Wnt4/β-Catenin Signaling Induces VSMC Proliferation and Is Associated With Intimal Thickening

Aikaterini Tsaousi, Helen Williams, Cressida A. Lyon, Victoria Taylor, Amanda Swain, Jason L. Johnson, Sarah J. George

Rationale: Vascular smooth muscle cell (VSMC) proliferation causes intimal thickening in atherosclerosis and restenosis. Previously, we demonstrated that Wnt/β-catenin signaling upregulates VSMC proliferation in vitro. Objective: We examined this pathway in vivo and investigated the involvement of specific Wnt proteins in VSMC proliferation.

Methods and Results: Left carotid arteries of TOPgal (β-catenin signaling reporter) transgenic mice were ligated to induce intimal thickening. β-Catenin signaling was induced in the media and intima at 3 and 28 days after ligation, respectively, and was associated with VSMC proliferation and cyclin D1 expression. In vivo, a Wnt agonist promoted mouse VSMC proliferation, whereas Wnt inhibitory factor (WIF)-1 retarded platelet-derived growth factor-BB (PDGF-BB)–induced VSMC proliferation. Microarray analysis and quantitative PCR detected a significant induction of Wnt2 and Wnt4 mRNA in PDGF-BB-treated (proliferating) VSMCs compared to quiescent VSMCs. Western blotting revealed this increase was only translated into protein for Wnt4. Specific silencing RNA knockdown of Wnt4, but not Wnt2, significantly reduced VSMC proliferation. Recombinant Wnt4, but not Wnt2, significantly increased VSMC proliferation by ~2-fold and silencing RNA knockdown revealed this is via Frizzled 1. Immunohistochemistry showed that increased Wnt4 protein correlated with increased VSMC proliferation and cyclin D1 expression (P<0.05 and P<0.001, respectively) during intimal thickening after rat carotid artery injury. Importantly, we also showed that intimal thickening and VSMC proliferation after carotid artery ligation was significantly retarded in Wnt4−/− compared to Wnt4+/+ mice.

Conclusions: This study demonstrates that Wnt/β-catenin signaling occurs in proliferating VSMCs during intimal thickening and indicates that this is a result of Wnt4 upregulation. (Circ Res. 2011;108:427-436.)

Key Words: smooth muscle ■ Wnt ■ β-catenin ■ proliferation ■ intimal thickening

Inappropriate vascular smooth muscle cell (VSMC) activation plays a key role in pathologies that involve intimal thickening, such as atherosclerosis and restenosis after angioplasty and vein grafting.1,2 Intimal thickening occurs as a result of augmented VSMC proliferation, in addition to increased VSMC migration, extracellular matrix synthesis and phenotypic change.3 Recently, we and others have proposed that the Wnt/β-catenin pathway is a novel regulator of VSMC proliferation and thereby intimal thickening.4-10 The Wnt family (comprised of 19 secreted, lipid-modified glycoproteins) plays a crucial role not only in the regulation of embryogenesis and development, but also in cell proliferation, differentiation, polarity, migration, and invasion (see review11). Wnt proteins activate downstream signaling pathways (in a paracrine and autocrine manner) after binding to cell surface Frizzled (Fzd) receptors.12 Abnormal Wnt signaling is associated with many human diseases, including cancer and degenerative diseases13; however, the involvement of Wnt signaling in atherosclerosis and restenosis remains to be fully elucidated.

Although Wnt signaling via β-catenin has a recognized role in controlling the proliferation of cells in human cancers,13 its role in VSMCs has only recently been considered. We previously showed that activation of β-catenin signaling, as a result of dismantling of N-cadherin–mediated cell–cell contacts, induced VSMC proliferation in vitro, via modulation of the expression of the cell cycle genes cyclin D1 and p21.6 Moreover, dismantling of cadherin/β-catenin complexes occurred in vivo during medial VSMC proliferation in balloon-injured rat carotid arteries, and was associated with increased expression of cyclin D1,5 indicating β-catenin signaling also regulates VSMC proliferation in vivo. This is corroborated by the detection of elevated levels of β-catenin after balloon injury in rat carotid arteries.7 There is also evidence that activation of the Wnt pathway induces VSMC proliferation.8-10 A Wnt inhibitor, secreted frizzled related
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This study, we investigated the role of Wnt/β-catenin signaling in VSMC proliferation and intimal thickening. We directly assessed whether Wnt/β-catenin signaling is activated during intimal thickening in vivo and aimed to identify which of the 19 Wnts are involved in the induction of VSMC proliferation.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Animal Models of Intimal Thickening
TOPgal transgenic mice containing the TOPFLASH reporter14 were a kind gift of Dr Yingzi Yang (National Human Genome Research Institute, Bethesda, MD). Heterozygous mutant Wnt4 (Wnt4+/−) and wild-type (Wnt4+/+) mice were generously provided by Dr Amanda Swain15 but were originally created in the laboratory of Andrew McMahon.16 Wnt4+/− mice were quiesced for 24 hours at 37°C, 5% CO2, in serum-free media (DMEM supplemented with 100 μg/mL penicillin, 100 IU/mL streptomycin, and 2 mM/L 1-glutamine). Proliferation was stimulated with 20 ng/mL platelet-derived growth factor (PDGF)-BB (or, in a few experiments, 20 ng/mL basic fibroblast growth factor).

In some cases, VSMCs were treated with 500 ng/mL recombinant Dickkopf (DKK)-1 protein (1096-DK, R&D Systems), 3 mg/mL WIF-1 recombinant protein (1341-WF, R&D Systems), 5 μmol/L Wnt agonist17 (681665, Calbiochem), or 500 ng/mL human (96.1% homology with mouse) Wnt2 recombinant protein (H0007472-P01, Abnova) or mouse Wnt4 recombinant protein (475-WN-005, R&D Systems).

In Vitro Cell Proliferation
VSMC proliferation was assessed by bromodeoxyuridine (BrdUrd) incorporation.

β-Catenin Activity
β-Catenin activity was quantified by measurement of β-galactosidase expression in TOPgal VSMCs.

