HHS Public Access

Author manuscript

Arterioscler Thromb Vasc Biol. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2015 June ; 35(6): 1472–1479. doi:10.1161/ATVBAHA.115.305534.

A myocardial infarction-associated SNP at 6p24 interferes with MEF2 binding and associates with *PHACTR1* expression levels in human coronary arteries

Mélissa Beaudoin[#1], **Rajat M. Gupta**[#2,3], **Hong-Hee Won**[4,5,6,7], **Ken Sin Lo**[1], **Ron Do**[4,5,6,7], **Christopher A. Henderson**[2], **Claire Lavoie-St-Amour**[1], **Simon Langlois**[1], **Daniel Rivas**[8], **Stephanie Lehoux**[8], **Sekar Kathiresan**[4,5,6,7], **Jean-Claude Tardif**[1,9], **Kiran Musunuru**[2,3], and **Guillaume Lettre**[1,9]

[1]Montreal Heart Institute, 5000 Bélanger Street, Montréal, Québec, H1T 1C8, Canada.

[2]Department of Stem Cell and Regenerative Biology, Harvard University, and Harvard Stem Cell Institute, Cambridge, Massachusetts 02138, USA.

[3]Division of Cardiovascular Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA.

[4]Center of Human Genetic Research, Massachusetts General Hospital, Boston, MA, USA.

[5]Cardiovascular Research Center, Massachusetts General Hospital, Boston, MA, USA.

[6]Department of Medicine, Harvard Medical School, Boston, MA, USA.

[7]Program in Medical and Population Genetics, Broad Institute, Cambridge, MA, USA.

[8]Lady Davis Institute for Medical Research, McGill University, 3755 Côte Sainte-Catherine, Montreal, Quebec, H3T 1E2, Canada.

[9]Department of Medicine, Université de Montréal, 2900 Boul. Édouard-Montpetit, Montréal, Québec, H3T 1J4, Canada.

[#] These authors contributed equally to this work.

Abstract

Objective—Coronary artery disease (CAD), including myocardial infarction (MI), is the main cause of death in the world. Genome-wide association studies (GWAS) have identified dozens of single nucleotide polymorphisms (SNPs) associated with CAD/MI. One of the most robust CAD/MI genetic associations is with intronic SNPs in the gene *PHACTR1* on chromosome 6p24. How these *PHACTR1* SNPs influence CAD/MI risk, and whether *PHACTR1* itself is the causal gene at the locus, is currently unknown.

Approach and results—Using genetic fine-mapping and DNA re-sequencing experiments, we prioritized an intronic SNP (rs9349379) in *PHACTR1* as causal variant.We showed that this

Corresponding author: Guillaume Lettre, Tel: 514-376-3330, Fax: 514-593-2539, guillaume.lettre@umontreal.ca..

Disclosures

None.

variant is an expression quantitative trait locus (eQTL) for *PHACTR1* expression in human coronary arteries. Experiments in endothelial cell extracts confirmed that alleles at rs9349379 are differentially bound by the transcription factors MEF2. We engineered a deletion of this MEF2 binding site using CRISPR/Cas9 genome-editing methodology. Heterozygous endothelial cells carrying this deletion express 35% less *PHACTR1*. Finally, we found no evidence that *PHACTR1* expression levels are induced when stimulating human endothelial cells with VEGF, TNFα or shear stress.

Conclusions—Our results establish a link between intronic SNPs in *PHACTR1*, MEF2 binding and transcriptional functions at the locus, *PHACTR1* expression levels in coronary arteries and CAD/MI risk. Because *PHACTR1* SNPs are not associated with the traditional risk factors for CAD/MI (*e.g.* blood lipids or pressure, diabetes), our results suggest that *PHACTR1* may influence CAD/MI risk through as yet unknown mechanisms in the vascular endothelium.

Keywords

PHACTR1; genetic association study; eQTL; myocardial infarction; coronary artery disease

Introduction

Coronary artery disease (CAD), including myocardial infarction (MI), remains the main cause of death and disability worldwide despite a large number of efficient drugs to manage the traditional risk factors (*e.g.* blood lipids, blood pressure, heart rate)[1]. Recently, several new drugs have failed to show efficacy in large-scale clinical trials[2-4], emphasizing the need for new therapeutic targets. CAD/MI is heritable and the functional study of its genetic determinants could yield new biological pathways important for disease etiology. Genome-wide association studies (GWAS) have already identified 45 single nucleotide polymorphisms (SNPs) robustly associated with CAD, including several markers that are not associated with the CAD epidemiological risk factors such as hypertension, dyslipidemia and type 2 diabetes[5]. One of these CAD/MI loci is located on chromosome 6p24 and is defined by a group of SNPs in linkage disequilibrium (LD) within the third intron of the gene *PHACTR1*. In addition to the genetic association between *PHACTR1* SNPs and MI[6] or CAD[5, 7-9], these 6p24 SNPs have also been robustly associated with coronary artery calcification[10], coronary artery stenosis[11], migraine[12], hemodynamic indexes[13], and cervical artery dissection[14].

It is currently unknown if *PHACTR1* is the causal gene at the locus, and if causal, what are the mechanisms underlying its effect on CAD/MI. The protein encoded by *PHACTR1* was initially identified in a yeast two-hybrid screen as a protein phosphatase 1 (PP1) interactor[15]. It is abundantly expressed in the nervous system, modulates PP1 phosphatase activity *in vitro* and also interacts with actin[15]. In human umbilical vein endothelial cells (HUVEC), it has been reported that *PHACTR1* expression is induced upon treatment with vascular endothelial growth factor (VEGF)[16] and that *PHACTR* depletion induces apoptosis and decreases tube formation[17]. In NIH3T3 fibroblasts, ectopically expressed *PHACTR1* translocates to the nucleus following serum stimulation and this translocation depends on the competition of PP1 and G-actin for the PHACTR1 RPEL motifs and C-terminal domain[18]. Disruption of these domains inhibits the cytoplasmic/nuclear localization of PHACTR1 and

impairs actomyosin assembly[18]. Recently, it was shown that PHACTR1 acts downstream of TGF-β to mediate actin reorganization and migration of breast cancer cells[19].

