Aging Cell (2011) **10,** pp220–232 Doi: 10.1111/j.1474-9726.2010.00661.x

The Wnt/beta-catenin pathway is activated during advanced arterial aging in humans

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Summary

Aging is the main risk factor for cardiovascular diseases, but the associated molecular mechanisms are poorly understood. The Wnt signaling pathway was shown to be induced during aging in muscle and in the skin, but the regulation and role of Wnt signaling in the aged vessel have not yet been addressed. While screening for age-related changes in gene expression in the intima/media of human mammary arteries, we observed that the expression of frizzled 4 (Fzd4), a Wnt receptor, and of several targets of the Wnt/β-catenin/TCF signaling pathway [Wnt-inducible secreted protein 1 (WISP1), versican, osteopontin (SPP1), insulin-like growth factor binding protein 2 (IGFBP-2), and p21] were modified with age, suggesting an activation of the Wnt/β-catenin pathway. In contrast, we did not observe any regulation of forkhead transcription factor (FoxO) target genes. Beta-catenin-activating phosphorylation at position Ser675 was increased in aging mammary arteries, confirming the activation of this pathway. We confirmed *in vitro* that Wnt3a or Wnt1 treatment of human vascular smooth muscle cells (VSMCs) induced β-catenin phosphorylation at Ser675 and WISP1, SPP1, and IGFBP-2 expression. *In vitro*, Wnt treatment induced proliferation and cyclin D1 expression in VSMC from young (6 weeks old) rats but not in cells from older rats (8 months old), even though low-density lipoprotein receptor–related protein 6 and β-catenin phosphorylation, and β-catenin nuclear translocation demonstrated β-catenin activation in both cell types. Beta-catenin silencing demonstrated that Wnt induction of cyclin D1 expression is β-catenin dependent. Altogether, our data show that the Wnt/β-catenin/TCF pathway is activated in aging human mammary artery cells, but fails to induce the proliferation of aging vascular cells.

Key words: Aging; arteries; cyclin D1; microarray; proliferation; rat; senescence; Wnt; Wnt3a.

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Accepted for publication *10 November 2010*

Introduction

Aging is the main risk factor for cardiovascular diseases, notably through lesser distensibility and increased stiffening of large conduit arteries (Lakatta, 2003). Vessel stiffness, independently of blood pressure, is associated with an enlarged lumen and increased intimal and medial width (Guyton *et al.*, 1983; Michel *et al.*, 1994). Matrix deposition and invading smooth muscle cells (SMCs) accumulate in the intima, while medial SMCs grow in size but are reduced in number, and shift from a contractile to a synthetic phenotype (Michel *et al.*, 1994; Li *et al.*, 1999). Extracellular matrix organization is altered with age, with gradual deposition of calcium salts and collagen, leading to a reduced elastin/collagen ratio, elastin fragmentation, and elevated quantities of fibrinogen, fibronectin, proteoglycans and advanced glycation end-products (AGEs), leading to collagen cross-linking and resistance to proteolysis (Cliff, 1970; Richardson *et al.*, 1988; Michel *et al.*, 1994; Li *et al.*, 1999).

Aging is also associated with increased intimal expression of adhesion molecules (Li *et al.*, 1999) and inflammatory molecules, increased TGF-β levels, and increased MMP-2 expression and activity (Wang & Lakatta, 2002). Endothelium-derived NO bioavailability is reduced (Moritoki *et al.*, 1986), while production of endothelial COX-derived contracting factors is increased (Heymes *et al.*, 2000), leading to impaired endothelium-dependent relaxation. The smooth muscle contractile response to agonists is variously reported to be reduced or maintained (for review see Marin, 1995). The proliferative and migratory capacities of human SMCs decrease with age (Ruiz-Torres *et al.*, 1999). In addition, cultured SMCs from old animals appear to respond less efficiently to growth factors, cytokines, and matrix components such as PDGF, TGF-β, and heparin (McCaffrey & Falcone, 1993). Despite these findings, aging-induced modifications in vascular cells, and the relative contributions of the different regulatory pathways, are not clearly understood.

Two recent studies have emphasized the role of Wnt during aging (Brack *et al.*, 2007; Liu *et al.*, 2007). In aged myogenic progenitors, conversion to a fibrogenic lineage was associated with an activation of the canonical Wnt signaling pathway and

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could be suppressed by Wnt inhibitors. The authors suggest that components of serum from aged mice that bind to the Frizzled family of proteins, which are Wnt receptors, may account for the elevated Wnt signaling in aged cells (Brack *et al.*, 2007). In another study, augmented Wnt signaling was observed in the skin of mice lacking klotho expression (Klotho mice) (Liu *et al.*, 2007). These mice have a shortened lifespan and exhibit a number of early-onset age-related changes, including arteriosclerosis, decreased fertility, and skin atrophy (Kuro-o *et al.*, 1997). In this model, continuous Wnt exposure triggered both *in vitro* and *in vivo* accelerated cellular senescence. There are no data on the Wnt pathway during aging of the vascular cells. However, the Wnt/beta-catenin/T-cell factor (TCF) signaling was shown to play a role in vascular smooth muscle cell (VSMC) proliferation through modulation of cyclin D1 and p21 and phosphorylation of beta-catenin (Quasnichka *et al.*, 2006; Taurin *et al.*, 2008).

We used DNA chip technology to identify SMCs genes and pathways that are modulated in the aging human mammary artery media that is largely spared by atherosclerosis. We compared the expression profile in patients under 60 years of age and patients over 75. One of the pathways that appeared the most differentially regulated was the Wnt pathway. We therefore studied the regulation and the role of the Wnt/β-catenin pathway in the aging vessel.

Results

Microarray analysis

We compared the expression profiles of the mammary artery media between eight middle-aged patients (M group) and seven older patients (group O). Except for age (54.8 ± 5.5 vs. 77.1 ± 2.2 years), the two groups were comparable (Table 1). We identified 529 genes that were differentially regulated in mammary artery during aging (Table S1) and efficiently discriminated between the two groups after unsupervised hierarchical clustering and principal components analysis (Fig. S1). Real-time PCR analysis of 26 of the genes with the strongest differential expression was applied to a larger pool of samples and confirmed 61.5% of the mRNA differences identified by microarray analysis (Table S2).

Table 1 Characteristics of all mammary artery donors

	M	O	*P* value
Patients	17	19	
Male, %	100	100	ns
Age, years (mean ± SE)	54.8 ± 5.5	77.1 ± 2.2	0.0001
Disorders			
AAS, %	100	89	ns
LVEF, % (mean ± SE)	53.8 ± 11.5	55 ± 10.9	ns
Stenosis, mean number/patient	3.1	2.7	ns
Bypass, mean number/patient	2.9	2.6	ns
Diabetes, %	29	37	ns
Medications, %			
Nitrates	0	5	ns
β-blockers	88	74	ns
Statins	88	89	ns
Insulin	24	32	ns
ACEI/ARB	35	32	ns
Clopidogrel	18	11	ns

AAS, ascending aortic stenosis; LVEF, left ventricular ejection fraction; ACEI/ARB, angiotensin I-converting-enzyme inhibitors/angiotensin II receptor blocker; ns, not significant.

Functional clustering of the regulated genes with gene ontology (GO terms) or Kyoto encyclopedia of genes and genomes (KEGG) pathways revealed strong representation of genes involved in transcription regulation, cell adhesion, junction and actin cytoskeleton organization, intracellular signal transduction, and protein degradation (Fig. 1and Fig. S2). Statistical enrichment of groups of functionally related genes was observed for pathways of interest, namely cell cycle regulation, protein kinase regulation, and focal adhesion (Table S3). Two protein families were also enriched in the age-regulated gene list, namely the Ras and cytochrome B561 families.

