Coronary artery disease (CAD) is the leading cause of death globally. Endothelial dysfunction has been recognized as an initiating factor for the formation of atherosclerotic lesions and associated with all stages of atherosclerosis. Vascular endothelium covers the luminal surface of the blood vasculature and provides a physical barrier that controls the traffic of plasma proteins and circulating cells across the blood vessel. Endothelial barrier dysfunction leads to lipoprotein leakage and monocyte extravasation into the vessel walls, thereby accelerating atherosclerosis and inducing atherosclerotic plaque rupture.

The tyrosine kinase receptor B (TrkB) is a high-affinity receptor for brain-derived neurotrophic factor (BDNF). The BDNF-TrkB pathway plays critical roles in the survival, growth, maintenance, and death of central and peripheral neurons. In addition to its nervous system functions, TrkB is also expressed in the cardiovascular system. The BDNF-TrkB axis has been reported to protect the myocardium against ischemic injury in conditional knockout mice model. The BDNF-TrkB axis also plays critical roles in cardiovascular development through promoting endothelial survival. Mice with a disrupted TrkB gene lack a significant proportion of intramyocardial blood vessels and showed early postnatal death; BDNF deficiency results in endothelial cell apoptosis, intraventricular wall hemorrhage, depressed cardiac contractility, and early postnatal death in mice; BDNF-TrkB pathway overexpression in developing mouse hearts resulted in increased cardiac capillary density; However, the role of BDNF-TrkB axis in development of CAD remains unknown.

In this study, we first examined the association between CAD and TrkB polymorphism –69C>G or IVS13+40G>A. Then we investigated its mechanism using ApoE+/− mice and human vascular endothelial cells.

**Materials and Methods**

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

TrkB –69C>G Polymorphism Is Associated With CAD and TrkB –69C Homozygotes, Which Corresponded to Decreased TrkB Expression, Showed Increased Risk for CAD

To investigate the association between TrkB and CAD, we compared CAD and nonCAD subjects for the genotype and allele frequency of TrkB polymorphisms –69C>G and IVS13+40G>A, which have been associated with eating disorders by screening the entire TrkB gene, but in 2 independent cohort from different geographic areas. The frequency of the TrkB –69C>G genotype and allele significantly differed between patients with CAD and controls in the Shandong group (all  P <0.05; Table). This difference was also observed in the Shanxi group (all  P <0.05; Table). Multiple logistic regression analysis revealed homozygous TrkB –69C, rather than TrkB –69G carriers, were associated with an increased risk of CAD in the Shandong group (odds ratio, 2.1; 95% confidence interval, 1.68–2.62;  P <0.05) and in the Shanxi group (odds ratio, 2.0; 95% confidence interval, 1.69–2.67;  P <0.05), after adjusting sex, age, and body mass index. There was no association between the TrkB IVS13+40G>A polymorphism and CAD ( P >0.05; Table). All genotype frequencies showed Hardy–Weinberg equilibrium. Statistical powers are all 100% in Shandong and Shanxi group. The clinical characteristics of the 2 cohorts of patients and controls are shown in Table I in the online-only Data Supplement.

Polymorphism –69C>G is located in the promoter region of TrkB. It has been reported that approximately one third of promoter variants may alter gene expression to a functionally relevant extent. We then constructed luciferase reporter vectors with TrkB promoter segments (–3258 to –11 bp) of contrasting genotypes (–69CC versus –69GG; Figure 1A and 1B) and tested the effect of TrkB –69C>G polymorphism on luciferase reporter gene expression. Regardless of genotype, the promoter significantly promoted luciferase expression (Figure 1C and 1D). Of note, luciferase activity was significantly lower with the –69C construct than with the –69G construct in HeLa cells and human vascular endothelial cells (ECs; Figure 1C and 1D). These data suggested that decreased TrkB expression might be associated with increased risk for CAD.

TrkB is Expressed in Aortic ECs of Atherosclerotic Lesions in Humans and ApoE–/– Mice

We then investigated the expression of TrkB in aorta. TrkB was prominently expressed in aortic ECs in normal C57BL/6 mice (Figure 2). We next examined TrkB expression in early atherosclerotic lesions of ApoE–/– mice and observed similar results, TrkB was prominently expressed in aortic ECs in early atherosclerotic lesions (Figure 2). Moreover, the expression of TrkB in endothelium is seemed to be reduced in atherosclerotic lesions compared with that in normal endothelium, and its underlining mechanism requires further investigation. We further investigated TrkB expression in the aortas of 10 patients with atherosclerotic lesions. In early atherosclerosis lesions, immunofluorescence staining showed that TrkB expression was prominent in ECs (Figure 2). In consistent with Kraemer et al, we found the expression of TrkB in smooth muscle cells in advanced atheroma. In addition to smooth muscle cells, we also found TrkB expression in ECs in advanced atheroma (Figure 1 in the online-only Data Supplement).

