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Genetic Insights Into Smooth Muscle Cell Contributions to Coronary Artery Disease

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Abstract

Coronary artery disease is a complex cardiovascular disease involving an interplay of genetic and environmental influences over a lifetime. Although considerable progress has been made in understanding lifestyle risk factors, genetic factors identified from genome-wide association studies may capture additional hidden risk undetected by traditional clinical tests. These genetic discoveries have highlighted many candidate genes and pathways dysregulated in the vessel wall, including those involving smooth muscle cell phenotypic modulation and injury responses. Here, we summarize experimental evidence for a few genome-wide significant loci supporting their roles in smooth muscle cell biology and disease. We also discuss molecular quantitative trait locus mapping as a powerful discovery and fine-mapping approach applied to smooth muscle cell and coronary artery disease-relevant tissues. We emphasize the critical need for alternative genetic strategies, including cis/trans-regulatory network analysis, genome editing, and perturbations, as well as single-cell sequencing in smooth muscle cell tissues and model organisms, under both normal and disease states. By integrating multiple experimental and analytical modalities, these multidimensional datasets should improve the interpretation of coronary artery disease genome-wide association studies and molecular quantitative trait locus signals and inform candidate targets for therapeutic intervention or risk prediction.

Keywords

coronary artery disease; gene editing; genome-wide association study; quantitative trait loci; risk factors; vascular smooth muscle cells

Coronary artery disease (CAD) remains the leading cause of mortality worldwide.[1] Despite advances in medical treatments and prevention strategies, there has been an increased prevalence in developing countries.[2,3] CAD is caused by a chronic accumulation of cholesterol deposits and inflammation in the blood vessel wall and often manifests in myocardial infarction (MI).[4] It is now well established that an array of interacting genetic

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Disclosures

None.

and environmental risk factors contribute equally to disease susceptibility.[5,6] Since the discovery of the first genetic association with CAD located at chromosome 9p21, genome-wide association studies (GWAS) have identified over 160 individual loci for CAD/MI.[7–9] Many of these loci harbor genes that are organized into discrete signaling pathways, such as lipoprotein metabolism, NO/cGMP (cyclic guanosine monophosphate), TGFB (transforming growth factor β), PDGF (platelet derived growth factor), extracellular matrix, inflammation, etc[8,10,11] Although it remains to be determined whether these genes are causally associated with CAD, many are already supported by in vitro or in vivo experimental evidence.[12–19] There still remain a significant number of disease loci containing genes with little or no relevant functional annotations, suggesting these loci may highlight novel disease processes.

Multiple cell types have been linked to CAD pathogenesis, including smooth muscle cells (SMCs), endothelial cells, and macrophages. Thus, it seems likely that CAD-associated loci also operate through cell type–dependent regulatory pathways. Given that the majority of associated loci involve noncoding genetic variants predicted to affect gene expression, large-scale genomic consortia, such as Genotype-Tissue Expression (GTEx),[20] Encyclopedia of DNA Elements,[21] and Roadmap Epigenomics,[22] have been critical to map these regulatory profiles across different human tissues. Still, significant gaps exist for which there are no regulatory profiles for specific cell types related to coronary atherosclerosis. We and others have developed cell-specific genomic resources to enable fine-mapping of CAD GWAS loci in the appropriate context.[23–25] Whereas many of these datasets involve bulk sequencing, more recent efforts have established single-cell approaches to identify novel cell types that may contribute to the disease.[26] Also, it is now appreciated that specific environmental contexts should be accounted for when developing these cell type–specific genomic datasets.[27] In this brief review, we discuss the contribution of smooth muscle cells (SMCs) towards CAD risk, as informed by recent genetic discoveries (Figure 1 and Figure 2A). We highlight a few loci (eg, 9p21, *TCF21*, *SMAD3*, *LMOD1*) with predicted functional roles in SMC. We also discuss methods to discover SMC mediated genetic mechanisms through quantitative trait loci (QTL) mapping (Figure 2B). Finally, we briefly discuss alternative approaches to prioritize SMC contributions to disease including CRISPR perturbations, network biology, and single-cell omics (Figure 2C through 2E). Together these methods may refine the role of SMC and intermediate cell types in relevant vessel wall signaling pathways and phenotypic transitions during CAD.

CAD GWAS Loci Harboring SMC Candidate Genes

9p21.3 (CDKN2B-AS1/CDKN2A/CDKN2B)

The first genetic locus associated with CAD risk was identified at chromosome 9p21.3 in 2007 by 3 independent groups.[39–41] Despite the highly reproducible nature of this locus association with CAD, MI, and other conditions,[42,43] the causal genes, variants, and underlying mechanisms in this region are still elusive. Adding to the intrigue, this locus is unlikely to be driven by traditional CAD risk factors, suggesting it encodes for hidden risk factors yet to be exploited for disease intervention. This locus contains many single nucleotide polymorphisms (SNPs) in high linkage disequilibrium located within the long noncoding RNA, *ANRIL* (antisense noncoding RNA in the *INK4* locus