Age-induced regulation of Wnt/β-catenin targets

One of the regulated pathways identified by functional clustering was the Wnt signaling pathway (Fig. 1). Wnt proteins belong to a large family of secreted glycoproteins that locally activate receptor-mediated signaling cascades and play important roles in development and tissue homeostasis. They were shown to play a role in aging in other systems (Brack *et al.*, 2007; Liu *et al.*, 2007). We therefore carefully analyzed the regulated gene list to identify Wnt/β-catenin targets. In addition to frizzled 4 (Fzd4), a Wnt receptor, we found that the following Wnt targets were regulated (Table 2): osteopontin (SPP1), WISP1, versican, CDKN1A (p21), and insulin-like growth factor binding protein 2 (IGFBP-2) (Xu *et al.*, 2000; El-Tanani *et al.*, 2001; Naishiro *et al.*, 2005; Rahmani *et al.*, 2005; Quasnichka *et al.*, 2006; Scheller *et al.*, 2008). RT–PCR confirmed the microarray data (Table 2). SPP1 (×5.2), WISP1 (×2.2), versican (×2.7), and IGFBP-2 (×1.7) mRNAs were significantly upregulated, while p21 mRNA was significantly downregulated (×0.53) with age. The cyclin D1 mRNA level was unchanged. Expression of Runx2, another Wnt target involved in vascular calcification (Gaur *et al.*, 2005; Byon *et al.*, 2008), was slightly but not significantly increased in the O group (×1.5). Western blot confirmed the significant regulation of SPP1 and IGFBP-2 at the protein level (respectively ×1.7 and ×2.2 in elderly patients), while WISP1 (×1.5) and versican (×1.5) regulation did not reach statistical significance, owing to the extreme variability of expression in each group (Fig. 2). In keeping with the mRNA results, cyclin D1 protein expression was unchanged. P21 protein expression was also unchanged, although p21 mRNA expression was decreased. Despite the absence of cyclin D1 regulation, these results point to an activation of the canonical Wnt signaling pathway in the elderly human mammary artery media.

The RT–PCR data also confirmed the microarray results for Fzd4 (×1.34 in the O group, $P < 0.05$) but no difference was observed between the O and M groups for dishevelled 1 (Dvl1),

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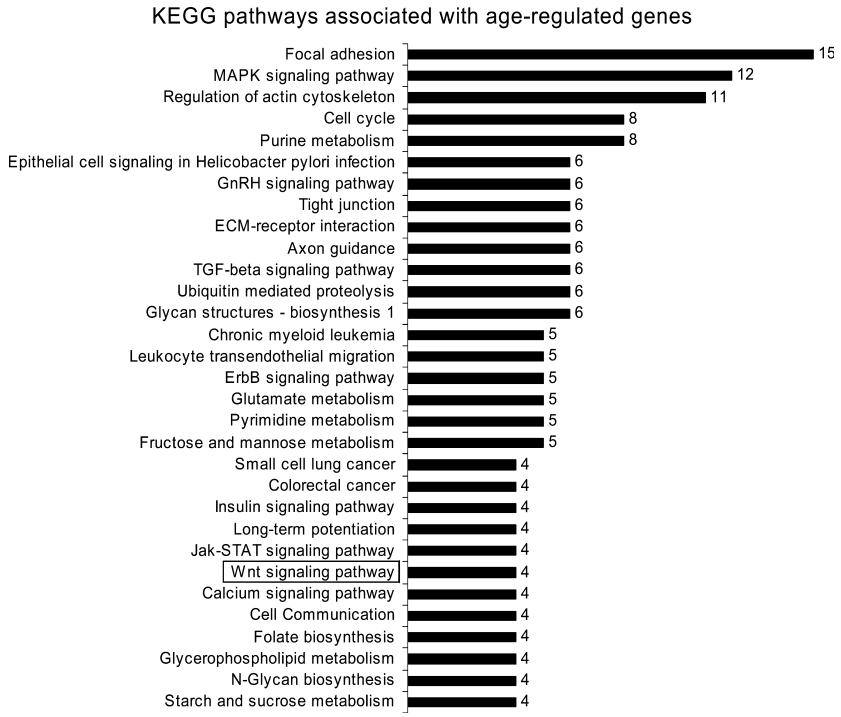


Fig. 1 Kyoto encyclopedia of genes and genomes (KEGG) pathway classification of age-regulated genes in human mammary arteries. Expression profiles of the mammary artery media/intima samples from the M group (n = 8) were compared with samples from the O group (n = 7). The 529 regulated genes were identified with the significant analysis of microarray method (SAM) and with a false discovery rate of 10%. KEGG pathway classification was performed with the DAVID tool.

β-catenin and for some of the Wnt effectors (Table 2). No Wnt1 expression was detected.

Aging-related phosphorylation of β-catenin in the human mammary artery

As β-catenin activity is controlled by phosphorylation, we measured β-catenin phosphorylation. We detected no serine 33/37 or threonine 41 phosphorylations that induce β-catenin degradation in the cytosol (data not shown). However, in the O group, we observed a significant increase in β-catenin phosphorylation at serine 675 (×4.3, $P < 0.01$) and no significant change in total β-catenin or β-catenin phosphorylation at serine 552 (Fig. 3). Low-density lipoprotein receptor–related protein 6 (LRP6) phosphorylation, another important step of β-catenin activation, was barely detected in elderly patients and totally absent in younger patients (Fig. S3). These phosphorylation steps (Ser675 and Ser552) have been shown to induce β-catenin nuclear localization and transcriptional activity, and our findings therefore suggest that β-catenin is activated during aging.

Several studies have shown that, during aging and oxidative stress, β-catenin signals by interacting with FoxO proteins rather than LEF/TCF, inducing genes such as catalase, Mn superoxide dismutase (SOD2), Gadd45A, and p27/Kip1 (Essers *et al.*, 2005; Almeida *et al.*, 2007; Li *et al.*, 2008). Microarray data, confirmed by real-time PCR, showed no difference in the expression of the FoxO target genes catalase, p27/Kip1, and SOD2 (Table S4). These results suggest that the β-catenin/FoxO3a signaling pathway is not activated in aging human mammary artery media.

In vitro Wnt treatment leads to β-catenin phosphorylation at Ser675

We then investigated whether Wnt stimulation could be responsible for β-catenin phosphorylation at Ser552 and Ser675 in the human mammary artery. Cultured human VSMCs were treated with Wnt3a or Wnt1 (100 ng mL^{-1}) (similar results were obtained with 200 ng mL^{-1}, data not shown) for 4 h (Fig. S4) and 24 h (Fig. 4). Treatment with Wnt3a or Wnt1 did not alter β-catenin or cyclin D1 expression but significantly induced β-catenin phosphorylation at Ser675 after 4 and 24 h. Phosphorylation at Ser552 was not significantly modified in the same conditions. Wnt1 or Wnt3a treatment for 24 h also increased the expression of the two Wnt targets that we found induced in aging human arteries, IGFBP-2 and SPP1, as well as the expression of the β-catenin target WISP1 (Fig. 4). These results are in good agreement with the results obtained *ex vivo* and demonstrate that *in vitro* Wnt stimulation induces β-catenin phosphorylation at Ser675 in VSMC and expression of Wnt/β-catenin targets. As the results obtained with Wnt1 and Wnt3a were not significantly different, we used mainly Wnt3a for further experiments and checked that the effect of Wnt 1 was similar for some of them (data not shown).

