Genetic deficiency of Phactr1 promotes atherosclerosis development via facilitating M1 macrophage polarization and foam cell formation

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Abstract
Genetic variants in phosphatase actin regulator-1 (Phactr1) is reported to be associated with arteriosclerotic cardiovascular disease. However, the function of Phactr1 in atherosclerosis remains unclear. Patients with acute coronary syndrome (ACS) who underwent coronary angiography and optical coherence tomography were enrolled and divided into non-ST segment elevation (NST-ACS) group and ST-ACS group. The expression of Phactr1 on monocytes was higher in NST-ACS and ST-ACS groups as compared to control group. Furthermore, NST-ACS patients who have more vulnerable features including TCFA and large lipid area showed higher levels of Phactr1 on monocytes than those with stable plaques. Through mouse models of atherosclerosis, Phactr1−/−Apoe−/− mice (DKO) developed more severe atherosclerotic plaques, recruiting more macrophages into subendothelium and having elevated levels of proinflammatory cytokines in plaques. Similarly, Apoe−/− mice receiving DKO bone marrow (BM) exhibited elevated plaque burden compared with Apoe−/− mice receiving Apoe−/− BM, indicating the protective effect of Phactr1 in hematopoietic cells. We found that depletion of Phactr1 in BM-derived macrophages (BMDMs) tended to differentiate into M1 phenotype, produced more proatherogenic cytokines and eventually converted into foam cells driven by oxidized low-density lipoprotein. Mechanistically, Phactr1 activated CREB signaling via directly binding to CREB, upregulating CREB phosphorylation and inducing KLF4 expression. Finally, overexpression of KLF4 partly rescued the excessive inflammation response and foam cell formation induced by deficiency of Phactr1. In conclusion, our study demonstrates that elevated Phactr1 in monocytes is a promising biomarker for vulnerable plaques, while increased Phactr1 attenuates atherosclerotic development.
via activation of CREB and M2 macrophage differentiation.

**Key Words:** acute coronary syndrome, Phactr1, macrophage polarization, foam cell, CREB signaling
Introduction
Arteriosclerotic cardiovascular disease (ASCVD), including coronary artery disease (CAD) and myocardial infarction, remains the leading cause of morbidity and mortality worldwide. Both hyperglycemia and hyperlipidemia accelerate vascular aging and atherosclerosis.(1) Monocyte infiltration, macrophage polarization, maladaptive inflammation and foam cell formation are features of arteriosclerotic development.(2) Increasing evidence suggests that atherosclerosis is mediated by multiple proinflammatory stimuli that act indirectly or directly to cause plaque vulnerability and even rupture. During these processes, macrophages are referred as prominent immune cells in response to oxidized low-density lipoprotein (ox-LDL). In addition, several specific intracellular pathways are necessary for establishing the association between ox-LDL and macrophage polarization, cholesterol influx and eventually foam cell formation.(3) It had been reported that cell surface receptors encountering modified LDL triggered signaling cascades that promoted the production of cytokines and the transcription of scavenger receptors by p38MAPK/CREB pathway in macrophages.(4) In this regard, lipoxygenase 1 (LOX-1) and scavenger receptors CD36 and SR-A are responsible for modified LDL uptake by macrophage.(5) Conversely, ABCA1 and ABCG1 play crucial roles in mediating cholesterol efflux from macrophages and protecting against atherogenesis.(6) Multiple cardiovascular centers have now identified that phosphatase and actin regulator 1 (Phactr1) is strongly associated with ischemic stroke, coronary artery calcification, spontaneous coronary artery dissection and myocardial infarction based on genome-wide association study (GWAS) and meta-analysis.(7-9) Phactr1 located within chromosome 6p24 is defined as an inhibitor of protein phosphatase 1, a ubiquitous serine and threonine phosphatase known to regulate multiple cellular processes, such as cytoskeleton formation, cell motility and oxidative stress.(10, 11) Two recent studies implicate the presence of Phactr1 in human atherosclerotic plaques.(12, 13) While Phactr1 is abundant in endothelial cells (ECs) and macrophages but not vascular smooth muscle cells (VSMCs), Reschen et al.(12) reported that pathogenic genetic variants on Phactr1 markedly suppressed the expression of short Phactr1 transcript in macrophages. Likewise, Zhang et al.(13) found that depletion of Phactr1 disrupted NF-κB activation and therefore alleviated oxidative stress and inflammatory response in ECs. However, the effect of Phactr1 on plaque vulnerability and the exact underlying mechanism remains elusive.
To better understand the role of Phactr1 in plaque stabilization and explore the potential mechanism of Phactr1 in atherosclerosis, we conducted a cross-sectional study in patients with acute coronary syndrome (ACS) and investigated ox-LDL-induced macrophage polarization, inflammation response and foam cell formation in Phactr1 deficiency mice.

Methods
Study population
We consecutively enrolled a study population comprising 90 unstable patients with suspected ACS who underwent coronary angiography from January 1, 2018 to
The diagnosis and classification of ACS conformed to the Third Universal Definition of Myocardial Infarction. ACS patients with significant coronary stenosis were referred as those with ≥ 50% stenosis in a major coronary artery ≥ 2.0 mm diameter by angiography. Individuals who were accompanied with congenital heart disease, cardiomyopathy, significant arrhythmia, acute infections, chronic obstructive pulmonary disease (COPD), pregnant state, severe kidney or liver diseases, and cancers were excluded from the study. Thirty sex- and age-matched participants without coronary stenosis after angiography were recruited as control group.

The clinical trial was approved by the First Hospital of Jilin University Ethics Committee. The informed written consent was obtained from the whole enrollment.

Protocol of coronary angiography and assessment of plaque morphology by OCT
Coronary segments were scored in a semi-quantitative manner with an 18-segment Society of Cardiovascular Computed Tomography model. The burdens of coronary plaques were quantified for luminal diameter stenosis as moderate (50% to 69%), severe (70% to 99%) or occlusion (100%). The number of stenotic vessels was also documented. In cases with multivessel diseases, the experts documented the highest degree of stenosis for further investigation.

For the purpose of accurate assessment of plaque morphology, we applied intracoronary optical coherence tomography (OCT, C7 Intravascular Imaging Systems, Abbott, USA) in non-ST segment elevation (NST-ACS) patients. Lipid-rich plaque was considered as a lesion with lipid arc over 90°. Thin-cap fibroatheroma (TCFA) was defined as a plaque with the thinnest part of the fibrous cap < 65 μm.

