**CDKN2B Regulates TGFβ Signaling and Smooth Muscle Cell Investment of Hypoxic Neovessels**

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**Rationale:** Genetic variation at the chromosome 9p21 cardiovascular risk locus has been associated with peripheral artery disease, but its mechanism remains unknown.

**Objective:** To determine whether this association is secondary to an increase in atherosclerosis, or it is the result of a separate angiogenesis-related mechanism.

**Methods and Results:** Quantitative evaluation of human vascular samples revealed that carriers of the 9p21 risk allele possess a significantly higher burden of immature intraplaque microvessels than carriers of the ancestral allele, irrespective of lesion size or patient comorbidity. To determine whether aberrant angiogenesis also occurs under nonatherosclerotic conditions, we performed femoral artery ligation surgery in mice lacking the 9p21 candidate gene, Cdkn2b. These animals developed advanced hindlimb ischemia and digital autoamputation, secondary to a defect in the capacity of the Cdkn2b-deficient smooth muscle cell to support the developing neovessel. Microarray studies identified impaired transforming growth factor β (TGFβ) signaling in cultured cyclin-dependent kinase inhibitor 2B (CDKN2B)–deficient cells, as well as TGFβ1 upregulation in the vasculature of 9p21 risk allele carriers. Molecular signaling studies indicated that loss of CDKN2B impairs the expression of the inhibitory factor, SMAD-7, which promotes downstream TGFβ activation. Ultimately, this manifests in the upregulation of a poorly studied effector molecule, TGFβ1-induced-1, which is a TGFβ-rheostat known to have antagonistic effects on the endothelial cell and smooth muscle cell. Dual knockdown studies confirmed the reversibility of the proposed mechanism, in vitro.

**Conclusions:** These results suggest that loss of CDKN2B may not only promote cardiovascular disease through the development of atherosclerosis but may also impair TGFβ signaling and hypoxic neovessel maturation. (Circ Res. 2016;118:230-240. DOI: 10.1161/CIRCRESAHA.115.307906.)

**Key Words:** atherosclerosis ■ cyclin-dependent kinase inhibitor p15 ■ genetic variation ■ pathologic angiogenesis ■ peripheral artery disease ■ smooth muscle cells

Cyclin-dependent kinase inhibitor 2B (CDKN2B) is a highly conserved cell-cycle regulator and tumor suppressor gene implicated in the pathogenesis of several malignancies.1 Recently, however, CDKN2B has also been implicated as a candidate gene, which may be responsible for a portion of the genetic risk concentrated at the chromosome 9p21 cardiovascular genome-wide association study locus.2,3 We previously reported that loss of Cdkn2b alters the blood vessel’s response to both mechanical injury4 and chronic atherosclerosis,5 potentially providing insights into how the 9p21 locus potentiates both abdominal aortic aneurysm disease and myocardial infarction.

**In This Issue, see p 183**

**Editorial, see p 190**

Interestingly, the 9p21 locus has now been associated with several other potentially nonoverlapping vascular phenotypes, including nonatherosclerotic intracranial berry aneurysms and peripheral artery disease (PAD).6,9 Among these, PAD is increasingly recognized as a growing public health concern, which now affects >200 million individuals worldwide and accounts for every fifth cardiovascular healthcare dollar spent.10,11 PAD is interesting in that it shares several atherosclerosis-related factors.
risk factors with coronary artery disease, but also seems to be driven by a variety of PAD-specific mechanisms, including pathways related to angiogenesis and the response to hypoxia. Accordingly, the aim of this study was to determine the role of Cdkn2b in a nonatherosclerotic ischemic injury model, with the goal of advancing our understanding of how the 9p21 locus simultaneously promotes an individual’s heritable risk for so many divergent clinical phenotypes.

Methods

Detailed methods are available in the Online Data Supplement.

Human Studies

Coronary arteries were dissected from the hearts of victims of sudden cardiac death, paraffin-embedded, serially sectioned at 3- to 4-mm intervals, processed for histological examination, and visualized at the CVPath Institute (Gaithersburg, MD), as previously described. Microvessel density was computed as the total number of CD31+CD34+ vessels/total area, and mural cell coverage as (α-smooth muscle actin (SMA)+ microvessel/total microvessel (Ulex)+). Genotyping for rs1333049 was confirmed using TaqMan Scanner and Feature Extractor (software v 9.5.1) and analyzed with Genespring GX 13.0.2 (Agilent). Confirmatory pairwise analysis was performed between conditions using Statistical Analysis of Microarrays (version 4.0) with false detection ratio <1.19 Genes that were consistently dysregulated in both ECs and SMCs were then subjected to bioinformatics analyses. Enrichment for Kyoto Encyclopedia of Genes and Genomes pathway categories and Gene Ontology biological process categories were determined using DAVID Bioinformatics Resources 6.7 (National Center for Biotechnology Information), with P<0.05 EASE cutoff. All microarray data were submitted to the National Center for Biotechnology Information’s Gene Expression Omnibus.

Statistical Analysis

Data are presented as mean±SE using GraphPad Prism 6. In experiments where 2 groups were compared, analysis was performed with unpaired 2-tailed t-testing assuming Gaussian distribution. In experiments with >2 groups, analysis was performed with ANOVA and post-testing performed using the method of Tukey. Differences were deemed significant when the P value was found to be <0.05. Data from each experiment was replicated at least 3x and all analyses were performed in a blinded fashion. All microarray studies employed Student’s t tests with correction for multiple comparisons according to the Sidak–Bonferroni method with correlations determined by Pearson analysis. In all figures: *P<0.05, **P<0.01, and ***P<0.001.