TrkB Prevented EC Barrier Leakage in ApoE–/– Mice

We then investigated the effects of TrkB on the endothelial barrier function in vivo. ApoE–/– mice were systemically infected with adeno-associated virus serotype-9 carrying a Zsgreen reporter gene (AAV9-control), AAV9 carrying small hairpin RNA-TrkB (AAV9-shTrkB), or AAV9-shTrkB plus AAV9 carrying the shRNA-resistant TrkB (AAV9-TrkB) via the tail vein, followed by chow feeding for 8 weeks. After systemic infection of AAV9 virus, which can integrate into genomic DNA

Table. Frequency of TrkB –69C/G and IVS13+40G>A Polymorphisms in Patients with CAD and Controls

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Group</th>
<th>Sample</th>
<th>Genotype Frequency</th>
<th>PValue</th>
<th>Allele Frequency</th>
<th>PValue</th>
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<tbody>
<tr>
<td>rs1187325</td>
<td>Shandong</td>
<td>(n=3144)</td>
<td>Positive Control</td>
<td>C</td>
<td>0.000 G</td>
<td>0.000</td>
</tr>
<tr>
<td>(~69C/G)</td>
<td>(n=1,451)</td>
<td>CC</td>
<td>422 (0.291)</td>
<td>0.978</td>
<td>0.552 C</td>
<td>0.448</td>
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<tr>
<td></td>
<td>(n=1,693)</td>
<td>CG</td>
<td>759 (0.523)</td>
<td>0.629</td>
<td>0.415 C</td>
<td>0.585</td>
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<tr>
<td></td>
<td>Shanxi</td>
<td>(n=911)</td>
<td>Non-CAD</td>
<td>CG</td>
<td>0.001 G</td>
<td>0.000</td>
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<tr>
<td></td>
<td>(n=453)</td>
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<td>0.270</td>
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<td>0.459</td>
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<tr>
<td>rs1047896</td>
<td>Shandong</td>
<td>(n=3144)</td>
<td>IVS13+40G&gt;A</td>
<td>G</td>
<td>0.978 A</td>
<td>0.896</td>
</tr>
<tr>
<td>(~69G/A)</td>
<td>(n=1,451)</td>
<td>GA</td>
<td>15 (0.010)</td>
<td>0.967</td>
<td>0.067 G</td>
<td>0.933</td>
</tr>
<tr>
<td></td>
<td>(n=1,693)</td>
<td>AA</td>
<td>163 (0.112)</td>
<td>0.967</td>
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<td>0.933</td>
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<td>GA</td>
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<td>2 (0.004)</td>
<td>0.967</td>
<td>0.065 A</td>
<td>0.935</td>
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</tbody>
</table>

CAD indicates coronary artery disease.
and long-term expression in the transduced cells, highly efficient expression of reporter gene-Zsgreen was observed in the aortic ECs of ApoE–/– mice (Figure 3A). The introduction of AAV9-shTrkB to ApoE–/– mice led to a 93% decrease in TrkB expression in endothelium, which was blocked by overexpressing AAV9-TrkB (Figure 3B–3D). We then assessed the endothelial barrier function using Evans blue assay. In ApoE–/– mice, the knockdown of TrkB expression in aortic ECs resulted in a 30% increase in Evans blue deposition in the aorta, and these effects were rescued by AAV9-TrkB (Figure 3E). We then examined effects of TrkB knockdown on vascular endothelial cadherin (VE-cadherin) expression. Immunofluorescence staining and real-time polymerase chain reaction revealed that VE-cadherin protein and mRNA expression were decreased in aortic endothelium in response to AAV9-shTrkB, and the effects were rescued by AAV9-TrkB (Figure 3F and 3G).

**Figure 1.** Effects of tyrosine kinase receptor B (TrkB) –69C>G polymorphism on TrkB promoter activity. **A,** TrkB promoter was cloned into a pGL3 basic vector. Site-directed mutagenesis was used to produce the change of C or G at –69. **B,** Arrows indicate the change with C or G. **C** and **D,** HeLa cells and human vascular endothelial cells (ECs) were transfected with pGL3 basic, pGL3-promoter-TrkB-AlleleC, or pGL3-promoter-TrkB-AlleleG for 48 h and analyzed for luciferase activity. Data are means±SEM from 3 samples of duplicate determinations in 3 separate experiments. *P<0.05.