Isolation of circulating monocytes by magnetic beads
Circulating monocytes were collected within 3 hours of blood sampling. Peripheral mononuclear cells from each patient were isolated from whole blood by density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare, USA). CD14+ circulating monocytes were purified using CD14-conjugated magnetic assisted cell sorting (MACS, Miltenyi Biotec, Germany) and a quadroMACS separator (Miltenyi Biotec, Germany) according to the manufacturer’s instructions.

Additionally, a total of 600 μl blood was collected from each mouse and circulating monocytes were magnetically labeled and separated via a quadroMACS separator and Monocyte Isolation Kit (Catalog 130-100-629, Miltenyi Biotec, Germany).

Generation of Phactr1⁻/⁻ Apoe⁻/⁻ mice and models of atherosclerosis
The animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011). All the experimental procedures were approved by the Institutional Animal Care and Use Committee of the First Hospital of Jilin University (No. 2017-056). The animal experiments were performed at Animal Centre of the First Hospital of Jilin University. Experimental mice were housed in a specific pathogen-free animal workshop and maintained at 24 °C on 12-hour light/dark cycle with free access to...
sterile food and water. Apoe knockout mice (Apoe<sup>−/−</sup>) with C57BL/6 background were obtained from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Phactr1 global knockout mice (Phactr1<sup>−/−</sup>) mice were generated by GeneCopoeia Incorporation (China). Briefly, a targeting vector was constructed by replacing a fragment including the Phactr1 open reading frame (exon 2-3) with a neomycin resistant gene cassette, an HSV-thymidine kinase gene inserted for negative selection. Recombinant embryonic stem cells containing targeting vectors were microinjected into female C57BL/6 mice. Phactr1<sup>−/−</sup> mice were obtained after heterozygous F1 progenies intercrossed. The genotyping primers are summarized in Supplementary Table 1. Phactr1 and Apoe double knockout mice (Phactr1<sup>−/−</sup> Apoe<sup>−/−</sup>, DKO) were generated by crossing Phactr1<sup>−/−</sup> and Apoe<sup>−/−</sup> mice. The littermate male Apoe<sup>−/−</sup> were used as control mice. Eight-week-old male DKO and Apoe<sup>−/−</sup> mice were fed on high-fat diet (16% fat and 1.3% cholesterol, SLAC Laboratory Co. Ltd., Shanghai, China) for 16 weeks. Myeloid cells specific KLF4 overexpression mice (KLF4<sup>δMC</sup>) were obtained from GeneCopoeia Incorporation (China). Briefly, the full-length KLF4 cDNA (Plasmid #15950, addgene, USA) was cloned downstream of the lysozyme 2 (LysM) promoter. The construct was microinjected into mouse embryos to generate KLF4<sup>δMC</sup> mice. The genotyping primers are summarized in Supplementary Table 1. The KLF4<sup>δMC</sup> mice was cross with Phactr1<sup>−/−</sup> Apoe<sup>−/−</sup> mice to produce KLF4<sup>δMC</sup>/Phactr1<sup>−/+</sup> Apoe<sup>−/−</sup> mice, which were then crossed back with Phactr1<sup>−/−</sup> Apoe<sup>−/−</sup> mice to generate KLF4<sup>δMC</sup>/Phactr1<sup>−/−</sup> Apoe<sup>−/−</sup> mice.

For bone marrow (BM) transplant models, 8 week-old recipient Apoe<sup>−/−</sup> mice were subjected to whole-body irradiation (7 Gy) followed by recovery for 6 weeks and then injected with fresh sterile BM donor cells (2 x 10<sup>5</sup> cells) by tail vein injection. Additionally, 8 week-old recipient Phactr1<sup>−/−</sup> Apoe<sup>−/−</sup> mice were subjected to whole-body irradiation and then injected with fresh sterile BM donor cells from Phactr1<sup>−/−</sup> Apoe<sup>−/−</sup> and KLF4<sup>δMC</sup>/Phactr1<sup>−/−</sup> Apoe<sup>−/−</sup> mice by tail vein injection.

Plasma from DKO and Apoe<sup>−/−</sup> mice fasted overnight prior to euthanasia were collected. Total triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured on AU480 Biochemistry Analyzer (Beckman, USA).

**Morphology analysis**

Mice were anaesthetized with pentobarbital sodium (60 mg/kg, Sigma, USA) by intraperitoneal injection before tissue harvest, then were perfused with ice-cold PBS followed with 4% paraformaldehyde after sacrifice. Atherosclerotic plaques in the aorta root where three aortic valves were visible from cryosections were stained with Oil Red O (Sigma-Aldrich, USA) for 30 minutes followed by 2 washes of 60% isopropanol. Masson’s trichrome staining was carried out to evaluate the plaque morphology. The burden of plaques, the area of necrotic core and the thickness of fibrous cap were calculated using Image J. Paraffin sections of the aorta root were prepared and immunohistochemistry staining was performed to detect collagen I expression using Collagen I antibody (Catalog ab90395, abcam, UK) in the atherosclerotic plaques. Frozen sections of the aorta root were prepared and
immunofluorescent staining was performed to detect macrophage and Phactr1 expression using Mac2 antibody (Catalog PA5-34819, Invitrogen, USA) and Phactr1 antibody (Catalog ab229120, abcam, UK) in the atherosclerotic plaques. The relative collagen areas and Phactr1+/Mac2+ cells were calculated using Image J.

**Induction of macrophage polarization**

Murine BM cells were obtained from femurs and tibias. For differentiation of BM-derived macrophages (BMDMs), the monocytes cultured in RPMI-1640 (Gibco, USA) were incubated with 50 ng/mL recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF, Peprotech, USA) for 7 days to differentiated into BMDMs. BMDMs were then polarized to M1 phenotype using 100 ng/ml LPS (Sigma-Aldrich, USA) and 20 ng/ml recombinant interferon gamma (IFN-γ, Peprotech Incorporation, USA), or to M2 phenotype using 20 ng/ml recombinant interleukin 4 (IL-4, Peprotech Incorporation, USA).

For macrophage differentiation, CD14+ monocytes from control group and ACS patients were maintained in RPMI-1640 (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 100 U/ml penicillin-streptomycin (Gibco, USA) for 5 days. Macrophages were washed and treated for 16 h with RPMI-1640 + 5% FBS alone, 100 ng/ml LPS and 20 ng/ml IFN-γ (R&D Systems, USA) for differentiation to the M1 phenotype, or 20 ng/ml recombinant human IL4 (R&D Systems, USA) for differentiation to the M2 phenotype.