RNA Extraction, Labeling, Amplification, and Reverse Transcription
Total RNA was extracted from mouse VSMCs and carotid arteries using the RNAeasy Kit (74104, Qiagen), according to the instructions of the manufacturer. Extracted RNA was then either directly labeled with Biotin-UTP (11388908910, Roche) and amplified using the TrueLabel AMP 2.0 Kit (GA-030, Superarray) for use in microarray analysis, or subjected to reverse transcription using the One-Step RT-PCR Kit (210210, Qiagen), according to the instructions of the manufacturer.

Microarray Analysis
Biotin-UTP–labeled RNA was cleaned using the RNAeasy kit (74104, Qiagen), according to the instructions of the manufacturer. For each sample, at least 1 μg of clean/extra pure (260:280 ratio >2.0 and 260:230 ratio >1.8) biotin-UTP–labeled RNA was added to 2 mL of Hybridization Solution (H-01, Superarray), and the mixture was incubated with mouse Wnt focused pathway spotted cDNA OligoGE array membranes (OMM-043-4, Superarray) at 37°C overnight, in a rotating cylinder. Hybridized oligonucleotide pairs were detected by an enhanced chemiluminescence kit (D-01, Superarray). Optical densities (OD/mm2) were normalized using housekeeping genes and concentration controls (GAPDH, BAS2C, Piaa, Hspcb, B2m, Rps27a, and 2 blank controls) on the array.

Real-Time Quantitative PCR
After reverse transcription, cDNA was subjected to quantitative (Q)-PCR for several Wnt genes using Quantitect primers (Qiagen; Online Table II) and the SYBR Green PCR Kit (204143, Qiagen), as described in the instructions of the manufacturer. Quantification was achieved after normalization using 18s ribosomal RNA values. The following primers for 18S ribosomal RNA were used: forward, 5’-CTCGATGCTCTAGTGAGT; reverse, 5’-CTTCAACCTCAGGTTCC (Sigma).

Western Blotting
VSMC proteins were extracted by SDS lysis, and the total protein concentration was determined using a bichinchoninic acid protein assay kit (Pierce). Equal total protein concentrations were loaded on 4% to 20% gradient gels and transferred to nitrocellulose membranes. Blots were blocked in 5% (wt/vol) skimmed milk powder and incubated overnight at 4°C with primary antibodies in blocking buffer. Bands were visualized using an enhanced chemiluminescence kit (32100, Roche). Optical densities (OD/mm2) were normalized using housekeeping genes (

Non-standard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BrdUrd</td>
<td>bromodeoxyuridine</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CM</td>
<td>conditioned media</td>
</tr>
<tr>
<td>DKK-1</td>
<td>Dickkopf-1</td>
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<tr>
<td>FP</td>
<td>fluorescent pixel</td>
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<td>Fzd</td>
<td>Frizzled</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>LRPs</td>
<td>low-density lipoprotein receptor–related protein-6</td>
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<tr>
<td>OD/mm²</td>
<td>optical density</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<td>Q-PCR</td>
<td>quantitative PCR</td>
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<td>Rfzb-1</td>
<td>Rat secreted frizzled-1</td>
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<tr>
<td>sFRP-1/FrzA</td>
<td>secreted frizzled-related protein-1</td>
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<tr>
<td>SFM</td>
<td>serum-free tissue culture media</td>
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<td>SIRNA</td>
<td>silencing RNA</td>
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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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<td>WIF-1</td>
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conjugated antibodies and enhanced chemiluminescence (Amersham International). Optical density of bands (OD × mm²) were quantified using a Bio-Rad GS-690 scanning densitometer (Bio-Rad) and then normalized with loading control OD × mm² values.

**Immunocytochemistry**

VSMCs grown on glass coverslips were quiesced in SFM for 24 hours before the addition of 20 ng/mL PDGF-BB and/or 10 μmol/L BrdUrd. VSMCs were fixed with ice-cold 4% (wt/vol) paraformaldehyde for 10 minutes. After incubation in 1% (vol/vol) Triton X-100 at 4°C for 5 minutes, VSMCs were incubated with 5 μg/mL goat antihouse Wnt4 antibody (AF475, R&D Systems) or 5 μg/mL nonimmune mouse IgG, and with 6 μg/mL anti-BrdUrd antibody (B-2531, Sigma) in 1% (wt/vol) BSA in PBS, overnight at 4°C. VSMCs were incubated for 45 minutes with rabbit anti-goat Alexa Fluor 488 diluted 1:200 in 1% (wt/vol) BSA in PBS, and then for 45 minutes with goat anti-mouse Alexa Fluor 594 diluted 1:200 in 1% (wt/vol) BSA in PBS. Coverslips were mounted on glass slides with Vectashield hard-set mounting medium (Vector Laboratories).

**Silencing RNA Knockdown**

Silencing RNA (SI) oligonucleotides (Online Table II) and scrambled oligonucleotides serving as a negative control (All Stars, Amaxa Inc, Cologne, Germany) were nucleofected into VSMCs with a Nucleofector device and a VSMC kit (API-1004, Amaxa Inc), according to the instructions of the manufacturer. Cells (8 × 10⁵ to 10 × 10⁵) were nucleofected with 250 pmol of Wnt, Fzd, or Allstar SIRNAs using program A-33 and analyzed 24 hours later. The knockdown efficiency was evaluated by Q-PCR.

**Wnt4 Conditioned Media**

Chinese Hamster Ovary (CHO) cells were transfected with 2 μg of mouse Wnt4 plasmid (17995, Addgene) or with 2 μg of green fluorescent protein (GFP) control plasmid (VSC 1001, Amaxa Inc) using a Nucleofector device and a CHO kit (VCA-1002, Amaxa Inc). After 3 days, GFP was verified using a fluorescent microscope and conditioned media collected.

**Statistics**

All values are expressed as means ± SEM. Results were analyzed by 1-sample t test versus 100, unpaired Student t tests or for multiple comparisons ANOVA and the Student–Newman–Keuls post test. Significant differences were accepted when *P* < 0.05.

