Wnt/β-Catenin Signaling Stimulates Chondrogenic and Inhibits Adipogenic Differentiation of Pericytes
Potential Relevance to Vascular Disease?

John Paul Kirton,* Nicola J. Crofts,* Sarah J. George, Keith Brennan, Ann E. Canfield

Abstract—The aberrant differentiation of pericytes along the adipogenic, chondrogenic, and osteogenic lineages may contribute to the development and progression of several vascular diseases, including atherosclerosis and calcific vasculopathies. However, the mechanisms controlling pericyte differentiation and, in particular, adipogenic and chondrogenic differentiation are poorly defined. Wnt/β-catenin signaling regulates cell differentiation during embryonic and postnatal development, and there is increasing evidence that it is involved in vascular pathology. Therefore, this study tested the hypothesis that Wnt/β-catenin signaling regulates the chondrogenic and adipogenic differentiation of pericytes. We demonstrate that pericytes express several Wnt receptors, including LDL receptor–related proteins 5 and 6, and Frizzled 1 to 4 and 7, 8, and 10, and that Wnt/β-catenin signaling is stimulated by both Wnt3a and LiCl. Furthermore, induction of Wnt/β-catenin signaling by LiCl enhances chondrogenesis in pericyte pellet cultures in the presence of transforming growth factor-β3, as demonstrated by increased Sox-9 expression and glycosaminoglycan accumulation into the matrix. In contrast, transduction of pericytes with a recombinant adenovirus encoding dominant-negative T-cell factor-4 (RAd/dnTCF), which blocks Wnt/β-catenin signaling, inhibited chondrogenesis, leading to reduced Sox-9 and type II collagen expression and less glycosaminoglycan accumulation. Together, these data demonstrate that transforming growth factor-β3 induces the chondrogenic differentiation of pericytes by inducing Wnt/β-catenin signaling and T-cell factor–induced gene transcription. Induction of Wnt/β-catenin signaling also attenuates adipogenic differentiation of pericytes in both pellet and monolayer cultures, as demonstrated by decreased staining with oil red O and reduced peroxisome proliferator-activated receptor γ2 expression. This effect was negated by transduction of pericytes with RAd/dnTCF. Together, these results demonstrate that Wnt/β-catenin signaling inhibits adipogenic and enhances chondrogenic differentiation of pericytes. (Circ Res. 2007;101:581-589.)

Key Words: pericytes ▪ differentiation ▪ Wnt signaling ▪ chondrogenesis ▪ vascular disease

There is compelling evidence that cells with multilineage potential (pericytes, calcifying vascular cells, smooth muscle cells, and adventitial myofibroblasts) are present within the walls of blood vessels and that the aberrant differentiation of these cells contributes to the development and progression of several vascular pathologies.1–5 For example, the acquisition of an adipogenic phenotype by some populations of vascular smooth muscle cells is thought to contribute both to the development of atherosclerotic lesions and to plaque instability.9 On the other hand, the differentiation of vascular progenitor cells into chondrocytes and osteoblasts is thought to result in the deposition of cartilage and bone in the blood vessel wall.1–8 This latter process, which has been given the generic name “vascular calcification,” is highly correlated with increased morbidity and mortality in patients with atherosclerosis, diabetes, end-stage renal disease, and calciphylaxis.1–4 The mechanisms controlling the osteogenic differentiation of these cells have been the subject of intensive investigations.1–4 However, little is known about how their differentiation along the adipogenic and chondrogenic lineages is regulated.

There is increasing interest in the potential role that pericytes may play in vascular disease because it is now recognized that these cells are present throughout the entire human vascular bed and are not just present in microvessels, as originally thought.7,10–12 Furthermore, pericytes are recruited to the intimal and medial layers of arteries as part of the angiogenic response associated with several diseases, including atherosclerosis,1,11,13 and these cells have been identified at sites of calcification in these vessels.13 The similarities between pericytes and other progenitor cells isolated from arteries have been highlighted by several
groups, although there is also some evidence to suggest that calcifying vascular cells have a more limited differentiation potential than pericytes. Currently, it is not clear how these different cell populations are related.