Results

Carriers of the 9p21 Cardiovascular Risk Allele Display Pathological Angiogenesis and Impaired Vessel Maturation Within Their Atherosclerotic Lesions

Among the 66 individuals genotyped in the autopsy cohort, 12 were found to be homozygous for the 9p21 risk allele rs1333049 (C/C), 24 had only the ancestral allele (G/G), and 29 were heterozygous (C/G). Importantly, the 9p21 genotype was not associated with any recorded clinical variable, including age, gender, body mass index, or cardiovascular risk factors, such as smoking, hypertension, diabetes mellitus, or dyslipidemia (P>0.21 for each phenotype measured, Online Table 1A). A total of 88 coronary arteries were analyzed (14 from C/C, 36 from G/G, and 38 from C/G), and intraplaque CD31+CD34+ microvessel density was found to
be significantly higher in the carriers of the risk allele than in those with the ancestral allele ($P$ for trend across genotype <0.05, Figure 1A). This trend occurred across the spectrum of lesion phenotypes and irrespective of plaque size (Online Figure I). Next, mural cell coverage of these microvessels was quantified by measuring the presence of $\alpha$-SMA–positive SMCs surrounding the Ulex-positive endothelial tubes, as previously described.\textsuperscript{13} This analysis revealed that intraplaque vessels present in lesions obtained from carriers of the risk allele were significantly less likely to be enveloped by adjacent SMCs, relative to those from carriers of the ancestral allele ($P$ for trend across genotype <0.05, Figure 1B), even after controlling for comorbidities (Online Table IB). Together, these findings suggest that the 9p21 risk allele may promote neovessel formation, but impair microvessel maturation within the atherosclerotic plaque.

**Loss of the 9p21 Candidate Gene, Cdkn2b, Promotes Critical Limb Ischemia and Impairs Ischemic Vessel Maturation, In Vivo**

To determine whether the microvascular phenotype observed above is restricted to the atherosclerotic plaque or if it may also occur under nonatherosclerotic conditions, we next subjected mice deficient in the 9p21 candidate gene, Cdkn2b, to femoral artery ligation surgeries. Importantly, Cdkn2b\textsuperscript{−/−} mice did not display a baseline defect in vascular anatomy, as quantified by absolute EC content (CD31 staining), SMC content ($\alpha$-SMA staining), and capillary density (CD31\textsuperscript{+} vessels/mm fiber) in unligated, nonischemic limbs, relative to those from carriers of the ancestral allele ($P$ for trend across genotype <0.05, Figure 1B), even after controlling for comorbidities (Online Table IB). Together, these findings suggest that the 9p21 risk allele may promote neovessel formation, but impair microvessel maturation within the atherosclerotic plaque.

Figure 1. Carriers of the 9p21 risk allele display pathological neoangiogenesis within their atherosclerotic plaques.

**A.** Quantitative morphometric analysis revealed that carriers of the representative 9p21 risk allele (C at rs1333049 in red) have a significantly higher burden of intraplaque microvessels than carriers of the ancestral allele (G in black, $P$ for trend=0.02), representative images of Movat pentachrome on left [2x] and CD31/CD34 endothelial cells on right [20x]). Black arrows indicate endothelial tubes.

**B.** Immunohistochemistry revealed that mural cell coverage of the lesional neovessel is significantly reduced among carriers of the 9p21 risk allele, such that 100% of these immature vessels do not become enveloped by adjacent smooth muscle cells (SMCs; $P$ for trend<0.05). Representative images shown at right for each (Ulex [brown]/$\alpha$-SMA [red] double staining [20x]). Red arrows represent endothelial tubes surrounded by SMCs and black arrows represent vessels not surrounded by SMCs.

**Loss of CDKN2B Promotes Angiogenic Behavior in ECs, But Impairs SMC Recruitment to Developing Vessels, In Vitro**

After confirming efficient knockdown (Online Figure IIA and IIB), we next performed a series of in vitro assays to understand how loss of Cdkn2b could impair vessel maturation under hypoxic conditions. We found that hypoxic CDKN2B-deficient (siCDKN2B) ECs displayed several proangiogenic features relative to control-transfected (siCont) cells, including: (1) enhanced migration in a scratch test assay (71.7% versus 43.1% wound healing, $P<0.001$, Figure 3A), (2) increased proliferation in an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (1.51 versus 1.01 relative proliferation units, $P<0.001$, Figure 3B), (3) greater expression of the angiogenic cytokine gamma interleukin-8 ($\gamma$-IL-8), which is thought to support EC survival and migration.

Quantitative histologic analysis revealed that the ischemic hindlimbs of Cdkn2b\textsuperscript{−/−} mice contained reduced vascular density (as assessed by the number of CD31\textsuperscript{+} vessels/mm fiber, Online Figure IIIB) and that there was a significantly higher burden of immature neovessels not invested by mature SMCs (as quantified by the ratio of $\alpha$-SMA-to-CD31 content in the ischemic hindlimb; 0.12 versus 0.57, $P<0.01$, Figure 2F). TUNEL (terminal deoxynucleotidyl transferase [TdT] dUTP nick-end labeling) staining failed to identify a difference in vascular apoptosis across genotypes in the hypoxic limbs (data not shown), but Evan Blue analyses revealed a trend toward increased vascular permeability in the ischemic hindlimbs of Cdkn2b\textsuperscript{−/−} mice (Online Figure IIC). Qualitative microCT scans suggest that Cdkn2b\textsuperscript{−/−} have lower vascular density than wild-type mice, after an ischemic insult (Online Figure IID).

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vascular endothelial growth factor (VEGF; 6.9- versus 2.3- fold increase relative to baseline, \(P<0.001\), Figure 3C), (4) increased tube formation in a Matrigel assay (1.07 versus 0.82 tubes/hpf, \(P<0.001\), Figure 3D), and (5) increased endothelial permeability in a barrier diffusion assay (54.7% increase, \(P<0.001\), Online Figure IIIC). Conversely, although we found that CDKN2B-deficient SMCs displayed significantly greater migration than control-transfected SMCs at baseline (58.8% increase, \(P<0.001\)), we found that this effect on chemokinesis was lost under hypoxic conditions (10.2% increase, \(P=\text{NS}\), Figure 3E). In an attempt to model vessel maturation in vitro, we then cocultured CDKN2B-deficient ECs and SMCs together in Matrigel, and quantified tube formation and investment by mural cells. In keeping with the findings provided above (Figures 1B and 2F), we found that hypoxic CDKN2B-deficient neovessels were thin walled and less likely to be