**Results**

**Activation of Wnt/β-Catenin Signaling In Vivo During Intimal Thickening**

Left carotid artery ligation was performed in TOPgal transgenic mice to induce VSMC proliferation and intimal thickening. The induction of Wnt/β-catenin pathway after carotid artery ligation in mice. Immunohistochemistry for β-galactosidase (green) was performed on carotids removed at 3 (A, C, E, and G) and 28 days (B, D, F, and H) after ligation to assess β-catenin signaling (n = 6). Dual immunohistochemistry for β-galactosidase (green) and PCNA (red) (E and F) or cyclin D1 (red) (G and H). E and G, Insets show high-power images. Dotted lines indicate the intimal:medial boundary, and solid lines indicate the medial:adventitial boundary. Scale bars: 25 μm (panels); 10 μm (insets).
These mice contain the β-galactosidase gene under the control of a promoter composed of β-catenin responsive units and therefore can be used to quantify β-catenin signaling. Carotid arteries were removed at 3 days to assess medial VSMC proliferation and at 28 days to examine intimal and medial VSMC proliferation. β-Catenin signaling, assessed by immunohistochemistry for β-galactosidase, was almost absent in control unligated arteries (0.4±0.4 fluorescent pixels [FP] per μm²; Figure 1A and 1B) but significantly increased in the media at 3 and 28 days (130±60 and 1.8±0.4 FP, respectively) and in the intima at 28 days (6600±600 FP) after ligation (Figure 1C and 1D). In addition, dual immunohistochemistry showed that β-catenin signaling was associated with proliferating cell nuclear antigen (PCNA), a proliferation marker and cyclin D1 (100±0% in media at 3 days and 100±0% in intima at 28 days, n=6 per time point; Figure 1E through 1H).

Induction of VSMC Proliferation by Wnt Pathway In Vitro
To test the effect of Wnt pathway stimulation on VSMC proliferation, we treated quiescent VSMCs with a Wnt agonist17 and assessed BrdUrd incorporation. The Wnt agonist significantly increased proliferation by ∼2.5-fold (Figure 2A), which was similar to that observed with PDGF-BB, a potent VSMC mitogen. Moreover, an additive effect was not observed when the Wnt agonist and PDGF-BB were added simultaneously. In addition, we observed that WIF-1, a Wnt antagonist, significantly attenuated PDGF-BB-stimulated proliferation (Figure 2B), suggesting that Wnt proteins contribute to the proliferative response.

Consequently, we aimed to determine which Wnt genes were upregulated in proliferating VSMCs. Using focused Wnt pathway microarray analysis (n=3) on pooled VSMC mRNA, we consistently detected Wnt2, -4, -6, and -10b (Figure 3A). Interestingly, we observed a significant upregulation of Wnt2, -4, and -10b mRNA in proliferating VSMCs compared to quiescent VSMCs. Using Q-PCR we confirmed a significant increase in Wnt2 and Wnt4 mRNA in proliferating compared to quiescent VSMCs (Figure 3B), whereas no significant difference in mRNA levels was detected for any of the other Wnts tested (Wnt6 and 10b, Figure 3B; and Wnt5a, -7a, -9b, and -11, data not shown) between quiescent and proliferating VSMCs. We were unable to detect a significant increase in Wnt2 protein after treatment with PDGF-BB (Figure 4A). In contrast, we observed an induction of Wnt4 expression after PDGF-BB treatment by Western blotting in lysates (0.43±0.03 versus 0.21±0.11 O.D.xmm², n=4, $P<$0.05; Figure 4A) and in CM (Figure 4B). Similar findings were observed with other mitogens (basic fibroblast growth factor and FCS; data not shown). The induction of Wnt4 protein in proliferating VSMCs was further confirmed by immunocytochemistry (Figure 4C through 4F).

Effect of SIRNA Knockdown of Wnt2 and -4 on VSMC Proliferation In Vitro
To assess the effect of depletion of Wnt2 or Wnt4 on PDGF-BB-induced proliferation, VSMCs were subjected to SIRNA knockdown of these genes. Q-PCR revealed that Wnt2 SIRNA effectively knocked down Wnt2 mRNA (>50%, $P<$0.05), without significantly affecting Wnt4 mRNA, and vice versa for Wnt4 SIRNA (Online Figure I, A). In addition, no effect on Wnt6 or Wnt10b mRNA levels (the other two Wnts detected in mouse VSMCs) was observed with either SIRNA (data not shown). Western blotting confirmed Wnt2 and Wnt4 proteins were significantly reduced by the SIRNA (67±6% and 74±15%, respectively, n=3, $P<$0.05; Online Figure I, B). Interestingly, whereas knockdown of Wnt4 significantly reduced VSMC proliferation by 54±9%, knockdown of Wnt2 had no effect (Figure 4G).

Figure 2. Wnts promote VSMC proliferation in vitro. Mouse aortic VSMCs were quiesced for 24 hours in SFM and then treated with 5 μmol/L Wnt agonist and/or 20 ng/mL PDGF-BB (A) or 20 ng/mL PDGF-BB with or without 3 μg/mL WIF-1 (B). Proliferation was assessed after 24 hours by BrdUrd incorporation. *$P<$0.05, significant difference from quiescent control; $\$P<$0.05, significant difference from PDGF-BB alone (n=4).

Treatment of VSMCs With Wnt2 and Wnt4 In Vitro
We next tested whether Wnt2 or 4 proteins induce VSMC proliferation in vitro. Wnt4 CM significantly augmented mouse VSMC proliferation by ∼3-fold, compared to GFP control CM (Figure 5A). 500 ng/mL Wnt4, but not Wnt2, significantly increased VSMC proliferation by ∼2-fold (Figure 5B), supporting our SIRNA results. In addition, combined treatment with 500 ng/mL Wnt2 and 500 ng/mL Wnt4 did not induce a synergistic effect, or alter the Wnt4 proliferative response (Figure 5B). Despite the lack of effect of Wnt2 on VSMC proliferation, Wnt2 induced a significant increase in β-catenin activity, indicative of canonical Wnt/β-catenin signaling, in a...
similar manner to Wnt4 (Figure 5C). Increased levels of active β-catenin (αABC-β-catenin) and cyclin D1 (a Wnt/β-catenin responsive gene) were detected via Western blotting after Wnt4 treatment (Figure 5D). Finally, when DKK-1 (an inhibitor of Wnt/β-catenin signaling) was added to cultures treated with Wnt4CM, the proliferation rate of VSMCs was significantly reduced (Figure 5E). Together these findings suggest that despite the increase in both Wnt2 and Wnt4 mRNA in VSMC treated with PDGF-BB, only Wnt4 plays a direct role in proliferation.