The Wnt/β-catenin signaling pathway is known to be a critical regulator of adipogenic, chondrogenic, and osteogenic differentiation, although the mechanisms by which this pathway exerts its effects are still not fully understood. In embryos, low levels of Wnt/β-catenin signaling stimulate, whereas high levels of Wnt/β-catenin signaling inhibit, chondrogenic differentiation of progenitor cells. However, Wnts have been shown to both inhibit and stimulate chondrogenic differentiation of adult progenitor cells; this apparent disparity may be caused by differences in the stage of differentiation of the target cells, the levels of Wnt activity, and or crosstalk between Wnt and other signaling pathways (eg, transforming growth factor (TGF)β and bone morphogenetic protein). Wnt signaling inhibits the adipogenic differentiation of predipocytes. However, there is still some confusion about the mechanism by which this occurs, with suggestions that it is mediated by both β-catenin-independent and –dependent signaling.

Evidence is now emerging to suggest that Wnt signaling is also a key regulator of several vascular pathologies. For example, recent studies have shown that the β-catenin/T-cell factor (TCF) signaling pathway regulates vascular smooth muscle cell proliferation, suggesting that it may be involved in intimal thickening. However, there is still some confusion about the mechanism by which this occurs, with suggestions that it is mediated by both β-catenin-independent and –dependent signaling.

In order to test the hypothesis that Wnt signaling regulates chondrogenic and adipogenic differentiation of pericytes and to determine whether this occurs in a β-catenin–dependent manner, we demonstrate that pericytes express several Wnt receptors and Wnt signals are activated as a consequence of bone morphogenetic protein-2-induced Msx2 expression within calcified plaques in LDLR mice, resulting in increased osteogenesis. Other studies also have shown that LDL receptor–related protein (LRP)5 expression is upregulated at sites of chondrogenesis and osteogenesis in human degenerative valves and that atorvastatin inhibits hypercholesterolemia-induced calcification of rabbit aortic valves by downregulating LRP5 and β-catenin levels. However, the contribution of Wnt signaling to the chondrogenic and adipogenic differentiation of progenitor cells within the vessel wall, which also plays a significant role in vascular dysfunction, has received little attention.

Therefore, the purpose of this study was to test this hypothesis that Wnt signaling regulates chondrogenic and adipogenic differentiation of pericytes and to determine whether this occurs in a β-catenin–dependent manner. We demonstrate that pericytes express several Wnt receptors and can respond to Wnt agonists by stabilizing cytoplasmic β-catenin. In addition, we demonstrate that activation of Wnt signaling enhances the chondrogenic differentiation of pericytes and that chondrogenesis is inhibited by using recombinant adenoviruses encoding dominant-negative TCF-4, confirming that TGF-β3 induces chondrogenesis via β-catenin/TCF-induced gene transcription. In parallel studies, we also show that Wnt signaling inhibits the adipogenic differentiation of pericytes. This study provides new evidence that Wnt signaling directly modulates pericyte differentiation and further highlights the potential use of antagonists/inhibitors of this pathway for the prevention of vascular disease.

Materials and Methods
Cell Culture and Adenoviral Infection
Bovine retinal pericytes were isolated, characterized, and cultured as described. These cells, like pericytes isolated from other vascular beds, can be induced to differentiate along the adipogenic, chondrogenic, and osteogenic lineages in vitro and in vivo. For experiments, cells were plated at 4 × 104 cells/cm² and cultured in Eagle’s minimal essential medium (MEM) containing 20% FCS, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 50 μg/mL ascorbic-2-phosphate, and nonessential amino acids (20% FCS-MEM). To activate the Wnt signaling pathway, cells were incubated with conditioned medium containing Wnt3a for 3 hours. This medium was collected from L-Cell fibroblasts stably expressing Wnt3a. Conditioned medium from a parental L-Cell cell line was used as a control. Alternatively, culture medium was supplemented with either 1 or 10 mmol/L LiCl (a Wnt mimetic); in these experiments, KCl (1 or 10 mmol/L, as appropriate) was used as a control. Pericytes were infected with recombinant adenoviruses (RAd) at 80% confluence using either (1) RAd encoding dominant negative TCF-4 (dnTCF) (RAd/dnTCF) or (2) RAd encoding β-galactosidase (RAd/β-Gal), which acted as viral infection control. A multiplicity of infection of 50, which routinely achieved 90% transfection efficiency without any cell toxicity, was used for all experiments. Following infection with RAd, culture medium supplemented with either 1 mmol/L LiCl or KCl was added, and the cells were incubated for a further 48 hours. To stimulate chondrogenic differentiation, 5 × 105 cells from each treatment were subsequently pelleted and cultured in chondrogenic medium supplemented with either 1 mmol/L LiCl or KCl for 16 days, as described. Adipogenic differentiation was induced in cell monolayers by culturing RAd-infected cells in MEM containing 15% rabbit serum (15% RS-MEM) containing 1 mmol/L LiCl or KCl for 8 days. Control cells were maintained in 20% FCS-MEM supplemented with 1 mmol/L LiCl or KCl.

