Hemodynamic Activation of β-Catenin and T-Cell-Specific Transcription Factor Signaling in Vascular Endothelium Regulates Fibronectin Expression

Bradley D. Gelfand, Julia Meller, Andrew W. Pryor, Michael Kahn, Pamela D. Schoppee Bortz, Brian R. Wamhoff, Brett R. Blackman

Objective—The goal of this study was to assess the activity of β-catenin/T-cell-specific transcription factor (TCF) signaling in atherosclerosis development and its regulation of fibronectin in vascular endothelium.

Methods and Results—Histological staining identified preferential nuclear localization of β-catenin in the endothelium of atheroprone aorta before and during lesion development. Transgenic reporter studies revealed that increased levels of TCF transcriptional activity in endothelium correlated anatomically with β-catenin nuclear localization and fibronectin deposition. Exposure of endothelial cells to human-derived atheroprone shear stress induced nuclear localization of β-catenin, transcriptional activation of TCF, and expression of fibronectin. Activation of fibronectin expression required β-catenin, TCF, and the transcriptional coactivator CRBP-binding protein. Finally, we identified platelet endothelial cell adhesion molecule-1 as a critical regulator of constitutive β-catenin and glycogen synthase kinase-3β activities.


Key Words: endothelium ■ extracellular matrix ■ β-catenin ■ shear stress ■ TCF/LEF

Beta-catenin (β-cat) is a highly conserved, multifunctional member of the armadillo family whose nuclear translocation and coactivation of the T-cell-specific transcription factor (TCF)/lymphoid enhancer-binding factor (LEF) family of transcription factors represents a critical step in a variety of cell processes, including development, epithelial-mesenchymal transition, angiogenesis, and differentiation.1 Studies have identified a role for TCF/LEF activity in several pathological features of advanced atherosclerotic lesions, including vascular calcification2-4 and smooth muscle cell proliferation.5 However, the involvement of this signaling pathway in the endothelium during early atherosclerosis development is poorly understood.

Cytosolic β-cat is constitutively targeted for ubiquitination-mediated degradation via glycogen synthase kinase 3β (GSK-3β)-dependent phosphorylation. On stimulation by various factors (including canonical Wnts and growth factors) GSK-3β activity is decreased, leading to nuclear accumulation of β-cat, followed by binding and activation of the TCF/LEF family of transcription factors. One target of TCF/LEF-dependent transcription is the extracellular matrix protein fibronectin.6 TCF-dependent fibronectin expression has been identified to play important roles in several cell contexts, including fibroblast differentiation,7 lung branching morphogenesis,8 and epithelial-mesenchymal transition.9 Fibronectin is also highly regulated in atherosclerotic tissue and is involved in atherosclerosis development through promotion of inflammation and endothelial permeability.9-12 However, the role of endothelial β-cat/TCF in this process remains unknown.

One prominent feature of the atherosclerotic environment is hemodynamic shear stress, which regulates the phenotype of endothelial cells (EC)13,14 and largely explains the regional bias of atherosclerosis development.15,16 Specifically, low-magnitude, reversing shear stress, such as that which occurs in branching and curved vessels,17,18 induces a chronic inflammatory phenotype in preatherosclerotic endothelium. GSK-3β inactivation occurs in response to onset of shear stress in a platelet endothelial cell adhesion molecule-1 (PECAM-1)-dependent manner,19 although the role of PECAM-1 in β-cat/TCF transcriptional regulation is not known. In this report, we describe the preferential activation...
of β-cat/TCF signaling in the atherosclerotic environment by hemodynamic shear stress, as well as its contribution to expression of the proatherogenic protein fibronectin. The overall goal of this work was to identify novel pathways involved in atherosclerosis development, inflammation, and shear stress that may provide valuable insight into the heterogeneous anatomic distribution of the disease, as well as providing new preventative or interventional opportunities.

Methods

Cell Culture
Primary human umbilical vein EC (HUVEC) were isolated as previously described20 and maintained in M199 (Lonza) with 10% fetal bovine serum (Gibco), 5 μg/mL EC growth supplement (Biomedical Technologies), 10 μg/mL heparin (Sigma-Aldrich), 2 mMol/L L-glutamine (Gibco), and 100 U penicillin/streptomycin (Invitrogen). Bovine aortic EC were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum, 2 mMol/L L-glutamine, and 100 U penicillin/streptomycin. β-cat null murine EC (described here24) were maintained in MCDB-131 (Gibco) supplemented with 20% fetal bovine serum, 5 μg/mL EC growth supplement, 10 μg/mL heparin, 2 mMol/L L-glutamine, and 100 U penicillin/streptomycin.