Expression of Frizzled Receptors in VSMCs
Using the Wnt pathway microarray we identified that Fzd-1 and -6 were the predominant Fzd receptors in mouse aortic VSMCs (Online Figure II, A). In fact, these were the only two Fzds detected on all three arrays. Fzd-1 protein, but not Fzd6, was increased in proliferating VSMCs compared to quiescent VSMCs (Online Figure II, B). Interestingly, we observed that SIRNA knockdown of Fzd1, but not Fzd6 (Online Figure II, C), significantly reduced VSMC proliferation (Figure 5F).

Wnt4 and Fzds In Vivo During Intimal Thickening
We confirmed that Wnt4, was significantly elevated in carotid arteries 28 days after ligation compared to unligated arteries (1.5±0.06 versus 1.0±0 fold, P=0.012), but no increase in Wnt2 mRNA was observed (data not shown). Immunohistochemistry for Wnt4 revealed Wnt4 protein was low in the media of control arteries (5±3 and 7±1 FP, and 28 days, respectively), but was significantly upregulated in ligated carotid arteries in the media after 3 days (5±3 versus 19±4 FP, n=3, P<0.05) and in the intima after 28 days (7±1 versus 57±9 FP, n=3, P<0.05) (Online Figure III). Moreover, Wnt4 protein expression correlated with β-catenin signaling (ie, β-galactosidase protein expression, P=0.049, n=33). In addition, we examined the expression of Wnt4 in the rat carotid artery balloon injury model (Figure 6 and Online Figure IV). Wnt4 protein was low in control arteries but significantly upregulated following balloon injury of the rat carotid artery in the media after 2 days and in the intima after 10 days. Dual immunohistochemistry revealed that the increased Wnt4 protein expression correlated with augmented VSMC proliferation, measured by BrdUrd incorporation, and cyclin D1 expression during intimal thickening (Figure 6 and Online Figure IV; P<0.05 and P<0.001, respectively, n=18).

Importantly, the intimal lesion size and intimal cell proliferation rate (detected by PCNA immunohistochemistry) were significantly smaller in Wnt4−/− mice than wild-type control mice (Figure 7). Significantly less Wnt4 and nuclear β-catenin proteins were detected in the intima of ligated carotid arteries in Wnt4−/− mice compared to the wild-type control mice (Figure 7).

Using Q-PCR, we confirmed the expression of both Fzd1 and 6 mRNA in mouse carotid arteries. No significant differences in the level of Fzd1 were detected between control and ligated arteries after 28days (0.7±0.3 versus 1±0 fold), whereas Fzd6 mRNA was significantly reduced in the ligated arteries com-
pared to the unligated controls (0.13±0.04 versus 1.0±0 fold, \( P = 0.0025 \)). Immunohistochemistry revealed that Fzd-1 protein was barely detectable in the media of control uninjured rat carotid arteries (Online Figure V, A), whereas it was significantly increased in the media at 2 days after balloon injury (0.54±0.17 versus 1.79±0.24 FP, \( P < 0.05, n = 5 \); Online Figure V, C) and in the intima at 10 days after injury (0.54±0.17 versus 12.70±2.05 FP, \( P < 0.05, n = 5 \); Online Figure V, D). No difference in Fzd-1 protein levels was observed between control medial layer and the medial layer of arteries 10 days after injury (0.54±0.17 versus 0.28±0.15 FP, \( n = 5 \); Online Figure V, A and D). In contrast, low levels of Fzd-6 protein were observed in the control uninjured arteries (0.22±0.05 FP) and in the media at 2 and 10 days after injury (0.18±0.07 and 0.19±0.03 FP, respectively) (Online Figure V, E, G, and H). Fzd-6 protein was significantly increased in the intima at 10 days after injury compared to the media of control uninjured arteries (0.70±0.1 versus 0.22±0.05 FP, \( P < 0.05, n = 5 \); Online Figure V, E and H); however, the amount detected was considerably less than Fzd1.

**Discussion**

In this study, we provide the first direct evidence that Wnt4-induced \( \beta \)-catenin signaling occurs in proliferating VSMCs during intimal thickening in vivo. Additionally, we show that Wnt4 upregulation contributes to the induction of VSMC proliferation and intimal thickening in vivo. Consequently, we propose that modification of the Wnt pathway is a potential target for inhibition of VSMC proliferation and intimal thickening.

The involvement of \( \beta \)-catenin signaling in the regulation of VSMC proliferation during intimal thickening is currently ill-defined. However, previous studies have indicated that \( \beta \)-catenin signaling is involved in the regulation of VSMC proliferation. Firstly, balloon injury of the rat carotid artery, which induces VSMC proliferation, also results in upregulation of \( \beta \)-catenin protein levels.\(^5\)\(^,\)\(^7\)\(^,\)\(^1^\)\(^8\) Additionally, we have demonstrated that growth factor stimulation of VSMC proliferation in vitro augmented active \( \beta \)-catenin levels and association of \( \beta \)-catenin with LRP6, a transmembrane coreceptor for Wnt, in rat VSMCs. Nonimmune IgG (IgG) was used as negative control (E and F). Scale bar represents 10 μm. G, Proliferation (BrdUrd incorporation) was quantified 24 hours after addition of 20 ng/mL PDGF and transfection of control, Wnt2, or Wnt4 SI (\( n = 4 \)). \( * P < 0.05 \), significant difference from control SI.

Before this study, direct evidence for the involvement of Wnt proteins in VSMC proliferation and intimal thickening was scant. It has been shown that LRP6, a transmembrane coreceptor for Wnt proteins, promotes VSMC proliferation through the Wnt signaling cascade.\(^8\) In addition, FrzA, a Wnt inhibitor, reduced VSMC entry into S-phase and the levels of cyclins (including cyclin D1) and cyclin-dependent kinases.\(^8\) Moreover, increased expression of Rfzb-1, an antagonist of the Wnt cascade, was observed in quiescent isolated VSMCs and in the rat arterial wall at 4 days and 3 weeks after in vivo injury; time points when VSMC proliferation is suppressed.\(^9\) This suggests that Rfzb-1 suppresses proliferation; hence Wnt signaling must have a positive role in proliferation. In this study, we show that
a Wnt agonist promotes VSMC proliferation, confirming the positive involvement of Wnt proteins in VSMC proliferation. Importantly, we observed that PDGF-BB stimulated VSMC proliferation was retarded by addition of WIF-1, a Wnt antagonist, indicating that the Wnt pathway plays a key role in proliferation. However, from this experiment, the precise Wnt proteins involved in modulation of VSMC proliferation are still unknown, as WIF-1 is a nonspecific Wnt inhibitor.