Figure 2. Loss of Cdkn2b impairs neovessel maturation and promotes hindlimb ischemia in a nonatherosclerotic model of peripheral artery disease. Compared with wild-type mice, Cdkn2b−/− mice developed advanced hindlimb ischemia after femoral artery ligation, as quantified by blinded critical limb ischemia scoring (A) and ambulatory impairment assessment (B, \(P<0.05\) for each). C, Laser Doppler imaging confirmed that the hindlimb ischemia phenotype was secondary to a perfusion defect observable as soon as 7 days after femoral artery ligation (\(P<0.0001\) via 2-way ANOVA; representative images shown in [D]). E, Representative images reveal that Cdkn2b−/− mice sustain a disproportionate amount of tissue loss and digital autoamputation (arrows), relative to Cdkn2b+ mice. F, Simultaneous immunofluorescence staining revealed that the ratio of smooth muscle cells (α-SMA, red) to ECs (CD31, green) is severely reduced in the ischemic hindlimb of Cdkn2b−/− mice relative to Cdkn2b+ mice (\(P<0.01\)), confirming that a vessel maturation defect occurs under nonatherosclerotic conditions. NS indicates not significant. +<0.05, #<0.01, **<0.001.
Figure 3. Loss of CDKN2B promotes endothelial cell (EC) angiogenesis, but inhibits smooth muscle cell (SMC) recruitment to hypoxic neovessels in vitro. Relative to control-transfected (siCont) cells, hypoxic CDKN2B-deficient (siCDKN2B) ECs displayed several proangiogenic features including: (A) increased wound healing in a scratch assay (P<0.001); (B) increased proliferation in an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (P<0.001); (C) upregulation of the angiogenic cytokine vascular endothelial growth factor (VEGF; P<0.001); and (D) increased tube formation in a Matrigel assay (P<0.001). Conversely, CDKN2B-deficient SMCs lose their baseline growth advantage (left, P<0.001) under hypoxic conditions (right, P=NS). (E) This defect correlates with an inability of CDKN2B-deficient endothelial tubes to mature into vessels covered by mural cells, when cocultured with CDKN2B-deficient SMCs (P<0.05 relative to assay performed with siCont ECs and SMCs). +<0.05, #<0.01, **<0.001.
supported by SMCs than control vessels (3.13 versus 5.35, \(P<0.01\), Figure 3F).

**Microarray Studies Implicate Impaired TGFβ Signaling in CDKN2B-Deficient Vascular Cells**

To pursue the molecular mechanism by which loss of *CDKN2B* leads to the observed angiogenic defect, we next performed a series of cDNA microarray assays in *CDKN2B*-deficient ECs and SMCs. Although each set of arrays identified a varying number of differentially regulated genes (Online Table II), we found a collection of 247 genes that were consistently and significantly dysregulated in all *CDKN2B*-deficient cells, relative to control-transfected cells (Figure 4A). Bioinformatic analyses performed on this common list of genes revealed that 5 of the 8 conditions predicted to be associated with loss of *CDKN2B* by the Genetic Association Database for Disease Associations are phenotypes previously associated with the 9p21 risk locus through genome-wide association study (Figure 4B, red).6,21 Kyoto Encyclopedia of Genes and Genomes Pathway analysis identified angiogenesis as the most significantly altered pathway in *CDKN2B*-deficient cells. Moreover, Panther and Gene Ontology analysis revealed that 12 of the 18 significantly enriched biological process terms associated with loss of *CDKN2B* involve angiogenesis-related processes (Figure 4B, brown) or specifically include dysregulation of a transforming growth factor β (TGFβ)-family signaling member (Figure 4B, blue), implicating this latter pathway in the observed phenotype.

**eQTL Studies Confirm Anticorrelation of CDKN2B and TGFβ1 in Human Vascular Tissue**

To determine whether TGFβ1 signaling is also perturbed in the vasculature of subjects with reduced *CDKN2B* expression, we next evaluated 127 carotid endarterectomy samples from the BiKE Biobank. Carriers of the representative 9p21 risk allele (G at rs1412829, who we previously showed have significantly reduced vascular *CDKN2B* expression), had a trend toward increased TGFβ1 expression within their atherosclerotic plaque (P for trend=0.07, Figure 4C). Coexpression analysis revealed that TGFβ1 and *CDKN2B* expression were inversely correlated \((r^2=-0.4972, P=0.0001, \text{Figure 4D})\), suggesting upregulation of TGFβ1 under conditions of reduced *CDKN2B* expression in the vessel wall.

**Molecular Signaling Studies Confirm That Loss of CDKN2B Promotes TGFβ Autoinduction and Upregulation of TGFβ1i1**

Given the data presented above, we performed confirmatory ELISA assays, and found that hypoxic *CDKN2B*-deficient ECs and SMCs both secreted more TGFβ1 than control-transfected cells \((P<0.01\) for each, Figure 4E). Although a number of downstream TGFβ-signaling family members were dysregulated in each of the EC and SMC microarrays performed above, the only factor that was consistently dysregulated (fold change >1.5, false detection ratio<1 by Statistical Analysis of Microarrays) in each condition tested was a poorly studied effector molecule known as TGFβ1-induced-1 (TGFβ1i1). Real-time polymerase chain reaction assays confirmed these microarray findings, and revealed that knockdown of *CDKN2B* led to a significant increase in the expression of TGFβ1i1 in both hypoxic ECs and SMCs \((P<0.05\) for each, Figure 4F). Subsequent Western blots directed at uncovering the mechanism by which loss of *CDKN2B* leads to the induction of TGFβ1i1 revealed that knockdown of *CDKN2B* in hypoxic cells results in: (1) down-regulation of the key inhibitory factor, SMAD7; (2) upregulation of TGFβ1; (3) increased downstream SMAD3 activation; and (4) an ultimate increase in TGFβ1i1 (Figure 4G and 4H). In vitro, the changes in SMAD7 and TGFβ1 seem to occur at the post-transcriptional level because no differences were observed in the mRNA levels of these genes across conditions (data not shown).