The aim of our study was to determine if *PHACTR1* is a good biological candidate for a role in CAD/MI. Here, we show that CAD/MI-associated SNPs at 6p24 correlate with *PHACTR1* expression levels in coronary arteries. This result suggests that changes in *PHACTR1* expression levels may modulate CAD/MI risk. We fine-mapped the association signal to a SNP in the third intron of *PHACTR1*, and showed that its alleles disrupt binding of the transcription factors MEF2. Deleting this MEF2 binding site using CRISPR/Cas9 reduces *PHACTR1* expression in endothelial cells. Our findings support a possible role for PHACTR1 in the vascular endothelium and provide a framework to further understand how *PHACTR1* SNPs, which are not associated with the epidemiological risk factors of cardiovascular diseases, can influence CAD/MI risk.

Materials and Methods

Materials and methods are available in the online-only Data Supplement.

Results

SNPs at *PHACTR1* associate with MI in French Canadians

To characterize the genetic risk factors of CAD/MI in French Canadians, we genotyped SNPs previously associated with MI or CAD in 1176 MI cases and 1996 controls selected from the Montreal Heart Institute (MHI) Biobank (**Supplemental Table I**)[5]. Of the 45 SNPs that we genotyped successfully, 35 had an odds ratio (OR) consistent with the literature (binomial $P=1.2\times10^{-4}$) and 12 of these 35 SNPs were nominally significant (one-tailed $P<0.05$, binomial $P=1.5\times10^{-6}$)(**Supplemental Table II**). The strongest genetic association with MI risk that we observed was with rs12526453, a SNP located in the third intron of *PHACTR1* (G-allele OR=0.79, $P=8.4\times10^{-4}$)[6]. To fine-map the genetic association between the *PHACTR1* locus and MI risk in our population, we selected and genotyped 13 additional SNPs that cover common genetic variation at the locus based on genetic variation patterns in European-ancestry individuals from the 1000 Genomes Project (**Supplemental Table III**)[20, 21]. We also imputed ungenotyped markers using reference haplotypes from the 1000 Genomes Project[21, 22]. After filtering on imputation quality, we analyzed association between MI and genotypes for 387 DNA sequence variants (**Supplemental Table IV**). The strongest association with MI was with a genotyped SNP, rs9349379, located ~24 kilobases (kb) downstream of rs12526453 but still in intron 3 of *PHACTR1* (G-allele OR=1.37, $P=8.4\times10^{-6}$)(**Figure 1** and **Table 1**)[23]. When we conditioned on genotypes at rs9349379, no additional DNA markers were significantly associated with MI at the *PHACTR1* locus ($P>0.10$).

We tested association between genotypes at *PHACTR1* rs9349379 and several risk factors for MI in the MHI Biobank samples: hypertension and blood pressure, type 2 diabetes, LDL- and HDL-cholesterol levels, and smoking[24]. After accounting for the number of phenotypes tested, none of the associations were significant (**Table 1**), consistent with results from large meta-analyses of genome-wide association results for these traits[25-27].

This suggests that genetic variation at the *PHACTR1* locus may influence MI risk through an unanticipated risk factor.

Coding variants in *PHACTR1* are not associated with MI

We tried to link genetic variation at the *PHACTR1* locus with a specific gene, focusing initially on the *PHACTR1* gene itself. Identification of rare and functional DNA sequence variants by exon re-sequencing can be used to establish gene causality[28, 29]. As part of a parallel study, we re-sequenced all 14 exons of *PHACTR1* in 500 early-onset MI cases and 500 matched controls from the MHI Biobank (**Supplemental Table V**)[30]. We identified four novel rare exonic variants: two missense variants (ss836901033 (p.Ser190Pro) and ss836901061 (p.Glu196Lys)), as well as a 3' splice site variant (exon 12, ss836901074) and a 3'UTR variant (ss836901090)(**Supplemental Table III**). We also queried data from the 1000 Genomes Project and found three additional rare non-synonymous DNA sequence variants: one frameshift indel (rs36000655) and two missense variants (rs61746695 (p.Arg94Pro) and rs17602409 (p.Ile247Met)).

We genotyped six of these markers (the 3' splice site variant ss836901074 failed assay design) in the MHI Biobank MI panel. The three 1000 Genomes Project variants were monomorphic in our DNA collection. After excluding the re-sequenced samples, the carrier frequencies for the remaining three *PHACTR1* variants (ss836901033, ss836901061, ss836901090) were 1.0% in MI cases and 0.7% in controls (0.3% and 0.1% in cases and controls, respectively, when restricting to missense variants only). The gene-based MI association results for the three coding variants or only the two missense variants were, respectively, P=0.38 and P=0.089 (**Materials and Methods**). We also genotyped the same three rare *PHACTR1* coding variants in an additional subset of the MHI Biobank (870 MI cases and 1494 controls). The carrier frequencies were 1.4% in cases and 1.3% in controls for the three variants (P=0.95) and 0.2% in cases and 0.3% in controls for the two missense variants alone (P=0.51). Consistent with our results, rare coding variants in *PHACTR1* were not associated with MI risk in a recent large-scale whole-exome sequencing project carried out in 4,703 MI cases and 5,090 controls (gene-based P>0.3, **Supplemental Table VI**)[31]. Thus, we cannot conclude that rare coding genetic variation implicates *PHACTR1* in MI.

PHACTR1 rs9349379 is an eQTL in human coronary arteries

Correlation between SNP genotypes and gene expression levels has also been used to identify causal genes involved in complex human diseases[32]. *PHACTR1* was originally identified as a gene highly expressed in the nervous system[15]. We screened several human tissues and cell lines and detected *PHACTR1* expression in the heart, aorta, and primary endothelial cells (HUVEC)(**Figure 2A**).

Based on these expression results and the reported association between *PHACTR1* SNPs and CAD/MI, we obtained and extracted RNA from 25 human right coronary arteries to measure *PHACTR1* transcript levels. These samples were from patients undergoing heart transplant at the MHI (**Supplemental Table VII**). We also genotyped *PHACTR1* rs9349379 in the DNA of the same patients. We detected an association between genotypes at rs9349379 and *PHACTR1* expression levels in this tissue (r^2=0.21, P=0.018)(**Figure 2B**). We also

measured in the same coronary artery samples the expression of all coding genes located 1 megabase on either side of rs9349379 and found no association with genotypes at this marker (**Supplemental Figure I**). Thus, the association between genotypes and expression levels seems to be specific to *PHACTR1* within this locus in human coronary arteries.

