Wnt2 and WISP-1/CCN4 Induce Intimal Thickening via Promotion of Smooth Muscle Cell Migration

Helen Williams, Carina A.E. Mill, Bethan A. Monk, Sarah Hulin-Curtis, Jason L. Johnson, Sarah J. George

Objective—Increased vascular smooth muscle cell (VSMC) migration leads to intimal thickening which acts as a soil for atherosclerosis, as well as causing coronary artery restenosis after stenting and vein graft failure. Investigating factors involved in VSMC migration may enable us to reduce intimal thickening and improve patient outcomes. In this study, we determined whether Wnt proteins regulate VSMC migration and thereby intimal thickening.

Approach and Results—Wnt2 mRNA and protein expression were specifically increased in migrating mouse aortic VSMCs. Moreover, VSMC migration was induced by recombinant Wnt2 in vitro. Addition of recombinant Wnt2 protein increased Wnt1-inducible signaling pathway protein-1 (WISP-1) mRNA by ≈1.7-fold, via β-catenin/T-cell factor signaling, whereas silencing RNA knockdown of Wnt-2 reduced WISP-1 mRNA by ≈65%. Treatment with rWISP-1 significantly increased VSMC migration by ≈1.5-fold, whereas WISP-1 silencing RNA knockdown reduced migration by ≈40%. Wnt2 and WISP-1 effects were integrin-dependent and not additive, indicating that Wnt2 promoted VSMC migration via WISP-1. Additionally, Wnt2 and WISP-1 were significantly increased and colocated in human coronary arteries with intimal thickening. Reduced Wnt2 and WISP-1 levels in mouse carotid arteries from Wnt2+/− and WISP-1−/− mice, respectively, significantly suppressed intimal thickening in response to carotid artery ligation. In contrast, elevation of plasma WISP-1 via an adenovirus encoding WISP-1 significantly increased intimal thickening by ≈1.5-fold compared with mice receiving control virus.

Conclusions—Upregulation of Wnt2 expression enhanced WISP-1 and promoted VSMC migration and thereby intimal thickening. As novel regulators of VSMC migration and intimal thickening, Wnt2 or WISP-1 may provide a potential therapy for restenosis and vein graft failure. (Arterioscler Thromb Vasc Biol. 2016;36:1417-1424. DOI: 10.1161/ATVBAHA.116.307626.)

Key Words: intimal thickening ■ restenosis ■ smooth muscle cell migration ■ WISP-1/CCN4 ■ Wnt2

Elevated rates of vascular smooth muscle cell (VSMC) migration and proliferation contribute to intimal thickening in vivo.1 Intimal thickening underlies complications, such as restenosis after angioplasty and stent implantation and late failure of vein grafts,2 and acts as the soil for atherosclerosis.3 VSMC migration into the intima is a key process in intimal thickening because if VSMCs do not enter the intima, there is no opportunity for accelerated VSMC proliferation within the intima. Currently, many approaches designed to reduce intimal thickening aim to retard VSMC proliferation, but antimigratory approaches may be equally desirable.

The Wnt signaling pathway has been implicated in intimal thickening and VSMC migration and proliferation by both our own group4-6 and others.7-10 Abnormal upregulation of the Wnt pathway in various disease states mimics the classic role of the Wnt pathway during embryonic development, where the pathway controls cell migration and proliferation during the formation of tissues and organs.11 The Wnt family consists of 19 highly conserved genes that encode secreted glycoproteins involved in cell signaling. The Wnt ligands bind to frizzled (Fzd) receptors and their coreceptors, the low-density lipoprotein–related proteins 5/6, to activate downstream pathways.12 Upregulation of the Wnt pathway has been well established in many cancer subtypes13 and in the development of osteoarthritis14; however, their role in atherosclerosis and restenosis is less well established. Evidence shows that the Wnt pathway, via the receptor low-density lipoprotein–related protein 6, regulates cell proliferation and survival in VSMCs,7 whereas a Wnt pathway antagonist reduced proliferation.15 We previously demonstrated that Wnt4 directly induced VSMC proliferation and caused intimal thickening in vivo, via Fzd1 activation and cyclin D1 upregulation.5 Additionally, in this study, we demonstrated that although Wnt2 mRNA was significantly elevated in proliferating VSMCs, this

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was not translated into protein, and recombinant Wnt2 (rWnt2) protein did not increase VSMC proliferation in vitro. The Wnt pathway has also been shown to have a role in cell migration, with Wnt3a involvement in both migration and adhesion of VSMCs through integrin linked kinase regulation of β1-integrin. However, on initiation of this study, it was unclear which Wnt proteins modulated VSMC growth factor–induced migration and whether Wnt-induced VSMC migration promoted intimal thickening in vivo. Many genes are upregulated by the Wnt pathway, some of which are known to modulate VSMC migration, including Wnt-1-inducible signaling pathway protein-1 (WISP-1/CCN4). WISP-1 is a member of the CCN family of genes, which regulates fibrosis and wound healing, angiogenesis, osteogenesis, and cancer. In cultured VSMCs, WISP-1 promotes survival, proliferation, and migration through integrin α5β1.