**Dual Knockdown Studies Suggest That the CDKN2B-Dependent Vascular Maturation Defect Is Reversible**

To investigate the reversibility of the observed phenomenon, and prove that TGFβ1i1 was the effector molecule responsible for the vascular maturation phenotype, we performed individual and simultaneous knockdown of *CDKN2B* and TGFβ1i1 in cultured ECs and SMCs. As shown in Figure 5A, *CDKN2B* and TGFβ1i1 individually had opposing effects in the Matrigel coculture assay (top 2 panels, \(P<0.05\) for each); however, simultaneous inhibition of both factors led to a normalization in tube formation and vessel maturation, as quantified by SMC:EC ratio \((P=0.92, \text{Figure 5A})\). Together, these data suggest that the hypoxic *CDKN2B*-dependent angiogenic defect could be reversed, at least in vitro. Similar effects were observed in cells overexpressing SMAD7 (Online Figure IIID and IIIE), further supporting the molecular pathway proposed in Figure 5B.

**Discussion**

The studies presented above suggest that carriers of the 9p21 risk allele, whom we have previously shown to have reduced vascular expression of *CDKN2B*, experience an imbalance in endothelial tube formation and neovessel maturation in the vessel wall. We find that this defect is not restricted to atherosclerotic conditions and show that loss of Cdkn2b impairs the ability of the SMC to support neovessel development and tissue perfusion in a nonatherosclerotic animal model of PAD. Molecular studies suggest that these defects are the result of a previously unappreciated TGFβ autoinduction cascade, which culminates in the upregulation of a complicated and poorly studied effector molecule, TGFβ1i1. Together, these findings provide a possible mechanism for how a top PAD genome-wide association study locus modifies the risk for peripheral vascular disease, and may do so independently of its described effects on atherosclerosis and other traditional cardiovascular risk factors.21

The finding that loss of *CDKN2B* leads to an increase in the production of TGFβ1 is interesting, given that TGFβ1 is generally considered to be a proangiogenic cytokine.22,23 However, it is important to note that this concept is based largely on embryonic development data, and that very few studies have examined the effect of postnatal TGFβ1 modulation in vascular disease models.24 Furthermore, it is now appreciated that TGFβ1 is a complex factor that induces cell- and context-specific effects, including a variety of seemingly
antagonistic cellular phenotypes.25,26 For example, TGF-β1 has well-described bifunctional and dose-dependent properties in the endothelium.27,28 In these cells, TGF-β1 can either promote migration and tube formation (if ALK1 and SMAD 1/5 signaling is triggered) or inhibit angiogenesis (if ALK5 and SMAD 2/3 signaling is triggered).24,28 TGF-β1 also has protean effects in the SMC, and it has separately been reported to promote differentiation (via CarG/SSF interactions),26 inhibit growth (via p38),24 stimulate proliferation (via PDGF-AA),23 suppress apoptosis,24 inhibit chemokinesis (independent of SMAD3),24 or induce extracellular matrix synthesis (including collagen),29 depending on the condition.
Figure 5. The CDKN2B-dependent defect in vessel maturation is reversible, in vitro. A, Compared with the opposing effects observed with individual knockdown of CDKN2B (41.4% reduction in vessel maturation, P<0.001) and TGFβ1i1 (86.1% increase in vascular maturation, P<0.02), simultaneous small interfering RNA (siRNA) knockdown of both CDKN2B and TGFβ1i1 led to a complete normalization of the baseline Matrigel tube maturation defect, indicating that the phenomenon may be reversible, at least in vitro (P=0.92, relative to control-transfected cells). B, Proposed molecular signaling mechanism for how CDKN2B may mediate its angiogenic effects. EC indicates endothelial cell; and SMC, smooth muscle cell.
One candidate that may partially specify the diverging vascular effects of TGFβ1 is TGFβ3i1i1. TGFβ3i1i1 is a focal adhesion molecule that is induced by TGFβ1, and it is known to regulate a wide variety of processes, including tumorigenesis, cellular senescence, extracellular matrix sensing, TGFβ autoinduction, and nuclear receptor signaling.30,31 In the vasculature, TGFβ3i1i1 seems to play a similarly complicated role, and it has been reported to have antagonistic effects on the endothelium and SMC,32 as observed in this study (Figure 3). For example, published gain-of-function and loss-of-function studies suggest that TGFβ3i1i1 enhances EC spreading and tube formation in Matrigel,23,33 but inhibits SMC proliferation and migration via myocardin-dependent differentiation.34,35 Indeed, TGFβ3i1i1 seems to serve as a TGFβ rheostat, given its ability to either promote (via SMAD3)36 or inhibit (via SMAD7)30 downstream signaling. Wang et al30 have proposed that the balance of these competing effects may determine whether TGFβ1 functions as a tumor suppressor or oncogene, and by extension, whether it promotes or inhibits cell cycling and angiogenesis.

An important finding in this report is that the observed angiogenic defect is not restricted to hypoxic skeletal muscle, but instead also occurs within human atherosclerotic plaque. This is a significant observation, given that the thin-walled and permeable neovessel has now been associated with leukocyte infiltration and erythrocyte extravasation, and ultimately with atherosclerotic lesion expansion over time.13,37 Our current eQTL findings suggest that carriers of the 9p21 risk allele may experience TGFβ1 upregulation in the arterial wall in vivo (Figure 4C and 4D) and develop vascular lesions with a disproportionately high number of endothelial tubes that are not stabilized by mural cells (Figure 1), irrespective of plaque size, composition, or medical comorbidity (Online Figure I and Table I). Future histopathology studies should investigate whether such individuals are prone to rupture of these immature neovessels, and if intraplaque hemorrhage is a contributor to the robust link between 9p21 risk allele status and coronary artery disease burden.

