Inhibition of Pathological Differentiation of Valvular Interstitial Cells by C-Type Natriuretic Peptide

Cindy Y.Y. Yip, Mark C. Blaser, Zahra Mirzaei, Xiao Zhong, Craig A. Simmons

Objective—Calcific aortic valve disease is associated with the differentiation of valvular interstitial cells (VICs) to myofibroblast and osteoblast-like cells, particularly in the fibrosa layer of the valve. Previous studies suggested that C-type natriuretic peptide (CNP) protects against calcific aortic valve disease to maintain homeostasis. We aimed to determine whether CNP inhibits VIC pathological differentiation as a mechanism to explain its protective effects.

Methods and Results—CNP expression was prominent in normal porcine aortic valves, particularly on the ventricular side, but reduced in sclerotic valves concomitant with the appearance of pathological VIC phenotypes in the fibrosa. In vitro, CNP inhibited calcified aggregate formation and bone-related transcript and protein expression by VICs grown in osteogenic conditions. Under myofibrogenic culture conditions, CNP reduced α-smooth muscle actin expression and cell-mediated gel contraction, indicating inhibition of myofibroblast differentiation. Similar to CNP, simvastatin inhibited VIC osteoblast and myofibroblast differentiation in vitro. Strikingly, simvastatin upregulated CNP expression in VICs cultured under myofibrogenic conditions, and small interfering RNA knockdown of natriuretic peptide receptor-b (a CNP receptor) significantly reduced the antifibrotic effect of simvastatin, suggesting that it acts in part via CNP/NPR-B autocrine/paracrine signaling.

Conclusion—CNP inhibits myofibroblast and osteoblast differentiation of VICs and is responsible in part for inhibition of VIC myofibroblast differentiation by statins, suggesting novel mechanisms to explain the protective effect of CNP and the pleiotropic effects of statins in the aortic valve. (Arterioscler Thromb Vasc Biol. 2011;31:1881-1889.)

Key Words: calcification ■ aortic valves ■ C-type natriuretic peptide ■ fibrosis ■ statins

Calcific aortic valve disease (CAVD) is characterized by fibrosis and calcification that lead to thickening of the valve leaflets and eventual impairment of leaflet motion. Pathological alterations in valve tissue in CAVD are mediated by valvular interstitial cells (VICs). The normal VIC population is heterogeneous, consisting primarily of fibroblasts, a subpopulation of which consists of mesenchymal progenitor cells with multilineage differentiation potential, and a small population (≈1% to 5%) of smooth muscle cells and myofibroblasts. VICs undergo myofibroblast and osteoblast differentiation during the progression of CAVD, contributing to fibrosis and calcification. Myofibroblasts, which are up to 30% of the total VIC population in sclerotic valves, are identified by prominent α-smooth muscle actin (α-SMA) stress fibers and are associated with increased collagen synthesis and cellular contractility. Osteoblast-like cells are also often found in calcified aortic valves, accompanied by an increase in the expression of bone-related transcripts and proteins.

Pathological differentiation of VICs has been shown to be inhibited by statins in vitro, however, clinical trials with statins have yielded conflicting results. Retrospective and prospective cohort studies show that statins delay progression of CAVD, whereas randomized, controlled trials such as Simvastatin and Ezetimibe for Aortic Stenosis and Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression found no benefit with statin therapy. An improved understanding of the molecular determinants leading to pathological differentiation of VICs may provide insights into the potential of statins or other pharmacological therapies to arrest CAVD, which will eventually aid in refining treatment regimens and patient selection criteria for clinical trials.

Clues to the molecular determinants of CAVD are likely to come from the side-dependent susceptibility of aortic valves to lesion formation. We previously found by microarray analysis of endothelial cells from healthy porcine aortic valves that C-type natriuretic peptide (CNP) is more highly expressed on the disease-protected ventricular side compared with the disease-prone aortic side. Subsequently, it was shown that CNP is constitutively expressed in normal human valve leaflets, but its expression and that of its activator, furin, are downregulated in sclerotic valves. Further, CNP was shown to suppress the formation of calcified multicellular aggregates by VICs in vitro. In nonvalve cells and
tissues, CNP regulates calcification and differentiation and is antifibrotic in vivo. Although the factors that regulate CNP expression in the valve are unknown, CNP expression is stimulated in vascular endothelial cells by cytokines, shear stress, and, notably, statins. Motivated by these findings, we investigated whether CNP suppresses the differentiation of VICs into myofibroblasts or osteoblasts and whether induction of CNP expression by statins is responsible in part for their inhibition of VIC pathological differentiation. Here we demonstrate that CNP: (1) is expressed in normal valves but is downregulated in sclerotic valves in vivo and in VICs undergoing pathological differentiation in vitro; (2) inhibits the differentiation of VICs to osteoblasts and myofibroblasts in vitro; (3) is induced in myofibroblastic but not osteoblastic VICs by simvastatin; and (4) is responsible, in part, for the inhibition of myofibroblast differentiation of VICs by simvastatin.