Hemodynamic Shear Stress Application
Shear stress profiles measured from healthy human subjects17 were applied to cells using a cone and plate viscometer as previously described.20,22,23 Shear stress was applied for 24 hours except where indicated to assess the role of TCF/LEF signaling in a shear stress–adapted phenotype.22–24

Mice
Male wild-type, apolipoprotein E (ApoE)−/− mice were isolated as previously described and maintained in a minimum of 3 mice in each condition. Heterozygous TOPGAL mice on a CD1 background (Jackson Laboratory) were crossed with B6 mice for 3 generations. To study TCF/LEF activity in reporter mice (TOPGAL) was measured. The expression of lacZ was greater in the atheroprone lesser curvature compared with the greater curvature (Figure 2A), suggesting that the atheroprone environment contributes to activation of TCF/LEF transcriptional activity. The activity of β-cat/TCF within developing atherosclerotic lesions was assessed in TOPGAL+/−/ApoE−/− mice (Figure 2B). LacZ expression was observed in endothelium superficial and adjacent to lesion development, which suggests that TCF/LEF-dependent transcription precedes lesion growth. Consistent with previous reports, fibronectin was strongly expressed in early atherosclerotic lesions (Figure 2C). Fibronectin expression and TCF/LEF activation exhibited parallel region-specific staining patterns. Cumulatively, these findings indicate that β-cat nuclear signaling preferentially occurs in regions predisposed to atherosclerosis.

Atheroprone Hemodynamics Promote β-cat/TCF Activation and Expression of Fibronectin
Because constitutive nuclear β-cat and TCF/LEF activities were observed in arterial regions that constantly experience low, reversing shear stress, we next tested relative activity of this pathway in response to hemodynamic forces. Cultured EC monolayers were subjected to human-derived atheroprone and atheroprotective shear stresses via a shear stress cell culture system (Figure 3A and Supplemental Figure II). Following shear stress exposure, levels of nuclear accumulation of β-cat were significantly increased by atheroprone compared with atheroprotective shear stress (Figure 3B). Furthermore, cells transected with a TCF-responsive luciferase reporter plasmid exhibited significantly increased TCF activity when exposed to the atheroprone flow compared with atheroprotective flow (Figure 3C).
To directly test the transcriptional capacity of β-catenin/TCF signaling in response to arterial hemodynamic simulation, we focused on a small cohort of known TCF-dependent, atherosclerosis-related genes. To identify the requirement of β-catenin/TCF signaling to gene expression, we analyzed mRNA transcripts from EC treated with adenovirus (Ad) containing either a dominant-negative (DN) TCF-4 lacking the N-terminal binding26 (Ad-DN-TCF4) or an empty cytomegalovirus promoter control and exposed to shear stress. Three candidate genes (CyclinD1, interleukin [IL]-8, and fibronectin) were identified as being both upregulated under atheroprone hemodynamics and inhibited by Ad-DN-TCF4 treatment (Figure 4A and Supplemental Figure III). Because of the critical role of fibronectin in endothelial biology, we focused on this target. Application of atheroprone hemodynamics increased expression of fibronectin mRNA by approximately 2-fold compared with atheroprotective flow (Figure 4A). Pretreatment of EC with Ad-DN-TCF4 signifi-
Significantly reduced expression of fibronectin under atheroprone flow. Protein analysis confirmed both the atheroprone hemodynamic-induced increase as well as the reduction in fibronectin protein levels by Ad-DN-TCF treatment (Figure 4B). This suggests a novel role for \( \gamma \)H9252\-cat/TCF regulation of EC fibronectin in response to hemodynamic stimulation. To determine whether \( \gamma \)H9252\-cat binds to the fibronectin promoter, we performed a chromatin immunoprecipitation assay. We observed a significant level of enrichment of the proximal fibronectin promoter when lysates were precipitated with a \( \gamma \)H9252\-cat antibody (Figure 4C and Supplemental Figure IV) compared with mock control and compared with a more upstream genomic region. This finding confirms the specific interaction between \( \beta \)-cat and the fibronectin transcriptional regulatory region.

To confirm the requirement of \( \beta \)-cat in fibronectin expression, we next interrogated the expression of fibronectin in \( \beta \)-cat knockout murine EC. Cells were reconstituted with \textit{Xenopus} \( \beta \)-cat in the presence or absence of a \textit{Drosophila} derived engrailed repressor domain fused to the C terminus (X\( \beta \)-cat-Eng) (Figure 4D). This construct has been shown to effectively inhibit \( \beta \)-cat/TCF transcriptional activity. Atheroprone hemodynamic-induced expression of fibronectin was rescued in knockout cells reconstituted with X\( \beta \)-cat, but not in cells expressing X\( \beta \)-cat-Eng (Figure 4E), providing direct evidence for the transcriptional requirement of \( \beta \)-cat in fibronectin expression. Thus, the requirement of \( \beta \)-cat/TCF signaling in shear stress-induced fibronectin expression was substantiated by loss- and gain-of-function experiments.