or *CDKN2B-AS1*), and multiple nearby genes encoding the tumor suppressors CDKN2A (cyclic-dependent kinase inhibitor 2A), CDKN2B (cyclic-dependent kinase inhibitor 2B), and MTAP (methylthioadenosine phosphorylase).[44] Several of the disease-associated SNPs located in the 3' region of ANRIL correlate with ANRIL expression but also nearby genes, *CDKN2A*, and *CDKN2B*.[44] Like many long noncoding RNA transcripts, *ANRIL* is capable of forming both linear and circular RNA.[45] Holdt et al demonstrated that linear *ANRIL* confers proatherogenic cell functions through *Alu* element–mediated transregulatory networks.[46,47] However, circular *ANRIL* inhibits atherogenic processes in SMC and macrophages by inducing nucleolar stress, p53 activation, and impairing ribosome biogenesis.[48] The lead risk allele at 9p21 was correlated with reduced *CDKN2B* expression and increased cell cycle regulation in whole blood.[44,46] Consistent with this mechanism, the CAD risk allele was associated with reduced *CDKN2A* and *CDKN2B* expression in vascular SMCs, and downregulated *CDKN2B* correlated with increased SMC proliferation and SMC content in atherosclerotic plaques.[12] Loss of *CDKN2B* in mice has been linked to p53 mediated SMC apoptosis and aortic aneurysms,[49] increased atherosclerosis through impaired efferocytosis,[13] as well as hypoxic neovessel formation through dysregulated TGFB signaling.[50] More recently, TALEN (transcription activator-like effector nuclease)-based genome editing of 9p21 risk haplotypes (including *ANRIL*) in induced pluripotent stem cell (iPSC)–derived SMCs revealed altered transcriptional networks associated with CAD genes and SMC functions.[51] Taken together, these studies highlight the complex and diverse roles of *ANRIL* and *CDKN2B*/*CDKN2A* in regulating SMC proliferation and related phenotypes during atherosclerosis.

6q23.2 (TCF21/TARID)

TCF21 (also known as Pod1/Epicardin/Capsulin) was first identified as a candidate causal gene for CAD-associated at chromosome 6q23.2 (P<1.1E-12).[52] TCF21 (transcription factor 21) is a basic helix-loop-helix TF (transcription factor) involved in the development of the coronary vasculature.[53,54] Two lead SNPs, rs12190287 and rs12524865, identified in Europeans and East Asians, respectively, were shown to regulate PDGFRB (platelet-derived growth factor receptor beta) mediated TCF21 expression in human coronary artery SMC (HCASMC) through 2 different AP-1 (activator protein 1) binding sites.[14] Interestingly, the protective allele of the 3'UTR (3' untranslated region) SNP, rs12190287, also perturbs a miR-224 (microRNA-224) seed binding site thereby stabilizing the *TCF21* mRNA transcript.[15] These 2 mechanisms are predicted to work in concert to fine-tune TCF21 levels. Chromatin immunoprecipitation (ChIP)-seq of TCF21 protein demonstrated genome-wide binding sites in genes related to cell migration and proliferation, which overlapped those of the promitogenic AP-1 TF family.[16] Interestingly, TCF21 target genes are highly enriched for CAD GWAS variants in high linkage disequilibrium with SNPs in TCF21 binding sites, suggesting that TCF21 acts as a master regulator of other CAD-associated genes.[16] RNA-seq of TCF21 knockdown in HCASMC revealed consistent target genes and implicated TCF21 in the suppression of SMC contractile markers and promotion of proliferation and migration.[17] Lineage tracing of TCF21 in mouse arterial lesions and human atherosclerotic samples revealed staining localized to the subcapsular space during late-stage disease suggesting a role in stabilizing the fibrous cap.[17] Recent CAD GWAS from the UK Biobank identified the lead SNP rs2327429 residing 0.5 kb 5′ of TCF21,

also located within the first exon of antisense long noncoding RNA, known as TARID (TCF21 antisense RNA inducing promoter demethylation). This variant was identified as an expression QTL (eQTL) for both *TCF21* and *TARID* in artery tissues in GTEx,[20] STARNET (Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task),[55] and our HCASMC dataset,[23] suggesting coordinated regulation associated with CAD.[56] Given the molecular interaction of TARID with the TCF21 promoter, this could potentially be exploited to deliver TARID (eg, via extracellular nanovesicles) to elevate TCF21 and promote plaque stability. Thus, future studies to directly modulate *TARID* in SMCs and CAD-related disease models are warranted.

15q22.33 (SMAD3)

As a pleiotropic signaling pathway involved in both coronary artery development and disease responses, the TGFB superfamily typically represses SMC migration, proliferation, and promotes extracellular matrix production.[57,58] Both the canonical TGFB ligand and downstream mediators of this pathway are highly enriched among CAD GWAS loci.[59–61] One of these mediators, *SMAD3* (mothers against decapentaplegic homolog 3) is hypothesized to be the causal gene at the CAD locus 15q22.33 based on epigenomic profiling and eQTL mapping in relevant tissues.[18,24] The risk allele of candidate causal variant rs17293632 forms an AP-1 TF binding site, while the protective allele disrupts this site, resulting in reduced *SMAD3* expression under PDGF-BB and TGFB stimulation in SMC.[18,24] Loss of SMAD3 protein in HCASMC increased proliferation and reduced expression of SMC contractile markers.[62] As a positive modulator of SMC differentiation, SMAD3 and TCF21 regulate opposing downstream transcriptional pathways.[62] ChIP-seq analyses of both factors demonstrated similar target genes with limited overlap in their binding sites, suggesting a model in which the TCF21 mediated recruitment of HDACs (histone deacetylases) prevent the binding of Smad3.[62,63] Consistent with the antimitogenic role of SMAD3 in vitro, in vivo experiments utilizing a femoral artery injury model showed loss of Smad3 increased proliferation and reduced collagen synthesis following vascular injury.[64]

1q32.1 (LMOD1)