**Cell transfection**

BMDMs were infected with an adenovirus expressing vector-transfected mouse cDNA of KLF4 (Ad-KLF4) or an empty sequence as a control (Ad-Control). The cells were infected with the adenoviruses at a multiplicity of infection of 100 and a transduction enhancer (Genepharma Incorporation, China). The following experiments were performed 48 hours after infection. Additionally, control small interfering RNA (siRNA) and Lox-1 siRNA (siRNA ID SASI_Mm01_00118143) were purchased from Sigma-Aldrich Incorporation (Merck, Germany). Control and Lox-1 siRNA duplex were mixed with RNAiMax reagent (Thermo Fisher, USA) in OPTI-MEM medium (Thermo Fisher, USA), and transiently transfected into BMDMs for 24 hours.

Human monocyte-derived macrophages (80% confluent monolayer) were transfected with control and Phactr1 siRNA duplex (siRNA ID SASI_Hs01_00014345, Merck, Germany) using RNAiMax reagent in OPTI-MEM medium for 24 hours. After 72 hours, transfection efficiency was determined by immunoblots.

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 100 U/ml penicillin-streptomycin (Gibco, USA). Flag-Phactr1 and HA-CREB were purchased from Genomeditech Incorporation (Shanghai, China). HEK293T cells were transfected with Flag-Phactr1, HA-CREB or Luciferase reporter vectors using Lipofectamine 2000 transfection reagents (Invitrogen, USA).
Measurement of intracellular cholesterol
Cholesterol extracts of BMDMs were assayed using the Cholesterol Quantification Kit (BioVision, USA) according to the manufacturer’s protocol. Data were normalized to total protein and measured by the Lowry assay.

Assessment of foam cell formation
After induction of ox-LDL for 24 hours, BMDMs were fixed with 4% paraformaldehyde for 15 minutes followed by 2 washes of PBS. Then the cells were stained with 0.5% Oil Red O solution at 37 °C for 20 minutes in darkness and rinsed with 60% isopropanol and PBS. Oil Red O-stained foam cells were observed under light microscope (Olympus Incorporation, Japan).

RNA purification and quantitative RT-PCR
Total RNA was isolated from cells using Trizol reagent (Thermo Fisher, USA) and reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Biotechnology, Dalian, China). The mRNA expression levels of the target genes were quantified by quantitative RT-PCR using SYBR Green Master Mix (Takara Biotechnology, Japan) with an ABI PRISM 7900 System (ABI, USA) as previously described. Each reaction was performed in triplicate and normalized to the β-actin as a housekeeping gene. Sequence specific PCR detection primers are summarized in Supplementary Table 1.

Flow cytometry
BMDMs were incubated with a mixture of antibodies at 4°C for 20 min. Anti-CD45-FITC (30F11.1, eBioscience, USA), anti-CD11b-PE (M1/70, eBioscience, USA), anti-Ly6C-PE-Cy7 (AL-21; BD Biosciences, USA), anti-Ly6G-PerCP-Cy5.5 (1A8; BD Biosciences, USA) and anti-CD206-APC (MR5D3; Biolegend, USA) antibodies were used for flow cytometric analysis. The obtained M1 and M2 subsets were expressed as the percent of total macrophages. Flow cytometric analysis were performed on a FACSAria I and III instrument (BD Biosciences, USA) and analyzed using FlowJo software (Tree Star, USA).

Mouse cytokine array
Proteome Profiler Mouse Cytokine Array (Panel A, R&D System, USA) was applied to measure the cytokine profile in BMDMs according to the manufacturer’s instruction.

Immunoblots
The proteins were extracted from BMDMs using a commercially available extraction reagent (RIPA Lysis and Extraction Buffer, ThermoFisher, USA) supplemented with protease inhibitors (Pierce Protease and Phosphatase Inhibitor, ThermoFisher, USA). Protein concentration was determined by a bicinchoninic acid protein assay kit (Beyotime, Haimen, China). The samples were then separated using 8% SDS-PAGE gel and transferred to PVDF membranes, followed by incubation with primary
antibodies overnight at 4 °C. After 3 washes of 0.1% PBST, the samples were incubated with HRP-conjugated secondary antibodies (Beyotime, Haimen, China) for 1 hour at room temperature followed by ECL detection (ThermoFisher, USA). The following primary antibodies were applied in our work: Phactr1 (Catalog ab229120, abcam, UK), LOX-1 (Catalog ab214427, abcam, UK), CD36 (Catalog sc-7308, Santa Cruz, USA), SR-A (Catalog sc-166184, Santa Cruz, USA), ABCA1 (Catalog ab66217, abcam, UK), ABCG1 (Catalog GTX30598, genetex, USA), phospho-IκBα (Catalog 5209, Cell Signaling Technology, USA), p38MAPK (Catalog 8690, Cell Signaling Technology, USA), phospho-p38MAPK (Catalog 4511, Cell Signaling Technology, USA), CREB (Catalog 9197, Cell Signaling Technology, USA), phospho-CREB (Catalog 9198, Cell Signaling Technology, USA), KLF4 (Catalog ab106629, abcam, UK) and β-actin (Catalog ab8227, abcam, UK).

Co-immunoprecipitation
BMDMs and HEK293T cells were lysed with RIPA Lysis and Extraction Buffer plus protease inhibitors. Cell lysates were incubated with specific primary antibodies by gentle rotation overnight at 4 °C. After incubation with protein A/G beads for 2 hours at 4 °C, protein A/G beads were collected by centrifugation at 2,000 rpm and immunoprecipitated proteins were eluted by RIPA Lysis buffer. Equal amount of samples was analyzed by immunoblotting with specific primary antibodies.

Dual Luciferase reporter assay
The pGM-CREB-Luciferase Reporter vector (GM-021030) was purchased from Genomeditech Incorporation (Shanghai, China). Flag-Phactr1, pGM-CREB-Luciferase Reporter vector and Renilla vector, which was used as an internal control, were co-transfected into HEK293T cells via Lipofectamine 3000 transfection assay (Invitrogen, USA). After 24 hours of transfection, Dual-Luciferase Reporter Assay system (Promega, USA) was applied to detect the activity of CREB responsive element according to the manufacturer’s instruction. In order to determine the effect of CREB on KLF4 transcription, we searched the KLF4 promoter for putative CREB binding sites (JASPAR database, http://jaspar.genereg.net/) and respectively cloned these fragments into pGL3-KLF4-Luc plasmids. Each experiment was performed in triplicate.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) assay was conducted according to the manufacturer’s instruction (EZ-ChIP kit, Merck, Germany). In brief, BMDMs and HEK293T cells were crosslinked with 0.4% formaldehyde at 37 °C for 15 minutes and lysed with glycine for an additional 10 minutes. Samples were then sonicated to shear the size of DNA fragment to 100-500 bp, followed by immunoprecipitation using phospho-CREB antibody or rabbit IgG overnight at 4 °C. The immunoprecipitated complex were reversed and chromatin DNA was purified. The CREB binding site within the promoter of KLF4 was recognized by JASPAR database. Primers predesigned for ChIP PCR are summarized in Supplementary Table.
Statistical analysis
Statistical analysis was performed with SPSS 16.0 software. Categorical outcomes were presented as counts and proportions (%). Continuous outcomes were expressed as mean ± standard deviation (SD). Comparisons of normally distributed variables between two groups were conducted by using Student t test. Differences between multiple groups were compared using One-way ANOVA test followed with Bonferroni comparison. Values of $P < 0.05$ were considered significant.

