



Effects of Coronary Artery Disease–Associated Variants on Vascular Smooth Muscle Cells

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BACKGROUND: Genome-wide association studies have identified many genetic loci that are robustly associated with coronary artery disease (CAD). However, the underlying biological mechanisms are still unknown for most of these loci, hindering the progress to medical translation. Evidence suggests that the genetic influence on CAD susceptibility may act partly through vascular smooth muscle cells (VSMCs).

METHODS: We undertook genotyping, RNA sequencing, and cell behavior assays on a large bank of VSMCs (n>1499). Expression quantitative trait locus and splicing quantitative trait locus analyses were performed to identify genes with an expression that was influenced by CAD-associated variants. To identify candidate causal genes for CAD, we ascertained colocalizations of VSMC expression quantitative trait locus signals with CAD association signals by performing causal variants identification in associated regions analysis and the summary data–based mendelian randomization test. Druggability analysis was then performed on the candidate causal genes. CAD risk variants were tested for associations with VSMC proliferation, migration, and apoptosis. Collective effects of multiple CAD-associated variants on VSMC behavior were estimated by polygenic scores.

RESULTS: Approximately 60% of the known CAD-associated variants showed statistically significant expression quantitative trait locus or splicing quantitative trait locus effects in VSMCs. Colocalization analyses identified 84 genes with expression quantitative trait locus signals that significantly colocalized with CAD association signals, identifying them as candidate causal genes. Druggability analysis indicated that 38 of the candidate causal genes were druggable, and 13 had evidence of drug-gene interactions. Of the CAD-associated variants tested, 139 showed suggestive associations with VSMC proliferation, migration, or apoptosis. A polygenic score model explained up to 5.94% of variation in several VSMC behavior parameters, consistent with polygenic influences on VSMC behavior.

CONCLUSIONS: This comprehensive analysis shows that a large percentage of CAD loci can modulate gene expression in VSMCs and influence VSMC behavior. Several candidate causal genes identified are likely to be druggable and thus represent potential therapeutic targets.

Key Words: coronary artery disease ■ genetics ■ muscle, smooth, vascular ■ transcriptomes

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Genome-wide association studies (GWASs) have identified common genetic variants at >190 loci that are robustly associated with coronary artery

disease (CAD).^{1–3} However, the underlying biological mechanisms are still unknown for most of these loci. This hinders translation from the genetic findings to a new

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Supplemental Material is available at <https://www.ahajournals.org/doi/suppl/10.1161/circulationaha.121.058389>.

For Sources of Funding and Disclosures, see page XXX.

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Clinical Perspective

What Is New?

- We have found numerous genes with an expression in vascular smooth muscle cells that is under genetic regulation of coronary artery disease (CAD)-associated genetic loci.
- The study has identified 84 candidate causal genes with expression quantitative trait loci signals in vascular smooth muscle cells that significantly colocalize with reported CAD-association signals, of which 38 are potentially druggable.
- Many of the candidate causal genes are located at CAD loci that show suggestive associations with vascular smooth muscle cell proliferation, migration, or apoptosis in this study.

What Are the Clinical Implications?

- Genetic influence on CAD risk likely, in part, acts through vascular smooth muscle cells, as indicated by this and other studies.
- The potentially druggable candidate causal genes at the CAD susceptibility loci represent promising therapeutic targets.

Nonstandard Abbreviations and Acronyms

CAD	coronary artery disease
eCAVIAR	expression quantitative trait locus and genome-wide association study causal variant identification in associated regions
eQTL	expression quantitative trait locus
GWAS	genome-wide association study
HEIDI	heterogeneity in dependent instruments
lncRNA	long noncoding RNA
RNA-Seq	RNA sequencing
SMR	summary data-based mendelian randomization
SNP	single nucleotide polymorphism
sQTL	splicing quantitative trait locus
TGFβ	transforming growth factor- β
VSMC	vascular smooth muscle cell
WGCNA	weighted gene coexpression network analysis

understanding of disease mechanisms and the development of new treatments.

About two-thirds of the CAD-associated loci/variants identified by GWASs are not associated with the conventional risk factors (eg, elevated low-density lipoprotein cholesterol level and raised blood pressure),^{4–6} suggesting that the large majority of CAD susceptibility loci do not act through the traditional pathways and thus are

not addressed by current treatment. Instead, many of the CAD loci identified by GWASs contain genes implicated in vascular cell biology,^{1,5,7,8} pointing to novel mechanisms directly involving cells of the blood vessel wall. Hence, identifying novel pathways and therapeutic targets in vascular cells can potentially aid the development of new treatments that complement current strategies that target conventional risk factors.

Vascular smooth muscle cells (VSMCs) are a major cell type in the blood vessel wall and play important roles in the development and progression of atherosclerosis,⁹ the pathological condition underlying CAD. Growing evidence suggests that the genetic influence on CAD risk may act, in part, through VSMCs. For example, genetic variants at a number of CAD loci have been shown to influence gene expression in VSMCs or affect VSMC behavior.^{10–19}

However, a systematic large-scale analysis that integrates genetic, transcriptomic, and phenotypic assays of a large biobank of VSMCs from different individuals has not been undertaken. Here, we report such an analysis and describe how the findings can be used to identify novel drug targets.



METHODS

The data, analytical methods, and study materials will be/have been made available to other researchers for purposes of reproducing the results or replicating the procedure. The RNA-sequencing (RNA-Seq) data from this study are available from the Gene Expression Omnibus²⁰ with the accession number GSE189300. The study was approved by the East Midlands–Derby Research Ethics Committee, and the parents of umbilical cord donors gave written informed consent.

The methods used in this study are summarized here and detailed in [Supplemental Methods](#).

VSMC Biobank

VSMCs were isolated from the artery of umbilical cords from 2114 different donors with a previously reported method.²¹ Immunocytochemical staining of cell-type markers was performed on randomly selected representative samples at passage 3 (n=231). The VSMC samples showed strong staining of the VSMC marker α -smooth muscle actin but not the endothelial cell marker von Willebrand factor or the fibroblast marker TE7 ([Figure S1](#)).

Genotyping and Imputation

A total of 1992 DNA samples were genotyped for 760 000 variants with Infinium Global Screening Array-24 2.0 BeadChips (Illumina), and imputation was performed to obtain genotypic information of 7 334 165 variants ([Supplemental Methods](#)).

Selection of CAD-Associated Variants

We compiled a list of single nucleotide polymorphisms (SNPs) reported by Erdmann et al,¹ Aragam et al,³ and the GWAS catalog² that had been previously associated with CAD at the

genome-wide significance level ($P < 5 \times 10^{-8}$). Linkage disequilibrium between the SNPs was assessed with the LDlink SNPclip tool (Supplemental Methods). In total, 511 independent CAD-associated SNPs were identified (Table S1). Of these, 424 were available in this study, which were either typed by the Global Screening Array or imputed.

RNA Sequencing

RNA-Seq was performed on VSMC RNA samples from 1499 donors. Total RNA was extracted from an aliquot of passage 3 VSMCs stored in RNeasy lysis solution. RNA concentration and integrity were assessed by RNA BR and RNA IQ assays. A strand-specific library with rRNA removal was prepared from total RNA of each of the 1499 VSMC samples, and 150-bp paired-end sequencing at a 30 million read depth was performed with the Illumina platform. RNA data were processed as described in the Supplemental Methods.

To determine whether RNA-Seq results were random or had repeatable differences between VSMCs from different individuals, VSMCs at passage 5 through 7 from 10 randomly selected samples of the VSMC bank were subjected to RNA-Seq using the strand-specific library preparation and paired-end sequencing methods mentioned previously. A hierarchical cluster analysis and a principal component analysis were performed on the RNA-Seq data (Supplemental Methods). The analysis showed that gene expression data from different passages (5–7) of VSMCs from each individual tended to cluster and to be more similar to each other than data from other individuals (Figure S2), indicating that RNA-Seq results had reproducible differences between VSMCs from different individuals.