We genotyped and imputed *PHACTR1* SNPs in the DNA extracted from the 25 right coronary artery donors, as described above (**Materials and Methods**, **Supplemental Table III**). When we conditioned on genotypes at rs9349379, no other markers were significantly associated with *PHACTR1* expression levels, consistent with the MI genetic association results (**Supplemental Table VIII**). This suggests that rs9349379 is an expression quantitative trait locus (eQTL) for *PHACTR1* in human right coronary arteries, providing an argument in favor of *PHACTR1* being at least one of the genes at the 6p24 locus implicated in CAD/MI development. Because we could not identify an independent collection of human coronary arteries to replicate this eQTL effect, we turned to functional characterization to understand how genotypes at rs9349379 may modulate *PHACTR1* expression levels.

rs9349379 alleles modulate MEF2 binding at the *PHACTR1* locus

There are no genetic variants in the 1000 Genomes Project European populations in strong LD with rs9349379 (no variants with $r^2 \geq 0.8$). We queried data from the ENCODE and Roadmap Epigenomics Projects to identify how genotypes at rs9349379 can influence *PHACTR1* expression[33]. There is no functional annotation in ENCODE for rs9349379. In the Roadmap Epigenomics data, chromatin state predictions based on histone tail modifications suggest that rs9349379 is located in a transcriptional enhancer in skeletal and stomach smooth muscle. However, we could not measure *PHACTR1* expression in intestine smooth muscle, highlighting the importance to validate these bioinformatic predictions by direct biological experiments (**Figure 2A**). Although the Roadmap Epigenomics Project plans on testing several relevant human tissues for CAD/MI, notably the heart and aorta, these results are not yet publicly available.

Additional *in silico* searches revealed that rs9349379 lies within a predicted binding site for the transcription factors myocyte enhancer factor-2 (MEF2). MEF2 is a family of transcription factors encoded by four genes in humans (*MEF2A-D*) and is important for cellular differentiation and stress response[34]. A previous report suggested that genetic variation in *MEF2A* might predispose to CAD risk [35], although this result is controversial[36, 37]. In the presence of the A-allele at rs9349379, the DNA sequence matches perfectly the canonical MEF2 binding site except for a C-to-A change at the 5' end of the motif (**Figure 3**). The G-allele at rs9349379, associated with lower *PHACTR1* expression in right coronary arteries, disrupts the MEF2 binding motif (**Figure 3**). To determine if rs9349379 is bound by MEF2, we performed electromobility shift assays (EMSA) with HUVEC nuclear extracts. These experiments showed that the probe with the A-allele at rs9349379, but not the G-allele, is shifted by proteins in the nuclear extract (**Figure 3**). When we added an antibody that recognizes either MEF2A or MEF2C, we observed a supershift of Probe A, indicating that MEF2 is one of the binding proteins (**Figure 3**). Furthermore, the interaction is specific as addition of excess unlabeled Probe A, but not

Probe G, could efficiently disrupt the shift (**Figure 3**). Although we cannot completely rule out a role for MEF2C in the observed supershift, siRNA-mediated knockdowns indicate that MEF2A is the most abundant MEF2 transcription factor in HUVEC that is recognized by the anti-MEF2 antibody (**Supplemental Figure II**). When we reduced the expression of *MEF2A* or *MEF2C* using siRNA in HUVEC, we did not detect a reduction in *PHACTR1* expression levels. However, the incomplete knockdowns of *MEF2A* and *MEF2C* (**Supplemental Figure II**), or other compensatory mechanisms, might explain the lack of effect on *PHACTR1* expression.

The MEF2 binding site at rs9349379 controls *PHACTR1* expression *in vivo*

To provide further *in vivo* evidence that the MEF2 binding site that overlaps with rs9349379 is important to modulate the expression of *PHACTR1* in endothelial cells, we engineered its deletion using the CRISPR/Cas9 system (**Figure 4A**). Starting with human embryonic stem cells (hESCs), we introduced a guide RNA that specifically targets rs9349379. After screening, we identified a clone that carries a heterozygous 34-base pairs (bp) deletion that removes rs9349379 as well as all but one bp of the MEF2 binding site (**Figure 4A**). We then differentiated wild-type and heterozygous hESCs into endothelial cells using an established protocol (**Materials and Methods**)[38, 39]. Endothelial cells that carry the 34-bp deletion at rs9349379 express 35% less *PHACTR1* than cells homozygous for the high-expressing A-allele at rs9349379 (**Figure 4B**). This represents a promising *in vivo* validation of the potential regulatory role of rs9349379 in the control of *PHACTR1* expression in the vascular endothelium. This result is also consistent with our eQTL result.

No ectopic induction of *PHACTR1* expression in HUVEC

In a previous report, VEGF treatment was shown to induce *PHACTR1* expression in HUVEC[17]. We attempted to reproduce this result using a similar protocol. First, we confirmed that our HUVEC can respond to VEGF by demonstrating the phosphorylation of the mitogen-activated protein kinase (MAPK) ERK upon VEGF treatment (**Supplemental Figure III**). However, we could not measure by quantitative PCR a significant difference in *PHACTR1* expression levels using different VEGF concentrations and induction times (**Figure 5A**). We also tested the effect of tumor necrosis factor (TNF)-α, an inflammatory molecule that can trigger endothelial dysfunction, and shear stress, a stimulus known to protect blood vessels from atherosclerosis, on *PHACTR1* expression levels in HUVEC. In both cases, we showed induction of the positive controls, *NFKB1* for TNFα and *KLF2* for shear stress. However, we again did not measure a significant change in *PHACTR1* expression levels (**Figures 5B-C**).

Discussion

The genetic association between intronic SNPs in *PHACTR1* and CAD/MI is robust, relatively strong for a GWAS finding (odds ratio for the risk allele at rs9349379 is 1.37 in the MHI Biobank) and pleiotropic (the same SNPs are also associated with coronary artery calcification and stenosis, migraine, hemodynamic indexes, and cervical artery dissection). It is different from many of the known CAD/MI-associated SNPs because it is not associated with the traditional risk factors such as lipid levels, blood pressure or diabetes.