Although we have focused on CDKN2B in this study, it is important to note that debate persists over which gene (or genes) is responsible for the risk encoded by the 9p21 locus. We and others have previously reported that carriers of the risk allele have reduced expression of CDKN2B in vivo, in circulating cells and cardiovascular tissue.23,38,39 However, other eQTL studies have reported alternative patterns, and recent work has increasingly associated risk variants with altered expression of a long noncoding RNA known as ANRIL (CDKN2B-AS1).40–42 Although studies are ongoing to determine whether this candidate gene may also have a direct effect on vascular tissue in humans, ANRIL is known to epigenetically suppress the CDKN2B promoter,43 suggesting that this may be 1 mechanism by which it could potentiate disease.42

A theme that has clearly emerged from the field of 9p21-related research, however, is that genetic variation at this locus seems to alter the behavior of the vascular SMC, and may do so in a context- and disease-specific manner. For example, we have reported that loss of Cdkn2b can induce SMC apoptosis in response to an acute injury4 or separately render an SMC resistant to efferocytic clearance under conditions of chronic inflammation.3 Others have studied animals deficient in the entire 9p21 murine ortholog (which display consistent reductions in Cdkn2b), but inconsistent changes in Cdkn2a, and found that these mice display altered SMC proliferation and senescence at baseline,44 and a predisposition toward medial degeneration when infused with angiotensin II.11,45 It may be that context-dependent regulation of TGFβ signaling explains the diverging phenotypes observed. For example, we observed a reduction in upstream SMAD7 expression under hypoxic conditions in this study, whereas Loinard et al45 reported an imbalance in canonical downstream SMAD2 signaling in the SMCs of their aneurysm model. These subtle differences in TGFβ signaling, which can have major effects on SMC behavior in vivo42,48, could help to explain how the 9p21 locus has been simultaneously associated with so many divergent clinical conditions, including nonatherosclerotic intracranial berry aneurysms and atherosclerotic plaque accumulation.6

In conclusion, loss of the PAD candidate gene, Cdkn2b, seems to impair the ability of the hypoxic neovessel to mature into an oxygen carrying artery, independent of its described effects on plaque accumulation. This process occurs through a novel pathway that involves fine-tuning of the TGFβ pathway, and importantly seems to be reversible, at least in vitro (Figure 5). Given the growing worldwide burden of PAD,10 and the potential relevance of these findings to atherosclerosis in general, these data may inform future translational studies for vascular disease patients.

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Disclosures

None.

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**Novelty and Significance**

**What Is Known?**

- Genome-wide association studies have unequivocally identified loci associated with cardiovascular disease.
- The mechanism by which the top genome-wide association study locus promotes disease remains unknown.

**What New Information Does This Article Contribute?**

- Subjects who carry the top cardiovascular genome-wide association study risk allele harbor atherosclerotic lesions with a high burden of immature intraplaque vessels.
- Mice deficient in a related candidate gene display a vascular maturation defect, even under nonatherosclerotic conditions.
- Genetic variation may promote cardiovascular disease not only by promoting plaque growth but also by impairing vascular maturation.

Peripheral artery disease results from defects in atherogenesis and angiogenesis. Genomic variants at the chromosome 9p21 cardiovascular genome-wide association study locus have been associated with the heritable risk for both coronary artery disease and peripheral artery disease. *CDKN2B* is a 9p21 candidate gene, which has previously been shown to regulate atherogenesis. Whether this gene also regulates angiogenesis is unknown. This study reveals that the 9p21 risk allele is correlated with microvessel density within the atherosclerotic plaque, independent of lesion size or medical comorbidity. Mice deficient in *Cdkn2b* develop advanced hindlimb ischemia because of a defect in the maturation of immature blood vessels, even under nonatherosclerotic conditions. Signaling studies indicate that this phenomenon occurs because of a previously unrecognized defect in transforming growth factor β (*TGFβ*)–dependent smooth muscle cell recruitment to the nascent blood vessel. This work may inform future translational studies aimed at: (1) promoting the development of oxygen-carrying blood vessels for patients with peripheral artery disease or (2) stabilizing rupture–prone intraplaque neovessels for patients with coronary artery disease.
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Supplemental Material

Detailed methods

Human atherosclerotic plaque collection, immunohistochemistry and quantitative morphometry – 88 randomly selected coronary atherosclerotic plaques were dissected from the hearts of 57 male and 9 female victims of sudden cardiac death sent to the CVPath Institute (Gaithersburg, Maryland) for diagnostic consultation from the Medical Examiner’s Office. Clinical variables including gender, age, BMI, smoking status, and the presence of diabetes, hypertension and hyperlipidemia were recorded. The arteries analyzed included the full spectrum of representative plaques, including 7 normal vessels, 12 with pathological intimal thickening, 11 with early fibroatheroma, 11 with late fibroatheroma, 15 with thin-cap fibroatheroma (TCFA), 18 vulnerable plaques and 14 plaque ruptures. Collection, storage, and use of tissue and patient data were performed in agreement with institutional ethical guidelines. The dissected segments of coronary arteries were serially sectioned at 3- to 4-mm intervals and processed for histological examination, as previously described. Sections were paraffin-embedded, stained with Movat pentachrome, and immunohistochemistry was performed to stain for endothelial cells (Ulex aeropaeus lectin-1 and CD31/CD34) and mural cells (α-smooth muscle actin), as previously described. Staining was visualized using 3,3'-diaminobenzidine tetrachloride (DAKO) tinted with 0.04% nickel chloride (Sigma-Aldrich). Microvessels were quantified on the basis of intraplaque region (close to the necrotic core and away from the media) using image processing software (IVision, Scanalytics). CD31+/CD34+ microvessels in the intraplaque regions were counted in ≤3 hotspots (each hotspot has ≥3 microvessels). Microvessel density (MVD) was computed as the total number of CD31+/CD34+ vessels/total area, and mural cell coverage as αSMA+ microvessel/total microvessel (Ulex+). The average across hotspots of each measurement was computed per region. DNA from these samples was extracted from either frozen tissue using Gentra Puregene Tissue Kit or formalin-fixed paraffin-embedded block sections using QIAamp DNA FFPE Tissue Kit (Qiagen) according to manufacturer’s instruction. Genotyping for rs1333049 was performed using TaqMan SNP allelic discrimination genotyping assay (Cat. # 4351379, Life Technologies) on an Applied Biosystems 7500 Fast Real-Time PCR System. All histomorphometric quantification were performed in a blinded fashion and locked into a database prior to performing genotyping and data analysis.