LMOD1 (leimodin 1) was one of the recent vascular wall loci associated with CAD (P=7.77E-10).[65] As a member of tropomodulin similar leiomodin family, LMOD1 functions as an actin filament nucleator that is highly enriched in smooth muscle containing visceral organs and arteries.[66,67] Loss of *Lmod1* in mice recapitulated a rare congenital disorder known as human megacystis microcolon intestinal hypoperistalsis syndrome, characterized by defective intestinal and bladder function.[67] *Lmod1* global knockout reduces filamentous actin content and impairs contraction in visceral SMCs.[67] Expression of *LMOD1* is regulated by the SRF (serum response factor)/myocardin complex with 2 canonical CArG box located in its promoter region.[66] Epigenomic, transcriptomic, and genetic fine-mapping studies pinpointed *LMOD1* as the causal gene and identified a mechanism for a candidate causal CAD variant at this locus.[19,24] The risk variant downregulates *LMOD1* expression in atherosclerotic carotid artery tissue, as well as normal tibial arteries by perturbing a FOXO3 (forkhead box O3) binding site.[19] Knockdown of *LMOD1* in HCASMC increased proliferation and migration while inhibiting contraction.[19] Complementary ex

vivo immunohistochemistry staining of carotid atherosclerotic lesions reveal LMOD1 coexpression with SMC contractile markers, ACTA2 (smooth muscle alpha-2 actin), MYH11 (myosin heavy chain 11), CNN1 (calponin 1), and MYOCD (myocardin) and potently downregulated in response to atherogenic stimuli, shear stress, and carotid artery injury.[68] In vivo data utilizing lineage traced mice localized LMOD1 expression to the fibrous cap with limited staining in the synthetic SMCs within the lesion.[19] Taken together, these studies support a role of LMOD1 in maintaining the contractile SMC phenotype.

Quantitative Trait Mapping to Prioritize SMC Mechanisms

Expression and Splicing QTL in Atherosclerotic Tissues and SMC

Although CAD GWAS have revealed tremendous insights into vessel wall biology, it has been challenging to define causal genes and mechanisms at many of these putative vascular loci. One powerful strategy to refine these candidate genes and mechanisms involves molecular quantitative trait locus mapping, such as eQTL (Figure 2B). By correlating genetic variation with gene expression as an intermediate phenotype, it is possible to prioritize biologically plausible target genes from these associations.[69–71] The GTEx project has provided large-scale mapping of eQTLs across 53 human tissues in an effort to define tissue-specific and broad insights into gene regulation.[20,72] However, the majority of the samples profiled in GTEx have been heterogeneous, bulk tissues from nondiseased individuals making it difficult to delineate cell-type specific CAD regulatory mechanisms. The STARNET study is another large-scale study,[55,73] which completed RNA-seq (RNA-sequencing) based eQTL analysis of 7 cardiometabolic tissues (atherosclerotic-lesion-free internal mammary artery, atherosclerotic aortic root, blood, subcutaneous fat, visceral abdominal fat, skeletal muscle, and liver) from ≈600 individuals undergoing coronary artery bypass graft procedures (Table). This dataset identified 2047 cardiometabolic GWAS SNPs with corresponding cis-eQTLs (≈61%; cis-eQTLs alter gene expression of nearby genes, while trans-eQTLs affect expression of distant genes, often located on different chromosomes). This is several fold more than the original GTEx dataset, emphasizing that not all disease regulatory variants can be detected in healthy tissues. Importantly this dataset was used to provide functional evidence for top candidate CAD regulatory variants identified from epigenomic profiling in HCASMC and coronary artery tissues.[24] Other groups have integrated these datasets with GWAS for multiple common diseases using summary-based Mendelian randomization to reveal general insights into gene-trait associations.[80] Additional disease-relevant cis-eQTL datasets have been generated from various cohorts, including STAGE (Stockholm Atherosclerosis Gene Expression),[75] ASAP (Advanced Study of Aortic Pathology),[77] and BiKE (Biobank of Karolinska Carotid Endarterectomies).[76] These cohorts include both nonatherosclerotic and atherosclerotic bulk artery tissues, whereas the ASAP cohort includes additional datasets for medial and adventitial layers of the ascending aorta. Other omics data are available for these cohorts (eg, plasma proteomics); however, it is worth noting that their clinical utility could be limited to European populations (Table).

Recently, we completed eQTL mapping of HCASMC by profiling RNA-seq and whole genomes in 52 unrelated, multiethnic donors[23] (Table). Despite the relatively small sample

size, we leveraged allele-specific expression to discover 1220 significant loci, which were enriched in regions of open chromatin in HCASMC.[23] Additionally, we identified 582 splicing QTLs, which may explain additional regulatory effects of genetic variants.[81] Colocalization of eQTL and CAD GWAS signals using 2 methods revealed 5 significant loci, including *FES*, *SMAD3*, *TCF21*, *PDGFRA*, and *SIPA1*. In this study, we also quantitated the contribution of HCASMC to CAD risk and provided evidence that these cells capture a large portion of CAD heritability.[23] Overall, these findings highlighted CAD risk genes related to vascular remodeling and phenotypic modulation in HCASMC. These data suggest that genetic upregulation of SMC genes (eg, *SMAD3*, *PDGFRA*, and *SIPA1*) may increase CAD risk, whereas upregulation of other SMC genes (eg, *TCF21* and *FES*) may be atheroprotective. Although eQTL data from other coronary artery cell types (eg, endothelial cells, macrophages) are not available, human aortic endothelial cells (HAEC) eQTLs from 147 transplant donors were previously used to identify 7 candidate causal CAD genes, including *PPAP2B*, *GALNT4*, *MAPKAPK5*, *TCTN1*, *SRR*, *SNF8*, and *ICAM1*.[78] Similarly, a collection of eQTL datasets from the GRASP (Genome-Wide Repository of Associations Between SNPs and Phenotypes),[82] STAGE study,[83] Massachusetts General Hospital liver/adipose study,[84] Cardiogenics consortium monocytes/macrophages study,[85] and HAEC[78] were used to identify 66 candidate CAD genes from 159 lead CAD SNPs.[86]

Additional gene regulatory mechanisms may be captured by profiling cells treated with disease-relevant environmental perturbations. While such studies have yet to be conducted in SMCs, gene x environment eQTLs have been identified in a cohort of 96 transplant donor HAEC treated with the proatherogenic oxidized phospholipid species, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine.[25,78,87] This dataset identified significant gene x environment eQTLs for about a third of the top 59 regulated transcripts, most of which were distal or trans-acting, but some of the strongest effects observed were cis-acting (eg, *FGD6*), suggesting that certain QTLs may be driven through specific response elements.[87] Similar perturbation studies in a study of primary monocytes from 432 donors stimulated with interferon-γ or lipopolysaccharide identified response QTLs in more than half of all cis-eQTLs and revealed multiple master regulatory trans-eQTLs, such as the major histocompatibility complex and resolved putative causal genes at GWAS loci related to innate immune responses (eg, *CARD9*, *ATM*, and *IRF8*).[88] Another study of 134 monocytes exposed to lipopolysaccharide or microbe-associated molecules identified 417 response QTLs across the different conditions.[89] Both of these monocyte studies demonstrated stronger enrichment of immune-related GWAS signals in response QTLs compared with constant eQTLs. Together, these studies emphasize that regulatory effects of genetic variants associated with complex diseases are likely modified by specific contextual stimuli related to disease pathogenesis.

