A Role for the β-Catenin/T-Cell Factor Signaling Cascade in Vascular Remodeling

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Abstract—β-Catenin and T cell factor (Tcf) are distal components of the highly conserved Wnt pathway that govern cell fate and proliferation in lower organisms. Thus, we hypothesized that the regulation of β-catenin and Tcf played a critical role in vascular remodeling. The first objective was to define β-catenin expression in vascular smooth muscle cells (VSMCs) after balloon injury. Indeed, β-catenin mRNA and protein were significantly elevated 7 days after balloon injury in the rat carotid artery. We hypothesized that β-catenin accumulation in response to vascular injury inhibited VSMC apoptosis. In line with our hypothesis, transfection of a degradation-resistant β-catenin transgene into rat VSMCs significantly inhibited apoptosis. Accumulation of β-catenin also resulted in a 10-fold increase in the activation of Tcf. To test if Tcf was necessary to confer β-catenin–induced survival, loss of function studies were carried out with a dominant negative Tcf-4 transgene lacking the β-catenin binding domain, Tcf4(N31). Indeed, loss of Tcf-4 activity abolished β-catenin–induced survival. We further postulated that β-catenin and Tcf promoted cell cycle progression by activating cyclin D1, a target gene of Tcf-4. β-Catenin activated cyclin D1, and this activation was partially blocked with loss of Tcf-4. In parallel, blockade of Tcf-4 resulted in inhibition of [3H]thymidine incorporation and partial blockade of the G1-S phase transition. In conclusion, β-catenin and Tcf-4 play a dual role in vascular remodeling by inhibiting VSMC apoptosis and promoting proliferation. (Circ Res 2002;90:340-347.)

Key Words: vascular smooth muscle cells • apoptosis • proliferation • vascular injury

The dynamic process of vascular remodeling involves numerous molecular signaling cascades governing VSMC migration, differentiation, proliferation, and fate.1–4 The highly conserved Wnt signaling cascade regulates many of these same processes in lower organisms.5–7 Wnts are a large family of secreted glycoproteins that bind to a class of 7 transmembrane receptors termed “frizzled.”5–7 Wnt binding activates disheveled leading to inactivation of the serine-threonine kinase glycogen synthase kinase 3β (GSK3β) and stabilization and accumulation of β-catenin.5–7 β-Catenin translocates to the nucleus and activates a family of transcription factors referred to as T-cell factors/lymphoid-enhancing factors that collectively include Lef-1, Tcf-1, Tcf-3, and Tcf-4.7 The roles of β-catenin and the Tcf family of HMG box-containing DNA-binding proteins have not been defined in VSMCs.

Recent work suggests a role for the Wnt/β-catenin signaling pathway in the pathophysiological remodeling within the cardiovascular system.8,10–13 We recently demonstrated differential inhibition of GSK3β in the intimal tissue following vascular injury that acted as a critical signal mediating VSMC survival.4 This confirmed previous work in other cell systems defining a role for GSK3β in regulating cell fate.9 Moreover, Blankesteijn et al10 demonstrated upregulated expression of the Wnt receptor isoform Frizzled-2 following myocardial infarction, suggesting a role for the Wnt pathway in the spatial control of cardiac wound repair. Finally, Mao and colleagues11 recently demonstrated altered expression of the Frizzled receptor family in VSMCs in response to vascular injury. Based on these collective findings in VSMCs and other tissues, we hypothesized that the inactivation of GSK3β in VSMCs after vascular injury lead to enhanced accumulation of β-catenin and activation of the transcription factor, Tcf.

In addition to its role in cell fate, the GSK3β/β-catenin/Tcf signaling pathway has been demonstrated to exert cell cycle control via regulation of a cell cycle regulatory protein, cyclin D1.14–16 Cyclin D1 contains a Tcf response element within its promoter region and is thought to be a rate-limiting mediator of the G1 to S phase transition in the cell cycle.14,17 The cyclin D1 gene encodes the regulatory subunit of the holoenzyme that phosphorylates the Rb protein resulting in its inactivation. In addition, cyclin D1 has also been shown to act as an oncogene.18 Thus, the second half of our working hypothesis states that the induction of VSMC proliferation in response to vascular injury is mediated in part through the
regulation of a novel β-catenin/Tcf signaling pathway. Taken together, we propose that the GSK3β/β-catenin/Tcf-4 signaling relay may play a dual role in vascular remodeling by inhibiting apoptosis and promoting cell cycle progression.