Results

Association between Phactr1 expression on circulating monocytes and plaque vulnerability
The baseline characteristics of the whole enrollments are presented in Table 1. As an initial step, we extracted purified monocytes, the most abundant immune cells observed in atherosclerotic plaques, from control and ACS groups respectively. The extent of ox-LDL uptake in ACS group was clearly much stronger than that in control group (Supplementary Figure 1A and 1B). It could be explained that ACS patients are more likely to have higher proportion of proinflammatory monocyte subsets (CD14++CD16+ monocytes), which subsequently contribute to foam cell and necrotic core formation in plaques.\m{17} The Phactr1 expression is remarkably higher in the NST-ACS and ST-ACS groups compared with control group (Figure 1A). In parallel with this finding, the expression of M1 markers (TNF-\(\alpha\), IL-6 and iNOS) was higher in macrophages derived from NST-ACS and ST-ACS groups than that from control group (Supplementary Figure 1C). By contrast, the expression of M2 markers (Arg-1, IL-10 and CD206) decreased in macrophages from NST-ACS and ST-ACS groups (Supplementary Figure 1D). In terms of the severity of atherosclerotic plaques, we found a gradual increase in the expression of Phactr1 in circulating monocytes as increase in the number of culprit vessels and the degree of stenosis (Figure 1B and 1C).

It is widely accepted that OCT can identify the features of rupture-prone plaques based on high-resolution anatomic images.\m{18} The prevalence of plaque characteristics by OCT are shown in Table 2. In this regard, Phactr1 in monocytes was significantly higher in patients with TCFA than those without TCFA (Figure 1D). Similarly, patients with prominent lipid-rich plaques (lipid arc >90°) had higher levels of Phactr1 in monocytes relative to those with stable plaques (Figure 1E). These data imply that Phactr1 is gradually increased in circulating monocytes as atherosclerosis worsen.

Phactr1 expression is increased in macrophages during murine atherogenesis
Apoe\(^{\text{--/--}}\) mice were fed on a normal chow diet for 8 weeks or a high-fat diet for 8-16 weeks to observe the expression and location of Phactr1 in plaques and in circulating monocytes. Double-label immunofluorescent staining revealed that Phactr1 fluorescence intensity was stronger in advanced atherosclerotic plaques and predominantly expressed in Mac2 positive cells, which was considered as a specific
marker of macrophage (Supplementary Figure 2A). We also found a marked increase in Phactr1 expression in circulating monocytes from Apoe⁻/⁻ mice with advanced lesions, whereas the expression of Phactr1 was virtually low in monocytes from Apoe⁻/⁻ mice with normal chow diet (Supplementary Figure 2B).

Depletion of Phactr1 aggravates atherosclerosis

We next sought to clarify whether Phactr1 directly affected atherosclerosis in mice. The genotype of Phactr1⁺/+ and Phactr1⁻/⁻ was confirmed by quantitative PCR (Supplementary Figure 3A). The expression of Phactr1 in aortas from Phactr1⁺/+ and Phactr1⁻/⁻ mice was further confirmed by immunoblot (Supplementary Figure 3B). After consumption of high-fat diet for 16 weeks, there was no difference in body weight and food intake between Phactr1⁺/+ and Phactr1⁻/⁻ mice (Supplementary Figure 3C and 3D). While Apoe⁻/⁻ mice on high-fat diet had high plasma levels of TC, TG and LDL-C, Phactr1⁻/⁻Apoe⁻/⁻ mice retained comparable plasma levels of the lipid profile to Apoe⁻/⁻ mice (Supplementary Figure 3E). Oil Red O staining on aortic sinus cross-sections showed that Phactr1⁻/⁻Apoe⁻/⁻ mice had stronger atherosclerotic plaques on the aortic sinus than those in Apoe⁻/⁻ mice (Figure 2A). Moreover, Phactr1⁻/⁻Apoe⁻/⁻ mice displayed larger area of necrotic core, a thinner fibrous cap and more accumulation of macrophages, although we did not detect significant difference in the percentage of VSMCs (Figure 2B-D). Accordingly, Phactr1 deficiency led to robust decreases in collagen accumulation within atherosclerotic plaques on the aortic sinus in Apoe⁻/⁻ mice (Figure 2E). Phactr1⁻/⁻Apoe⁻/⁻ mice showed more abundance of TNF-α and IL-6 in the aorta (Figure 2F). Plasma levels of TNF-α and IL-6 are also elevated in Phactr1⁻/⁻Apoe⁻/⁻ mice (Figure 2G). Collectively, these data suggest that loss of Phactr1 accelerates the progression toward a vulnerable plaque phenotype.

To study Phactr1 function specifically in macrophages in the model of atherosclerosis, we transplanted BM from Apoe⁻/⁻ or Phactr1⁻/⁻Apoe⁻/⁻ mice to reconstitute irradiated Apoe⁻/⁻ mice (Figure 3A). Both Phactr1⁺/+ and Phactr1⁻/⁻ alleles shown by genotyping indicated that there was mixed chimerism in Apoe⁻/⁻ mice receiving Phactr1⁻/⁻Apoe⁻/⁻ BM (Supplementary Figure 4A). The success rate of BM transplantation was confirmed by quantitative PCR in BM cells and whole blood cells (Supplementary Figure 4B and 4C). Consistently, Apoe⁻/⁻ mice receiving Phactr1⁻/⁻Apoe⁻/⁻ BM cells developed more aggressive plaques as compared with those receiving Apoe⁻/⁻ BM cells (Figure 3B). The area of lipid-rich necrotic core was markedly increased and the thickness of fibrous cap was accordingly decreased in Apoe⁻/⁻ mice transplanted with Phactr1⁻/⁻Apoe⁻/⁻ BM cells (Figure 3C). Immunofluorescent staining showed an increase in macrophage burden in Apoe⁻/⁻ mice receiving Phactr1⁻/⁻Apoe⁻/⁻ BM cells (Figure 3D). Accordingly, the expression of TNF-α and IL-6 was significantly increased and plasma levels of TNF-α and IL-6 were elevated in Apoe⁻/⁻ mice receiving Phactr1⁻/⁻Apoe⁻/⁻ BM cells as well (Figure 3E and 3F). Collectively, these findings validate that the specific role of Phactr1 on macrophages is anti-atherogenic and anti-inflammatory.