Reverse Transcriptase–Polymerase Chain Reaction
Total RNA was extracted from confluent monolayer pericytes using TRIzol (Invitrogen, Paisley, UK) according to the instructions of the manufacturer. Pelleted pericytes were placed directly into RiboLyser tubes (Anachem, Luton, UK) containing 600 μL of TRIzol and vortexed in a Hybaid RiboLyser for 40 seconds. RNA extraction was then performed as described above for monolayer pericytes. Reagents used for RT-PCR were obtained from Roche and reactions conducted following the guidelines of the manufacturer. Reverse transcription (RT) reactions were performed using 2 μg of RNA, with and without enzyme. PCR was performed with 5 μL of RT product, 300 nmol/L each gene-specific primer, 10% dimethyl sulfoxide and 5 U of Taq polymerase. The conditions for amplification were as follows: 95°C (2 minutes); 30 cycles of 95°C (15 seconds), 50°C (30 seconds), 72°C (1 minute); and a final 7-minute extension at 72°C. The sequences of all primers used together with amplicon size generated are shown in Table 1 in the online data supplement, available at http://circres.ahajournals.org.

Real-Time PCR
A standard quantitative real-time PCR was prepared using 5 μL of RT product and the TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, UK), following the guidelines of the manufacturer. In addition, control reactions were set up for each gene that included no template (water) and unknown cDNA samples (12.5 ng/μL). An Applied Biosystems ABI Prism 7000 real-time PCR machine was used to quantify gene expression. This was expressed as a value normalized to levels of 18S RNA. TaqMan probes and primers to bovine Sox-9, aggrecan, collagen type II, and 18S RNA.
were designed using the Applied Biosystems Primer Express design software and are shown in Table II of the online data supplement. PCR cycling parameters were as follows: 50°C (2 minutes) followed by 95°C (10 minutes); 35 cycles of 94°C (20 seconds), 55°C (20 seconds), and 72°C (20 seconds).

**Immunohistochemical Staining and Transmission Electron Microscopy**
Pericytes cultured in monolayer were washed in PBS and fixed in 10% formaldehyde/100 mmol/L CaCl₂ for 20 minutes and stained for lipid accumulation by using oil red O as described previously. Pellets were analyzed by immunohistochemical staining and transmission electron microscopy (TEM) as detailed in the expanded Materials and Methods section in the online data supplement.

**Biochemical Analysis of Pellets**
Glycosaminoglycan and DNA quantification was conducted as described previously. (See the online data supplement.)

**Protein Extraction and Western Blot Analysis**
Pellets (4 per treatment) were placed into a RiboLyser tube (Hybaid green matrix) containing 400 μL of lysis buffer (0.0625 mol/L Tris/HCl, 2% SDS, 2 mol/L urea, 2 mmol/L N-ethylmaleimide, 2 mmol/L phenylmethylsulphonylfluoride [PMSF]) and were vortexed in a Hybaid RiboLyser for 40 seconds, and the supernatant was collected for further analysis. The supernatant was centrifuged (15000 g, 5 minutes). The supernatant containing conditioned medium or LiCl was used as a control. Cells were fractionated, and the cytosolic protein fraction. Cell lysates were maintained at 4°C throughout both protein extraction procedures. Protein contents were quantified using a BCA assay (Pierce, Cramlington, UK) and analyzed by Western blotting for levels of β-catenin (Figure 1B and 1C, upper bands). Results showed that β-catenin levels were detected in the levels of collagen II gene expression in these cultures (Figure 2C). In contrast, very low levels of Sox-9, aggrecan, and collagen II mRNA were detected when cells

**Results**

**Pericytes Express Frizzled and LRP Receptors and Respond to Wnt Agonists by Accumulating β-Catenin**
To establish which Wnt receptors were expressed by pericytes, RNA was extracted from confluent cells, and RT-PCR was performed. Figure 1A shows that pericytes express mRNA for LRP5 and -6, in addition to Frizzled receptors 1 to 4, and 7, 8, and 10; Frizzled receptors 5, 6, and 9 were not detected using this procedure (data not shown). Confirmation that the correct target genes had been amplified was achieved by sequencing (data not shown).