![Figure 2. Preferential TCF/LEF activation in the atherosclerotic prone aorta. A, En face analysis of TCF/LEF reporter TOPGAL transgenic mice. Mixed background CD1:B6 heterozygous transgenic reporter mice were assessed for the relative expression of lacZ within the atheroprotected greater and atheroprone lesser curvature of the aortic arch. Aortic rings were fluorescently stained for lacZ expression, and staining intensity was quantified. (n=3, \( * \)P<0.05). B, TCF/LEF activation in the aorta during early atherosclerosis. LacZ expression was assessed in early atherosclerotic lesions of chow diet-fed 12- to 20-week-old TOPGAL\(^{+/+}\)/ApoE\(^{-/-}\) mice using immunohistochemistry in paraffin-embedded cross-sections. i, Representative aortic cross section of an early atherosclerotic lesion. ii and iii, Higher magnification images of lacZ expression. iv, No primary antibody staining control. v, TOPGAL-null ApoE\(^{-/-}\) littermate stained for lacZ expression as a nonspecific control. Ab indicates antibody. C, Fibronectin expression correlates with TCF/LEF activity in early atherosclerosis. Sections adjacent to those presented in B were stained for fibronectin expression. ii and iii, Higher magnification images of fibronectin expression. iv, Isotype staining control.](http://atvb.ahajournals.org/doi/10.1161/01.ATV.0000379640.11700.94)
To achieve maximal activation, β-catenin must recruit histone acetyl transferases, including CREB-binding protein (CBP) and p300, to the transcription complex. Human EC were exposed to specific inhibitors of β-catenin/CBP and β-catenin/p300 interactions (ICG-001 and IQ-1 respectively). Inhibition of β-catenin/CBP interactions inhibited both fibronectin gene and protein expression (Figure 4F). Pharmacological inhibition of β-catenin/p300 had no statistically significant effect, though we cannot eliminate the possibility of β-catenin/p300 regulation of fibronectin expression. This suggests that recruitment of CBP to the β-catenin/TCF complex is required for expression of fibronectin in EC induced by atheroprone hemodynamics.

**DN-TCF4 Inhibits Shear Stress–Induced EC Inflammation**

The transcription factor complex NF-κB represents a major inflammatory mediator in EC and atherosclerosis. Atheroprone shear stress enhances NF-κB activity in vitro to prime EC toward a proinflammatory phenotype. Here we wanted to test the hypothesis that downstream activation of β-catenin/TCF contributes the “primed” NF-κB activation in response to atheroprone flow. Infection with Ad-DN-TCF4 reduced atheroprone flow-stimulated NF-κB activity by 70% (Figure 4G), supporting the physiological importance of β-catenin/TCF signaling in the atheroprone environment.

**PECAM-1 Is Required for Constitutive β-catenin Nuclear Localization**

Having established that activation of β-catenin/TCF signaling occurs in response to hemodynamics, we next interrogated the importance of a critical shear stress–sensing protein PECAM-1. Immunostained aortic sections from young (8-week-old) PECAM-1−/− ApoE−/− mice exhibited no nuclear localized β-catenin compared with age-matched ApoE−/− mice (Figure 5A). In addition, 8- to 13-week-old PECAM-1−/− ApoE−/− mice, whose ApoE−/− counterparts exhibited early lesion development, showed no nuclear β-catenin staining in atheroprone regions (Figure 5B). Together, these observations point toward a novel regulatory role for PECAM-1 in the constitutive regional activation of β-catenin nuclear translocation.

**Shear Stress–Induced Inhibition of Endothelial GSK-3β Depends on PECAM-1**

The presence of nuclear β-catenin and active TCF/LEF in endothelium of atherosclerotic lesions suggests inhibition of the β-catenin degradation pathway. Following exposure to atheroprone hemodynamics, phospho-serine-GSK-3β (pS-GSK-3β) levels were found to depend on PECAM-1 expression (Figure 5C).

**Atherosclerotic Lesions Exhibit Elevated GSK-3β Inhibition, Which Depends on PECAM-1**

We next assessed the relative abundance of inactivated GSK-3β in atherosclerotic lesions. pS-GSK-3β levels were elevated at sites of atherosclerotic lesion in ApoE−/− mice (Figure 5D, ii versus iii). In PECAM-1−/− ApoE−/− aortas, pS-GSK-3β levels were homogeneous around the aortic circumference, regardless of lesion burden (Figure 5D, vi versus vii). Thus, in PECAM-1−/−, the endothelium exists in 2 distinct populations of pS-GSK-3β expression, whereas genetic deletion of PECAM-1 imparts uniformity along the vessel lumen (Figure 5D, iv versus viii). pS-GSK-3β levels correlate with both nuclear β-catenin colocalization (Supplemental Figure II), as well as fibronectin expression. This implicates PECAM-1 as a critical regulator of pS-GSK-3β in atherosclerotic endothelium.