**Materials and Methods**

An expanded Materials and Methods section is available in the Supplemental Materials section, available online at http://atvb.ahajournals.org. Unless otherwise stated, all materials were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

**VIC Isolation and Animal Model**

Primary porcine aortic VICs were isolated from normal aortic valves by collagenase digestion. Sclerotic leaflets were obtained from pigs fed an atherogenic diet for 6 months. This hypercholesterolemic model develops valvular lesions preferentially in the fibrosa (refer to the online supplement to Simmons et al). Normal and sclerotic valve leaflets were fixed, paraffin embedded, and serial sectioned for immunohistochemical staining with polyclonal rabbit anti-mouse Runt-related transcription factor 2 (Runx2) (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-human osteocalcin (clone 1A4) antibody (Affinity BioReagents, Golden, CO), and is antifibrotic in vivo. Although the factors that regulate CNP expression in the valve are unknown, CNP expression is stimulated in vascular endothelial cells by cytokines, shear stress, and, notably, statins. Motivated by these findings, we investigated whether CNP suppresses the differentiation of VICs into myofibroblasts or osteoblasts and whether induction of CNP expression by statins is responsible in part for their inhibition of VIC pathological differentiation. Here we demonstrate that CNP: (1) is expressed in normal valves but is downregulated in sclerotic valves in vivo and in VICs undergoing pathological differentiation in vitro; (2) inhibits the differentiation of VICs to osteoblasts and myofibroblasts in vitro; (3) is induced in myofibroblastic but not osteoblastic VICs by simvastatin; and (4) is responsible, in part, for the inhibition of myofibroblast differentiation of VICs by simvastatin.

**Calcium Deposition**

VIC cultures were stained with 40 mmol/L alizarin red S solution. The number of alizarin red S-positive aggregates was counted after 14 days of culture.

**Quantitative Real-Time Polymerase Chain Reaction**

At days 3, 8, and 16 of culture, transcript expression of Runx2, osteonectin, and osteocalcin was quantified by quantitative reverse transcription polymerase chain reaction using SYBR green for detection and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. Gene expression was quantified by comparative Ct (cycle threshold) method ($2^{ΔΔCt}$).

**Colony-Forming Unit–ALP Assay**

VICs were seeded at 0.2 cells/well into 96-well plates. Cells were stained for ALP after 3 weeks of incubation in osteogenic medium with or without CNP. The negative natural logarithm of the ratio of wells without ALP-positive aggregates to the total number of wells was calculated as the colony-forming unit–ALP (CFU-ALP)/osteogen gel model. In brief, 10,000 VICs/cm² VICs were cultured on constrained, compliant collagen gels for 4 days in complete medium with or without CNP. Myofibroblast differentiation of VICs was induced by treating the cells with 1 ng/mL of transforming growth factor-β1 (TGF-β1) for 48 hours, after which the gels were released to allow for contraction. Images of the collagen gels were taken every 30 minutes, and the gel areas were determined using ImageJ.

**Simvastatin Treatment**

Cells were cultured in complete medium or osteogenic media with (1 μmol/L) or without activated simvastatin for up to 14 days. CNP transcript expression was determined by quantitative reverse transcription–polymerase chain reaction and the comparative Ct method after 3 days of simvastatin treatment. Relative CNP transcript expression was expressed as the ratio of simvastatin-treated VICs to VICs without simvastatin. A ratio greater than 1 indicates higher expression of CNP transcript with simvastatin treatment.

**Small Interfering RNA for NPR-B**

Because of the lack of an available natriuretic peptide receptor-B (NPR-B) antagonist, specific inhibition of CNP signaling was achieved with custom designed Silencer small interfering RNAs (siRNAs) (Applied Biosystems, Foster City, CA) targeted to porcine NPR-B. VICs were transfected with 100 nmol/L of NPR-B siRNAs or Silencer negative control siRNAs using Oligofectamine (Invitrogen, Burlington, Ontario, Canada) for 12 hours. Cells were subsequently incubated in complete medium with 100 nmol/L CNP or 1 μmol/L activated simvastatin for 4 days, followed by formalin fixation and immunostaining for α-SMA.

