Inhibition of Smooth Muscle β-Catenin Hinders Neointima Formation After Vascular Injury

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Objective—Smooth muscle cells (SMCs) contribute to neointima formation after vascular injury. Although β-catenin expression is induced after injury, whether its function is essential in SMCs for neointimal growth is unknown. Moreover, although inhibitors of β-catenin have been developed, their effects on SMC growth have not been tested. We assessed the requirement for SMC β-catenin in short-term vascular homeostasis and in response to arterial injury and investigated the effects of β-catenin inhibitors on vascular SMC growth.

Approach and Results—We used an inducible, conditional genetic deletion of β-catenin in SMCs of adult mice. Uninjured arteries from adult mice lacking SMC β-catenin were indistinguishable from controls in terms of structure and SMC marker gene expression. After carotid artery ligation, however, vessels from mice lacking SMC β-catenin developed smaller neointimas, with lower neointimal cell proliferation and increased apoptosis. SMCs lacking β-catenin showed decreased mRNA expression of Mmp2, Mmp9, Sphk1, and Slpr1 (genes that promote neointima formation), higher levels of Jag1 and Gja1 (genes that inhibit neointima formation), decreased Mmp2 protein expression and secretion, and reduced cell invasion in vitro. Moreover, β-catenin inhibitors PKF118-310 and ICG-001 limited growth of mouse and human vascular SMCs in a dose-dependent manner.

Conclusions—SMC β-catenin is dispensable for maintenance of the structure and state of differentiation of uninjured adult arteries, but is required for neointima formation after vascular injury. Pharmacological β-catenin inhibitors hinder growth of human vascular SMCs. Thus, inhibiting β-catenin has potential as a therapy to limit SMC accumulation and vascular obstruction.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:879-888. DOI: 10.1161/ATVBAHA.116.308643.)

Key Words: beta-catenin ■ beta-catenin inhibitors ■ myocytes, smooth muscle ■ neointima ■ vascular injury

The smooth muscle cell (SMC) is a key participant in adult vascular remodeling because of its remarkable phenotypic plasticity.1 This flexibility allows the SMC to switch—in response to environmental cues triggered by injury or disease—from a contractile, differentiated, and quiescent state in normal vessels to a secretory, proliferative, and migratory form in injured vessels, usually associated with a reduction in SMC-selective marker gene expression.1,2 SMC phenotypic plasticity is recognized as a major factor in the pathogenesis of prevalent cardiovascular diseases such as atherosclerosis, hypertension, restenosis, and saphenous vein graft disease.2,3 Current models of vascular SMC phenotypic modulation are based on the integration of several complex environmental factors: increased growth factor and cytokine activity, disruption of cell–extracellular matrix interactions, neuronal influences, and mechanical forces.1 The in vivo molecular mechanisms that underlie this process, however, are not fully elucidated.

The protein β-catenin plays a dual function in the cell: it works as a transcriptional coactivator in the canonical Wnt signaling pathway and a structural component of the cadherin–catenin complex that mediates cell–cell adhesion.4 β-catenin is known to play critical roles during development, adult homeostasis, and disease, particularly in cancer biology.5 Interestingly, studies performed in the past 15 years suggest that β-catenin may also be a key regulator of SMC biology during adult vascular remodeling. β-Catenin protein levels increase in rat carotid arteries 7 days after balloon injury; this expression decreases by day 14 and is almost absent by day 28.6 Overexpression of a degradation-resistant β-catenin inhibits apoptosis of vascular SMCs in culture and activates cyclin D1, and this effect is lost after expressing a dominant-negative version of T-cell factor 4 (Tcf4, also known as Tcf7l2); moreover, expression of this dominant-negative Tcf4 reduces the G1 to S transition of the cell cycle in vascular SMCs.6 On the contrary, overexpression of N-cadherin, inhibitor of β-catenin and Tcf (ICAT, also known as Ctnnbip1), or a dominant-negative Tcf4 reduces proliferation of vascular SMCs, associated with decreased cyclin D1 expression and increased p21 (also known as Cdkn1a) levels.7 Other cell culture studies support the idea that Wnt4 acting on frizzled class receptor 1 activates...
β-catenin signaling and vascular SMC proliferation. Carotid artery ligation in mice increases β-catenin signaling, which is evident 3 and 28 days after ligation in the media and intima, respectively, and vascular injury also induces Wnt4 and cyclin D1 expression, whereas loss of 1 Wnt4 allele in mice (Wnt4+/-) reduces neointima formation and nuclear β-catenin expression. In human aortic SMCs in culture, oxidized low-density lipoprotein promotes β-catenin stabilization, nuclear translocation, and cyclin D1 expression, and β-catenin is required for oxidized low-density lipoprotein–induced proliferation. Moreover, β-catenin expression correlates with proliferation markers in human atherosclerotic plaques. More recent studies have complemented the earlier observations mentioned above, further supporting the idea that SMC β-catenin has a critical role in adult vascular remodeling. Several studies associate decreased vascular remodeling to factors that may decrease canonical Wnt signaling: (1) genetic loss of transglutaminase 2 reduces vascular SMC proliferation and migration, inhibits platelet-derived growth factor receptor (PDGFR)/Akt1 and β-catenin activation, and attenuates neointima formation after carotid artery ligation; (2) Wnt2−/− and WNT1-inducible-signaling pathway protein 1 knockout (Wisp1−/−) mice exhibit reduced intimal thickening after carotid artery ligation; (3) knockdown of kindlin-2 (also known as Fermi2) reduces carotid intimal hyperplasia after balloon injury in rats and suppresses in vitro Wnt5a–induced vascular SMC proliferation and migration and expression of β-catenin target genes cyclin D1 and c-myc; (4) Emodin, a plant-derived anthraquinone, inhibits carotid intimal hyperplasia after balloon injury associated with reduction of Wnt4, Dvl-1, and β-catenin protein levels and seems to require micro–RNA-126 for its action; (5) the orphan nuclear receptor Nur77 (also known as Nrun1) opposes angiotensin II–induced vascular SMC proliferation, migration, and phenotypic switching by attenuating β-catenin signaling; and (6) the long noncoding RNA growth arrest–specific 5 regulates hypertension-induced vascular remodeling, while interacting with β-catenin and limiting its nuclear translocation in endothelial cells and SMCs in vitro.