**Discussion**

This study identifies for the first time the activation of β-catenin/TCF signaling in the endothelium before and during early atherogenesis. Our data suggest that atheroprone shear stress serves as a potent activator of endothelial β-catenin/TCF signaling through a PECAM-1/GSK-3β-dependent mechanism and that this in turn drives transcription of fibronectin, a critical regulator of endothelial phenotype.

Activation of β-catenin/TCF signaling is known to occur in response to a diverse set of cellular cues. Classically, initiation of TCF activity occurs through canonical Wnt signaling, where soluble Wnt factors bind to the Frizzled family receptors, leading to repression of the GSK-3β/Axin/adenomatous polyposis coli complex. Nuclear translocation of β-catenin in EC by canonical Wnt signaling within the vascular wall is conceivable, given that they express multiple Frizzled receptors. However, the presence of canonical Wnt ligands in the vessel wall during atherosclerosis is unknown. A recent
study points to the presence of Wnt pathway antagonist Dickkopf-1 in atherosclerotic plaques. In addition, the noncanonical Wnt-5a was found to be abundantly expressed in plaques and may inhibit canonical Wnt signaling. In addition to shear stress, other atherogenic stimuli induce activation of β-catenin/TCF signaling in EC. IL-1β alone or in combination with tobacco smoke extract activates TCF/LEF through AKT/GSK-3β. Tumor necrosis factor-α drives paracrine Wnt signaling to promote osteogenesis in arterial smooth muscle cells. In addition to IL-1β, lipopolysaccharide also induced β-catenin nuclear accumulation (Supplemental Figures V and VI), suggesting that activation of this pathway may represent a generic inflammatory response. In the context of the present study, the activation of TCF/LEF likely occurs as a result of a balance between promoting and antagonizing signals.

Our findings support the idea that PECAM-1 is a critical regulator of nuclear accumulation of β-catenin in EC within developing lesions. Previous work documents a role for PECAM-1 in regulating β-catenin localization in the absence of shear stress and atherosclerotic burden. However, divergent responses were reported depending on cell type, suggesting that this process depends on cell-specific factors, including SHP-2 activity. In the context of atherosclerosis develop-
Figure 5. Regulation of nuclear β-caten/TCF localization and GSK-3 activity by platelet endothelial cell adhesion molecule-1 (PECAM-1) preceding and during atherosclerosis. A, En face fluorescent staining of β-caten and PECAM-1 in atheropane regions of chow diet–fed ApoE−/− and PECAM−1−/−ApoE−/− aortas before atherosclerotic plaque development (8 weeks). B, Cross-sections of the lesser curvature of aortas of 8- to 13-week-old chow diet–fed PECAM−1−/−ApoE−/− mice. Tissue was immune-stained and imaged for β-caten (red), nuclei (blue), and tissue autofluorescence (green) (i). Single-channel images of nuclei (ii) and β-caten (iii) reveal decreased nuclei/β-caten colocalization (compare with Figure 1B). Arrows denote individual EC. C, HUVEC treated with either scrambled small interfering RNA (siCtl) or small interfering RNA targeted against PECAM-1 (siPECAM-1) were exposed to 24 hours of prone shear stress and assessed for relative levels of pS-GSK-3β by Western blot. **P<0.001, n=4 to 6. D, Aortas of Western diet–fed 22-week-old ApoE−/− (i to iii) and PECAM−1−/−ApoE−/− (iv to vii) mice were stained for pS-GSK-3β. i and v, Low-magnification images of IgG control-stained (left) and working-stained (right) aortic cross-sections. Expression was assessed in lesion-burdened (ii and vi) and non–lesion-burdened (ie, healthy) (iii and vii) endothelium. A histogram of pixel intensities of lesion and healthy EC pS-GSK-3β expression for ApoE−/− (iv) and PECAM−1−/−ApoE−/− (vii) revealed increased pS-GSK-3β levels in atherosclerotic plaques of ApoE−/− but not in PECAM−1−/−ApoE−/− plaques (the rightward shift of pS-GSK-3β intensity in lesion-burdened EC in ApoE−/− was absent in PECAM−1−/−ApoE−/−).
Fibronectin expression, shown here to require CBP/β-catenin/TCF under atheroprose shear stress, exerts multiple proatherogenic effects. Shear stress activation of NF-κB and JUN N-terminal kinase is enhanced on cells plated on fibronectin matrices.11,47 Fibronectin also contributes to shear stress–induced endothelial barrier dysfunction via activation of p21-activated kinase.48 The mechanism by which β-catenin/TCF signaling drives transcription of fibronectin gene expression is not completely known. A consensus TCF/LEF binding site was identified in the Xenopus fibronectin promoter; however, our analysis of the human fibronectin promoter revealed no putative proximal TCF/LEF consensus binding site. β-catenin has been shown to bind to the fibronectin promoter independently of TCF/LEF in SW480 cells.49 In this regard, the CBP/β-catenin/TCF complex may be critical for fibronectin expression, independently of TCF/DNA binding. We confirmed via chromatin immunoprecipitation assay that β-catenin binds to the proximal fibronectin promoter in EC. Together, this supports that regulation of fibronectin by β-catenin may occur via both direct TCF-independent and indirect TCF-dependent transcriptional regulators.