The purpose of this study was 2-fold: (1) to define the regulation of distal signaling elements mediated by the posttranslational modifications of GSK3β in the vasculature after injury and (2) to further characterize the role of this pathway in mediating VSMC survival and cell cycle regulation.

Materials and Methods

Materials
Wild-type β-catenin construct (gift from S. Byers), β-catenin mutant construct in which the conserved serine/threonine residues in the amino terminus of β-catenin were mutated to alanines19 (gift from D. Kimelman, a dominant-negative form of Tcf-4, known as Tcf-4(N31) (Tcf4(N31)) lacking the N-terminal 31 aa as well as the Tcf-4 control vector (pPGS-CMV-CITE-neo)20 (gifts from E. Fearon), the reporter constructs Topflash and Fopflash, containing either 3 copies of the optimal Tcf motif CCTTTGATC or 3 copies of the mutant motif CCTTTGGCC upstream of a minimal c-Fos promoter driving luciferase expression (gifts from B. Vogelstein),21 cyclin D1 luciferase reporter construct22 (gift from R. Pestell and C. Albanese).

Cell Culture
The clonal A7r5 rat aortic VSMCs were purchased from American Type Culture Collection.

Rat Carotid Artery Balloon Injury Model
Balloon injury was performed as previously described with a 2f balloon catheter in the carotid arteries of 10- to 14-week-old male Sprague-Dawley rats (350 g, n = 35; Harlan, Madison, Wis) in accordance with protocols approved by the Standing Committee on Animals at Morehouse School of Medicine.8,18,22

RNA Isolation and cDNA Preparation
Total RNA was isolated with RNeasy columns with RNase-free DNase treatment. Reverse transcription (RT) reactions were performed using oligo (dT)18 as a primer.

Quantitative Real-Time RT-PCR
Changes in mRNA levels under different experimental conditions were compared by real-time quantitative RT–polymerase chain reaction (PCR) analysis, using the Light Cycler thermocycler (Roche Diagnostics Corp). Reactions were prepared in the presence of the fluorescent dye SYBR green I for specific detection of double-stranded DNA. Quantification was performed at the log-linear phase of the reaction and cycle numbers obtained at this point were plotted against a standard curve prepared with serially diluted control samples.

Transfection
Retrovirus-Mediated Transfection
AmphoPack 293 cells were transfected with the pPGS-CMV-CITE-neo vector (control) or the pPGS-CMV-CITE-neo vector containing the dominant negative Tcf4 mutant lacking the N terminal 31 aa β-catenin binding domain (Tcf4(N31))20 via calcium phosphate as previously described.8 Virus produced from the 293 cells was transfected into A7r5 VSMCs and stable lines were created.

Transient Transfection
A7r5 VSMCs were transiently transfected with Effectene with a total of 0.3 μg DNA/well according to the manufacturer’s directions.

Reporter Assays
Luciferase and EGFP activities in cell extracts were determined according to the manufacturer’s directions. All data are expressed as fold activation of luciferase activity/EGFP fluorescence over values for control transfected cells under baseline conditions.

Quantitation of Apoptosis
Apoptosis was assessed by staining with H33342 and quantitating the percentage of apoptotic nuclei (100 cells counted/sample) in the transfected subset by identifying cells cotransfected with pDsRed1-Mito as previously described.8,23 Our laboratory has extensively cross-validated the use of H33342 staining with other apoptotic indices.8,18,22,23

[^3H]-Thymidine Incorporation
VSMCs stably expressing a control vector or Tcf4(N31) were placed in DMEM plus 1% FBS to induce a quiescent state followed by stimulation with 10% FBS for 18 hours. During the final 4 hours, [3H]thymidine (1.0 μCi/mL) was added.

Cell Cycle Analysis
Cell cycle analysis was assessed by FACS in VSMCs with constitutive expression of Tcf4(N31) or a control vector after a 24-hour exposure to media containing 1% FBS followed by stimulation with 10% FBS for 18 hours.

Western Blotting
SDS-PAGE and Western blotting was carried out as previously described.8 The membranes were re-probed with a mouse monoclonal vimentin antibody to verify equal loading.