Chromatin Accessibility and TF Binding QTL in SMC

Given that the majority of genetic variation discovered through GWAS reside in noncoding regions, they are predicted to regulate gene expression through specific regulatory elements. Thus chromatin accessibility QTLs (caQTLs) may provide an additional layer of regulation to interpret these signals and could more directly explain cis-acting mechanisms. To date, there have not been any large caQTL studies in SMC; however, there have been a number

of such studies in lymphoblastoid cell lines.[81,90–92] Importantly, these studies demonstrated that 56% of candidate causal GWAS regulatory SNPs reside within the region of open chromatin itself.[90] This is consistent with findings that 60% of all regulatory variants in these cells alter chromatin level phenotypes.[81] Although initial studies mapped chromatin accessible sites using DNase I hypersensitivity sequencing,[93–95] more recent efforts have leveraged the Tn5 transposase based assay for transposase accessible chromatin (ATAC)-seq,[96] which requires a fraction of the starting material (≈50 000 cells) and time (≈3 h) to prepare libraries for sequencing, compared with previous approaches. We recently used ATAC-seq in growth factor (TGFB, PDGF-BB, PDGF-DD) stimulated HCASMC and frozen human coronary artery segments to map candidate regulatory variants and mechanisms for CAD loci.[24] With the reduced search space (≈200–300 bp open chromatin region) and single-nucleotide resolution of this assay, we were able to prioritize 64 CAD-associated regulatory variants and provided experimental follow-up and candidate TF binding mechanisms (eg, TCF21 and AP-1) for 7 variants in these loci (*SMAD3*, 9p21.3, *PDGFD*, *CCDC97*/*TGFB1*, *LMOD1*, *IL6R*, and *BMP1*).[24]

Similar ATAC-seq studies have been conducted in 56 HAEC donors to elucidate the function of an intronic variant, rs17114036, in the *PLPP3* CAD and ischemic stroke locus, encoding phospholipid phosphatase 3 (also known as PPAP2B [phosphatidic acid phosphatase type 2B]).[79,97] This study used the combined haplotype test,[98,99] to perform caQTL mapping by comparing heterozygous and homozygous individuals at rs17114036. ATAC-seq signals spanning this region were strongly correlated with *PLPP3* expression in HAEC, supporting a potential role as an enhancer of *PLPP3*. These authors provide additional evidence using luciferase reporter, ChIP, and CRISPR/Cas9 editing assays and demonstrate regulatory effects on PLPP3 through mechanosensing KLF2 (Kruppel-like factor 2) in response to unidirectional flow.[79] caQTL mapping represents a powerful epigenomic approach to fine-map regulatory variants associated with CAD and other complex traits. However, additional assays, such as ChIP-seq, may be necessary to pinpoint true functional variants that alter TF binding.[100]

Other Molecular QTL Discoveries in SMC

To efficiently capture long-range interactions between enhancers and promoters, improved chromosome conformation capture (3C) or HiChIP (high-throughput chromatin immunoprecipitation) based assays such as HiChIP[101] and Promoter Capture HiC[101,102]) have been developed. HiChIP was recently used in HCASMC using H3K27ac histone modification as bait to reveal genome-wide insights into the connectome in these cells.[103] This study identified a number of enhancer-promoter interactions that may be influenced by CAD-associated genetic variants (eg, 9p21). Promoter capture HiC was recently used to identify interactions at 31 253 promoters in 17 different primary blood cell types.[102] With the higher resolution of this approach, the distal impact of regulatory elements can be investigated in primary SMC to elucidate the effects of noncoding CAD regulatory variants. Other molecular QTLs have been discovered in blood plasma that have been associated with CAD. For instance, a large study of 6861 Framingham Heart Study participants identified 16 000 plasma protein QTLs of which a subset was tested for causal links to CAD.[104] Another study identified 52 916 cis-methylation QTL and 2025 transmethylation QTL in whole

blood with significant enrichment for CAD.[105] More relevant to CAD pathology, the large Athero-Express Genomics study (N=1439), which has methylation, SNP genotyping, and histopathology data (Table), identified a number of variants associated with atherosclerotic plaque phenotypes, including the *ALOX5AP* locus associated with intraplaque vessel density and SMC content.[106] More recent findings from this cohort identified 21 variants nominally associated with 7 histopathologic characteristics, demonstrating the impact of risk variants in a heterogeneous disease environment.[74] Finally, as described above for eQTL associations, molecular QTL discoveries may be expanded by investigating the impact of genetic variants in more tightly controlled environments. A study in 17 human umbilical vein endothelial cell donors identified eQTLs, caQTLs, and candidate response elements associated with specific perturbations in these cells.[27] Interestingly, some exposures were shown to amplify the underlying genetic effects, whereas others were buffered, pointing to the complex nature of these interactions on molecular phenotypes.

