Wnt signalling in smooth muscle cells and its role in cardiovascular disorders

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Abstract

Vascular smooth muscle cells (SMCs) are the major cell type within blood vessels. SMCs exhibit low rates of proliferation, migration, and apoptosis in normal blood vessels. However, increased SMC proliferation, migration, and apoptosis rates radically alter the composition and structure of the blood vessel wall and contribute to cardiovascular diseases, such as atherosclerosis, and restenosis that occur after coronary artery vein grafting and stent implantation. Consequently, therapies that modulate SMC proliferation, migration, and apoptosis may be useful for treating cardiovascular diseases. The family of Wnt proteins, which were first identified in the wingless drosophila, has a well-established role in embryogenesis and development. It is now emerging that Wnt proteins also regulate SMC proliferation, migration, and survival. In this review article, we discuss recently emerging research that has revealed that Wnt proteins are important regulators of SMC behaviour via activation of β-catenin-dependent and β-catenin-independent Wnt signalling pathways.

Keywords

Vascular smooth muscle • Proliferation • Migration • Apoptosis • Wnt

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1. Role of SMCs in cardiovascular disease

Cardiovascular diseases (CVD) are the leading cause of mortality in Western countries, accounting for over 16 million deaths per year.1 Atherosclerosis is the pathology behind the development of CVD, including myocardial infarction, angina, and stroke, and it is caused by the thickening of the arterial wall leading to reduced blood flow and ischaemia.2 The coronary arteries are composed of three layers: tunica intima, tunica media, and tunica adventitia. The smooth muscle cells (SMCs) reside in the medial layer and exhibit a contractile phenotype with low proliferative rates;3 however, during atherosclerosis endothelial dysfunction and vessel wall injury results in activation and de-differentiation of arterial SMCs (synthetic phenotype).4,5 This phenotypic switch permits arterial SMC migration into the intima where they proliferate and deposit extracellular matrix (ECM).5 The resultant thickened intima is the soil for the progression of the atherosclerotic plaque.6 Intimal thickening, occurring in 30–50% of patients, is also an undesirable injury response after balloon angioplasty, bypass vein grafting, and stent implantation.3

Paradoxically, fibrous cap SMC migration and proliferation is considered beneficial at the later stages of atherosclerosis, as it is important for the formation of the fibrous cap (a structure composed primarily of SMCs and ECM but also inflammatory cells such as macrophages and lymphocytes, and foam cells) that is responsible for plaque rupture resistance. Conversely, reduced ECM synthesis leading to decreased tensile strength of the fibrous cap occurs as a result of fibrous cap SMC apoptosis in unstable plaques.7 In normal arteries, SMC apoptosis is rare; however, increased apoptosis (~1%) occurs in unstable atherosclerotic plaques.8 Arterial SMC apoptosis is an important modulator of aneurysm formation,9 a small but significant (~2%) cause of sudden death in the UK.10

In summary, SMCs play a significant role in the formation and stability of the atherosclerotic plaque and in intimal thickening, which leads to restenosis after treatments of atherosclerosis. Consequently, a clear understanding of the mechanisms underlying SMC migration, proliferation, and apoptosis may be useful for devising new improved therapies for atherosclerosis and restenosis. Wnt proteins regulate proliferation, migration, and apoptosis of numerous cells types and therefore, if expressed during atherosclerosis and restenosis, are likely mediators of the cellular processes associated with these cardiovascular disorders. This review will focus on the regulation of SMC behaviour by Wnt proteins with particular translation to their functional significance in atherosclerosis and restenosis.
2. Introduction to Wnt signalling