Immunofluorescence
A7r5 VSMCs were incubated in serum free media in the presence and absence of lithium chloride (50 mmol/L) for 6 hours. Cells were fixed, blocked, and incubated in primary β-catenin antibody (1:100) or an equal concentration of anti–mouse-IgG control. Fluorescein-conjugated secondary antibody (1:100) was then added and incubated for 1 hour.

Immunohistochemistry
Paraffin sections (5 μm) were mounted on aminopropyltriethoxy-silane-coated slides. Diluted (1:500) monoclonal anti-β-catenin antibody or equal concentrations of a control mouse IgG1 were incubated with sections overnight at room temperature. The proliferating cell nuclear antigen (PCNA, 50 μg/mL) (DAKO) was stained according to manufacturer’s directions.

Statistical Analysis
Comparisons between 2 groups were analyzed via a Student’s t test (P<0.05), whereas comparisons between 3 groups were analyzed by an analysis of variance (ANOVA) with a Student-Newman-Keuls post hoc test (P<0.05). Data are presented as mean±SE.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results
Regulation of GSK3β/β-Catenin Signaling Pathway After Balloon Injury in the Rat
We utilized real-time quantitative RT-PCR, Western blotting, and immunohistochemistry to define the expression profile of β-catenin after vascular injury. β-Catenin mRNA was significantly increased 7 days after balloon injury in rat carotid arteries (data are expressed as a normalized ratio of β-catenin to GAPDH expression levels) (uninjured control vessel 1.2±0.1; injured vessel, 2.2±0.3; n = 9, P<0.01) (Figure 1A). GAPDH mRNA was not significantly different between
Injured and control (uninjured) vessels. Moreover, the accumulation of β-catenin protein was also increased at 7 days after balloon injury as shown by both Western blotting and immunohistochemistry (Figures 1B and 2). A defined time course analysis demonstrated significant accumulation of β-catenin at 7 days after injury that was reduced at day 14 and nearly absent at 28 days (Figure 2). No staining was seen with the negative mouse IgG1 control antibody (Figure 2).

β-Catenin is negatively regulated by GSK3β. Phosphorylation of the regulatory serine 9 site of GSK3β inactivates the kinase and promotes the accumulation of β-catenin in cells.5-7 We utilized a phosphospecific GSK3β antibody specific for the serine 9 site to determine phosphorylation state of GSK3β after 7 days of balloon injury in the rat carotid artery. In line with the accumulation of β-catenin at 7 days, the phosphorylated and inactive form of GSK3β was differentially increased in the injured vessels compared with the control uninjured vessels (Figure 3, top). The membrane was re-probed with vimentin to verify equal loading (Figure 3, bottom). Total GSK3β protein expression was unchanged (data not shown).

**β-Catenin Stimulates an Antiapoptotic Signaling Pathway**

Earlier work from our laboratory demonstrated that the activation state of GSK3β was an important control site regulating VSMC fate.8 Activation of GSK3β was sufficient to induce apoptosis, whereas inactivation of GSK3β, as seen in the intimal tissue, promoted survival.8 We hypothesized that activation of GSK3β degraded β-catenin expression and that β-catenin degradation was an important distal event in the proapoptotic pathway. To test this we blocked β-catenin degradation with a degradation-resistant β-catenin mutant transgene in which the GSK3β serine phosphorylation sites have been mutated to alamines9 and determined the apoptotic rate in response to GSK3β activation (see Materials and Methods). In line with our hypothesis, transfection of the degradation-resistant β-catenin transgene significantly inhibited GSK3β-induced apoptosis (control transfected, 13±2%; GSK3β transfection, 29±2%; GSK3β+β-catenin mutant, 11±1%; n=6, P<0.001) (Figure 4A). This indirectly suggested that GSK3β-induced degradation of β-catenin was important in mediating the proapoptotic response. To directly test this hypothesis we examined the ability of a wild-type β-catenin transgene (degradable by GSK3β) to block GSK3β-induced apoptosis. In accord with our hypothesis, upregulation of a wild-type β-catenin gene was ineffective in blocking GSK3β-induced death (27±3%, n=6) (Figure 4A).

