, we investigated the hypothesis that reduced expression of \( Cdkn2a \) cell proliferation inhibitor transcripts in BM-derived cells leads to accelerated atherosclerosis via increased proliferation of inflammatory monocyte/macrophages.

**Materials and Methods**

**Mice.** B6.MOLF-Athsq1 congenic mice were bred using marker-assisted congenic strain production as described (162) and chapter 2 herein. B6.129S7-Ldlr\(^{\text{tm1Her}}\) (B6-Ldlr\(^{\text{–/–}}\)) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Subcongenic mice were derived from the 54-Mb strain; recombinants were identified and intercrossed at the N14 generation. B6-Cdkn2a\(^{\text{–/–}}\) mice were a kind gift of Dr. Sean Morrison (University of Michigan). The targeted mutation replaced exons 2 and 3 with a neo cassette, therefore knocking out both \( p16^{\text{INK4a}} \) and \( p19^{\text{ARF}} \). Mice were bred to B6-Ldlr\(^{\text{–/–}}\) mice in our colony. Mice were fed WTD containing 21% milk fat and 0.15% cholesterol (TD 88137, Harlan Teklad) for the indicated time periods before sacrifice. All procedures were in accordance with institutional guidelines.

**DNA extraction and genotyping.** DNA was extracted from tail tips, and microsatellite markers and \( Ldlr \) alleles were typed by polymerase chain reaction (PCR) as previously described (157, 162). \( Cdkn2a \) alleles were commercially typed by PCR (Gene Typer LLC).
**Gene expression profiling.** Peritoneal monocyte/macrophages were isolated from B6-
*Ldlr*\(^{-/-}\) or 54-Mb congenic mice three days after no treatment (resident) or intra-peritoneal
injection of concavalin A to elicit monocytes to the peritoneal cavity. Cells were plated
and washed one hour later to remove non-adherent cells, followed by overnight
incubation in DMEM (Dulbecco's modified Eagle's medium, 25 mM glucose), 10%FBS,
and 1% penicillin/streptomycin. Splenic monocyte/macrophages were isolated to >90%
purity by Magnetic Activated Cell Sorting (MACS) using an anti-CD11b antibody (Miltenyi)
as described (104, 183). RNA was isolated using an RNA micro kit (Qiagen) according to
the manufacturer’s protocol.

Microarray analysis was performed as described (184). Merck/Affymetrix mouse 1.0
custom arrays monitoring 38,384 individual transcripts (25,846 Entrez genes, NCBI build
37) were used. Raw intensity was normalized using the robust multi array average
algorithm (185). ANOVA analysis identified differentially expressed genes using a 1.2-
fold change threshold (congenics vs controls, \(p < 0.05\)). Raw data were deposited in the
NCBI gene expression and hybridization array data repository, GEO and are available
under accession # GSE 24342.

**Transcript-specific real-time PCR.** Transcript-specific real-time PCR (rtPCR) was
performed to confirm results. Briefly, 1 \(\mu\)g of purified total RNA was used for reverse
transcription with SuperScript II Reverse Transcriptase (Invitrogen) according to the
manufacturer’s instructions. Transcript expression was measured using TaqMan
strategies specific for *p16\(^{INK4a}\)*, *p19\(^{ARF}\)*, *p15\(^{INK4b}\)*, and other indicated transcripts as
previously reported(186). Expression levels were normalized to 18S ribosomal RNA.
**Atherosclerotic Lesion Measurements.** Paraffin-embedded serial sections were prepared from the aortic root as described (157, 162). Lesion area was quantified by morphometric analysis of H&E stained sections, and average lesion size determined from six sections per mouse.

**BM Transplantation.** Irradiated recipient B6-\(Ldlr^{-/-}\) mice injected with either B6-\(Ldlr^{+/+}\) or B6-\(Ldlr^{-/-}\), \(Cdkn2a^{+/+}\) BM. Reconstitution of the BM with donor cells was confirmed at six weeks (wks) post-injection with microsatellite markers for the congenic interval or allele-specific \(Cdkn2a\) and \(Ldlr\) primers, respectively. Mice were then fed 10-11 wk WTD before euthanasia.

**Bromodeoxyuridine (BrdU) labeling.** Mice were injected intraperitoneally with 200 ul of 2 mg BrdU/mouse. The pulse was 18 hrs for the analysis of circulating monocytes in the BM transplantation experiment. The dose and duration were based on studies investigating the proliferative activity of murine monocytes(187, 188). To assess proliferation of tissue macrophages, a longer BrdU dosing protocol was used. For assessment of peritoneal macrophage proliferation, cells were harvested after three consecutive injections of BrdU over 72 hrs following a 24-hr concavalinA injection.

**Flow cytometry analysis.** Flow cytometry analysis was performed as previously described (25). Briefly, 100 µl of blood was collected into EDTA tubes followed by red blood cell lysis. Cells were washed with HBSS (Hank's Buffered Salt Solution, 0.1% BSA w/v, 5 mM EDTA) and stained on ice using an antibody cocktail including CD45-APC-Cy7 (pan –leukocytes), CD115-APC (monocytes),Ly6C-PerCP (monocyte subsets), and BrdU-FITC (all from BD Pharmingen). Monocytes were identified as CD45\(^+\) CD115\(^+\) cells and further gated as Ly6C\(^{hi}\) (inflammatory monocytes) or Ly6C\(^{lo}\) (patrolling monocytes).
Peritoneal monocyte/macrophages were freshly collected and stained with CD45, CD115 and F4/80-PE (mature macrophage marker). Multiparameter analyses were performed using a BD LSR II flow cytometer (Becton Dickinson) with DiVa software. Data were analyzed using FlowJo software (Tree Star, Inc.).