To determine changes in gene expression in VSMCs with knockdown of *YIPF6* or *SLC25A36*, RNA-Seq was performed on VSMCs transfected with *YIPF6* siRNA, *SLC25A36* siRNA, or negative control siRNA, as described in the Supplemental Methods.

Multidimensional Scaling Analysis

We used multidimensional scaling analysis to determine the relationship between the VSMC RNA-Seq data and other publicly available bulk RNA-Seq data and single-cell RNA-Seq data (Supplemental Methods). Details about the data sets included in the multidimensional scaling plot are available in Table S2.

Expression Quantitative Trait Locus and Splicing Quantitative Trait Locus Analyses

We performed expression quantitative trait locus (eQTL) and splicing quantitative trait locus (sQTL) analyses on 1486 VSMC samples that had both genotype and gene expression data (Supplemental Methods). The numbers of protein coding genes, long noncoding RNA (lncRNA) genes, pseudogenes, and other genes in the eQTL and sQTL analyses are given in Table S3. We used the eigenMT-BH method²² for multiple testing correction.

Colocalization With CAD GWAS

We used eQTL and GWAS causal variant identification in associated regions (eCAVIAR)²³ to test for colocalization of CAD GWASs with VSMC *cis*-eQTL or *cis*-sQTL

(Supplemental Methods). The eCAVIAR analysis used only SNPs that had been reported to associate with CAD at the genome-wide significance level ($P < 5 \times 10^{-8}$) and had genome-wide significant quantitative trait loci in VSMCs in our study. The top quantitative trait locus SNPs (ie, SNPs with the lowest *P* value) of genes or splice clusters were selected, and SNPs within 500 kb of the top SNP were overlapped with GWAS summary data. The eCAVIAR colocalization test was performed for genes/splice clusters that had >4 overlapping SNPs with GWAS summary data. The number of causal SNPs was set to 2, and colocalization events with >0.05 colocalization posterior probability were considered significant.

We also used summary data-based mendelian randomization (SMR)/heterogeneity in dependent instruments (HEIDI)²⁴ to test for colocalization between CAD GWAS and VSMC *cis*-eQTL and *cis*-sQTL (Supplemental Methods). Although we ran transcriptome-wide SMR/HEIDI analysis, we focused only on SNPs that had been reported to associate with CAD at the genome-wide significance level ($P < 5 \times 10^{-8}$) in GWASs. We applied a threshold of $P_{\text{SMR}} < 0.05$ and $P_{\text{HEIDI}} > 0.05$ to identify significant colocalizations of CAD GWAS signals with VSMC eQTL or sQTL signals.

Druggability and Drug–Gene Interaction Analyses

Genes with eQTL signals in VSMCs that showed significant colocalization with CAD GWAS signals in the eCAVIAR or SMR analyses were interrogated for druggability and drug-gene interactions in the Drug Gene Interaction Database.²⁵

Pathway Analyses

Genes with eQTL signals in VSMCs that showed significant colocalization with CAD GWAS signals in the eCAVIAR or SMR analyses were subjected to functional pathway analyses performed with DAVID version 6.8, Panther version 17.0, and the ToppFun function of ToppGene.

VSMC Behavior Assays

VSMCs at passage 3 were subjected to proliferation, migration, and apoptosis assays with the use of an Operetta CLS High-Content Analysis System (PerkinElmer; Supplemental Methods). In each of these assays, each VSMC preparation was analyzed in 4 replicate wells on a 96-well plate. All 3 assays have low coefficient of variation: 2.99% (95% CI, 2.12%–3.87%) for the proliferation assay, 1.81% (95% CI, 1.50%–2.12%) for the migration assay, and 3.01% (95% CI, 1.62%–4.53%) for the apoptosis assay. To verify that the cell behavior data generated from this study are reproducible, we repeated the assays on a random selection of the VSMC preparations ($n=50$). For each preparation, the 2 experiments were performed at 2 different times (1 at passage 3 and the other at passage 4). The results of the 2 independent assays were highly consistent (Figure S3), indicating high reproducibility of the data generated.

Weighted Gene Coexpression Network Analysis

We used weighted gene coexpression network analysis (WGCNA)²⁶ to identify gene coexpression modules that



were correlated with VSMC behavior, as detailed in the [Supplemental Methods](#).

siRNA Transfection

VSMCs were transfected with *YIPF6* siRNA, *SLC25A36* siRNA, or negative control siRNA ([Supplemental Methods](#)). Successful knockdown of *YIPF6* and *SLC25A36* was confirmed by reverse-transcription polymerase chain reaction and Western blot analyses ([Figures S5 and S6](#)).

Statistical Analysis of CAD Risk Variants in Relation to VSMC Behavior Parameters

We ascertained associations of the aforementioned 424 CAD SNPs with each VSMC behavior parameter in an additive model ([Supplemental Methods](#)).

Polygenic Score Analysis

A polygenic score analysis was carried out using variants that had been reported to be associated with CAD in GWASs^{1–3} and that were nominally associated ($P < 0.05$) with VSMC behavior in our study ([Supplemental Methods](#)).

RESULTS

The design of this study is summarized in Figure 1. For this study, we developed a large bank of VSMCs derived from the umbilical artery of 2114 individuals. We genotyped a total of 1992 samples of this VSMC biobank using Infinium Global Screening Array-24 2.0 Bead-Chips for 760 000 variants and performed imputation to obtain genotypic information for 7 334 165 variants. We also undertook RNA-Seq on 1499 VSMC samples (at culture passage 3) at a 30 million read depth (150 bp paired end) using a strand-specific library with ribosomal RNA removal prepared from each sample. Furthermore, assays of VSMC proliferation ($n=2025$ samples), migration ($n=2019$), and apoptosis ($n=2075$) were performed with passage 3 cells. The resulting data sets of genotypes, RNA-Seq, and VSMC behavior parameters were analyzed as outlined in Figure 1 and described here.

Influences of CAD-Associated Variants on Gene Expression/Splicing in VSMCs

Using data from the RNA-Seq mentioned previously, we performed multidimensional scaling analysis comparing the transcriptomes of VSMCs in this study with reported transcriptomic data from human coronary artery smooth muscle cells¹⁶ and transcriptomic data of other types of cell/tissue. The analysis showed that the gene expression profile of VSMCs in this study mapped closer to human coronary artery smooth muscle cells than any other cell/tissue types (Figure 2).

Because the vast majority of disease-associated genetic variants identified by GWASs are located in non-

coding regions of the genome,^{8,27} a prevailing hypothesis is that these variants alter the individual's disease risk through their effect on gene expression.^{8,27} Using the genotypic and RNA-Seq data sets from VSMCs in this study, we performed eQTL and sQTL analyses to systematically identify genetic variants that were associated with gene expression or splicing and to determine the genes with an expression or splicing that was associated with these variants.

These analyses generated a comprehensive compilation of eQTLs and sQTLs in VSMCs, with stringent statistical significance (summarized in [Table S3](#)). We then looked up genetic variants that had been reported to be associated with CAD in GWASs^{1–3} in this eQTL/sQTL catalog. Approximately 60% of the CAD-associated variants^{1–3} analyzed in this study showed statistically significant eQTL and/or sQTL effects (summarized in [Figures S7 and S8](#) and detailed in [Tables S4–S6](#)). Two-thirds of these variants had eQTL effects on >1 gene. The CAD risk allele of $\approx 20\%$ of such variants had higher expression (positive β value) of all of the associated genes, although the CAD risk allele of some ($\approx 25\%$) variants had lower expression (negative β value) of all associated genes. However, the CAD risk allele of the majority ($\approx 55\%$) of such variants was associated with higher expression of some gene(s) but lower expression of other gene(s). Of the associated genes, $\approx 80\%$ were protein coding and $\approx 20\%$ were of lncRNAs (highlighted in green and yellow, respectively, in [Tables S4–S6](#)).