We hypothesized that treatment of VSMCs with growth factors increases Wnt protein expression, which contributes to induction of proliferation. We therefore investigated Wnt mRNA expression in quiescent versus proliferating mouse aortic VSMCs, to mimic quiescent VSMCs in the normal blood vessel wall and proliferating VSMCs in the diseased blood vessel wall after injury and exposure to growth factors. To identify the specific Wnt proteins involved in VSMC proliferation in vitro we performed focused microarray and Q-PCR analysis. We show that although Wnt2 and Wnt4 mRNA are both significantly increased in proliferating VSMCs in vitro, we only observe upregulation of the Wnt4 protein. This may indicate that Wnt2 is controlled by microRNAs in a similar manner as that observed by others previously for Wnt1.19 In addition, we observed that specific and efficient Wnt4 SIRNA knockdown significantly retarded VSMC proliferation, whereas treatment with Wnt4 protein significantly increased VSMC proliferation. Contrastingly, no effect on VSMC proliferation was observed after Wnt2 SIRNA knockdown or treatment with Wnt2 recombinant protein, despite its ability to activate β-catenin signaling. Moreover, we confirmed that treatment with Wnt4 protein resulted in activation of the Wnt/β-catenin pathway and increased expression of the β-catenin responsive gene cyclin D1. Together, these results show that Wnt4 causes activation of the canonical Wnt/β-catenin pathway, resulting in increased cyclin D1 expression and VSMC proliferation. This is supported by the observation that DKK-1, a specific inhibitor of canonical Wnt/β-catenin signaling, significantly reduced Wnt4-induced VSMC proliferation. The discrepant findings between Wnt2 and Wnt4 may possibly be attributable to the ability of Wnt4 to activate other (noncanonical) Wnt pathways in addition to canonical Wnt/β-catenin signaling.

This study is the first to demonstrate Wnt4 expression in VSMCs in vitro and during intimal thickening in vivo. To directly assess the role of Wnt4 in intimal thickening, we subjected Wnt4−/− and Wnt4+/+ mice to carotid ligations. Importantly, using immunohistochemistry, we observed that reduced levels of Wnt4 were present in ligated carotid arteries of Wnt4−/− mice versus wild-type controls. In addition, Wnt activity, assessed by quantification of nuclear β-catenin, was significantly reduced in Wnt4−/− mice compared to wild-type controls, as was intimal cell proliferation and intimal lesion area, illustrating Wnt4 promotes intimal thickening. Wnt4 has a defined role in embryogenesis, particularly in kidney develop-
Wnt signaling is dependent on Frizzled receptors expression. We showed that both Fzd-1 and Fzd-6 are present in both quiescent and proliferating VSMCs, indicating that these cells can bind Wnt4. However, Fzd1 knockdown, and not Fzd6, retarded VSMC proliferation. Moreover, only Fzd-1 protein was significantly increased in proliferating compared to quiescent VSMCs. Additionally, Fzd1 protein was more readily detectable in rat carotid arteries and was significantly increased in the intima at 10 days and in the media at 2 days after injury. These findings contrast with a previous study, where Fzd-1 mRNA was significantly reduced in the media at 2 days after injury. This apparent contradiction in findings may be explained by the fact that the previous study by Mao et al assessed mRNA, whereas we have also measured protein. Nevertheless, our findings suggest that Fzd-1, present...
in the arterial wall cells at all time points, may bind Wnt4 and thus induce VSMC proliferation and intimal thickening.

Our data suggest that inhibition of Wnt4 is an attractive therapeutic approach for intimal thickening. However, a Wnt4-specific antagonist that can be locally delivered to vein grafts or stented arteries is desirable, because global Wnt inhibition may be detrimental. Currently, approaches for stimulation of the Wnt pathway such as small molecule inhibitors of GSK-3 and neutralizing antibodies to DKK-1 and secreted Fzds, are under consideration for bone disorders. However, the converse is required for treatment of intimal thickening. Several academic groups have published proof-of-concept reports on small-molecule Wnt pathway inhibitors, and a few biotech and pharmaceutical companies are actively developing these and other compounds. However, Wnt4-specific agents are currently not available.

In summary, we propose that exposure to PDGF-BB in response to injury to the blood vessel (for example after stenting or vein grafting) causes increased expression of Wnt4 in VSMCs, which leads to activation of Wnt/β-catenin signaling via Fzd1 and thereby modulates the expression of β-catenin responsive genes, including cyclin D1, that cause VSMC proliferation. Aberrant VSMC proliferation during intimal thickening may therefore be reduced by Wnt4-specific inhibition.

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Disclosures

None.

References

Proliferation of vascular smooth muscle cells (VSMCs) leads to thickening of the inner layer (intima) of blood vessels, thereby contributing to disease pathologies such as restenosis and atherosclerosis.

VSMCs proliferate following cleavage of N-cadherin–mediated cell–cell contacts on the cell membrane, after which β-catenin is released from the membrane into the cell, permitting movement to the cell nucleus and regulation of the expression of many genes.

It has also been postulated that β-catenin signaling promotes VSMC proliferation via Wnt proteins (canonical pathway), but the Wnt proteins involved are unknown.

What New Information Does This Article Contribute?

- Identification of the specific Wnt protein (Wnt4) involved in the regulation of VSMC proliferation both in vitro and in vivo.
- Wnt4 is upregulated in response to growth factors and promotes VSMC proliferation via the Frizzled receptor 1 and β-catenin.
- Wnt/β-catenin pathway is activated in vivo during the thickening of the inner layer of blood vessels (intimal thickening).