**Statistical analysis.** ANOVA and t tests were performed using STATVIEW 5.0 (Abacus Concepts, Inc). Both males and females were included in lesion analyses using genotype and sex as factors in the ANOVA. The threshold for significance was p < 0.05. Data shown are mean ± SD or SEM, as indicated.

**Results**

**Decreased macrophage expression of p16\(^{INK4a}\) and p19\(^{ARF}\), but not p15\(^{INK4b}\), in the **Athsq1** conegenic mice.** In parallel to the mapping effort, we performed microarray analysis to narrow down the list of candidate genes in the atherosclerosis susceptibility subcongenic interval. Gene expression analysis was performed to search for genes that are differentially expressed between the congenics and controls. Elicited peritoneal macrophages derived from full (54-Mb) congenics and non-congenic controls were collected after 6-wk WTD feeding. Differentially-expressed genes were defined as exhibiting ≥20% difference in expression level with a significance threshold corrected for multiple testing (184). Only 2-8 of the differentially expressed transcripts reside within the narrowed 5.4-9-Mb interval and could be considered as causal candidate genes (Table 3-1). Strikingly, the most significant difference was a ~6-fold decrease in Cdkn2a levels in congenic compared to control macrophages. Cdkn2a encodes two transcripts involved in cell proliferation regulation: p16\(^{INK4a}\) and p19\(^{ARF}\) (170).
Cdkn2a is the most differentially expressed macrophage-derived gene residing in the 5.4-9-Mb mapped susceptibility interval.

<table>
<thead>
<tr>
<th>Genesymbol</th>
<th>Gene name</th>
<th>Position (Mb)</th>
<th>Fold change (relative to b/b)</th>
<th>ANOVA p-value</th>
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<td>methythioadenosinephosphorlase</td>
<td>88.8</td>
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<td>7.77E-05</td>
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<tr>
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<tr>
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<td>Jun oncogene</td>
<td>94.7</td>
<td>-1.39</td>
<td>0.0028</td>
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<td>cyclin-dependent kinase inhibitor 2A</td>
<td>88.9</td>
<td>-5.98</td>
<td>1.25E-01</td>
</tr>
</tbody>
</table>

Fold-change listed for all transcripts encoded within the 5.4-9-Mb interval and exhibiting a ≥20% difference in expression level in peritoneal macrophages derived from Athsq1 54-Mb full congenic or non-congenic mice fed 6-wk WTD. N = 10 mice/group. Note that Ptplad2 is listed twice (+1.24, -1.30 fold changes), likely the result of splice variants occurring within the array.

On the right is a schematic of the 5.4-9-Mb narrowed susceptibility interval with microsatellite markers, and the corresponding genome coordinates in megabases (Mb). The white rectangle denotes the minimally mapped susceptibility locus derived from MOLF/Ei. The black rectangles denotes alleles derived from B6.

The two transcripts from the Cdkn2a locus-p16^{INK4a} and p19^{ARF} utilize different promoters and alternative reading frames resulting in proteins with no amino acid homology(169). Because of the Cdkn2a exonic structure, the microarray could not discern p16^{INK4a} vs p19^{ARF} transcripts. Therefore, we further examined Cdkn2a expression using transcript-specific rtPCR, and confirmed the results in the 17-Mb subcongenic strain.
Expression of both $p16^{\text{INK4a}}$ and $p19^{\text{ARF}}$, but not the adjacent $p15^{\text{INK4b}}$ transcript, were reduced in macrophages from the atherosclerosis-prone 54- and 17-Mb congenics compared to non-congenic controls (Figure 3-1, A). Similar results were observed for both transcripts in resident peritoneal macrophages, and for $p16^{\text{INK4a}}$ in splenic monocyte/macrophages, from 54- and 17-Mb congenic mice compared to controls (Figure 3-1, B-C). Thus, an atherosclerosis-prone murine strain, identified through an unbiased genetic screen, exhibits decreased expression of the $p16^{\text{INK4a}}$ and $p19^{\text{ARF}}$ tumor suppressor genes.
Figure 3-1  Decreased expression of $p16^{INK4a}$ and $p19^{ARF}$, but not $p15^{INK4b}$, cell proliferation inhibitor transcripts in BM-derived cells from Athsq1congenic mice compared to controls.