In this study, we aimed to identify whether any of the 19 Wnts are involved in VSMC migration in vitro and thereby intimal thickening in vivo. In addition, if a candidate Wnt was confirmed, to investigate the downstream Wnt pathway targets that promote VSMC migration in vitro and intimal thickening in vivo.

Materials and Methods

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

**Figure 1.** Wnt2 was upregulated in migrating smooth muscle cells in vitro. **A**, Wnt2 mRNA was quantified by oligonucleotide array, n=3, and quantitative polymerase chain reaction (QPCR), n=6. Data were normalized with standard housekeeping genes for the array and with 18S for QPCR and then expressed as a fold change from control (unwounded/nonmigratory). * indicates a significant difference from control, P<0.05, 1-sample t test. **B**, Western blotting for Wnt2 protein in control and migrating (wounded, W) smooth muscle cells normalized with β-actin. * indicates a significant difference from control, P<0.05, 1-sample t test, n=4. **C**, Wounded vascular smooth muscle cells (VSMCs) were treated with recombinant Wnt2 protein or subjected to Wnt2 silencing with siRNA (Si) and the distance migrated measured. Controls were treated with Allstars siRNA controls. * indicates a significant difference from control, P<0.05, analysis of variance (ANOVA), and Student Newman Keuls post test, n=4. **D**, Wnt2-induced migration of cells subjected to siRNA for Frizzled 1 and 6, expressed as a % of the Allstars siRNA control. * indicates a significant difference from control, P<0.05, 1-sample t test, n=3.
Results

Wnt2 Was Upregulated in Migrating VSMCs In Vitro

Wnt mRNA levels during VSMC migration were assessed using an in vitro scratch wound assay with multiple wounds to stimulate migration of the VSMCs. mRNA was extracted from VSMCs and applied to a focused Wnt pathway microarray to assess whether the level of expression changed during migration. A significant increase was only observed in Wnt2 mRNA (Figure 1A), and this change was confirmed using quantitative polymerase chain reaction (Figure 1A). No significant change was seen in the mRNA levels of any other Wnts (see Table III in the online-only Data Supplement). It was observed by Western blotting (Figure 1B) and immunocytochemistry (Figure 1 in the online-only Data Supplement) that the increase in Wnt2 mRNA was translated into augmented Wnt2 protein levels in migrating VSMCs. Migrating VSMCs on the wound edge could be seen to express higher levels of Wnt2 protein than nonmigratory VSMCs further away from the wound edge (Figure 1 in the online-only Data Supplement).

Wnt2 Promoted VSMC Migration In Vitro

Addition of rWnt2 protein significantly increased VSMC migration in vitro, whereas knockdown of Wnt2 using silencing RNA (siRNA) inhibited migration (Figure 1C). When rWnt2 was added back to VSMCs subjected to Wnt2 knockdown, the inhibitory effect of Wnt2 siRNA was reversed (Figure 1C). rWnt2 also increased migration in the transwell assay (92.8±10.4 cells versus 47.3±5.8 in control, n=4; P<0.05 student t test). We previously showed that Fzds 1 and 6 are the predominant Fzds expressed in VSMCs. Knockdown of Fzdz6 using siRNA resulted in a significant reduction in migration, whereas Fzd1 knockdown had no significant effect on migration (Figure 1D).

Wnt2 Was Upregulated During Intimal Thickening

To corroborate these in vitro changes in a relevant in vivo model of intimal thickening, mice were subjected to left carotid artery ligation. Carotids were removed after 28 days and stained for the presence of Wnt2 using immunofluorescence (Figure 2A–2C). Elevated levels of Wnt2 protein were detected in the media, and the intima of ligated carotids was compared with the unligated control arteries (auto-fluorescence from red blood cells was observed in the nonimmune immunoglobulin-negative controls and was excluded from analysis of Wnt2, only intimal fluorescence and not luminal fluorescence was measured).