Other Approaches to Prioritize SMC Contributions to CAD

Network Analysis

Several studies have leveraged network analysis and unbiased systems genetics to uncover vascular wall genes with key biological roles in the development of CAD.[59,61,75,107] As described above, cis- and trans-QTL discovery in the STARNET cohort were used to construct tissue-specific and shared regulatory networks for cardiometabolic traits[55] (Figure 2C). Interestingly, trans-eQTL interactions between tissues involving CAD-associated SMC genes, such as *TCF21*, provided validation for epigenomic based molecular interactions in HCASMC.[24,55] Recently, Lempiäinen et al[108] used a network-based approach that first used a combination of genetic association, chromatin interactions, eQTL data, and mouse phenotypic data to prioritize causal genes at CAD loci. These CAD candidate genes were integrated with regulatory gene networks from the STAGE study and protein-protein interaction data to determine if these genes play regulatory roles in gene-protein modules (ie, subnetworks). Many of these modules are of functional relevance to SMC biology, including extracellular matrix organization and disassembly. These CAD candidate genes and modules were also scored for druggability. Notably, many of the top modules contained genes coding for druggable protein kinases and G-protein coupled receptors.[108]

CRISPR/Cas9 Perturbations

The development of genome editing technologies, such as CRISPR, now facilitates direct perturbations of CAD-associated risk variants or risk haplotypes. Although CRISPR-Cas9 has been used to disrupt genes with roles in dyslipidemia,[109] there is a lack of CRISPR studies in SMCs in the context of CAD. Direct genome editing and clonal selection are difficult for human primary coronary artery SMCs because these cells often become senescent in culture and are pheno-typically heterogeneous across individuals and disease states. An alternative is to use human iPSCs to disrupt a CAD region of interest using genome editing and subsequently differentiate successfully edited cells into SMCs. As described above, TALEN editing of the complex 9p21.3 CAD locus in human iPSCs differentiated to SMCs highlighted precisely how this region alters SMC gene expression and function.[51] Successfully, CRISPR/Cas9 editing human iPSCs and differentiating

into macrophages has already been performed.[110] Protocols now exist for successful differentiation of human iPSCs into vascular SMCs.[111–113] While wild-type Cas9 cuts DNA, the advent of endonuclease dead Cas9 (dCas9) allows dCas9 and guide RNAs to target transcriptional activators (eg, dCas9-SAM, dCas9-p300[114,115]) or transcriptional repressors (eg, dCas9-KRAB [Kruppel-associated box][116]) to specific regions of the genome (Figure 2D). This dCas9 transcriptional activation/repression will enable direct perturbation of CAD genes or CAD-associated variants in SMCs. Also, fusion of dCas9 or Cas9 nickase with cytidine or adenine deaminases has allowed for single base pair changes in the genome to be made.[117–119] These small perturbations to the DNA sequence, particularly if located in TF binding sites, are sufficient to induce physiologically relevant transcriptional change. For example, a single point mutation in the CArG box in the CNN1 promoter completely ablated expression of this protein in vivo.[120] Similarly, a 13bp deletion of a GATA1 (GATA-binding protein 1) binding site in the intronic region of *ALAS2* significantly reduced its expression during erythroid cell differentiation.[121]

Single-Cell Sequencing

Recently, new single-cell technologies, such as single-cell RNA-seq and CyTOF (mass cytometry), have been used to better understand the heterogeneity of the atherosclerotic plaque including heterogeneity within specific immune cell types.[122–124] There are now many commercially available and adopted single-cell sequencing systems (eg, 10X Genomics, DropSeq), which involve droplet-based cell capture. So far, with respect to CAD, scRNA-seq has been applied to leukocytes in the plaque such as macrophages but not SMCs. It is expected that scRNA-seq (single-cell RNA-Seq) analysis of human normal and diseased coronary arteries will (1) decipher what CAD candidate genes are co-expressed in cells expressing SMC markers and (2) define different subsets of SMCs in diseased coronary artery that can shed more light on disease pathogenesis (Figure 2E). Similarly, single-cell epigenomics (such as single-cell ATAC-seq) has gained substantial traction recently and can be applied to trace SMC transitions as atherosclerosis progresses.[125,126] Single-cell epigenomics also can allow CAD-associated variants to be linked to open chromatin regions (eg, enhancers, promoters) in specific cell types that will facilitate more targeted follow-up functional studies. Finally, integration of single-cell approaches with lineage tracing studies in mice in vivo,[127,128] may clarify the precise identity and origin of SMC-derived cells in the atherosclerotic plaque which lack traditional SMC markers.[129] Such studies may also improve our understanding of the clonal nature of SMC infiltration and expansion in atherosclerotic plaques.[128,130–132]

Limitations and Challenges of Genetic Association Studies

It is worth acknowledging the various obstacles in conducting genetic-based association studies for CAD/MI that may confound biological interpretation of the results. For example, quantitation of stable and unstable CAD using angiography still remains a challenge and could produce different results influencing the case definitions of these studies. Also, MI is an equally heterogeneous condition with ≈70% of cases due to plaque rupture, ≈30% due to plaque erosion and a minority due to calcified nodules.[133,134] Due to the distinct causes of these pathological phenotypes, the clinical end points may only provide genetic associations

for the most prevalent pathology (eg, rupture) while obscuring the others. The impact of demographics, such as age, sex, and ethnicity, while often corrected in these studies, should be specifically addressed to better understand why certain groups are more susceptible to different pathologies (eg, younger women are often more at risk for plaque erosions than rupture).[133] It is also worth noting that the widespread use of statins in recent years has led to more MI cases associated with plaque erosion versus rupture,[135] thus making it difficult to extrapolate relevant clinical findings from historical cohorts.

It is now well appreciated that the majority of GWAS and QTL based associations represent common variants with modest effects that could impact CAD and related phenotypes over a lifetime. However, human disease manifests through a spectrum of genetic variants and in some individuals CAD susceptibility may be driven by less common alleles with intermediate or large effects that arose through recent ancestors (referred to as Clan Genomics).[136] By accounting for the entire catalog of genomic variation in individuals, we can establish the true impact of multiple variants on CAD, and better inform polygenic risk scores for patient stratification and interventional follow-up. Nonetheless, like most genetic discoveries these findings still require experimental functional studies in disease-relevant cell and animal models to elucidate causal genes and mechanisms. Given that many associations mark developmental genes or transition states, it will be important to study these pathways in the appropriate environmental contexts to evaluate their putative functions.