Transcript-specific rtPCR results for Cdkn2a ($p16^{INK4a}$ and $p19^{ARF}$) and Cdkn2b ($p15^{INK4b}$) expression in (A) concavalin A-elicited peritoneal macrophages, (B) resident peritoneal macrophages, or (C) splenic CD11b+ monocyte/macrophages derived from B6-Ldlr$^{-/-}$ (b/b) or subcongenic (17-Mb) mice fed 6-wk WTD. ANOVA performed with log transformation. Data are shown as mean ± SD. *$p \leq 0.05$, **$p \leq 0.005$, p ≤ 0.0005 compared to b/b controls. N=7-12/group.
Expansion of the circulating Ly6C<sup>hi</sup> inflammatory subset of monocytes in 17-Mb congenics compared to controls. To test potential atherogenic mechanisms consistent with decreased \(p16^{\text{INK4a}}\) and/or \(p19^{\text{ARF}}\) expression, we assessed monocytosis in 17-Mb subcongenics and controls. No differences were observed in circulating white blood cell counts or total monocytes in blood derived from 54-Mb congenics compared to controls (Figure 3-2, A-D). Pro-inflammatory monocyte subsets have been associated with hypercholesterolemia and CHD events (35, 37, 38). Therefore we further investigated monocyte subsets by using a Ly6C antibody to differentiate inflammatory monocytes (Ly6C<sup>hi</sup>) vs patrolling monocytes (Ly6C<sup>lo</sup>). We observed increased percentages of Ly6C<sup>hi</sup>, and decreased Ly6C<sup>lo</sup>monocyte subsets in congenics compared to controls after 3- (pre-lesional), 6- and 9-wk WTD feeding (Figure 3-3, A-B). This resulted in an increased ratio of Ly6C<sup>hi</sup>: Ly6C<sup>lo</sup> monocytes in the circulation (Figure 3-3, C). These data suggested that expansion of the Ly6C<sup>hi</sup> inflammatory subset of circulating monocytes might underlie the accelerated atherosclerosis phenotype of the 17-Mb congenic mice. This mechanism is consistent with decreased expression of \(p16^{\text{INK4a}}\) and/or \(p19^{\text{ARF}}\), the latter of which has been shown to regulate macrophage proliferation in other settings (189).
Figure 3-2  No differences in white blood cell counts in the circulation of 54-Mb congenics compared to non-congenic controls.

White blood cell counts were measured after 4-, 8- and 15-wk WTD feeding. No differences were observed in the levels of (A) total white blood cell counts, (B) neutrophils, (C) monocytes or (D) lymphocytes.
Figure 3-3  Expansion of the inflammatory Ly6C<sup>hi</sup> monocyte subset in the circulation of 17-Mb congenics compared to non-congenic controls.

(A) Flow cytometry analysis of blood monocytes after various periods of WTD feeding. Note that the 3-wk timepoint is pre-lesional. Monocytes were gated as CD45<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>hi</sup> or CD45<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>lo</sup> cells and representative scatter plots for each timepoint are shown. (B) Quantification of the percentages of Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> monocytes among total CD45<sup>+</sup>CD115<sup>+</sup> cells in control (b/b) and 17-Mb subcongenics. (C) Ratio of Ly6C<sup>hi</sup>:Ly6C<sup>lo</sup> monocyte subsets in the circulation after various periods of WTD feeding. Data are shown as mean ± SEM. *p ≤ 0.05, **p ≤ 0.005.
Heterozygous deletion of *Cdkn2a* in BM-derived cells results in accelerated atherosclerosis, expansion of the Ly6C<sup>hi</sup> monocyte pool, and increased BrdU incorporation into monocytes and macrophages. To directly test the hypothesis that decreased *Cdkn2a* expression in BM-derived cells is pro-atherogenic, we performed a BM transplantation study using a previously described *Cdkn2a*-deficient mouse. The targeted mutation replaces exons 2/3 with a neo cassette, knocking out both *p16<sup>INK4a</sup>* and *p19<sup>ARF</sup>* expression (190), and has been crossed into a uniform B6 background. In an effort to simulate the two- to four-fold reduction of *p16<sup>INK4a</sup>* and *p19<sup>ARF</sup>* expression observed in congenics compared to controls, we used donor B6-*Ldlr<sup>+/−</sup>* mice heterozygous for the *Cdkn2a* allele. Lethally irradiated B6-*Ldlr<sup>+/−</sup>* recipients were injected with BM derived from B6-*Ldlr<sup>+/−</sup>* or B6-*Ldlr<sup>−/−</sup>, *Cdkn2a<sup>+/−</sup>*, mice. Following repopulation of the BM, mice were fed 10-wk WTD. No differences were observed in body weight, plasma total cholesterol, HDL or triglycerides (Table 3-2).

**Table 3-2**  Body weight and plasma lipoprotein profiles of B6-*Ldlr<sup>+/−</sup>* mice receiving *Ldlr<sup>−/−</sup>* or *Ldlr<sup>+/−</sup>, Cdkn2a<sup>+/−</sup>* BM.

<table>
<thead>
<tr>
<th>BM donor genotype</th>
<th>BWT (g)</th>
<th>TC (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>Non-HDL-C (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>26.5±3.1</td>
<td>1230±339</td>
<td>83±22</td>
<td>445±12</td>
<td>1147±338</td>
</tr>
<tr>
<td><em>Ldlr&lt;sup&gt;+/−&lt;/sup&gt;, Cdkn2a&lt;sup&gt;+/−&lt;/sup&gt;</em></td>
<td>26.4±4.4</td>
<td>1363±229</td>
<td>82±14</td>
<td>424±11</td>
<td>1276±232</td>
</tr>
</tbody>
</table>