**Statistical Analysis**

Results are presented as mean ± standard error. Unpaired Student’s t-tests or ANOVA and Fisher’s least significant difference tests were used as appropriate.

**Results**

**Expression of CNP and Pathological Markers in Normal and Sclerotic Aortic Valves**

Consistent with our previous report of side-dependent expression of CNP mRNA in aortic valve endothelium, immunofluorescent staining revealed expression of CNP by the endothelium on the ventricular side but not the aortic side of the valve (Figure 1A and Supplemental Figure IA to IC).
However, CNP was also expressed abundantly throughout the interstitium, with the highest expression deep within the ventricularis layer (Figure 1A). Much of the expression in situ appeared to be cell specific, and immunostaining confirmed that freshly isolated VICs expressed CNP (Supplemental Figure 1D). α-SMA positive cells were rare in normal leaflets (Figure 1D), but numerous cells in the fibrosa of sclerotic valves stained strongly positive for α-SMA (Figure 1E and 1F), indicative of VIC myofibroblast differentiation. In these regions, CNP expression was weak or absent (Figure 1H and 1I), which accompanied by low expression of CNP (Figure 1B and 1C). In the ventricularis of sclerotic leaflets, CNP and α-SMA were rarely detected (Supplemental Figure IIA and IIB), and Runx2 was never detected (Supplemental Figure IIC). CNP was occasionally observed focally within the interstitium of sclerotic valves (Supplemental Figure IID), often adjacent to regions with myofibroblasts. α-SMA-positive cells were occasionally observed near the ventricularis surface (Supplemental Figure IIE).

Cellular Proliferation and Morphological Changes

To test the role of CNP in regulating myofibroblast and osteoblast differentiation, 100 nmol/L CNP was added to cultures of VICs grown in complete or osteogenic medium, respectively. CNP had no effect on proliferation, whether cells were cultured in complete (Supplemental Figure IIIA) or osteogenic (Supplemental Figure IIIB) medium (P ≥ 0.4 between non-CNP-treated and CNP-treated samples at each time point). In complete medium, VICs displayed typical (myo)fibroblastic morphology with elongated processes, and no substantial morphological differences were observed between CNP-treated and non-CNP-treated cells (data not shown). However, when cells were cultured in osteogenic media for more than 10 days, formation of multicellular aggregates was prominent only in the absence of CNP (Figure 2A and 2B). Because CNP had no effect on cellular proliferation in all culture conditions, cell density was similar in all cases and was unlikely to lead to the observed differences in aggregation or other phenotypic characteristics.

CNP Inhibits Calcification and Osteoblast Differentiation of VICs

VICs formed multicellular aggregates when cultured in osteogenic media (Figure 2B). In non-CNP-treated cultures, the aggregates contained calcium as shown by intense positive staining of alizarin red S (Figure 2D and 2F). CNP-treated VICs stained diffusely for calcium, with minimal aggregate formation (Figure 2C). The few aggregates that did form in the CNP-treated culture stained weakly for alizarin red S (Figure 2E). Quantification of the number of alizarin red S-positive aggregates confirmed that CNP treatment inhibited calcified aggregate formation (Figure 2G).

Formation of calcified VIC aggregates occurs in vitro through at least 2 processes, one associated with myofibroblast-induced contraction and the other associated with osteoblast differentiation. These processes produce aggregates with distinct morphologies, both of which were observed in the non-CNP-treated cultures when VICs were grown on uncoated tissue culture-treated polystyrene (Figure 2D), indicating calcification in the non-CNP-treated VICs was due in part to osteoblast differentiation. Notably, transcript expression of Runx2, osteonectin, and osteocalcin in CNP-treated cells was lower than that of non-CNP-treated VICs over the culture duration, with significant inhibition of osteonectin expression as early as 8 days of culture (Figure 3A to 3C). Consistent with the transcriptional profile, expression of bone-related proteins was also reduced with CNP treatment. ALP activity (Figure 3D) and osteocalcin expression (Figure 3F) were low within CNP-treated cultures but
high within aggregates in the non-CNP-treated cultures (Figure 3E and 3G). We confirmed that CNP inhibited aggregation associated with osteogenetic processes by growing VICs on compliant collagen substrates that favor osteoblast differentiation34 (Supplemental Figure IV). To further understand the cellular target of the antiosteogenic effect of CNP, we determined the CFU-ALP frequency as a measure of the differentiation of single osteoprogenitor cells.2 Under CNP treatment, the CFU-ALP frequency was significantly reduced compared with the non-CNP-treated culture (P<0.05; Figure 3H), suggesting that CNP suppressed the differentiation of osteoprogenitor cells within the VIC population.