Colocalization of CAD Association With eQTL Signals in VSMCs

Colocalization between disease GWASs and eQTL signals has been used as a fine-mapping approach to successfully identify candidate causal variants and candidate causal genes at disease risk loci.^{16,23,24,28,29} Therefore, we carried out colocalization tests using publicly available CAD GWAS summary statistics^{7,30,31} and VSMC eQTL data from our study. We applied 2 colocalization tools: eCAVIAR²² and SMR/HEIDI.²⁴ The loci/variants/genes identified from these analyses are described in [Tables S7 and S8](#). A recent colocalization study¹⁶ of human coronary artery smooth muscle cells reported 5 genes (*SIPA1*, *TCF21*, *SMAD3*, *FES*, and *PDGFRA*) with eQTL signals that showed significant colocalization with CAD GWAS signals in either eCAVIAR or SMR analysis. Three of these genes (*TCF21*, *SMAD3*, and *FES*) showed significant colocalization in both the eCAVIAR and SMR analyses in our study. Furthermore, our study detected significant colocalizations of eQTL signals of 81 other genes (thus, 84 genes in total, including *TCF21*, *SMAD3*, and *FES*) with CAD GWAS signals in eCAVIAR or SMR analyses, among which 18 showed significant colocalization in both analyses ([Tables S7 and S8](#)). The

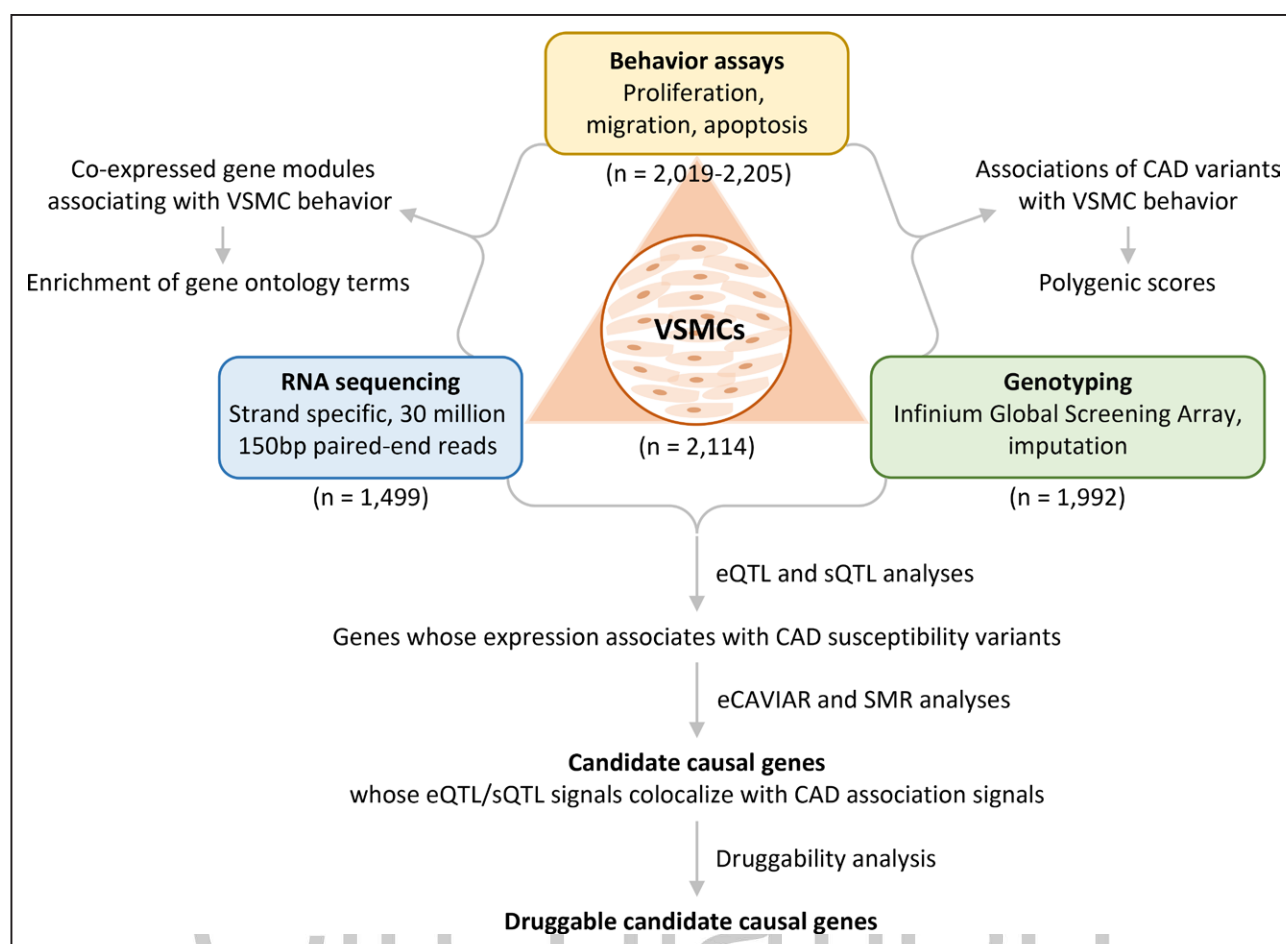


Figure 1. Flowchart of the study design.

CAD indicates coronary artery disease; eCAVIAR, expression quantitative trait locus and genome-wide association study causal variant identification in associated regions; eQTL, expression quantitative trait locus; SMR, summary-data based mendelian randomization; sQTL, splicing quantitative trait locus; and VSMC, vascular smooth muscle cell.

colocalizations between VSMC eQTL and CAD GWAS signals in several representative loci observed in this study are presented in Figure 3. The chromosomal locations of the 84 candidate causal genes are shown in Figure 4A. Functional pathway analyses revealed enrichment of several biological pathways, for example, the transforming growth factor- β (TGF β) signaling pathway, which included *TGFB1*, *BMP2*, *BMP1*, and *SMAD3* (Figure 4B and Table S9).

Druggability of Candidate Causal Genes

An interrogation of the Drug Gene Interaction Database²⁵ of the 84 candidate causal genes with eQTL signals in VSMCs that showed significant colocalization with CAD GWAS signals in the eCAVIAR or SMR analyses identified 38 genes that were druggable (Figure 5 and Table S10), indicating that they are potential therapeutic targets. Furthermore, the interrogation of the Drug Gene Interaction Database showed that 13 of the 84 candidate causal genes had evidence of drug-gene interactions (Figure 5 and Table S11).

Coexpressed Gene Modules Associated With VSMC Behavior

Using the VSMC transcriptomic and cell behavior data sets, we investigated possible associations between gene expression levels and VSMC behavior. Because it is expected that VSMC behavior is influenced by many genes concurrently, we performed a WGCNA.²⁶ The WGCNA algorithm clustered the VSMC transcriptome into 32 coexpression modules, each of which was given a name by the WGCNA program with a prefix ME (Module Eigengene) followed by a color such as MEred, MEyellow, and so on. WGCNA²⁶ usually assigns genes (typically housekeeping genes) with an expression that has little variation throughout the data set to the MEgray module. In this study, the gray module contained 65% of all of the genes analyzed. The numbers of genes and the identity of the hub gene in each of the modules are described in Table S12.