Mice were fed 10-wk WTD. Data are mean ± SD, n=17-19/group. Abbreviations: BWT, body weight; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; Non-HDL-C, non-HDL cholesterol.
Resident peritoneal macrophages exhibited a 2.5-fold decrease in expression of $p16^{\text{INK4a}}$ but not $p19^{\text{ARF}}$ or $p18^{\text{INK4c}}$ (another INK4-class gene encoded by a distant region of mouse chr 4) in B6-Ldlr$^{+/−}$, Cdkn2a$^{+/−}$ recipients compared to controls (Figure 3-4,B). Similarly, splenic CD11b$^+$ monocyte/macrophages exhibited ~4-fold decreases in both $p16^{\text{INK4a}}$ and $p19^{\text{ARF}}$ but not $p18^{\text{INK4c}}$ (Figure 3-4,C). Importantly, mean atherosclerotic lesion area was increased 34% and 17%, in males and females respectively, in B6-Ldlr$^{+/−}$, Cdkn2a$^{+/−}$ recipients compared to controls ($p < 0.05$ for combined data) (Figure 3-4, A). Thus, heterozygous deficiency of Cdkn2a in BM-derived cells is sufficient to confer an accelerated atherosclerosis phenotype in mice.
Heterozygous deficiency of Cdkn2a in BM-derived cells is sufficient to promote atherosclerosis in the B6-Ldlr<sup>−/−</sup> mouse model.

(A) Mean lesion areas from lethally-irradiated B6-Ldlr<sup>−/−</sup> mice injected with BM derived from B6-Ldr<sup>+/−</sup>, Cdkn2a<sup>+/−</sup> or B6-Ldr<sup>−/−</sup> mice. Following repopulation of the BM, mice were fed 10-wk WTD. ANOVA performed with square root transformation. Horizontal bars indicate mean values for males (circles) and females (triangles) for each genotypic group. (B-C) Transcript-specific rtPCR results for Cdkn2a (p16<sup>INK4a</sup> and p19<sup>ARF</sup>) and p18<sup>INK4c</sup> (another INK4-class gene encoded by a distant region of mouse chr 4) expression in (B) resident peritoneal macrophages or (C) splenic CD11b<sup>+</sup> monocyte/macrophages. ANOVA performed with log transformation. Data are shown as mean ± SD. *p ≤ 0.05.
We determined whether the increased lesion area in mice receiving Cdkn2a<sup>+/</sup> BM was associated with elevated levels of versican accumulation. Versican staining in mice receiving B6-Ldlr<sup>+/</sup>, Cdkn2a<sup>+/</sup> or control BM did not differ (Figure 3-5). We then investigated whether the pro-atherosclerotic mechanism involved apoptosis or monocytosis. TUNEL staining of atherosclerotic lesions revealed few positive cells and no difference between B6-Ldlr<sup>+/</sup>, Cdkn2a<sup>+/</sup> recipients compared to controls (data not shown). However, flow cytometry analysis of blood monocytes revealed an expansion of the Ly6C<sup>hi</sup> subset in B6-Ldlr<sup>+/</sup>, Cdkn2a<sup>+/</sup> recipients similar to that observed in the 17-Mb congenics. Increased percentages of Ly6C<sup>hi</sup> and decreased percentages of Ly6C<sup>lo</sup> subsets were observed in B6-Ldlr<sup>+/</sup>, Cdkn2a<sup>+/</sup> recipients compared to controls at each timepoint (0-10 wk WTD feeding) (Figure 3-6, A-B). This resulted in a significant increase in the ratio of Ly6C<sup>hi</sup>: Ly6C<sup>lo</sup> monocytes in the
circulation (Figure 3-6,C). To test for a direct effect of heterozygous Cdkn2a deficiency on monocyte proliferation, we injected mice with BrdU before sacrifice at the 10-wk timepoint. B6-Ldlr<sup>-/-</sup>, Cdkn2a<sup>+/+</sup> recipients exhibited an increase in the percentage of BrdU<sup>+</sup> monocytes compared to controls, indicating increased monocyte proliferation (Figure 3-6,D-E). Moreover, the increase in BrdU incorporation occurred mainly in the Ly6C<sup>hi</sup> subset (Figure 3-6,E left panel).

Figure 3-5 No difference in versican staining in mice receiving Cdkn2a<sup>++</sup> or Cdkn2a<sup>+/−</sup>BM. Prominent accumulation of versican was observed at the media: intima interface of plaques in some mice receiving either (A) Cdkn2a<sup>++</sup> or (B) Cdkn2a<sup>+/−</sup> BM.
Figure 3-6 Expansion of the inflammatory Ly6C<sup>hi</sup> monocyte subset, mediated by increased cell proliferation, in the circulation of B6-Ldlr<sup>−/−</sup> mice transplanted with Ldlr<sup>−/−</sup>, Cdkn2a<sup>−/−</sup> BM compared to controls.
(A) Flow cytometry analysis of blood monocytes from Ldlr<sup>+/−</sup>, Cdkn2a<sup>+/−</sup> or Ldlr<sup>+/−</sup> BM recipients after various periods of WTD feeding. (B) Monocytes were gated as CD45<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>hi</sup> or CD45<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>lo</sup> and representative scatter plots for each timepoint are shown. (C) Increased ratio of Ly6C<sup>hi</sup>: Ly6C<sup>lo</sup> monocytes in the circulation of B6-Ldlr<sup>−/−</sup> mice injected with BM derived from B6-Ldlr<sup>−/−</sup>, Cdkn2a<sup>+/−</sup> mice compared to controls injected with B6-Ldlr<sup>−/−</sup> BM. Blood was collected following 0-10 weeks of WTD feeding. (D) Quantification of the percentages of Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> cells among total CD45<sup>+</sup>CD115<sup>+</sup> cells. (E) Analysis of proliferating CD45<sup>+</sup>CD115<sup>+</sup> monocytes at the 10-wk timepoint. Cells were gated as Ly6C<sup>hi</sup>BrdU<sup>+</sup> and representative plots are shown. E, Quantification of the percentage of BrdU<sup>+</sup> cells among total CD45<sup>+</sup>CD115<sup>+</sup> monocytes. Data are shown as mean ± SEM. *p ≤ 0.05, **p ≤ 0.005, †p ≤ 0.0005.