Depletion of Phactr1 in macrophages accelerates M1 macrophage polarization, inflammation and foam cell formation

To investigate the in vitro relevance of Phactr1 on macrophage polarization and
inflammation, we first purified and induced BMDMs from Phactr1+/+ and Phactr1−/− mice. Consistent with the previous report, the expression of Phactr1 was upregulated in the presence of ox-LDL and reached the peak at 12-hour treatment (Figure 4A). Lox-1 is the principle receptor that is abundant in the surface of macrophages. It could bind to ox-LDL and in turn initiate intracellular NF-κB signaling pathway. \(^{(19)}\) In this respect, we found that both depletion of Lox-1 and inhibition of classical NF-κB pathway by EVP4593 mitigated the increased expression of Phactr1 induced by ox-LDL in BMDMs, suggesting that ox-LDL induced Phactr1 expression, at least in part, through Lox-1/NF-κB pathway (Supplementary Figure 5).

Quantitative RT-PCR showed that loss of Phactr1 prominently promoted the expression of M1 macrophage markers such as TNF-α and IL-6 in BMDMs in the presence of LPS and IFN-γ, while the expression of M2 phenotype markers such as Arg1 and CD206 was dramatically decreased in Phactr1−/− BMDMs induced by IL-4 (Figure 4B and 4C). As shown in Supplementary Figure 6A, we presented the gating strategy for pro-inflammatory M1 macrophages (CD11b+Ly6G−Ly6C+CD206−) and anti-inflammatory M2 macrophages (CD11b+Ly6G+Ly6C+CD206+). We observed an enhanced proportion of M1 macrophages in Phactr1−/− BMDMs induced by LPS and IFN-γ, and a suppressed proportion of M2 macrophages (Supplementary Figure 6B and 6C). Similarly, knockdown of Phactr1 in macrophages from control and ACS groups exhibited increased expression of M1 phenotype markers and decreased expression of M2 phenotype markers (Supplementary Figure 7A and 7B). Mouse cytokine array indicated the higher levels of proatherogenic cytokines, including TNF-α, IL-6, RANTES, CCL2, IL-1β, IFN-γ and M-CSF, in Phactr1−/− BMDMs as compared to those in Phactr1+/+ BMDMs (Figure 4D). Quantitative RT-PCR further corroborated that silencing of Phactr1 in BMDMs enhanced the expression of these cytokines induced by ox-LDL (Figure 4E).

To determine whether Phactr1 is a pivotal modulator of foam cell formation, we evaluated the intracellular cholesterol content of BMDMs and performed Oil Red O staining. Loss of Phactr1 induced a significant increase in intracellular lipid content stained by Oil Red O when compared to that in Phactr1+/+ BMDMs (Figure 4F). Consistent with these findings, the intracellular cholesterol content in Phactr1+/− BMDMs was substantially higher than that in Phactr1+/+ BMDMs (Figure 4G). When compared with Phactr1+/+ BMDMs, Phactr1 knockout led to increase levels of LOX-1, SR-A and CD36 in BMDMs, whereas Phactr1 knockout had no marked effect on the expression of ABCA1 and ABCG1, the hallmark of cholesterol efflux (Figure 4H). These data collectively suggest that Phactr1 is a critical mediator of macrophage-mediated inflammation, macrophage polarization and foam cell formation.

**Phactr1 facilitates CREB activation and KLF4 transcription**

Emerging evidence indicates that both MAPK and CREB are critical pathways to drive macrophage polarization and foam cell formation. Purified CD14+ monocytes from ACS group displayed a significant elevation of CREB phosphorylation versus control group (Supplementary Figure 8A). We then hypothesized that Phactr1 promoted M2 phenotype switch via upregulating p38MAPK/CREB signaling. As
shown in Figure 5A, depletion of Phactr1 in BMDMs resulted in suppression of CREB phosphorylation but did not affect the phosphorylation of p38MAPK. Consistently, knockdown of Phactr1 in CD14+ monocyte-derived macrophages of control and ACS groups substantially alleviated CREB phosphorylation (Supplementary Figure 8B). Co-immunoprecipitation revealed that Phactr1 and CREB appeared to physically bind together in BMDMs (Figure 5B). The direct interaction between Phactr1 and CREB was further confirmed by co-transfection of Flag-Phactr1 and HA-CREB and co-immunoprecipitation in HEK293T cells (Figure 5C). Functionally, overexpression of Phactr1 augmented the activity of CREB responsive element measured by Luciferase assay (Figure 5D).

Based on the Jaspar database (Matrix ID: MA0018.1, http://jaspar.genereg.net/matrix/MA0018.1/) and the promoter sequence retrieved from UCSC website, we predicted a potential binding site of CREB at the promoter of KLF4 (Figure 6A). Toward this end, both quantitative RT-PCR and immunoblots confirmed decreased expression of KLF4 in Phactr1−/−BMDMs (Figure 6B and 6C). Mechanistically, ChIP-qPCR conducted at the promoter of KLF4 using CREB antibody pulldown demonstrated that depletion of Phactr1 blocked the abundance of CREB at the promoter of KLF4 (CREB binding motif within Fragment 1) in BMDMs (Figure 6D). In addition, Dual Luciferase assay further confirmed that the transcription activity of KLF4 was substantially strengthen after Phactr1 overexpression in HEK293T cells (Figure 6E). We then evaluated whether the induction of KLF4 is essential for macrophage polarization in the presence of Phactr1. In fact, overexpression of KLF4 via transduction of KLF4 adenovirus (Ad-KLF4) alleviated the expression of M1 markers induced by loss of Phactr1 but restored the expression of M2 markers (Figure 6F and 6G). Accordingly, overexpression of KLF4 mitigated the increased expression of scavenger receptors, Lox-1, CD36 and SR-A, in Phactr1-deficient BMDMs (Figure 6H-J). Likewise, the content of intracellular cholesterol in Phactr1−/−BMDMs was significantly reduced by KLF4 upregulation (Figure 6K). Results of Oil Red O staining indicated that transduction of Ad-KLF4 led to a decrease in foam cell formation induced by Phactr1 deficiency (Figure 6L). These data suggest that KLF4 could reverse M1 phenotype switch, promote macrophages to polarize into M2 phenotype and suppress foam cell formation stimulated by Phactr1 deficiency. To further corroborate the protective effect of KLF4 on plaque burden in Phactr1-deficient mice, we generated KLF4δMC/Phactr1−/−Apoe−/− mice, conducted BM transplant models and confirmed KLF4 expression in VSMCs and BMDMs by immunoblots (Figure 6M). Measurements of Oil Red O-stained areas displayed that Phactr1−/−Apoe−/− mice receiving KLF4δMC/Phactr1−/−Apoe−/− BM cells had decreased plaque areas as compared with those in Phactr1−/−Apoe−/− mice receiving Phactr1−/−Apoe−/− BM cells (Figure 6N), suggesting that KLF4 overexpression in BM-derived cells is able to partly counteract the aggravated effect of Phactr1 deficiency on atherosclerotic plaques.