CNP Inhibits Myofibroblast Differentiation of VICs

To evaluate the effect of CNP on myofibroblast differentiation of VICs, we cultured freshly isolated VICs on stiff tissue culture-treated polystyrene with complete medium. Only zero-passage primary VICs were tested, as subculturing induces myofibroblast differentiation and phenotypic drift.3 The majority of freshly isolated VICs expressed CNP (Supplemental Figure ID). We confirmed that VICs and not contaminating endothelial cells were primarily responsible for CNP expression in culture (Figure 4A). In some experiments, VICs were treated with 1 ng/mL TGF-β1 for 6 (Figure 4B) or 8 (Figure 4C) days. Some VICs differentiated into myofibroblasts with prominent α-SMA stress fibers after 6 days, whereas others that did not express α-SMA continued to express CNP (Figure 4B). Some VICs expressed both α-SMA and CNP (Figure 4B, bold arrow). After 8 days of TGF-β1 treatment, most VICs were α-SMA-positive, and CNP expression was low (Figure 4C). This mimicked the loss of CNP and appearance of α-SMA-positive myofibroblasts observed in fibrosa lesions in sclerotic aortic valve leaflets (Figure 1A to 1F).

In VICs treated with exogenous CNP, α-SMA expression was suppressed in a dose-dependent manner (Figure 4D). CNP treatment also suppressed collagen synthesis compared with non-CNP-treated cells (P<0.05; Supplemental Figure V). To evaluate the effect of CNP on myofibroblast-induced contractility, we cultured TGF-β1-treated VICs on constrained collagen gels and then measured gel contraction on release. Gels treated with CNP contracted significantly less than non-CNP-treated gels (P<0.05 for 1.0 and 1.5 hours after gel release; Figure 4E). Inhibition of myofibroblast differentiation by CNP was specifically mediated via its receptor NPR-B, as siRNA knockdown of NPR-B reversed the suppression of myofibroblast differentiation by CNP (Figure 4F and Supplemental Figure VI).

Simvastatin Upregulates CNP Transcript Expression and Inhibits Myofibroblast Differentiation of VICs via Autocrine/Paracrine CNP/NPR-B Signaling

We next tested whether CNP expression by VICs was inducible by statins, as it is in vascular endothelial cells.33 We found that 1 μmol/L simvastatin treatment for 3 days had no detectable effect on CNP transcript expression in VICs cultured in osteogenic medium (Figure 5A), although it did inhibit aggregate formation after up to 14 days in culture (P<0.05; Supplemental Figure VII), consistent with previous reports.39 In contrast, when VICs were grown in complete medium, simvastatin treatment significantly upregulated CNP transcript expression in VICs by 2.78±0.11 fold (P<0.05; Figure 5A) and reduced the fraction of myofibroblasts (identified by α-SMA stress fibers) by 7.35±0.51 fold (P<0.05; Figure 5B, 5C, and 5E) relative to non-statin-treated controls.

To test whether induction of CNP expression and suppression of myofibroblast differentiation by statins were linked, VICs transfected with NPR-B siRNA were treated with simvastatin. Under these conditions, the fraction of myofibroblasts increased significantly over VICs treated with statins alone.
Discussion

CNP is expressed in disease-protected regions of normal porcine valves and in normal human valves, but its expression is downregulated in calcified human aortic valves, suggesting that CNP protects against CAVD. Here we found that CNP expression in vivo is reduced in sclerotic porcine valves concomitant with increases in pathological cell markers, and we confirmed in vitro that CNP inhibits differentiation of VICs to myofibroblasts and osteoblasts, phenotypes associated with CAVD. In addition, we showed that the inhibition of myofibroblast differentiation of VICs by simvastatin is, in part, dependent on CNP/NPR-B signaling. Our current findings provide a cellular basis responsible for the putative homeostatic role of CNP and suggest a novel mechanism by which statins inhibit myofibroblast differentiation of VICs.