Other reports associate increased canonical Wnt signaling with enhanced SMC activities involved in vascular remodeling. These studies show that (1) treatment of vascular SMCs in culture with recombinant Wnt2b, Wnt4, Wnt5a, or Wnt9a, but not Wnt11, increases β-catenin protein levels and cell proliferation; (2) treatment with recombinant Wnt2 increases Wisp1 mRNA levels and induces SMC migration; (3) balloon injury of carotid arteries in diabetic rats results in neointima hyperplasia associated with increased Wnt4, Dvl-1, β-catenin, and cyclin D1 expression and reduced p21 levels, a phenotype suppressed by upregulation of micro–RNA-24; (4) aortas from rats fed a high-fat diet have increased numbers of SMCs and lipid droplets, associated with higher mRNA levels for Wnt3a, β-catenin, Tcf4, and cyclin D1, suggesting a role for Wnt/β-catenin signaling in hyperlipidemia-induced SMC proliferation; and (5) in a model of angiotensin II–induced arterial hypertension, complement C1q activates β-catenin signaling, which is required for vascular SMC proliferation.

Overall, these studies are consistent with a role for the canonical Wnt signaling pathway in SMC biology during adult vascular remodeling; however, the absence of in vivo studies using a SMC-specific, β-catenin loss-of-function approach, particularly in the response to vascular injury (for instance, after carotid artery ligation or balloon injury), limits conclusions as to the direct and essential nature of β-catenin’s involvement in this context. Moreover, whether SMC β-catenin is essential during adult vascular remodeling has therapeutic implications. Inhibitors of β-catenin have been developed, so pharmacological inhibition of β-catenin function is feasible; this strategy would be ineffective if the biological role of β-catenin in adult SMC biology is redundant. On the contrary, if SMC β-catenin is essential in adult vascular remodeling, pharmacologically targeting β-catenin would have potential as a novel therapy for cardiovascular disease. We have recently shown that SMC β-catenin is required in vivo during mammalian development because its loss precludes arterial wall formation and embryonic survival. Here, we have used a tamoxifen-inducible and tissue-specific genetic approach in the mouse to delete SMC β-catenin in adulthood, which has allowed us to test whether it is required in the response to vascular injury. These studies show that SMC β-catenin is dispensable for the maintenance of uninjured adult vessels, but is required for neointimal formation after vascular injury. Moreover, β-catenin is required for expression of a set of genes reported to promote SMC invasion and neointimal growth, including matrix metalloproteinase 2 (Mmp2), and is necessary for SMC invasion in vitro; this complements the proproliferative and prosurvival roles of SMC β-catenin reported previously. Finally, we found that inhibitors of β-catenin effectively reduced growth of mouse and human vascular SMCs in culture.

**Materials and Methods**

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

**Results**

**Adult Vascular Remodeling Is Associated With Induced β-Catenin Expression in SMCs**

β-catenin is expressed in and required by vascular SMCs during developmental artery formation, so we wondered whether it was still expressed in these cells in mature, uninjured arteries of adult mice. We harvested uninjured carotid arteries from 8- to 9-week-old wild-type mice and evaluated β-catenin and SM22α expression by immunofluorescence. We found a clear signal for β-catenin in the endothelial layer but could not detect β-catenin expression in the media of normal arteries (Figure 1A). Then, we induced vascular remodeling in 8- to 9-week-old mice by carotid artery ligation and found β-catenin expression in not only endothelium but also SM22α+ cells in the neointima (Figure 1B). To complement
these studies, we also evaluated β-catenin expression by Western blotting in lysates isolated from carotid arteries and found increased β-catenin levels in ligated vessels compared with uninjured arteries (Figure 1C; Figure IA in the online-only Data Supplement). In addition, when we isolated SMCs from uninjured carotid arteries or aortas and cultured them under standard growth-promoting conditions, we observed β-catenin expression in these primary SMCs at cell–cell contacts and in the cytosol and nucleus (Figure 1D; Figure IB in the online-only Data Supplement). Therefore, quiescent SMCs within an uninjured adult artery do not express detectable levels of β-catenin, in contrast to SMCs in active vascular remodeling or under growth-promoting conditions in culture.

**β-Catenin Is Not Required in SMCs for Maintaining the Structure or Differentiation State of Arteries in Adulthood**

To test the significance of SMC β-catenin expression during vascular remodeling in adulthood, we pursued a loss-of-function approach in the mouse. Because loss of SMC β-catenin causes embryonic lethality, we had to overcome developmental demise by crossing Myh11-CreER T2 (tamoxifen-inducible SMC-selective Cre) mice23 with β-cateninflox/flox mice. Tamoxifen induced Cre-mediated recombination in arteries and rendered a β-catenin (Ctnnb1) null allele (Figure 2A). Moreover, this recombination event was specific to SMC-rich blood vessels—the null allele was not detected in other tissues such as brain, liver, lung, or tail (Figure IIA in the online-only Data Supplement). The presence of the null allele correlated with a significant reduction in β-catenin protein expression in total arterial lysates from a pool of 3 aortas per group (Figure 2B). Reduction in β-catenin protein expression in aortas was still evident 28 and 35 days after the first tamoxifen injection (Figure IIB in the online-only Data Supplement). In addition, β-catenin protein levels were also reduced in the bladder (an organ enriched in SMCs) of iSMβCKO mice but were not affected in the tail (not particularly enriched in SMCs) or in the lung (an organ enriched in endothelial cells; Figure IIC in the online-only Data Supplement). These findings are consistent with effective, SMC-selective inactivation of β-catenin. Then, we tested whether the loss of β-catenin in SMCs in adulthood had any repercussions for overall mouse health or on the structure of uninjured arteries. We followed up iSMβCKO and control mice for 12 weeks after the first tamoxifen injection and did not see lethality or morbidity in unchallenged animals (data not shown). Moreover, carotid arteries from iSMβCKO and control mice harvested 3, 4, and 5 weeks after the first tamoxifen injection did not look different in histological analysis (Figure 2C). In addition, immunostaining of carotid arteries from both groups showed clear expression of β-catenin in the endothelium, but did not detect β-catenin in SMCs within medial layers (Figure 2D). Finally, expression of SM22α, a marker of smooth muscle differentiation, appeared equal in iSMβCKO and control arteries (Figure 2D). These observations suggest that expression of β-catenin is minimal in SMCs of uninjured adult arteries and that SMC β-catenin is dispensable for at least 5 weeks in mice for maintenance of SMC differentiation state and the structure of the arterial wall under normal conditions.