In addition to fibronectin, more than 50 genes have been identified as being TCF/LEF dependent.50,51 Among these are several atherosclerosis-associated genes, including CyclinD152 and IL-8,53 whose transcript levels were elevated by atheroprose hemodynamics and significantly inhibited by DN-TCF-4 treatment, suggesting that β-catenin/TCF activity contributes broadly to endothelial gene expression in atheroprose regions. It is appreciated that endothelial turnover at atherogenic sites is significantly elevated. It is conceivable that β-catenin/TCF-dependent CyclinD1 expression plays a role in this process.54 Furthermore, although elevated TCF/LEF reporter activity in atherosclerotic lesions was isolated to the endothelium and subendothelial foam cells, we cannot exclude that activation of this pathway in other cells contributes to lesion development. We observed β-catenin levels in smooth muscle cells, as well as several circulating cells that were likely monocyes.

Collectively, this study identifies a novel pathway activated in the early atheroprose environment, as well as revealing the regulation of a key player in arterial remodeling during atherosclerosis development. We demonstrate that β-catenin nuclear localization and TCF/LEF transcriptional activation in the aortic endothelium occurs in response to atheroprose hemodynamic stimulation and precedes lesion development or advancement. This phenomenon may have diverse effects on atherosclerotic lesion biology in addition to its role in the regulation of the proinflammatory molecule fibronectin, and future study is warranted to identify other β-catenin/TCF signaling targets in atherosclerosis.

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Disclosures
None.

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DETAILED METHODS

Cell culture

Cells were cultured on 1% gelatin coated, tissue treated plastic. All cells were used below passage 4. For gene expression studies, HUVEC were plated at 80,000 cell/cm², treated with either DN-TCF4 or Ad-Empty adenovirus (Vector Bio Labs) at 100 MOI, and incubated for 24hr.

Media during the flow experiment comprised of M199 (HUVEC), DMEM (BAEC) or MCDB-131 (MEC) supplemented with 4%w/v dextran (Sigma) such that the viscosity was increased to 4cP, 2% fetal bovine serum, 2mM L-glutamine, 100U penicillin/streptomycin, 5μg/ml endothelial cell growth supplement, and 10μg/ml heparin. Fresh media was continuously exchanged in the culture dish, and the environment was humidified and maintained at 37°C and 5%CO₂.

Other reagents used include recombinant human IL-1β (Peprotech, 5nM) and GSK-3β inhibitor IX (BIO) (Calbiochem, 1.4μM), ICG-001 and IQ-1 (generous gifts from Dr. Michael Kahn, University of Southern California). For ICG-001 and IQ-1 studies, media containing 5μM of either compound or equivalent volume of DMSO (1:20,000) was continuously supplied to cells throughout the duration of the flow experiment.

siRNA Knockdown

For gene silencing experiments, HUVEC were plated at 40,000 cells/cm², and transfected with either scrambled control oligonucleotides, or pooled siRNA directed against the human PECAM-1 gene (Dharmacon, OnTargetPlus) at a concentration of 50mM using Oligofectamine (Invitrogen) according to the manufacturer’s instructions. Treatment with siRNA yielded a roughly 80% reduction in PECAM-1 levels. Cells were allowed to recover for 48hr prior to shear stress application.

Reporter studies

For TCF/LEF reporter experiments, approximately 2 million cells were transfected with 4μg of Super8x-TOPFlash (a gift from Dr. Randall Moon) using Lipofectamine2000 (Invitrogen) according to the manufacturer’s instructions. Cells were allowed to recover for 48hr. For NF-κB reporter studies, cells were infected with NF-κB luciferase reporter adenovirus (Vector Bio Labs) at 50MOI 24hr prior to shear stress stimulation. Following shear stress application, cells were lysed using Passive Lysis Buffer (Promega). Lysis samples were injected with Luciferase Assay Reagent (Promega) and luciferase activity was assessed (BMG Fluostar). Values were normalized by protein concentration using the Bradford Protein Assay (Thermo Scientific).