Wnt2 Promoted Intimal Thickening In Vivo

To determine whether Wnt2 regulated intimal thickening in vivo, Wnt2−/− mice were subjected to left carotid artery ligation. In unligated carotid arteries, Wnt2 protein was undetectable in both the Wnt2−/− and Wnt2+/+ mice (Figure II A and II B in the online-only Data Supplement). After carotid ligation, we observed reduced levels of Wnt2 protein in the ligated arteries of Wnt2−/− mice using immunofluorescence on the carotid arteries (Figure IIC in the online-only Data Supplement), compared with ligated arteries of control wild-type (Figure IID in the online-only Data Supplement); the amount of staining was quantified, showing that the amount of Wnt2 protein within the intima was significantly reduced after 3 and 28 days of carotid ligation (Figure IIE and IIF in the online-only Data Supplement). This reduction in Wnt2 was associated with attenuated intimal thickening in the Wnt2−/− mice (Figure 2D). No significant difference was observed in the percentage of proliferating cells (measured using bromodeoxyuridine staining) at either 3 or 28 days (Figure III in the online-only Data Supplement) in either the intima (A) or the media (B).

Wnt2 Affected WISP-1 Levels, Both In Vitro and In Vivo

Treatment of VSMCs with rWnt2 induced WISP-1 mRNA, whereas Wnt2 knockdown using siRNA resulted in a down-regulation of WISP-1 mRNA quantified with quantitative polymerase chain reaction (Figure 3A). However, treatment of VSMCs with rWnt4 did not induce WISP-1 mRNA (1±0 versus 0.93±0.05). Treatment with rWnt2 caused no significant change in any other downstream Wnt/β-catenin downstream targets that we analyzed (see Table IV in the online-only Data Supplement). Induction of WISP-1 by Wnt2 treatment was inhibited using the β-catenin inhibitor CCT03134-hydrobromide (Figure 3B). Knockdown of WISP-1 led to a significant reduction in migration in vitro, which was of a similar extent to that seen with knockdown of Wnt2 (Figure 3C). Simultaneous knockdown of both Wnt and WISP-1 did not produce an additive effect, suggesting that WISP-1 and Wnt2 are working in series rather than in parallel, with most likely WISP-1 downstream of Wnt2. Levels of Wnt2 and WISP-1
also appeared to be related in vivo, where levels of WISP-1 were significantly reduced in the intimal lesions of Wnt2+/− mice compared with the wild-type controls (Figure 3D).

Wnt2 Induced WISP-1 via β-Catenin
Migration of cells in response to rWnt2, but not rWISP-1, was inhibited by the β-catenin inhibitor, CCT03134-hydrobromide (Figure 3E and 3F). This indicated that Wnt2 acts via β-catenin in migrating cells but that WISP-1-induced migration is β-catenin-independent.

Wnt2 and WISP-1 Both Required Integrins
Cell migration in response to both rWnt2 (Figure 3E) and rWISP-1 (Figure 3F) and wounding were both inhibited by the integrin inhibitor, RGD peptide, but not by a control peptide, suggesting that integrins are downstream of both Wnt2 and WISP-1.

Wnt2 and WISP-1 Colocated Within Intimal Thickening
To investigate the association of Wnt2 with WISP-1, we examined whether these proteins were colocalated in mouse-ligated carotid arteries (Figure 4A–4D; Figure V in the online-only Data Supplement) and early neointimal thickenings (without foam cells and plaque formation) in human coronary arteries (Figure 4E and 4F) by staining for Wnt2 and WISP-1 using immunofluorescence. We observed that Wnt2 and WISP-1 proteins were indeed colocalated in both mouse and human arteries during intimal thickening. In human coronary arteries, the amount of Wnt2 and WISP-1 proteins were significantly
higher in arteries with early intimal thickenings compared with normal control undiseased arteries (Figure 4G). In addition, there was a significant correlation between the amount of Wnt2 and WISP-1 proteins in the vessels ($r=0.647$ and $r^2=0.419$, $n=13$; $P<0.05$ and $P<0.02$). There was no significant difference in expression of either Wnt2 or WISP-1 between the intima and the media.