Discussion
There are two principal findings of the study that help advance our understanding of
the association of Phactr1 with atherosclerosis and the underlying mechanism. Given the robust association between genetic variants on Phactr1 locus and ASCVD identified by several GWAS, a growing number of researches sought to determine the role of Phactr1 in ASCVD. (8, 20, 21) Interestingly, of six different transcripts generated using Rapid Amplification of cDNA Ends (RACE), both the major intermediate transcript and the short transcript are expressed in monocytes and macrophages. (22) However, Codina-Fauteux et al. (22) declared that genetic variant on Phactr1 locus is able to modulate the expression of the intermediate transcript in ECs and VSMCs but not the short transcript in macrophages. Previous reports have consistently indicated an increase in the expression of Phactr1 in atherosclerotic plaques. (12, 13) Consistent with the prior finding in plaques, we find a significant elevation of Phactr1 in purified circulating monocytes of patients with ACS as compared to control group. When TCFA overlying lipid-rich plaque with increased macrophage infiltration obtained from OCT is a critical feature of plaque vulnerability, high proportion of circulating CD14+ monocytes is also associated with rupture-prone plaques and independently predicts increased incidence of adverse cardiovascular events. (23, 24) Our results further reveal that Phactr1 overexpression on circulating CD14+ monocytes is positively correlated with plaque vulnerability. It is likely that proatherogenic stimuli provoke Phactr1 expression on monocytes, which in turn incorporate into coronary plaques and regulate the development of atherosclerosis. Extreme macrophage infiltration and activation has been shown to promote atherosclerotic progression. Recent evidence depicts that Phactr1 lead to endothelial dysfunction through enhancement of oxidative stress and inflammation. Additionally, Jing et al. (25) reported that Phactr1 knockdown impaired ECs via inhibition of EC proliferation and migration but promotion of EC apoptosis. A post-GWAS study unveiled the functional characterization of rs9349379 within Phactr1 intron and identified its distal regulatory effect on endothelin-1, which possesses pleiotropic influence on vasculature. (26) Nevertheless, whether Phactr1 exerted influence on atherosclerosis and macrophage function was unknown. In our study, although increased Phactr1 in circulating monocytes appears to be a biomarker of more advanced plaques, Phactr1 deficiency aggravates atherosclerotic burden accompanied with excessive macrophage recruitment and inflammation, implying that it plays a beneficial role rather than a detrimental role in atherosclerosis. Moreover, based on BM transplantation, we verify the specific role of hematopoietic Phactr1 in mice plaques. Because BM transplantation replaces all types of hematopoietic cells including lymphocytes, neutrophils, monocytes and dendritic cells and mixed chimerism of Apoe−/− mice receiving Phactr1+/− Apoe−/− BM cells, we could not totally point out the specific role of Phactr1 in macrophages in atherosclerosis. Myeloid cell-specific Phactr1 transgenic mice is required to further validate the effect of Phactr1 in monocytes and macrophages in vivo. It is well known that macrophage-derived foam cells are formed due to excessive accumulation of ox-LDL. (3) In this regard, Phactr1 is upregulated in ECs and macrophage in the presence of ox-LDL. (13) Here we present that Phactr1 controls macrophage inflammation, polarization and foam cell formation. It is widely accepted
that increases in M1 phenotype and decreases in M2 phenotype are accompanied with the progression of atherosclerosis.\(^{(27, 28)}\) The suppression of M2 polarized macrophages by knockdown of Phactr1 leads to reciprocal increases in M1 macrophage phenotype in atherosclerosis that aggravates inflammation. In parallel with M1 phenotype switch and inflammation, Phactr1 deficiency amplifies the formation of foam cells. We also show that Phactr1 deficiency contributes to an increase in the expression of LOX-1, CD36 and SR-A, all of which are hallmarks that mediate the uptake of ox-LDL in a variety of cell types. Unlike cholesterol influx, we find no effect of Phactr1 deficiency on ABCA1 and ABCG1 that administrate cholesterol efflux to apoA-I or HDL.\(^{(29)}\) These results highlight the importance of Phactr1 in regulating macrophage homeostasis that is critical to plaque development and rupture.

Functionally, CREB administrates a plethora of diverse cellular processes, including cell proliferation, inflammation, differentiation, apoptosis and autophagy. A recent study underscored that p38MAPK/CREB pathway was responsive to modified LDL and regulated lipid accumulation in M1 macrophages.\(^{(4)}\) In our recent study, we illustrate that Phactr1 is able to bind to CREB and activates CREB phosphorylation without affecting p38MAPK phosphorylation. Following the activation, CREB binding sites at the promoter of KLF4 appeared to be enhanced and KLF4 transcription was in turn upregulated.\(^{(30)}\) KLF4 is referred as an important transcription factor and centrally integrates numerous intracellular signal inputs in vascular remodeling and atherogenesis.\(^{(31, 32)}\) For instance, induction of KLF4 promotes the phenotypic switch of VSMCs and macrophages in response to diverse atherogenic stimuli.\(^{(32, 33)}\) Li et al.\(^{(34)}\) concluded that KLF4 promoted M1 to M2 phenotypic transition and eventually alleviated atherosclerosis. It therefore seems to be reasonable to assume that Phactr1 augments KLF4 expression via direct interaction with CREB. In support of this hypothesis, we conduct ChIP-qPCR and Luciferase assay, finding that Phactr1 enhances KLF4 transcript via interaction with transcription factor CREB. Furthermore, KLF4 activation is required for recovery of M1 polarization, suppression of excessive expression of scavenger receptors and foam cell formation induced by Phactr1 knockout. In vivo observations also support that KLF4 upregulation in BM-derived cells reverses increased atherosclerotic plaques resulting from Phactr1 deficiency. Regarding to its clinical perspective as a therapeutic target against atherosclerosis, activating Phactr1 rather than inhibiting it may have the beneficial effect of retarding atherosclerosis development, although the expression of Phactr1 in circulating monocytes is proved to be positively associated with the severity of atherosclerotic plaques.