Plasmid Transfections

Approximately 1 million β-catenin knockout MECs were plated at a density of 80,000 cells/cm² and transfected with 3μg of either Xβ-cat or Xβ-cat-Eng using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were allowed to recover for 24hrs prior to exposure to atheroprone hemodynamics.

Western blotting

Samples were lysed in 62.5mM Tris, 2%w/v SDS, 10% glycerol, 50mM DTT, 0.01%w/v bromophenol blue. Blots were blocked in 5% bovine serum albumin, and incubated with the following primary antibodies: anti-Fibronectin (BD Transduction Laboratories), anti α-tubulin (Sigma), anti-PECAM-1 (Santa Cruz), anti-phospho-GSK-3β (Cell Signaling), and anti-TATA binding protein (BD Transduction Laboratories).

Nuclear isolation

Nuclear extracts were isolated as previously described. Briefly, cells were lysed in hypotonic lysis buffer (10 mM HEPES/KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT), homogenized and centrifuged at 2,000 RPM 4°C for 10min. The pellet containing intact nuclei was treated with nuclear
extraction buffer (20mM HEPES/KOH, pH7.9, 420 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 25% Glycerol, Protease Inhibitor Cocktail) and centrifuged at 15,000xg for 30 min at 4ºC. The supernatant containing nuclear protein was supplemented with 3x glycerol-free sample buffer (150 mM Tris-HCl pH 6.8, 6% SDS, 3% β-mercaptoethanol, 0.06% bromophenol blue), gel loaded, and Western blotted.

RNA isolation and PCR

RNA was extracted using the PureLink Kit (Invitrogen), and genes were assessed using RT-PCR with the following reagents: AmpliTaq Gold (Applied Biosystems), SYBR Green (Bio-Rad). The primers used are described below. Gene expression levels were normalized to the control gene β-2-microglobulin.

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Immuno-Staining

Cells were plated on gelatin coated treated plastic coverslips, stimulated, then fixed in 4% paraformaldehyde. Cells were permeabilized with 0.2% Triton-X for 5 min, incubated with either mouse-anti-β-catenin (BD Biosciences) or mouse-anti-Engrailed (Santa Cruz Biotechnology) followed by fluorescent secondary antibody (Alexa Fluor 546, Molecular Probes).

For mouse studies, animals were sacrificed and perfusion fixed in 4% paraformaldehyde at the indicated times. For en face imaging, aortic rings were excised such that orientation (lesser/greater curvature) was maintained. For slides, 5µm thick sections were taken from paraffin embedded tissue. For en face imaging, tissue was treated boiled in water with antigen retrieval solution (Vector Labs) and permeabilized with 0.2% Triton-X for 5 min, incubated with primary antibody overnight (goat-anti-PECAM-1, Santa Cruz), followed by incubating with the nuclear stain TOTO III (Invitrogen) and fluorescently conjugated secondary antibody. For p65 staining, a mouse-anti-p65 antibody (Chemicon) was fluorescently tagged using Alexa Fluor 546 Monoclonal Antibody Labeling Kit (Invitrogen). Fluorescent lacZ staining was accomplished using an anti-β-galactosidase primary antibody (Cortex Biochem) and FITC-conjugated Tyramide Signal Amplification Kit (PerkinElmer) according to the manufacturer’s instructions.

Cross-section staining was performed as follows: Paraffin-embedded aortic sections were incubated with rabbit polyclonal primary antibodies against β-galactosidase (Cortex Biochem) or β-catenin (Cell Signaling). Sections were mounted in DAPI for visualization of cell nuclei. For immunohistochemistry, pressure cooker antigen retrieval, streptavidin-conjugated secondary antibody anti-rabbit IgG (Sigma), Vectstain Elite Kit (Vector Labs) and diaminobenzidine (Dako) were used for antigen visualization. Staining was quantified by segmenting the endothelial layer through image processing, binning cells as either lesion or non-lesion by visual inspection and plotting gray-scaled pixel intensity.