Summary and Perspective

Large-scale genetic studies have uncovered a myriad of new candidate genes with experimental evidence supporting their contributions to vascular functions and CAD risk. However, additional work involving higher resolution assays in other cell types and model systems may be necessary to establish causal mechanisms. Colocalizing GWAS data with molecular quantitative trait locus and other high-throughput multiomics datasets from relevant cells and tissues represents a powerful statistical approach to prioritize causal genes and pathways underlying associations.[23,137] Such studies will likely be augmented by other systems-based network analyses to infer directionality of risk loci with upstream and downstream signaling pathways.[108] Larger cohorts will likely be required to map transregulatory networks responsible for disease associations.[138,139] Complementary CRISPR/Cas9 genome editing and perturbation experiments may provide more empirical evidence for candidate genes/pathways impacting disease-relevant phenotypes.[140] Nonetheless, CAD remains a complex trait involving interactions of multiple cell types not limited to SMC, including macrophages, endothelial cells, fibroblasts, and other immune cells, and intermediate cells. Single-cell sequencing promises to deconstruct novel SMC and non-SMC markers and hierarchies during atherosclerotic phenotypic transitions.[26] Integration with in vivo lineage tracing could reconcile some of the controversial origins of these cells in early and advanced lesions. Together, these genetically driven insights into SMC roles may ultimately inform new treatments, biomarkers, or risk stratification for many patients affected by CAD and related diseases.

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Nonstandard Abbreviations and Acronyms

ASAP	Advanced Study of Aortic Pathology
ATAC	assay for transposase accessible chromatin
BiKE	Biobank of Karolinska Carotid Endarterectomies
CAD	coronary artery disease
caQTL	chromatin accessibility quantitative trait locus
ChIP	chromatin immunoprecipitation
eQTL	expression quantitative trait locus
GRASP	Genome-Wide Repository of Associations Between SNPs and Phenotypes
GTEx	Genotype-Tissue Expression
GWAS	genome-wide association study
HAEC	human aortic endothelial cells
HCASMC	human coronary artery smooth muscle cells
iPSC	induced pluripotent stem cell
MI	myocardial infarction
PDGF	platelet-derived growth factor
SMC	smooth muscle cells
SNP	single nucleotide polymorphism
STAGE	Stockholm Atherosclerosis Gene Expression
STARNET	Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task
TF	transcription factor
TGFB	transforming growth factor β

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Highlights

- In this brief review, we highlight several coronary artery disease genome-wide association study loci that harbor genes with functional experimental evidence in smooth muscle cells.
- We discuss the power of molecular quantitative trait locus strategies in vascular tissues and cell types to discover genetic regulators of molecular phenotypes and to prioritize genome-wide association study loci.
- We also describe studies that utilize quantitative trait locus based network analyses to identify driver genes and interactions across vascular tissues and to predict effects of genetic loci on vascular phenotypes.
- We discuss modified CRISPR/dCas9 approaches to target specific regulatory regions at coronary artery disease loci.
- Finally, we emphasize single-cell genomic approaches to uncover new cell populations and resolve the heterogeneity of genetic associations.