This observation is promising, inasmuch as understanding how this genetic variation influences CAD/MI risk may yield new insights into the biology of atherosclerosis and potentially, in the long-term, new therapeutic strategies.

To translate this genetic discovery, we first need to connect genetic variants with genes. Our own DNA re-sequencing project and a large whole-exome sequencing effort failed to identify coding variants in *PHACTR1* that might directly implicate this gene in MI[30, 31]. Previous eQTL experiments to link CAD/MI-associated *PHACTR1* SNPs with its expression levels have equally been unsuccessful despite large sample sizes and a wide variety of tissues tested (leukocytes (including monocytes), liver, fat, skin, omentum, aortic media and adventitia, mammary artery, and lymphoblastoid cell lines)[5, 40]. Reasoning that the transcriptional effect might be tissue-specific, we measured an association between genotypes at rs9349379 and *PHACTR1* expression levels in human coronary arteries. This is an important result because it supports *PHACTR1* as a potential causal gene at the locus and suggests that low *PHACTR1* expression levels in coronary arteries increase CAD/MI risk. Although coronary artery is not a homogenous tissue, the lack of *PHACTR1* expression in smooth muscle and of eQTL effect in leukocytes support the idea that the vascular endothelium may be the most relevant tissue to study how PHACTR1 functions influence CAD/MI.

A recent report showed that the G-allele at *PHACTR1*-rs9349379 is associated with lower risk of cervical artery dissection[14]. The same G-allele is also associated with reduced migraine risk[12], a known risk factor for cervical artery dissection[41]. These results are in sharp contrast with the reported association between the G-allele at rs9349379 and increased risk of CAD or MI. This is not the first report of a genome-wide genetic association of opposite effects of the same SNP on two diseases[42]. We will need to understand PHACTR1 biological activities to explain its opposite roles in cervical artery dissection and CAD/MI. Maybe PHACTR1 modulates a downstream pathway differently in carotid or cervical arteries than in coronary arteries? Or maybe it affects different biological pathways in these different artery tissues? Although to our knowledge it has not been tested, we would predict that rs9349379 would be an eQTL for *PHACTR1* expression levels in human carotid and/or cervical arteries.

High-density genotyping and bioinformatic analyses pinpointed rs9349379 as a potential causal variant at the locus. Using *in vitro* assays, we showed that alleles at this intronic *PHACTR1* SNP bind differentially members of the MEF2 transcription factor family. Furthermore, deletion of the MEF2 binding site that overlaps with rs9349379 reduced *PHACTR1* expression in endothelial cells. A small exonic 21-bp deletion in *MEF2A* was originally described to cause familial CAD, although this result has not been widely replicated[35-37]. It is important to emphasize that our results are not invalidated by the lack of consistent association between genetic variation at *MEF2A* and CAD. First, we do not know if MEF2A or MEF2C (or both) recognize *in vivo* the binding motif that we characterized at rs9349379. Second, we do not need variation in *MEF2* expression levels or activities to explain the variation in *PHACTR1* expression levels. Changes in *PHACTR1* expression levels are controlled, at least in part, by genotypes at rs9349379. MEF2 transcription factors

remain good candidates for a role in atherosclerosis as they are present in the endothelium and are involved in maintaining vascular integrity[34].

A previous report suggested that *PHACTR1* expression levels in HUVEC can be induced upon VEGF treatment, but we were not able to reproduce this result despite trying different VEGF concentrations and induction times[17]. More generally, we explored if "classic" endothelial stimuli (VEGF, TNFα, and shear stress) can induce *PHACTR1* expression. Under our stimulation protocols, we failed to ectopically change *PHACTR1* expression levels in HUVEC. These negative observations are informative in guiding future experiments to determine if other endothelial stresses, potentially through MEF2 activation, may act synergistically with genotypes at rs9349379 to influence CAD/MI risk. In conclusion, our results provide an initial model (CAD/MI-associated SNPs at 6p24 affects MEF2 binding and consequently *PHACTR1* expression levels) and a candidate tissue (vascular endothelium) to further characterize how PHACTR1 influences atherosclerosis independently of the traditional CAD/MI risk factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all participants and staff of the Montreal Heart Institute (MHI) Biobank. We acknowledge the technical support of the Beaulieu-Saucier MHI Pharmacogenomic Center for DNA sequencing and genotyping. We thank Chantal St-Cyr from the "Réseau d'échange de tissus et d'échantillons biologiques (RÉTEB)" for providing human coronary artery samples.

Sources of funding

This work was funded by the Centre of Excellence in Personalized Medicine (CEPMed), the "Fonds de Recherche Santé Québec" (FRQS), the Canadian Institute of Health Research (CIHR-MOP136979) and the MHI Foundation (to G.L.), and the CIHR-MOP102489 (to S. Lehoux). S. Lehoux, J.C.T. and G.L. are Canada Research Chair holders. Sequencing of *PHACTR1* was performed by the Myocardial Infarction Genetics Exome Sequencing Consortium with support from the National Heart, Lung, and Blood Institute (NHLBI) and the National Human Genome Research Institute (NHGRI) of the U.S. National Institutes of Health (NIH). Grant support included RC2 HL-103010, RC2 HL-102923, RC2 HL-102924, RC2 HL-102925, RC2 HL-102926 and 5U54HG003067-11. S.K. is supported by a Research Scholar award from the Massachusetts General Hospital (MGH), the Howard Goodman Fellowship from MGH, the Donovan Family Foundation, R01HL107816, and a Transatlantic Networks of Excellence award from Fondation Leducq. R.D. is supported by a Banting Fellowship from the CIHR. K.M. is supported by Grant R01-GM104464 from the United States National Institutes of Health (NIH) and a Cardiovascular Program Pilot Grant from the Harvard Stem Cell Institute. R.G. is supported by the LaDue Fellowship at Harvard University and the Sarnoff Foundation Scholar Award. H.-H. Won is supported by a postdoctoral award from the American Heart Association (15POST23280019).