**Figure 2.** Tyrosine kinase receptor B (TrkB) expression on early atherosclerotic lesions in human and ApoE–/– mice aorta. Image of double-immunofluorescence staining for TrkB and CD31 in aorta of normal CA57BL/6 mice (**top**). Aortic root sections of ApoE–/– mice fed a high-cholesterol diet for 8 wk were performed with immunofluorescence staining for TrkB and CD31 (**middle**). Image of double-immunofluorescence staining for TrkB and vascular endothelial-cadherin (endothelial cell marker) in early atherosclerotic lesions of human (**bottom**). Insets are control sections. EC indicates human vascular endothelial cells.
TrkB Protected Endothelial Barrier Integrity in VE-Cadherin–Dependent Manner

Then, we investigated whether TrkB protected endothelial barrier integrity dependent on VE-cadherin in human aortic endothelial cells. The TrkB siRNA efficiently abrogated the expression of the TrkB (Figure II in the online-only Data Supplement). Knockdown of TrkB expression significantly increased fluorescein isothiocyanate-dextran diffusion compared with controls (Figure 4A). VE-cadherin gap was also significantly increased after TrkB knockdown (Figure 4B). Transendothelial migration of leukocytes is a critical event for inflammation, and VE-cadherin gap formation...
Therefore, we investigated whether TrkB knockdown would enhance the migration of T cells across a monolayer of endothelial cells in a transwell system. Our data showed that TrkB knockdown resulted in the increased transendothelial migration of human T cells (Figure 4C). TrkB knockdown also resulted in significantly decreased mRNA and protein expression of VE-cadherin, and overexpression of TrkB rescued the effects (Figure 4D and 4E). Importantly, the increases in fluorescein isothiocyanate-dextran diffusion, gaps and migration of T cells induced by depleting TrkB were all prevented by overexpressing VE-cadherin using lentivirus (*P<0.05; Figure 4A–4C). We also examined the effects on the proliferation of endothelial cells. We performed the Edu experiments and found that the knockdown or overexpression of TrkB did not significantly affect the endothelial cell proliferation in our study (Figure III in the online-only Data Supplement).

TrkB Promoted Expression of VE-Cadherin Through Induction and Activation of Ets1 Transcriptional Factor

It has been found that Ets1 transcriptional factor was essential for the expression of VE-cadherin in endothelial cells. Moreover, Ets1 is the most abundant Ets factors in ECs. We next investigated effects of TrkB signaling on the expression of Ets1 transcriptional factor. TrkB knockdown also led to significantly decreased expression of Ets1 in ECs (Figure 5A), whereas BDNF caused rapid phosphorylation of TrkB (Figure 5B and 5C) and increased expression of Ets1 and VE-cadherin at different time periods after the addition of BDNF (Figure 5D). Next, we examined whether Ets1 would be necessary for the BDNF-mediated upregulation of VE-cadherin by Ets1 knockdown. The Ets1 siRNA effectively abrogated the expression of the Ets1 (Figure IV in the online-only Data Supplement). The Ets1 siRNA effectively blocked the BDNF-stimulated expression of VE-cadherin (Figure 5E). Therefore, Ets1 is indispensable for the upregulation of VE-cadherin by BDNF. Next, analysis with anti-Ets1 immunofluorescence technique showed that BDNF treatment not only increased Ets1 expression but also stimulated the nuclear localization of Ets1 proteins, whereas TrkB knockdown led to decreased Ets1 expression and nuclear localization (Figure 5F). Ets1 promoted expression of VE-cadherin by binding to the 2 Ets-binding sites in the VE-cadherin gene promoter in endothelial cells. Then, we investigated whether BDNF promoted the Ets1-binding activity. We analyzed nuclear extracts of ECs stimulated with or without BDNF pulled-down using anti-Ets1 antibody.
Figure 5. Ets-1 was involved in tyrosine kinase receptor B (TrkB)-regulated expression of VE-cadherin. The human aortic endothelial cells were transfected with the indicated siRNA for 12 h, followed by addition of brain-derived neurotrophic factor (BDNF) (50 ng/mL). A, Western blot analysis of the Ets1 protein levels by TrkB knockdown. B and C, Western blot analysis of the phosphorylated TrkB levels after addition of BDNF with different time or dose. Lysates were immunoprecipitated with TrkB antibody and the immunoprecipitates were then immunoblotted using the anti-Phospho-Tyr antibody. D, Western blot analysis of the Ets1 and VE-cadherin protein levels after addition of BDNF. E, Effects of Ets1 knockdown on BDNF-induced VE-cadherin. F, Immunofluorescence analysis with Ets1 antibody for nuclear translocation of Ets1 after incubation of the cells with BDNF and blocking the pathway by TrkB knockdown. Bar represents 100 μm. G, ChIP assay for Ets1-binding activation. Chromatin was prepared from the cells, and Ets1-DNA complex was pulled-down using anti-Ets1 antibody. Subsequently, the VE-cadherin promoter was amplified by real-time polymerase chain reaction. All experiments were performed in triplicate. *P<0.05.
BDNF induced a significantly increased level of the immunoprecipitated VE-cadherin promoter.