Novelty and Significance

VSMC proliferation leads to thickening of the inner layer of blood vessels, thereby contributing to atherogenesis and restenosis of vein grafts and stented arteries. Although previous studies suggest the involvement of the Wnt/β-catenin signaling pathway in VSMC proliferation and neointimal thickening, this has not been directly demonstrated in vivo and the precise Wnt protein(s) involved remain unknown. We provide direct evidence showing that Wnt/β-catenin signaling pathway promotes VSMC proliferation in vivo. We demonstrate for the first time that both in vitro and in vivo that Wnt4 is specifically upregulated by growth factors and that it promotes VSMC proliferation and activation of the canonical Wnt/β-catenin pathway through the Frizzled receptor 1. Our findings provide a greater understanding of the regulation of VSMC proliferation by growth factors and identify Frizzled-1 as the predominant Wnt4 receptor in this cell type. Additionally, we identify Wnt4 and Frizzled-1 as potential targets for new therapeutic approaches to retard VSMC proliferation to prevent intimal thickening and thereby attenuate atherosclerosis and restenosis.
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Expanded Materials and Methods

Animal models of intimal thickening

The housing and care of all the animals, and all the procedures used in these studies, were performed in accordance with the guidelines and regulations of the University of Bristol and the United Kingdom Home Office.

TOPgal transgenic mice containing the TOPFLASH reporter (i.e. the β-galactosidase gene under the control of a β-catenin responsive promoter) were a kind gift of Dr Yingzi Yang (National Human Genome Research Institute, Bethesda, MD, USA). These mice were generated on a CD1 background by backcrossing on CD1 >10 times.

Heterozygous mutant Wnt4 (Wnt4⁺⁻) mice and wild-type (Wnt4⁺⁺) littermate control mice were generously provided by Dr Amanda Swain[1] but were originally created in Andrew McMahon's laboratory. Wnt4⁺⁻ mice were generated on a 129/Sv background by backcrossing on 129/Sv >10 times. Wnt4⁺⁻ and Wnt4⁺⁺ mice were bred to obtain sufficient offspring for experiments and identification of heterozygous and wild-type mice was performed by PCR and subsequent agaroze gel electrophoresis (AGE), according to Jaxmice protocol Wnt4-tm1Amc. The wild-type Wnt4 allele was detected using a forward primer (5’-CTGAGGAAGAGCAGGGTCAC-3’) which anneals at a region common/shared between the two alleles, and a reverse primer (5’-ATGGTCACCCCCATTTTACA-3’) which anneals at a region existing only on the wild-type Wnt4 cDNA. This wild-type Wnt4 amplicon size was approximately 300bp. The targeted (mutated) Wnt4 allele was detected using the same forward primer and a reverse primer (5’-TGGATGTGGAATGTGTGCGAG-3’) which anneals at a region existing only on the mutant Wnt4 cDNA. This targeted/mutated Wnt4 amplicon size was approximately 400bp. PCR reactions (i.e. 45 cycles of: 1min at 93°C, 30sec at 65°C, 30sec at 72°C followed by 5 min at 72°C) for the amplification of either the
wildtype or the mutant Wnt4 allele cDNA fragment were performed separately. PCR products (wild-type and mutant bands) were visualised after AGE (1.5% agaroze w/v in 1xTBE Buffer containing EtBr).

All animals were anesthetized by inhalation of isofluorane (2%). The left common carotid artery was dissected and ligated near the carotid bifurcation to induce intima formation. Vetricgesic (1.5ug/mouse) was used for pain relief.

Rat carotid arteries were subjected to balloon injury as follows. Male Sprague Dawley rats (325-375 g) were obtained from Charles River (Manston, Kent, UK). Animals were anaesthetized by intraperitoneal injection with a mixture of ketamine hydrochloride (70 mg/kg of bodyweight) and xylazine (5 mg/kg of bodyweight), given under light inhalational anaesthesia with halothane. Three rotating passes of a saline-inflated size 2F arterial embolectomy balloon catheter (Actamed, Wakefield, West Yorkshire, UK) were used to injury the left common carotid artery and aorta. After injury the artery was ligated, and the skin wound was closed with stainless steel clips. Rats were killed at the designated time-points by intravenous injection of sodium pentobarbital and then immediately perfused with PBS at 120 mm Hg. Vessels taken for histology were perfusion fixed with 0.1 mol/L phosphate-buffered 10% (v/v) formaldehyde. Control rats were subjected to a sham operation, which involved the entire procedure apart from insertion of the balloon catheter. Vessels were removed from control (sham) rats at 0.25 hours. Proliferating cells were labeled with bromodeoxyuridine (BrdU) for the last 24 hours prior to sacrifice by placement of a 50 mg BrdU pellet (Boehringer Mannheim, Lewes, UK) under the skin.
Histochemistry and Immunohistochemistry

To assess intimal lesion size in ligated carotid arteries from Wnt4+/− and Wnt4+/+ mice, arteries were removed after 21 days and embedded longitudinally in paraffin-wax. After staining with elastin van Gieson stain, the intimal area was measured by image analysis.

Immunohistochemistry was performed on mouse and rat carotid artery paraffin-embedded sections using the antibodies detailed in supplementary table I, as described below. Paraffin-wax embedded sections (3 µm) were subjected to antigen retrieval by microwaving for 2 x 5 minutes in 10 mmol/L citrate buffer (pH 6.0) and then blocked by incubation in Image Fx (Invitrogen) for 30 minutes. For single detection of Wnt4, Fzd-1, Fzd-6, β-galactosidase and PCNA the sections were incubated overnight with primary antibodies diluted in 1% BSA/PBS as outlined in Supplementary Table I. After incubation with Alexa Fluor 488 conjugated secondary antibodies diluted 1:200 in 1% BSA/PBS, the sections were mounted in Prolong Gold mountant containing DAPI (Invitrogen). Dual immunohistochemistry for Wnt4 protein with cyclin D1 protein or incorporated BrdU, and β-galactosidase protein with PCNA or cyclin D1 proteins was performed on balloon-injured carotids. Firstly, immunohistochemistry for Wnt4 or β-galactosidase was carried out using primary antibodies diluted in 1% BSA/PBS as outlined in Supplementary Table I, and secondary antibodies labelled with Alexa Fluor 488 (diluted 1:200 in 1% BSA/PBS). Then cyclin D1, PCNA or BrdU was detected using primary antibodies diluted in 1% BSA/PBS as outlined in Supplementary Table I and Alexa Fluor 596 secondary antibodies (diluted 1:200 in 1% BSA/PBS). In the case of BrdU staining, sections were pre-incubated with 4N HCL at 37°C for 30 minutes. The number of positive pixels was quantified per section using image analysis (Image ProPlus) and expressed as a percentage of the total number of pixels in the area. Non-immune immunoglobulin G (IgG) was used as a negative control.
VSMCs culture

