Wnt2 and WISP-1/CCN4 induce intimal thickening via promotion of smooth muscle cell migration

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Abstract:

Objective: Increased vascular smooth muscle cell (VSMC) migration leads to intimal thickening in coronary artery restenosis and vein graft failure as well as acting as a soil for atherosclerosis. 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 (rWnt2) *in vitro*. Addition of rWnt2 protein increased Wnt1-inducible-signalling pathway protein-1 (WISP-1) mRNA by ~1.7-fold, via β-catenin/TCF signalling, while siRNA knockdown of Wnt-2 reduced WISP-1 mRNA by ~65%. Treatment with rWISP-1, significantly increased VSMC migration by ~1.5-fold, while WISP-1 siRNA 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 co-located in human coronary arteries with intimal thickening. Reduced Wnt2 and WISP-1 levels in mouse carotid arteries from $Wnt2^{+/-}$ and $WISP\text{-}1^{-/-}$ 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 to 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.

Keywords:
Wnt2, WISP-1/CCN4, intimal thickening, restenosis, smooth muscle cell migration.

Non-standard Abbreviations and Acronyms	
BrdU	bromodeoxyuridine
CABG	Coronary artery bypass graft
Fzd	frizzled receptor
LRP	low density lipoprotein receptor
PDGF	platelet derived growth factor
Q-PCR	quantitative PCR
SFM	serum free medium
SiRNA	silencing RNA
VSMC	vascular smooth muscle cell
WISP-1	Wnt-1 inducible signalling pathway protein 1

Introduction

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 following 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 since 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 anti-migratory approaches may be equally desirable.

The Wnt signalling pathway has been implicated in intimal thickening and VSMC migration and proliferation by both our own group [4-6] 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 signalling. The Wnt ligands bind to Frizzled (Fzd) receptors and their co-receptors the LDL related proteins 5/6 (LRP5/6) to activate downstream pathways [12]. Upregulation of the Wnt pathway has been well established in many cancer subtypes [13] and in the development of osteoarthritis [14], however their role in atherosclerosis and restenosis is less well established. Evidence shows that the Wnt pathway, via the receptor LRP6, regulates cell proliferation and survival in VSMCs [7], while 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 was not translated into protein and recombinant Wnt2 protein did not increase VSMC proliferation *in vitro* [5]. 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 ILK regulation of β1-integrin [10]. 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 signalling pathway protein-1 (WISP-1/CCN4) [16]. WISP-1 is a member of the CCN family of genes [17] which regulates fibrosis and wound healing [18], angiogenesis [19], osteogenesis [20] and cancer [21]. In cultured VSMCs WISP-1 promotes survival [19], proliferation [22], and migration through integrin α5β1 [23].

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 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.

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 focussed 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 Q-PCR (Figure 1A). No significant change was seen the mRNA levels of any other Wnts (see Supplementary Table III). It was observed by Western blotting (Figure 1B) and immunocytochemistry (Supplementary Figure I) 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 non-migratory VSMCs further away from the wound edge (Supplementary Figure 1).

Wnt2 promoted VSMC migration *in vitro*

Addition of rWnt2 protein significantly increased VSMC migration *in vitro*, while knockdown of Wnt2 using 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). Recombinant Wnt2 also increased migration in the transwell assay (92.8±10.4 cells vs. 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 [5]. Knockdown of Fzd6 using siRNA resulted in a significant reduction in migration, while 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-C). Elevated levels of Wnt2 protein were detected in the media and the intima of ligated carotids compared to the unligated control arteries (autofluorescence from red blood cells was observed in the non-immune 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*

In order 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 (Supplementary Figure IIA and B). Following carotid ligation we observed reduced levels of Wnt2 protein in the ligated arteries of $Wnt2^{+/-}$ mice using immunofluorescence on the carotid arteries (Supplementary Figure IIC), compared to ligated arteries of control wild type (Supplementary Figure IID), 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 (Supplementary Figure IIE and F). 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 BrdU staining) at either 3 or 28 days (Supplementary Figure III) 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, while Wnt2 knockdown using siRNA resulted in a down-regulation of WISP-1 mRNA quantified with Q-PCR (Figure 3A). However, treatment of VSMCs with rWnt4 did not induce WISP-1 mRNA (1±) vs. 0.93±0.05). Treatment with rWnt2 caused no significant change in any other downstream Wnt/β-catenin downstream targets that we analysed (see Supplementary Table IV). 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 Wnt2 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 to 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 F). 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 co-located within intimal thickenings

To investigate the association of Wnt2 with WISP-1, we examined whether these proteins were co-located in mouse ligated carotid arteries (Figure 4A-D and Supplementary Figure V) and early neointimal thickenings (without foam cells and plaque formation) in human coronary arteries (Figure 4E-F) by staining for Wnt2 and WISP-1 using immunofluorescence. We observed that Wnt2 and WISP-1 proteins were indeed co-located 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 to 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 ($r=0.647$, r2 0.419, $n=13$, $p<0.05$, $p<0.02$). 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 (Supplementary Figure IV) and this resulted in a significant increase in intimal formation in the carotid artery following ligation (Figure 5B). This confirmed that WISP-1 promoted intimal thickening in the mouse *in vivo.* Sections were stained with ISEL to identify apoptotic cells. Half of all sections examined had no apoptosis, while others had one or two apoptotic cells. No significant difference in apoptosis rates between the control and WISP-1 deficient mice (data not shown). The percentage of proliferating cells (measured by PCNA) was significantly reduced in the vessels of WISP-1 deficient animals compared to the wildtype 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 β-catenin dependent signalling. Additionally, we demonstrated that WISP-1 and Wnt2 promote VSMC migration via interaction with integrins. Consequently we suggest Wnt2 and WISP-1 are novel regulators of intimal thickening and thereby potential targets for reducing intimal thickening in patients following coronary artery bypass grafts (CABG) 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 characterised 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], while inhibition of Wnt2 in mouse non-small cell lung cancer cells resulted in reduced tumour cell growth [26]. In scleroderma patients Wnt2 was found to be elevated and associated with activation of β-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 al.* [10] 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 cell proliferation and intimal thickening [5]. 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 recombinant Wnt2 and Wnt4 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 two studies in our group is summarised in Figure 6 and suggests that regulation of VSMC migration and proliferation via β-catenin dependant signalling, are controlled by different Wnt protein members, with Wnt2 controlling migration predominantly via Fzd 6 (although the involvement of Fzd 1 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 signalling 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 recombinant Wnt2 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 co-located in ligated mouse carotid arteries, confirming that activation of WISP-1 by Wnt2 is physiologically relevant as WISP-1 is found alongside Wnt2.