**Proatherosclerotic Factor-Induced Endothelial Hyperpermeability was Attenuated by TrkB Activation**

We then investigated whether TrkB signal protected endothelial barrier integrity against endothelial hyperpermeability induced by proatherosclerotic factors, tumor necrosis factor α or oxidized low-density lipoprotein. We found that TrkB activation by BDNF prevented the tumor necrosis factor α-induced increase in fluorescein isothiocyanate-dextran diffusion (Figure 6A), T-cell transendothelial migration (Figure 6B), and gap formation (Figure 6C and 6D). Moreover, TrkB activation blocked the reduced mRNA and protein expression of VE-cadherin induced by tumor necrosis factor α in ECs, and the effects was abrogated by Ets1 siRNA (Figure 6E and 6F). Furthermore, the protective effects of TrkB activation in tumor necrosis factor α-induced endothelial hyperpermeability were all abrogated by VE-cadherin knockdown (Figure 6A–6D). We then examined the effects of TrkB signal on oxidized low-density lipoprotein–induced endothelial hyperpermeability and observed similar results (data not shown).

**Discussion**

CAD is a complex disorder. Although conventional risk factors are important, both rare and common genetic variants account for >50% of susceptibility to CAD. However, identifying the genomic loci associated with increased CAD susceptibility has been a challenge. In this study, our data showed −69C>G polymorphisms of TrkB gene significantly associated with CAD by a case–control study in 2 independent cohorts of Chinese subjects, indicating TrkB may be a novel candidate gene for CAD. We also found that −69C>G is a novel functional polymorphism of TrkB. TrkB −69C homozygotes, which corresponded to decreased TrkB expression, showed increased risk for CAD, suggesting that TrkB have a protective role in CAD. Our data demonstrate that TrkB plays a previously unknown protective role in development of CAD. However, there are some limitations in our case–control study. We cannot characterize subjects as controls with stress echo or exercise tolerance test. Because of limited medical resource, the asymptomatic healthy subjects are not suggested to accept the stress echo or exercise tolerance test in China. Therefore, we choose the exclusion criteria as Rossi et al. Although a cohort fulfilling these criteria is expected to have a low prevalence of asymptomatic CAD, a small percentage of asymptomatic coronary patients may have been wrongly assigned to controls. Second, there are significant baseline differences...
in case and control group. To eliminate the effects of confounders, including sex, age, and body mass index, we performed a multiple logistic regression analysis, so the effects of these confounders are eliminated. Considering smoking, hypertension, diabetes mellitus, hypercholesterolemia, and hypertriglyceridemia may account for the observed results, we further investigated the relationship of TrkB –69C/G and the confounders in the patients with CAD and did not find any association (data not shown). Although these baseline differences are seemed not account for the observed results in our case–control study, our results also require further verification in other population. Finally, in this study, we investigated the relationship between TrkB and CAD by examining 2 single nucleotide polymorphisms in 2 independent cohort from different geographic areas. CAD is a complex disorder, several genes in combination and environmental factors can affect atherogenesis. Therefore, the interaction between TrkB and other genes or environmental factors requires further investigation.