**Loss of β-Catenin in SMCs Restrains Neointima Formation After Arterial Ligation Injury**

Because SMC β-catenin expression is induced during adult vascular remodeling (Figure 1B), we wondered whether...
SMC β-catenin expression was indeed essential for neo-intima formation. We performed carotid artery ligation injury in iSMβCKO and control mice 7 days after the first tamoxifen injection and evaluated β-catenin expression and neointima formation. By immunostaining, β-catenin expression was induced in SMCs within the neointima of control mice (Figure 3A), as seen before (Figure 1B); in contrast, β-catenin expression was abrogated in SMCs of iSMβCKO mice but readily apparent in the endothelial cell layer (Figure 3A). The latter was present in both uninjured and injured arteries lining the lumen of the vessel and did not look different between control and iSMβCKO animals (Figure IIIA in the online-only Data Supplement). Interestingly, we found a significant reduction in neointima formation in iSMβCKO compared with control mice 14 days after injury (Figure 3B). We also treated Myh11-CreERT2;β-cateninWT/WT mice with the same tamoxifen and carotid artery ligation protocols (tamoxifen control group) and did not observe a reduction in neointima formation (Figure 3B). Thus, the decrease in neointima formation seen in iSMβCKO animals was not because of off-target, β-catenin–independent effects of tamoxifen or CreERT2, but rather resulted from genetic inactivation of β-catenin in vascular SMCs. This reduction in neointima formation seemed not to be a simple delay in injury response, but more likely a...
reduction in its magnitude because smaller neointimas were still seen 21 days after ligation (Figure 3C). These observations show that β-catenin expression in SMCs is required for neointima formation after injury in adulthood. To assess relevance to clinical cardiovascular disease, we looked for β-catenin expression in human arteries undergoing vascular remodeling after injury. We evaluated diseased postmortem human coronary arteries proximate to sites of previous stent placement, and we found β-catenin expression that coincided in some areas with smooth muscle actin-α (also known as ACTA2, a marker of SMCs; Figure 3D; Figure IIIB in the online-only Data Supplement). These observations support the idea that β-catenin may contribute to clinical vascular pathogenesis.

**SMC β-Catenin Promotes Proliferation and Prevents Apoptosis in the Neointima**

In previous studies, we found that proproliferative and antiapoptotic functions of SMC β-catenin are essential, in part, by restraining p53 activity, for artery formation during embryogenesis, so we wondered whether β-catenin induced during adult vascular remodeling also promotes proliferation and survival of SMCs. We evaluated cell proliferation by measuring the expression of a marker of mitosis, phosphorylated histone H3, by immunohistochemistry, and assessing the expression of Ki67, a marker of cell proliferation, by immunofluorescence. We found a reduced percentage of phosphorylated histone H3+ cells or Ki67+ cells in the neointima of iSMβCKO compared with controls (Figure 4A; Figure IVA in the online-only Data Supplement). We also evaluated apoptosis by expression of cleaved caspase 3 and a terminal deoxynucleotidyl transferase dUTP end labeling assay and found an increased percentage of caspase 3+ or terminal deoxynucleotidyl transferase dUTP end labeling+ cells in the neointima of iSMβCKO mice (Figure 4B; Figure IVB in the online-only Data Supplement). These findings support a proliferative and prosurvival functions of β-catenin in SMCs during vascular remodeling in adulthood. We could not detect cells expressing markers of the monocyte/macrophage lineage or neutrophils by immunostaining for colony-stimulating factor 1 receptor or CD68 within the vessel wall 14 days after injury (Figure IVC–IVE in the online-only Data Supplement), suggesting that inflammatory infiltrates are not a hallmark of this particular model of vascular injury.

**Loss of β-Catenin in SMCs Results in a Gene Expression Pattern Consistent With a Quiescent, Noninvasive, and Apoptosis-Prone Phenotype**

We previously found that vascular SMCs lacking β-catenin show defective growth in culture are arrested in G<sub>s</sub>/G<sub>1</sub> phase of the cell cycle and are prone to cell death; this phenotype is associated with increases in p53 acetylation, transcriptional activity, and expression of the p53 target genes p21 (Cdkn1a) and Bax. Moreover, loss of p53 in SMCs in vivo suppresses the effect of β-catenin inactivation and substantially restores arterial wall formation; this rescue effect, although significant, is not complete, suggesting that additional p53-independent mechanisms operate downstream of β-catenin. Consistent with this idea, we found that SMCs lacking β-catenin (Figure 5A) have decreased expression of several genes positively associated with vascular remodeling, including Mmp2, Mmp9, and sphingosine-1-phosphate signaling components—sphingosine kinase 1 (Sphk1) and sphingosine-1-phosphate receptor 1 (S1pr1; Figure 5B). Interestingly, these genes have been shown to promote vascular SMC migration or invasion and neointima formation after vascular injury, and S1pr1 also promotes vascular SMC proliferation. In contrast, loss of β-catenin resulted in higher expression of jagged 1 (Jag1) and connexin 43 (Gja1; Figure 5C), which have been shown to promote a contractile SMC phenotype and to prevent neointima formation. On the contrary, we did not find differences in expression of platelet-derived growth factor receptor β, tissue inhibitor of metalloproteinase 1 (Timp1), Timp2, or N-cadherin between vascular SMCs lacking β-catenin and control cells (Figure VA–VC in the online-only Data Supplement). Altogether, these observations indicate that β-catenin is required in SMCs to promote a proliferative, antiapoptotic, and invasive gene expression signature that favors neointima formation after vascular injury.

Moreover, SMCs lacking β-catenin also showed decreased Mmp2 protein expression measured by Western blotting (Figure 5D) and rendered a conditioned media with decreased levels of Mmp2, which were measured by assessing the proteolytic activity (gelatinase activity) of the conditioned media by zymography (Figure 5E and VE in
the online-only Data Supplement). These findings indicate that β-catenin is necessary in vascular SMCs for expression and secretion of Mmp2. It has been shown that Mmp2 is required for migration of vascular SMCs through a basement membrane barrier\(^{20}\) and also necessary for human vascular SMC migration and invasion in vitro.\(^{31,32}\) In previous transwell assays without a barrier, we found no differences in migration between SMCs lacking β-catenin and control cells,\(^{21}\) so this time we assessed vascular SMC invasion, and we found a reduced ability of β-catenin–deficient SMCs for crossing a basement membrane barrier in vitro (Figure 5E). Thus, β-catenin promotes expression and secretion of Mmp2 and cell invasion in vascular SMCs. As movement of SMCs from the media to the intima is a key feature of the response to vascular injury, it is possible that this proinvasion function of β-catenin (Figure 5E) plus its proliferative and antiapoptotic activities (Figure 4; Figure IV A and IV B in the online-only Data Supplement) contribute to neointima formation.