The vertebrate Wnt family is a highly conserved group of 19 genes that encode for cysteine-rich-secreted glycoproteins that act as important extracellular signalling ligands. Wnt genes were first identified in mutant wingless Drosophila melanogaster in 1978 and were thus termed Wingless genes.11 Subsequently, a similar sequence homology was found in the int-1 gene of vertebrates and the two terms were eventually combined in 1991 to form the nomenclature ‘Wnt’.12 The Wnt signalling pathway is highly conserved in eukaryotes and is essential for embryogenesis and development, where it regulates diverse processes including cell proliferation, differentiation, cell polarity, and migration. Additionally, post-natal Wnt signalling regulates numerous biological processes involved in cancer and neurological, metabolic, and inflammatory disorders.13

Wnt ligands fine-tune cellular behaviour through intricate signalling pathways involving their interaction with transmembrane-spanning Frizzled receptors, of which 10 have been classified, as well as specific co-receptors. The respective Wnt proteins, Frizzled receptors, and co-receptors are differentially expressed among cells in a spatio-temporal manner to allow the highly specific and elaborate regulation of both embryonic development and disease progression. The appearance and complexity of Wnt signalling is revealed by the myriad of both lethal and viable phenotypes resulting from Wnt-null mice, as is described in detail by van Amerongen and Berns in their comprehensive review.14

Wnt signalling has been classically defined, for convenience, into two intracellular pathways: canonical Wnt signalling, which is β-catenin dependent and non-canonical Wnt signalling, which is β-catenin independent. The activation of either pathway is largely dependent on the subsequent associations of Frizzled receptors with endogenous co-receptors including the low-density lipoprotein (LDL)-related proteins 5 and 6 (LRP5/6) for canonical Wnt signalling and receptor tyrosine kinase-like orphan receptor 2 (ROR2) for non-canonical Wnt signalling. Thus, the specific pathway that is activated upon Wnt ligand stimulation is highly dependent on cellular context and the complement of cell surface Wnt receptor subtypes.15 Activation of the canonical Wnt pathway leads to the modulation of the transcription of >50 genes in mammals (a comprehensive list can be found at http://www.stanford.edu/group/nusselab/cgi-bin/wnt). Binding of Wnt proteins to the Frizzled–LRP5/6-receptor complex activates Dishevelled (Dvl) proteins, causing the disassembly of the β-catenin destruction complex [composed of axin, adenomatous polyposis coli, and glycosyn thase kinase-β (GSK-3β)], which usually phosphorylates β-catenin, targeting it to the proteasome for degradation (Figure 1). β-Catenin is an multi-faceted protein, with signalling capacity, but also contributes to the adherens junction complex, mediating homophilic cell-to-cell adhesion through interactions with cadherin transmembrane proteins. Upon disassembly, the β-catenin degradation complex can no longer phosphorylate β-catenin, therefore resulting in the rapid accumulation of dephosphorylated β-catenin and eventual translocation from the cytoplasm to the nucleus. Once in the nucleus β-catenin displaces the transcriptional co-repressor proteins Groucho/transducin-like enhancer of split from the T-cell factor (TCF) or lymphoid enhancer-binding factor (LEF) transcriptional factors, leading to TCF regulation of split from the T-cell factor (TCF) or lymphoid enhancer-binding factor (LEF) transcriptional factors, leading to TCF regulation of target genes such as those involved with cell-cycle activation (cyclin D1,16 c-Myc17), survival ( survivin,18 Wnt-1-induced secreted protein-1 (WISP-1),19 and insulin-like growth factor-1 (IGF-1)).20

Moreover, Wnt signalling regulates the expression of several ECM components including fibronectin21 and versican,22 and matrix-degrading mettalloproteinases (MMPs) such as MMP-723,24, MMP-225, MMP-926, MMP-1327, MMP-1428, and MMP-26.29 Both ECM composition and MMP activity regulate SMC migration, proliferation, and apoptosis (see review)30 and as a consequence canonical Wnt/TCF signalling may alter SMC behaviour during intimal thickening and atherosclerosis via modulation of the expression of matrix proteins and MMPs. However, there is little direct evidence for this in the literature except that highlighted in the section 5 for MMP-7.31