Endothelial barrier dysfunction accelerates atherosclerosis and induces atherosclerotic plaque rupture. We found that TrkB is expressed in aortic ECs of atherosclerotic lesions in humans and ApoE–/– mice and has important roles in protecting endothelial barrier’s integrity during atherogenesis. TrkB knockdown in endothelium led to vascular leakage in ApoE–/– mice and TrkB activity protected against endothelial hyperpermeability induced by proatherosclerotic factors in ECs. Our present results showed that 1 major mechanism underlying the protective effect of TrkB on CAD may be through promoting endothelial barrier’s integrity. Similarly, as an angiogenic factor, angiopoietin-114 and angiopoietin-2 15 have been reported to protect adult vasculature integrity against atherosclerosis. TrkB has also been found in smooth muscle cell of advanced atherosclerotic lesions and to promote smooth muscle cell activity. 16 Smooth muscle cell activity appears to have the dual characteristic of promoting atherosclerotic lesions and stabilizing atherosclerotic plaques. Haplodeficient expression of TrkB in ApoE–/– mice led to decreased smooth muscle cell and collagen content, and increased macrophage accumulation in the lesions. 9 Therefore, TrkB may stabilize advanced atherosclerotic plaque via simultaneously maintaining endothelial barrier integrity and promoting smooth muscle cell activity. BDNF circulates systemically. It has been reported that the level of plasma BDNF decreased in the aged 16 and patients with CAD. 17 Consistently, decreased BDNF expression in vascular endothelium was associated with hypertension, 18 a common complication of CAD. Reduced TrkB expression in tissues was also found during aging. 19,20 Considering that peripheral administration of BDNF activated cardiac TrkB and significantly restored the cardiac dysfunction after myocardial infarction in neuronal BDNF-deficient mice, 4 enhancing vascular TrkB activation by increasing plasma BDNF levels may be a useful therapeutic strategy for CAD.

In this study, we identify a new pathway for regulating endothelial permeability via TrkB/Ets1/VE-cadherin. Our data provide the evidence that TrkB is required for regulation of VE-cadherin expression and TrkB signal promoted-synthesis of VE-cadherin is through induction and activation of Ets1 transcriptional factor. VE-cadherin–mediated adhesion junctions are essential to the endothelial barrier function. Many proteases have been found to be induced under atherosclerotic conditions, leading to VE-cadherin disorganization and hyperpermeability in the ECs. 21,22 However, normal endothelial cells have the capacity to restore the junctions over the course of several hours because the destruction of the homotypic interactions between the extracellular domains of VE-cadherin induces a rapid resynthesis of VE-cadherin, leading to the restoration of endothelial cell–cell contacts. 23 So, in addition to VE-cadherin disorganization, decreased capacity to synthesize VE-cadherin is another important reason of endothelial hyperpermeability during atherogenesis. In this study, we demonstrated that TrkB signaling can promote the synthesis of VE-cadherin and restore the endothelial barrier’s integrity against CAD. However, we cannot exclude the possibility that TrkB signal is also involved in the expression of other transcriptional factors and endothelial cell junction molecules that are also important for vascular permeability and may directly or indirectly contribute to the observed effect.

Systemic gene transfer with virus vectors is an effective tool for investigating gene functions in vivo. However, the aortic wall has been notoriously difficult to transduce with virus vectors. Here, as in Bostick et al, 24 we were able to efficiently transduce AA V9 vectors into the aortic ECs of mice. Consistent with Bostick et al, 24 limited reporter gene expression was observed in other cell components of the aorta. The poor transduction in other cell components of the aorta might be the result of the intrinsic biological properties of these cells. 24 AAVs vectors, which can integrate into mouse genomic DNA and long-term express in the transduced cells, are increasingly being evaluated as part of clinical gene therapy trial. 25–27 Recent study showed that AAVs are potentially safe. 28 Therefore, systemic infection with AAV9 via the tail vein is an ideal method for investigating gene functions in aortic ECs in mice.

Our data demonstrate that TrkB plays a previously unknown protective role in development of CAD and maintains endothelial integrity during atherogenesis by promoting Ets1-mediated VE-cadherin expression.

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Disclosures

None.

References

We conclude that enhancing endothelial TrkB activation to protect endothelial barrier integrity may be a useful therapeutic strategy for CAD. Our findings link TrkB to the CAD and extend our knowledge on molecular regulation of endothelial barrier function during atherogenesis. Through promoting Ets1-mediated vascular endothelial cadherin expression, indicating potential mechanisms of TrkB protecting against CAD.

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Tyrosine Kinase Receptor B Protects Against Coronary Artery Disease and Promotes Adult Vasculature Integrity by Regulating Ets1-Mediated VE-Cadherin Expression
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Materials and Methods

Reagents and Antibodies
Lipofectamine 2000 was purchased from Invitrogen; collagen-coated transwell filters were purchased from Costar; fluorescein isothiocyanate (FITC)-dextran (Mr 40 000) was purchased from Sigma; TNF-α was purchased from Invitrogen; the dual-luciferase reporter assay system was purchased from Promega; endothelial cell growth medium was purchased from Sciencell; anti-mouse endothelial cell monoclonal antibodies (CD31) were purchased from Abcam; antibodies against TrkB, VE-cadherin [C19 and BV9], Ets1, p-Tyr antibody, VE-cadherin siRNA and Ets1 siRNA were purchased from Santa Cruz Biotechnology.