WISP-1 has been shown previously by our own group [19] to be important in VSMC survival and by Liu *et al.* [23] 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], while Reddy *et al.* [22] showed than in human VSMCs IL-18 increased survival and proliferation, via TCF/LEF and WISP-1. WISP-1 then acted via AP-1 to upregulate MMP2, 9 and 14 mRNA. Our study therefore corroborates the findings of Liu and colleagues [23], showing similar mechanisms in both rat and mouse VSMCs in culture, however we do not see the upregulation of MMP2, 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 to healthy cells [33] and to stimulate migration via NFκB activation of MMP2. Our study shows that Wnt2 acts via β-catenin to upregulate WISP-1, which then acts through integrins to increase cell migration. This confirms that a similar pathway is utilised 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 αvβ3 integrin receptor [30], while in osteogenesis WISP-1 acts via α5β1 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 co-located and increased in diseased coronary

vessels compared to 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 promoted VSMC migration *in vitro* and intimal thickening *in vivo.* We identified that downstream, Wnt2 activates β-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 both 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. WISP-1 may provide a more suitable target for drug intervention compared to Wnt2 as 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 effects other cell types; so ideally a tissue targeted WISP-1 inhibitor would be preferable, such as stent-coating or *ex vivo* vein graft application prior to 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 CABG and stent implantation and therefore increase the longevity of these treatments.

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Disclosures

None.

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Highlights

- Wnt2 is upregulated in migrating cells and promoted VSMC migration *in vitro*, and intimal thickening *in vivo.*
- Wnt2 induced migration is dependent on β-catenin activation, leading to an upregulation of WISP-1, which in turn promoted VSMC migration via integrins.
- WISP-1 promoted intimal thickening *in vivo*.
- This is the first demonstration that Wnt2 promoted migration via WISP-1.
- WISP-1 may be a suitable target for reducing intimal thickening after therapies such as CABG and stent implantation and therefore increase the longevity of these treatments.

Figure Legends

Figure 1: Wnt2 was upregulated in migrating smooth muscle cells *in vitro.*

Wnt2 mRNA was quantified by oligonucleotide array, n=3 and quantitative PCR (Q-PCR), n=6 (A). Data was normalised with standard housekeeping genes for the array and with 18S for Q-PCR and then expressed as a fold change from control (unwounded/non-migratory). * indicates a significant difference from control, $p<0.05$, one-sample t-test. (B) Western blotting for Wnt2 protein in control (C) and migrating (wounded, W) smooth muscle cells normalised with b-actin. * indicates a significant difference from control, $p<0.05$, one-sample t-test, n=4. (C) Wounded 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, $p<0.05$, ANOVA and Student Newman Keul's 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, $p<0.05$, one-sample t-test, n=3.

Figure 2: Wnt2 promoted intimal thickening.

Immunofluorescence for Wnt2 protein in carotid arteries 28 days after ligation and control unligated control arteries. (A) Ligated left carotid artery with intimal thickening at 28 days after ligation. (B) Unligated control carotid artery. (C) Non-immune IgG negative control (ligated artery). Wnt2 protein (green), nuclei blue (DAPI). Red dotted line indicates the intimal:medial boundary. (D) Intima area in wild type ($Wnt2^{+/+}$) and Wnt2 heterozygous knockout mice ($Wnt2^{+/-}$), * indicates $p<0.05$, t-test n=14 per group. Images are EVG staining for elastin in longitudinal sections. Scale bar represents 50 μm in A, B and C and 100 μm in D.

Figure 3: WISP-1 was induced by Wnt2 and WISP-1 promoted cell migration.

(A) Relative expression of WISP-1 mRNA in VSMCs with and without Wnt2 protein treatment, n=4 and after silencing of Wnt2 with siRNA (Si), n=5. (B) WISP-1 mRNA following treatment with rWnt2 and CCT, n=3. (C) Migration of VSMCs after silencing Wnt2 and/or WISP-1 using scratch wound assay, n=5. (D) The percentage of WISP-1 positive intimal cells in wild type ($Wnt2^{+/+}$) and $Wnt2^{+/-}$ mouse carotid arteries 28 days after ligation, n=7. Migration of cells following treatment with either rWnt2 (E) or rWISP-1 (F), with or without

CCT or RGD peptide or control peptide, n=4. * indicates significant difference vs. control P<0.05 $ indicates significant difference from rWnt2/rWISP-1 treatment, using either t-test or ANOVA as applicable.

Figure 4: Colocation of Wnt2 and WISP-1 in mouse intimal lesions and human coronary artery disease.

Immunofluorescence for Wnt2 (A: green) and WISP-1 (B: red) proteins and merged images (C: yellow). Non-immune IgG negative control (D). White dotted line indicates the intima:media boundary. Scale bar in (A) represents 50 μm and applies to panels A-D. Human early coronary lesions were subjected to immunofluorescence for Wnt2 protein (E: green) and WISP-1 protein (F: red), nuclei are blue (DAPI) in human coronary artery with intima. Scale bar in (E) represents 50 μm and applies to panels E and F. Quantification of intimal and medial Wnt2 and WISP-1 proteins in control undiseased coronary arteries and coronary arteries with early intimal thickening (G). * indicates significant difference from controls (ANOVA and Kruskal Wallis post test, n=5 controls, n=5 with intimal thickening, p<0.05).

Figure 5: WISP-1 promoted intimal thickening.
(A) Intima area within carotid arteries 28 days after ligation in wild type controls and WISP-1$^{-/-}$ mice, n=12. (B) Intima area within carotid arteries 28 days after ligation in mice infected with control adenovirus and adenovirus to overexpress WISP-1, n=10. Scale bars represents 100 μm. * indicates significant difference vs. control P<0.05. (C) Percentage of proliferating cells in the intima and media measured by PCNA staining * indicates significant difference vs. control P<0.05.

Figure 6: Summary of the roles of Wnt2, Wnt4 and Wnt5a in the regulation of VSMC behaviour.

Arterial injury leads to the increased expression of Wnts 2 and 4. Wnt2 activates β-catenin/TCF signalling predominantly via Fzd6, leading to the induction of WISP-1 which promotes VSMC migration via integrins. Wnt4 activates β-catenin/TCF signalling via Fzd1, leading to the induction of cyclin D1 which promotes VSMC proliferation. In the presence of oxidative stress Wnt5a activates β-catenin/CREB signalling, leading to the induction of WISP-1 which promotes VSMC survival, whether this is via integrins is unknown. Summarised from data within this paper as well as previous work by Tsaousi *et al* and Mill *et al* [5 & 19].

Figure 1:

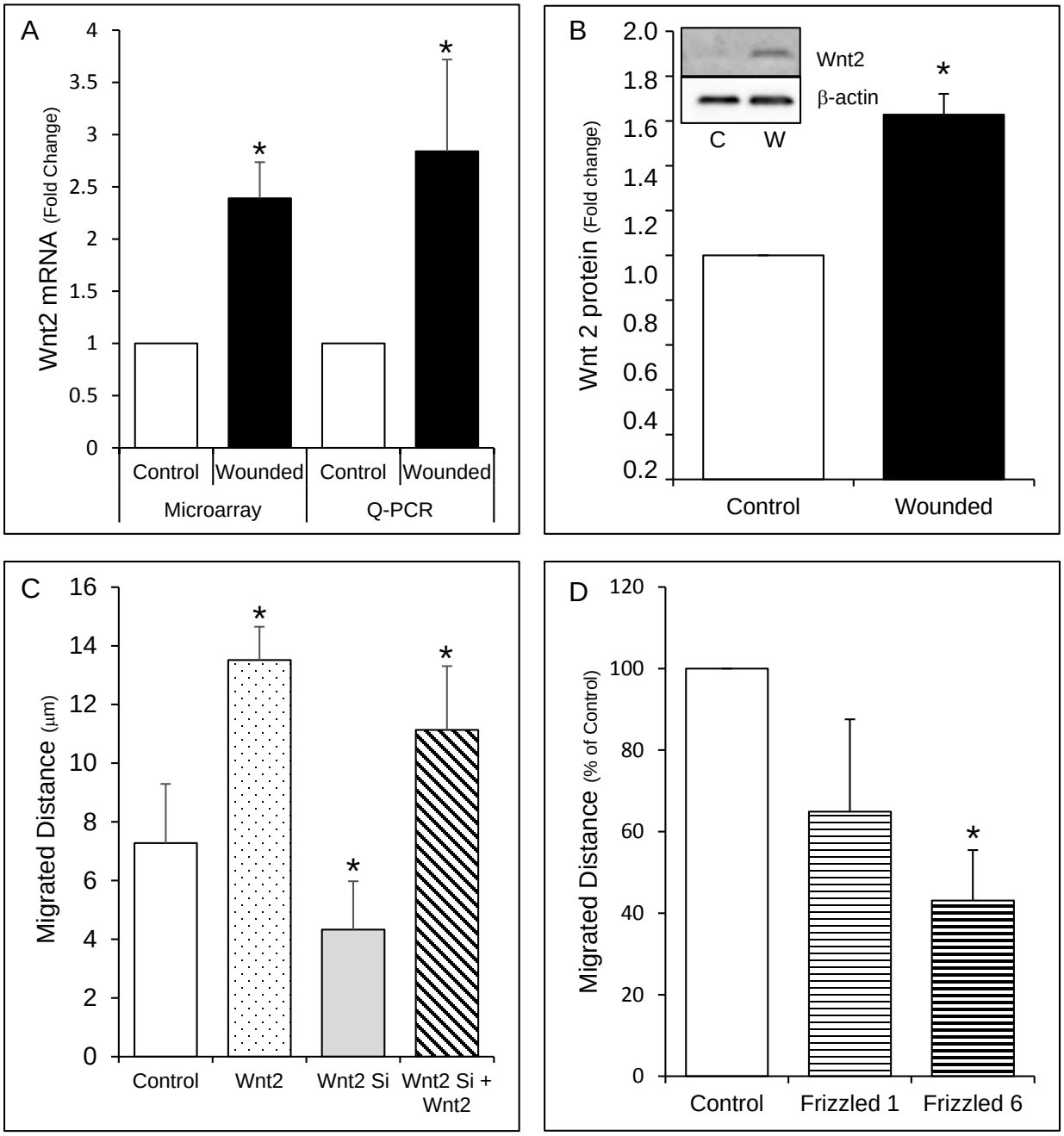

A
Wnt2 mRNA (Fold Change)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
Control
Wounded
Control
Wounded
Microarray
Q-PCR
B
Wnt 2 protein (Fold change)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Wnt2
β-actin
C
W
*
Control
Wounded
C
Migrated Distance (μm)
16
14
12
10
8
6
4
2
0
*
*
*
Control
Wnt2
Wnt2 Si
Wnt2 Si + Wnt2
D
Migrated Distance (% of Control)
120
100
80
60
40
20
0
*
Control
Frizzled 1
Frizzled 6

Figure 2:

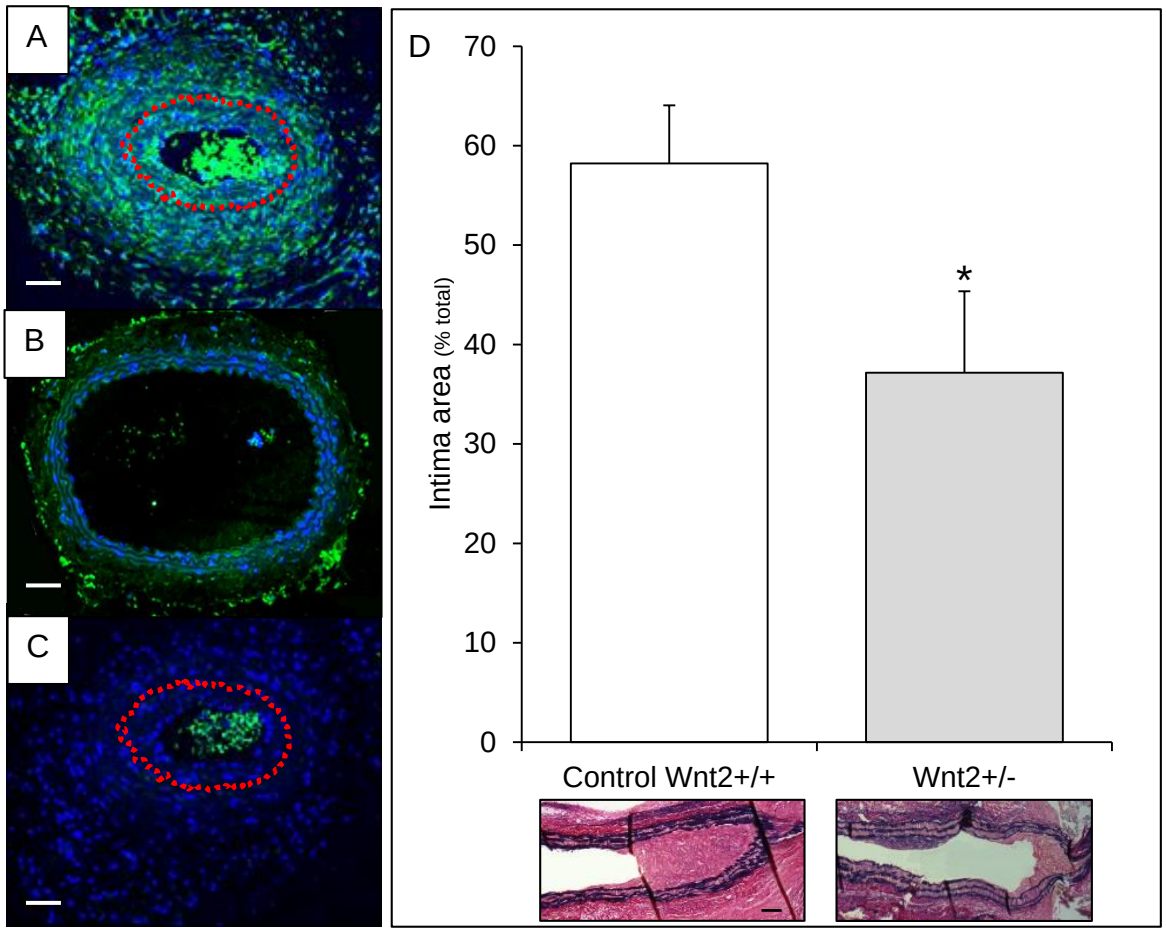


Figure 3:

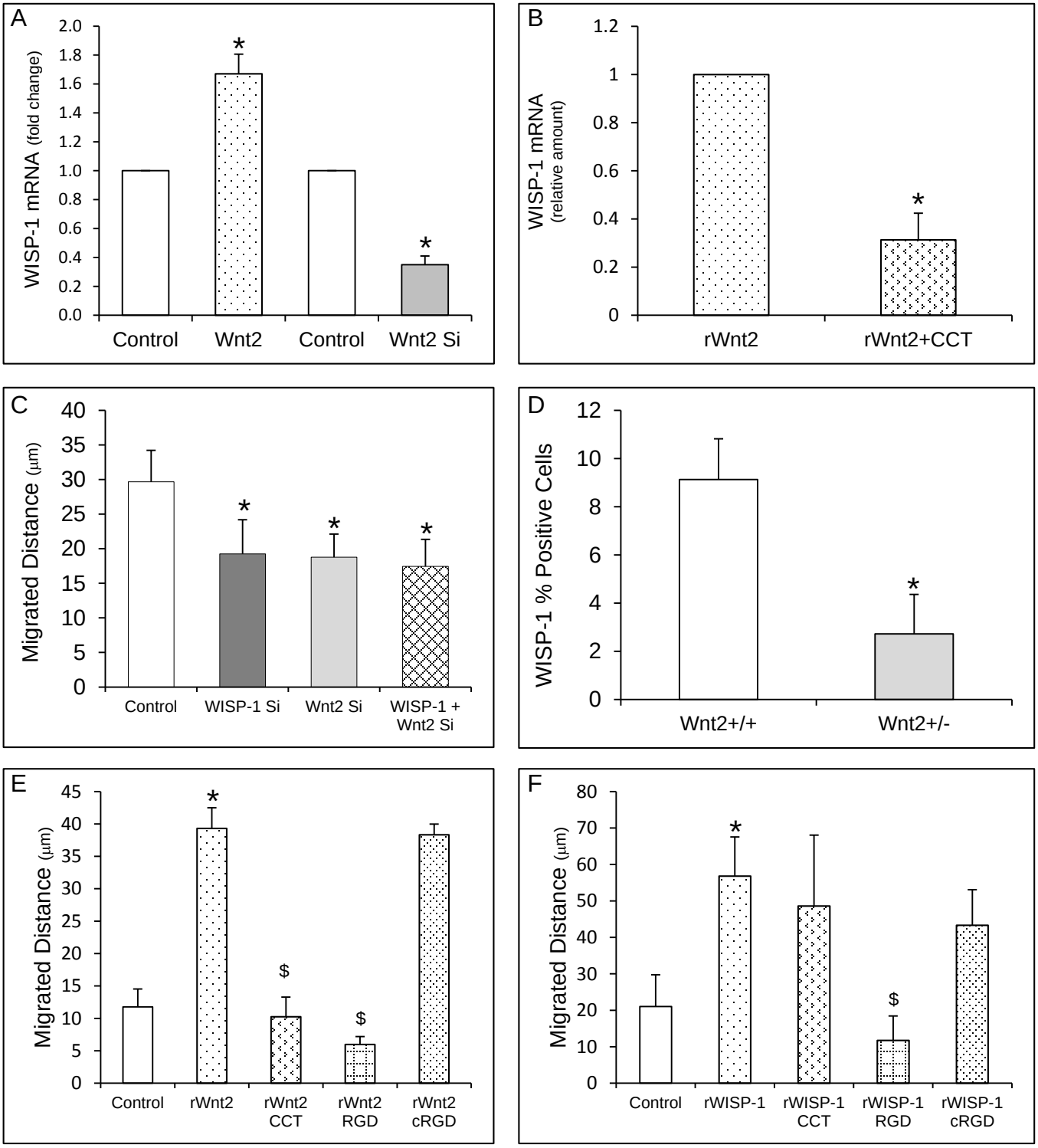

A
WISP-1 mRNA (fold change)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Control
Wnt2
Control
Wnt2 Si
B
WISP-1 mRNA
(relative amount)
1.2
1
0.8
0.6
0.4
0.2
0
*
rWnt2
rWnt2+CCT
C
Migrated Distance (μm)
40
35
30
25
20
15
10
5
0
*
*
*
Control
WISP-1 Si
Wnt2 Si
WISP-1 +
Wnt2 Si
D
WISP-1 % Positive Cells
12
10
8
6
4
2
0
*
Wnt2+/+
Wnt2+/-
E
Migrated Distance (μm)
45
40
35
30
25
20
15
10
5
0
*
$
$
Control
rWnt2
rWnt2
CCT
rWnt2
RGD
rWnt2
cRGD
F
Migrated Distance (μm)
80
70
60
50
40
30
20
10
0
*
$
Control
rWISP-1
rWISP-1
CCT
rWISP-1
RGD
rWISP-1
cRGD

Figure 4:

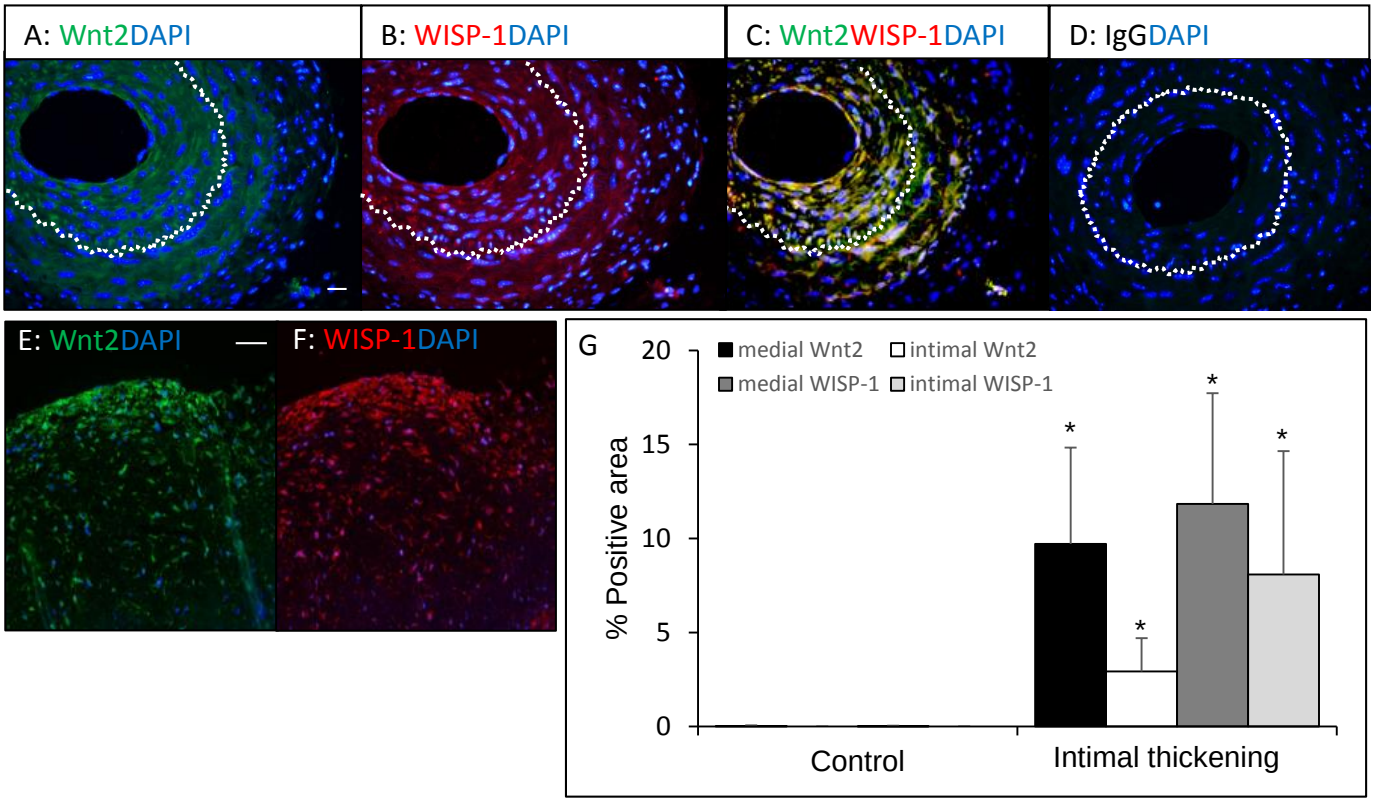
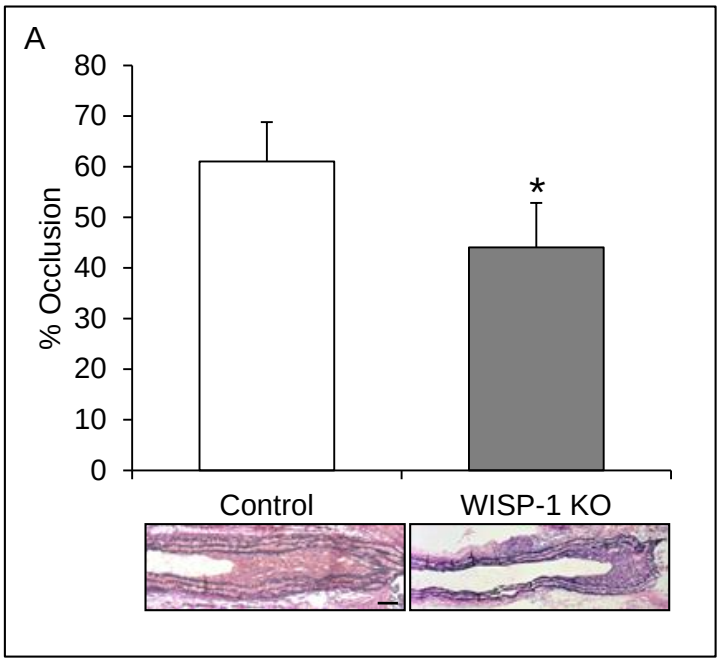
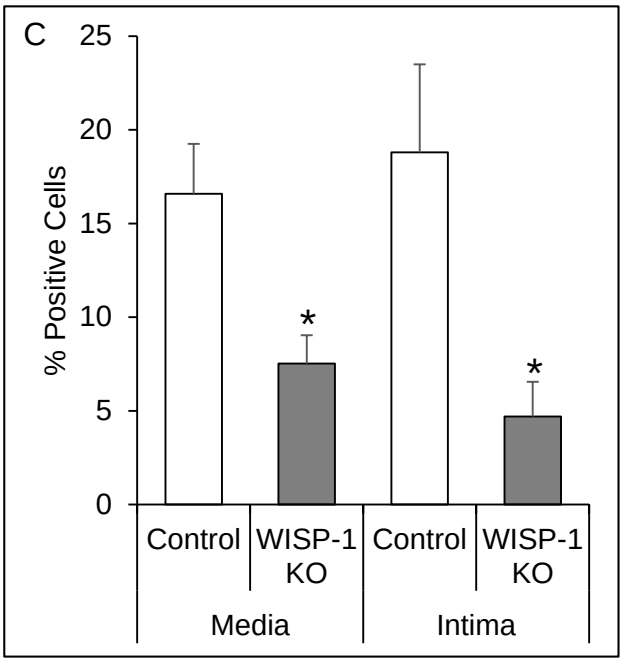

A: Wnt2DAPI
B: WISP-1DAPI
C: Wnt2WISP-1DAPI
D: IgGDAPI
E: Wnt2DAPI
F: WISP-1DAPI
G
medial Wnt2
intimal Wnt2
medial WISP-1
intimal WISP-1
% Positive area
20
15
10
5
0
*
*
*
*
Control
Intimal thickening

Figure 5:


A
% Occlusion
80
70
60
50
40
30
20
10
0
*
Control
WISP-1 KO


C
% Positive Cells
25
20
15
10
5
0
*
*
Control
WISP-1 KO
Control
WISP-1 KO
Media
Intima