Abbreviations

bp	base pairs
CAD	coronary artery disease
CEU	Utah Residents with Northern and Western European Ancestry
EMSA	electromobility shift assay
eQTL	expression quantitative trait locus

GWAS	genome-wide association study
gDNA	genomic DNA
HDL	high-density lipoprotein
HRM	high-resolution melting
hESC	human embryonic stem cell
HUVEC	human umbilical vein endothelial cells
KLF2	kruppel-like factor-2
LDL	low-density lipoprotein
MAF	minor allele frequency
MAPK	mitogen activated protein kinase
MHI	Montreal Heart Institute
MI	myocardial infarction
MEF2	myocyte enhancer factor-2
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
PHACTR1	phosphatase and actin regulator-1
siRNA	short interfering RNA
SKAT	sequence kernel association test
SNP	single nucleotide polymorphism
TBP	TATA-box binding protein
TGF-β	transforming growth factor-β
TNFα	tumor necrosis factor-α
VEGF	vascular endothelial growth factor

References

1. Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ. Global and regional burden of disease and risk factors, 2001: Systematic analysis of population health data. Lancet. 2006; 367:1747–1757. [PubMed: 16731270]
2. Barter PJ, Caulfield M, Eriksson M, et al. Effects of torcetrapib in patients at high risk for coronary events. The New England journal of medicine. 2007; 357:2109–2122. [PubMed: 17984165]
3. Nissen SE, Tardif JC, Nicholls SJ, Revkin JH, Shear CL, Duggan WT, Ruzyllo W, Bachinsky WB, Lasala GP, Tuzcu EM. Effect of torcetrapib on the progression of coronary atherosclerosis. The New England journal of medicine. 2007; 356:1304–1316. [PubMed: 17387129]
4. Schwartz GG, Olsson AG, Abt M, et al. Effects of dalcetrapib in patients with a recent acute coronary syndrome. The New England journal of medicine. 2012; 367:2089–2099. [PubMed: 23126252]
5. Deloukas P, Kanoni S, Willenborg C, et al. Large-scale association analysis identifies new risk loci for coronary artery disease. Nature genetics. 2013; 45:25–33. [PubMed: 23202125]

6. Kathiresan S, Voight BF, Purcell S, et al. Genome-wide association of early-onset myocardial infarction with single nucleotide polymorphisms and copy number variants. Nature genetics. 2009; 41:334–341. [PubMed: 19198609]
7. Schunkert H, Konig IR, Kathiresan S, et al. Large-scale association analysis identifies 13 new susceptibility loci for coronary artery disease. Nature genetics. 2011; 43:333–338. [PubMed: 21378990]
8. Peden JF, Hopewell JC, Saleheen D, Chambers JC, Hager J, Soranzo N. A genome-wide association study in europeans and south asians identifies five new loci for coronary artery disease. Nature genetics. 2011; 43:339–344. [PubMed: 21378988]
9. Lu X, Wang L, Chen S, et al. Genome-wide association study in han chinese identifies four new susceptibility loci for coronary artery disease. Nature genetics. 2012; 44:890–894. [PubMed: 22751097]
10. O'Donnell CJ, Kavousi M, Smith AV, et al. Genome-wide association study for coronary artery calcification with follow-up in myocardial infarction. Circulation. 2011; 124:2855–2864. [PubMed: 22144573]
11. Hager J, Kamatani Y, Cazier JB, et al. Genome-wide association study in a lebanese cohort confirms phactr1 as a major determinant of coronary artery stenosis. PloS one. 2012; 7:e38663. [PubMed: 22745674]
12. Freilinger T, Anttila V, de Vries B, et al. Genome-wide association analysis identifies susceptibility loci for migraine without aura. Nature genetics. 2012; 44:777–782. [PubMed: 22683712]
13. Patel RS, Morris AA, Ahmed Y, Kavtaradze N, Sher S, Su S, Zafari AM, Din-Dzietham R, Waddy SP, Vaccarino V, Alexander RW, Gibbons G, Quyyumi AA. A genetic risk variant for myocardial infarction on chromosome 6p24 is associated with impaired central hemodynamic indexes. American journal of hypertension. 2012; 25:797–803. [PubMed: 22513829]
14. Debette S, Kamatani Y, Metso TM, et al. Common variation in phactr1 is associated with susceptibility to cervical artery dissection. Nature genetics. 2015; 47:78–83. [PubMed: 25420145]
15. Allen PB, Greenfield AT, Svenningsson P, Haspeslagh DC, Greengard P. Phactrs 1-4: A family of protein phosphatase 1 and actin regulatory proteins. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:7187–7192. [PubMed: 15107502]
16. Allain B, Jarray R, Borriello L, Leforban B, Dufour S, Liu WQ, Pamonsinlapatham P, Bianco S, Larghero J, Hadj-Slimane R, Garbay C, Raynaud F, Lepelletier Y. Neuropilin-1 regulates a new vegf-induced gene, phactr-1, which controls tubulogenesis and modulates lamellipodial dynamics in human endothelial cells. Cellular signalling. 2011; 24:214–223. [PubMed: 21939755]
17. Jarray R, Allain B, Borriello L, Biard D, Loukaci A, Larghero J, Hadj-Slimane R, Garbay C, Lepelletier Y, Raynaud F. Depletion of the novel protein phactr-1 from human endothelial cells abolishes tube formation and induces cell death receptor apoptosis. Biochimie. 2011; 93:1668–1675. [PubMed: 21798305]
18. Wiezlak M, Diring J, Abella J, Mouilleron S, Way M, McDonald NQ, Treisman R. G-actin regulates the shuttling and pp1 binding of the rpel protein phactr1 to control actomyosin assembly. Journal of cell science. 2012; 125:5860–5872. [PubMed: 22976292]
19. Fils-Aime N, Dai M, Guo J, El-Mousawi M, Kahramangil B, Neel JC, Lebrun JJ. Microrna-584 and the protein phosphatase and actin regulator 1 (phactr1), a new signaling route through which transforming growth factor-beta mediates the migration and actin dynamics of breast cancer cells. The Journal of biological chemistry. 2013; 288:11807–11823. [PubMed: 23479725]
20. 1000 Genomes Project Consortium. A map of human genome variation from population-scale sequencing. Nature. 2010; 467:1061–1073. [PubMed: 20981092]
21. 1000 Genomes Project Consortium. An integrated map of genetic variation from 1,092 human genomes. Nature. 2012; 491:56–65. [PubMed: 23128226]
22. Li Y, Willer CJ, Ding J, Scheet P, Abecasis GR. Mach: Using sequence and genotype data to estimate haplotypes and unobserved genotypes. Genetic epidemiology. 2010; 34:816–834. [PubMed: 21058334]