To confirm that Wnt signaling could be activated in pericytes, confluent cells were incubated with either Wnt3a-containing conditioned medium or LiCl. Control conditioned medium or KCl was used as a control. Cells were fractionated, and the cytosolic fraction was analyzed by Western blotting for levels of β-catenin (Figure 1B and 1C, upper bands). β-Tubulin was used as a loading control (Figure 1B and 1C, lower bands). Results showed that β-catenin levels were enhanced in pericytes incubated in Wnt3a conditioned medium and LiCl in comparison with controls.

**Canonical Wnt Signaling Enhances Chondrogenic Differentiation of Pericytes**
We have previously demonstrated that pericytes undergo chondrogenic differentiation when cultured as high-density pellets in medium containing TGF-β3. To investigate the role of Wnt signaling in the chondrogenic differentiation of pericytes, cells were infected with either RAd/dnTCF (which blocks β-catenin-induced TCF-4 transcription) or RAd/βGal (control) and incubated in 20% FCS-MEM supplemented with 1 mmol/L LiCl (a Wnt mimetic that inhibits glycogen synthase kinase-3β and thus activates β-catenin/TCF transcription) or 1 mmol/L KCl (control) for 48 hours. Cells were then pelleted, cultured in chondrogenic medium containing either 1 mmol/L LiCl or KCl for 16 days, and analyzed.

The effect of modulating Wnt signaling on the expression of chondrogenic markers was first analyzed using real-time PCR. Activating Wnt signaling using LiCl significantly increased both Sox-9 and aggrecan mRNA abundance in pellets infected with the control virus, compared with KCl-treated controls (Figure 2A and 2B). No difference was detected in the levels of collagen II gene expression in these cultures (Figure 2C).
were preincubated with RAd/dnTCF and then maintained in chondrogenic medium (Figure 2).

The deposition of type II collagen within the pellets was examined further using immunohistochemistry. In control (RAd/βGal-infected) pericyte pellets, the presence of LiCl in the chondrogenic medium stimulated an increase in alcian blue and safranin O staining (which detects sulfated proteoglycans and negatively charged glycosaminoglycans [GAGs], respectively) and enhanced metachromatic staining with toluidine blue (not shown) when compared with KCl-treated pellets (Figure 3A, compare d and e; compare g and h). On the other hand, pellets infected with RAd/dnTCF displayed less intense alcian blue staining in comparison with control pellets, particularly at the periphery (Figure 3A, compare d and f). In addition, reduced safranin O staining was observed, making the methyl green counterstain much more apparent in the RAd/dnTCF pellets (Figure 3A, compare g and i).

GAG content within pellets was also quantified and expressed relative to DNA content. Figure 3B shows that stimulation of the Wnt signaling pathway using LiCl significantly increased GAG abundance. A significant reduction in the GAG content of pellets was also observed following infection with RAd/dnTCF, in comparison with the control virus. Together, these results demonstrate that TGF-β-stimulated chondrogenic differentiation of pericytes occurs through activation of Wnt/β-catenin signaling. In addition, they demonstrate that the effects of TGF-β and Wnt signaling on chondrogenesis are additive.

**Canonical Wnt Signaling Inhibits Adipogenic Differentiation of Pericytes**

Further analysis of pericyte pellets using immunohistochemistry and TEM demonstrated that some cells within the pellets resembled adipocytes, displaying the classic “signet ring” morphology and containing lipid droplets (Figure 4a and 4b), suggesting that both chondrogenesis and adipogenesis were occurring in these cultures. Lipid accumulation within the pellets was confirmed by staining with oil red O (Figure 4c); no staining was detected when adjacent control sections were delipidized before incubation with oil red O (Figure 4d). We have previously shown that pericytes in monolayer culture undergo adipogenic differentiation and accumulate lipid in the presence of rabbit serum.6 Because activation of the Wnt pathway has been shown to inhibit adipogenic differentiation of preadipocytes,25–29 we therefore investigated whether Wnt/β-catenin signaling would inhibit the adipogenic differentiation of pericytes both in pellet cultures and in monolayers.