**WISP-1 Promoted Intimal Thickening In Vivo**

Deficiency of the WISP-1 gene produced a significant reduction in the intima formation (Figure 5A). In contrast, using an adenovirus encoding WISP-1 protein, we increased plasma levels of WISP-1 protein at 3 and 7 days post infection (Figure IV in the online-only Data Supplement), and this resulted in a significant increase in intimal formation in the carotid artery after ligation (Figure 5B). This confirmed that WISP-1 promoted intimal thickening in the mouse in vivo. Sections were stained with in situ end labelling to identify apoptotic cells. Half of all sections examined had no apoptosis, whereas others had 1 or 2 apoptotic cells. No significant difference was seen in apoptosis rates between the control and WISP-1-deficient mice (data not shown). The percentage of proliferating cells (measured by proliferating cell nuclear antigen) was significantly reduced in the vessels of WISP-1-deficient animals compared with the wild-type controls in both the media and the intima of the vessels (Figure 5C).

**Discussion**

This study demonstrates that Wnt2 and WISP-1 are upregulated in migrating VSMCs in vitro and in intimal cells in vivo. We have also shown that modulating the levels of either Wnt2 or WISP-1 directly affects VSMC migration in vitro and intimal thickening in vivo. We have shown that Wnt2 upregulates WISP-1 and promotes VSMC migration, via $\beta$-catenin-dependent signaling. Additionally, we demonstrated that WISP-1 and Wnt2 promote VSMC migration via interaction with integrins. Consequently, we suggest that Wnt2 and WISP-1 are novel regulators of intimal thickening and, thereby, potential targets for reducing intimal thickening in patients after coronary artery bypass grafts and stent implantation.

Although this is the first study to demonstrate the involvement of Wnt2 in adult VSMC migration and intimal thickening in vivo, Wnt2 does have a well-characterized role in cell migration in other scenarios such as during embryonic development; for example, mouse embryo VSMCs also express Wnt2 as they migrate to form the heart, vasculature, and lungs.24 Wnt2 also has a role in cell migration in various cancers. Wnt2 levels were linked to metastasis in pancreatic cancer,25 whereas inhibition of Wnt2 in mouse nonsmall cell lung cancer cells resulted in reduced tumor cell growth.26 In scleroderma patients, Wnt2 was found to be elevated and associated with activation of $\beta$-catenin,27 as we have shown in our study. Therefore, it is possible that the disease states of intimal thickening or cancers lead to a reawakening of this embryonic pathway, which is usually quiescent in mature cells, leading to Wnt2-dependent migration of cells.

Other members of the Wnt family have also been implicated in intimal thickening, both by our own group and others. For example, Wu et al10 reported that Wnt3a stimulated migration, adhesion, and proliferation of rat VSMCs in culture. Previous work in our group using a similar approach to this study showed that Wnt4 was increased during VSMC proliferation and that modulation of Wnt4 led to changes in
Moreover, we demonstrated that although Wnt2 mRNA, as well as Wnt4 mRNA, was increased in proliferating cells, this only translated into an increase in protein expression for Wnt4, and not for Wnt2. We also tested addition of both rWnt2 and rWnt4 proteins to VSMCs in culture to assess whether they could directly affect proliferation. We observed that only Wnt4 induced VSMC proliferation and Wnt2 had no significant effect on proliferation. Additionally, in our previous study, we demonstrated that Wnt4 protein levels were induced during intimal thickening in vivo, and intimal thickening induced by carotid artery ligation was significantly reduced in Wnt4+/− mice. A comparison of these 2 studies in our group is summarized in Figure 6 and suggests that regulation of VSMC migration and proliferation via β-catenin-dependant signaling are controlled by different Wnt protein members, with Wnt2 controlling migration predominantly via Fzd6 (although the involvement of Fzd1 cannot be ruled out) and Wnt4 promoting proliferation via Fzd1.

We investigated the mechanism of action and discovered that Wnt2 promoted migration via the β-catenin-dependent canonical signaling pathway, which increased WISP-1 levels. Knockdown of either Wnt2 or WISP-1 in vitro using siRNA resulted in a similar reduction in cell migration. However, when both were added, this offered no increase in effect. This suggested that they were acting in series rather than in parallel. To investigate this further, we looked at the effect of adding rWnt2 and found that this significantly increased the levels of WISP-1 in vitro, in contrast to Wnt4 which did not affect WISP-1 levels. We also showed that WISP-1 was reduced in Wnt2 knockout mouse–ligated carotids in vivo. This shows that WISP-1 can be regulated directly by Wnt2, and so WISP-1 is most likely to be downstream of Wnt2. Wnt2 and WISP-1 were also found to be collocated in ligated mouse carotid arteries, confirming that activation of WISP-1 by Wnt2 is physiologically relevant because WISP-1 is found alongside Wnt2.