Previous work from our laboratory and others has demonstrated that growth factor withdrawal activates GSK3β.8,9,24 Thus, as another means of testing our working hypothesis that β-catenin blocked GSK3β-induced apoptosis, we subjected VSMCs to serum withdrawal to activate GSK3β and assessed the ability of β-catenin accumulation to inhibit apoptosis. In line with our previous data, upregulation of the degradation-resistant β-catenin mutant resulted in significant blockade of serum withdrawal-induced apoptosis (control transfected, 26±3%; β-catenin transfected, 13±1%; n=7, P<0.001). There was no significant change in the percentage of apoptotic nuclei in serum.

**Figure 1.** β-Catenin mRNA and protein expression in rat carotid arteries 7 days after balloon injury. A, β-Catenin mRNA assessed by real-time quantitative RT-PCR is expressed as a normalized ratio of β-catenin to GAPDH expression (uninjured control vessel 1.2±0.1; injured vessel, 2.2±0.3; n=9, P<0.01). B, Representative Western blot of β-catenin protein 7 days after balloon injury (Top); same blot re-probed with vimentin to control for equal loading (Bottom). C indicates control; I, injury.

**Figure 2.** Time course of β-catenin accumulation assessed by immunohistochemistry at 40× magnification. A, Negative control for β-catenin (a mouse monoclonal antibody IgG1) in the injured vessel at 7 days. B through D, β-Catenin staining in the injured rat carotid artery at 7 (B), 14 (C), and 28 (D) days after injury.

**Figure 3.** Representative Western blot demonstrating enhanced phosphorylated (ser9) GSK3β protein expression in injured vessels 7 days after injury compared with uninjured control vessels. Phosphorylated GSK3β protein expression assessed by Western blotting 7 days after balloon injury (Top); same blot re-probed with vimentin to control for equal loading (Bottom). C indicates control; I, injury.
As a final test of our hypothesis that β-catenin was a critical distal mediator of VSMC fate, we utilized the well-characterized pharmacological inhibitor of GSK3β, LiCl. Immunofluorescence studies demonstrated that LiCl resulted in a significant translocation of β-catenin into the nucleus of VSMCs (Figures 4B through 4G). In line with our hypothesis, LiCl induced nuclear translocation of endogenous β-catenin coincided with a significant inhibition of GSK3β-induced apoptosis (12±2%; n=6) (Figures 4A through 4G). These results suggest that accumulation and nuclear translocation of β-catenin block a proapoptotic signaling pathway induced by growth factor withdrawal and GSK3β activation.

Role of Tcf-4 in β-Catenin–Mediated VSMC Survival

We hypothesized that β-catenin–induced activation of Tcf-4 was an important distal signaling event in the survival pathway. Initial studies with real-time quantitative RT-PCR demonstrated that the Tcf-4 isoform was the predominant Tcf isoform in cultured rat and human VSMCs as well as the rat carotid artery. To test the ability of β-catenin to stimulate Tcf-4, we transiently transfected a Tcf-4 reporter gene construct (Topflash)\(^{21}\) along with a β-catenin degradation-resistant mutant or a control gene into rat VSMCs. Indeed, upregulation of the β-catenin degradation-resistant mutant resulted in significant activation of the Tcf-4 reporter construct (data expressed as fold activation of luciferase/EGFP) (control+Topflash, 1.0±0.1; β-catenin mutant+Topflash, 22.3±1.1; n=6, P<0.001) (Figure 5A). β-catenin–induced activation of Topflash was 10-fold greater than the mutated Tcf reporter construct Fopflash (2.4±0.1, n=6), thereby demonstrating specificity of the response.