23. Pruim RJ, Welch RP, Sanna S, Teslovich TM, Chines PS, Gliedt TP, Boehnke M, Abecasis GR, Willer CJ. Locuszoom: Regional visualization of genome-wide association scan results. Bioinformatics. 2010; 26:2336–2337. [PubMed: 20634204]
24. Thomas J, Thomas DJ, Pearson T, Klag M, Mead L. Cardiovascular disease in african american and white physicians: The meharry cohort and meharry-hopkins cohort studies. Journal of health care for the poor and underserved. 1997; 8:270–283. discussion 284. [PubMed: 9253218]
25. Teslovich TM, Musunuru K, Smith AV, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature. 2010; 466:707–713. [PubMed: 20686565]
26. Ehret GB, Munroe PB, Rice KM, et al. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature. 2011; 478:103–109. [PubMed: 21909115]
27. Morris AP, Voight BF, Teslovich TM, et al. Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. Nature genetics. 2012; 44:981–990. [PubMed: 22885922]
28. Nejentsev S, Walker N, Riches D, Egholm M, Todd JA. Rare variants of ifih1, a gene implicated in antiviral responses, protect against type 1 diabetes. Science. 2009; 324:387–389. [PubMed: 19264985]
29. Galarneau G, Palmer CD, Sankaran VG, Orkin SH, Hirschhorn JN, Lettre G. Fine-mapping at three loci known to affect fetal hemoglobin levels explains additional genetic variation. Nature genetics. 2010; 42:1049–1051. [PubMed: 21057501]
30. Beaudoin M, Lo KS, N'Diaye A, Rivas MA, Dube MP, Laplante N, Phillips MS, Rioux JD, Tardif JC, Lettre G. Pooled DNA resequencing of 68 myocardial infarction candidate genes in french canadians. Circulation. Cardiovascular genetics. 2012; 5:547–554. [PubMed: 22923420]
31. Do R, Stitziel NO, Won HH, et al. Exome sequencing identifies rare ldlr and apoa5 alleles conferring risk for myocardial infarction. Nature. 2015; 518:102–106. [PubMed: 25487149]
32. Musunuru K, Strong A, Frank-Kamenetsky M, et al. From noncoding variant to phenotype via sort1 at the 1p13 cholesterol locus. Nature. 2010; 466:714–719. [PubMed: 20686566]
33. Ward LD, Kellis M. Haploreg: A resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. Nucleic Acids Res. 2012; 40:D930–934. [PubMed: 22064851]
34. Potthoff MJ, Olson EN. Mef2: A central regulator of diverse developmental programs. Development. 2007; 134:4131–4140. [PubMed: 17959722]
35. Wang L, Fan C, Topol SE, Topol EJ, Wang Q. Mutation of mef2a in an inherited disorder with features of coronary artery disease. Science. 2003; 302:1578–1581. [PubMed: 14645853]
36. Liu Y, Niu W, Wu Z, Su X, Chen Q, Lu L, Jin W. Variants in exon 11 of mef2a gene and coronary artery disease: Evidence from a case-control study, systematic review, and meta-analysis. PloS one. 2012; 7:e31406. [PubMed: 22363637]
37. Weng L, Kavaslar N, Ustaszewska A, Doelle H, Schackwitz W, Hebert S, Cohen JC, McPherson R, Pennacchio LA. Lack of mef2a mutations in coronary artery disease. The Journal of clinical investigation. 2005; 115:1016–1020. [PubMed: 15841183]
38. White MP, Rufaihah AJ, Liu L, Ghebremariam YT, Ivey KN, Cooke JP, Srivastava D. Limited gene expression variation in human embryonic stem cell and induced pluripotent stem cell-derived endothelial cells. Stem cells. 2013; 31:92–103. [PubMed: 23079999]
39. Rufaihah AJ, Huang NF, Jame S, Lee JC, Nguyen HN, Byers B, De A, Okogbaa J, Rollins M, Reijo-Pera R, Gambhir SS, Cooke JP. Endothelial cells derived from human ipscs increase capillary density and improve perfusion in a mouse model of peripheral arterial disease. Arteriosclerosis, thrombosis, and vascular biology. 2011; 31:e72–79.
40. Zhang X, Johnson AD, Hendricks AE, Hwang SJ, Tanriverdi K, Ganesh SK, Smith NL, Peyser PA, Freedman JE, O'Donnell CJ. Genetic associations with expression for genes implicated in gwas studies for atherosclerotic cardiovascular disease and blood phenotypes. Human molecular genetics. 2013; 23:782–795. [PubMed: 24057673]
41. Debette S, Leys D. Cervical-artery dissections: Predisposing factors, diagnosis, and outcome. The Lancet. Neurology. 2009; 8:668–678. [PubMed: 19539238]

42. Smyth DJ, Plagnol V, Walker NM, et al. Shared and distinct genetic variants in type 1 diabetes and celiac disease. The New England journal of medicine. 2008; 359:2767–2777. [PubMed: 19073967]

Significance

Despite having efficient therapies and prevention strategies, coronary artery diseases remain one of the main causes of death in the World. Recent failed or futile clinical trials have highlighted the need to identify new therapeutic targets. Unbiased human genetic studies can provide such new entry points into disease pathophysiology. Genome-wide association studies for coronary artery disease risk have identified a robust signal for DNA polymorphisms within the *PHACTR1* gene on chromosome 6. *PHACTR1* single nucleotides polymorphisms are not associated with traditional atherosclerosis risk factors, such as blood lipids, hypertension or diabetes. Our work establishes a link between these genetic variants and *PHACTR1* expression levels in human coronary arteries through a potential effect on the binding of the transcription factors MEF2 at the locus. These results set the stage to explore how PHACTR1 functions in the vascular endothelium influence coronary artery disease risk.