WISP-1 has been shown previously by our own group19 to be important in VSMC survival and by Liu et al23 to promote both VSMC migration and proliferation in cultured rat VSMCs. Marchand et al confirmed the presence of WISP-1 in human mammary arterial cells,28 whereas Reddy et al22 showed than in human VSMCs, interleukin-18 increased survival and proliferation, via T-cell factor/LEF and WISP-1. WISP-1 then acted via activator protein-1 (AP-1) to upregulate matrix-degrading metalloproteinase (MMP)-2, -9 and -14 mRNA. Our study therefore corroborates the findings of Liu et al,23 showing similar mechanisms in both rat and mouse VSMCs in culture; however, we do not see the upregulation of MMP-2, -9, and -14 by WISP-1. This is the first study to show that WISP-1 leads to intimal thickening in vivo and the first to examine the mechanism of action. The interplay between Wnt5a and Wnt2, which are both able to act via β-catenin to activate WISP-1 (Figure 6), reveals the complexity of how various Wnts may act synergistically by overlapping pathways to produce different effects. More work is needed in this area to define exactly how these pathways may act either separately or together in different disease models.

As with Wnt2, WISP-1 has been studied in cancer cells, where it has been shown to be a chemoattractant,20 encourage metastasis,29 increase invasiveness, and decrease chances of survival.30–32 WISP-1 has also been shown in human cancer cells to be upregulated compared with healthy cells31 and to stimulate migration via NFκB activation of MMP-2. Our
WISP-1 may provide a more suitable target for drug intervention compared with Wnt2 because the effects may be more specific further downstream in the pathway, and as we have shown WISP-1 promotes both migration and proliferation in this model, so could be a preferable candidate. However, as noted previously, WISP-1 affects other cell types; so ideally, a tissue-targeted WISP-1 inhibitor would be preferable, such as stent coating or ex vivo vein graft application before bypass surgery. Targeting migration and proliferation of VSMCs from the media of the vessel into the intima should retard the formation of a neointima after therapies, such as coronary artery bypass grafts and stent implantation, and therefore increase the longevity of these treatments.

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Disclosures
None.

References

Figure 6. Summary of the roles of Wnt2, Wnt4, and Wnt5a in the regulation of vascular smooth muscle cell (VSMC) behavior. Arterial injury leads to the increased expression of Wnts 2 and 4. Wnt2 activates beta-catenin/T-cell factor (TCF) signaling predominantly via Fzd6, leading to the induction of Wnt1-like inducing signaling pathway protein-1 (WISP-1), which promotes VSMC migration via integrins. Wnt4 activates beta-catenin/T-cell factor signaling via Fzd1, leading to the induction of cyclin D1, which promotes VSMC proliferation. In the presence of oxidative stress, Wnt5a activates beta-catenin/CREB signaling, leading to the induction of WISP-1, which promotes VSMC survival, whereas this is via integrins is unknown. Summarized from data within this article, as well as previous work by Tsauosi et al26 and Mill et al.19 Fzd indicates frizzled receptor.

study shows that Wnt2 acts via beta-catenin to upregulate WISP-1, which then acts through integrins to increase cell migration. This confirms that a similar pathway is utilized by WISP-1 in VSMCs as in other cell types. For example, in human oral squamous cell carcinoma cells, WISP-1 acts through AP-1 and the alpha3/beta3 integrin receptor, whereas in osteogenesis, WISP-1 acts via alpha5/beta1 integrin.20

To establish whether this Wnt2-WISP-1 association was present in human intimal lesions as well as in mice, we stained sections from human coronary arteries with early-stage intimal thickening (without atherosclerotic plaques) to locate Wnt2 and WISP-1. We found that Wnt2 and WISP-1 was colocalized and increased in diseased coronary vessels compared with control vessels, and the increase in the levels of Wnt2 and WISP-1 in diseased vessels were correlated. This suggested that as Wnt2 increased, this led to an associated increase in WISP-1 as we observed in vitro.

In summary, we have found that Wnt2 is upregulated in migrating cells both in vitro and in vivo and that Wnt2 promotes VSMC migration in vitro and intimal thickening in vivo. We identified that downstream, Wnt2 activates beta-catenin, leading to an upregulation of WISP-1, which like Wnt2 also has the ability to stimulate migration of cells in vitro and intimal thickening in vivo, via integrins. Although both Wnt2 and WISP-1 have been separately implicated to be involved in cell migration in other cell types, the action of Wnt2 promoting migration via WISP-1 had not been previously reported.