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Table 2 Comparison of mRNA expression of Wnt/β-catenin signaling molecules and targets in human mammary artery media from middle-aged and old patients

	HUGO symbol	Gene name	Microarray fold change (O/M) (8M–7O)	qRT–PCR fold change (O/M) (11M–9O)
Wnt targets				
Osteopontin	SPP1	Secreted phosphoprotein 1 (osteopontin)	3.0**	5.25***
Versican	VCAN	Versican	2.2**	2.75*
WISP1	WISP1	WNT1 inducible signaling pathway protein 1	1.7**	2.18*
IGFBP-2	IGFBP2	Insulin-like growth factor binding protein 2, 36 kDa	1.7**	1.71*
p21	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	−2.4**	−1.91*
Cyclin D1	CCND1	Cyclin D1	0.9^{ns}	1.14^{ns}
RUNX2	RUNX2	Runt-related transcription factor 2	1.1^{ns}	1.48^{ns}
Wnt pathway				
Wnt3a	WNT3A	Wingless-type MMTV integration site family, member 3a	ND	1.17^{ns}
Wnt4	WNT4	Wingless-type MMTV integration site family, member 4	ND	-1.17^{ns}
Fzd4	FZD4	Frizzled homolog 4 (Drosophila)	1.4**	1.34*
Dvl1	DVL1	Dishevelled, dsh homolog 1 (Drosophila)	1.0^{ns}	-1.10^{ns}
β-catenin	CTNNB1	Catenin (cadherin-associated protein), beta 1, 88 kDa	1.1^{ns}	1.17^{ns}

Microarrays were applied to eight samples from the M group and seven samples from the O group, and qRT–PCR to 11 M and 9 O samples. Fold changes were calculated as the ratio between the mean value of the O group and the mean value of the M group. Statistical analysis of the microarray results was performed with significant analysis of microarray (SAM), implemented with the IlluminaGUI suite in R. A false discovery rate of 10% was chosen. A two-independent-sample Wilcoxon Mann–Whitney test was used for qRT–PCR results. Microarray $^{**}P < 0.001$; RT–PCR $^{*}P < 0.05$; $^{***}P < 0.005$, ns, not significant. ND, not detected on the microarrays.

Wnt/β-catenin activation does not induce cyclin D1 expression and proliferation in aged vascular smooth muscle cells (VSMC)

To examine the effect of β-catenin activation in aged VSMC, we compared the proliferative effect of Wnt3a on VSMC from young (6 weeks) and older (8 months) rat. Aging was confirmed by a strong senescence-associated β-galactosidase staining in VSMC from 8-month-old rats (Fig. S5). In response to serum, proliferation of VSMC from young and old rats was not significantly different (Fig. 5A). However, Wnt3a treatment significantly enhanced the proliferation of VSMC from young rats but not from old rats (Fig. 5A). In parallel, Cyclin D1 mRNA and protein expressions were induced by Wnt3a in VSMC from young animals but not from old ones (Fig. 5B,C). We therefore checked on β-catenin activation. Wnt3a activated phosphorylation of LRP6 and of β-catenin on Ser675 in VSMC from both groups (Fig. 6). Wnt3a treatment affected β-catenin subcellular localization similarly in VSMC from young and old rats as revealed by immunocytochemistry (Fig. 7). In both groups, at basal state, β-catenin staining was observed at the membrane, in the cytosol, and in the nucleus. Wnt3a treatment completely abolished the cytosolic β-catenin staining while reinforcing the nuclear staining. Taken together, the similar effects of Wnt3a on β-catenin phosphorylation and localization between the two cell types indicate that β-catenin is similarly activated in VSMC from young and old rats upon Wnt3a treatment.

These results were also observed using *in vitro* aged VSMCs. *In vitro* aging abolished Wnt3a-induced proliferation while β-catenin nuclear translocation confirmed its activation (Fig. S6).

Finally, we checked the role of β-catenin on cyclin D1 induction by Wnt3a using a siRNA that repressed β-catenin mRNA expression by 70% (Fig. S7). Beta-catenin depletion completely abolished the effect of Wnt3a on induction of cyclin D1 mRNA expression in VSMC from young rats (Fig. 8). Thus, in aged VSMC, β-catenin activation is unable to induce cyclin D1 expression and proliferation.

Discussion

Aging has been associated with an increased Wnt signaling that could participate in the senescence process of the muscle and the skin (Brack *et al.*, 2007; Liu *et al.*, 2007). In the vascular system, Wnt is known to induce VSMC proliferation but there are no data on Wnt in the aging vessel. Our study is based on differential gene expression found in mammary artery tissue from middle-aged patients (43–60 years old, group M) and aged patients (75–83 years old, group O). Although we could not obtain tissue from younger patients, who do not undergo surgery for coronary artery disease, the age difference is sufficient between the two groups to see a significant differential expression (see Fig. S1), indicating a rapid and important change in gene expression within 20–30 years of aging. We also report here that the expression of multiple known Wnt/β-catenin target genes is modulated in the aging human vascular wall, suggesting an activation of the Wnt pathway. Activation of the Wnt/β-catenin pathway in aged vessels is also supported by an increased phosphorylation of a β-catenin-activating site. Induction of these targets and of β-catenin phosphorylation by Wnt was confirmed *in vitro* on human VSMC from 55- to 60-year-old patients. In our hands, VSMC from mammary or coronary

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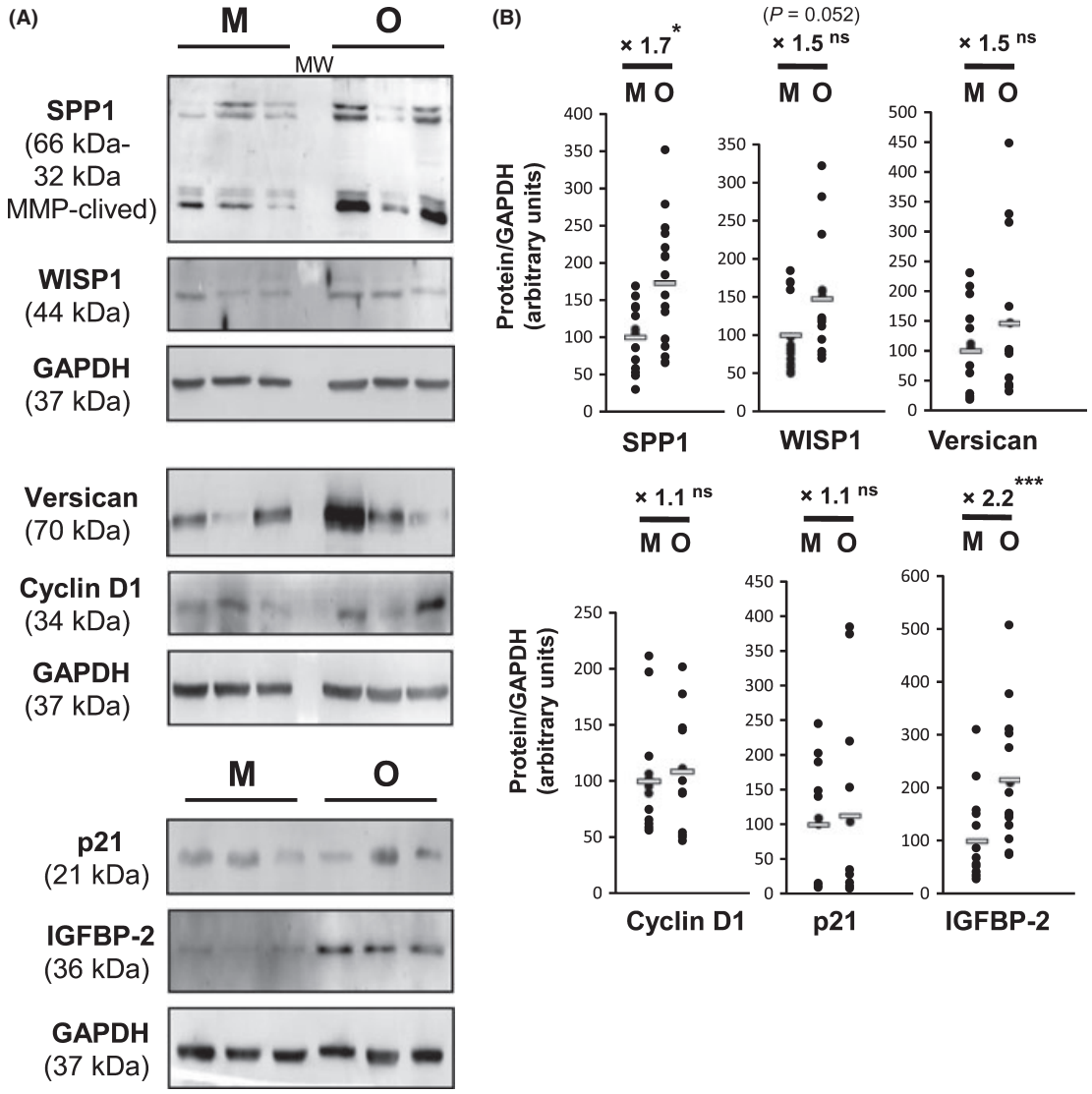