To test for an effect of BM-derived Cdkn2a deficiency on tissue macrophage proliferation, we assayed concavalin A-elicited peritoneal macrophages by flow cytometry following intraperitoneal BrdU injection. While there was no difference in the total number of CD45<sup>+</sup>CD115<sup>+</sup>F4/80<sup>+</sup> macrophages (Figure 3-7, A), there was a significant increase in the percentage of macrophages staining positive for BrdU in B6-Ldlr<sup>−/−</sup>, Cdkn2a<sup>−/−</sup> mice compared to B6-Ldlr<sup>−/−</sup> controls (p < 0.04) and a trend in the 17-Mb congenics compared to controls (p = 0.1) (Figure 3-7, B-C). Together, these data provide direct evidence for a suppressive effect of p16<sup>INK4a</sup> and/or p19<sup>ARF</sup> expression in BM-derived cells on inflammatory monocyte/macrophage proliferation.
Figure 3-7 Increased proliferation of peritoneal macrophages derived from B6-Ldlr<sup>−/−</sup> mice transplanted with Ldlr<sup>−/−</sup>, Cdkn2a<sup>−/−</sup> BM compared to controls.

(A) Flow cytometry analysis of concavalin A-elicited macrophages from B6-Ldlr<sup>−/−</sup>, 17-Mb congenic, or B6-Ldlr<sup>−/−</sup>, Cdkn2a<sup>−/−</sup> mice after 4-5 wks of WTD feeding. Macrophages were gated as CD45<sup>+</sup>CD115<sup>+</sup>F4/80<sup>+</sup> and representative scatter plots for each genotypic group are shown. (B) Analysis of proliferating CD45<sup>+</sup>CD115<sup>+</sup>F4/80<sup>+</sup> macrophages at the 4-5 wk timepoint. Cells were gated as BrdU<sup>+</sup> and representative plots are shown. (C) Quantification of the percentage of BrdU<sup>+</sup> cells among total CD45<sup>+</sup>CD115<sup>+</sup>F4/80<sup>+</sup> macrophages. Data are shown as mean ± SEM. *p ≤ 0.05

Discussion

Prior to the identification of 9p21 as a risk locus for human CVD, we used an unbiased murine genetic approach to identify the homologous region of mouse chr 4 as
a modifier of atherosclerosis susceptibility in chapter two. In the current study, we show that Cdkn2a mediates some or all of the altered atherosclerosis susceptibility of this region through altered transcript expression in monocyte/macrophages. In support of this model, heterozygous deficiency of Cdkn2a transcripts in BM-derived cells was found to be sufficient to confer accelerated atherogenesis in the B6-Ldlr\(^{-/-}\) background. The modest (~2-4-fold) reduction in p16\(^{INK4a}\) and p19\(^{ARF}\) expression associated with increased atherosclerosis in these murine models is consistent with the reduction of p16\(^{INK4a}\) and p19\(^{ARF}\) expression in carriers of the 9p21 risk allele (104). Along with other data (104, 105, 166, 172), this strongly suggests that decreased expression of p16\(^{INK4a}\) and/or p19\(^{ARF}\) may be responsible for the accelerated atherosclerosis associated with chr 9p21 in humans. Moreover, our study strongly suggests that the underlying pathogenic mechanism involves increased proliferation/expansion of the Ly6C\(^{hi}\) inflammatory monocyte subset in the circulation as well as increased proliferation of tissue macrophages. This provides a plausible mechanism to account for accelerated atherogenesis and, possibly, other vascular phenotypes associated with 9p21 in humans (see below).

Consistent with our BM transplantation experiment, whole body p19\(^{ARF}\)- deficiency was recently shown to be pro-atherosclerotic in the B6-Apoe\(^{-/-}\) background (172). The mechanism of action suggested in the Apoe\(^{-/-}\) model was decreased apoptosis, although the culprit cell type was not identified. We did not observe differences in apoptosis in either the Athsq1congenic model or mice carrying BM-deficiency of Cdkn2a compared to respective controls. The discrepancy between the two studies could be due to different experimental designs (BM vs whole body deficiency) or mouse models (Ldlr\(^{-/-}\) vs Apoe\(^{-/-}\)). However, apoptosis leads to necrotic core formation and increased inflammatory state of
lesions, likely resulting in increased plaque vulnerability to rupture. Thus, while decreased apoptosis may accelerate early lesion formation, increased apoptosis likely contributes to lesion progression to advanced plaques with clinically significant consequences [191].

Evidence for an association between elevated WBC counts and CVD risk has been documented in more than a dozen prospective epidemiological studies [192]. In particular, high levels of circulating CD14⁺CD16⁻ inflammatory monocytes have been associated with hypercholesterolemia [36] and CAD events [39]. There is also substantial evidence in animal models for a causal relationship between monocytosis and atherogenesis [193-196]. In mouse models of diet-induced atherosclerosis, the subset of Ly6C⁺ expressing monocytes expands with hypercholesterolemia and selectively enters sites of inflammation including atherosclerotic lesions, compared to the Ly6C⁻ subset [54, 187]. In addition, single gene mutations resulting in reduced blood monocytes also reduced atherosclerosis independent of plasma cholesterol levels [197-200]. While the relationship between monocytosis and atherosclerosis has been well-documented, detailed mechanisms are lacking. Consistent with a recent finding [25], our study suggests that the control of myeloid cell proliferation during hypercholesterolemia is an important factor determining magnitude of leukocytosis and the atherogenic response.