Patients and Controls
We recruited two independent cohorts from different geographic areas. The first cohort of 3,144 Chinese subjects (1,693 with and 1,451 without CAD) was recruited from Qilu Hospital, Shandong University, Shandong Province, China. The second cohort included 458 patients with CAD and 453 controls recruited from the First Affiliated Hospital, School of Medicine, Xian Jiao Tong University, Shanxi Province, China. All of the CAD patients had ≥50% stenotic lesions in at least one major coronary vessel, as determined by coronary angiography. In the control subjects, it was unethical to perform coronary angiography to rule out the presence of asymptomatic CAD. Therefore, the inclusion criteria for controls were: no abnormal Q wave or ST-T changes on electrocardiography; negative family history of CAD and stroke; nonsmoking status; absence of hypercholesterolemia, hypertriglyceridemia, diabetes mellitus, and hypertension. Based on available data from epidemiologic and family studies, a cohort fulfilling these criteria is expected to have a very low prevalence of asymptomatic CAD. Written informed consent was obtained from all subjects before enrollment. The study was approved by the Medical Research Ethics Committee of all participating universities and was conducted in accordance with the Declaration of Helsinki (1996) of the World Health Organization.

Genotype Analysis
Genomic DNA was isolated from whole blood using standard procedures. All of the genotyping involved real-time PCR with Taqman probes. The primer sequences for TrkB -69C/G (rs1187325) were forward, 5’-CGA CAT CCC TAG CAG CCA GT-3’, and reverse, 5’-CAA CAA GCA CCG AGG AGT TAA GA-3’; probe1, 5’-CCA CCC GTG CGG GGA GG-3’; probe2, 5’-CCA CCC GTG CCG GGA GG-3’; and TrkB IVS13+40G>A forward, 5’-AGA TGA AAA AGT CAA ACC CTC TGA A-3’, and reverse, 5’-TGC TTT TTT ACC CTA CTGGAC AT-3’; probe1: 5’-AGA AAA TCA TAC TAA AAT ATG T-3’; probe2: 5’-T AGA AAA TCA CAC TAA AAT A’.

Reporter Gene Assays
The promoter for TrkB (-3258 to -11 bp) including the -69 position was amplified with the following primers: forward, 5’-GCTAGC GAA GTA GGA CCG CAA A TC CTA ATT TTA AGT GGG AG-3’ and reverse, 5’-AAGCTT CAG TGC CAG CCC GAG TGC CTG TCC C-3’ with the High Fidelity PCR system (Roche). The amplified product was digested with NheI and HindIII for cloning into the pGL3 basic vector. The reporter constructs underwent site-directed mutagenesis. Sequencing was performed to confirm the change with C or G at the -69 position.

The reporter gene vector pGL3-Basic was used as a background control for the luciferase assays. Renilla luciferase control plasmid was used as an internal control to correct for transfection efficiency among samples. The reporter constructs were transfected in HeLa cells and ECs with the Lipofectamine 2000 for 48 h, and the cells were harvested with a passive luciferase lysis buffer and analyzed on a fluorescence plate reader. All assays were performed in triplicate.

Analysis of TrkB expression in human and mouse atherosclerotic plaques
Aortic samples of patients with atherosclerosis were collected from ten cadaver donors. Mouse aortic
samples were taken from apoE-/- mice fed a high-cholesterol diet (containing 42% fat and 0.2% cholesterol) for 8 weeks. Eight-week-old male ApoE-/- mice were obtained from the Department of Laboratory Animal Science, Peking University Health Science Center (PUHSC), China. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No.85-23, revised 1996) and were approved by the Ethics Committee of Shandong University. The protein expression of TrkB in the aorta was detected using conventional immunofluorescence with anti-TrkB antibody.