To test whether activation of Tcf-4 was necessary to confer the β-catenin–mediated survival pathway in VSMCs, we utilized a retroviral strategy to establish a line of VSMCs with stable upregulation of a FLAG-tagged mutant Tcf-4 gene lacking the β-catenin binding domain (Tcf4(N31)). Characterization of these VSMCs by real-time quantitative RT-PCR and Western blotting with an anti-FLAG antibody confirmed that the mutant Tcf-4 transgene was constitutively upregulated (Figures 5B and 5C). In addition, functional assays using the Tcf-4 reporter construct, Topflash, demonstrated that β-catenin–induced Tcf-4 transactivation in Tcf4(N31) VSMCs was significantly abolished (Figure 5D). In line with our hypothesis, loss of β-catenin–induced Tcf-4 transactivation, significantly inhibited β-catenin–mediated survival in response to serum withdrawal (stable control VSMC lines transiently transfected with a control transgene (pcDNA3.1) 30±2%; or a β-catenin mutant, 15±2%; Tcf4(N31) stable VSMC lines transfected with a control transgene, 30±1%; or a β-catenin mutant, 26±2%; n=7, P<0.001) (Figure 5E). Similar inhibition of Tcf activation and β-catenin–induced survival was seen in transient cotransfection experiments in which a degradation-resistant mutant β-catenin was cotransfected along with the Tcf-4 mutant (control, 36±3%; β-catenin mutant, 17±1%; Tcf4(N31)+control, 29±1%; Tcf4(N31)+β-catenin mutant, 31±2%; n=6, P<0.001).

Role of β-Catenin/Tcf-4 in Cell Cycle Regulation

Previous work in other cell systems has demonstrated that the β-catenin/Tcf signaling pathway regulated cell cycle control.\(^{14–16}\) Indeed, the cell cycle regulatory protein cyclin D1 contains a Tcf-responsive element in its promoter region.\(^{14}\) However, the role of β-catenin and Tcf-4 in governing proliferative signaling pathways in VSMCs has not been determined. To test the ability of β-catenin to stimulate cyclin D1, we utilized a cyclin D1 promoter reporter construct.\(^{16}\) In line with previous work in other cell systems, transfection of a degradation-resistant β-catenin mutant resulted in an increase in cyclin D1 promoter activation (control, 1.0±0.0; β-catenin mutant, 2.3±0.2; n=6, P<0.001). Parallel experiments carried out in Tcf4(N31) VSMCs demonstrated a
significant blockade of β-catenin–induced cyclin D1 activation (control, 0.7 ± 0.0; Tcf4(N31) + β-catenin mutant, 1.5 ± 0.4; n = 6, P < 0.001) (Figure 5F); suggesting that cyclin D1 expression was regulated via a β-catenin/Tcf–dependent signaling pathway.

To better understand the specific role of Tcf-4 in proliferative signaling pathways, we performed [3H]-thymidine experiments in VSMCs with stable upregulation of the Tcf4(N31) mutant. Upregulation of Tcf4(N31) under conditions of 10% FBS resulted in a significant inhibition of thymidine incorporation (Control, 32 671 ± 1689 cpm/min; Tcf4(N31), 24 549 ± 272 cpm/min; n = 12, P < 0.001). This suggested that the proliferative response of VSMCs after vascular injury might be partially governed by the upregulation of β-catenin and activation of Tcf-4.

D-type cyclins have been suggested to be rate-limiting mediators of the G1 to S phase transition in the cell cycle. To directly test this, we utilized FACS analysis to determine the percentage of cells in the S phase of the cell cycle. In line with our earlier data, we saw a 37% reduction in the percentage of cells in the S phase of the cycle in the stable Tcf4(N31) VSMC line compared with the stable control.