Although less well understood, Wnts also activate divergent (β-catenin independent) pathways collectively named under the umbrella term, non-canonical signalling (Figure 2). These pathways involve the alternative binding of Wnt ligands to Frizzled receptors alone or in complex with discrete co-receptors (other than LRP5 and 6). Non-canonical Wnt pathway activation can involve the release of Ca2+ from intracellular stores to activate co-effector kinases such as protein kinase C (PKC), calcium- and calmodulin-dependent kinase II (CAMKII), and Jun N-terminal kinase (JNK) or can activate JNK directly. Individually these kinases regulate the
transcription factors nuclear factor of activated T cell (NFAT), activating protein-1, and nuclear factor k-B (NFκB) leading to the coordination of gene transcription to regulate a multitude of cellular behaviours including cytoskeletal organization, cell polarity, and cell motility. Rho and Rac-kinases can also be activated by Wnt via a non-canonical pathway known as the planar cell pathway (PCP), which can in turn activate JNK or rho-associated protein kinase (ROCK). There is increasing evidence of cross-talk between these pathways, for example, the activation of the non-canonical pathways can lead to the inhibition of canonical Wnt signalling outcomes (see review).

Both canonical and non-canonical Wnt signalling pathways are controlled by several endogenous inhibitory proteins such as secreted Frizzled-related proteins (SFRPs), which act as decoy receptors, and Wnt-inhibitory factor-1, which binds directly to the Frizzled receptor. Additionally, the canonical Wnt pathway is specifically inhibited by members of the Dickkopf (DKK) family, whose inhibitory mechanism involves binding to the canonical specific LRPS/6 co-receptor.

3. Wnt signalling in SMC proliferation

The important role that Wnt-β-catenin signalling plays in SMC proliferation has been demonstrated in a number of important studies over the last decade. This has been reviewed comprehensively in the following review. Briefly, β-catenin activation and TCF signalling up-regulates pro-proliferative genes including cyclin D1 and down-regulates p21 in arterial and venous SMCs. Moreover, enhanced proliferative rates are associated with the activation of β-catenin signalling in atherosclerotic plaques in vivo; however, in this study, it was not determined in which cell type this occurred. Retarding Wnt...
signalling using a dominant negative LRP significantly reduced arterial SMC proliferation and a sFRP (FrzA), which acts as a decoy receptor for Wnt ligands, delayed arterial SMC entry into S-phase. Since β-catenin is regulated downstream of Wnt activation, this suggests that canonical Wnt signalling may play a role in arterial and venous SMC proliferation.

Direct evidence for the role of Wnt signalling in SMC proliferation has been provided in a small number of studies. Wnt1 and Wnt3a are capable of inducing β-catenin signalling and cyclin D1 expression in arterial SMCs in vitro, but there is no evidence for expression of these Wnt proteins by proliferating SMCs in vitro or in vivo. However, our group has recently identified that Wnt4 is a critical modulator of arterial SMC proliferation in vitro and contributes to pathological intimal thickening in vivo. In addition, Wnt4 is expressed in human internal mammary artery SMCs. Wnt4 was the only Wnt protein that is significantly induced in arterial SMCs when stimulated to proliferate with growth factors in vitro. The important role that Wnt4 plays in proliferation and intimal thickening in vivo was demonstrated using a Wnt4 heterozygous mouse, which experienced significantly reduced intimal thickening and proliferation following ligation of the right carotid compared with wild-type controls. Interestingly, this proliferative effect of Wnts may be regulated by age since the pro-proliferative effects of Wnt1 and Wnt3a were lost with increasing age despite sustained Wnt signalling. Since Wnt-β-catenin signalling can regulate the expression of many genes, it is likely that other genes in addition to cyclin D1 and p21 are regulated by this pathway and may therefore affect SMC proliferation. In fact recently, Reddy et al. demonstrated that interleukin-8-induced venous SMC proliferation is dependent on the Wnt-β-catenin responsive gene WISP-1, a member of the family of connective tissue growth factors.