Regulation of CDKN2A/B gene expression by the 9p21 CVD locus in humans is unknown. It has been suggested to occur via cis or trans mechanisms involving either the structurally overlapping non-coding RNA, ANRIL [106, 201], or other regulatory motifs residing within the 58-kb risk locus [108, 167]. The mouse genome does not contain a contiguous sequence with homology to ANRIL. However, markedly decreased
expression of Cdkn2a/b transcripts in the 70-kb deletion mutant suggests the existence of a cis-acting enhancer (166). Expression of CDKN2A/B transcripts are highly correlated in humans (106, 183). In our Athsq1 congenic mouse, there is a loss of coordinate regulation with decreased expression of Cdkn2a (p16^{INK4a} and p19^{ARF}) transcripts compared to non-congenic controls but not Cdkn2b (p15^{INK4b}). Thus, the causal variant is likely to be cis-acting and specific to the Cdkn2a locus.

The 9p21 locus has been associated with multiple vascular phenotypes. These include myocardial infarction (91), abdominal aortic and intracranial aneurysms (98), ischemic stroke (202, 203) and peripheral artery disease (204). In mice, mutations in monocyte/macrophage chemoattractants or their receptors, such as monocyte chemoattractant-1 (Ccl2 in mice; MCP1 in humans) and fractalkine receptor-1 (Cx3cr1), result in reduced blood monocyte counts and proportionate reductions in atherosclerosis (199). Interestingly, monocyte/macrophage recruitment is an important process in cerebral aneurysm formation and Ccl2 deficiency has been shown to inhibit macrophage accumulation in aneurysmal walls and significantly decrease aneurysm formation in an experimentally induced mouse model (205). In another study, increased immune-positivity for CD68 antigen was observed in intracranial aneurysms compared to control tissue (206). In ischemic stroke, macrophage accumulation has been associated with severity of brain injury (207). Thus, alterations in monocyte/macrophage proliferation may provide a common underlying mechanism for vascular phenotypes associated with the 9p21 locus.
Chapter 4. Conclusions and Discussion
The goal of this thesis was to fine map the \textit{Athsq1} locus and elucidate the mechanism(s) leading to atherosclerosis. Here we report refined mapping of the \textit{Athsq1} locus by creating a series of subcongenic strains and identify the existence of two susceptibility loci within the interval, with BM-derived cells shown to play an important role for the accelerated atherosclerosis. The proximal interval was further narrowed to a genomic region containing 8-21 genes, including the region of homology with a human CHD risk interval on chr 9p21, with \textit{Cdkn2a} as a candidate locus within the interval. Interestingly, macrophages exhibited markedly decreased \textit{Cdkn2a} (\textit{p16}^{\text{INK4a}} and \textit{p19}^{\text{ARF}}) expression in congenic mice compared to controls. Gene-targeted knockout alleles of tumor suppressor/cell cycle regulation genes (\textit{p16}^{\text{INK4a}} and \textit{p19}^{\text{ARF}}) in BM-derived cells lead to increased atherosclerosis, monocyte/macrophage proliferation and expansion of Ly6C\textsuperscript{hi} inflammatory monocytes. These findings provide a potential mechanism to explain the most consistent and striking new genetic marker for arterial disease risk emerging from GWASs.

Mapping susceptible loci underlying atherosclerosis with the identification of BM-derived cells playing an important role was the main focus in chapter 2. Fine mapping of the \textit{Athsq1} interval using subcongenic strains revealed a distal locus showing proatherogenic activity during early atherosclerosis development (6-wk WTD) and a proximal locus conferring accelerated atherosclerosis at a later timepoint (9-wk WTD). The identification of two susceptibility loci under one linkage peak is not-unusual. Typical linkage peaks are very large and contains hundreds of genes. For example, a locus affecting seizure susceptibility in mice composed of multiple loci each having small effects (4). Another example was the identification of \textit{Alox5} in the congenic interval (124) having two loci each with a large effect on atherosclerotic lesion development (123).
Thus, the mapping of disease susceptibility locus should be performed with caution as multiple alleles would either work together to increase the phenotype or cancel out opposing effects.

Animal models are important tools for biomedical research because their environmental exposure can be easily controlled and manipulated. The finding of loci acting at different phases during disease development stresses the utility of mouse models to define the role of genes in disease etiology, as this can be examined by time course experiments with the characterization of specific markers or morphological features during different phases of the disease. Similarly, mouse models enable us to define culprit cells types involved in the pathogenesis of the disease. For instance, reciprocal BM transplantation experiments revealed that BM-derived cells are responsible for the atherosclerotic phenotype observed in the 17-Mb congenics. Thus, mouse models facilitate our understanding of the pathophysiology underlying diseases.