**Inhibitors of β-Catenin Prevent Growth of Mouse and Human Arterial SMCs in Culture**

Because genetic inhibition of SMC β-catenin prevents SMC investment during artery formation\(^{21}\) and restrains neointimal growth after arterial ligation (Figure 3B and 3C), we hypothesized that pharmacological inhibition of β-catenin would inhibit growth of arterial SMCs. We treated mouse aortic SMCs (MASMCs) with increasing concentrations of validated β-catenin inhibitors, ICG-001\(^{18}\) or PKF118-310\(^{19}\)—these inhibitors effectively reduce β-catenin/TCF transcriptional activity assessed with a TOPflash reporter assay in arterial SMCs.\(^{21}\) Both inhibitors opposed MASMC growth in a dose–responsive manner (Figure 6A; Figure VIA in the online-only Data Supplement). Notably, ICG-001 and PKF118-310 also inhibited human coronary artery SMC growth (Figure 6B; Figure VIB in the online-only Data Supplement). PKF118-310 seemed more potent than ICG-001 in both human and mouse SMCs (Figure 6A and 6B). At higher concentrations of PKF118-310, cell number actually decreased, consistent with induction of cell death. The inhibitory effect on SMC population growth observed with higher doses of ICG-001 and PKF118-310 seemed to be stronger than that observed with genetic deletion of β-catenin in vascular SMCs, which we have reported previously,\(^{21}\) suggesting that these chemicals might mediate additional β-catenin–independent mechanisms that block SMC population growth as their concentration increases. To test this idea, we assessed the effect of these inhibitors on growth of control and β-catenin–deficient MASMCs within the same assay. We found that β-catenin–deficient SMCs consistently exhibited impaired growth compared with control SMCs when both groups were treated with only vehicle (Figure VIC in the online-only Data Supplement). We also observed that treatment with 0.1 µmol/L of PKF118-310 or 1 µmol/L of ICG-001 only inhibited growth of control cells but did not affect that of β-catenin–deficient MASMCs (Figure VIC in the online-only Data Supplement), indicating a β-catenin–specific effect. In contrast, treatment with 0.5 µmol/L of PKF118-310 or 10 µmol/L of ICG-001 affected growth of both control and β-catenin–deficient MASMCs (Figure VIC in the online-only Data Supplement), indicating additional β-catenin–independent inhibitory mechanisms. Thus, there is a concentration threshold at which these β-catenin inhibitors gain additional inhibitory mechanisms; in our hands, in MASMCs in culture, that threshold seems to be between 0.1 and 0.5 µmol/L for PKF118-310 and between 1 and 10 µmol/L.
for ICG-001. Overall our observations show that known pharmacological inhibitors of β-catenin limit growth of mouse and human vascular SMCs in culture.

**Discussion**

These studies provide direct evidence that adult SMC β-catenin is not essential in vascular SMCs for the maintenance of the arterial wall under normal conditions in adulthood, but is required for neointimal formation after vascular injury. Although previous reports have shown that β-catenin expression and its transcriptional activity are enhanced after vascular injury,6,8 whether β-catenin is actually essential for neointima formation had not been tested. The CreER driver we have used relies on regulatory elements for the most specific SMC marker gene, Myh11, with tamoxifen induction providing temporal control; β-catenin inactivation through this genetic intervention inhibits neointima formation after arterial ligation, in association with decreased cell proliferation and increased apoptosis. Together with our previous observations that SMC β-catenin is essential for arterial wall formation during embryogenesis,21 these results define β-catenin as a key factor in vascular SMCs required for the formation and maturation of the arterial wall but dispensable for the maintenance of the mature state once it has been achieved. When the adult arterial wall is perturbed by injury or disease, however, β-catenin again becomes an essential promoter of SMC proliferation and survival. This differential requirement for β-catenin in normal versus injured arteries in adulthood suggests that a therapeutic strategy aimed at inhibiting β-catenin function in SMCs should have a specific effect on injured vessels—that is, it would modulate remodeling of injured vessels but would not affect normal vasculature.

During adult homeostasis, the main function of SMCs in mature arteries is contraction, which regulates the vessel tone and diameter important for the conduction of blood to different organs, and the regulation of blood flow, vascular resistance, and blood pressure. This function requires the expression of proteins involved in the contractile function and an appropriate extracellular matrix that confers the necessary mechanical properties. Disruption of those proteins and matrix would affect vascular homeostasis. We have shown that within this setting, characterized by quiescent nonmigratory SMCs, SMC β-catenin is hardly expressed and not required. In contrast, SMC β-catenin is expressed and required in settings characterized by enhanced SMC proliferation and migration, such as during developmental formation and maturation of the arterial wall21 or the response to vascular injury (Figure 3).

How SMC β-catenin expression is induced and maintained during development, how it is repressed after the vessel wall matures, and how it is induced after vascular injury are not fully understood.
Our studies of vascular injury in adulthood also support the idea that the requirement for β-catenin in vascular remodeling originates with cells that express Myh11, that is, differentiated medial SMCs. According to our study design, tamoxifen activation of Myh11-CreERT2-mediated recombination was completed before arterial injury; neointimal cells arising from a progenitor or stem cell, or by transdifferentiation from a different cell type, would not have expressed Myh11-CreERT2 at the time of tamoxifen administration and would not lose the ability to express β-catenin. Thus, our observations are consistent with the idea that most neointimal cells that accumulate after injury derive from medial SMCs, as opposed to resident or circulating progenitors or via transdifferentiation from another mature cell type.40

We found that β-catenin expression is induced in the SMC-rich neointima after injury and is associated with enhanced cell proliferation and reduced apoptosis. We have recently shown that β-catenin represses p53 activity in vascular SMCs in vivo during artery formation.41 Interestingly, both loss- and gain-of-function studies indicate that p53 opposes neointima formation after vascular injury because of its antiproliferative and proapoptotic effects.41–46 It has also been suggested that mitogen-induced inactivation of p53 in vascular SMCs precedes the initiation of proliferation and migration of these cells from the media.47 As SMC phenotype during vascular remodeling after injury may resemble that found during embryogenesis,3 it is very likely that β-catenin–mediated repression of p53 in SMCs is also operative in the context of vascular injury—this would explain, in part, the proproliferative and antiapoptotic functions of β-catenin. However, our recent developmental studies also showed that inhibition of p53 was not the only β-catenin–dependent mechanism during artery formation because the loss of p53 resulted in a partial suppression of the phenotype observed with loss of β-catenin;21 suggesting that β-catenin may play roles beyond regulation of cell cycle and apoptosis in SMCs. Here, we have shown that loss of β-catenin in primary arterial SMCs reduces the expression of a set of genes that promote migration/invasion or extracellular matrix remodeling and enhance neointima formation after injury—Mnp2, Mnp9, Sphk1, and S1pr125–35; at the same time, β-catenin loss increases the expression of genes that promote a contractile phenotype of SMC and inhibit neointima formation—Jag1 and Gja1.36,37 We have also shown that β-catenin promotes Mnp2 protein expression and secretion and vascular SMC invasion in vitro. Altogether, these findings, plus observations in previous studies from our group and others, argue that β-catenin expression in SMCs supports a gene expression signature that broadly affects SMC phenotype and favors neointima formation after injury.