Figure 5. A, Upregulation of β-catenin activates Tcf. VSMCs were transiently cotransfected with a control vector or a β-catenin degradation-resistant mutant, the Tcf-luciferase reporter construct Topflash, or the mutated reporter construct Fopflash, and the reporter construct pEGFP-C1. Data are expressed as fold activation of luciferase/EGFP over control transfected cells (mean ± SE) (control + Topflash, 1.0 ± 0.1; β-catenin mutant + Topflash, 22.3 ± 1.1; control + Fopflash, 1.3 ± 0.1; β-catenin mutant + Fopflash, 2.4 ± 0.1; n = 6, P < 0.001). B, Tcf-4 mRNA expression assessed by real-time quantitative RT-PCR and expressed as a ratio of Tcf-4 to GAPDH expression in control transfected rat VSMCs as well as VSMCs with constitutive upregulation of the Tcf4(N31) transgene. Primers were designed to the middle region of the gene thus recognizing both the native Tcf-4 as well as the constitutive overexpression of the Tcf4(N31) dominant negative gene. C, Representative Western blot for FLAG epitope expression in VSMCs with constitutive upregulation of the FLAG-tagged Tcf4(N31) construct. D, β-Catenin–induced Tcf-4 transactivation as measured by a luciferase promoter reporter construct is blocked in VSMCs with constitutive upregulation of Tcf4(N31), providing functional characterization (Tcf-4 control at baseline, 1.0 ± 0.0; Tcf-4 control + β-catenin mutant, 22.4 ± 0.6; Tcf4(N31) at baseline, 1.0 ± 0.1; Tcf4(N31) + β-catenin mutant, 1.1 ± 0.1; n = 6, P < 0.001). Data are expressed as fold activation of luciferase/EGFP in control cells. E, β-Catenin–induced VSMC survival is mediated through a Tcf-4–dependent pathway. VSMCs with constitutive upregulation of a control vector or Tcf4(N31) were transiently transfected with β-catenin and the reporter construct mitotracker red. Apoptosis was assessed in response to serum withdrawal in the transfected subset (Control at baseline, 30 ± 2%; apoptotic nuclei; Control + β-catenin mutant, 15 ± 2%; Tcf4(N31) at baseline, 30 ± 1%; Tcf4(N31) + β-catenin mutant, 26 ± 2%; n = 7, P < 0.001). Data are expressed as mean ± SE. F, Upregulation of β-catenin stimulates cyclin D1 transactivation through a Tcf-4–dependent signaling pathway. VSMCs with constitutive upregulation of a control vector or Tcf4(N31) were transiently transfected with β-catenin, a cyclin D1 luciferase promoter reporter construct and the reporter construct pEGFP-C1. Data are expressed as fold activation of luciferase/EGFP over control cells. Expression was determined under conditions of 10% serum (control at baseline, 1.0 ± 0.0; control + β-catenin mutant, 2.3 ± 0.2; Tcf4(N31) at baseline, 0.7 ± 0.0; Tcf4(N31) + β-catenin mutant, 1.5 ± 0.4; n = 6, P < 0.001). Data are presented as mean ± SE.
VSMC line (Control VSMC, 15.6±0.4% in S phase; Tcf4(N31) VSMC, 9.9±0.5% in S phase; n=8, P<0.001). Thus taken together, we have identified a novel signaling pathway regulating cell survival and cell cycle control in response to vascular injury.

To determine if β-catenin expression was colocalized in proliferating VSMCs within the developing neointima, we stained for proliferating cell nuclear antigen (PCNA), the delta accessory protein of DNA polymerase synthesized in late G1 and S phases of the cell cycle, and β-catenin 7 days after balloon injury in the same vessel. As seen in Figure 6, β-catenin expression is partially colocalized in actively proliferating VSMCs. Taken together, our data suggests that β-catenin accumulation plays a critical role in VSMC proliferation in the context of vascular remodeling.

Discussion

The dynamic process of vascular remodeling involves numerous molecular signaling cascades governing VSMC migration, differentiation, proliferation, and fate. We hypothesized that β-catenin played a critical regulatory role in remodeling of the vessel wall in response to balloon injury. In accord with our hypothesis, we demonstrated significant accumulation of β-catenin as well as inactivation of GSK3β, an upstream negative regulator of β-catenin, 7 days after balloon injury. Transfection of a degradation-resistant β-catenin transgene as well as translocation of endogenous β-catenin to the nucleus resulted in a significant inhibition of apoptosis as well as activation of the downstream transcription factor Tcf. To test if Tcf-4 activation was necessary to confer the antiapoptotic effect of β-catenin stabilization, we performed loss of function studies in which VSMCs expressing a Tcf-4 dominant negative mutant transgene lacking the β-catenin binding domain were transfected with the degradation-resistant β-catenin transgene. In accord with our hypothesis, loss of Tcf-4 activation abolished β-catenin–induced survival.

Prior work from our laboratory and others has demonstrated that mitogens, nutrient signals, or receptor ligand systems that induce an antiapoptotic effect in VSMCs also have the capability to promote proliferation. Furthermore, recent work has demonstrated that the GSK3β/β-catenin/Tcf signaling promotes activation of cyclin D1. Based on these findings, we further hypothesized that injury-induced activation of β-catenin and Tcf-4 played a dual role in the course of vascular remodeling: stimulating cyclin D1 as well as inhibiting apoptosis. Indeed, upregulation of β-catenin resulted in a significant activation of cyclin D1. The β-catenin–induced activation of cyclin D1 was lost in VSMCs expressing the mutant Tcf-4 gene lacking the β-catenin binding domain, suggesting that Tcf-4 was mediating the β-catenin–induced increase in cyclin D1. To our knowledge, this is the first study to demonstrate a role for the β-catenin/Tcf-4 signaling pathway in VSMCs.