**Adeno-associated virus serotype-9 (AAV9) construction**

The sequences for the shRNA specifically targeting the tyrosine kinsase domain of the mouse TrkB gene were 5’-gaa cat caa gag cat cca ctt caa gag agt gga tgc tct tga tgt tct ttt tt-3’ and 5’-aaa aaa gaa cat caa gag cat cca ctc tct tga agt gga tgc tct tga tgt tc-3’. The mouse full-length TrkB cDNA was cloned. The shRNA-resistant full-length TrkB was modified on the targeting site by site-directed mutagenesis (wild-type: aagaacatcaagagcatccac to mutant-type: aagaattataaaaagcatccac), which did not change the encoded amino acids. The recombinant Adeno-associated virus serotype-9 (AAV9)-encoding shRNA-resistant full-length TrkB (AAV9-TrkB), empty AAV9 carrying a Zsgreen reporter gene (AAV9-control), and AAV9 carrying the shRNA (AAV9-shTrkB) were generated at Shenzhen Baienwei Company, China.

**In vivo endothelial barrier assays**

All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No.85-23, revised 1996) and were approved by the Ethics Committee of Shandong University. Eight-week-old male ApoE-/- mice were obtained from the Department of Laboratory Animal Science, Peking University Health Science Center (PUHSC), China. In vivo endothelial barrier function was measured using Evans blue assay with modifications. Briefly, eight-week-old ApoE-/- mice were injected via the tail vein with AAV9-control, AAV9-shTrkB or AAV9-shTrkB+ AAV9-TrkB, followed by chow feeding. After 8 weeks, the mice were injected via the tail vein with Evans blue at a dose of 20 mg/kg body weight in 0.2 ml PBS. After 45 minutes, the mice were perfused through the left ventricle with 10 ml of PBS in 3 minutes, and the aorta was excised. For quantification, the aortas were weighed after drying at 80°C for 18 hours. Evans blue was extracted by incubating each aorta in 0.15 ml of formamide for 24 h at 60°C. Absorbance was measured at 620 nm. The amount of dye was determined with a standard curve and normalized to the dry weight of the aorta.

**Cell Culture**

Human aortic endothelial cells (HAEC) were purchased from ATCC (Manassas, VA). The HAECs were cultured in endothelial cell medium. HeLa cells and 293T cells were grown in DMEM supplemented with 10% fetal bovine serum. All of the cells were cultured at 37°C with 5% CO2. All assays were performed in triplicate.

**Paracellular Permeability in vitro**

The permeability of the ECs was studied as described elsewhere. In brief, the cells were grown to confluent monolayers on collagen-coated transwell filters (0.4-µm pore size, Costar) in 24-well dishes. Fluorescein isothiocyanate (FITC)-dextran (40,000 Mr; Sigma) at a final concentration of 1 mg/mL was added to the upper chamber. At the indicated times, 50-µL samples were taken from the lower compartment and replaced with the same volume of medium. The fluorescent content of the samples was measured with a fluorescence plate reader at an excitation of 485 nm and an emission of 530 nm (Thermo Scientific Varioskan® Flash).

**Transendothelial migration**

ECs were grown to confluent monolayers on collagen-coated transwell tissue culture inserts of 3-µm pore size. Phytohemagglutinin (PHA)-stimulated T cells (PHA-blasts) were resuspended in endothelial cell growth medium; 5×105 cells were added into each insert and left to migrate through the monolayers for 24 h, and the cells in the lower chamber were counted. The results are expressed as the percentage of
transmigrated cells in 3 independent counts.

**siRNA-mediated Gene-specific Suppression**

The sequences for the siRNA duplexes specifically targeting the tyrosine kinase domain of the human TrkB gene were as follows: 5'- AAC CAC UGG GAU CAG CUG GUG UU-3 and 5'- AAC ACC AGC UGA UCC CAG UGG UU-3. For the negative control, the sequences for an siRNA that does not target any known mammalian gene were 5-UUC UCC GAA CGU GUC ACG U-3 and 5-ACG UGA CAC GUU CGG AGA A-3. The siRNA duplex was transfected using the Lipofectamine 2000 method.

**Lentiviral construction**

The rat full-length TrkB cDNA carrying an HA-flag or mouse VE-cadherin cDNA were subcloned into a pCCL.PGK.TetLinker.WPRE vector. The plasmid was mixed with ENV plasmid (VSV-G), packaging plasmid (pMDLg/pRRE), and pRSV-REV and transfected into 293T cells with the Lipofectamine 2000. The collection and transfection of lentivirus followed the manufacturer’s protocol. The cells were used 72 h after transfection.