Quantification of subcellular βcat distribution

Images of immunostained cells or tissue was processed to determine relative βcat distribution. Regions of interest were taken such that junctional, cytosolic and nuclear subcellular fractions were represented within a narrow field, and image intensity was plotted as a function of unidirectional spatial distribution. Sub-regions were separated as containing either cell junction, cytosol or nucleus and average staining intensity was calculated. Cell ROIs were chosen blindly, and calculations were performed in a custom automated MATLAB program.
**ChIP Asssay**

Confluent HUVEC cultures were fixed with 1% formaldehyde at 37°C for 10 min. Fixation was terminated at room temperature with 125 mM Glycine for 5 min. Hereafter, the cells, chromatin preparations and ChIP reactions where handled at 4°C as previously described with modifications. The cells were washed once with ice cold Dulbecco’s phosphate buffered saline then harvested by scraping in cold immunoprecipitation (IP) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% nonidet-P40, 0.1% Triton-X 100) containing 1.5% of a protease/phosphatase inhibitor solution (HALT #78440, Pierce; Rockford, IL). The cells were pelleted by centrifugation (2000 × g, 5 min), washed twice with IP buffer, diluted to approximately 10⁷ cells/mL then sonicated at 300 watts for 30 – 35 min in 30 sec bursts using a Bioruptor™ (Diagenode; Sparta, NJ). Aliquots of supernatant containing sheared chromatin from approximately 3 × 10⁶ cells were transferred to reaction tubes after the cellular solution was cleared by centrifugation (12,000 × g, 10 min). Specific ChIP reactions were performed with a rabbit polyclonal antibody (1.2 µg; Cell Signaling Technologies, #9587; Danvers, MA) targeting the carboxy terminus of human β-Catenin. ChIP reactions containing either rabbit anti-mouse RNA polymerase II (5 µg; Santa Cruz Biotechrntologies, #sc-899X; Santa Cruz, CA), or non-immune rabbit IgG (5 µg; Millipore #PP64; Danvers, MA) served as the positive and negative controls, respectively. The reactions (400 µL final volume) were incubated on a rotating platform for 18 h then cleared by centrifugation before the supernatant containing the antibody-protein-chromatin complexes was transferred to tubes containing Protein A agarose beads block with salmon sperm DNA (40 µL of a 50% slurry; Millipore, #16-157). The immunoprecipitation tubes were incubated with rotation for 2 h after which the antibody-bead complexes were pelleted by centrifugation (2000 × g, 30 sec) and the supernatant was discarded. The beads were washed five times with 1 mL of cold IP buffer without protease or phosphatase inhibitors. ChIP DNA and “Total” control DNA (ethanol-precipitated from 50 µL of each starting chromatin preparation) were purified at room temperature using Chelex 100 as described.

**Quantification of ChIP DNA**

The DNA concentration of the final ChIP and Total DNA samples was determined in triplicate using a FLUOSStar Omega (BMG Labtech; Durham, NC) microplate reader and PicoGreen® dsDNA dye (#P7581, Invitrogen; Carlsbad, CA). Briefly, 5 µL of sample was diluted in 400 µL of TE solution (10 mM Tris, 1 mM EDTA, pH 7.5) containing PicoGreen® dye diluted 1:400. The samples were compared to a DNA standard curve generated with sheared DNA extracted from sonicated HuVEC cells using the phenol|chloroform|isoamyl alcohol (AC327115000, Fisher Scientific; Pittsburgh, PA) method and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

**Real-time PCR**

Real-time PCR was performed in triplicate using 3 ng of ChIP DNA in a 25 µL reaction containing 1X iQ™ SYBR® Green supermix (Bio-Rad; Hercules, CA) and 400 nM each of forward and reverse primers. Specific primers were designed to amplify bases –182 to –445 within the human Fibronectin 1 (FN1) promoter region (Forward: 5’-445-TCCACCCCCGAAGAGGTTGACGCA-3’; Reverse: 5’-182-CACGGGGGACTGTGGGTTC-GC-3’). In addition, PCR was also performed on 3 ng of ChIP DNA using control primers directed at a region approximately 2 kb upstream from the FN1 transcriptional start site (Forward: 5’-1960-TCCCTCCCCCAGAATCAATGAA-3’; Reverse: 5’-
GGGAAGCCGAGTGTTTCTTCC-3’). The sheared DNA used as a standard in the PicoGreen assay was further diluted and used to generate a standard curve in each real-time PCR assay so that assay efficiencies and sample starting quantities (SQ) could be determined. The data are analyzed using SQ for β-Catenin, RNA polymerase II (positive control) or Mock (negative control) ChIP samples divided by SQ for the Total DNA control and presented as relative enrichment of the FNI promoter region or the upstream control region.

Statistics
Data were analyzed using SigmaStat software. Pair-wise comparisons were made using a student’s t-test or a Rank Sum test where appropriate.