We provide evidence from multiple experiments that accumulation of β-catenin in the neointima and activation of Tcf-4 plays a critical role in VSMC proliferation. Brabletz et al demonstrated that β-catenin expression in human tumors did not correlate with proliferative indices but rather with hypertrophy. However, regulation of Tcf-4 was not examined in this study. We speculate that the ability of β-catenin to transduce numerous signals to the cell including survival, hypertrophy, and proliferation is coupled to the downstream transcription factor(s) that it regulates. This likely depends on the balance of outside signals, including growth factors, etc. It is noteworthy that blockade of β-catenin binding to Tcf-4 at baseline does not significantly affect VSMC proliferation as evident from the ability of our line of VSMCs with constitutive expression of Tcf4(N31) to proliferate normally. These findings suggest that Tcf-4 plays a significant role under conditions of vascular remodeling when β-catenin is elevated but likely plays a minor role at baseline. It is noteworthy that mice lacking Tcf-4 die shortly after birth with the single histopathological abnormality in these animals being a lack of cell proliferation in the crypt regions within the small intestine, with no denoted abnormalities in the vasculature.

The role of β-catenin in apoptotic regulation appears to be cell-type specific. In line with our data, upregulation of the β-catenin/Tcf-4 signaling pathway has been demonstrated to inhibit apoptosis in fibroblasts. However, recent work has reported that β-catenin promotes apoptosis in other cell types. The disparity concerning the pro- or antiapoptotic role of β-catenin in different cell types is not surprising to the field of vascular biology given that numerous factors, including nitric oxide, glucose, and TGFβ, have opposing effects on vascular endothelial cells compared with VSMCs. Our work demonstrates that the ability of β-catenin to promote survival and activate cyclin D1 was mediated through Tcf-4. Work in other cell systems has established that upregulation of β-catenin activates several transcription factors, including Tcf-4, lymphoid enhancing factor, TATA-binding protein, Pontin, Teashirt, Sox13 and Sox17, and CREB. It is likely that the role of β-catenin in regulating cell fate in
different tissues is mediated through the complex regulation of multiple transcription factors and co-binding factors.

It is also noteworthy that we see a discrepancy between the time course of β-catenin accumulation and GSK3β phosphorylation state in the developing lesion. We previously demonstrated that 28 days after balloon injury GSK3β remains inactivated; however, β-catenin mRNA (data not shown) and protein in the injured vessel had returned to baseline values. This suggests that the regulation of the complex of GSK3β, β-catenin, axin, glycogen binding protein, APC, and yet to be defined factors is clearly dependent on multiple lines of regulation in addition to the phosphorylation state of GSK3β. Moreover, parallel signaling elements including the cytoskeletal cadherin family are also likely to be regulating β-catenin accumulation. Future studies will need to be completed to more fully understand the multitude of factors regulating β-catenin expression.

The most widely studied areas of research involving the Wnt cascade in mammals have been cancer biology, development, and stem cell differentiation. Recent work defines a role for β-catenin in stem cell differentiation into follicular keratinocytes. Furthermore, β-catenin appears to be necessary in the development of skin and hair. To our knowledge this is the first work to date to define a role for β-catenin in the vasculature. Preliminary data from our laboratory suggests an upregulation of the upstream Wnt signaling mediator, disheveled 1, by in situ hybridization in the developing lesion. Paired with work by others demonstrating expression of Wnt and Frizzled family members in the vasculature, we speculate that β-catenin stabilization and Tcf activation in the remodeled vessel are regulated in part through the Wnt signaling pathway. However, future studies will be needed to directly implicate a role of Wnts and the frizzled family of receptors in vascular disease.

In conclusion, we have identified an integral role for β-catenin and Tcf-4 in the regulation of vascular remodeling. To our knowledge, this is the first evidence of a role for these well-conserved genes in the process of vascular remodeling.

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References


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