During evaluation of β-catenin protein expression in normal and injured arteries, we consistently saw strong β-catenin expression in the endothelial layer. β-Catenin function has been studied in endothelial cell biology and shown to be important for angiogenesis, arterial specification, and blood–brain barrier formation.48,49 Interestingly, a recent report shows that genetically induced, endothelial-specific deletion of β-catenin in adult mice results in systemic depletion of endothelial β-catenin but disrupts the endothelial barrier function only in the central nervous system circulation, leading to neurological deficits and lethality within 2 weeks.50 This study suggest that with the exception of the central nervous system circulation, endothelial β-catenin function in adult vascular beds is dispensable for maintaining vascular homeostasis. The authors, however, evaluated only the pulmonary circulation as a reference and did not examine other vascular beds in detail. As far as we know, no studies have evaluated the requirement of endothelial β-catenin in the response to vascular injury, but it is possible that β-catenin is also dispensable in this setting for vessels outside of the central nervous system. If this is the case, and keeping in mind our findings that SMC β-catenin is required for neointima formation after vascular injury, it suggests that local inhibition of β-catenin in arteries outside the central nervous system, for instance, in the coronary circulation, may mediate a cell type–specific inhibitory effect in the vessel wall—that is, β-catenin inhibition would block growth of SMCs but would not affect endothelial homeostasis.

We have also shown that inhibitors of β-catenin function are effective growth inhibitors of cultured mouse aortic SMCs and human coronary SMCs. The 2 inhibitors we tested target distinct β-catenin interactions—with TCF4 for PFK118-310 and with CREB-binding protein for ICG-001. Disrupting either interaction proved to be effective. These strategies inhibit different aspects of the transcriptional function of β-catenin, but do not affect its cell adhesion function, which is consistent with genetic studies showing that the β-catenin’s signaling function is essential, whereas its cell-adhesion function is not sufficient for artery formation.21 As SMC growth plays a key role in the pathogenesis of atherosclerosis, restenosis after angioplasty and stent placement, vein graft disease, and transplant arteriosclerosis,1,51 inhibition of β-catenin could serve as a therapeutic strategy especially for those pathologies. Our studies also show that, depending on the concentration, these inhibitors can have β-catenin–independent effects. Overall our observations with β-catenin inhibitors offer a strong rationale to study the efficacy and toxicity of this kind of pharmacological compounds in animal models of vascular injury.

In conclusion, we have demonstrated that β-catenin is dispensable in SMCs to maintain the structure and differentiation state of the arterial wall in uninjured adult vessels, but is essential for neointima formation after vascular injury. We have also shown that β-catenin promotes cell proliferation and prevents apoptosis in the neointima and is required for expression of a gene signature in SMCs that supports functions associated with neointima formation, including SMC invasion. Finally, pharmacological inhibition of β-catenin hinders growth of mouse and human SMCs in culture. Together, these observations support the idea that inhibition of β-catenin has potential as a therapeutic strategy in cardiovascular disease associated with intimal thickening.

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Disclosures
None.

References


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**Highlights**

- beta-catenin is dispensable in smooth muscle cells to maintain the structure and differentiation state of uninjured adult arteries.
- beta-catenin is required in smooth muscle cells for neointima formation after vascular injury.
- beta-catenin inhibitors hinder growth of mouse and human vascular smooth muscle cells.
Inhibition of Smooth Muscle β-Catenin Hinders Neointima Formation After Vascular Injury
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Materials and Methods

Mice

β-catenin<sup>fl/fl</sup> mice have been described<sup>1</sup>, and were obtained from the Jackson Laboratory (B6.129-<i>Ctnnb1<sup>tm2Kem</sup>/KnwJ</i>). <i>Myh11-CreER<sup>T2</sup></i> mice were generated and validated in a previous study<sup>2</sup>, and were kindly provided by Dr. Stefan Offermanns. These mouse lines were crossed to generate <i>Myh11-CreER<sup>T2</sup>;β-catenin<sup>fl/fl</sup></i> mice. Seven to 8 week old male <i>Myh11-CreER<sup>T2</sup>;β-catenin<sup>fl/fl</sup></i> littermates were injected with tamoxifen or vehicle to generate smooth muscle β-catenin knockout (iSMβCKO) or control mice, respectively; mice underwent carotid artery ligation one week after the first injection. Animals were genotyped using tail DNA and validated published PCR protocols for β-catenin-null, floxed, and WT alleles<sup>1</sup> and the Cre transgene<sup>2</sup>. All animals were housed in pathogen-free conditions, and procedures followed the rules and regulations of the AAALAC and were approved by the Institutional Animal Care and Use Committee (IACUC) of Albert Einstein College of Medicine.

Tamoxifen Administration

Under pathogen-free conditions, 100 mg of tamoxifen free base (Sigma No. T5648) was dissolved in 0.5 ml of ethanol (Decon Laboratories, Inc. 22032601), and 9.5 ml of corn oil (Sigma C8267) was added to achieve a final concentration of 10 mg/ml. The same preparation of ethanol and corn oil without tamoxifen was used as vehicle. Seven to 8 week old male mice —<i>Myh11-CreER<sup>T2</sup>;β-catenin<sup>fl/fl</sup></i> or <i>Myh11-CreER<sup>T2</sup>;β-catenin<sup>WT/WT</sup></i> mice— were given 100 µl (1 mg) of tamoxifen or vehicle solution via
intraperitoneal injection daily for 5 consecutive days. Carotid artery ligation was performed 1 week after the first injection.

**Carotid Artery Ligation**

Carotid artery ligation has already been described. Briefly, tamoxifen or vehicle treated 8–9 week old male mice were anesthetized with ketamine/xylazine (90 mg/kg and 10 mg/kg, respectively) via intraperitoneal injection. A 5 mm skin incision on the base of the neck and blunt dissection were performed until the left common carotid artery was exposed. The artery was separated from surrounding tissues and ligated with 6-0 silk (Ethicon K889H) just proximal to the bifurcation. The right common carotid artery served as control. The incision was closed and mice were allowed to recover. Carotid arteries were harvested 7, 14, 21, and 28 days after ligation. Mice were euthanized by ketamine/xylazine injection plus thoracotomy, and the systemic circulation was flushed with phosphate buffer solution (PBS) and then perfused with 10% formalin phosphate buffer (Fisher Scientific SF100-4) for 7 minutes. Left and right carotid arteries were removed and post-fixed in 10% formalin phosphate buffer overnight. Tissues required for DNA, RNA, or protein isolation were harvested, snap frozen, and kept at -80 ºC until processing.