The WGCNA analysis revealed highly significant associations of several of the coexpressed gene modules with the various VSMC behavior parameters (Figures 6 and 7

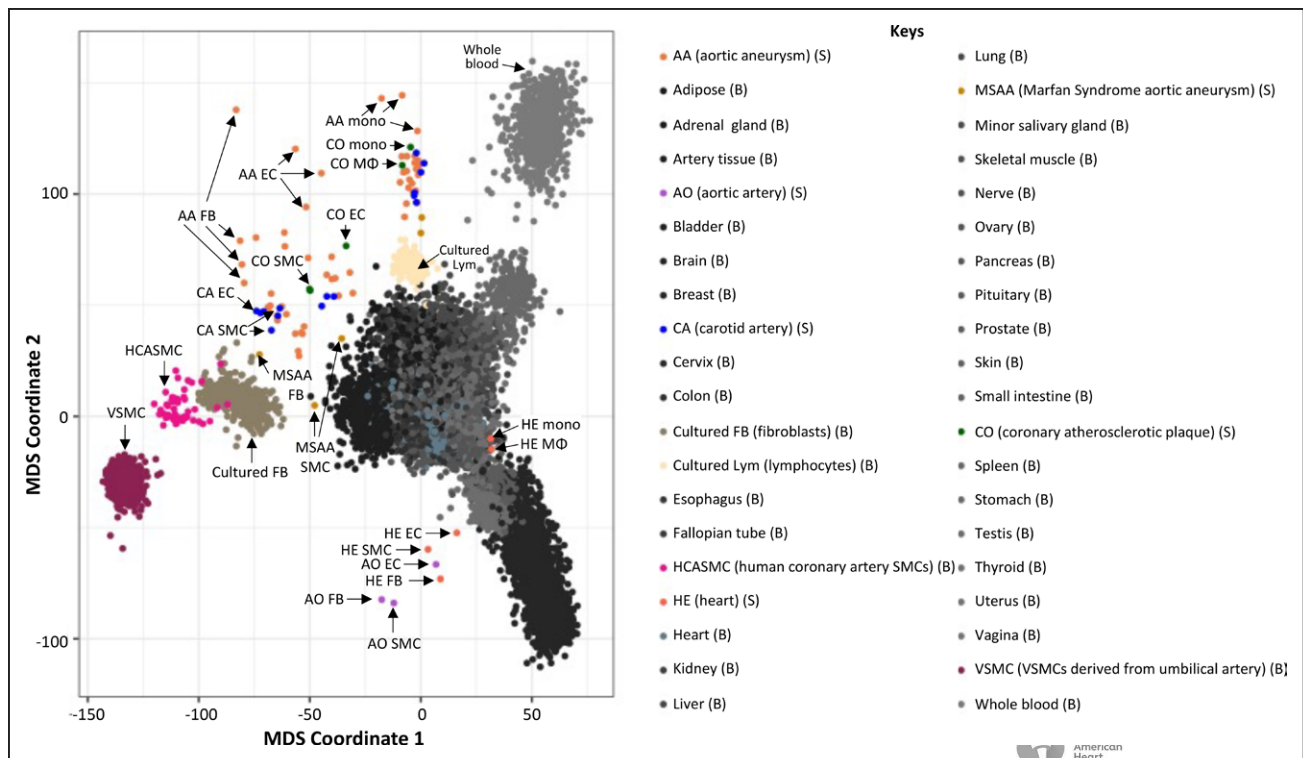


Figure 2. Gene expression profiles of VSMCs in this study compared with those of human coronary artery SMCs and other types of cell/tissue.

Multidimensional scaling (MDS) analysis comparing the transcriptomes of vascular smooth muscle cell (VSMC) samples of this study with reported transcriptomic data from human coronary artery (CA) smooth muscle cells (SMCs) and transcriptomic data of other types of cell/tissue (data sources described in Table S2). B indicates data from bulk RNA sequencing (RNA-Seq); EC, endothelial cells; FB, fibroblasts; Mono, monocytes; MΦ, macrophages; and S, data from single cell RNA-Seq.

and Figure S9); for example, the MEgreen module was inversely correlated with VSMC proliferation (Figure 6A and 6B), whereas the METurquoise module was inversely associated with apoptosis (Figure 7A and 7B). To test the effect of the MEgreen module hub gene *YIPF6* on VSMC proliferation and the effect of the METurquoise module hub gene *SLC25A36* on apoptosis, we performed a proliferation assay in VSMCs with siRNA-mediated knockdown of *YIPF6* and an apoptosis assay in VSMCs with siRNA-mediated knockdown of *SLC25A36*. The experiments demonstrated that *YIPF6* knockdown increased VSMC proliferation (Figure 6C), whereas *SLC25A36* knockdown promoted VSMC apoptosis (Figure 7C). An RNA-Seq analysis showed that *YIPF6* knockdown altered the expression of a panel of other genes (Figure S10 and Table S13), whereas *SLC25A36* knockdown changed the expression of another panel of genes (Figure S11 and Table S14).

A gene ontology analysis showed that the various coexpressed gene modules had significant enrichment of genes in particular biological processes, cellular components, molecular functions, and functional pathways. For example, the MEgreen module had significant enrichment of genes in pathways related to phagosome, membrane trafficking, and vesicle-mediated transport (Figure 6D and Table S15), whereas the METurquoise module had significant enrichment of genes in pathways

involved in cadherin signaling, Wnt signaling, and the ensemble of genes encoding extracellular matrix proteins (Figure 7D and Table S16).

Effects of CAD-Associated Variants on VSMC Behavior

Using genotype and VSMC behavior assay data, we ascertained whether there were relationships between VSMC behavior and genetic variants that had been reported to be associated with CAD in GWASs.^{1–3} At the Bonferroni-corrected *P* value threshold of 8.42×10^{-3} ($0.05/14$ behavior parameters $\times 424$ variants), none of the CAD variants showed significant association with the VSMC behavior parameters measured in this study. However, 139 CAD variants showed nominal associations with at least 1 of the VSMC behavior parameters ($P < 0.05$; Table S17). An interrogation of the VSMC eQTL and colocalization data sets described earlier showed that 63 of these 139 variants had eQTL effects on the expression of genes in VSMCs and that the eQTL signals of 38 of these genes significantly colocalized with CAD GWAS signals (Figure S12).

Because genetic variants at multiple CAD loci showed suggestive association with VSMC behavior in our study, we calculated polygenic scores to ascertain and estimate possible additive effects of different CAD loci. The