Our study exists limitations that deserve further consideration. There were several Phactr1 isoforms existing in human samples. Although genetic variants and ox-LDL stimulation caused opposite regulation of short and intermediate Phactr1 transcripts, the protein expression of Phactr1 was consistently upregulated in atherosclerotic plaques and macrophages.\(^{(12)}\) It should be noted that because of the technical limitations of the available Phactr1 antibodies, we were unable to distinguish the protein expression from different endogenous transcripts. Future investigations
focusing on the role of specific Phactr1 transcript on macrophages and atherosclerotic plaques are required.

Clinical Perspective

- Genetic variants on Phactr1 is linked to the susceptibility and prognosis of ASCVD, whereas the underlying mechanism of Phactr1 in atherosclerosis remains elusive.
- Phactr1 is overexpressed in circulating monocytes of patients with ACS and unstable plaques. Mechanistically, Phactr1 inhibits M1 proinflammatory phenotype and eventually retards atherosclerotic development via facilitating CREB-KLF4 signaling.
- Our results explain the specific role of macrophage Phactr1 in atherosclerosis and Phactr1 could be proposed as a potential therapeutic target for ASCVD.
Author Contribution

Shudong Wang and Jian Kong designed the research and wrote the paper; Te Li, Lijuan Ding and Yonggang Wang performed the experiments and statistical analysis; Te Li and Yonggang Wang collected the clinical data; Yonggang Wang, Ou Yang and Shudong Wang performed OCT and angiography.

Conflict of interest

The authors had no conflicts of interest to declare in relation to this article.

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Figure Legends

Figure 1. Comparison of Phactr1 expression in monocytes in ACS patients according to the severity of atherosclerotic plaques.
A. Comparison of Phactr1 expression in monocytes among controls and patients with NST-ACS and ST-ACS. B. Comparison of Phactr1 expression in monocytes according to the degree of stenosis. C. Comparison of Phactr1 expression in monocytes according to the number of culprit vessels. D. Comparison of Phactr1 expression in monocytes of NST-ACS patients with and without lipid-rich plaques. E. Comparison of Phactr1 expression in monocytes of NST-ACS patients with and without TCFA. ACS = acute coronary syndrome, LC = lipid core, NST-ACS = Non-ST segment elevation ACS, ST-ACS = ST elevation ACS, TCFA = Thin-cap fibroatheroma. * P<0.05.

Figure 2. Deficiency of Phactr1 accelerates atherosclerosis.
A. Representative images of Oil Red O-stained aortic sinus cross-sections and quantification of plaque burden in Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice. Scale bar = 200 μm. B. Representative images of Masson’s trichrome-stained aortic sinus cross-sections and quantification of necrotic core and fibrous cap thickness in Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice. Scale bar = 50 μm. C. Representative immunofluorescent staining and quantification of macrophages labeled with Mac-2 antibodies (Green) in plaques of Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice. Scale bar = 20 μm. D. Representative immunofluorescent staining and quantification of smooth muscle cells labeled with α-SMA antibodies (Red) in plaques of Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice. Scale bar = 20 μm. E. Representative immunohistochemistry staining and quantification of collagen I area labeled with collagen I antibodies in plaques of Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice. Scale bar = 40 μm. F. The mRNA expression of TNF-α and IL-6 in aorta of Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice was determined by quantitative RT-PCR. G. Plasma levels of TNF-α and IL-6 in Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice were analyzed by ELISA. Data are mean ± SD from 6 mice in each group. * P<0.05.

Figure 3. Deficiency of hematopoietic Phactr1 promotes atherosclerosis.
A. Strategy for bone marrow (BM) transplantation. B. Representative images of Oil Red O-stained aortic sinus cross-sections and quantification of plaque burden in Apoe<sup>−/−</sup> mice transplanted with BM cells from Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice. Scale bar = 200 μm. C. Representative images of Masson’s trichrome-stained aortic sinus cross-sections and quantification of necrotic core and fibrous cap thickness in Apoe<sup>−/−</sup> mice transplanted with BM cells from Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice. Scale bar = 50 μm. D. Representative immunofluorescent staining of macrophages labeled with Mac-2 antibodies (Green) in plaques of Apoe<sup>−/−</sup> mice transplanted with BM cells from Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice. Scale bar = 20 μm. E. The mRNA expression of TNF-α and IL-6 in aorta of Apoe<sup>−/−</sup> mice transplanted with BM cells from Apoe<sup>−/−</sup> and Phactr1<sup>−/−</sup>Apo<sup>−/−</sup> mice was determined by quantitative RT-PCR. F. Plasma levels of TNF-α and IL-6 in Apoe<sup>−/−</sup> mice transplanted with BM cells from Apoe<sup>−/−</sup> and
Phactr1\(^{-}/\) Apoe\(^{-}/\) mice were analyzed by ELISA. Data are mean ± SD from 6 mice in each group. * P<0.05.

**Figure 4. Depletion of Phactr1 promotes M1 macrophage polarization and inflammation.**

A. Phactr1 was activated in bone-marrow-derived macrophages (BMDMs) by ox-LDL induction. * P>0.05 vs. BMDMs without ox-LDL induction. B. Quantitative RT-PCR showed the expression of M1 phenotype markers (TNF-α and IL-6) in BMDMs treated with LPS (100 ng/ml) and IFN-γ (20 ng/ml) for M1 polarization. C. Quantitative RT-PCR showed the expression of M2 phenotype markers (Arg1 and CD206) in BMDMs treated with IL-4 (20 ng/ml) for M2 polarization. D. Heatmap generated from cytokine array in BMDMs of Phactr1\(^{+/+}\) and Phactr1\(^{-/-}\) mice. Data are mean ± SD from 3 batches of BMDMs. E. Quantitative RT-PCR was performed to measure the expression levels of RANTES, CCL2, IL-1β and M-CSF in BMDMs of Phactr1\(^{+/+}\) and Phactr1\(^{-/-}\) mice. F. BMDMs of Phactr1\(^{+/+}\) and Phactr1\(^{-/-}\) mice were treated with ox-LDL (30 μg/ml) for 24 hours and then stained with Oil Red O to observe the foam cell formation. G. Effect of Phactr1 deficiency on intracellular cholesterol in BMDMs treated with ox-LDL (30 μg/ml) for 24 hours. H and I. Representative immunoblotting and quantitative analysis showed the protein expression of LOX-1, SR-A, CD36, ABCA1 and ABCG1 in Phactr1\(^{+/+}\) and Phactr1\(^{-/-}\) BMDMs treated with ox-LDL (30 μg/ml) for 24 hours. * P<0.05. **Figure 5. Phactr1 is required for CREB phosphorylation and activation.**