Fig. 2 Induction of Wnt/β-catenin targets expression in aged human mammary arteries. (A) Western blots of Wnt/β-catenin targets (osteopontin (SPP1), WISP1, versican, IGFBP-2, p21, and cyclin D1) and GAPDH of three patients representative from each group. (B) Protein signals were quantified and normalized to GAPDH levels. The graphs show all values (black points) obtained in the two groups for each Wnt/β-catenin target (M, n = 14; O, n = 16; except for p21 and cyclin D1: M, n = 11; O, n = 13). Fold changes (O group mean/M group mean) and statistical significance are indicated above the graphs. The gray line shows the mean value in each group (*$P < 0.05$, ***$P < 0.005$; ns, not significant).

arteries obtained from older patients could not be maintained in culture, and therefore, we could not compare their ability to respond to Wnt. This could be explained by a decrease in human VSMC proliferative capacities with aging (Ruiz-Torres *et al.*, 1999).

We also demonstrate that Wnt treatment failed to trigger proliferation of VSMC from old rats although β-catenin is activated by phosphorylation in these cells. Wnt-induced β-catenin nuclear translocation in VSMC from both young and old rats is suggested by the disappearance of the cytosolic β-catenin staining while there was no change in total β-catenin levels. This is associated, in VSMC from young rats, with β-catenin phosphorylation, cell proliferation, and cyclin D1 induction, indicating that in our experiments, extinction of cytosolic β-catenin staining reflects β-catenin activation. As similar effects on β-catenin localization were obtained in VSMC from old rats, this strongly suggests, together with β-catenin phosphorylation, that β-catenin is indeed activated in these cells. Interestingly, Wnt effects, in particular on proliferation, were dependent on the presence of serum. This could reflect an interaction of factors from the serum with low-density lipoprotein receptor–related proteins (LRP5 or LRP6) whose critical role in the regulation of Wnt signaling has been established (for review see He *et al.*, 2004).

Finally, we show that Wnt-induced cyclin D1 upregulation observed in VSMC from young rats is dependent on β-catenin activation and does not occur in VSMC from older rats, indicating a shift in the targets of the Wnt/β-catenin pathway with age. Taken together, all these results suggest that the Wnt/

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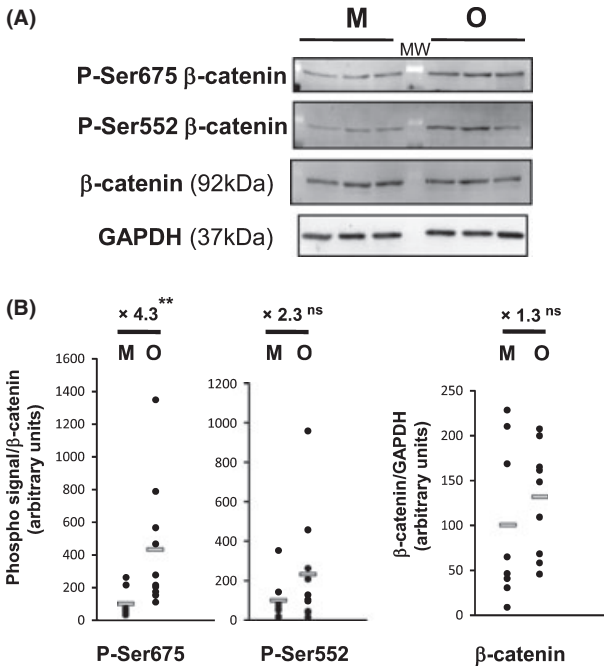


Fig. 3 Activation of β-catenin phosphorylation in aged human mammary arteries. (A) Western blots showing Ser552/Ser675-phosphorylated β-catenin, total β-catenin, and GAPDH protein expression in three representative patients from each group. (B) Protein signals were quantified and normalized to GAPDH levels. β-catenin phosphorylation signals were normalized to the total β-catenin signal. The graphs show all the values (black points) obtained in the two groups (M, n = 8; O, n = 10). Fold changes (O group mean/M group mean) and statistical significance are indicated above the graphs. The gray line shows the mean value in each group (**$P < 0.01$; ns, not significant).

β-catenin pathway is activated during human vascular aging but that the proliferative response to Wnt is altered downstream of β-catenin activation.

We found that the expression of Fzd4, a Wnt receptor, was increased and that several known direct target genes of the Wnt/β-catenin pathway were modulated during aging, pointing to increased Wnt stimulation in elderly subjects. Wisp1, versican, IGFBP-2, and SPP1 were upregulated and p21 was downregulated, as already observed during *in vitro* Wnt treatment in this study and in others (Xu *et al.*, 2000; El-Tanani *et al.*, 2001; Naishiro *et al.*, 2005; Rahmani *et al.*, 2005; Quasnichka *et al.*, 2006; Scheller *et al.*, 2008). Our data are in agreement with two other recent studies showing enhanced Wnt signaling during aging (Brack *et al.*, 2007; Liu *et al.*, 2007). Increased Wnt activity was observed in the serum of aged animals, (Brack *et al.*, 2007) possibly because of diminished interaction with Klotho protein, a Wnt antagonist (Liu *et al.*, 2007).

The major downstream Wnt signaling target is β-catenin. Activation of the β-catenin signaling pathway could occur by inhibition of GSK3β phosphorylation of the N-terminal part of β-catenin (which leads to a rapid degradation of the protein) but also by phosphorylation in the C-terminal part of the protein at serine 552 and serine 675, which facilitate the translocation of β-catenin to the nucleus and enhance its transcriptional activity, as it has been recently shown *in vitro* upon Akt or PKA activation (Fang *et al.*, 2007; Taurin *et al.*, 2008), including in VSMC (Taurin *et al.*, 2008). We observed an enhanced β-catenin phosphorylation at Ser675 in the aging vascular wall and also demonstrated *in vitro,* in human and rat VSMC, that Wnt enhances β-catenin phosphorylation at Ser675. Altogether, the β-catenin phosphorylation and gene expression regulations observed *in vitro* upon Wnt treatment are very similar to those obtained on aging human mammary arteries. Therefore, although we cannot exclude a role of PKA, Akt or other signaling pathways in β-catenin phosphorylation, our data strongly suggest an activation of the Wnt/β-catenin pathway during vascular aging.

In the nucleus, β-catenin heterodimerizes with different transcription factors such as T-cell factor/lymphoid enhancer factor (TCF/LEF) or FoxO. Bone aging has been linked to the activation by reactive oxygen species of the FoxO factor, which would then interact with β-catenin and compete with TCF factors for a limited pool of β-catenin. This shift from a β-catenin/TCF complex to a β-catenin/FoxO complex thus enhances the expression of FoxO target genes and antagonizes the expression of target genes of the canonical Wnt/β-catenin pathway (Almeida *et al.*, 2007). In contrast, we observed a regulation of β-catenin/TCF target genes in mammary arteries from older patients but no change in the expression of catalase, SOD2 or p27/Kip1, known FoxO direct transcriptional target genes (reviewed in Hoogeboom & Burgering, 2009). In addition, our microarray data do not show any regulation of the other known FoxO target genes. Therefore, although aging is characterized by an enhanced oxidative stress and although we cannot exclude that other FoxO target genes may be regulated, our data do not support increased FoxO activity or β-catenin/FoxO interaction in nonatherosclerotic vessel walls during advanced human aging. In support for this result, Funato *et al.* (2006) reported that H_2O_2-induced oxidative stress activates Dishevelled, a Wnt signaling molecule, leading to β-catenin/TCF activation. It is also possible that Wnt stimulation during aging could inhibit FoxO activity, as observed in cancer cells (Dehner *et al.*, 2008).