Roles for CNP in fibrosis and bone formation have been reported in tissues other than the heart valve. For example, administration of CNP in animal models reduced fibrosis associated with vascular intimal thickening, pulmonary fibrosis, and myocardial infarction, and an age-related decline in circulating CNP associated with reciprocal increase in left ventricular fibrosis. Mouse models with either targeted disruption of nppc43, the gene for natriuretic peptide precursor C, or a loss-of-function mutation in the NPR-B receptor display skeletal defects due to disturbed chondrogenesis during endochondral ossification. The mechanisms by which CNP influences fibrotic and bone formation processes are believed to result from binding of CNP to NPR-B, a transmembrane guanylyl cyclase that catalyzes synthesis of cyclic guanosine monophosphate (cGMP) and initiates cGMP-dependent signaling cascades. Preferential affinity of CNP for NPR-B and dose-dependent induction of
cGMP by CNP were previously reported in nonvalve cells.47 We also have observed that CNP treatment induces cGMP synthesis in VICs (C. Yip, PhD; K. Wei, MD, PhD; M. Blaser, B.Eng; C. Simmons, PhD; unpublished observations, 2011), which, together with our observation that silencing of NPR-B made VICs insensitive to CNP treatment, supports a similar CNP/NPR-B/cGMP signaling mechanism in VICs. Presumably, regulators downstream of cGMP signaling determine the specific cellular functions of CNP. The cGMP/protein kinase G pathway may be critical for blocking myofibroblast differentiation, as protein kinase G was recently shown to interact with phosphorylated Smad3 to inhibit fibrosis.48 Myofibroblast differentiation of VICs is also associated with phosphorylation of Smad3,49 suggesting a plausible downstream signaling pathway, the details of which are currently under investigation.

The observation that CNP inhibited VIC osteoblast differentiation is notable, as CNP has been reported to have reciprocal effects on calcification by bone versus vascular cells: in MC3T3-E1 preosteoblasts24–26 and primary rat calvarial cells,27,50 CNP increased calcium deposition and the expression of ALP and osteocalcin, whereas it reduced calcified aggregate formation in rat vascular smooth muscle cells25 and VICs.23 Formation of calcified aggregates in vitro can occur via osteoblast differentiation or through a nonskeletal processes involving myofibroblast differentiation leading to contraction- and apoptosis-dependent calcification34; it is unclear which mechanism was suppressed in previous reports of CNP-treatment of vascular smooth muscle cells and VICs. Here, by manipulating the culture conditions to promote osteoblast differentiation, we showed definitively that CNP inhibits osteoblast differentiation, as demonstrated by attenuation of osteoblast differentiation of single-valve mesenchymal progenitor cells, lower expression of bone-related transcripts and proteins, and less calcium deposition than non-CNP-treated cells. Of note, attenuation of osteoblast-related marker expression occurred before aggregate formation in CNP-treated cells, indicating that CNP modulated osteoblast differentiation directly and not by inhibiting aggregate formation. The mechanism by which CNP inhibits osteoblast differentiation of VICs is not known but might involve cGMP activation of type II cGMP-
dependent protein kinases, which play a critical role in CNP-mediated endochondral ossification. The effects of CNP are cell type specific, even when the cells originate from a common precursor. For example, ROB-C26 cells (a rat mesenchymal progenitor line) induced by bone morphogenetic protein 2 to undergo osteoblast differentiation displayed high levels of CNP-mediated cGMP activity, whereas the same cells committed to the adipogenic lineage with dexamethasone treatment exhibited marked reduction of CNP-mediated cGMP activity. Similarly, Inoue et al observed suppression of bone matrix transcript expression in primary newborn rat calvarial cells (representing a mix of early progenitors and more committed osteoblasts) but the opposite effect in the same cells after their commitment to the bone lineage in osteogenic culture conditions and in MC3T3-E1 preosteoblasts. Thus, the reciprocal effects of CNP on calcification by VICs versus bone cells may reflect differences in the extent of commitment of the 2 populations, with the freshly isolated VICs being rich in early progenitors and the bone cell lines being more committed. Alternatively, the reciprocal CNP effect may be an example of the unexplained paradoxical relationship between cardiovascular and skeletal calcification, similar to that observed by Wu et al, who found that simvastatin and pravastatin suppressed calcified aggregate formation by VICs but stimulated calcification by a mouse marrow stromal-derived cell line.