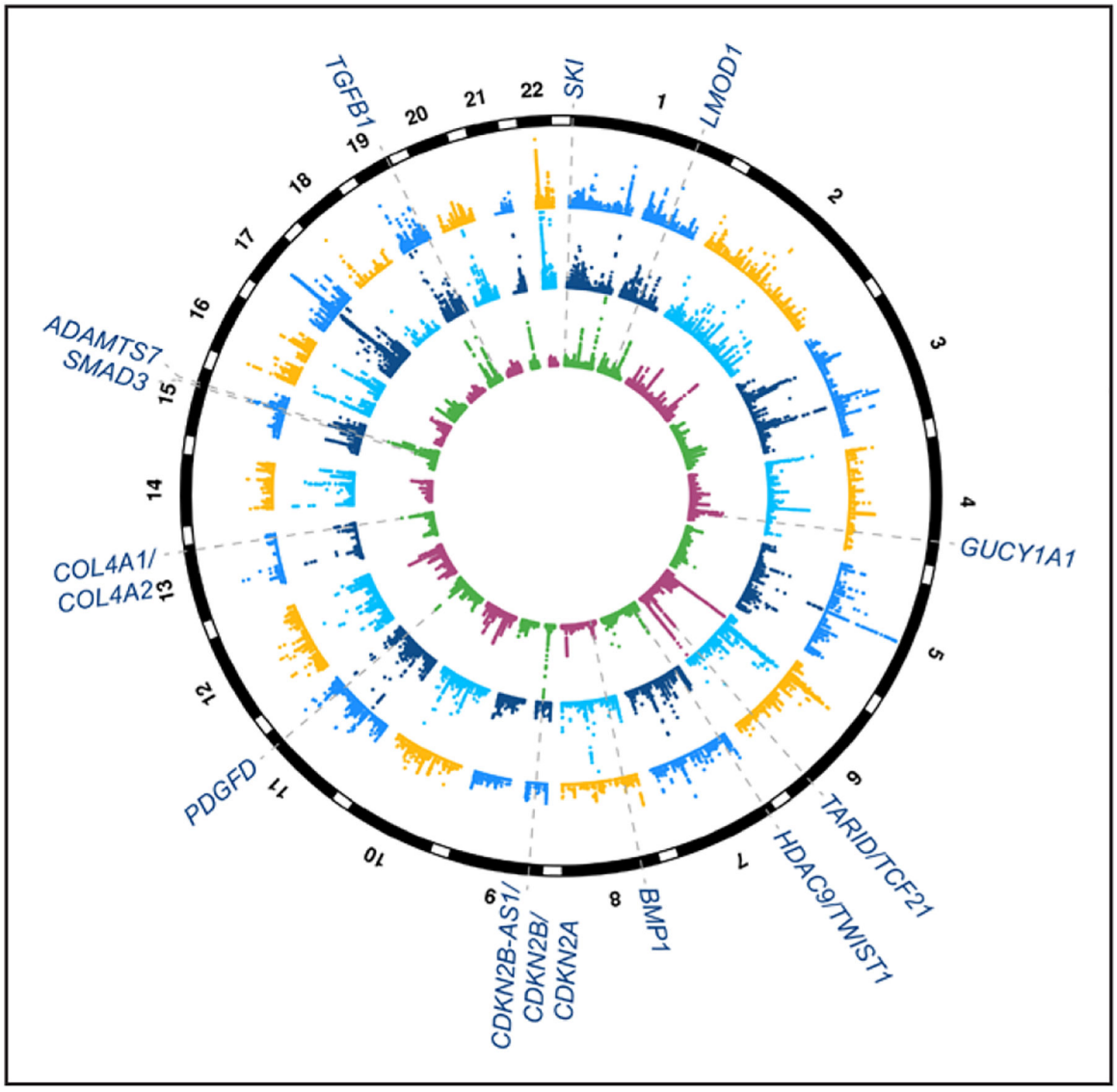

Figure 1.

Summary of coronary artery disease (CAD) genome-wide association study (GWAS) and expression quantitative trait locus (eQTL) targeting genes with evidence of smooth muscle functions. Circular Manhattan plot depicting genome-wide significant loci associated with CAD from meta-analyses of CARDIoGRAMplusC4D (Coronary Artery Disease Genome Wide and Replication and Meta-Analysis Plus The Coronary Artery Disease Genetics Consortium) and UK Biobank data.[7] Inner circle shows $-\log_{10} P$ values for CAD GWAS loci (maximum set to 50), and dashed lines and text highlight candidate causal genes with evidence of smooth muscle cell functions.[28–38] Middle circle shows $-\log_{10} P$ values for eQTLs (maximum set to 100) identified from tibial artery in Genotype-Tissue Expression (GTEx; largest sample size for artery samples in this database) and outer circle shows $-\log_{10} P$ values for liver eQTLs (maximum set to 30) in GTEx. Note: these candidate target genes are based on existing experimental studies in the literature and may be subject to change with additional statistical and/or functional fine-mapping efforts.

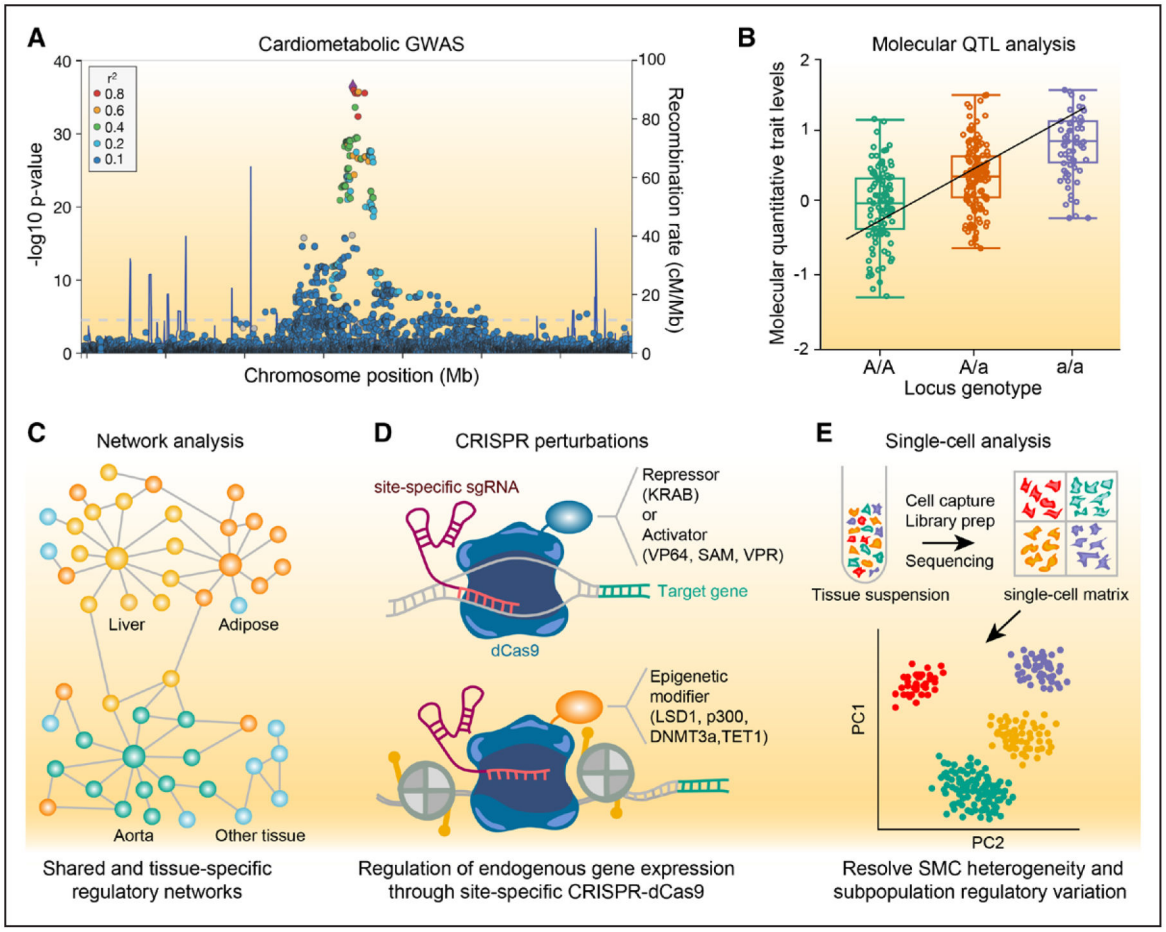

Figure 2.