**Processing and Morphometric Analysis of Carotid Arteries**

Fixed carotid arteries were trimmed under a Stemi 2000-C stereomicroscope (Zeiss) such that 3 mm of vessel adjacent to the ligation point was left available for analysis. Trimmed arteries were processed and paraffin embedded with the ligature on top. Paraffin blocks were trimmed until a full cross-section of the ligated artery was
visualized; thereafter, in sequence, the next 600 µm of tissue was discarded, 180 µm was collected for analysis (5 µm-thick sections, 3 sections per slide, 12 slides), 700 µm discarded, 180 µm collected for analysis, 600 µm discarded, and 180 µm collected for analysis. This protocol allowed the evaluation of vascular remodeling at three consistent distances from the ligation site in each artery. Arterial cross-sections were stained with hematoxylin and eosin (H&E), and photographed with a digital microscope (COOLSCOPE, Nikon). Morphometric analysis of carotid arteries was performed using ImageJ software as follows: the area of the lumen (L), the area inside the internal elastic lamina (IEL), and the area inside the external elastic lamina (EEL) were measured in pixels. The area of the intima was calculated by subtracting the area of the lumen from the area inside the IEL. The area of the media was calculated by subtracting the area inside the IEL from the area inside the EEL. Finally, the intima/media ratio was calculated.

**Immunofluorescence of Carotid Arteries**

Artery sections were deparaffinized and rehydrated, and antigens retrieved by boiling in sodium citrate solution (Vector Labs H-3300). Arteries were blocked in 0.3% triton-X-100 and 5% normal serum (same species of secondary antibody) in PBS for 2 hours at room temperature; and incubated overnight at 4º C with anti-β-catenin (Santa Cruz sc-7963, 1:50 dilution) and anti-SM22α (Abcam 14106, 1:100 dilution) antibody mixture in 0.3% triton-X-100 and 2% bovine serum albumin in PBS. After washing, sections were incubated for 1 hour at room temperature with fluorochrome-conjugated secondary antibodies Alexa 546 goat anti-mouse and Alexa 488 goat anti-rabbit IgGs (Molecular Probes, 1:500 dilution) in 0.3% triton-X-100 and 2% bovine serum albumin in PBS. After
washing, sections were stained for DAPI during mounting of coverslips using FLUORO-GEL II with DAPI (Electron Microscopy Sciences # 17985-50). Fluorescent signals were visualized with an Axio Observer.Z1 microscope (Zeiss). Subsequent image processing was performed with ImageJ. Omission of primary antibodies or use of normal IgGs instead of primaries were routinely employed as controls. Other primary antibodies used were anti-CD68 (BIO RAD MCA1957GA, 1:100 dilution), anti-CSF1R (Santa Cruz sc-692, 1:100 dilution), anti-Myh11 (Kamiya biomedical company MC-352, 1:50 dilution), anti-CD31 (Abcam 28364, 1:50 dilution), anti-SMA (Santa Cruz sc-7963, 1:200 dilution), anti-Ki67 (Abcam 15580, 1:50 dilution), and anti-cleaved caspase 3 (Cell signaling 9661, 1:50 dilution).

**Immunohistochemistry of Carotid Arteries**

Artery sections were deparaffinized and rehydrated, endogenous peroxidase activity neutralized, and antigens retrieved by boiling in sodium citrate solution (Vector Labs H-3300). Tissue sections were blocked for 1 hour at room temperature with 10% normal serum (same species of secondary antibody), 2% bovine serum albumin and avidin blocking solution (Vector Labs SP-2001) in PBS, and then incubated overnight at 4 °C with anti-phospho histone H3 (pHH3) (Cell Signaling 9701, 1:50 dilution) in 10% normal serum, 2% bovine serum albumin and biotin blocking solution (Vector Labs SP-2001). After washing, sections were incubated with biotinylated goat anti-rabbit IgG (Vector Labs BA-1000) for 1 hour at room temperature, washed again and then incubated with ABC reagent (Vector Labs PK-6100) for 30 minutes. After washing, DAB substrate (Dako K3467) was added to each section until specific signal was observed, and the reaction stopped by dipping slides in water. Sections were counterstained with
hematoxylin (Vector Labs H-3404) and coverslips mounted with toluene solution (Fisher Scientific SP15-100). Specificity of the staining was tested by omission of the primary antibody. Images were obtained using a digital microscope (COOLSCOPE, Nikon).

**TUNEL assay**

An *in situ* cell death detection kit with fluorescein readout (Roche 11684795910) was used to test apoptosis as per the manufacturer's instructions. Briefly, artery sections were deparaffinized and rehydrated, permeabilized by microwave irradiation in 0.1 M citrate buffer (pH 6), labeled with TUNEL reaction mixture (label solution and terminal transferase), counterstained with DAPI during mounting using FLUORO-GEL II with DAPI (Electron Microscopy Sciences # 17985-50), and analyzed by fluorescence microscopy in an Axio Observer.Z1 microscope (Zeiss). Sections incubated with label solution without terminal transferase were used as negative controls. Sections incubated with DNase I before labeling served as positive controls.

**Vascular Smooth Muscle Cells**

Primary mouse aortic smooth muscle cells (MASMCs) were isolated from 5–6 week old \(\beta\)-catenin\(^{loxp/loxp}\) mice using collagenase-elastase digestion as described before\(^4\), maintained in Dulbecco's modified eagle medium (DMEM) plus 20% fetal bovine serum (FBS), 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM L-glutamine, and subcultured weekly. Passages 2 or 3 were transduced with adenovirus expressing GFP or Cre (Ad5.CMV-GFP or Ad5.CMV-Cre) to generate control or cells lacking \(\beta\)-catenin, respectively. Passages 4 to 8 were used for experiments. Human coronary artery smooth muscle cells (HCASMCs) were obtained from LIFELINE cell technology (FC-
0031) and cultured with the same medium and conditions as for MASMCs. Passages 3 to 5 were used for experiments. To evaluate the effect of β-catenin inhibitors on growth of MASMCs and HCASMCs, $4 \times 10^3$ cells per well were plated in 96-well plates with a minimum of four independent wells per treatment and cell type, four independent plates were tested for most of the concentrations and inhibitors. PKF118-310 (Santa Cruz Biotechnology sc-364590) and ICG-001 (Cayman Chemical Company 16257) were dissolved in DMSO to obtain a stock solution at 10 mM. Dilutions from the stock solution in culture medium were performed to test final concentrations from 0.01 to 10 µM for ICG-001 and from 0.01 to 1 µM for PKF118-310. Same dilution protocols were used with only DMSO to generate vehicle controls specific for each concentration and inhibitor. Evaluation of cell growth in culture was performed with the AlamarBlue assay (BUF012A, BIO-RAD), absorbances at 570 nm and 600 nm were measured on a Synergy 2 microplate reader (BioTek). AlamarBlue reduction correlates with the number of viable cells, and was calculated according to the manufacturer's instructions and expressed as fold-change with respect to baseline.