Would an increase in Wnt/β-catenin signaling during aging be protective or deleterious? In Klotho knockout mice, which age prematurely, continuous Wnt exposure induces cellular senescence and a marked decrease in cell proliferation (Liu *et al.*, 2007). In keeping with these observations, our data show that the proliferative response to β-catenin activation is abolished in VSMC from old animals, as well as *in vitro*-aged VSMC. As Wnt-induced VSMC proliferation seem to depend on cyclin D1 increase (Quasnichka *et al.*, 2006), this alteration could be due, at least in part, to the absence of cyclin D1 upregulation by Wnt/β-catenin signaling in aged VSMC although we cannot exclude that other cyclins or proliferation factors modulated by Wnt/β-catenin signaling could also be implicated. This result suggests that VSMCs in vessels of elderly subjects may not proliferate in response to β-catenin activation contributing to the impaired proliferative and migratory capacities of VSMCs from elderly subjects (Ruiz-Torres *et al.*, 1999). In addition to this

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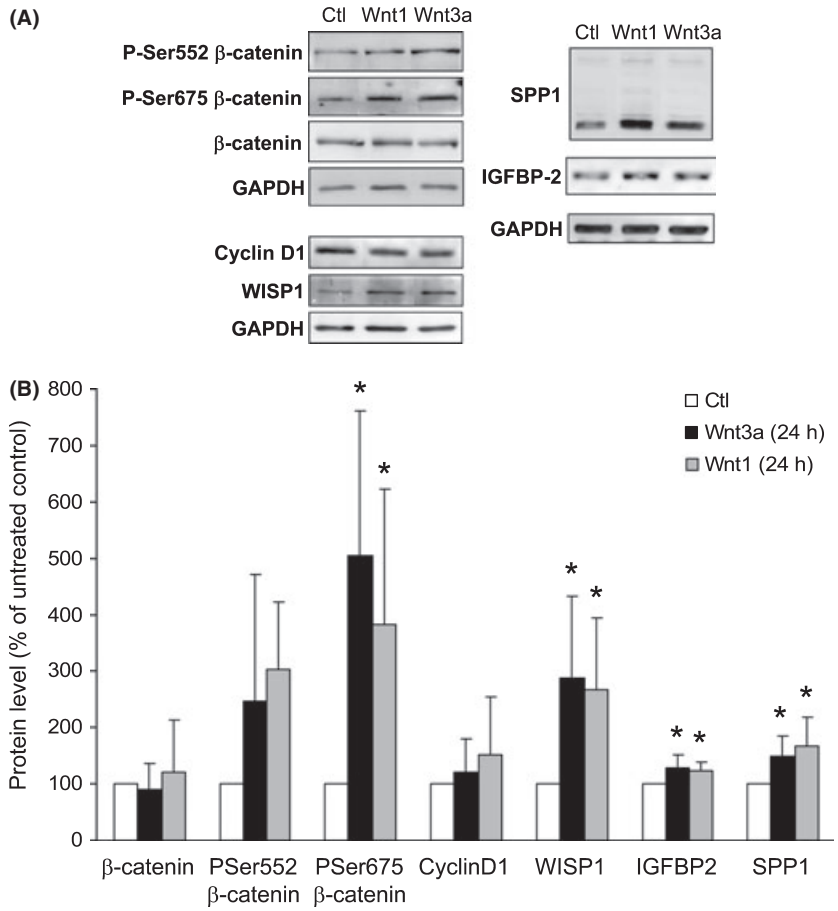


Fig. 4 Wnt activates β-catenin phosphorylation on serine 675 in human vascular smooth muscle cells (VSMCs) *in vitro*. Cultured human VSMCs were treated with vehicle (Ctl) or recombinant Wnt1 or Wnt3a (100 ng mL^{-1}) for 24 h, and protein expression was then measured. (A) Western blots showing Ser552 or Ser675 phosphorylated β-catenin, total β-catenin, cyclin D1, WISP1, IGFBP-2, and SPP1 proteins in control and Wnt-treated cells. (B) Protein signals were quantified and normalized to GAPDH levels. β-catenin phosphorylation signals were normalized to the total β-catenin signal. Data are means ± SD of five independent experiments (ns, not significant and $*P < 0.05$ vs. untreated control).

switch from a proliferative to a nonproliferative response to β-catenin activation during aging, regulation of other targets than cyclin D1 could participate in the inhibition of VSMC proliferation. We observed a clear induction of IGFBP-2 protein expression in aged human arterial cells and in cultured VSMC after Wnt treatment. IGFBP-2 has been shown to inhibit IGF-I-stimulated DNA synthesis and VSMC migration (Hsieh *et al.*, 2003), and its induction in human arteries from older patients could therefore contribute to the loss of VSMC proliferative capacity.

The aging-related modification of downstream Wnt target genes such as SPP1 and WISP1 could participate to the development of a pre-pathological state. Increased expression of SPP1, a matricellular protein with proproliferative and proinflammatory properties (Scatena *et al.*, 2007), has been linked to medial thickening (Isoda *et al.*, 2002). Moreover, SPP1 is found associated with mineralized deposits in human arteries and considered as a calcification marker although it seems to inhibit calcification (for review, Almeida *et al.*, 2007; Scatena *et al.*, 2007; Blom *et al.*, 2009). Similarly, WISP1 is described as a profibrotic and proinflammatory factor and is implicated in osteo-arthritis (Blom *et al.*, 2009; Konigshoff *et al.*, 2009). So Wnt/β-catenin signaling in aged VSMC could participate in reprogramming the cells towards a more osteogenic phenotype. However, we did not see in aged human mammary arteries any regulation of RunX2, a key transcription factor in osteoblast differentiation, which participates in the VSMC calcifying process (Gaur *et al.*, 2005; Byon *et al.*, 2008; Speer *et al.*, 2009). Altogether, our observations are in line with recent publications showing that senescent VSMC can transdifferentiate into osteochondrogenic precursors, an early step in vascular calcification (Burton *et al.*, 2009; Nakano-Kurimoto *et al.*, 2009; Speer *et al.*, 2009).