Together these findings clearly demonstrate that canonical Wnt signalling is pro-proliferative in arterial and venous SMCs, both in vitro and in vivo. However, despite the lack of direct evidence there is indirect evidence for the involvement of β-catenin-independent non-canonical Wnt signalling in the promotion of SMC proliferation. For example, NFATc1 is essential for human aortic SMC proliferation in vitro, via cyclin D1 gene expression, and after a balloon-induced vascular wall injury in vivo. In addition, CAMKII promotes rat aortic SMC proliferation and JNK is activated in proliferating SMCs after a balloon injury; however, these findings are not directly related to pathway activation by Wnt proteins. Moreover, it has been revealed that SMC proliferation is retarded by bone morphogenetic protein 2 (BMP2) via a novel tandem inter-dependent activation of both Wnt-β-catenin and Wnt-JNK pathways, which converge to ultimately activate RhoA-Rac1-dependent arterial SMC motility, but simultaneously repress further β-catenin accumulation and proliferation. These studies highlight the complexity of Wnt signalling pathways and their potential to cross-talk in order to achieve fine regulation of SMC cell behaviour. Nevertheless, it remains to be determined whether and which Wnt proteins cause the activation of the non-canonical pathways via NFAT, CAMKII, and JNK during SMC proliferation in vivo.

Characterization of the subtypes of Wnt and Frizzled receptors involved in SMC proliferation is incomplete. We observed expression of both Frizzled-1 and Frizzled-6 in quiescent and proliferating mouse arterial SMCs and in rat intimal SMCs in the carotid artery after a balloon injury. Interestingly, however, only the silencing of Frizzled-1 retarded Wnt-4-induced arterial SMC proliferation. Puzzlingly though, the down-regulation of Frizzled-1 and Frizzled-2 was observed at 1 and 4 h after an arterial injury of the carotid artery in rats and in proliferative arterial SMCs in vitro, suggesting that they may have a negative relationship to proliferation. On the other hand, increased expression of Fzb-1, an endogenous antagonist of the Wnt cascade, was observed in the rat arterial wall at 4 days and 3 weeks after an in vivo injury and in quiescent isolated SMCs. Since Fzb-1 is a sFRP that inhibits Wnt signalling by binding and thereby preventing their interaction with Fzds, it was suggested that this increased Fzb-1 expression, when an arrest of arterial SMC proliferation is observed in the media and neointima, indicates that Fzb-1 may suppress proliferation. Consequently this observation also suggests that Wnt signalling has a positive role in proliferation prior to the up-regulation of Fzb-1.

In summary, the data presented above provides strong evidence for the role of Wnt proteins in the stimulation of arterial and venous SMC proliferation; however, considerably more remains to be learnt concerning the precise mechanisms underlying the induction of SMC proliferation. The manipulation of Wnt proteins and Frizzled receptors in animal models of proliferative cardiovascular disorders in future studies may help to elucidate the precise role of the Wnt family in the pathogenesis of atherosclerosis and intimal thickening. Although the majority of the data so far are gained from arterial and venous SMCs, it is possible that Wnts may regulate venule SMC proliferation. Interestingly, DKK-1 promoted SMC proliferation in rat mesenteric microvasculature, which may indicate differential regulation of proliferation in SMCs from different blood vessel types.

4. Wnt proteins in SMC migration

In comparison with proliferation, less is known about the role that Wnt proteins play in the promotion of SMC migration. However, the involvement of the Wnt-β-catenin pathway appears likely, since animal models of intimal thickening (in which both SMC proliferation and migration occur) have shown increased β-catenin levels. Moreover, it is likely that Wnt proteins will play a role in SMC migration since they promote the migration of other cell types during development and migration of other cell types such as monocytes and endothelial cells. We currently have studies underway to directly examine the role of Wnt-β-catenin signalling in arterial SMC migration and have demonstrated that arterial SMC migration is regulated by Wnt2. To date the role of the non-canonical Wnt pathways (Wnt-Ca2+ and Wnt-JNK) in SMC migration has not been examined directly; however, indirect evidence suggests that these pathways may be involved. For example, NFATc1 promotes the arterial SMC response to injury, and the inhibition of GSK3β is required for the activation of NFAT during arterial SMC migration in wound repair. Additionally, CAMKII is required for arterial SMC migration, and JNK is activated after a rat carotid artery balloon injury when SMC migration is induced. Furthermore, BMP-2 consecutively and interdependently activates the Wnt-β-catenin and Wnt-non-canonical planar cell polarity (PCP) signalling pathways to facilitate arterial SMC motility.