**Immunofluorescence of Cells**

Vascular SMCs were plated in chamber slides (BD Bioscience) 24 hours before staining, washed with PBS and fixed with cold methanol, blocked for 1 hour at room temperature with 3% normal goat serum, 1% bovine serum albumin and 0.01% triton X-100 in PBS, and then incubated overnight at 4° C with anti-β-catenin (Santa Cruz sc-7963, 1:100 dilution) and anti-SM22α (Abcam 14106, 1:200 dilution) antibody mixture in blocking solution. After washing, cells were incubated for 1 hour at room temperature with fluorochrome-conjugated secondary antibodies Alexa 546 goat anti-mouse and
Alexa 488 goat anti-rabbit IgGs (Molecular Probes, 1:500 dilution) in blocking solution. After washing, nuclei were stained with DAPI during mounting of coverslips using FLUORO-GEL II with DAPI (Electron Microscopy Sciences # 17985-50). Images were taken with an Axio Observer.Z1 microscope (Zeiss) and subsequent image processing was done with ImageJ. Routine control experiments included omission of primary antibodies.

**Western Blotting**

Protein lysates were extracted from SMCs or blood vessels using RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 150 mM NaCl) plus protease inhibitors (Complete mini Roche Life Science 04693159001). A BCA protein assay kit (Pierce 23335) was used to measure protein concentrations, and equal amounts of protein were loaded (20 – 60 µg) and separated by 10% polyacrylamide gel electrophoresis. Proteins were transferred (Trans-Blot SD cell, BIO-RAD 170-3940) to 0.45 µm pore size PVDF membranes (Immobilon-P, Millipore), blocked for 1 hour at room temperature with TBST (Tris pH8.0, NaCl 150 mM, 0.1 % Tween 20) plus 4 % (wt/vol) nonfat milk, and incubated overnight at 4 ºC with anti-β-catenin (Santa Cruz sc-7963, 1:500 dilution), anti-GAPDH (Santa Cruz sc-25778, 1:5000 dilution), anti-PDGFRβ (Cell signaling 3165, 1:1000 dilution), anti-Timp1 (Thermo fisher scientific PA5-13668, 1:2000 dilution), anti-Timp2 (Thermo fisher scientific MA1-774, 1:2000 dilution), anti-Mmp2 (Thermo fisher scientific PA1-1667, 1:1500 dilution), anti-N-cadherin (Cell signaling 4061, 1:500 dilution), or anti-β-actin (Abcam 8226, 1:5000 dilution) as primary antibody in blocking solution. After washing, membranes were incubated with HRP-conjugated secondary antibodies for 1 hour at
room temperature. After washing, signals were detected by adding ECL substrate (ECL Western Blotting Substrate, Pierce 32106) and exposing films to membranes. Densitometric analysis was performed with Image J.

**Real Time PCR**

Total RNA was extracted from control and β-catenin-deficient mouse aortic SMCs by homogenization in TRIzol (ThermoFisher scientific 15596026), treated with DNase I (1 U/µl, Promega) and used for first-strand cDNA synthesis (SuperScriptIII, Invitrogen). Quantitative PCR was performed using a SYBR Green qPCR kit (Stratagene) and Mx3000P Real-Time PCR system (Stratagene). Levels of RNA were normalized to Rps13 and expressed as relative values in comparison to control cells using the comparative ∆∆Ct method (User Bulletin #2, ABI Prims 7700, Applied Biosystems). The following primer sequences were used: Axin2 (F 5’-gagagtgcggagcacagc-3’, R 5’-cggctgaccttctctcct-3’), ribosomal protein S13 (Rps13) (F 5’-tgctccccagcctaatggaa-3’, R 5’-cgggtgacacacacacagcattt-3’), Mmp2 (F 5’-taacctggatgccgctgt-3’, R 5’-ttcggtgctacagccttga-3’), Mmp9 (F 5’-acgagcatagacggcatca-3’, R 5’-gctggctgtcttgggtgttg-3’), Sphk1 (F 5’-agcctcttcaggggctgt-3’, R 5’-gctggctgtcttgggtgttg-3’), S1pr1 (F 5’-cgggtgtagaccagctcct-3’, R 5’-agctttctctctgggtagag-3’), Jag1 (F 5’-gagggctctctgtgagaaa-3’, R 5’-acgagaagccactgtcctttac-3’), Spp1 (F 5’-gagggcaagggcaagggcaagggcaagggcaagggca-3’), and Gja1 (F 5’-ttctttgacttccagcctca-3’, R 5’-ccatgtctgggccacctc-3’).

**Cell Fractionation**
Control and β-catenin deficient mouse aortic SMCs were harvested by trypsinization and collected by centrifugation. Isolation of cellular fractions was done using the Qproteome nuclear protein kit (QIAGEN 37582) and following manufacturer’s instructions. In brief, 4x10^6 cells were resuspended in hypotonic Lysis buffer NL, and plasma membranes disrupted by adding detergent and vortexing. The cytosolic fraction (supernatant) was separated from nuclei (pellet) by centrifugation. The nuclear pellet was washed and incubated in a high-salt buffer. The nucleic-acid-binding proteins (supernatant) were separated from the nuclear debris (including genomic DNA) by centrifugation. A buffer containing Benzonase was used to release proteins intimately associated to DNA.

**Zymography**

MASMCs, control and β-catenin-deficient, were incubated with serum free media for 48 hours, and conditioned media collected. Samples with 10 µg of total protein were prepared using BIO RAD zymogram sample buffer (BIO RAD 161-0764). Protein electrophoresis was performed using Novex 10% zymogram protein gels (EC6175) and carried out at 4°C under non-reducing conditions. The gel was washed twice in 1X renaturation buffer (BIO RAD 161-0765) for 15 minutes each, incubated overnight at 37°C in developing buffer (BIO RAD 161-0766), stained with Coomassie Brilliant Blue R-250 solution (BIO RAD 161-0436) for 1 hour, and de-stain using 40% Methanol,10% acetic acid. Human recombinant MMP-2 (EMD Millipore PF037) was used as a control. Densitometric analysis was performed with Image J.

**Cell Invasion**
Vascular SMC invasion was evaluated using a cell invasion assay kit (EMD Millipore ECM550) following the manufacturer’s instructions. In brief, control and β-catenin deficient MASMCs were incubated in serum-free media 24 hours before collection. A cell suspension containing $3 \times 10^5$ cells in 300 µl of serum-free media was prepared for each cell group. Invasion chambers were adjusted to room temperature and the extracellular matrix layer was rehydrated by adding serum free medium to the inserts for 2 hours. After rehydration the media was removed. Media containing 20 % fetal bovine serum was added to the lower chamber, and 300 µl of cell suspension were added to each insert. Invasion chambers were incubated for 24 hours in a tissue culture incubator. Non-invading cells were removed with a cotton-tipped swab, and invasive cells were stained for 20 minutes. Inserts were washed several times with water and allowed to air dry. Cell invasion was quantified by dissolving stained cells in 10% acetic acid and measuring the optical density of the dye/solute mixture at 560 nm with a Synergy 2 microplate reader (BioTek).