Human vascular plaque harvest, genotyping and gene expression analysis: Human plaque samples were harvested from n = 127 patients undergoing surgery for symptomatic (unstable) or asymptomatic (stable) carotid stenosis as part of the Biobank of Karolinska Endarterectomy (BiKE) study. Control arteries (n=9 iliac and 1 aorta) were obtained from organ donors without any history of cardiovascular disease. All samples were collected with informed consent from patients, organ donors or their guardians. The study was approved by the Ethical Committee of the Northern Stockholm. DNA and RNA was extracted from the carotid endarterectomy and control artery specimens and analyzed by Affymetrix HG-U133 plus 2.0 microarrays and Illumina 610w-QuadBead SNP-chips, as previously described and deposited in Gene Expression Omnibus (accession number GSE21545). Robust multi-array average (RMA) normalization was performed and processed gene expression data was returned in log2-scale. Student’s T-test with correction for multiple comparisons according to Sidak-Bonferroni method was used for statistical analyses of microarray data. Pearson’s correlations were calculated to determine association between expression of gene of interest and other genes from microarrays. For genes with multiple probe sets, probes with the highest sensitivity and specificity according to GeneAnnot were chosen (TGFβ1: 203084_at; CDKN2B: 207530_s). A P-value <0.05 was considered to indicate significance.

Murine hindlimb ischemia studies – All studies were approved by the Stanford University Administrative Panel on Laboratory Animal Care. Animals used in this study included 40 male, 6-8 month old, wild type and Cdkn2b−/− mice on a C57Bl/6 background (n/genotype =10 for histology, 7 for laser Doppler, 3 for microCT). In order to induce hindlimb ischemia (HLI), mice were subjected to femoral artery ligation and excision, as previously described. The contralateral limb was used as an internal control for non-
ischemic conditions. Perfusion to the hindlimbs was assessed using the PeriScan PIM3 System (Perimed) laser Doppler imager and the accompanying data acquisition and analysis software PimSoft (Perimed) at serial time points, as previously described. Fourteen days post-surgery, the extent of hindlimb ischemia and ambulatory movement impairment was quantified by an independent blinded observer, as previously described. In some mice, AltaBlu silicone casting material was perfused into the vasculature via direct intracardiac injection, and then mice were shipped to Numira Incorporated for micro-CT angiography, as previously described. In other mice, 1% Evans blue dye (2mL/kg of body weight) was injected intraperitoneal one hour prior to sacrifice. All other mice were euthanized and the hindlimb muscles were explanted and processed for immunohistochemistry and gene expression analyses.

Tissue preparation, Immunohistochemistry and Evan’s Blue analysis – Skeletal muscle samples from the control (unligated) and ischemic (ligated) limbs were dissected, rinsed with PBS, embedded in OCT (Sakura) and sectioned at 10μm thickness. Prior to immunohistochemistry, frozen tissue sections were air dried and subsequently fixed using pre chilled 70% acetone. Once fixed, tissue sections were incubated with primary antibodies recognizing CD31 (1:50; BD Pharmingen) or ACTA2 (1:100; Abcam) overnight at 4°C. After rinsing with PBS, tissue sections were incubated with corresponding species specific fluorescent labeled secondary antibodies (1:500; Life Technologies) for 1 h at room temperature. Images were captured with Zeiss’s Axioplan2 inverted microscope, quantified using Adobe Photoshop CS5.1 and graphed using GraphPad Prism 6. For mice injected with Evan’s Blue Dye, skeletal muscle sample were formamide-treated for 48 hrs and then subjected to spectrophotometric analysis at 610nm in a 96 well plate reader, as previously described. In vivo vascular permeability was quantified as the ratio of extravasated Evans blue dye per gram of tissue in the ligated and unligated limbs.

Cell culture – Commercially available human coronary artery SMCs (HCASMCs, hereafter referred to as SMCs) and human umbilical vein ECs (HUVECs, hereafter referred to as ECs) were purchased from Lonza and cultured in SMC basal medium (SmBM) supplemented with SmGM-2 SingleQuots kit and Endothelial Cell Basal-Medium-2 (EBM-2) supplemented with EGM-2 MV SingleQuots kit, respectively. SMC integrity was established by reports of positive smooth muscle α actin staining and negative von Willebrand (factor VIII) performed by Lonza and expressed as a fold change in gene expression. Information on the pr

mRNA isolation, cDNA generation and quantitative RT-PCR – Total RNA was extracted from indicated cell lines using the miRNeasy mini kit (Qiagen) as per the manufacturer’s instructions. RNA was quantified using a nanodrop (NanoDrop technologies) following which cDNA was generated using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies) or High Capacity RNA to cDNA (Life Technologies). cDNA was amplified and quantified using Applied Biosystem’s ViiA™7 RT-PCR system. Data generated was normalized to 18S as previously described and expressed as a fold-change in in gene expression. Information on the probes used to assess expression of target genes are listed in the Supplemental Figure III A.

Transfections – For knockdown experiments, cells were transfected with 10 nM of negative control (siCont, Catalog # 4390846) or siRNA to CDKN2B (siCDKN2B, Catalog # s2843) using Lipofectamine RNAiMAX (Life Technologies). After transfection (14-16h), cells were re-fed with fresh growth medium and thereafter maintained as per the experimental design. In some experiments, simultaneous transfection of siCDKN2B and an siRNA to TGFβ1-induced-1 (siTGFβ1i1, Life Technologies, Catalog # s14057) or an expression plasmid carrying a SMAD7 transcript (Addgene, Catalog # 11733) was performed. siRNA mediated knockdown efficiency was confirmed at the mRNA and protein level (Supplemental Figure III A).