When Wnt signaling was stimulated using LiCl, lipid deposition in the periphery of the pellets was reduced; however some staining was still detected in the center (Figure 5A, compare a and b). To determine whether LiCl reduced adipogenesis by modulating β-catenin–induced gene transcription, cells were infected with RAd/dnTCF before pelletting, and adipogenic differentiation within the pellets was assessed. As shown in Figure 5A, incubation of the cells with RAd/dnTCF prevented the LiCl-induced decrease in oil red O staining (Figure 5A, compare b and c), confirming that LiCl was exerting its effects via the Wnt/β-catenin pathway. The expression of peroxisome proliferator-activated receptor (PPAR)-γ (an adipocyte-specific transcription factor) was also analyzed in pellet cultures using Western blotting; β-actin was used as a loading control. Figure 5B shows that

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**Figure 2.** Real-time RT-PCR data showing levels of expression of Sox-9, aggrecan, and collagen type II mRNA in pericyte pellets. Pericytes were infected with either RAd/βGal or RAd/dnTCF, pelleted, and cultured for 16 days in chondrogenic medium containing either 1 mmol/L LiCl or KCl. RNA was isolated from pellets, reverse transcribed, and used for real-time PCR analysis with primers specific for the chondrogenic markers Sox-9 (A), aggrecan (B), and collagen type II (C). Gene expression is shown relative to 18S rRNA (control) levels. Bars represent ±SD. *P<0.05.
PPAR-γ levels were markedly reduced in RAd/βGal-infected pellets when Wnt signaling was activated using LiCl. Inhibition of Wnt/β-catenin signaling using RAd/dnTCF abolished the effect of LiCl and PPAR-γ protein was detected (Figure 5B).

To further investigate the effect of Wnt signaling on adipogenic differentiation of pericytes, confluent monolayer cells were infected with either RAd/dnTCF or RAd/βGal, cultured in 20% FCS-MEM for 48 hours, and then incubated for an additional 8 days with either 15% RS-MEM or 20% FCS-MEM containing either 1 mmol/L LiCl or KCl. Adipogenic differentiation was then assessed as detailed above. Figure 6A (a) shows intense oil red O staining in cells infected with RAd/βGal and cultured with 15% RS-MEM plus 1 mmol/L LiCl stained positively with oil red O (Figure 6A, c), confirming that inhibition of adipogenesis by LiCl occurred via β-catenin/TCF-induced gene transcription. Cells cultured with 20% FCS-MEM did not undergo adipogenic differentiation, irrespective of previous viral infection or LiCl/KCl treatment (data not shown).

Western blot analysis was used to detect the presence of PPAR-γ in nuclear-enriched fractions obtained from each treatment. As Figure 6B shows, this transcription factor was detected in cells treated with 15% RS-MEM, and its expression was markedly reduced in cells infected with control virus and cultured in 1 mmol/L LiCl. This reduction in expression did not occur in the presence of RAd/dnTCF, demonstrating that LiCl was acting through the canonical Wnt pathway and regulating gene transcription. PPAR-γ was not detected in cells incubated in 20% FCS-MEM in the presence or absence of LiCl. 

Figure 3. Effects of canonical Wnt signaling on chondrogenic differentiation of pericytes. Pericytes were infected with RAd/βGal or RAd/dnTCF, pelleted, and cultured as stated in the legend to Figure 2. A, Pellets were fixed, sectioned, and incubated with antibodies against type II collagen (a through c) or stained with alcian blue (d through f) or safranin O (g through i). Scale bar=100 μm. B, Four pellets from each treatment were papain digested and assayed for GAG and DNA content. GAG accumulation is shown relative to DNA content per pellet (n=4). Bar represents ±SD. *P<0.05.
Discussion

These studies demonstrate for the first time that Wnt/β-catenin/TCF signaling regulates the adipogenic and chondrogenic differentiation of pericytes, further highlighting the potential importance of this pathway in regulating many facets of vascular dysfunction, including atherosclerosis and the calcific vasculopathies. We have previously shown that culturing pericytes as pellets in a defined medium containing TGF-β3 stimulates their chondrogenic differentiation.6 We now demonstrate that Wnt signaling is required for the induction of chondrogenesis by TGF-β3 and that the effects of TGF-β3 and Wnt signaling on chondrogenesis are additive (Figures 2 and 3). The Wnt agonist LiCl promoted chondrogenesis in pericyte pellet cultures, as demonstrated by increased mRNA expression of Sox-9 and aggrecan, and increased accumulation of sulfated proteoglycans and negatively charged GAG in the matrix, as detected by alcian blue, toluidine blue, and safranin O staining. Interestingly, collagen II expression was not induced further by Wnt signaling, suggesting that its expression was already maximal. Transduction of pericytes with RAd/dnTCF, which inhibits β-catenin/TCF-induced gene transcription, inhibited the effects of TGF-β3 on chondrogenesis, resulting in decreased Sox9 mRNA transcription, decreased expression of type II collagen mRNA and protein, reduced aggrecan mRNA abundance, and weaker alcin blue, toluidine blue, and safranin O staining. These data demonstrate for the first time that the mechanism by which TGF-β3 induces the chondrogenic differentiation of pericytes is by activation of Wnt/β-catenin/TCF-induced signaling and the induction of Sox-9 expression. These results extend previous work showing that TGF-β upregulates Wnt and LRP5 gene expression and stabilizes β-catenin in adult human mesenchymal stem cells.25 However, this latter study did not show that blocking Wnt/β-catenin pathway prevents chondrogenesis.