Similar to our observations with CNP, statins suppress aggregate formation that is associated with myofibroblast and osteoblast differentiation of VICs in vitro. Here we demonstrated that, as in endothelial cells, simvastatin upregulated CNP transcript expression in VICs; however, this occurred only in culture conditions that favored myofibroblast differentiation and not osteogenic conditions. Strikingly, we found that the inhibition of myofibroblast differentiation by statins was largely dependent on signaling through the NPR-B receptor. These data suggest a novel mechanism to explain the pleiotropic effects of statins, in which CNP production is augmented in quiescent VICs treated with statins, leading to suppression of myofibroblast differentiation by autocrine/paracrine effects of endogenous CNP. The cell type specificity of the effects of CNP and its role in mediating VIC response to statins suggest that the therapeutic efficacy of CNP or agents such as statins that mediate their effects by augmenting endogenous CNP levels will depend on the stage and nature of the disease. Indeed, statin treatment has been shown to be effective in slowing the progression of CAVD in the early stages of the disease but not in moderate aortic stenosis.

In summary, CNP inhibits myofibroblast and osteoblast differentiation of VICs and is responsible in part for inhibition of VIC myofibroblast differentiation by statins. These data suggest a cellular mechanism by which CNP maintains valve homeostasis to protect against CAVD and a novel mechanism to explain the pleiotropic effects of statins in VICs. This fundamental knowledge enables future studies aimed at defining the molecular mechanisms of the putative protective actions of CNP and its potential therapeutic value.

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Disclosures

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Inhibition of pathological differentiation of valve interstitial cells by C-type natriuretic peptide

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Detailed materials and methods

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

VIC isolation and animal model. Normal aortic valves were obtained from eight month old pigs immediately after death (Quality Meat Packers, Toronto, ON). VICs were isolated by collagenase digestion as described previously¹ and were at least 85% viable in all cases. Normal valve leaflets were also obtained for histological analysis. Sclerotic leaflets were obtained for histological analysis from pigs fed an atherogenic diet for six months². This hypercholesterolemic diet induces focal calcification preferentially in the aortic side of the valves, similar to those observed in human sclerotic valves³. Leaflets were fixed in 10% neutral-buffered formalin (NBF), paraffin-embedded and serial sectioned for immunofluorescent staining with polyclonal rabbit anti-mouse Runt-related transcription factor 2 (Runx2) (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-human α-SMA (clone 1A4), or polyclonal goat anti-human CNP (Santa Cruz Biotechnology) antibodies.

VIC culture. Cells were seeded on uncoated tissue culture-treated polystyrene (TCP) at a density of 10,000 cells/cm², and medium was changed every two days unless otherwise stated. To evaluate osteoblast differentiation potential, VICs were cultured in osteogenic medium consisting of complete medium (Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 10,000 Units/mL penicillin, and 10 mg/mL
streptomycin) supplemented with 10 mmol/L β-glycerophosphate, 10 μg/mL ascorbic acid and 10 nmol/L dexamethesone for up to 21 days at 37 °C and 5% CO₂. To evaluate myofibroblast differentiation potential, cells were cultured on TCP in complete medium for seven days at 37 °C and 5% CO₂. In treated samples, CNP (CNP-22, Bachem, Torrance, CA) was added with every media change at a concentration of 100 nmol/L, unless stated otherwise. This concentration was used because it was shown in preliminary experiments to be the most effective in mediating detectable cellular responses.

**Alkaline phosphatase activity.** VICs were cultured for 10 days in osteogenic medium with (100 nmol/L) or without CNP followed by fixation with 10% NBF. Alkaline phosphatase activity was detected by staining with Naphthol AS MX-PO₄ (Fisher Scientific, Ottawa, ON), N, N-Dimethylformamide, Trizma-hydrochloride acid, and Fast Red Violet LB salt. The stained samples were rinsed with distilled water three times and examined under a light microscope. Positively stained cells display a reddish/purple colour.