A. Representative immunoblotting and quantitative analysis showed the phosphorylation of p38MAPK and CREB in Phactr1\(^{+/+}\) and Phactr1\(^{-/-}\) BMDMs treated with ox-LDL (30 μg/ml) for 15 minutes. B. Interaction between Phactr1 and CREB in BMDMs was confirmed by co-immunoprecipitation. C. Co-immunoprecipitation of Flag and HA tagged protein in HEK293T cells co-transfected with Flag-Phactr1 and HA-CREB. D. CREB transcription activity was upregulated by overexpression of Phactr1 in HEK293T cells. The luciferase activity of CREB responsive element was detected by Dual-Luciferase Reporter Assay system. Data are mean ± SD from triplicate independent experiments. * P<0.05. **Figure 6. Phactr1-CREB regulates macrophage polarization via promoting KLF4 transcription.**

A. The base sequence of CREB responsive elements (Upper panel); the putative fragments at KLF4 promoter represented CREB-binding elements (Lower panel). B. Quantitative RT-PCR showed the mRNA expression of KLF4 in Phactr1\(^{+/+}\) and Phactr1\(^{-/-}\) BMDMs stimulated with ox-LDL (30 μg/ml) for 24 hours. C. Representative immunoblotting and quantitative analysis showed the protein expression of KLF4 in Phactr1\(^{+/+}\) and Phactr1\(^{-/-}\) BMDMs stimulated with ox-LDL (30 μg/ml) for 24 hours. D. ChIP-qPCR was performed to determine the binding ability of CREB at the indicated fragments of KLF4 promoter in Phactr1\(^{+/+}\) and Phactr1\(^{-/-}\).
BMDMs. **E.** The luciferase activity of KLF4 promoter was measured using Dual Luciferase reporter assay after co-transfection of Flag-Phactr1 and pGL3-KLF4-Promoter-Luci vectors for 24 hours in HEK293T cells. **F.** Quantitative RT-PCR showed the expression of M1 phenotype markers (TNF-α and IL-6) in Phactr1+/+ and Phactr1−/− BMDMs transduced with empty vector (Ad-Control) or KLF4 adenovirus (Ad-KLF4). **G.** Quantitative RT-PCR showed the expression of M2 phenotype markers (Arg1 and CD206) in Phactr1+/+ and Phactr1−/− BMDMs transduced with Ad-Control or Ad-KLF4. **H-J.** Quantitative RT-PCR showed the expression of scavenger receptors (Lox-1, CD36 and SR-A) in Phactr1+/+ and Phactr1−/− BMDMs transduced with Ad-Control or Ad-KLF4. **K.** Comparison of the content of intracellular cholesterol in Phactr1+/+ and Phactr1−/− BMDMs transduced with Ad-KLF4. **L.** Phactr1+/+ and Phactr1−/− BMDMs transduced with Ad-Control or Ad-KLF4 were treated with ox-LDL (30 μg/ml) for 24 hours and then stained with Oil Red O to observe the foam cell formation. **M.** The protein expression of KLF4 in vascular smooth muscle cells (VSMCs) and BMDMs derived from Phactr1−/−Apoe−/− and KLF4−/−/Phactr1−/−Apoe−/− mice were evaluated by immunoblots. **N.** Representative images of Oil Red O-stained aortic sinus cross-sections and quantification of plaque burden in Phactr1−/−Apoe−/− mice transplanted with BM cells from Phactr1−/−Apoe−/− and KLF4−/−/Phactr1−/−Apoe−/− mice. Scale bar = 200 μm. Data are mean ± SD from triplicate independent experiments. * P<0.05.
Table 1. Clinical characteristics of patients with and without acute coronary syndrome.

|                      | Control (n=30) | NST-ACS (n=60) | ST-ACS (n=30) | P value |
|----------------------|----------------|----------------|---------------|---------|
| Age, yrs             | 58.6 ± 8.4     | 59.3 ± 7.6     | 61.5 ± 7.2    | 0.30    |
| Male (n, %)          | 19 (63.3)      | 41 (68.3)      | 19 (63.3)     | 0.85    |
| BMI, kg/m²           | 24.8 ± 4.7     | 24.3 ± 4.2     | 23.9 ± 4.4    | 0.73    |
| Smoking (n, %)       | 9 (30.0)       | 15 (25.0)      | 10 (33.3)     | 0.69    |
| Diabetes (n, %)      | 11 (36.7)      | 18 (30.0)      | 12 (40.0)     | 0.61    |
| Hypertension (n, %)  | 13 (43.3)      | 25 (41.7)      | 14 (46.7)     | 0.90    |
| Triglyceride, mmol/L | 1.7 ± 0.4      | 1.8 ± 0.5      | 1.7 ± 0.5     | 0.52    |
| Cholesterol, mmol/L  | 4.6 ± 1.6      | 4.9 ± 1.7      | 5.0 ± 1.6     | 0.61    |
| LDL-C, mmol/L        | 2.5 ± 0.8      | 2.9 ± 0.7      | 3.2 ± 0.8     | 0.13    |
| HDL-C, mmol/L        | 1.2 ± 0.3      | 1.4 ± 0.4      | 1.3 ± 0.4     | 0.45    |

Data are mean ± SD or n (%).

Abbreviations: ACS = acute coronary syndrome; BMI = body mass index; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; NST-ACS = non-ST segment elevation ACS; ST-ACS = ST segment elevation ACS.
Table 2. Plaque morphology in patients with non-ST segment elevation acute coronary syndrome by optical coherence tomography.

|                          | NST-ACS (n=60) |
|--------------------------|----------------|
| Lipid-rich plaques (n, %)| 34 (56.7)      |
| TCFA (n, %)              | 30 (50.0)      |
| Plaque rupture (n, %)    | 4 (6.7)        |
| Calcification (n, %)     | 18 (30.0)      |
| Thrombus presence (n, %) | 5 (8.3)        |

Abbreviations: NST-ACS = non-ST segment elevation ACS; TCFA = thin-cap fibroatheroma.