**Highlights**

- Wnt2 is upregulated in migrating cells, and it promoted vascular smooth muscle cell migration in vitro and intimal thickening in vivo.
- Wnt2-induced migration is dependent on β-catenin activation, leading to an upregulation of Wnt1-inducible signaling pathway protein-1, which in turn promoted vascular smooth muscle cell migration via integrins.
- Wnt1-inducible signaling pathway protein-1 promoted intimal thickening in vivo.
- This is the first demonstration that Wnt2 promoted migration via Wnt1-inducible signaling pathway protein-1.
- Wnt1-inducible signaling pathway protein-1 may be a suitable target for reducing intimal thickening after therapies such as coronary artery bypass grafts and stent implantation and, therefore, increase the longevity of these treatments.
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Materials and Methods

Animals

The housing and care of all the animals and 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. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Transgenic mice. Wnt2 heterozygous knockout mice (Wnt2+/−) on a pure CD-1 background from more than 10 backcrosses, were kindly provided by Dr Richard Lang (Cincinnati Children’s Hospital, Cincinnati, OH). WISP-1 homozygous knockout mice (WISP-1−/−) on a pure C57/bl6J background from more than 10 backcrosses, were kindly provided by Dr Marian Young (NIH, Bethesda, Maryland). Mice were bred within the University of Bristol animal unit to provide sufficient numbers of male Wnt2+/− or WISP-1−/− mice and their wild type littermate controls (Wnt2+/+ or WISP-1+/+). Homozygous Wnt2−/− animals could not be used as a result of perinatal death due to placental defects 1.

Elevation of plasma WISP-1. To overexpress WISP-1, 30 mice were anaesthetised using inhaled isoflurane and warmed on a heated plate, to increase the prominence of the tail vein, before receiving a tail vein injection of RAd66 empty virus (8 x 10^{10} particles/mouse1.1 x 10^{9} pfu/mouse) as previously described 2-4. Mice were allowed to recover on a heat mat before returning them to their cage. Four hours later the procedure was repeated and mice were given a further injection of either RAd66 or RAdWISP-1 (15 mice in each group, 2.2 x 10^{8} pfu/mouse). The RAdWISP-1 encoding virus was kindly supplied by Marian Young and was previously described 5.

Carotid ligation. Left carotid artery ligations were performed to induce intimal thickening, as described previously 6. Thirty mice were anaesthetised using inhaled isoflurane with oxygen and intraperitoneal injection of buprenorphine hydrochloride (1.5μg) and prepared for sterile surgery. The left common carotid artery was dissected and ligated just proximal to the carotid bifurcation to induce intimal formation. Mice also received an implanted minipump (Alzet 2004) in the subcutaneous space on their back containing BrdU (25mg/ml) to enable identification of proliferating cells.

Histochemistry, Immunohistochemistry and Immunofluorescence

Carotid arteries were removed either 3 or 21 days following ligation surgery, formalin fixed for 24 hours and embedded longitudinally or transversely in agar before processing and embedding in paraffin wax. 3 μm sections were cut onto Superfrost slides for elastin van Gieson (EVG) staining and on to Superfrost Plus slides for immunohistochemistry and immunofluorescence. EVG stained sections were used for visualisation and measurement of the vessel and lesion by image analysis (Image Pro). Immunohistochemistry was performed to visualise BrdU incorporation (Sigma: B2531; 8.6μg/ml) and PCNA staining (Abcam 18197; 1μg/ml); and immunofluorescence was utilised to localise Wnt2 (Santa Cruz: SC5208; 1.25μg/ml), WISP-1 (R&D: AF1680; 1μg/ml), and cleaved caspase 3 (R&D Systems: MAB835; 10μg/ml). Non-immune IgG of the same species as the primary was used as negative control in all protocols at the same concentration as the primary antibody to demonstrate the specificity of the protocol. Apoptosis was measured using ISEL as
described previously \(^7\). To investigate the association of Wnt2 with WISP-1 during intimal thickening in humans, we examined whether these proteins were co-located in early neointimal thickenings (without foam cells and plaque formation) in human coronary arteries by staining for Wnt2 and WISP-1 using immunofluorescence. Human right coronary arteries were removed from hearts collected for valve retrieval from deceased donors (Local ethical approval REC#08/H0107/48).