**Immunostaining.** To detect osteocalcin expression in vitro, cells were cultured in osteogenic media for 21 days, fixed in 10% NBF, and immunostained with 20 μg/mL mouse anti-bovine osteocalcin antibody (clone OCG4; Affinity BioReagents, Golden, CO), secondary biotinylated antibody, and 3,3’-diaminobenzidine substrate. α-SMA expression was detected in cells cultured in complete DMEM with (100 nmol/L) or without CNP by fixation with 10% NBF, permeabilization with 0.1% Triton-X and immunostained with 20 μg/mL monoclonal mouse anti-human α-SMA (clone 1A4) primary antibody, Alexa Fluor ® 568 secondary antibody, and counterstained with Hoechst 33242 for nuclei. To determine the myofibroblast content of freshly isolated VICs, an aliquot of isolated cells was cytopspun onto microscope glass slides, fixed, permeabilized, and stained for α-SMA. CNP was detected in fixed and permeabilized cells by immunostaining with polyclonal goat anti-human CNP (Santa Cruz Biotechnology) and Alexa Fluor ® 488 secondary antibody. For co-staining with CNP, a FITC-conjugated monoclonal mouse anti-human α-SMA antibody (clone 1A4; Sigma) was used. CD31 was detected in fixed and permeabilized cells immunostained with RPE-conjugated monoclonal mouse anti-pig CD31 (clone LCI-4; AbD Serotec). Nuclei were counterstained with Hoechst 33242.
**Immunoblotting.** Cell cultures were washed with ice-cold PBS followed by the addition of 1× cell lysis buffer (Cell Signaling, Danver, MA). Cells were scraped and transferred to pre-chilled tubes, followed by 30 minutes incubation on ice. Cell lysates were obtained by centrifugation at 16,200 × g for 15 minutes at 4 °C. The protein concentrations of cell lysates were determined by micro BCA protein assay (Pierce, Rockford, IL). 10 µg of protein extract from each treatment group was loaded onto 10% SDS-polyacrylamide gels for electrophoresis. Samples were then transferred to polyvinylidene fluoride membranes, followed by immunoblotting for α-SMA and GAPDH (mouse monoclonal antibody, Stressgen, Ann Arbor, MI). Expression of α-SMA was quantified by densitometry using ImageJ Software (NIH, Bethesda, MD) and normalized to GAPDH expression.

**Quantitative real-time PCR.** RNA extraction was performed after three, eight and 16 days of culture. Transcript expression of Runx2 (TIGR database: TC248152, forward primer: 5’-cccttggtctcatttctca –3’and reverse primer: 5’- cccagacctacgaatcaga -3’); osteonectin (ON; accession number: AW436132, forward primer: 5’-tcggatatctttcttctttcttc-3’ and reverse primer: 5’-ccctcacgctgggaagaggtttc-3’); and osteocalcin (OC; accession number: AW346755, forward primer: 5’-tcaaccccgactgcgacgag-3’ and reverse primer: 5’-ttggacagtggagatggta-3’) were quantified by real-time polymerase chain reaction (PCR) using SYBR green for detection and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; accession number: AF017079, forward primer: 5’- tgtaccaccaactgtttgtggtg-3’ and reverse primer: 5’ – ggcatggactgtggtcatgag –3’) as the housekeeping gene. Gene expression was quantified by standard comparative Ct (cycle threshold) method ($2^{-\Delta\Delta Ct}$). Relative gene expression was expressed as the ratio of CNP-treated samples to non-CNP treated samples, such that a ratio less than one indicates lower expression of the specific transcript with CNP treatment.

**Collagen synthesis.** Collagen content was measured with the Sirius Red dye release method\(^4\). Briefly, cultured cells were fixed with 10% NBF, followed by one hour incubation at room temperature with 0.1% Sirius Red F3BA reconstituted in saturated picric acid. Stained samples were washed five times with 10 mmol/L hydrochloric acid and then rinsed with distilled water. For quantification, Sirius Red dye was released by 0.1 mol/L sodium hydroxide for five minutes.
The absorbance of the supernatant containing the released dye was measured at 540 nm. Absorbance was normalized by total cell number determined by DNA content, which was measured using PicoGreen (Molecular Probes, Eugene, OR).

**Simvastatin treatment.** Simvastatin was activated prior to use by alkaline hydrolysis with NaOH and ethanol\(^5\). Cells were cultured in complete or osteogenic media with (1 \(\mu\)mol/L) or without activated simvastatin for up to 14 days. Morphological changes were evaluated with bright field microscopy. RNA was extracted after three days of simvastatin treatment and transcript expression of CNP (accession number: M64768, forward primer: 5’-accgactccagca-3’ and reverse primer: 5’-ataaagtggccagc-3’) was determined by qRT-PCR using SYBR green and the comparative Ct method, as previously described. Relative gene expression was expressed as the ratio of simvastatin-treated samples to non-simvastatin treated samples, such that a ratio greater than one indicates greater expression of the CNP transcript with simvastatin treatment.