In conclusion, we observed an increase in β-catenin-activating phosphorylation and a regulation of Wnt targets during human arterial wall aging, pointing to enhanced Wnt stimulation in aged mammary arteries. We also found that during aging, VSMC switch from a proliferative to a nonproliferative response to Wnt/β-catenin activation by the loss of cyclin D1 upregulation. In addition, other targets of the Wnt/β-catenin pathway are increased like IGFBP-2, SPP1, and WISP1. These results are consistent with the diminished capacities to proliferate and migrate of VSMC from elderly patients and the recent observations of an enhanced Wnt signaling during aging. Moreover,

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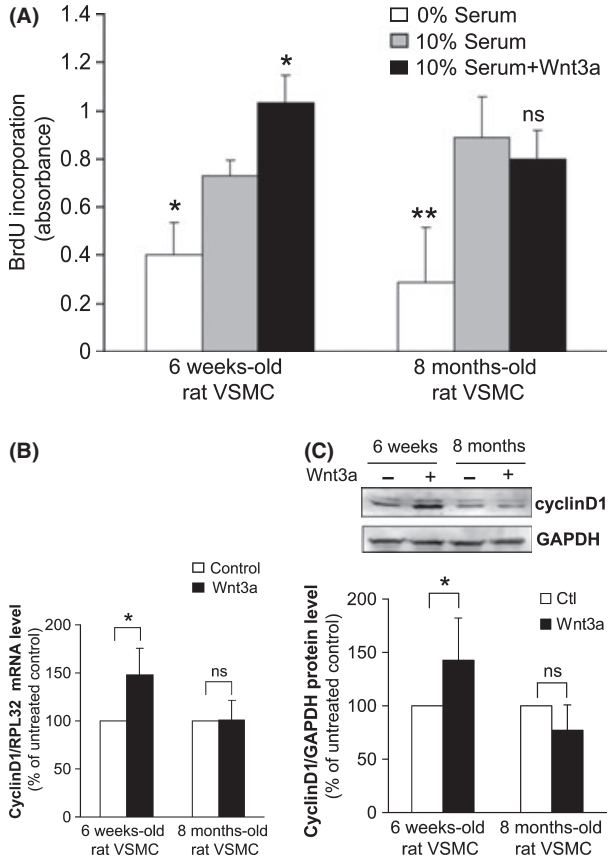


Fig. 5 Aging impairs Wnt-induced proliferation and cyclin D1 expression in rat vascular smooth muscle cells (VSMCs). Rat VSMCs (passage 2–5) from 6-week- and 8-month-old rats were cultured with 10% serum (or 0.1% serum for nonproliferative controls) and incubated with Wnt3a (100 ng mL^{-1}) for 24 h. (A) Proliferation was assayed by measuring bromodeoxy-uridine incorporation using a colorimetric assay. Cell proliferation was measured and expressed as absorbance value. Data are means ± SD of four independent experiments (ns, not significant and $^{**}P < 0.01$, vs. 10% serum in each group). (B) mRNA levels of Cyclin D1 were determined by qRT–PCR and normalized to RPL32 levels. In each group, data are expressed as % of the untreated value and are the means ± SD of five independent experiments (ns, not significant; $^{*}P < 0.05$ vs. untreated respective control). (C) Protein levels of Cyclin D1 were determined by western blot and normalized to GAPDH levels. In each group, data are expressed as % of the untreated control value and are the means ± SD of five independent experiments (ns, not significant and $^{*}P < 0.05$ vs. untreated respective control).

some of the Wnt/β-catenin upregulated targets could contribute to altering the VSMC phenotype towards a more fibrotic/osteogenic phenotype. Deciphering the mechanisms involved in the activation of this pathway, and also in the switch from a proliferative to a nonproliferative signaling pathway, should help to develop strategies to prevent aging-related vascular diseases.

Experimental procedures

Human media and sample preparation

Waste fragments of internal mammary arteries were obtained from patients undergoing coronary artery bypass surgery at the Cardiology Institute of Hôpital Pitié-Salpêtrière, Paris, France, in accordance with French legislation (L.1211-3-9). On the same day, the mammary artery segments, kept in physiological solution at 4 °C, were dissected by mechanically dissociating the media from the adventitia and were immediately frozen in liquid nitrogen.

The patients were subdivided into those under 60 years of age (M group, 43–60 years, 14 patients) and those over 75 (O group, 75–83 years, 16 patients). All the patients included in this study were male, and most had aortic occlusion; there was no significant difference in the indications or treatments between the groups (see Table 1).

RNA isolation and quality measurement

Frozen medias (usually 50–150 mg) were crushed on ice in six volumes of lysis binding buffer from the *mir*Vana miRNA isolation kit (Ambion, Applied Biosystems, Courtaboeuf, France), and total RNA (5–20 μg) was prepared according to the manufacturer's instructions.

To avoid genomic contamination, each RNA was treated with rDNase I (DNA-free™ DNase) and with removal reagents (all from Ambion). RNA concentrations were determined with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Courtaboeuf, France). RNA quality was determined using the RNA nano LabChip® and a 2100 Bioanalyzer (Agilent Technologies, Massy, France). Only patients with an RNA integrity number higher than 7 (as determined by the Bioanalyzer) and a 28S/18S ratio above 1 were selected for the following studies.

RNA from rat VSMCs were prepared using RNeasy Mini kit from Qiagen (Courtaboeuf, France) according to the manufacturer's instructions.

Expression profiling

Expression profiling was performed with the HumanRef-8 v2 BeadChip kit (Illumina Inc., San Diego,CA, USA) representing ~24 500 genes and expressed sequence tags (ESTs), as recommended by the manufacturer. Briefly, 250 ng of total RNA from eight samples in group M and seven samples in group O were amplified with the TotalPrep RNA amplification kit (Ambion), and biotin-16-UTP was incorporated. The concentration and quality of amplified RNA were determined as described previously. The DirectHyb assay was applied to 750 ng of each amplified RNA: the RNA was labeled with streptavidin-Cy3 and directly hybridized on the Beadchips for 16 h at 58 °C. The Beadchips were then washed and scanned on the Illumina BeadXpress® platform (http://www.p3s.chups.jussieu.fr/), following the manufacturer's protocol. Each chip was first analyzed with Illumina's BeadScan software. The arrays were then checked for background, hybridization intensities, and homogeneity and were quantile-normalized using the IlluminaGUI R-package (Schultze & Eggle, 2007). The normalized log-ratio values were used to compare gene expression between the O group and the M group. Statistical analysis and genes and samples clustering were performed with IlluminaGUI, using

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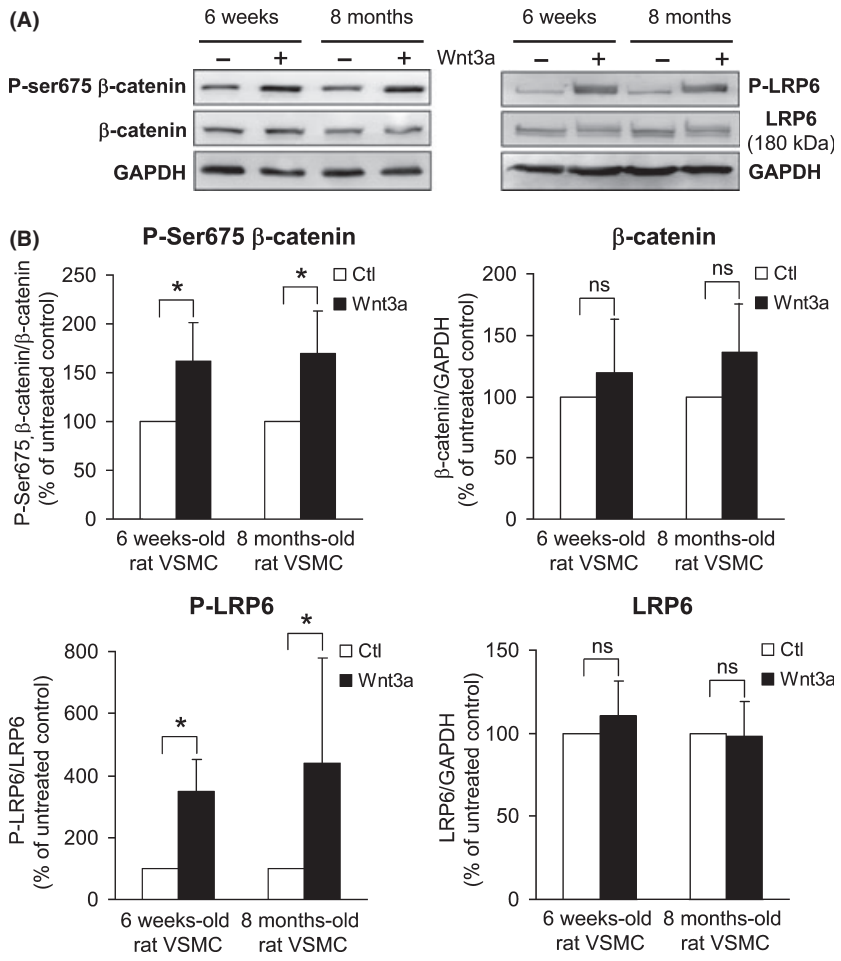