Wnts can either inhibit or promote chondrocyte differentiation and/or hypertrophy in different in vivo and in vitro models.18–25 The different effects of Wnt signaling appears to be critically dependent on stage of development and the cell type used.15,21 For example, activation of Wnt signaling using Wnt3a represses chondrogenesis of the prechondrocyte cell line ATDC540 but promotes chondrogenic differentiation of undifferentiated mesenchymal cells.32 Therefore, the demonstration that Wnt signaling promotes the chondrogenic differentiation of pericytes in this study is consistent with our previous work, which has suggested that pericytes are uncommitted progenitor cells of a mesenchymal origin.41 Furthermore, differences in cell type may also explain why TGF-β3–induced Wnt/β-catenin signaling induces chondrogenesis (this study), whereas bone morphogenetic protein-2–induced Wnt/β-catenin induces osteogenesis.32

Immunohistochemical and TEM analysis suggested that adipogenesis, in addition to chondrogenesis, was occurring in pericyte pellets (Figure 4). This finding was confirmed by positive oil red O staining, which detects lipid accumulation, and by Western blot analysis, which demonstrated the expression of PPAR-γ protein within the pellets (Figures 4 and 5). Similar findings have been reported by Brachvogel et al, who demonstrated lipid deposition and PPAR-γ2 expression by mouse perivascular cells cultured in chondrogenic medium.35 We now demonstrate for the first time that Wnt signaling inhibits the adipogenic differentiation of pericytes, as demonstrated by decreased oil red O staining and PPAR-γ protein expression within pericyte pellets and monolayer.
cultures (Figures 5 and 6). This effect was prevented by incubating cells with RAd/dnTCF, confirming that the inhibition of adipogenesis by LiCl was occurring via β-catenin/TCF-induced gene transcription. Wnt signaling has consistently been reported to prevent adipogenesis in a wide range of cell types.25–29,42 However, there is still some confusion about the mechanisms by which Wnt signaling inhibits adipogenesis, with suggestions that both β-catenin–independent and –dependent signaling are involved.29 Within pericytes, our data clearly demonstrate that Wnt/β-catenin signaling attenuates adipogenesis in these cells. However, further experiments will be required to show whether β-catenin–independent Wnt signals can also attenuate adipogenesis. Furthermore, our results demonstrate that within the pericyte pellet culture system activation of the Wnt pathway promotes chondrogenic differentiation and at the expense of adipogenic differentiation.

Given that pericytes and vascular smooth muscle cells9 can adopt an adipogenic phenotype and accumulate lipid, it is possible that the aberrant differentiation of these cells may contribute directly to the deposition of ectopic fat that is associated with atherosclerosis. However, it remains to be determined whether pericytes become fully differentiated into adipocytes or whether, like vascular smooth muscle cells, their differentiation is reversible.9 Furthermore, the relationship between these cells, adipocytes, and foam cells within atherosclerotic lesions is not known and remains an important area for future investigation.

Multipotent progenitor cells have been isolated from a wide range of vascular beds.1–9 However, the relationship of these cells to each other is unclear. The demonstration in this study that Wnt signaling promotes chondrogenic differentiation and inhibits adipogenic differentiation of pericytes may provide some clues about the relationship between calcifying vascular cells and pericytes. Demer and colleagues have shown that calcifying vascular cells resemble pericytes in terms of the markers they express, namely α-smooth muscle actin and 3G5.7 However, these cells exhibit differing differentiation profiles; calcifying vascular cells undergo chondrogenic and osteogenic differentiation but not adipogenic differentiation,8 whereas pericytes can differentiate along all 3 lineages.1,5,6 It is, therefore, tempting to speculate that calci-
fying vascular cells are pericytes that have been exposed to a Wnt signal.