**Small-interfering RNA (siRNA) for NPR-B.** Because of the lack of an NPR-B antagonist, specific inhibition of CNP signaling was achieved with custom designed Silencer® siRNAs (Applied Biosystems, Foster City, CA) targeted to porcine NPR-B (sense sequence: 5’-GUAUCUGGAUGCUCGCACAtt-3’ and anti-sense sequence: 5’-UGUGCGAGCAUCCAGAUACAG-3’). VICs were transfected with 100 nmol/L of NPR-B siRNAs or Silencer® negative control siRNAs using Oligofectamine (Invitrogen, Burlington, ON) for 12 hours. After transfection, the cells were incubated in complete media. Cells were allowed to proliferate for 48 hours, and the efficacy of siRNA knockdown was assessed by qRT-PCR. In a subset of cultures, 12 hours after transfection, cells were incubated in complete media with 100 nmol/L CNP or 1 \(\mu\)mol/L activated simvastatin. Cells were allowed to proliferate for four days, followed by formalin fixation and immunostaining for \(\alpha\)-SMA. Cells incubated in complete media without siRNA transfection served as a positive control. Cultures transfected with scrambled siRNA or NPR-B siRNA without oligofectamine served as negative controls.

**Compliant and stiff collagen matrices.** Compliant and stiff collagen matrices were constructed following procedures described previously\(^1\). VICs readily form osteoblastic aggregates on compliant matrices, whereas myofibroblastic aggregates are often found on stiff collagen
matrices. VICs were seeded on collagen matrices at 10,000 cells/cm² with (100 nM) or without CNP. Morphology analysis and aggregate count were evaluated after nine days in culture.

**Statistical Analysis.** Results are presented as mean ± standard error. Samples sizes were at least three in all cases, and experiments were repeated at least three times. Unpaired Student’s t-tests were used for comparisons between two groups. ANOVA and Fisher’s least significant difference tests were used to evaluate statistically significant differences in multiple group comparisons.

**References**

Supplemental Figures

Supplemental Figure I. CNP expression in normal aortic valve leaflets and freshly isolated VICs. Immunostaining of CNP (red) in (A) normal aortic valve, (B) the endothelium on the aortic side and (C) on the ventricular side (white arrows indicates CNP-positive endothelial cells), and (D) freshly isolated VICs. Nuclei are stained blue. Scale bar is 100 µm in panel (A); 50 µm in panels (B) and (C); and 20 µm in panel (D).
Supplemental Figure II. *Expression of CNP, α-SMA, and Runx2 in sclerotic valves.* (A) CNP, (B) α-SMA, and (C) Runx2 were generally not detected in the ventricularis of sclerotic porcine valves, although on occasion (D) CNP was expressed focally in regions of the interstitium (fibrosa shown) and (E) α-SMA was expressed in the focal regions of the ventricularis. Scale bars 50 µm in all panels. Indicated protein is red and nuclei are blue.
Supplemental Figure III. *Effect of CNP on cellular proliferation.* Proliferation of VICs from four hours to 15 days in (A) complete medium and (B) osteogenic supplemented (OS) medium.
Supplemental Figure IV. *CNP inhibits osteogenic and myofibroblastic aggregate formation.*

Osteogenic vs. myofibroblastic aggregates form more readily on compliant vs. stiff collagen matrices, respectively. (A) Relief phase contrast images of VICs cultured on compliant matrices with CNP treatment and (B) without CNP treatment. (C) Relief phase contrast images of VICs cultured on stiff matrices with CNP treatment and (D) without CNP treatment. (E) Quantitative comparison of aggregate formation in the presence or absence of CNP. “1” and “2” are the mean number of aggregates for the condition indicated.
Supplemental Figure V. *CNP inhibits collagen production*. Collagen production of CNP-treated and non-CNP treated cultures quantified with Sirius Red dye (* p < 0.05).
Supplemental Figure VI. *siRNA-based knockdown of NPR-B abrogates the inhibitory effect of CNP.* The percentage of cells expressing α-SMA stress fibers in VICs with and without NPR-B knockdown. (* p < 0.05 between non-treated and CNP-treated VICs; # p < 0.05 between transfected and non-transfected cultures).
Supplemental Figure VII. Inhibition of aggregate formation by simvastatin treatment. (A and C) Few aggregates were formed by VICs cultured in osteogenic media with simvastatin by day 9 and 14. (B and D) In the absence of simvastatin, aggregates formed readily by day 9 and appeared to increase in size by day 14. (E) Statin treatment significantly reduced aggregate formation by VICs cultured in osteogenic media in comparison to those of non-CNP treated samples (* p < 0.05).