**Real-Time PCR**

The primer sequences for human TrkB were forward, 5'-TGG CAT CAC CAA CAG TCA GC-3', and reverse, 5'- CCT CCA CGC AGA CGC CAT A-3'; for human VE-cadherin, forward, 5'-AGC CCA AAG TGT GTG AGA ACG-3', and reverse, 5'-GAG ATG ACC ACG GGT AGG AAG-3'; for human GAPDH, forward, 5'-AAG AAG GTG GTG AAG CAG GC -3', and reverse, 5'- TCC ACC ACC CTG TTG CTG TA -3'; for mouse VE-cadherin, forward, 5'-AGC AGG GAA ACA TCT ATA ACG-3' and reverse, 5'-CTT GAA CTT TGG GTT TAC TGG-3'; and for mouse GAPDH, forward, 5’–TGT CTC CTG CGA CTT CAA CA-3’ and reverse, 5’-GTT GGT CCA GGG TTT CTT ACT-3’; for mouse VE-cadherin promoter, forward, 5’-CTG CCT ATC TGC AGC CAG C-3’ and reverse, 5’-TCA GCC GAC CGT CCT TGG A-3’. SYBR green real-time PCR and quantitative assays used the Light Cycler 2.0 (Roche Diagnostics, Mannheim, Germany). The relative expression of genes was obtained by 2-∆∆Ct calculation.

**Western Blot Analysis**

The proteins were separated using 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then incubated with antibodies for anti-TrkB, anti-VE-cadherin, or anti-tubulin, followed by horseradish peroxidase-conjugated secondary antibody incubation. The bands were developed using an enhanced chemiluminescent reagent, detected with AlphaChem 9900, and quantified with AlphaChem software.

**ChIP assay**

Chromatin was purified from the ECs, and then immunoprecipitated using anti-Ets1 antibody and IgG was used as control. VE-cadherin promoter was amplified by real-time PCR, and its level was plotted relative to the input, and GAPDH was used as negative control. These experiments were performed in triplicate.

**Immunofluorescence**

Cryosections were fixed in 4% paraformaldehyde for 10 minute. After blocking nonspecific binding with normal serum, the tissues were incubated overnight with antibodies against TrkB, CD31, and VE-cadherin. The negative control was added with the respective nonimmune IgGs. After incubation with the appropriate FITC-conjugated or TRITC-conjugated secondary antibodies, the sections were observed using a fluorescence microscope. The cell contents of the intimal lesions were determined by measuring the percentage of positive areas with ImagePro-Plus.

**Statistical analysis**

Differences in allelic or genotypic frequencies between the CAD cases and controls were compared using the chi-squared test. The Hardy-Weinberg equilibrium was also tested with the chi-squared test. To evaluate the associations between the genotypes and CAD risk, odds ratios and 95% confidence intervals were calculated using logistic regression analysis. Animal and cell study data were presented as the mean ± SEM. The Kolmogorov-Smirnov test was used to test for normal distribution. Between-group comparisons of
continuous variables were compared by Student t tests or Mann-Whitney U test; for comparisons among multiple groups, ANOVA was used. Categorical variables were compared by chi-squared tests. All of the statistical tests were 2-tailed with $P<0.05$ set as the significance level and were performed with SPSS 15.0 software (SPSS, Chicago, IL).

References

**Suppl. Table I.** Patient characteristics

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<th>Shanxi (n=911)</th>
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<td>Control (n=1,451)</td>
<td>CHD (n=1,693)</td>
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<td>Age, yr</td>
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<td>Sex, M/F (%)</td>
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<td>Systolic blood pressure</td>
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<td>Hypertensive (%)</td>
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**Supplemental Figure legends**

**Suppl. Figure I.** Expression of TrkB in advanced atherosclerotic lesions of human was determined using immunofluorescence staining with TrkB and VE-cadherin (endothelial cell marker) antibody. The solid and hollow arrows indicate ECs and smooth muscle cells, respectively.

**Suppl. Figure II.** Efficiency of TrkB siRNA. Western blots analysis of TrkB protein in ECs transfected with TrkB siRNA or control siRNA.

**Suppl. Figure III.** Effects of knock-down and overexpression of TrkB on endothelial cell proliferation. Cells were treated as indicated and the incorporation of Edu was detected according to the manufacturer’s instructions.

**Suppl. Figure IV.** Efficiency of Ets1 siRNA. Western blots analysis of Ets1 protein in ECs transfected with Ets1 siRNA or control siRNA.
Suppl. Figure I
Suppl. Figure II

siRNA: control  TrkB

TrkB

Tubulin

TrkB protein expression (% control)

control siRNA  TrkB siRNA

*
Suppl. Figure III

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![Graph](graph.png)

Edu-positive cells/controls

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