The aberrant differentiation of progenitor cells present in the walls of blood vessels is thought to result in the deposition of ectopic fat, cartilage, and bone in these vessels, leading to increased morbidity and mortality in patients with atherosclerosis, diabetes, end-stage renal disease, and calciphylaxis.1–4,17 Controlling the differentiation of these cells may, therefore, represent a strategy to treat these diseases. The results presented herein suggest that the inappropriate activation of Wnt signaling drives the chondrogenic differentiation of progenitor cells present in the walls of blood vessels. Currently, we do not know whether Wnt signaling will inhibit or promote the osteogenic differentiation of pericytes. However, previous studies have demonstrated that activation of the Wnt pathway in response to bone morphogenetic protein-2/Msx2 signaling stimulates bone formation in the vessels of LDLR/H11002/H11002 mice32 and that the expression of the Wnt receptor LRP5 is upregulated at sites of both chondrogenesis and osteogenesis in human degenerative valves.33 Taken together, these studies highlight, therefore, the potential use of antagonists/inhibitors of the Wnt pathway for the prevention of vascular diseases involving intimal thickening, and the ectopic formation of cartilage and bone. However, this may also need to be approached with some caution, as our data also indicate that blocking Wnt signaling may, under certain conditions, promote adipogenic differentiation.

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Disclosures
None.

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EXPANDED METHODS:

**Immunohistochemical Staining of Cell Pellets**

Cell pellets were fixed in 4% formaldehyde/PBS, embedded in wax and sections (5 µm) stained with alcian blue, toluidine blue, safranin O or used for immunohistochemical staining as previously described\(^1\),\(^2\). Alternatively, pellets were placed directly into OCT (Raymond Lamb, Sussex, UK) embedding medium and snap-frozen using liquid nitrogen. Cryosections (5 µm) of pellets were fixed in 3% formaldehyde/PBS and stained with Oil Red O for 20 minutes and then rinsed in distilled water. The Oil Red O solution comprised filtered 1% (w/v) Oil Red O (Sigma) in isopropanol mixed in a 6:4 ratio with 1% Dextrin (Sigma). As a control, adjacent sections were delipidised in chloroform/methanol solution (2:1 ratio) prior to Oil Red O staining. Coverslips were applied using aqueous mountant (Dako, UK) and sections were visualised using an Olympus IX51 microscope.

**Transmission Electron Microscopy**

Pellets were fixed in 2.5% glutaraldehyde, in 0.1 mol/L phosphate buffer for 5 minutes at room temperature, followed by 1 hour at 4°C. Pellets were then washed in 0.1 mol/L phosphate buffer for 5 minutes. A secondary fix of 2% gluteraldehyde and 1% osmium tetroxide in 0.1 mol/L phosphate buffer, for 1 hour at 4°C was conducted, followed by several 5 minute rinses in distilled water. An enbloc stain of 2% uranyl acetate was applied overnight followed by a further series of 5 minute washes in distilled water. The samples were then dehydrated in acetone at 10%, 25%, 50%, 70%, 90%, and 95% all for 10 minutes each and 3 changes in 100% acetone for 15 minutes each. The samples were placed into propylene oxide for 2 changes, of 5
minutes each, propylene oxide/resin 3:1 mix, for 3 hours, propylene oxide/resin at a 1:1 mix overnight, propylene oxide/resin 1:3 for 2 changes over 2 hours and then into the full resin mix overnight, followed by 3 changes of 2 hours each. Samples were then placed in a 60°C oven overnight.

The resin embedded material was cut using a fine toothed, jewellers saw, and trimmed with razor blades. Sections were cut and the required area was selected and ultra-thin sections were cut on a Leica Ultracut UCT Ultracryomicrotome. Sections were collected across 3 mm copper mesh grids, and placed in petri dishes on filter paper to dry. These grids were then stained for electron microscopy; 30 minutes in uranyl acetate, rinsed in distilled water, followed by 5-10 minutes in lead citrate, rinsed in distilled water, and allowed to dry. The grids were examined on the Phillips 400 transmission electron microscope and photographed.

**Biochemical Analysis of Pellets**

Pellets were digested for 16 hours at 60°C in a 100 units/ml papain (Sigma), 100 mmol/L sodium acetate, 2.4 mmol/L ethylenediaminetetraacetic acid (EDTA), 5 mmol/L L-cysteine, pH 5.8 at 60°C. Glycosaminoglycan (GAG) content was determined using a colorimetric assay following addition of DMB solution (38.5 μmol/L 1-9 dimethylmethylene blue (Sigma), 2.9 mmol/L Sodium Formate (Sigma), 0.2% (v/v) Formic acid, pH 3.5) to each sample. Solutions were analysed at 570 nm and concentration of each sample calculated using a chondroitin sulphate (Sigma) standard curve. DNA concentrations were calculated using a Hoefer DyNA Quant 200 photometer. To the papain digest solution of each sample, an equal volume of DNA assay solution (0.1 ug/ml Hoechst 33258 dye (Sigma), 10X TNE buffer (200
mmol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA) was added and the concentration quantified relative to a DNA standard solution.