The panel of subcongenic strains enabled the refinement of the locus to a region that contains 8-21 genes including the region of homology with the highly replicated human CHD locus on chr9p21. The narrowing of the susceptibility locus in our congenic strain was based on the inclusion of pro-atherosclerotic 11-Mb interval and exclusion of the 4-Mb interval that does not have an effect on atherosclerosis. However, exclusion of the 4-Mb proximal tip as a causal candidate region alone does not rule out the possibility of a more complex genetic architecture. Epistasis, as defined by genetic interaction of the genotype at one locus that affects phenotypic expression determined by another locus, has been documented in several disease states (208-211). An example of synergism between loci has been demonstrated in a mouse model of systemic lupus erythematosus (212). When two susceptibility alleles are present in one genome, a fatal
systemic autoimmunity develops while each allele alone fails to confer severe autoimmunity (212). These data suggest that epistasis between two susceptibility alleles leads to a greater incidence in disease severity than individual loci. Therefore, the 4-Mb congenic might need in conjunction with some other distant locus/loci to confer the atherosclerosis phenotype.

We have shown that the mice deficient in Cdkn2a (p16\textsuperscript{INK4a} and p19\textsuperscript{ARF}) results in increased atherosclerosis and is associated with increased proliferation of monocytes and expansion of Ly6C\textsuperscript{hi} inflammatory subset. The proteins encoded from the Cdkn2a/b (p16\textsuperscript{INK4a}, p19\textsuperscript{ARF} and p15\textsuperscript{INK4b}) locus are cell cycle inhibitors and have been investigated actively and intensively in the field of cancer. On the other hand, recent studies have shed light of understanding genes from this locus and its relationship to CHD (104, 166, 167, 172). Perhaps atherosclerosis and cancer can be viewed as similar diseases having some common molecular pathways leading to disease development and progression. Macrophages play a key role in the pathogenesis of both atherosclerosis and cancer. In cancer, they are directly involved in tumor progression and metastasis (213). Macrophages are found at regions of basement-membrane breakdown during the transition from malignant to advanced tumors (213). The secretion of matrix metalloproteinases (MMPs) allows the invasion of tumor cells into the basement membrane (214). In atherosclerosis, vulnerable plaques and thrombosis have been linked to MMP secretion by macrophages. Thus, matrix remodeling is an example of a common pathway shared by both diseases. Decreased expression of p16\textsuperscript{INK4a} in human lung cancer cell lines and glioma has been associated with increased MMP secretion (215, 216). Although we have not examined MMP secretion in the Cdkn2a\textsuperscript{+/-} (p16\textsuperscript{INK4a}}
and $p19^{ARF}$) mice, it is possible that accelerated atherosclerosis phenotype observed in these mice might be due to increased MMP secretion.

Progressive angiogenesis is another common process in cancer and atherosclerosis. Angiogenesis in human atherosclerotic lesions has been associated with plaque expansion and vulnerability, as a result plaque rupture and thrombosis occurs (217). Tumor angiogenesis is associated with several malignancies such as breast cancer and prostate cancer. New blood vessel formation promotes the transition from hyperplasia (cellular multiplication) to neoplasia (uncontrolled proliferative state) (218). Neovascularization also facilitates tumor cell extravasation (exit from vessels) and metastasis to other organs. Perhaps loss of function of $p16^{NK4a}$ or $p19^{ARF}$ in the hematopoietic cells modulating angiogenesis in cancer (219, 220) may be one pathway of promoting atherosclerotic plaque development. Angiogenesis is not a common feature of mouse atherosclerotic plaques but can be observed in long term cholesterol-fed mice (111). This would be a great model to test mice deficient in $p16^{NK4a}$ and $p19^{ARF}$ exacerbates atheromas via promoting angiogenesis.

A number of genes involved in cell cycle regulation have been shown to play a role in atherosclerosis. Various cell cycle regulators, including transcriptional regulators $p53$ (221-224), retinoblastoma ($Rb$) (225), and the Cip/Kip family members, $p27$ (226-229) and $p21$ (230, 231) have been assessed its role in atherosclerosis by using genetically-altered animal models. There are evidence suggest that $p53$ plays a role in atherosclerosis development. Guevara et al. (221) first reported that Apoe-null mice with global inactivation of $p53$ exhibit accelerated atheroma development in the aorta with increased cell proliferation. Subsequent studies demonstrated a role for hematopoietic cells in conferring the accelerated atherosclerosis (222, 223) but the mechanisms was
not identified. Apoptosis was ruled out as a pathogenic mechanism in this model (224). Thus it is not clear whether p53 deficiency leads to atherosclerosis via proliferation or apoptosis. Inactivation of Rb in macrophages of Apoe-null mice leads to a 51% increase in atherosclerotic lesion area, with a 2.6 fold increase in lesional macrophage proliferation, compare to Apoe-null controls (225). p27 has been shown to be athero-protective via inhibiting macrophage and SMC proliferation (227, 228). On the other hand, another cell cycle inhibitor, p21, hsa been shown to confer athero-protection (231) or atherosclerosis susceptibility (230) in various studies. Thus, our findings of increased atherosclerosis in Ldlr-null mice with haplo-insufficiency of p16\textsuperscript{NK4a} and p19\textsuperscript{ARF} is consistent with studies in the literature regarding a role for regulators of cell proliferation in atherogenesis.