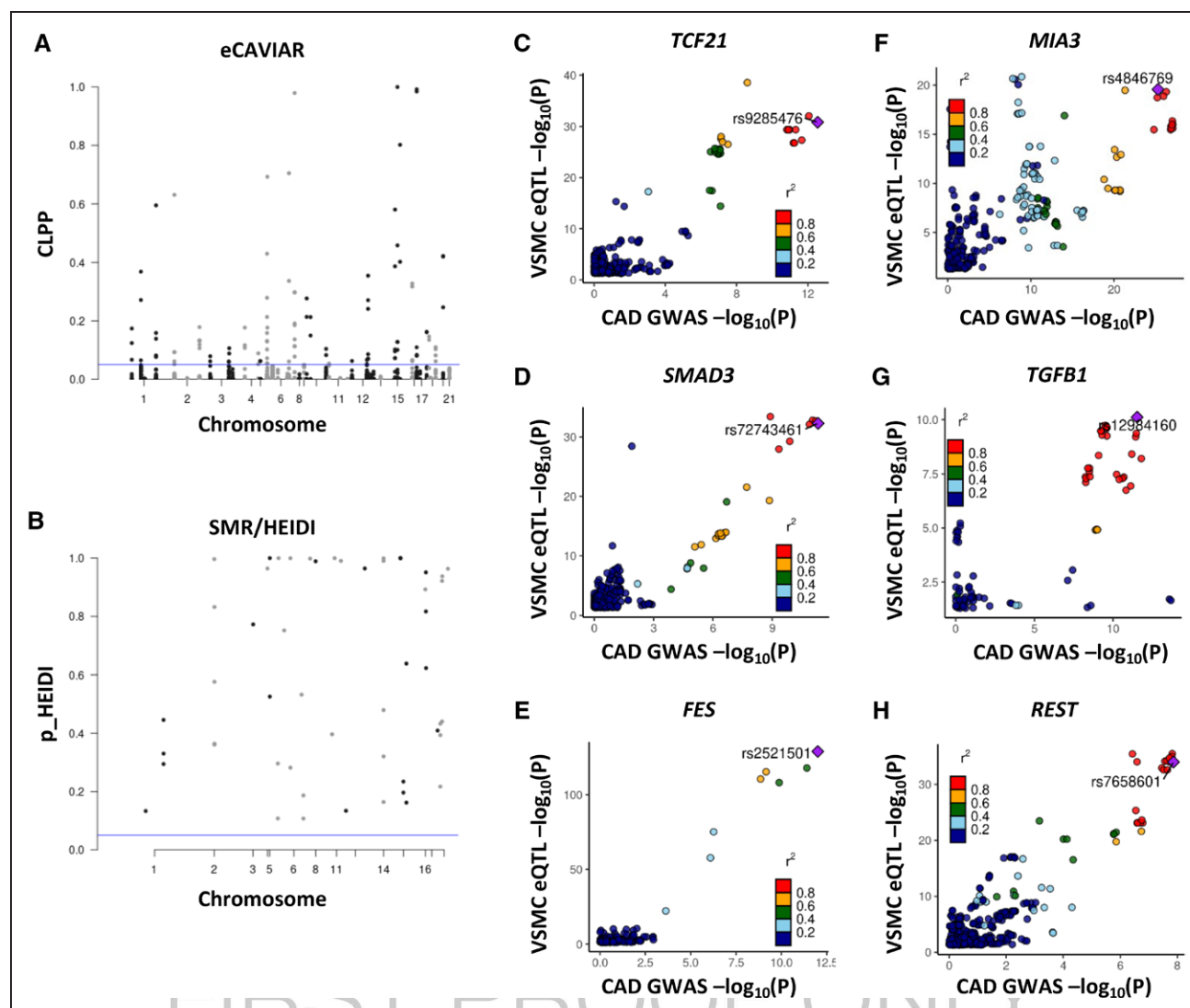


Figure 3. Colocalization between CAD GWAS and VSMC eQTL signals.

A, Results of expression quantitative trait locus (eQTL) and genome-wide association study (GWAS) causal variant identification in associated regions (eCAVIAR) analysis of colocalization between coronary artery disease (CAD) GWAS and vascular smooth muscle cell (VSMC) eQTL signals. Blue line indicates the colocalization posterior probability (CLPP) of 0.05. Each dot represents a genetic variant, and those above the blue line ($CLPP > 0.05$) represent variants with significant colocalization. **B**, Results of summary data-based mendelian randomization/heterogeneity in dependent instruments (SMR/HEIDI) analysis. Blue line indicates $P_{HEIDI} = 0.05$. Each dot represents a genetic variant, and those above the blue line represent variants with significant colocalization ($P_{SMR} < 0.05$ and $P_{HEIDI} > 0.05$). **C**, Colocalization of *TCF21* eQTL signal in VSMCs with CAD GWAS signal at the *TCF21* locus. **D**, Colocalization of *SMAD3* eQTL signal in VSMCs with CAD GWAS signal at the *SMAD3* locus. **E**, Colocalization of *FES* eQTL signal in VSMCs with CAD GWAS signal at the *FES* locus. **F**, Colocalization of *MIA3* eQTL signal in VSMCs with CAD GWAS signal at the *MIA3* locus. **G**, Colocalization of *TGFB1* eQTL signal in VSMCs with CAD GWAS signal at the *TGFB1* locus. **H**, Colocalization of *REST* eQTL signal in VSMCs with CAD GWAS signal at the *REST* locus.

polygenic score model was statistically significant for VSMC proliferation, migration straightness, and migration distance (Table S18). The polygenic score model explained up to 5.94%, 3.24%, and 5.02% of VSMC proliferation, migration straightness, and migration distance, respectively (Figure S13 and Table S18).

DISCUSSION

For most of the CAD-associated loci identified by GWAS to date, the biological mechanisms through which the

genetic variants influence CAD risk are unclear. The pathogenesis of atherosclerosis involves several different cell types, and it is plausible that different CAD loci may affect different cell types. To understand the biological mechanisms underlying the genetic associations with CAD and to translate the genetic findings into the development of new treatments, further studies to identify the cell types involved and the causal genes at the different CAD loci are required.

Previous studies have investigated some of the CAD loci individually in VSMCs, including *LMOD1* at

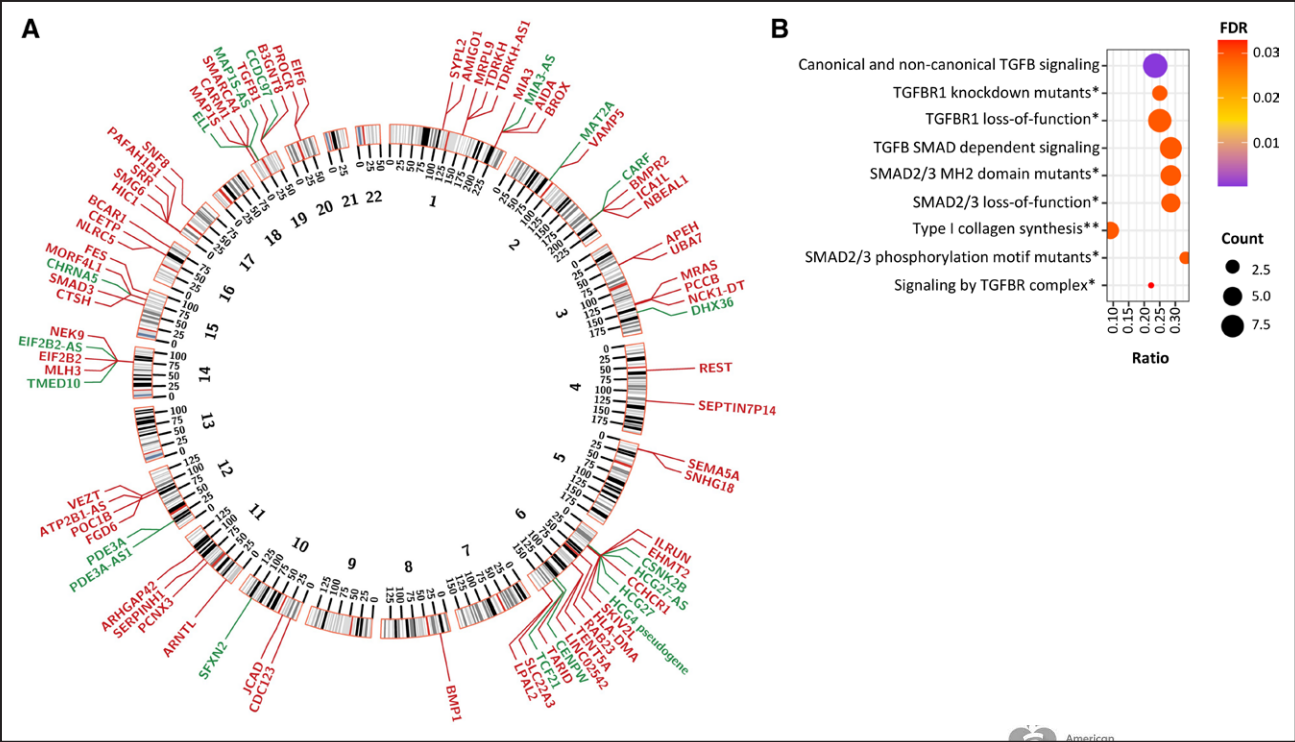


Figure 4. Chromosomal locations (A) and functional pathways (B) of candidate causal genes.
A. Boxes designate different chromosomes that are numbered from 1 to 22, with cytogenetic bands shown inside each box and chromosomal positions labeled by tick marks placed every 25 million bp. Lines on the outer aspect of the boxes symbolize coronary artery disease-associated variants that had expression quantitative trait locus (eQTL) or splicing quantitative trait locus (sQTL) effects in vascular smooth muscle cells. Each locus with both cis-eQTL and cis-sQTL effects is indicated by a red line, and each locus with only cis-eQTL is indicated by a green line. **B.** Dot plot showing identified functional pathways with enrichment of some of the candidate causal genes. Data shown are from a pathway analysis performed with ToppGene.³² FDR indicates false discovery rate. *Originally identified in cancer. **Originally identified in osteogenesis imperfecta.