Overview of genetic approaches to investigate smooth muscle disease mechanisms. **A**, Example locus association plot showing a genome-wide association study (GWAS) signal for a cardiometabolic trait. Circles indicate individual single nucleotide polymorphisms (SNPs) associated at a given *P* value and location across the genome. SNPs are color-coded for degree of linkage disequilibrium (r^2) in a European population. Blue lines indicate recombination rate. **B**, Example molecular quantitative trait locus (molQTL) box plot showing the levels of a given molecular trait (eg, gene expression) that are correlated with genotype at a specific GWAS locus. Black line indicates linear regression for molecular trait-genotype association. **C**, Schematic of shared and tissue-specific gene regulatory networks derived from molQTLs related to cardiometabolic traits (eg, STARNET [Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task]). Yellow, orange, and green colored nodes indicate liver, adipose, and aorta-enriched regulatory signals, respectively, while blue colored nodes indicate shared or nontissue enriched signals. **D**, Clustered regularly interspaced short palindromic repeats (CRISPR) approaches to perturb candidate gene expression levels using site-specific single guide RNA (sgRNA) and nuclease dead Cas9 (dCas9) fusion proteins to either repress/silence, activate, or epigenetically modify a particular regulatory region. **E**, Schematic of single-cell analysis (eg, single-cell RNA sequencing), showing single-cell gene matrix generated from bulk

population of cells after single-cell capture, reverse transcription, library preparation and next-generation sequencing. Different colors represent uniquely labeled cell type based on cell-specific gene expression levels. Clustering shown for principal components (PC) 1 and 2 which can be used to resolve smooth muscle cell (SMC) heterogeneity and various subpopulations potentially altered by regulatory genetic variation. DNMT3a indicates DNA methyltransferase 3 alpha; KRAB, Kruppel-associated box; LSD1, lysine-specific histone demethylase 1A; p300, histone acetyltransferase p300; QTL, quantitative trait locus; SAM, synergistic activation mediator; TET1, ten-eleven translocation methylcytosine dioxygenase 1; VP64, viral protein 64; and VPR, viral protein R.

Table.

Human Artery Tissue and Cellular eQTL Data Resources Available

Study	Tissue(s)	Sample size	Datasets	Platforms	Age (mean±SD)	Sex	Ancestry
Athero-Express Biobank Study[74]	Atherosclerotic carotid artery plaque	1439	Histological plaque characteristics, SNP genotyping, DNA methylation, proteomics (plasma, urine, plaque)	Sirius Red, α-actin, CD34, CD68, and H&E staining, Affymetrix and Illumina SNP arrays (genotyping), Illumina 450K Methylation array, mass spectrometry and Luminex (proteomics)	68.8±9.3	67.9% male	100% European
STARNET[55]	Atherosclerotic aortic root, nonatherosclerotic internal mammary artery, blood, subcutaneous adipose, abdominal adipose, liver, skeletal muscle	≈600	SNP genotyping, gene expression, cis/trans-eQTLs, networks	Human OmniExpressExome 8v1 array (genotyping), RNA-seq (~15–30M reads/sample)	65.8±8.7	70.3% male	100% European
STAGE[75]	Atheroscelrotic aortic root, nonatherosclerotic arterial wall internal mammary artery, liver, skeletal muscle, pericardial mediastinal visceral fat, carotid artery lesions	109	Gene expression, cis-eQTLs	Affymetrix, GenomeWideSNP_6 array (genotyping), Affymetrix Human Genome U133 Plus 2.0 Array (gene expression)	66±8	90% male	100% European
BiKE[76]	Atherosclerotic carotid artery plaque from endoarterectomy and nonatherosclerotic control artery, plasma	127 (plaque); 15 (control)	SNP genotyping, Gene expression, proteomics (plaque and plasma), cis-eQTLs, histology	Illumina Human 610 W- Quad Beadarrays (genotyping), Genotyping array, Affymetrix HT HU133A Plus array (gene expression), LC-MS/MS (proteomics)	70.0±8.9	80.2% male	100% European
ASAP[77]	liver, mammary artery (medial and adventitial), and dilated and nondilated ascending aorta (medial and adventitial)	117	SNP genotyping, gene expression, plasma proteomics, cis-eQTLs	Illumina Human 610 W- Quad Beadarrays (genotyping), Affymetrix Human Exon 1.0 ST array (gene expression), LC-MS/MS (proteomics)	63.2±11.5	≈70.2% male	100% European
HAEC[78,79]	HAECs from ascending aortic segment of transplant donors (control and Ox-PAPC treatment)	149	SNP genotyping, gene expression, coexpression network analysis, chromatin accessibility	Affymetrix Human SNP Array 6.0 (genotyping), Affymetrix HT HU133A array (gene expression), ATAC-seq	N/A	N/A	N/A
HCASMC[23,24]	HCASMC from nondiseased donors	52	SNP genotyping, gene expression, cis-eQTL, sQTL, chromatin accessibility	Whole genome sequencing (30X), RNA-seq (≈50M reads/sample), ATAC-seq (≈45M reads/sample)	39.0±15.4	65.3% male	78% European, 10% Hispanic, 10% African, 2% Asian

ASAP indicates Advanced Study of Aortic Pathology; ATAC-Seq, assay for transposase-accessible chromatin-sequencing; BiKE, Biobank of Karolinska Carotid Endarterectomies; eQTL, expression quantitative trait locus; H&E, hematoxylin and eosin; HAEC, human aortic endothelial cell; HCASMC, Human coronary artery smooth muscle cells; LC-MS/MS, liquid chromatography with tandem mass spectrometry; N/A, not applicable; Ox-PAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine; SNP, single nucleotide polymorphism; sQTL, splicing quantitative trait locus; STAGE, Stockholm Atherosclerosis Gene Expression; and STARNET, Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task.