**Tissue culture and in vitro migration assays**

Primary cultured VSMCs were obtained by culturing explants from aortae from C57/bl6 mice as described previously \(^6\). Cells were used from passage 3-9 and at least 3 different preparations were used for each experiment. VSMCs were quiesced for 24 hours at 37°C and 5% CO\(_2\) in serum free medium (SFM) before either treatment with 500ng/ml recombinant Wnt2 or being subjected to either a single or multiple scratch wound assay as described previously \(^8\), in SFM supplemented with 20ng/ml PDGF and 2\(\mu\)M hydroxyurea (to inhibit proliferation). To quantify migration, VSMCs were grown in a 24 well plate and subjected to two perpendicular wounds to form a cross. Wells were photographed immediately following wounding and then again 24 hours after wounding to measure the distance migrated by the VSMCs. For measurement of mRNA or protein, VSMCs were cultured in a 6 well plate and subjected to 20 wounds in two directions to form a grid pattern. VSMCs were left to migrate for either 6 hours (for mRNA) or 24 hours (for protein) before lysing the VSMCs in the relevant lysis buffer. In some experiments VSMCs were cultured in the presence of 500 ng/ml recombinant Wnt2, or recombinant WISP-1 and the inhibitors CCT03134-hydrobromide (Tocris, 20nM) and RGD peptide (Peptides International, 250\(\mu\)M) were also added. Cells were also seeded into a transwell cell culture inserts with 8\(\mu\)m pores (Millipore) and the number of VSMCs that migrated across the membrane in response to Wnt2 after 24 hours was measured as previously described \(^8\).

**RNA extraction and reverse transcription**

RNA was extracted from VSMCs using the RNAeasy kit (Qiagen) according to the manufacturer's instructions. RNA was then processed for Q-PCR analysis using reverse transcription using the RT-PCR Kit (Qiagen) or for focussed microarray analysis as described below.

**Focussed Microarray Analysis**

RNA was directly labelled with Biotin-UTP (Roche) and amplified using the TrueLabel AMP 2.0 Kit (SuperArray: GA-030). The Biotin-UTP labelled RNA was cleaned using the RNAeasy kit (Qiagen). A mouse Wnt pathway microarray (SuperArray: OMM-043-4) was left rotating overnight at 37°C in a hybridising oven with 1\(\mu\)g of cleaned, purified and labelled mRNA in 2ml of hybridisation solution. Hybridised oligonucleotides were detected using an enhanced luminescence kit (SuperArray: D-01,) according to the instructions, after washing twice for 30 minutes with SSC containing buffers (Sigma: 85635) at room temperature. The GEAnalysis software (SuperArray: GA-021) was used for quantification following normalisation using housekeeping genes and concentration controls (GAPDH, BAS2C, Ppia, Hspcb, B2m, Rps27a, and two blank controls) on the array.

**Quantitative PCR (Q-PCR)**
cDNA was subjected to Q-PCR for Wnt pathway genes using specific primers (see Supplementary Table I) as described previously 6. Results were normalised to 18s ribosomal RNA.

**Western Blotting**

SDS lysis buffer was used to extract VSMC proteins and total protein concentration was measured using a bixinchroninic acid protein assay kit (Pierce). Equal protein concentrations were loaded on 4-12% gradient gels (Novex Bis-Tris gel) and transferred on to 0.2µm nitrocellulose membranes. Blots were blocked with 5% (w/v) skimmed milk powder and incubated overnight at 4°C with anti-Wnt2 antibody (Abcam: ab27794; 4µg/ml) diluted in starting block (Pierce). Primary antibodies were detected using HRP conjugated secondary antibodies and enhanced chemiluminescence reagent (ECL, Amersham). Optical density of bands was quantified using densitometry (Quantity One) and normalised to a β-actin loading control (Sigma: A5316; 110ng/ml).

**Immunocytochemistry**

Following the *in vitro* migration assay VSMCs were fixed in 3% (w/v) paraformaldehyde for 10 minutes, washed with 0.1% (v/v) triton in PBS and blocked with 20% (v/v) rabbit serum for 1 hour. VSMCs were then and treated with Wnt2 antibody (Santa Cruz: SC5208; 5µg/ml) or IgG used at 200 µg/ml in 1% (w/v) BSA in PBS overnight at 4°C. The next day VSMCs were washed and treated with rabbit anti goat Alexa flour 488 at 1:200 for 45 minutes, wrapped in foil before washing and mounting with Prolong gold with 4',6-diamidino-2-phenylindole (DAPI).