The plethora of genes/variants identified through GWASs has been fruitful. However, we are currently at the early stage of understanding the biological basis of these variants. Mouse models have been informative to understand genes identified by GWASs and its underlying mechanisms for metabolic syndromes (139, 166, 167, 172, 181, 182), with the goal of their clinical usage in the near future. This project started with an unbiased approach (157, 162) to identify a locus on mouse chr 4 that confers susceptibility for atherosclerosis. The thesis further focused on a region that contains the region of homology with the human CHD locus on chr 9p21. Here, we provide a link between accelerated atherosclerosis, in which the mechanism of action is through the elevation of inflammatory monocytes. Complementing the well known risk factors such as plasma lipid levels and the establishment of C-reactive protein as an inflammatory marker, this will help propel us to an era in which the biology of atherosclerosis translates into clinical application.
Chapter 5. Future Directions
Many studies have revealed the importance of different monocyte subsets in association with CHD related complications (33-40, 159). Monocytosis has been shown to be an independent risk factor for CHD (33, 34). The CD14+CD16− subset has been shown to correlate with risk factors and family history in patients with stable CHD (35), levels of LDL and lipoprotein(a) (36) and correlate inversely to left ventricular recovery after MI (37). On the other hand, CD14+CD16+ monocyte counts have been associated with uptake of oxLDL (38) and increased cardiovascular events (39, 40). Chronic kidney patients have an increased risk of CVD events, and this is associated with increased levels of CD14+CD16+ (159). There is no clear homolog of monocyte subsets between mouse and human (32). In our study, we have found increased proliferation of monocytes and levels of Ly6C^hi subset with mice deficient in Cdkn2a. Given the clear role monocytosis plays in the development of atherosclerosis in the clinical setting, it remains important to determine if chr 9p21 risk allele carriers in humans have increases in specific monocyte subsets. In collaboration with Dr. Angali Ganda, we are trying to see whether 9p21 risk allele carriers with chronic kidney disease have increased levels of certain monocyte subsets. In addition, it would be interesting to see whether there is increased ex vivo proliferative activity of the human monocyte subsets.

To test whether there is increased proliferative activity of monocyte/macrophages in atherosclerotic plaques derived from Cdkn2a-deficient mice, we examined lesions from a small cohort of B6-Ldlr−/−, Cdkn2a+/− mice compared to controls by examining BrdU incorporation within lesions using immunohistochemistry. While ample numbers of cells (mean = 42-47 cells) stained positively following a 7-day pulse, the within-group variation was high and power calculations indicated that we would need to study 50 mice/group to have an 80% chance of detecting a significant difference (p< 0.05) comparable in
magnitude to the difference in lesion area (i.e. ~30%). Therefore in order to achieve a significant biological effect, we would use homozygous Cdkn2a\(^{-/-}\) compared to Cdkn2a\(^{+/+}\) mice (on a B6-Ldlr\(^{-/-}\) background) to examine the proliferative activity within the atherosclerotic lesions.

In our study, we have found that deficiency of Cdkn2a leads to atherosclerosis via the action of BM-derived cells. To test this hypothesis that the expression levels of Cdkn2a in BM affects atherosclerosis development post-transplantionally in humans, it would be intriguing to see whether patients receiving BM transplantation have an increased risk of developing CHD dependent on donor genotype at the the 9p21 risk allele. To test the genotypic effect of the host’s environment to CHD development, including vascular wall cells of the recipient, hormonal effects of the host, it is possible to genotype the recipients for the 9p21 risk allele and see whether recipient with the risk allele is associated with exacerbated CHD development.

Rheumatoid arthritis (RA) is associated with an increased risk for CVD events, such as MI and stroke (232). Developing rheumatoid arthritis is 50% attributable to genetic factors (233). Systemic inflammation has played a central role in driving the increased CHD risk in RA patients (233). Many risk alleles associated with RA has been identified by GWASs (234), with the associated SNPs located near genes with known immune function. Interestingly, many associations are located near genes that encode proteins that are important for T-cell function, activation and responsiveness (234). In our studies, we found an increased level of inflammatory monocyte subset in a mouse model simulating 9p21 risk allele carriers in humans. The expression levels of Cdkn2a in relationship to RA and CHD is not understood. To understand this relationship, this could be done by genotyping for the 9p21 risk allele in the RA patients and perform a
retrospective or prospective study to see whether 9p21 risk allele carriers in RA patients have increased risk of developing CHD.

The 9p21 CHD risk locus is associated with abdominal and intracranial aneurysms (98) and ischemic stroke (202, 203). Aneurysmal diseases arise in the setting of atherosclerosis (235). The main histological feature of aneurysm is chronic medial degradation, which is mainly caused by proteases (matrix metalloproteinases, serine proteases and cysteine proteases) secreted by macrophages and SMCs (236). In ischemic stroke, macrophage accumulation has been associated with severity of brain injury (206) and that the deficiency of MCP-1 (monocyte chemoattractant protein-1) in mice leads to decreased cerebral aneurysm formation and macrophage accumulation. Thus, it would be interesting to examine whether Cdkn2a\(^{-}\) mice have any morphological features of aneurysm and stroke in the vessel wall, including medial degradation and increased macrophage accumulation, and whether these mice secrete more proteases in the arterial wall.

In this thesis, we have demonstrated a mechanistic link between decreased Cdkn2a expression, increased monocytes/macrophages proliferation with the expansion of an inflammatory monocyte subset and increased atherosclerosis. Together, these data illustrate the feasibility of mouse models as tools to elucidate causal gene(s)/loci and potential pathogenic mechanisms underlying genome-wide association studies-identified loci for human disease.


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