Aortas were obtained from TOPgal transgenic mice and VSMCs were grown from aortic explants. Briefly, aortas were chopped using scissors into approximately 1mm squares and after allowing to adhere to the tissue culture plate for 30 minutes, cultured in Dulbecco’s modified essential media (DMEM) supplemented with 10% FCS, 100µg/mL of penicillin, 100IU/mL streptomycin, 2mM L-glutamine and used at passages 3–9. VSMCs were quiesced for 24 hours at 37°C, 5% CO₂ in serum free tissue culture media (SFM), (Dulbecco’s modified essential media supplemented with 100µg/mL of penicillin, 100IU/mL streptomycin, 2mM L-glutamine).

Proliferation was stimulated with 20ng/mL PDGF-BB (or in a few experiments 20ng/mL b-FGF) in SFM. In some cases, VSMCs were treated with 500ng/mL recombinant DKK-1 protein (1096-DK, R&D Systems), 3mg/mL Wnt inhibitory factor-1 (WIF-1) recombinant protein (1341-WF, R&D Systems), 5µM Wnt agonist[2] (681665, Calbiochem), or 50 and 500ng/mL human (96.1% homology with mouse) Wnt2 recombinant protein (H00007472-P01, Abnova) or mouse Wnt4 recombinant protein (475-WN-005, R&D Systems).

In vitro cell proliferation

VSMC proliferation was assessed by bromodeoxyuridine (BrdU) incorporation. VSMCs, seeded in 24 well plates, were washed with PBS and then incubated in serum free tissue culture media (SFM) supplemented with 10 µM BrdU and various treatments including Wnt proteins, Wnt agonist, PDGF-BB, DKK-1 or WIF-1 for 24 hours. For experiments with siRNA, VSMCs were subjected to siRNA nucleofection prior to seeding in 24 well plates. Cells were fixed with 4% (v/v) paraformaldehyde and then permeabilised with 1% (v/v)
Triton-X-100 in PBS. To denature DNA cells were incubated in 4N HCl for 30 minutes at 37°C. Cells were then incubated with 3 μg/mL mouse anti-BrdU antibody (Sigma) in 1% BSA/PBS overnight at 4°C. Finally cells were incubated with goat-anti-mouse immunoglobulin G (DAKO) diluted 1:200 in 1% BSA/PBS for 30 minutes at room temperature and then Extravidin™ horseradish peroxidase diluted 1:200 in 1% BSA/PBS for 30 minutes at room temperature. Bound antibodies were detected by incubation in Fast DAB (3,3′-Diaminobenzidine, Sigma) for 10 minutes at room temperature. The percentage of BrdU positive cells in ten low power fields was determined per sample and the mean number of BrdU positive cells per sample calculated (i.e. the proliferative rate).

β-catenin activity

β-catenin activity was quantified by assessment of β-galactosidase expression in TOPGAL VSMCs. Following seeding in 24 well plates, TOPGAL VSMCs were quiesced for 24 hours in SFM and then stimulated with Wnt proteins for 24 hours. VSMCs were lysed and β-galactosidase activity determined by using the Galacto-Light Plus kit (Tropix, Bedford, UK) following the manufacturer’s instructions. Luminescence was measured by a Glomax luminometer (Promega).

RNA extraction, labelling, amplification and reverse transcription.

Total RNA was extracted from VSMCs and mouse carotid arteries using the RNAeasy Kit (74104, Qiagen), according to the manufacturer’s instructions. Extracted RNA was then either directly labelled with Biotin-UTP (11388908910, Roche) and amplified using the TrueLabel AMP 2.0 Kit (GA-030, Superarray) for use in microarray analysis, or subjected to reverse transcription using the One-Step RT-PCR Kit (210210, Qiagen), according to the manufacturer’s instructions.
**Microarray Analysis**

Biotin-UTP-labelled RNA was cleaned using the RNAeasy kit (Qiagen), according to the manufacturer’s instructions. For each sample, at least 1µg of clean/extra pure (260:280 ratio >2.00 and 260:230 ratio >1.8) biotin-UTP labelled RNA was added to 2mL of Hybridization Solution (H-01, Superarray) and the mix was incubated with mouse Wnt focused pathway spotted cDNA OligoGE array membranes (OMM-043-4, Superarray) at 37°C overnight, in a rotating cylinder. Hybridized oligonucleotide pairs were detected by an enhanced chemiluminescence kit (D-01, Superarray) according to the manufacturer’s instructions, following two 30 minute stringent washes with SSC containing buffers (85635, Sigma) at room temperature. Detected clove-shaped spots were quantified using the GEAnalysis Suite software package (GA-021, Superarray). Optical densities (ODxmm²) were normalized using housekeeping genes and concentration controls (GAPDH, BAS2C, Ppia, Hspcb, B2m, Rps27a, and two blank controls) on the array.

**Real Time Quantitative PCR**

After reverse transcription, cDNA was subjected to quantitative PCR (Q-PCR) for several Wnt genes using Quantitect primers (Qiagen, Supplementary Table II) and the SYBR Green PCR Kit (204143, Qiagen), as described in the manufacturer’s instructions. Quantification was achieved after normalisation using 18s ribosomal RNA values. The following primers for 18s ribosomal RNA were used (Forward 5’-CTCGATGCTCTTAGCTGAGT & Reverse 5’-CTTCAAACCTCGACTTTTCG, Sigma).

**Western Blotting**

VSMC proteins were extracted by SDS lysis and the total protein concentration was
determined using a bicinchoninic acid protein assay kit (Pierce), as described in the manufacturer’s instructions. Equal total protein concentrations were loaded on 4–12% Novex Bis-Tris gels (Invitrogen) and transferred to 0.2μm nitrocellulose membranes. Blots were blocked in 5% (w/v) skimmed milk powder and incubated overnight at 4°C in Starting block with primary antibodies as outlined in Supplementary Table III (37538, Pierce). Bound primary antibodies were detected by secondary horseradish peroxidise-conjugated antibodies and enhanced chemiluminescence (Amersham International). Optical density of each band (ODxmm²) bands was quantified using a Bio-Rad GS-690 scanning densitometer (Bio-Rad) and then normalised with loading control ODxmm² values.