the 1q32.1 locus,¹⁷ *GUCY1A3* at 4q32,¹⁵ *PDGFRA* at 4q12,¹⁶ *TCF21* at 6q23,^{12,18} *CDKN2A/CDKN2B/ANRIL* at 9p21,^{10,13} *SIPA1* at 11q13,¹⁶ *COL4A1/COL4A2* at 13q34,¹⁴ *SMAD3* at 15q22.33,¹⁸ *ADAMTS7* at 15q25,¹¹ and *FES* at 15q26.¹⁶ In addition, a recent study¹⁶ interrogated 95 CAD loci in relation to gene expression in 52 coronary artery smooth muscle cell lines, and another recent study¹⁹ examined 163 CAD loci in relation to

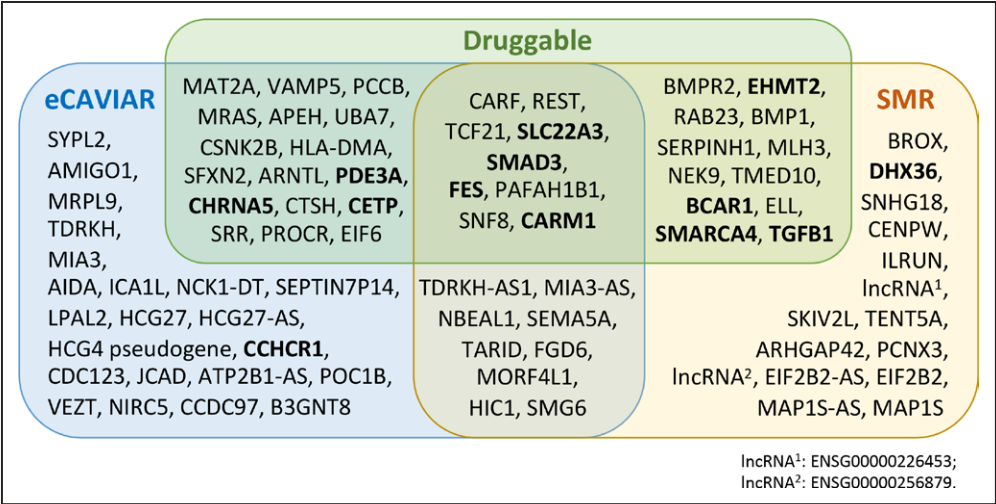


Figure 5. Druggability of genes with eQTL signals in VSMCs that significantly colocalized with CAD GWAS signals.
Shown is a summary of results of an interrogation in the Drug Gene Interaction Database²⁴ of candidate causal genes with expression quantitative trait locus (eQTL) signals in vascular smooth muscle cells (VSMCs) that showed significant colocalization with coronary artery disease (CAD) genome-wide association study (GWAS) signals in eQTL and GWAS causal variant identification in associated regions (eCAVIAR) or summary data-based mendelian randomization (SMR) analyses. Genes with evidence of drug-gene interactions are highlighted in bold. Details of druggability and drug-gene interactions are described in Tables S10 and S11, respectively.

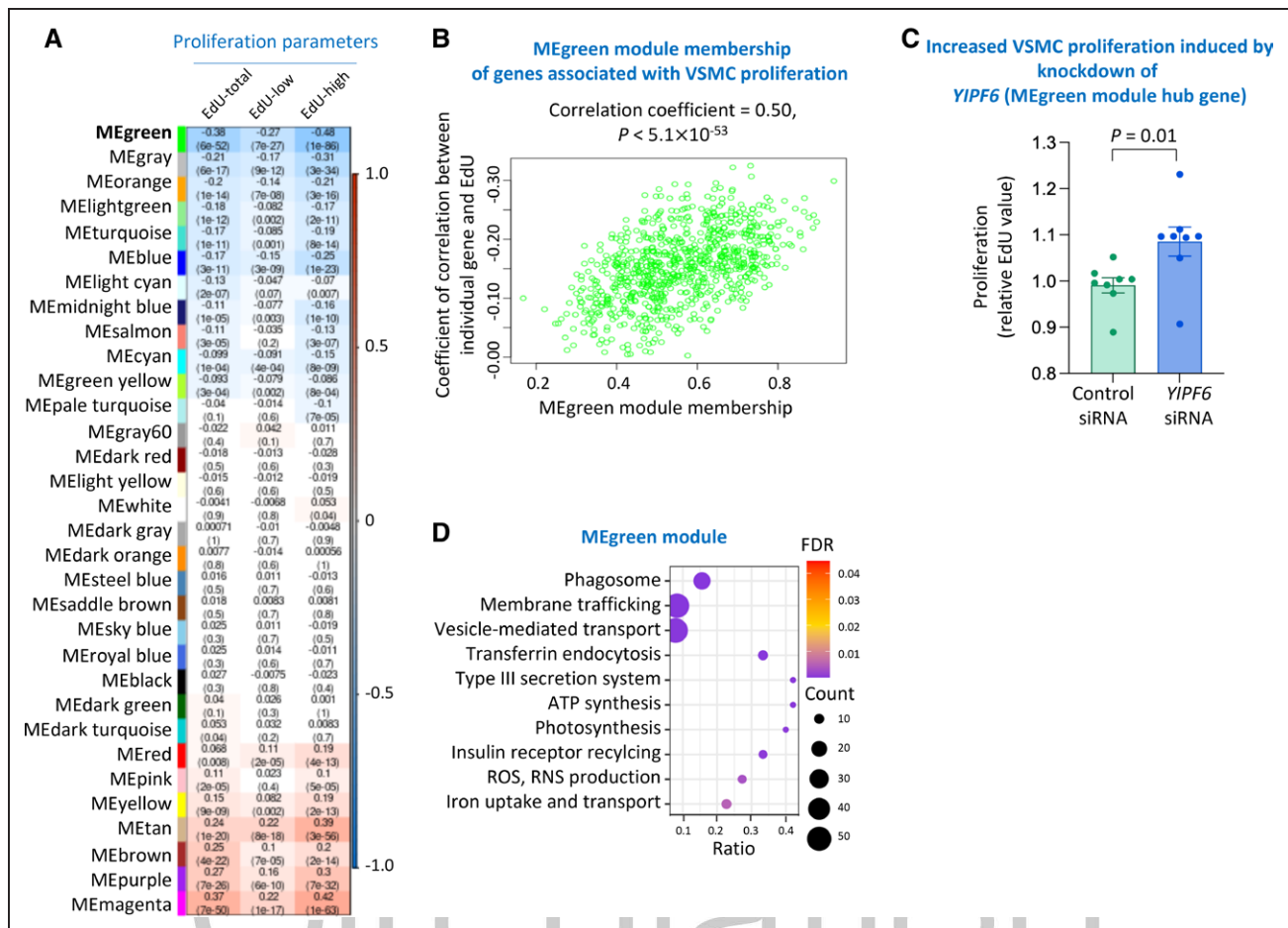


Figure 6. Coexpressed gene modules in relation to VSMC proliferation and functional pathways.