Endothelial Cell Basal-Medium-2 (EBM-2) supplemented with EGM-2 MV SingleQuots kit, respectively. SMC integrity was established by reports of positive smooth muscle α actin staining and negative von Willebrand (factor VIII) performed by Lonza. In certain experiments, SMCs were incubated with EC conditioned medium isolated from transfected ECs for 24 h to mimic EC-SMC crosstalk. In order to simulate hypoxic conditions, cultured cells were incubated in a sealed chamber filled with 2% oxygen for at least 6h after which mRNA or protein was isolated, as described below. Cells not subjected to such conditions were considered to be cultured in normoxic conditions.

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Scratch wound assay – Cell migration was assessed by performing a scratch wound assay as previously described. Briefly, transfected ECs were plated in a 6-well dish and cultured in growth medium for at least 24 h, following which at least two parallel scratch wounds were made across each well using a P200 pipet tip. Differences in cell migration were quantified on serial images taken with a phase contrast inverted microscope (Nikon).

EC permeability assay – In vitro EC permeability was detected by modifying the previously described Tracer Flux Assay (21874459). Briefly, transfected ECs were plated in the upper chamber of Transwell filter inserts (Cat#3401, Corning) and incubated at 37°C overnight to form an endothelial monolayer. 1mg/ml FITC-dextran (Cat#D-1821, Life Technologies) was then added to the top chamber for 10 minutes, and then EC permeability was quantified by measuring the amount of FITC-dextran which had defused to the lower chamber, using a 96-well plate fluorescent plate reader (TECAN Infinite M1000, TECAN).

Cell Viability Assay – Cell viability was determined by conducting a MTT (3-[4,5-dimethyl-thiazol-2-y]-2,5-diphenyltetrazolium bromide) assay, as previously described. In short, transfected ECs were grown in 96-well plates for 24 h following which they were incubated with 10 µL of MTT AB solution (Millipore) for 4 h at 37°C in conditions simulating either hypoxia or normoxia. Once complete, 100 µL of isopropanol containing 0.04N HCl was added to each well and the absorbance was measured using an ELISA plate reader set at 570 nm (reference wavelength 630 nm).

Tube Formation Assay – Angiogenesis was studied by performing a tube formation assay in uncoated µ slides (ibidi), as previously described. Briefly, transfected ECs were plated in µ slides precoated with Matrigel (BD Biosciences), exposed to conditions simulating hypoxia or normoxia for 24 h, then photographed using a phase contrast inverted microscope (Nikon), prior to a blinded quantification of new tubes formed.

Boyden chamber chemotaxis assay – SMC migration was assessed using a standard Boyden Chamber assay, as previously described. Briefly, transfected SMCs were serum starved for 14-16h prior to transfer into 8.0 µm trans-well migration chambers (BD Biosciences). Once trypsinized and resuspended in SmBM, equal number of transfected SMCs were distributed onto the upper chamber and allowed to migrate into the lower chamber containing SmBM with growth supplements for the following 24 h. Cells that migrated to the lower chamber were fixed in cold methanol, stained with hematoxylin, and manually counted in a blinded manner.

Matrigel co-culture assays – Transfected ECs and SMCs were co-cultured in 15-well 1µ microscopy chamber slides specifically designed to study angiogenesis, as per the manufacturer’s instructions (ibidi). Briefly, 10 µL of freshly thawed Matrigel (BD Biosciences) was first added to each well of the slide and allowed to solidify in a humidified incubator set at 37°C. Transfected ECs and SMCs were next incubated with a green or orange cell tracking dye (Life Technologies), respectively, in serum-free medium for 45 min, prior to rinsing and reincubation in their respective growth mediums for an additional 30 min. Cells were subsequently trypsinized and resuspended in growth media, and then plated in equal numbers within the Matrigel-containing wells. As per the experimental design, the chamber slide was exposed to hypoxic conditions for 16 h, after which the difference in cell co-localization was assessed using an upright fluorescence microscope. Differences in SMC-EC co-localization were quantified using Adobe PhotoshopCS5.1

Microarray assays – RNA samples from primary cell cultures were verified by Agilent 2100 Bioanalyzer (Agilent Technologies, 260/280 ratios >2.0, RNA integrity numbers ≥ 9 for inclusion). Samples were pooled by treatment group, labeled and hybridized to the Human HT-12v4 Beadchip Platform (Illumina) per the manufacturer’s protocol by the Stanford Functional Genomics Core Facility. Arrays (n = 4 per condition) were scanned using a Microarray Scanner and Feature Extractor (software v 9.5.1). QC reports
were generated by the Feature Extraction software, and rank-invariant normalized array data were analyzed with Genespring GX 13.0.2 (Agilent). Arrays were examined with QC metrics, principal components analysis (PCA), and hierarchical clustering analysis (HCA), leading to the exclusion of three arrays. The remaining arrays (at least three per treatment group) were subjected to moderated t-testing (cutoff of p < 0.05), with Benjamini-Hochberg correction for multiple testing. Significantly regulated genes were additionally required to show > 1.5-fold expression change from control. Confirmatory pairwise analysis was performed between conditions using Statistical Analysis of Microarrays (SAM v 4.0) with false detection ratio (FDR) < 1. Genes which were consistently dysregulated in both ECs and SMCs were then subjected to bioinformatics analyses. Enrichment for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway categories and GO (Gene Ontology) biological process categories were determined using DAVID Bioinformatics Resources 6.7 (NCBI), with p < 0.05 EASE cutoff. All microarray data were submitted to the National Center for Biotechnology Information’s Gene Expression Omnibus.

**TGFβ1 ELISA** – In certain *in vitro* experiments, TGFβ1 was measured in the culture media isolated from transfected cells using the TGFβ1 ELISA kit (R&D Systems) as per the manufacturer’s instructions. Data generated was graphed using GraphPad Prism 6.