**Silencing RNA (SiRNA)**

SiRNA oligonucleotides for Wnt2, WISP-1 and Frizzleds 1 and 6 (Supplementary Table II) were purchased from Qiagen along with Allstars scrambled negative control (Qiagen SI03650318) and introduced into VSMCs using an AMAXA nucleofector device and VSMC kit (API-1004, Lonza) according to the manufacturer’s instructions. VSMCs (1 x 10^6) were subjected to nucleofection with 250pmol of Wnt2, WISP-1, Fzds or Allstars control siRNAs using program A33 and analysed after 24 hours. The knockdown efficiency was quantified using Q-PCR of target mRNA and found to be significantly reduced by <50%, as previously published for Wnt2 6, WISP-1 9 and Fzds 6.

**Statistics**

Results are expressed as mean±SEM. All data was checked for normal distribution and since this was the case for all data, they were analysed by student t-test for comparison of two groups, one-sample t-test for one group analyses and two-way ANOVA with Kruskal Wallis post-test for multiple comparisons with more than two groups. A significant difference was accepted when p<0.05.
References


Supplementary Figure I:
Wnt2 was increased in migrating smooth muscle cells *in vitro*.
Immunofluorescence for Wnt2 protein (green) in VSMCs 24 hours after subjecting to wound injury (A). Red line indicates the position of the wound.
(B) Migrating cells on edge of wound, (C) Non-migratory cells, (D) Non-immune IgG negative control. Nuclei are blue (DAPI). Scale bar represents 200 µm in (A) and 20 µm in (B) and applies to panels B-D.
Supplementary Figure II:
Wnt2 protein was reduced in Wnt2+-/ mice.
Immunofluorescence for Wnt2 protein in control non-ligated carotid arteries (A and B) and ligated carotid arteries (C and D) 28 days following ligation. Wnt2+/-(A and C) and wild type mice (B and D: Wnt2+++) dotted lines show the intima/media boundaries, i = intima, m = media.
Quantification of Wnt2 protein in media of ligated carotid arteries after 3 days (E) and in intima and media after 28 days (F) in Wnt2+-/ and control Wnt2+++/ mice, n=5, p<0.05 using t-test and Mann-Whitney test. Scale bar = 50 μm.
Supplementary Figure III:
Proliferation in ligated arteries.
The percentage of proliferating cells in the intima (A) and media (B) was quantified by BrdU incorporation and compared in Wnt2^{+/+} and Wnt2^{+/−} mice at 3 days and 28 days after ligation, n=6.
Supplementary Figure IV:
Treatment with WISP-1 virus increased plasma WISP-1.
Plasma WISP-1 was quantified after 3, 7 and 28 days using ELISA.
* indicates a significant difference from control (n=4, p<0.05, Mann-Whitney test).
Supplementary Figure V:
Wnt2 and WISP-1 are upregulated at 7 days
Representative images of immunofluorescence for Wnt2 (A) and WISP-1 (C) proteins (green) in serial sections of carotid arteries 7 days after ligation. Non-immune immunoglobulin controls are shown in (B) and (D), respectively. Nuclei are stained blue with DAPI.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Source</th>
<th>Primer sequence Forward</th>
<th>Primer sequence Reverse</th>
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<tr>
<td>Brachyury</td>
<td>Sigma</td>
<td>GCTTCAAGGAGCTAACTAACGAG</td>
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<td>Quantitect QT01061571</td>
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**Supplementary Table II: SiRNA probes used for knockdown of WISP-1 and Wnt2**

<table>
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<th>Catalogue No</th>
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Supplementary Table III: Fold change of Wnt pathway genes quantified by microarrays.

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<th>Wnt pathway member</th>
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<th>One sample t-test vs. 1 p value</th>
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<td>Wnt1</td>
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<tr>
<td>Wnt2</td>
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<td>p&lt;0.05</td>
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<tr>
<td>Wnt2b</td>
<td>2.36±1.09</td>
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<tr>
<td>Wnt3</td>
<td>0.64±1.10</td>
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<td>Wnt3a</td>
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<td>Wnt4</td>
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<td>Wnt6</td>
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<tr>
<td>Wnt16</td>
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Supplementary Table IV: Fold change of potential Wnt2 responsive downstream genes following addition of recombinant Wnt2: mRNA quantified by Q-PCR.

<table>
<thead>
<tr>
<th>Wnt2 downstream target</th>
<th>Average fold change ± sem (n=3-6)</th>
<th>One sample t-test vs. 1</th>
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<td>Senp2</td>
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<tr>
<td>Sox17</td>
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<tr>
<td>WISP-1</td>
<td>1.67±0.14</td>
<td>*P&lt;0.05</td>
</tr>
<tr>
<td>WISP-2</td>
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</tbody>
</table>
Arterial Injury

Wnt2

Fzd

β-catenin/TCF

WISP-1

Integrins

VSMC MIGRATION