**Immunocytochemistry**

VSMCs grown on glass coverslips were quiesced in SFM for 24h prior to the addition of 20ng/mL PDGF-BB and/or 10μM BrdU. VSMCs were fixed with ice-cold 4% (w/v) paraformaldehyde for 10min. After incubation in 1% (v/v) Triton X-100 at 4°C for 5min, VSMCs were incubated with 5μg/mL goat anti-mouse Wnt4 antibody (AF475, R&D Systems) or 5μg/mL non-immune mouse IgG, and with 6μg/mL anti-BrdU antibody (B-2531, Sigma) in 1% (w/v) BSA in PBS, overnight at 4°C. VSMCs were incubated for 45min with rabbit anti-goat Alexa Fluor 488 diluted 1:200 in 1% (w/v) BSA in PBS, and then for 45min with goat anti-mouse Alexa Fluor 594 diluted 1:200 in 1% (w/v) BSA in PBS. Coverslips were mounted on glass slides with Vectashield® hard-set mounting medium (Vector Laboratories).

**SiRNA knockdown**

Silencing RNA (siRNA) oligonucleotides (Supplementary Table II) and scrambled oligonucleotides serving as a negative control (All Stars, Amaxa Inc., Cologne, Germany)
were nucleofected into VSMCs with a Nucleofector device and a VSMC kit (API-1004, Amaza, Inc.), according to the manufacturer's instructions. Briefly 8–10x10^5 cells were subjected to nucleofection with 250pmol of Wnt/Fzd or Allstar siRNAs (using program A-33), re-seeded onto culture plates and analysed 24 hours after nucleofection. The efficiency of RNA knockdown was evaluated by Q-PCR.

**Generation of Wnt4 Conditioned Media**

Chinese Hamster Ovary (CHO) cells were transfected with 2µg mouse Wnt4 plasmid (17995, Addgene) or with 2µg GFP control plasmid (VSC 1001, Amaza Inc), using a Nucleofector device and a CHO kit (VCA-1002, Amaza Inc.), following the manufacturer's instructions. After 3 days GFP was verified using a fluorescent microscope and conditioned media (CM) was collected.

**Statistics**

All values are expressed as mean±SEM. Analysis of 2 groups was performed by the unpaired Student’s t-test. For multiple comparisons, data were analyzed by ANOVA and the Student-Newman-Keuls post-test. Significant differences were accepted when p<0.05.

**References**

Supplementary Figure II

A

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B

- Fzd-1: 71 kDa
- β-actin: 42 kDa
- Fzd-6: 79 kDa
- β-actin: 42 kDa

C

- Fzd1 mRNA
- Fzd6 mRNA

D

- Control SI
- Fzd1 SI
- Fzd6 SI

- GAPDH: 35 kDa
- Fzd1: 71 kDa
- Fzd6: 79 kDa
Supplementary Figure IV

The diagram shows the % Area positive for different conditions:
- **Control media**
- **2 days media**
- **10 days media**
- **10 days intima**

The values are represented by bars, with * indicating statistical significance. The conditions are differentiated by symbols:
- BrdU
- Wnt4
- CD1

The y-axis represents the % Area positive, ranging from 0.00 to 70.00.

The x-axis lists the different time points and media conditions.
Supplementary Figure I: siRNA knockdown of Wnt2 and 4.

(A) Q-PCR was performed to assess Wnt2 and Wnt4 mRNA levels after treatment with Wnt2 and 4 siRNA (SI) compared to Allstars SiRNA control, n=3. * Indicates significant difference from control SI, p<0.05. (B) Western blotting for Wnt2 and Wnt4 proteins after siRNA treatments. β-tubulin is shown as loading control.

Supplementary Figure II: Expression of Frizzled receptors in vitro

(A) Normalised ODxmm² values (mean ± SEM) for all Fzd genes on the microarray. (B) Western Blot showing protein expression of Fzd-1 and Fzd-6 in quiescent versus proliferating VSMCs. β-actin is shown as the loading control. Bar chart shows densitometric analysis of Western blots, n=3, * indicates significant difference from control, p<0.05. (C) Q-PCR was performed to assess Fzd-1 and Fzd-6 mRNA levels after treatment with Fzd-1 and Fzd-6 siRNA (SI), n=3. * Indicates significant difference from control SI treated VSMCs. (D) Western blotting for Fzd-1 and Fzd-6 proteins after siRNA treatments. GAPDH is shown as loading control.

Supplementary Figure III: Wnt4 protein expression after ligation of the mouse carotid artery.

Left carotid arteries of TOPgal mice were ligated to induce VSMC proliferation and intimal thickening. Immunohistochemistry for Wnt4 (green) was performed on carotids removed after 3 (A and C) and 28 days (B and D). Nuclei were stained blue with DAPI. Dotted lines indicate the intimal:medial boundary, solid lines indicate the medial:adventitial boundary. Substitution of primary antibody with non-immune IgG was performed as a negative control (data not shown).
Supplementary Figure IV: Quantification of Wnt4, BrdU and cyclin D1 in rat aorta after balloon injury.

The percentage of the intima or media positive staining Wnt4, BrdU and cyclin D1 detected by immunohistochemistry was quantified in control uninjured carotid arteries and in balloon injured carotid arteries at 2 and 10 days after balloon injury, n=5 per timepoint, * indicates a significant difference from control media, p<0.05.

Supplementary Figure V: Expression of Frizzled receptors in vivo

Immunohistochemistry for Fzd-1 (green, A-D) and Fzd-6 (green, E-H) was performed on sham control carotid arteries or on carotid arteries removed at 2 and 10 days after injury. Nuclei were stained blue with DAPI. Dotted lines indicate the intimal:medial boundary, solid lines indicate the medial:adventitial boundary. Scale bar represents 20μm. Substitution of primary antibody with non-immune IgG was performed as a negative control for 10 days.
### Supplementary Table I: Antibodies used for immunohistochemistry

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### Supplementary Table II: Q-PCR primers and SiRNA (Qiagen). N/A = not applicable

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