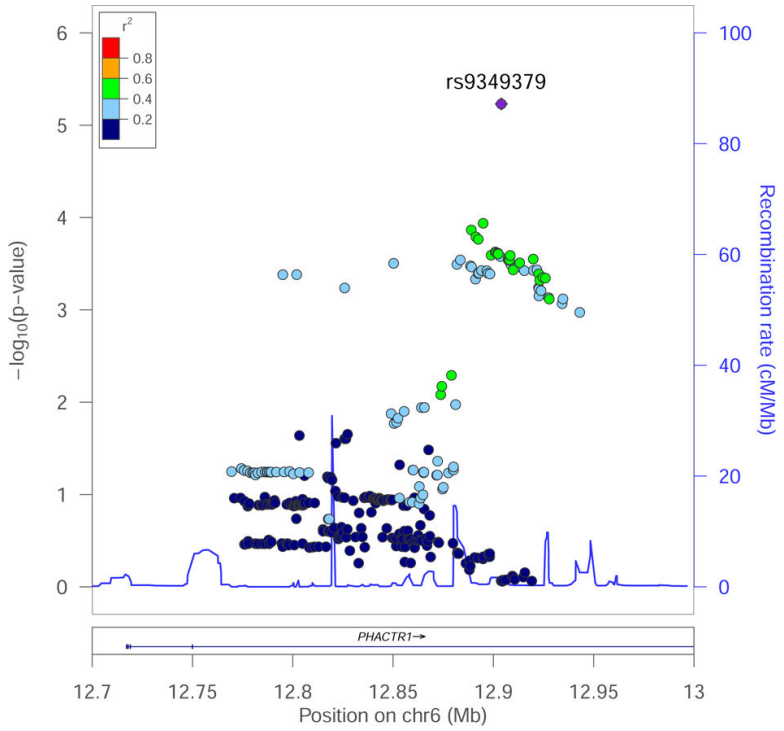

Figure 1.

Association results between 387 genotyped or imputed DNA markers in *PHACTR1* and myocardial infarction status in 3,172 French Canadians from the Montreal Heart Institute Biobank. rs9349379 has the strongest association signal (P=8.4×10^{-6}). We used the LocusZoom tool to plot association results.

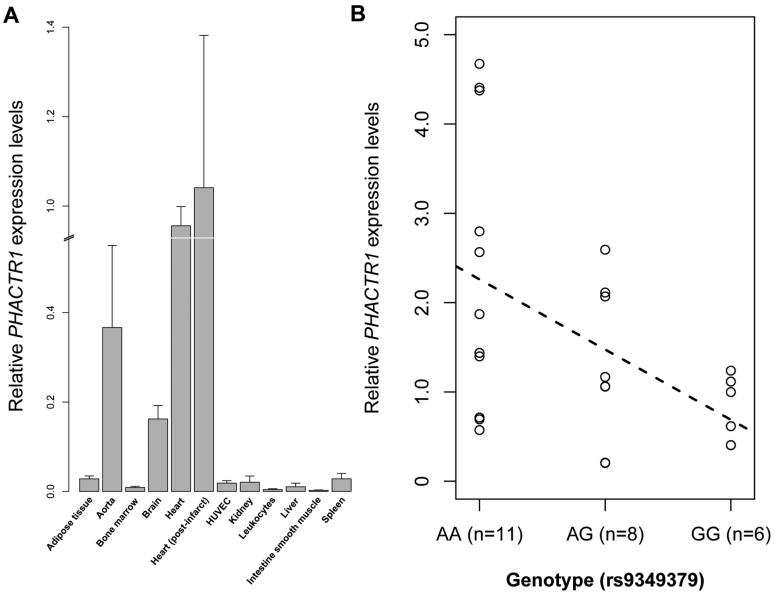

Figure 2.

PHACTR1 gene expression in human tissues. (**A**) *PHACTR1* expression levels were measured by quantitative PCR (experiment done in quadruplicates). Results were normalized on the housekeeping gene *HPRT* and calibrated on the expression in the heart (post-infarct). Error bars represent standard deviations. (**B**) rs9349379 is an expression quantitative trait locus (eQTL) for *PHACTR1* in human right coronary arteries (total n=25). The dashed line represents the best-fit regression line for *PHACTR1* expression levels.

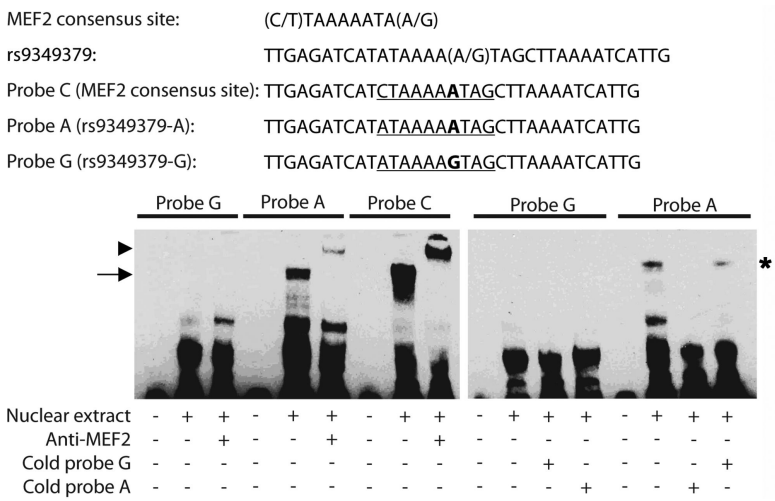

Figure 3.

PHACTR1-rs9349379 is differentially bound by MEF2. Electrophoretic mobility shift assays (EMSA) with human umbilical vein endothelial cell (HUVEC) nuclear extract. Probe C contains the canonical MEF2 binding site and acts as positive control. Probes A and G differ by their respective allele at rs9349379 : the G-allele disrupts the MEF2 consensus binding site. Only Probe A shifts in the presence of nuclear extract (arrow, left panel), and the complex supershifts when an antibody against MEF2 is added (arrowhead, left panel). The binding between Probe A and MEF2 is specific: excess unlabeled (cold) Probe A, but not unlabeled Probe G, competes and disrupts the interaction between labeled Probe A and MEF2 (star, right panel).