**Human Artery Studies**

Formalin-fixed paraffin-embedded human coronary artery atherosclerotic plaque sections were obtained from the CVPath Institute Sudden Cardiac Death Registry. Artery segments adjacent to the site of a bare-metal stent implanted within the preceding 30 days were chosen for analysis. Movat and H&E stains and immunohistochemistry for β-catenin and smooth muscle α-actin (SMA) were performed. The immunohistochemical stain was developed by NovaRED kit (Vector Laboratories, Burlingame, CA). An Axio Scan.Z1 (Zeiss, Germany) microscope was used to acquire
the images using a 20X objective. The HALO image analysis platform (Indica Labs, Corrales, NM) was used to set up the panel of images.

**Statistical Analysis**

Experiments shown were at least done three independent times and thus are representative of at least three independent experiments. Comparisons between two groups were assessed by t-test, and comparisons between three or more groups evaluated by analysis of variance (ANOVA) followed by multiple comparison test when appropriate. Specific statistical tests are mentioned in each figure legend. Significance was accepted for values of p < 0.05.
References


Figure I. β-Catenin expression is induced after vascular injury and is observed in the cytosolic and nuclear fractions of vascular SMCs.

A. Quantification of β-catenin protein levels in carotid arteries, normalized to loading control (GAPDH), by densitometric analysis of the Western blotting shown in Figure 1C. B. Cellular fractionation of control and β-catenin deficient (KO) mouse aortic smooth muscle cells (MASMCs), and Western blotting for indicated proteins. Lamin A/C, nuclear protein control. WCL, whole cell lysate. Cyto, cytosolic fraction. Nuclear, nuclear fraction.
Figure II. Presence of a β-catenin null allele and selective reduction of β-catenin protein levels in SMC-enriched tissues in iSMβCKO mice.

A, PCR for indicated β-catenin alleles. DNA isolated from tissues of iSMβCKO mice (Myh11-CreERT2;β-catenin\textsuperscript{flox/flox} plus tamoxifen). B, Western blotting for indicated proteins in total arterial protein lysates isolated from a pool of 3 aortas per mouse group 28 and 35 days (d) after vehicle or tamoxifen injection. C, Western blotting for indicated proteins in total lysates from tail (composed by several cell types, not particularly enriched in SMCs), bladder (organ enriched in SMCs), and lung (organ enriched in endothelial cells).
Figure III. The endothelial layer appears similar in control and iSMβCKO mice, and β-catenin is expressed in human restenotic arteries.

A, Immunostaining for CD31, a marker of endothelial cells, in uninjured and ligated carotid arteries, 14 days after ligation. Representative of 3 arteries per condition. L, lumen. Scale bar = 40 µm. B, Immunohistochemistry for β-catenin and smooth muscle α actin (SMA), and H&E and Movat stains in human coronary arteries adjacent to a previous stent placement. Representative of 3 arteries (see also Figure 3D). The squares indicate the regions magnified on the right panel for each stain.
Figure IV. SMC β-catenin promotes proliferation and survival in the neointima.

A. Left: Immunostaining for Ki67, a marker of cell proliferation, and smooth muscle α actin (SMA), a SMC marker, in carotid arteries 14 days after ligation. Arrowheads indicate Ki67+ cells in the neointima. Scale bar = 50 µm. Right: Percentage of
neointimal cells positive for Ki67. n=3 for both groups. *, p<0.05 by two-tailed t test. Data shown as mean±s.e.m. B, Left: TUNEL assay in carotid arteries 14 days after ligation. Arrowheads indicate TUNEL+ cells. Dotted line marks the internal elastic lamina. L, lumen. Scale bar, 25 µm. Right: percentage of neointimal cells positive for TUNEL. n=12 for both groups. *, p<0.05, by two-tailed t test. Data shown as mean±s.e.m. C and D, Immunostaining for colony stimulating factor 1 receptor (Csf1r), expressed in the monocyte/macrophage lineage, and Myh11, a SMC marker, in carotid arteries 14 days after ligation. Csf1r+ cells were observed in the perivascular tissue (C), but not within the arteries (D). The square indicates the area magnified in the insert showing a Csf1r+ cell. Images representative of 3 arteries per group, scale bar = 50 µm. E, Immunostaining for CD68, expressed in monocyte/macrophages and neutrophils, and SMA in carotid arteries 14 days after ligation. CD68+ cells were observed in the perivascular tissue, but not in the arteries. The squares indicate the areas magnified in the inserts showing CD68+ cells. Images representative of 3 vessels per group. Scale bar = 50 µm.
Figure V. Expression profile in mouse aortic SMCs and evaluation of gelatinase activity in conditioned media.

A and B, Western blotting for indicated proteins in total cell lysates isolated from control and β-catenin deficient (KO) mouse aortic smooth muscle cells (MASMCs). C, Quantification of levels of indicated proteins normalized to loading control (β-actin) by densitometric analysis of western blots shown in A and B. n=3 for every group. NS, not significant, by two-tailed t test. Data shown as mean±s.e.m. D, Evaluation of gelatinase activity by zymography in the condition media from control and KO MASMCs. Recombinant Mmp2 was used as control. E, Quantification of gelatinase activity by densitometric analysis of the zymogram shown in D. n=3 for both groups. **, p<0.01 by two-tailed t test. Data shown as mean±s.e.m.
Figure VI

**A**, Cell population growth of mouse aortic SMCs (MASMCs) in the presence of 10 nM of β-catenin inhibitors, ICG-001 or PKF118-310, or vehicle control. n=12 for every group. **B**, Cell population growth of human coronary artery SMCs (HCASMCs) treated as in **A**. n=16 for every group. In **A** and **B**, *, p<0.05 by two-way ANOVA. Data shown as mean±s.e.m. **C**, Cell population growth of control (WT) and β-catenin-deficient SMCs (KO) treated with indicated inhibitors and concentrations or only vehicle. n=6 for every group and condition. Black *, ** and **** indicate p<0.05, p<0.01 and p<0.0001, respectively, comparing WT+vehicle vs. WT+Inhibitor. Gray NS and **** indicate not significant and p<0.0001, respectively, comparing KO+vehicle vs. KO+Inhibitor. Analysis done by two-way ANOVA. Data shown as mean±s.e.m.