A, Heat map representation of correlations of co-expression gene modules with parameters of vascular smooth muscle cell (VSMC) proliferation. Values shown are correlation coefficients and P values (in brackets). The prefix ME in each module name stands for module eigengene. **B**, Scatterplot of MEgreen module membership (x axis) vs coefficient of correlation between individual gene expression level and percentage of VSMCs positive for EdU staining at low intensity (between 2000 and 10000 arbitrary units; Edu-low; y axis). Each gene is indicated by an open dot in green. **C**, Effect of the MEgreen module hub gene *YIPF6* on VSMC proliferation. Data shown are mean (\pm SEM) values of Edu-low in VSMCs transfected with either *YIPF6* siRNA or negative control siRNA relative to the average value of nontransfected VSMCs, control siRNA-transfected VSMCs, and *YIPF6* siRNA-transfected VSMCs ($n=8$; VSMCs from 8 different individuals; P value is from a 2-tailed Mann-Whitney test). *YIPF6* knockdown in VSMCs transfected cells is shown in Figure S5. **D**, Biological pathways enriched in the MEgreen module. Edu-high indicates percentage of VSMCs positive for EdU staining at high intensity (>10000 arbitrary units); Edu-total, percentage of VSMCs positive for EdU staining; and FDR, false discovery rate.

VSMC phenotypes in cells derived from 151 heart transplant donors. The results from these previous and recent studies indicate that genetic influences on the transcriptome and behavior of VSMCs can be an important mechanism at many of the CAD loci.

Using a systematic approach including genetic, transcriptomic and cell behavior assays, and a larger VSMC bioresource, here we examined all CAD loci identified by GWASs to date. Our study shows that at the vast majority of the currently known CAD loci, the disease-associated variants have eQTL effects on gene expression in VSMCs. This provides a comprehensive catalog of genes with an expression that is influenced by CAD-associated variants, informing which of the CAD loci influence gene expression in VSMCs and which genes are modulated at those loci.

For loci for which the disease-associated variant influences the expression of >1 gene, evidence of colocalization of the disease-association signal with the eQTL signal can indicate which gene is causative. In a recent genetic study of coronary artery smooth muscle cells, Liu et al¹⁶ identified 5 genes (*SIPA1*, *TCF21*, *SMAD3*, *FES*, and *PDGFRA*) with eQTL signals that significantly colocalized with CAD GWAS signals detected by either eCAVIAR or SMR analysis, suggesting that these genes are likely to be causal at their respective CAD loci. Our study confirmed the colocalization of 3 of these genes (*TCF21*, *SMAD3*, and *FES*) by both eCAVIAR and SMR analyses. In another recent genetic study of VSMCs, Aherrahrou et al¹⁹ found an important role of *MIA3* in CAD through modulation of VSMC behavior. In support, we observed colocalization of the *MIA3* eQTL signal with

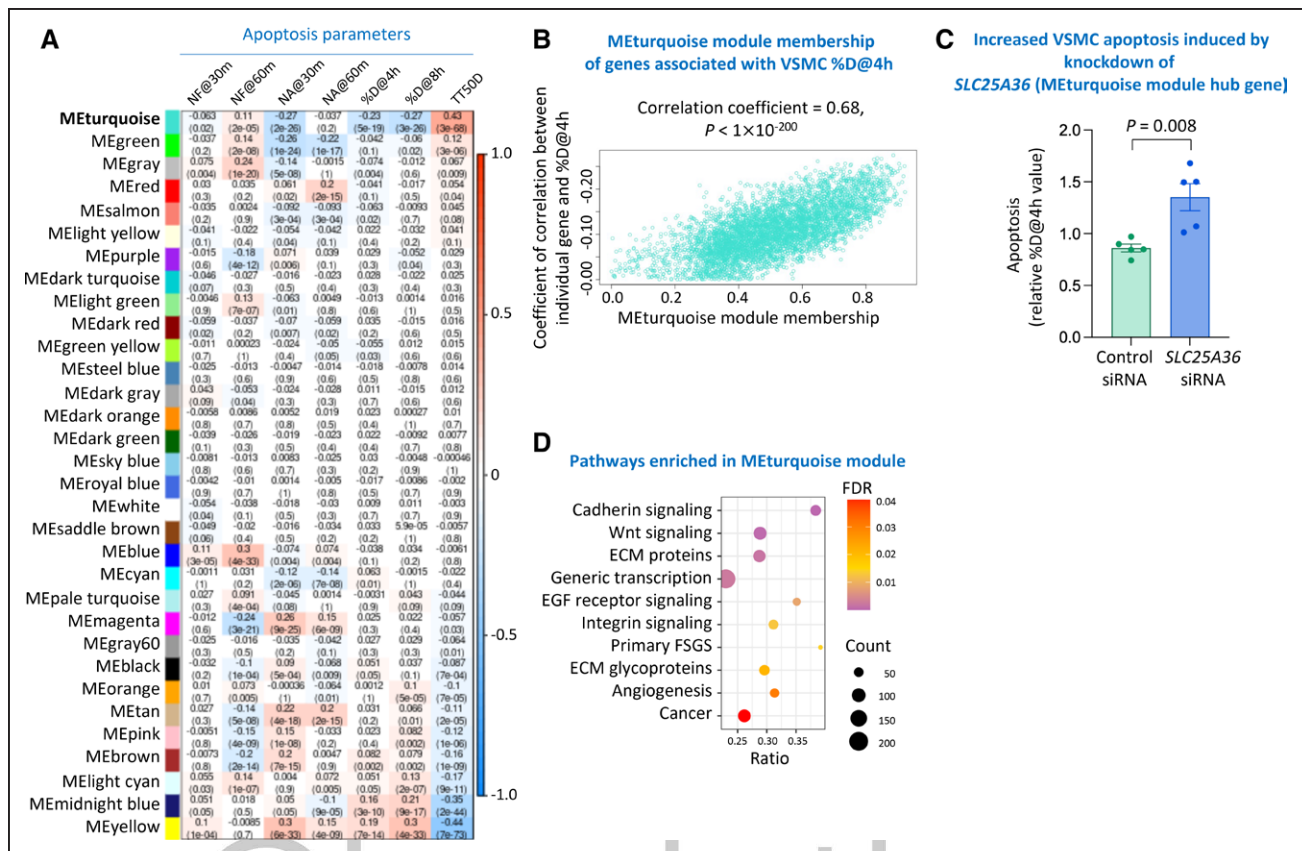


Figure 7. Coexpressed gene modules in relation to VSMC apoptosis and functional pathways.

A, Heat map representation of correlations of coexpression gene modules with parameters of vascular smooth muscle cell (VSMC) apoptosis. Values shown are correlation coefficients and P values (in brackets). The prefix ME in each module name stands for module eigengene. **B**, Scatterplot of METurquoise module membership (x axis) vs coefficient of correlation between individual gene expression level and %D@4h (y axis). Each gene is indicated by an open dot in turquoise. **C**, Effect of the METurquoise module hub gene *SLC25A36* on VSMC apoptosis. Data shown are mean (\pm SEM) values of the percentage of dead cells (propidium iodide positive) at 4 hours after staurosporine treatment (%D@4h) of VSMCs transfected with either *SLC25A36* siRNA or negative control siRNA relative to nontransfected VSMCs ($n=5$; VSMCs from 5 different individuals; P value is from a 2-tailed Mann-Whitney test). *SLC25A36* knockdown in VSMCs transfected cells is shown in Figure S6. **D**, Biological pathways enriched in the METurquoise module. ECM indicates extracellular matrix; EGF, epidermal growth factor; FDR, false discovery rate; FSGS, focal segmental glomerulosclerosis; NA@30m and NA@60m, change in nuclear area at 30 and 60 minutes after staurosporine treatment; NF@30m and NF@60m, change in nuclear fragmentation index at 30 and 60 minutes after treatment with the apoptosis inducer staurosporine; %D@8h, percentage of dead cells (propidium iodide positive) at 8 hours after staurosporine treatment; and TT50D, time in minutes for 50% of cells to become propidium iodide positive after staurosporine treatment.

the CAD GWAS signal. Furthermore, our study identified 80 other genes with eQTL signals that significantly colocalized with CAD signals, which have not been reported in the literature and thus represent novel findings.