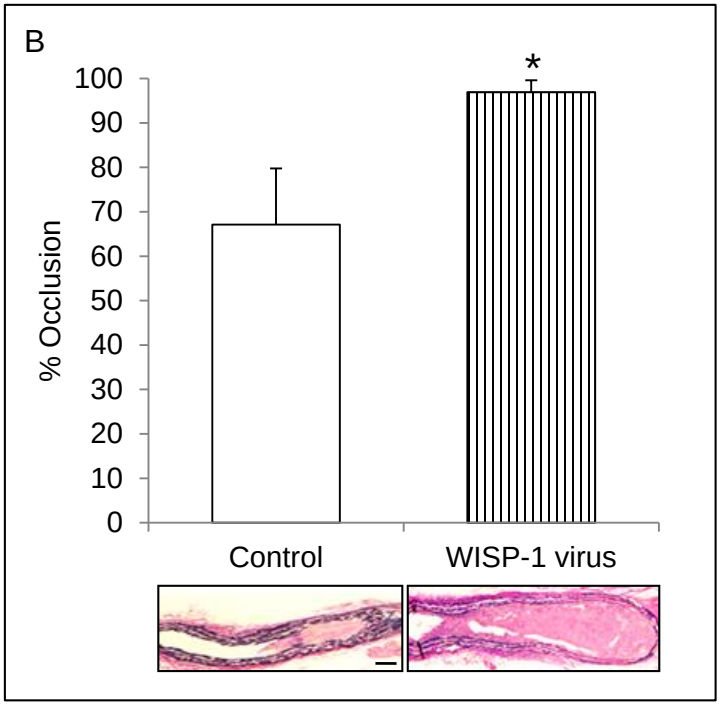
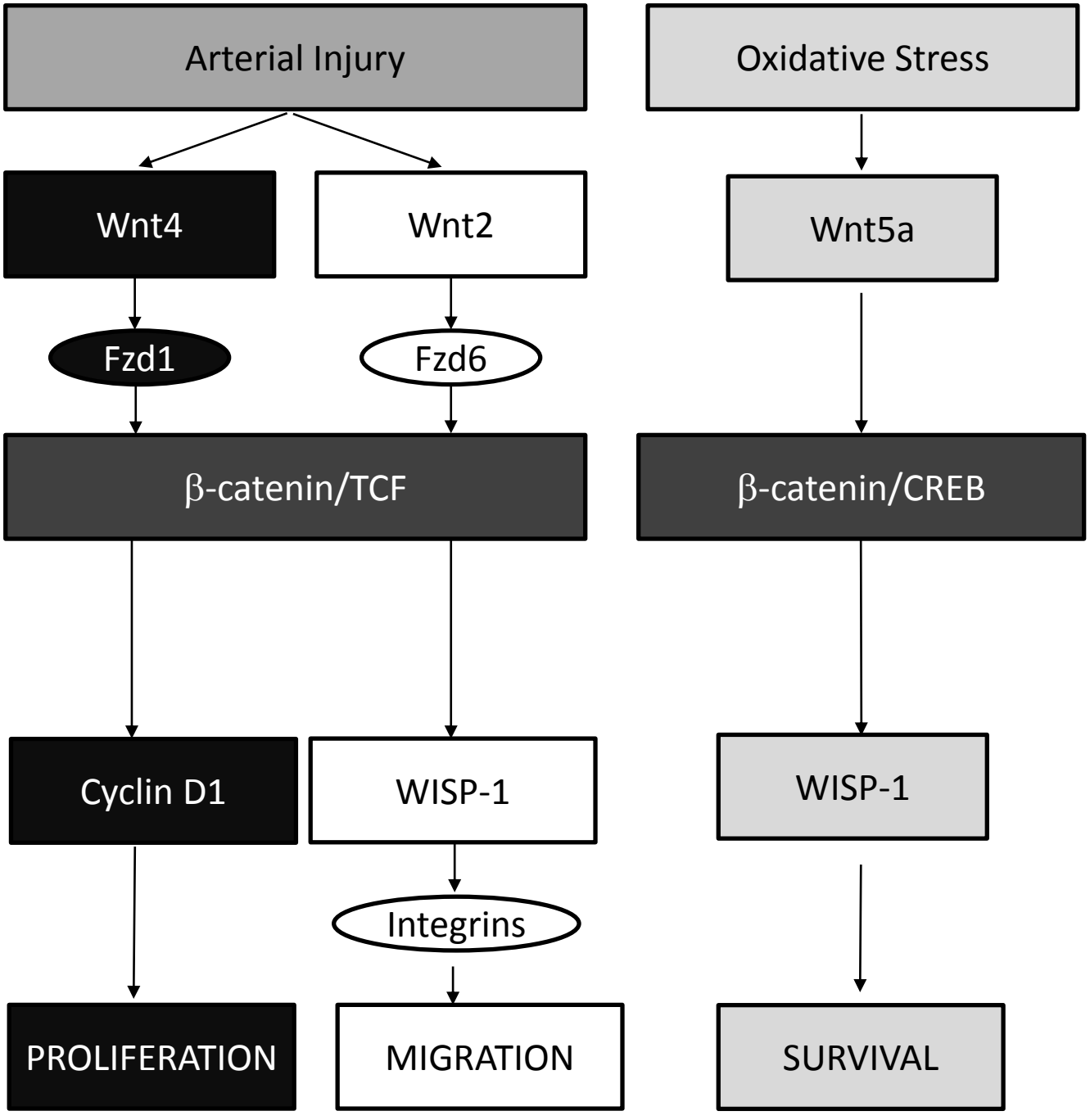

B
% Occlusion
100
90
80
70
60
50
40
30
20
10
0
*
Control
WISP-1 virus

Figure 6:


Arterial Injury
Oxidative Stress
Wnt4
Wnt2
Wnt5a
Fzd1
Fzd6
β-catenin/TCF
β-catenin/CREB
Cyclin D1
WISP-1
WISP-1
Integrins
PROLIFERATION
MIGRATION
SURVIVAL

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 ($WISP\text{-}1^{-/-}$) 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 $WISP\text{-}1^{-/-}$ mice and their wild type littermate controls ($Wnt2^{+/+}$ or $WISP\text{-}1^{+/+}$). 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 (8×10^{10} particles/mouse1.1×10^{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, 2.2×10^{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].

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 37ºC 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 2μM 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, 250μM) were also added. Cells were also seeded into a transwell cell culture inserts with 8μm 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 37ºC in a hybridising oven with 1μg 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 bicinchroninic 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^{o}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

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4. Johnson JL, Baker AH, Oka K, Chan L, Newby AC, Jackson CL, George SJ. Suppression of atherosclerotic plaque progression and instability by tissue inhibitor of metalloproteinase-2: Involvement of macrophage migration and apoptosis. *Circulation*. 2006;113:2435-2444
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6. Tsaousi A, Williams H, Lyon C, Taylor V, Swain A, Johnson J, George S. Wnt4/β-catenin signalling induces vsmc proliferation and is associated with intimal thickening. *Circ Res*. 2011;108:427-436
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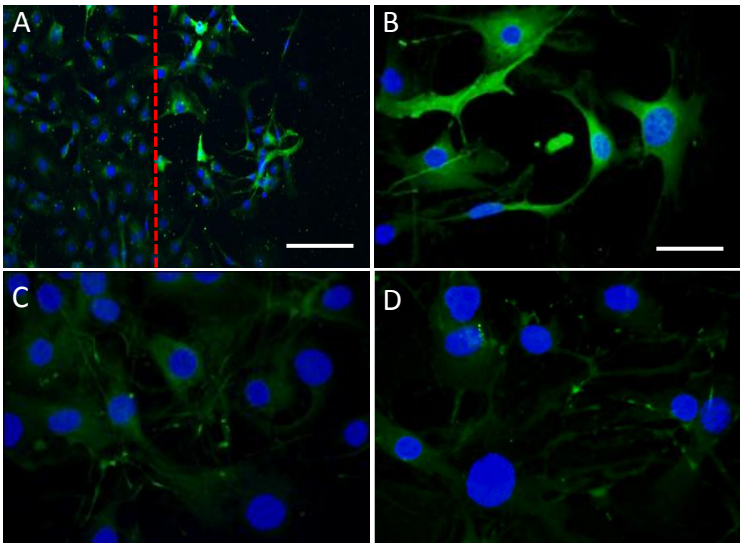
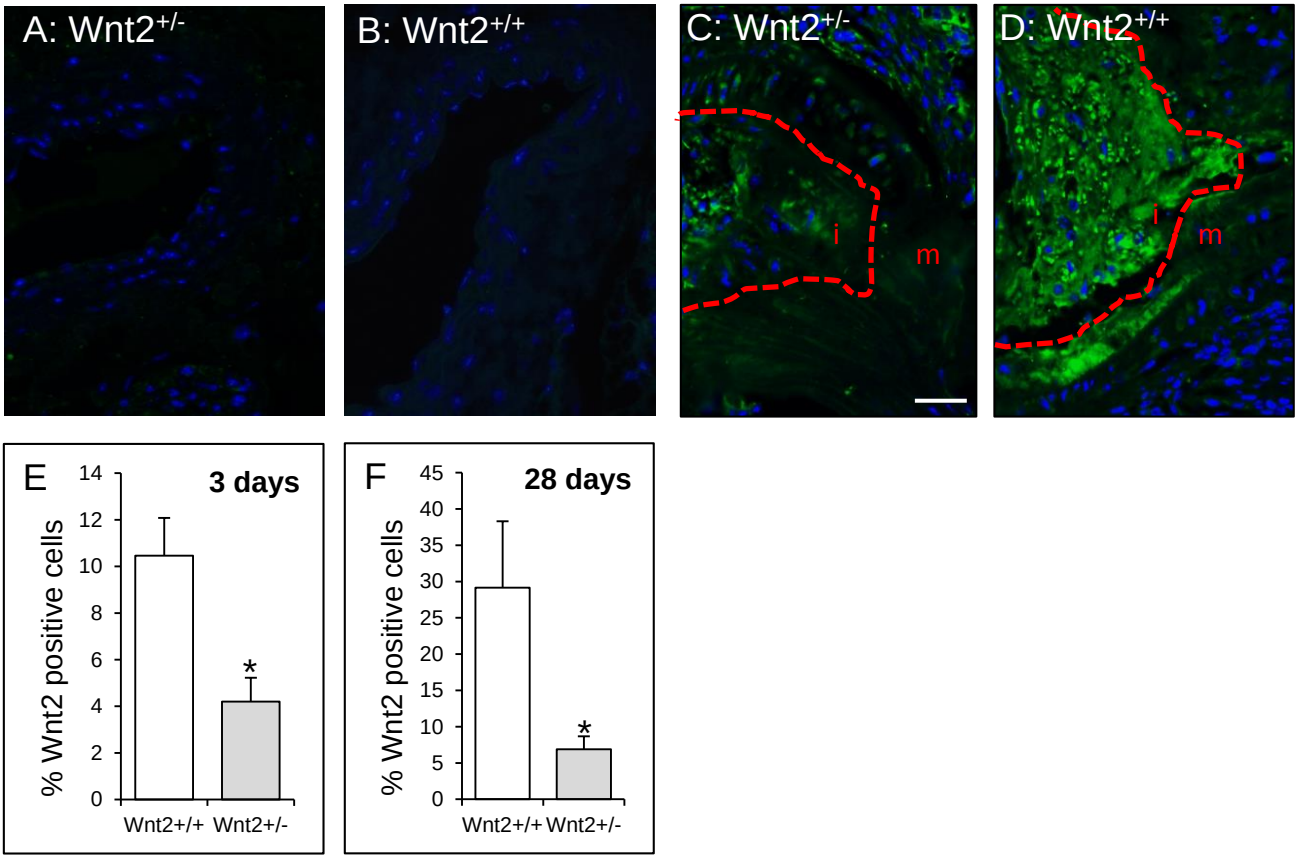
8. Dwivedi A, Sala-Newby GB, Newby AC, George SJ. Integrin-linked kinase is increased in intimal thickening and modulates cell-matrix and cell-cell interactions. *Eur Heart J*. 2004;25:748
9. Mill C, Williams H, Monk B, Jeremy J, Johnson J, George S. Wnt5a signalling promotes survival via wisp-1. *Arterioscler Thromb Vasc Biol*. 2014;34:2449-2456