5. Wnt proteins in vascular smooth muscle apoptosis

The apoptosis of SMCs in CVD can be induced by a number of extra-cellular agents and inflammatory mediators, including oxidative stress...
induced by reactive oxygen species, 59–61 reactive nitrogen species, 62 inflammatory mediators/cytokines such as IL-1β and IFN-γ, 63 and oxidized low-density lipoprotein (oxLDL). 59,64,65 As discussed previously, fibrous cap SMC apoptosis leads to thinning and weakening of the fibrous cap and contributes to aneurysm formation. Consequently, the identification of factors that provide an anti-apoptotic signal for fibrous cap SMCs may be useful for reducing plaque rupture and aneurysm formation. In addition, a pro-apoptotic strategy may be useful for the reduction in intimal thickening by inducing the apoptosis of arterial or venous SMCs that are responsible for intimal thickening. Wnt-dependent survival signalling predominantly concerns the activation of the β-catenin-dependent canonical Wnt signalling pathway. Over 54 β-catenin target genes have been identified, of which a handful of genes play a direct defined role in cell survival including survivin, IGF-1, and members of the connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family of growth factors. However, numerous transcription factors, including c-jun, fra-1, 5ox9, and ID2, are β-catenin responsive genes, which could activate the indirect expression of other genes involved in cell survival.

We and others have demonstrated that in arterial SMCs, Wnt-β-catenin signalling promotes SMC survival. 39,42,66,67 Recent findings suggest that a reduction in β-catenin protein levels through the inhibition of the cell cycle protein peptidyl-prolyl cis/trans isomerase (Pin1) in arterial SMC increases the rate of apoptosis. 67 The perturbation of Wnt-β-catenin signalling, using a dominant negative form of TCF-4 or LRP, increased arterial SMC apoptosis. 39,42 although the precise mechanisms of action were not investigated. We showed that Wnt5a treatment of mouse aortic SMCs results in β-catenin activation and enhanced TCF signalling. 66 Interestingly, when challenged with H₂O₂, a pro-apoptotic mimic of oxidative stress associated with CVD, we demonstrated that Wnt5a reversed H₂O₂-induced apoptosis of mouse aortic SMCs. 66 This effect was reversed by canonical Wnt pathway inhibition using the gene silencing of LRP5/6 canonical co-receptors and treatment with the canonical-specific pathway inhibitor DKK-1. 66 Intriguingly, in these experiments, the survival of arterial SMCs in the presence of Wnt5a and H₂O₂ was paralleled with a reduction in TCF signalling, suggesting that there is downstream inhibition of the canonical Wnt pathway by H₂O₂. 66 In these conditions, arterial SMC survival appeared to be dependent on the up-regulation of the β-catenin-dependent gene, WISP-1, which was regulated by an alternative Wnt5a-dependent pathway involving CREB transcription factor activation. 66 Further studies are, however, essential to completely dissect the precise role of β-catenin signalling in fibrous cap SMC apoptosis.