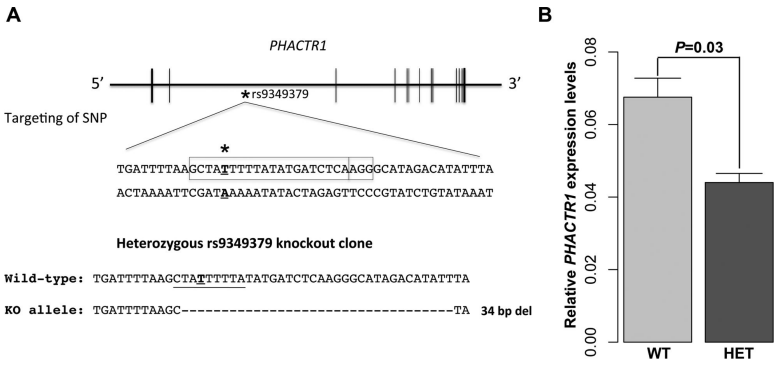

Figure 4.

A CRISPR/Cas9-induced deletion that encompasses rs9349379 and the MEF2 binding site reduces *PHACTR1* expression in endothelial cells. (**A**) Schematic representation of the *PHACTR1* locus. The star (bold, underline) corresponds to rs9349379 in intron 3 of *PHACTR1* and the box highlights the DNA sequence targeted by the CRISPR guide RNA. We isolated a heterozygous clone that carries a 34-bp deletion (KO allele) that removes rs9349379 (bold) and most of the MEF2 binding site (underline). (**B**) Human endothelial cells that are heterozygous for the *PHACTR1* 34-bp deletion (HET) express 35% less *PHACTR1* than wild-type cells (WT)(*t*-test *P*=0.03). Data shown is mean ± standard deviations. The experiment was done in triplicate.

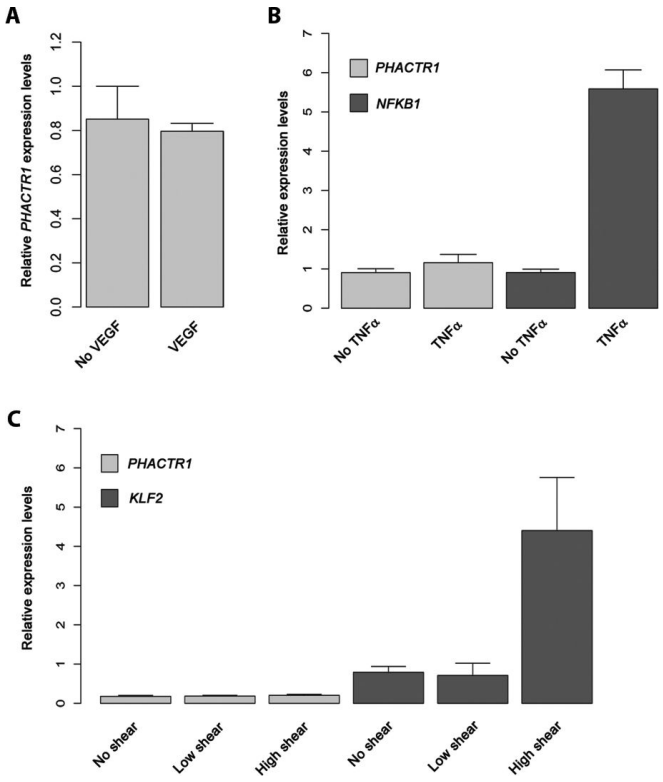

Figure 5.

Different endothelial cell stimuli do not induce *PHACTR1* expression levels as measured by quantitative PCR. (**A**) Serum-starved HUVEC were treated with VEGF (20ng/mL) for five minutes before RNA was extracted and *PHACTR1* transcript levels measured. See **Supplemental Figure III** for the positive control of this experiment. (**B**) HUVEC were treated with TNFα (10ng/mL) for 16 hours. RNA was extracted and the levels of *PHACTR1* and *NFKB1* (positive control) were quantified by quantitative PCR. (**C**) HUVEC were treated with no, low or high shear stress. RNA was extracted six hours after the beginning of the experiments, and *PHACTR1* and *KLF2* (positive control) expression levels were measured by quantitative PCR. The same results were observed after 24 hours of shear stress. For all panels, data shown is mean ± standard deviations. The VEGF and TNFα experiments were done in triplicates; we had four replicates for the shear stress experiment. All comparisons are non-significant (*t*-test $P>0.05$), except for *NFKB1* without and with TNFα treatment ($P=7.8\times10^{-5}$) and for *KLF2* without and with high shear stress ($P=0.0012$).

Table 1

Association results between rs9349379 and myocardial infarction (MI) in the Montreal Heart Institute (MHI) Biobank. Genetic associations were also tested between rs9349379 and several MI risk factors: hypertension, type 2 diabetes, smoking, systolic and diastolic blood pressure, and LDL- and HDL-cholesterol levels. For dichotomous traits (MI, hypertension, type 2 diabetes), odds ratios (OR) and 95% confidence intervals (95% CI) are provided. For blood pressure measures and cholesterol levels, effect sizes (Beta) and standard errors (SE) are in mmHg and mmol/L, respectively. The direction of effect is given for the G-allele on the positive strand (NCBI build 37.1). The rs9349379 G-allele frequency in the MHI Biobank is 37%.

Phenotype	**N**	**rs9349379 (chr6:12903957)**	
		OR (95% CI) or Beta (SE)	**P-value**
Myocardial infarction	1176 cases / 1996 controls	1.37 (1.19-1.57)	8.4×10^{-6}
Hypertension	1543 cases / 1534 controls	0.93 (0.83-1.05)	0.25
Type 2 diabetes	473 cases / 2604 controls	0.95 (0.81-1.10)	0.49
Smoking	2018 cases / 1075 controls	0.96 (0.86-1.07)	0.46
Systolic blood pressure	3060	−1.08 (0.51)	0.035
Diastolic blood pressure	3058	−0.37 (0.32)	0.25
LDL-cholesterol	1668	−0.013 (0.031)	0.67
HDL-cholesterol	1679	0.0002 (0.012)	0.99