Supplemental References

Supplementary Figure I. Endothelial NF-κB staining in the aortic arch. Adjacent aortic sections from 8-13wk ApoE⁺⁻ (from Figure 1B) were assessed for relative nuclear localization of the p65 subunit of the NF-κB transcription factor complex by immunofluorescence. To detect nuclear accumulation, aortic cross-sections were fluorescently stained for NF-κB (red), nuclei (blue) and tissue autofluorescence (green). Single channel images of nuclei (ii,v) and NF-κB (iii,vi) reveal that both enhanced NF-κB nuclear localization and βcat nuclear accumulation occur preferentially in the atheroprone lesser curvature compared to the atheroprotective greater curvature. Arrows denote individual endothelial nuclei.
Supplementary Figure II. Healthy and atherosclerotic regions of 22wk old Western diet-fed ApoE-/-(A) and PECAM-1+/ApoE+-(B) exhibit unique βcat nuclear localization patterns. High magnification immunofluorescent images of βcat (red) and nuclei (blue) were obtained in regions of free from atherosclerosis (i) and laden with atherosclerotic lesions (iv). Single channel images of nuclei (ii,v) and βcat (iii,vi) reveal unique staining pattern. Atherosclerotic ECs exhibit enhanced nuclear βcat co-localization in ApoE-/ (compare A-vi to A-iii), but not in PECAM-1+/ApoE+ (B-vi versus B-iii). Staining patterns of nuclear βcat correlate well with pS-GSK-3β levels (Figure 7B).
Supplementary Figure III. Selected TCF-target genes are upregulated by atheroprone hemodynamics in a TCF-dependent manner. HUVEC treated with either Ad-Empty or Ad-DN-TCF4 were exposed to atheroprotective or atheroprone shear stress for 24hr. mRNA levels were assessed by RT-PCR and normalized to β-2-microglobulin. *p<0.05, †p<0.01, n=4-6.
**Supplemental Figure IV.** PCR amplification of the proximal human fibronectin promoter was performed following pull-down with antibodies targeting Pol II and rabbit IgG (mock) antibodies which served as positive and negative controls respectively. Upstream refers to PCR amplification of a region 2kb upstream of the proximal fibronectin promoter. †p<0.01, n=6.
Supplementary Figure V

A

B

C

D

NF-κb/p65

Protected + Veh

Protected + LPS

Prone + Veh

Prone + LPS

β-catenin

Protected + Veh

Protected + LPS

Prone + Veh

Prone + LPS
Supplemental Figure V. βcat nuclear localization and TCF/LEF transcriptional activation in response to IL-1β and LPS. (A) *En face* staining of βcat in endothelium of IL-1β injected chow diet fed ApoE−/− mice. The descending abdominal aorta (left column) and lesser aortic arch (right column) were assessed for βcat localization upon injection of 10μg of recombinant murine IL-1β for indicated times. (B) Primary cultured HUVEC were treated with either 5ng/ml recombinant human IL-1β for 0, 30, or 60 min (i, ii, iii) or 1.4μM of the GSK-3β inhibitor BIO (iv) and fluorescently stained for βcat. (C) TCF/LEF transcriptional activation in the aorta following IL-1β injection. Chow diet fed TOPGAL+/− mice were injected with either vehicle (i, ii) or 10μg IL-1β (iii, iv) for 8hr, and stained for lacZ expression. Arrow indicates lacZ positive endothelial cell. (D) *En face* sections of protected and prone regions of chow diet fed ApoE−/− mouse aortas were assessed for endothelial distribution of the p65 subunit of NF-κB and βcat. Mice were injected with either 110μg LPS or vehicle for 4hr and fluorescently immune-stained for p65 (i-iv) or βcat (v-viii).
Supplementary Figure VI. Redistribution of βcat by IL-1β and LPS treatment. Immunofluorescent images of HUVEC monolayers were analyzed for geometric distribution of βcat. (A) A narrow region of interest (ROI) (indicated by white box) containing cell junctions, cytosol and nucleus was assessed for βcat signal intensity. (B) Intensity profiles across the ROI for four individual cells reveal unique staining patterns based on the treatment. Whereas in the control (unstimulated) condition, intensity peaks appear at the cell junction, BIO treated cells exhibit strong nuclear βcat staining. IL-1β-treated ECs exhibit modest peaks in the nuclear region. (C) βcat signal intensities within separate cell compartments were quantified. *p<0.05, n=4. (D) Images from the study presented in Figure 2A & D were similarly assessed for subcellular distribution of βcat. ApoE−/− mice treated with either vehicle, IL-1β or LPS (4hr) were analyzed for βcat distribution. ECs from atheroprone regions exhibited significantly increased levels of nuclear βcat compared to atheroprotected. Further, IL-1β enhanced nuclear βcat levels in atheroprotected ECs and LPS increased nuclear βcat in both atheroprotected and atheroprone situated ECs. At least 6 cells from each condition were assessed. *p<0.05 compared to atheroprotected/nuclear/control. †p<0.05 compared to atheroprone/nuclear/control.