The novel finding of >80 candidate causal genes provides a broader understanding of the biological effects underlying the genetic associations with CAD. Currently known functions (if any) of these genes are summarized in Table S19. Pathway analyses of the 84 candidate causal genes showed enrichment of important functional pathways. Of particular interest is the TGF β signaling pathway, which includes the candidate causal genes *TGFB1*, *SMAD3*, *BMP1*, and *BMPR2*. TGF β signaling acts through the intracellular mediator SMAD3 and can interact with the BMPR2-mediated BMP signaling pathway.³³ Previous investigations have indicated that TGF β and BMP signaling can modulate

VSMC behavior and is implicated in atherosclerosis and CAD.^{34,35} Another pathway of interest is related to type I collagen synthesis, which includes the candidate causal genes *BMP1*, *MIA3*, and *SERPINH1*. Studies have shown that type I collagen affects VSMC proliferation³⁶ and plays a key role in atherogenesis.³⁷ Our present study provides genetic data further indicating the importance of these pathways/genes in modulating VSMC behavior and in atherosclerosis.

Furthermore, several other candidate causal genes are also known to influence VSMC behavior and are involved in atherosclerosis. For example, a recent study revealed a role for TCF21 in VSMC phenotypic modulation, demonstrated that TCF21 plays a protective role against atherosclerosis, and showed that increased TCF21 expression is associated with decreased CAD risk.³⁸ In support, our study observed an eQTL effect of

the reported CAD-associated variant rs2327429³¹ on *TCF21* expression in VSMCs, with the nonrisk allele (C) having higher *TCF21* expression than the CAD risk (T).

Growing evidence indicates that lncRNAs play important roles in many biological processes and diseases, including atherosclerosis,³⁹ and a number of lncRNAs have been identified as potential therapeutic targets.⁴⁰ In this study, we observed eQTL effects of CAD-associated variants on lncRNA expression at many loci, for example, *CDKN2B-AS1* (Table S4) on chromosome 9p21, the locus that is more significantly associated with CAD in GWASs than any other locus.^{1,3,30,31} Furthermore, analyses of colocalizations of CAD association with eQTL signals in VSMCs indicated that several lncRNAs (*TDRKH-AS1*, *MIA3-AS*, *ATP2B1-AS*, *EIF2B2-AS*, *MAP1S-AS*, *HCG27-AS*, ENSG00000226453, and ENSG00000256879) are candidate causal genes. In addition, our study suggests a relationship of some of these lncRNA genes with VSMC behavior. For example, we observed that the CAD risk allele of rs11810571^{30,31} was associated with decreased *TDRKH-AS1* expression in VSMCs and that *TDRKH-AS1*, which was one of the genes in the MEgreen module in the WGCNA analysis, was inversely associated with VSMC proliferation.

Among the 84 candidate causal genes, 38 are classified as druggable in the Drug Gene Interaction Database,²⁵ indicating that these genes are potential therapeutic targets. The candidate causal genes in the TGFβ/BMP signaling pathway (*TGFB1*, *SMAD3*, *BMP1*, and *BMPR2*) and *TCF21* highlighted previously are among the list of druggable genes. Furthermore, 13 of the 84 candidate causal genes had existing evidence of drug-gene interactions. These findings are likely to have utility in therapeutics development; recent studies have indicated that using genetic data to select the best targets can significantly increase the likelihood of success in the development of new drugs.^{41–43}

In a recent study, Aherrahrou et al¹⁹ observed nominal associations of 79 CAD loci with VSMC phenotypes. Our study also detected suggestive associations of variants at 139 CAD loci with parameters of VSMC behavior. However, the experimental conditions and measurements of VSMC behavior of these 2 studies were different. Aherrahrou et al¹⁹ measured VSMC proliferation in response to platelet-derived growth factor-BB, TGFβ1, or interleukin-1β; VSMC migration induced by platelet-derived growth factor-BB; and VSMC calcification. The VSMC behavior assays of our study were proliferation of nonstimulated cells, migration of nontreated cells, and apoptosis. Therefore, the results of these 2 studies are not directly comparable. Nevertheless, both studies have provided new information indicating that a considerable number of CAD loci likely act, in part, through genetic influences on VSMC behavior.

Studies have indicated that a large proportion of CAD heritability in humans is attributable to the collective poly-

genic effect of variants at many genetic loci, each having a moderate contribution.⁸ It is plausible that the polygenic influence of CAD susceptibility is a result of polygenic effects on the function/behavior of the various types of cells that are involved in the pathogenesis of CAD. The presence of a polygenic influence on VSMC behavior is evident from previous and our present studies, which have collectively shown the influences of genetic variants at many different CAD loci on VSMC behavior.^{10–19} The polygenic score analysis of the CAD-associated variants that showed nominal associations with VSMC behavior in the present study indicated that these variants collectively explained up to 5.94% of the between-sample variation of VSMC behavior. This is likely to be an underestimate because of the in vitro nature of the VSMC behavior assays. Nonetheless, these results support the notion of a polygenic influence on VSMC behavior.

The WGCNA of this study likely represents the most comprehensive analysis of coexpressed gene modules in VSMCs to date. The results of this analysis indicate that VSMC proliferation, migration, and apoptosis are associated with various coexpressed gene modules; for example, proliferation is inversely associated with the MEgreen module, whereas apoptosis is inversely correlated with the MEturquoise module. We experimentally validated the effects of the hub gene (*YIPF6*) of the MEgreen module and the hub gene (*SLC25A36*) of the MEturquoise module, demonstrating that knockdown of *YIPF6* increased VSMC proliferation and knockdown of *SLC25A36* promoted VSMC apoptosis. Therefore, the results of our study suggest that *YIPF6* inhibits cell proliferation, which is in line with the finding of a previous study that overexpressing *YIPF6* in a prostate carcinoma epithelial cell line reduced cell proliferation.⁴⁴ It has been shown that *YIPF6* interferes with the reassembly of the Golgi apparatus,⁴⁵ which could be a reason for the inhibitory effect of *YIPF6* on cell proliferation. With regard to *SLC25A36*, the results of our study indicate that it has an antiapoptotic effect. *SLC25A36* is an intracellular transporter that imports/exports pyrimidine nucleotides into and from mitochondria, thereby playing an important role in mitochondrial DNA and RNA synthesis.⁴⁶ A recent study has shown that *SLC25A36* deficiency causes a reduction in mitochondrial DNA and a decrease of mitochondrial membrane potential.⁴⁷ It is well established that the loss of mitochondrial membrane potential is a key trigger of apoptosis.⁴⁸

Conclusions

This study, with integrative analysis of genetic, transcriptomic, and cell behavior data from a large bank of VSMCs, identified 84 genes that may modulate CAD risk through VSMCs, a number of which (eg, *TGFB1*, *SMAD3*, *BMP1*, and *BMPR2*) are likely connected in functional pathways that influence VSMC behavior.

Among the candidate causal genes identified, 38 are potentially druggable and therefore represent promising therapeutic targets. These findings could be used to inform the development of new treatments that locally target VSMCs and complement current interventions that address conventional CAD risk factors.

ARTICLE INFORMATION

Received November 16, 2021; accepted May 24, 2022.

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Acknowledgments

The authors thank Martha Hardy for her technical assistance in DNA and RNA extraction from VSMCs and the Oxford Genomics Centre at the Wellcome Centre for Human Genetics (funded by Wellcome Trust grant reference 203141/Z/16/Z) for the generation and initial processing of the Global Screening Array data.

Sources of Funding

This work was funded by the British Heart Foundation (RG/16/13/32609, RG/19/9/34655, PG/16/9/31995, PG/18/73/34059, and SP/19/2/344612), the National University of Singapore and the National University Health System Internal Grant Funding (NUHSRO/2022/004/Startup/01), and the National Natural Science Foundation of China (81370202, 82070466, and 82000341). This work falls under the portfolio of research conducted within the National Institute for Health Research Leicester Biomedical Research Centre.

Disclosures

None.

Supplemental Material

Expanded Methods

Figures S1–S13

Tables S1–S19

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