**Protein extraction and Western blotting** – Total protein was isolated from hypoxic cultured cell lines and its expression was thereafter using 1X RIPA Buffer (Millipore) supplemented with 1X Halt Protease & Phosphatase Single-Use Inhibitor Cocktail (Thermo Scientific), as previously described. The protein concentration in each sample was measured using Pierce BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein were loaded and separated on precast gels (Bio-Rad) and thereafter transferred onto PVDF membranes (Bio-Rad). Following a 1h incubation in 5% skim milk solution prepared in 1X TBST, these membranes were probed with commercially available antibodies designed to recognize endogenous SMAD7 (1:1000; R&D Systems), TGFβ1 (1:1000; R&D systems), TGFβ1i (1:1000; Proteintech), SMAD3 (1:1000; Cell Signaling Technologies), TUBA (1:1000; Cell Signaling Technologies) and GAPD (1:1000; Cell Signaling Technologies) overnight at 4°C. Membranes were rinsed with TBS and incubated with appropriately matched HRP conjugated anti mouse (1:5000; Life Technologies) or anti rabbit (1:5000; Life Technologies) antibodies for 1 h, before protein expression was detected using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). Exposed films were scanned and relative protein expression was quantified using ImageJ software, normalized to TUBA or GAPD.
References


Supplemental Table I. 9p21 genotype correlates with intraplaque vessel maturation independently of clinical comorbidities.

A. 9p21 risk allele status (‘C’ at rs1333049) does not correlate with any cardiovascular risk factor or clinical demographic. B. Multivariable linear regression analysis indicates that 9p21 risk allele status correlates with intraplaque vessel maturation, even after controlling for clinical variables.
**Supplemental Table IA** - Characterization of human subjects according to 9p21 genotype (risk allele for rs1333049 = C) and correlation with clinical risk factors and demographics.

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<th>C/G</th>
<th>C/C</th>
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<td></td>
</tr>
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<td>Male, % (n)</td>
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<td>100% (29)</td>
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<tr>
<td>Age, yrs</td>
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<td>Weight, kg</td>
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<td>BMI</td>
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<td>Heart Weight, g</td>
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P values for age, weight, BMI and heart weight were from one-way ANOVA. P values for others were from logistic regression analysis.

**Supplemental Table IB** - Multivariable linear regression for the association of the 9p21 genotype (risk allele for rs1333049 = C) with human plaque microvessel immaturity.

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<th>Coefficient</th>
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<tr>
<td>31.57</td>
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CI, confidence interval

Adjusted for gender, age, body mass index, diabetes status, smoking status and presence hyperlipidemia or hypertension
Supplemental Figure I. Correlation between 9p21 risk allele status and intraplaque microvessel density across the spectrum of lesion types and plaque sizes.

A. Correlation plots associating each subject’s atherosclerotic plaque size and microvessel density indicates a steeper relationship amongst carriers of the risk allele (‘C’) than carriers of the ancestral allele (‘G’), regardless of plaque size (r = 10.87 for ‘C/C’, r = 8.79 for ‘G/C’, and r = 8.28 for ‘G/G’). 

B. Plaque phenotyping according to Virmani classification indicates that the full spectrum of plaque morphologies was represented in each genotype. 

C. Qualitative assessment suggests that microvessel density is increased in lesions obtained from carriers of the 9p21 risk allele across several plaque phenotypes. 

D. Additional representative examples of the increased microvessel density staining (brown, top) and vessel immaturity (brown vessels without red SMCs surrounding, bottom) in carriers of the 9p21 risk allele, relative to those with the ancestral allele.
A

Supplemental Figure I

B

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C

D
Supplemental Figure II. Additional \textit{in vivo} data suggesting that loss of $Cdkn2b$ has no effect on baseline vascular anatomy, but impairs vessel maturation in response to an ischemic insult.

A. Phenotypic analyses of non-ischemic, unligated hindlimbs from wild type and $Cdkn2b^{-/-}$ mice identified no difference in baseline endothelial cell content (CD31), smooth muscle cell content ($\alpha$-SMA) or capillary density (microvessels/mm fiber; $P > 0.53$ for each assay). B. 14 days after femoral artery ligation, however, $Cdkn2b^{-/-}$ mice displayed a reduction in vascular density compared to wild type mice (0.59 vs 1.17 microvessels/mm fiber, $P < 0.001$). C. Vascular permeability assays revealed a trend towards increased extravasation of Evan’s Blue Dye into the ischemic skeletal muscle of $Cdkn2b^{-/-}$ mice relative to controls (60.4% increase, $P = 0.12$). D. Qualitative microCT angiograms indicate reduced vascular density after femoral ligation in $Cdkn2b^{-/-}$ mice compared to wild type animals.
Supplemental Figure II

A. Baseline Endothelial Cell Content

B. Vascular Density 14 Days After Ischemic Insult

C. Evan’s Blue Vascular Permeability Assay

D. Representative MicroCT 14 Days After Ischemic Insult
Supplemental Figure III. Additional in vitro data suggesting that loss of CDKN2B impairs vascular maturation via a SMAD7 and TGFβ1i1–dependent mechanism.

A. siRNA knockdown efficiency for CDKN2B and TGFβ1i1 in ECs and SMCs. B. Genotyping primers and Taqman probes utilized. C. In vitro endothelial permeability assays revealed that loss of CDKN2B increases the permeability of ECs under both normoxic (36.4% increase, $P < 0.01$) and hypoxic conditions (54.7% increase, $P < 0.001$). D. While Cdkn2b-deficient (siCDKN2B, black) cells express more TGFβ1i1 than control-transfected (siCont, white) cells, concomitant overexpression of SMAD7 (grey) normalizes the defect, indicating the importance of this factor at a mechanistic level. E. Similarly, SMAD7 overexpression (grey) normalizes the baseline defect in vascular maturation observed after knockdown of CDKN2B (black), again indicating the upstream role of SMAD7 in the proposed signaling pathway.
Supplemental Figure III

A) Knockdown Efficiency

B) Primers and Probes Used

C) In Vitro Endothelial Permeability Assay

D) SMAD7 mRNA Rescue Assay

E) SMAD7 Matrigel Rescue Assay
Supplemental Table II. Common set of genes upregulated in hypoxic endothelial cells, normoxic smooth muscle cells and hypoxic smooth muscle cells after knockdown of CDKN2B.
Supplemental Table II - List of common genes upregulated in CDKN2B deficient hypoxic endothelial cells, normoxic smooth muscle cells and hypoxic smooth muscle cells.

<table>
<thead>
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