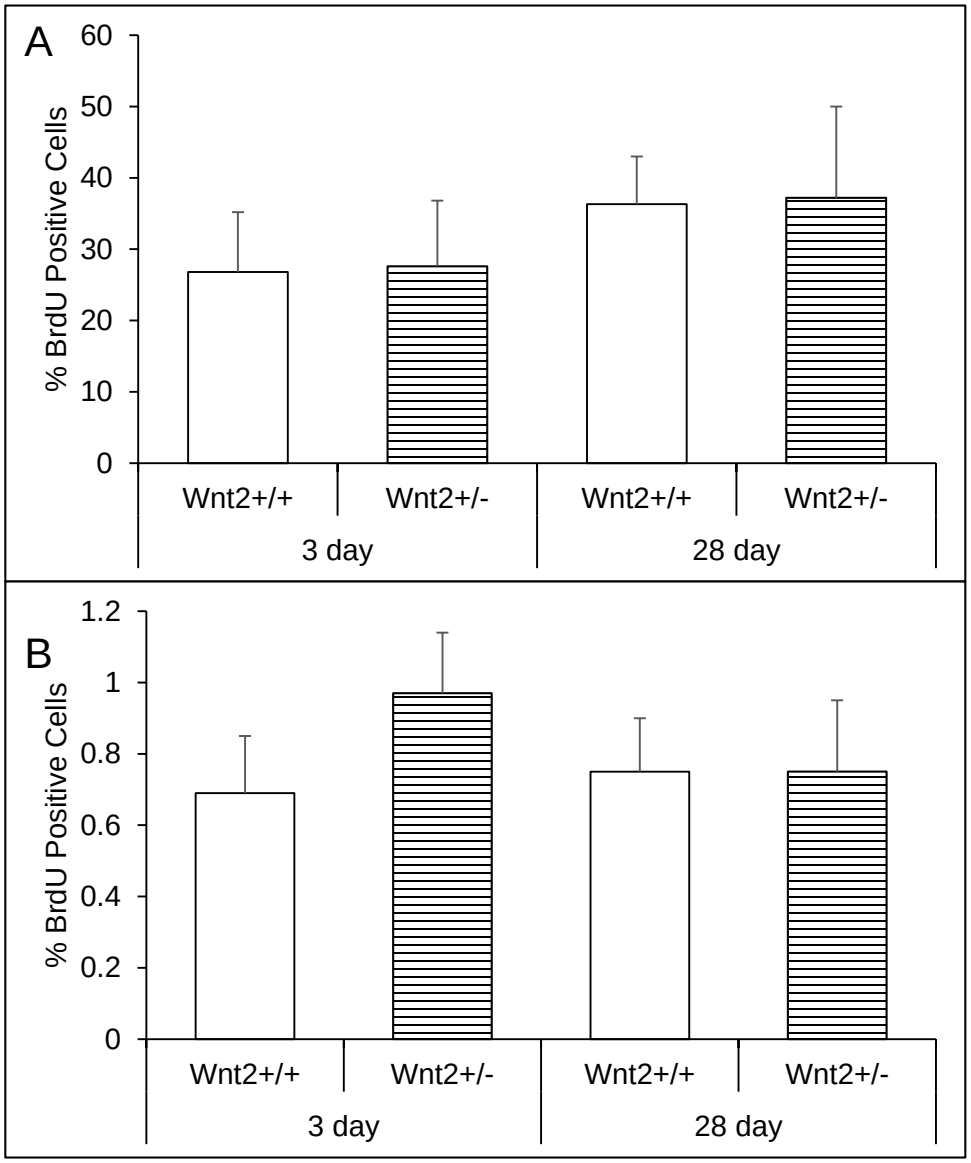
Supplementary Figure I:
Wnt2 was increased in migrating smooth muscle cells *in vitro.*
Immunofluorescence for Wnt2 protein (green) in VSMCs 24 hours after subjecting to wound injury (A). Red line indicates the position of the wound.
(B) Migrating cells on edge of wound, (C) Non-migratory cells, (D) 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.