6. Wnt and SMC differentiation

Differentiation of venous and arterial SMC may also be regulated by Wnts; however, this research area has received little attention. The canonical Wnt/TCF pathway has been identified as a strong SMC lineage inducer in the chick embryo. 68 Moreover, the Notch pathway acts together with the BMP and Wnt pathways in coordinating mesodermal ventralization, ventral mesodermal lineage induction, and lineage segregation events. 68 Specifically, Wnt3a (but not Wnt1 or 5a) promotes the expression of the early myofibroblast marker SM-22α. 69 Studies in the lung have also highlighted Wnts as potentially important regulators of SMC differentiation. Specifically, Wnt7b is thought to play a role during the early events of pulmonary SMC differentiation, through canonical signalling via Fzd1 and 10 and LRP5. 70 Interestingly, Wnt7b-/- embryos and newborn mice exhibit severe defects in the smooth muscle component of the major pulmonary vessels, due to increased apoptosis of SMCs. 71

7. Regulation of Wnt and Frizzled expression

Although the key regulators of Wnt and Frizzled expression have not been determined in CVD, previous studies in other cell types have indicated possible mechanisms of regulation. The tumour suppressors p63/p73, 72 and Wilms tumour 179 positively regulate Wnt4 expression, whereas p21 has the opposite effect, 80 suggesting that the maintenance of appropriate Wnt4 expression is potentially modulated by...
several factors. Also there was evidence that the ELL-associated factor and Wnt4 form an auto-regulatory negative feedback loop in vivo.\textsuperscript{81} WNT5A is also transcribed due to multiple mechanisms, such as NFkB, Hedgehog, JAK-STAT3, TGF-\(\beta\), and Notch signalling activation.\textsuperscript{82} Demethylation of the promoter region of Wnts may also be an important regulator of Wnt gene expression in SMC.\textsuperscript{83,84}

Although the data regarding Frizzled promoter regulation is not extensive, it is apparent that differential regulation of the Frizzled may occur. ERG promotes Frizzled4 gene,\textsuperscript{85} SRY-related HMG-box 4 (Sox4); POU5F1 and POU2F subfamily members regulate Frizzled5 expression,\textsuperscript{86,87} and ELK1- and PAX4-binding sites were conserved in Frizzled8 promoters,\textsuperscript{88} whereas the binding sites for PU.1, SP1/Krüppel-like, CCAAT-box, and TCF/LEF/SOX transcription factors were conserved among the 5'-promoter in Frizzled7 orthologs.\textsuperscript{89}

The triggers for the activation of Wnts in arterial and venous SMCs in CVD are currently unknown. Evidence in the literature from other cell types suggest various regulatory factors, for example, retinoic acid,\textsuperscript{90} cytokines including IL-6,\textsuperscript{91} low oxygen,\textsuperscript{91} toll-like receptor activators, for example, lipopolysaccharide,\textsuperscript{92} and LDL may be regulators of Wnt activity.\textsuperscript{93} Further analyses are essential to determine which of these regulators are important for the modulation of Wnt gene expression in SMC during intimal thickening and atherosclerosis.

In this review, we have described Wnt–Frizzled signalling in SMCs in a variety of processes associated with cardiovascular disorders. Our understanding of the underlying mechanisms of Wnt proteins in cardiovascular disorders, however, remains incomplete. The exploitation of small-molecule Wnt inhibitors may advance our understanding of the Wnt pathways in CVD, but also facilitate the development of therapeutic reagents for the treatment of CVD, associated with deregulation of normal Wnt signalling such as atherosclerosis and intimal thickening. Recent studies have proposed that many of the components of the Wnt signalling pathway are ‘druggable’ (Figure 3). A number of synthetic inhibitors that act at different steps in the Wnt/\(\beta\)-catenin signal transduction pathway have been identified.\textsuperscript{94–106}

![Figure 3](https://academic.oup.com/cardiovascres/article-abstract/95/2/233/293764)

**Figure 3** Endogenous and synthetic inhibitors of the Wnt/\(\beta\)-catenin signalling pathway. The Wnt/\(\beta\)-catenin signalling pathway is regulated by several endogenous inhibitors shown in the diagram and in the upper table. Recently described small-molecule inhibitors are also shown in the diagram (A–H), with their sites of action detailed in the lower table.
the potential of various inhibitors such as CRT on intimal thickening and atherosclerosis in future studies.

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