Fig. 6 Aging does not impair β-catenin and low-density lipoprotein receptor–related protein 6 (LRP6) activation in Wnt-treated vascular smooth muscle cell (VSMC). (A) Representative western blots showing Ser675-phosphorylated β-catenin, total β-catenin, phospho-LRP6, total LRP6 and GAPDH in untreated and Wnt3a-treated VSMC from 6-weeks- and 8-months-old rats. Rat VSMCs (passage 2–5) from 6-weeks- and 8-months-old rats were treated with Wnt3a (100 ng mL^{-1}) for 24 h. (B) Protein signals were quantified and normalized to GAPDH. Phosphorylation signals were normalized to their respective total protein signal. In each group, data are expressed as % of the untreated control value and are the means ± SD of 4–5 independent experiments (ns, not significant; $*P < 0.05$ vs. untreated respective control).

significance analysis of microarrays (SAM) with two classes and unpaired data (Tusher *et al.*, 2001), hierarchical clustering, and principal component analysis. Genes that were differentially expressed between the two groups were selected with SAM, with a false discovery rate (FDR) threshold of 10%. Functional clustering was performed with the DAVID tool (http://david.abcc.ncifcrf.gov/) (Dennis *et al.*, 2003; Huang da *et al.*, 2009). The microarray data have been deposited in the GEO repository (http://www.ncbi.nlm.nih.gov/geo/) with the record number GSE16487.

RT–PCR

Reverse transcriptase–PCR analysis was performed as described elsewhere (Dupuis *et al.*, 2004), using the Absolute QPCR SYBR green mix (ABgene, Courtaboeuf, France) on an MX3005P QPCR system (Stratagene, Agilent Technologies, Massy, France). Each primer pair (list available in Table S5) was designed with Primer Express software (Applied Biosystems) or previously published and was checked by using a standard curve to ensure that the reaction efficiency was above 90%. Melt curves were analyzed to check the specificity of each RT–PCR amplification. Transcript levels were normalized to the RPL32 mRNA content, and the relative transcript level between two samples was calculated by using the $2^{-\Delta\Delta CT}$ method.

Protein preparation and western blot

Frozen media samples from human mammary arteries were homogenized in six volumes of cold Cell Disruption Buffer from the mirVana PARIS kit (Ambion) supplemented with protease inhibitors (Sigma-Aldrich, Saint-Quentin Fallavier, France) and phosphatase inhibitors (Sigma-aldrich), using a Polytron homogenizer. The total protein concentration was determined with the Bio-Rad assay (Bio-Rad, Marne-la-coquette, France). Alternatively, protein extracts from rat VSMCs were prepared

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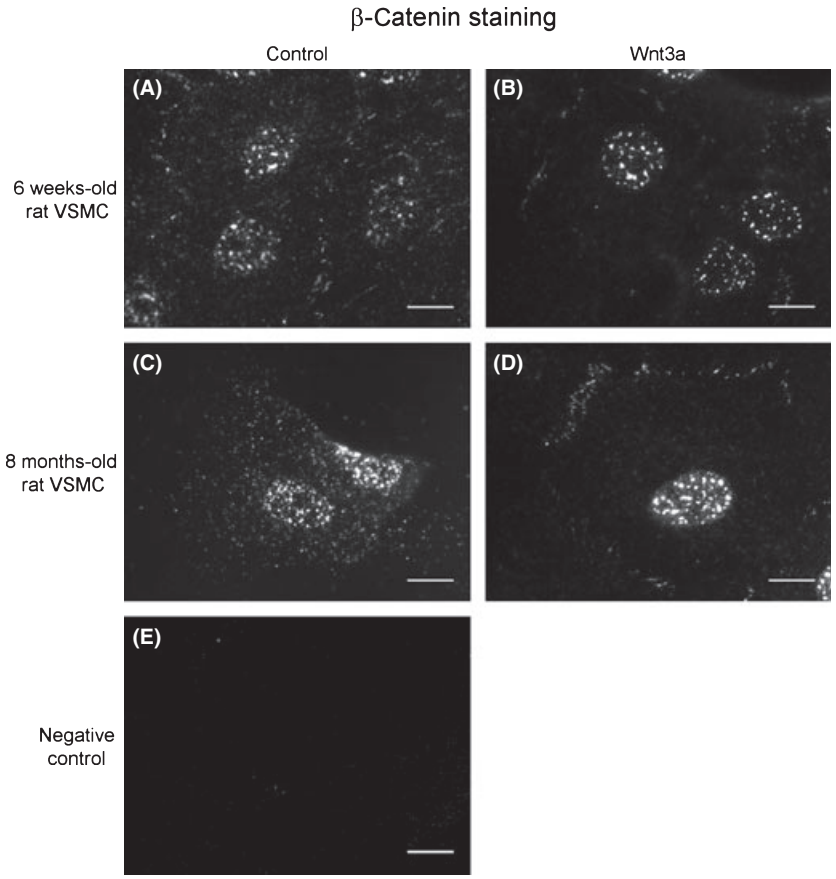


Fig. 7 Aging does not impair β-catenin translocation in Wnt-treated rat vascular smooth muscle cell (VSMC). Immunofluorescence localization of β-catenin in untreated or Wnt3a (100 ng mL^{-1} for 24 h)-treated VSMC from 6-weeks-old rats (panels A, B) and 8-months-old rats (panels C, D). Panel E, negative control for immunofluorescence labeling (anti-rabbit IgG). Scale bar is 10 μm for all panels. At basal state, β-catenin staining (gray) is observed at the plasma membrane, in the cytosol, and in the nucleus in both young and old VSMC (panels A, C), whereas it is clearly absent from the cytosol in Wnt3a-treated cells (panels B, D). The data were confirmed in three different experiments.

using Promokine Mammalian Whole Cell Extraction kit (PromoCell GMBH, Heidelberg, Germany) and phosphatase inhibitors.

Equal amounts (50 μg) of total protein were separated by 7.5% or 10% SDS–PAGE followed by immunoblotting with a Trans-Blot Semi-Dry (Bio-Rad), following the manufacturer's protocol. The following antibodies were used: rabbit polyclonal antibodies to SPP1 (ab8448, 1/2000), p21 (ab18209, 1/500), WISP1 (ab10737, 1/500), and versican (ab19345, 1/2000) were from Abcam (Paris, France), mouse anti-human cyclin D1 (556470, 1/1000) was from BD Biosciences (Le Pont de Claix, France), rabbit anti-Phospho-LRP6(Ser1490) (#2568, 1/500), anti-LRP6(C5C7) (#2560, 1/500), anti-IGFBP-2 (#3922, 1/1000), and anti-β-catenin sampler kit (#2951) containing rabbit anti-β-catenin(6B3) (1/1000), anti-phospho-β-catenin (Ser33/37/Thr41) (1/1000), anti-phospho-β-catenin (Ser552) (1/1000), and anti-phospho-β-catenin (Ser675) (1/1000) were from Cell Signaling, Ozyme (Saint Quentin en Yvelines, France). Western blots were revealed with the ECLplus reagent (GE Healthcare, Diegem, Belgique) and scanned with an Ettan-DIGE Imager (GE Healthcare). Densitometric analysis was performed

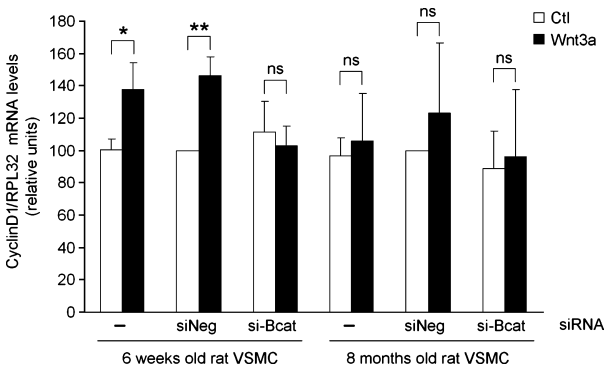


Fig. 8 Wnt induction of cyclin D1 in rat vascular smooth muscle cell (VSMC) is β-catenin dependent. Cyclin D1 mRNA levels were measured in untreated and Wnt3a-treated VSMC and normalized to RPL32 mRNA levels. VSMC from young (6 weeks old) or old (8 months old) rats were not transfected or transfected with a negative siRNA (siNeg) or with a β-catenin siRNA (si-Bcat). Beta-catenin depletion was checked by PCR (see Fig. S8). In each group, data are expressed as % of the value of untreated siNeg-transfected cells and are the means ± SD of five independent experiments (ns, not significant; $*P < 0.05$ and $**P < 0.01$ vs. untreated corresponding control).