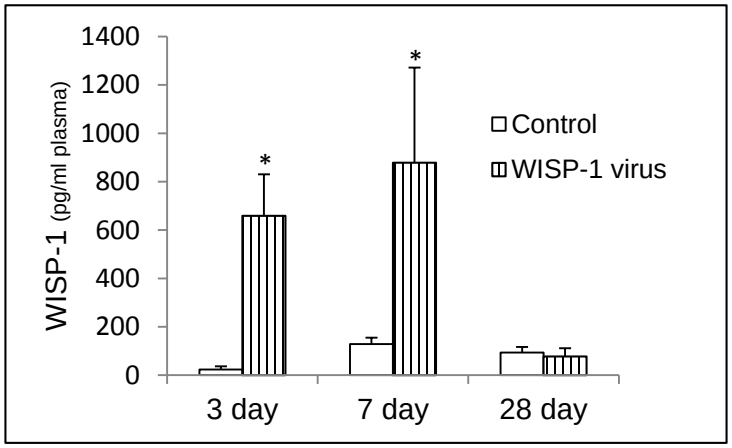
Supplementary Figure II:
Wnt2 protein was reduced in Wnt2+/- mice.
Immunofluorescence for Wnt2 protein in control non-ligated carotid arteries (A and B) and ligated carotid arteries (C and D) 28 days following ligation. $Wnt2^{+/-}$ (A and C) and wild type mice (B and D: $Wnt2^{+/+}$) dotted lines show 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.



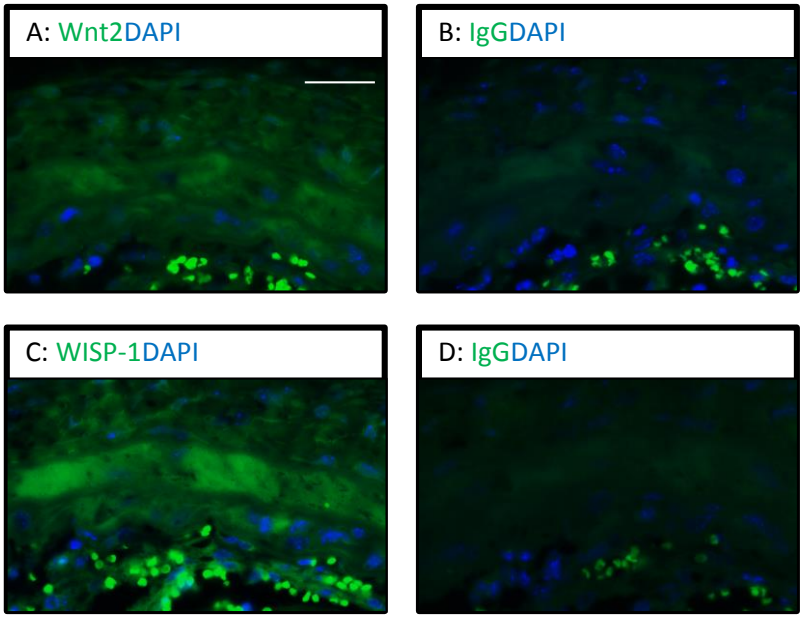
Supplementary Figure III:
Proliferation in ligated arteries.
The percentage of proliferating cells in the intima (A) and media (B) was quantified by BrdU incorporation and compared in $Wnt2^{+/+}$ and $Wnt2^{+/-}$ mice at 3 days and 28 days after ligation, n=6.



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.



Supplementary Tables

Supplementary Table I: Primer sequences used for Q-PCR

Gene	Source	Primer sequence Forward	Primer sequence Reverse
Brachyury	Sigma	GCTTCAAGGAGCTAACTAACGAG	CCAGCAAGAAAGAGTACATGGC
Frizzled 7	Sigma	GTCCCACCGCCTACCCTACTG	GTGAGCACCGTGAAGAGCGTC
Frizzled 10	Sigma	TGGTACGCATAGGGGTCTTC	TCAGGCAGTCAGGTGTCTTG
FoxN1	Sigma	CACTGGAAGCCTTTGAGGAG	AAGGCAGGCTGAGAAGAACA
MMP2	Sigma	GGCTGACATCATGATCAACTTTGG	GCCATCAGCGTTCCCATACTTTAC
MMP7	Sigma	AATGGCATTCCAGAATTGTCACCT	GATCTCTCCTTGCGAAGCCAATTA
MMP9	Sigma	GAGAACACCACCGAGCTATCCACT	AGAGAGGAGTCTGGGGTCTGGTTT
MMP14	Sigma	ACCACAAGGACTTTGCCTCTGAAG	CACTGAGCTGTGAGATTCCCTTGA
Porcupine	Sigma	TGGAGTTCATGGGCTACCTCT	CGTTTCTTGTTGCGAAGGAGT
Sox17	Sigma	GATGCGGGATACGCCAGTG	CCACCACCTCGCCTTTCAC
Senp2	Sigma	GCTGGCTAAGGTTCTCGGC	CTGGGATCTCATCAGTGTCCA
WISP-1	Sigma	CGTGGAGCAACGGTATGAG	GAGAGTGAAGTTCGTGGCC
WISP-2	Qiagen	Quantitect QT01061571	

Supplementary Table II: SiRNA probes used for knockdown of WISP-1 and Wnt2

Gene	Source	Catalogue No	Catalogue No
WISP-1 (Mouse)	Qiagen	S100212702	S102673370
Wnt2 (mouse)	Qiagen	S101472653	S101472660
Frizzled 1	Qiagen	S100218771	S102674252
Frizzled 6	Qiagen	S102666979	S102708510

Supplementary Table III: Fold change of Wnt pathway genes quantified by microarrays.

Wnt pathway member	Average fold change (n=3 arrays)	One sample t-test vs. 1 p value
Wnt1	2.01±0.63	NS
Wnt2	2.39±0.35	p<0.05
Wnt2b	2.36±1.09	NS
Wnt3	0.64±1.10	NS
Wnt3a	0.05±0.98	NS
Wnt4	0.45±0.54	NS
Wnt5a	0.32±0.23	NS
Wnt5b	1.20±0.78	NS
Wnt6	1.28±0.34	NS
Wnt7a	0.40±0.39	NS
Wnt7b	1.50±1.22	NS
Wnt8a	1.45±0.96	NS
Wnt8b	2.88±1.86	NS
Wnt9a	not detectable	
Wnt9b	5.34±3.36	NS
Wnt10a	0.12±0.55	NS
Wnt10b	1.11±0.28	NS
Wnt11	1.46±0.67	NS
Wnt16	0.96±0.42	NS

Supplementary Table IV: Fold change of potential Wnt2 responsive downstream genes following addition of recombinant Wnt2: mRNA quantified by Q-PCR.

Wnt2 downstream target	Average fold change ± sem (n=3-6)	One sample t-test vs. 1
Brachyury	1.19±0.52	NS
Frizzled 7	2.52±1.46	NS
Frizzled 10	1.25±0.60	NS
MMP2	9.79±9.47	NS
MMP7	3.43±1.75	NS
MMP9	1.35±0.52	NS
MMP14	4.45±2.20	NS
Porcupine	1.21±0.33	NS
Senp2	1.22±0.32	NS
Sox17	1.32±0.36	NS
WISP-1	1.67±0.14	*P<0.05
WISP-2	0.79±0.57	NS