**Western Blot Analysis**

Proteins (5-10 µg) were electrophoresed on reducing 15% sodium dodecyl sulfate–polyacrylamide gels and electrotransferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked using 5% Marvel/PBS-Tween (0.1%) for 16 hours at 4°C and incubated with antibodies against PPAR-γ (Abcam; ab19481), β-tubulin (Santa Cruz; sc-5274), β-catenin (BD Transduction lab; 610153) or β-actin (Sigma; A1978) at 22°C for 1 hour (dilution 1:4000, 1:1000, 1:1000, and 1:10000 respectively). Western blot analysis was performed using a horseradish peroxidase–conjugated swine anti-rabbit or rabbit anti-mouse secondary antibody (dilution 1:2000; Dako, Denmark) and results were visualized by enhanced chemiluminescence and autoradiography (ECL Plus, GE Healthcare).

**REFERENCES**


## Online Table I: Primers used for standard PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward Primer 5’→ 3’</th>
<th>Reverse Primer 5’→ 3’</th>
<th>Size of amplicon (base pairs)</th>
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<td>GGGTGGTGTGTGAACGACAAG</td>
<td>CTG GCCATGTGAAGAAGTA</td>
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<td>GCGTCTTCTCGGTCTCTTAC</td>
<td>GATGGCTAGGCTCTTGGCAGT</td>
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<td>Frizzled 3</td>
<td>CCGTCTGCTACATGATGGTG</td>
<td>AGATCCCTTGGTCACGTTG</td>
<td>119</td>
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<td>Frizzled 4</td>
<td>TTGAAGAGGCAGCAGAACCT</td>
<td>GAAGCTGGGCATCCAGAAAAA</td>
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<td>Frizzled 5</td>
<td>ACCAGTTCAACCATGACACG</td>
<td>ATGGGCGTGTACATAGCAAGA</td>
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<td>Frizzled 6</td>
<td>GGGTTGAAGCAAGAAAAAG</td>
<td>TTGGAATGACCTTCCAGTC</td>
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<td>Frizzled 7</td>
<td>TCCTGTCGGGGTTGCTACTTC</td>
<td>CGAAGAAGTAAACCAACCATCGA</td>
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<td>Frizzled 8</td>
<td>AGTGGGGTACCATGATTGA</td>
<td>TGCTGCACAGGAAGAGAAAGA</td>
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<td>Frizzled 9</td>
<td>AGCCACCTGCGCTTCTTGA</td>
<td>GAAGGCGTGAGGAAGAAGAAGA</td>
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<td>Frizzled 10</td>
<td>CAACAGGAACCCAGCAACT</td>
<td>GACAAACCAGGAAGGTC</td>
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<td>TGGGCAAGAACCTCATTGG</td>
<td>CATGTTGGTGTCAGGGCC</td>
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<tr>
<td>LRP6</td>
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<td>CACTTCTCCCCAGTGTCC</td>
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## Online Table II: Primers used for Real-Time PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward Primer 5’→ 3’</th>
<th>Reverse Primer 5’→ 3’</th>
<th>Probe 5’→ 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type II</td>
<td>GGGCCTGTCTGCTTTCTGTA</td>
<td>GGGTCTTCTGGTTGGTAAG</td>
<td>AACCCGAAACCCAGAA CCAACACACATCC</td>
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<td>Sox-9</td>
<td>AGTACCCGACCACTCTACA</td>
<td>CACGGAACGGCCATGC</td>
<td>CCGAGCTCACAGGACA CTCTGGCCA</td>
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<tr>
<td>Aggrecan</td>
<td>GCAGGTTCCGGGTCA</td>
<td>GTAGAATCCCGGGATG</td>
<td>CGCCCTAAAGACAA GGTCAAGCT</td>
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<tr>
<td>18S RNA</td>
<td>GATCCATTTGAGGAGGTCA</td>
<td>GCAGACACTTTATATAGCCA</td>
<td>CAGCAGCGCCGGTTA TTCCAGC</td>
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