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with ImageQuant TL software (GE Healthcare) or NIH Image/ImageJ, and the expression level of each protein was normalized to GAPDH.

Human mammary artery smooth muscle cell (SMC) culture and treatment

Smooth muscle cells were isolated from the media of mammary arteries from 55–60 years old patients, using a protocol described for rat aortic SMCs (Vallot *et al.*, 2000). Cells were resuspended in human SMC basal medium containing 20% of supplement mix (basic fibroblast growth factor, epidermal growth factor, insulin, and fetal calf serum) (Promocell GMBH) and 1% GIBCO Antibiotic-antimycotic (Invitrogen, Cergy Pontoise, France). After 2 days, the medium was replaced with basal medium containing 5% supplement mix. Cells at passages 2–4 were used for the experiments. Recombinant human Wnt1 was from PromoKine (Promocell GMBH) and recombinant human Wnt3a was from R&D Systems (Lille, France). Both were used at 100 ng mL^{-1} (from a solution at 200 μg mL^{-1} in sterile water or PBS) for the times indicated in the figures. Total RNA and protein were then isolated with the *mir*Vana PARIS kit (Ambion, Applied Biosystems) following the manufacturer's instructions.

Proliferation of VSMCs from young (6 weeks) and old (8 months) rats

Rat VSMCs were prepared as previously described (Vallot *et al.*, 2000) using aortas from 6-week- or 8-month-old rats and used at passages 2–5. Rat aortic SMCs were cultured in 96-well tissue culture plates for 2 days in DMEM (Gibco, Invitrogen, Cergy-Pontoise, France) supplemented with 10% or 0% fetal bovine serum (Biowest, Nuaillé, France) and 1% penicillin/streptomycin (Gibco). Cells were incubated with recombinant human Wnt1 or Wnt3a (100 ng mL^{-1}) for 24 h and bromodeoxy-uridine (BrdU) was added for the last 16 h. The plates were then washed and a colorimetric BrdU cell proliferation assay was applied, as recommended by the manufacturer (Roche Diagnostics, Meylan, France).

Beta-catenin knockdown and Wnt treatment

Silencer select negative control 1 siRNA (siNeg) and Silencer select predesigned siRNA against ctnnB1 gene (s136460) (siBeta-catenin) were from Ambion (Applied Biosystems). SiRNAs were retrotransfected using Lipofectamine 2000 transfection reagent (Invitrogen). Briefly, 6-well plates were coated with 25 nM siRNA (6 μL from 5 μM stock solution) mixed with 3 μL Lipofectamine in 300 μL Opti-MEM (Invitrogen). Rat VSMCs (10^5 cells) from 6-week- or 8-month-old animals resuspended in 10% FCS–DMEM were then added. Medium was changed 24 h later. In some cases, after 48 h, a Wnt3a treatment at 100 or 200 ng mL^{-1} was performed. Cells were harvested 72 h after transfection and total RNA was prepared.

Senescence-associated β-galactosidase staining

Cells were cultured in 10-mm-diameter dishes, washed with PBS, and fixed for 10 min in 2% formaldehyde solution. After washing in PBS, cells were incubated in senescence-associated β-galactosidase staining solution [X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) 1 mg mL^{-1}; 40 mM citric acid–sodium phosphate buffer pH 6.0; 5 mM ferrocyanide; 5 mM ferricyanide; 150 mM NaCl and 2 mM $MgCl_2$] for 16 h at 37 °C (Dimri *et al.*, 1995).

Immunofluorescence

Rat VSMCs were maintained for 3 days in serum-free medium. They were incubated for 24 h with Wnt3a at 100 ng mL^{-1}, then fixed with cold methanol, blocked in PBS + 5% BSA, and incubated for 18 h at 4 °C with anti-beta-catenin (from Cell signaling, 1/50 dilution in PBS + 1% BSA). The secondary goat anti-rabbit antibody Alexa594 (Invitrogen; 1/500 dilution in PBS + 1% BSA) was added for 45 min at 37 °C before mounting in DAKO fluorescent mounting medium (Dako France, Trappes, France).

Images were acquired with a cooled CoolSnap camera (Roper Scientific, Evry, France) on an Olympus epifluorescence microscope (60×, UPlanSApo, 0.17) using Metamorph software (Molecular Devices, Sunnyvale, CA, USA)

Statistical analysis

Data are expressed as means ± SD. Patient characteristics were compared by chi-squared or nonparametric Mann–Whitney analysis between the two groups. Microarrays results were analyzed with the SAM method. Experiments with two groups were analyzed with the nonparametric Mann–Whitney test. When several pairwise comparisons were performed with the Mann and Whitney test within the same experiment, we used a Bonferroni-adjusted *P* value (*P* value × number of tests). Multiple comparisons used two-way ANOVA, followed by Student Newman–Keuls post hoc test. *P* values below 0.05 were considered to denote significant differences.

Acknowledgment

We thank Cécile Duplàa for helpful discussions.

Sources of funding

This work was supported by INSERM, Université Pierre et Marie Curie, and Fondation pour la Recherche Médicale (FRM). Dr Marchand was supported by Fondation pour la Recherche Médicale (FRM) and Fondation Lefoulon-Delalande. Dr Nadaud was supported by the European Vascular Gene Network (EVGN) and by IFR14. Dr Lompré was supported by the Leducq Foundation.

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Author contributions

Dr A. Marchand designed the research and acquired the data; F. Atassi and A. Gaaya helped to acquire the data; Dr P. Leprince and Dr C. LeFeuvre provided human tissues and patient characteristics; Pr F. Soubrier made critical revision of the manuscript; Dr S. Nadaud and Dr A.-M. Lompré conceived the research, handled funding and supervision and helped to analyze the data; D. A. Marchand, S. Nadaud and A-M Lompré wrote the manuscript. All the authors have read and criticized the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Unsupervised hierarchical clustering and principal components analysis of microarray data for O vs. M human mammary arteries.

Fig. S2 Gene ontology classification of age-regulated genes in human mammary arteries.

Fig. S3 Phospho-LRP6 detection in human mammary arteries from elderly patients.

Fig. S4 Wnt activates β-catenin phosphorylation on serine 675 in human VSMCs *in vitro* after 4 h of treatment.

Fig. S5 Senescence-associated β-galactosidase staining in VSMC from 8 months-old rats.

Fig. S6 *In vitro* aging of VSMC impairs Wnt-induced cell proliferation but not β-catenin activation.

Fig. S7 Inhibition of β-catenin expression by β-catenin siRNA transfection.

Table S1 Microarray results: genes regulated in aging human mammary artery media.

Table S2 RT-PCR confirmation of microarray data.

Table S3 Functional annotation clustering analysis of age-regulated genes in human mammary arteries.

Table S4 Expression of FoxO targets genes in aged human mammary arteries.

Table S5 Real-time PCR primers used in this study.

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