CCN3 Inhibits Neointimal Hyperplasia Through Modulation of Smooth Muscle Cell Growth and Migration

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Objective—CCN3 belongs to the CCN family, which constitutes multifunctional secreted proteins that act as matrix cellular regulators. We investigated the pathophysiological roles of CCN3 in the vessels.

Methods and Results—We examined the effects of CCN3 on the proliferation and migration of rat vascular smooth muscle cells (VSMC). CCN3 knockout mice were created, and vascular phenotypes and neointimal hyperplasia induced by photochemically induced thrombosis were investigated. CCN3 suppressed the VSMC proliferation induced by fetal bovine serum. The neutralizing antibody for transforming growth factor-β did not affect the growth inhibitory effect of CCN3. Moreover, CCN3 enhanced the mRNA expression of cyclin-dependent kinase inhibitors, p21 and p15. Gamma secretase inhibitor, an inhibitor of Notch signaling, partially inhibited the enhanced expression of p21 induced by CCN3. CCN3 also inhibited the VSMC migration. Finally, the histopathologic evaluation of the arteries 21 days after the endothelial injury revealed a 6-fold enhancement of neointimal thickening in the null mice compared with the wild-type mice.

Conclusion—CCN3 suppresses neointimal thickening through the inhibition of VSMC migration and proliferation. Our findings indicate the involvement of CCN3 in vascular homeostasis, especially on injury, and the potential usefulness of this molecule in the modulation of atherosclerotic vascular disease. (Arterioscler Thromb Vasc Biol. 2010;30:675-682.)

Key Words: growth factors ■ vascular biology ■ vascular muscle

CN3/NOV belongs to a family of multicellular growth regulators originally referred to as the acronym CCN (cysteine-rich protein, Cys61/CCN1; connective tissue growth factor, CTGF/CCN2; and nephroblastoma overexpressed, NOV/CCN3), which now includes 3 additional genes (Wnt-1–induced secreted proteins 1 to 3; WISP1–3/CCN4–6). The CCN3 gene was first isolated from avian nephroblastomas induced by viral infections. Its antiproliferative activity in a variety of cells is now well-established, especially in tumor cells.4,5

See accompanying article on page 667

Although other members of the CCN family, such as CCN1 and CCN2, are strongly expressed in a wide range of tissues,6,7 CCN3 mRNA is highly and restrictively expressed in rat aortas and carotid arteries.8 This specific expression pattern in vessels indicates that CCN3 plays an important role in vascular homeostasis.

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The knockdown of CCN1/Cyr61 in mice suppresses neointimal hyperplasia in a rat artery balloon injury model.9 CCN2/CTGF also accumulates in the shoulders of human rupture-prone atherosclerotic plaques.10 Because CTGF induces mononuclear cell chemotaxis in a dose-dependent manner in vitro, CTGF may also have a role in atherogenesis. However, despite the similarity of the amino acid sequence of CCN3 and CCN1 and CCN2,4 the pathophysiological roles of CCN3 in vessels has not been fully elucidated. The present study confirmed the expression of CCN3 in medial layer of mouse aortas. Therefore, the effects of CCN3 on vascular smooth muscle cell (VSMC) proliferation and migration were investigated. Finally, CCN3-null mice were created and the physiological and pathological roles of CCN3 in vessels were determined.

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Materials and Methods

Reagents
The reagents used are described in the expanded Supplementary Materials and Methods section (available online at http://atvb.ahajournals.org).

Cell Culture
Primary cultures of rat aortic smooth muscle cells were isolated from 250- to 300-gram Wister Rats (QLEA Japan, Inc.) as described previously.11 See the Supplementary Materials and Methods section for details.

Semiquantitative Reverse-Transcription Polymerases Chain Reaction
Reverse-transcription polymerase chain reaction is described in the Supplementary Materials and Methods section.

Proliferation Assay
VSMC proliferation was quantified by direct cell counting and by 5-bromo-2'-deoxyuridine (BrdU) incorporation using a commercially available enzyme-linked immunosorbent assay kit (Roche Diagnostics). See the Supplementary Materials and Methods section for details.

Migration Assay
The ability of smooth muscle cells to migrate toward CCN3 was examined using a 96-well chemotaxis chamber (AB96; Neuro Probe).12 See the Supplementary Materials and Methods section for details.

Western Blotting
Western blotting was performed essentially as described previously.13 See the Supplementary Materials and Methods section for details.

Generation of CCN3 Mutant Mice
A knockout mouse line of the CCN3 gene was generated according to methods previously described.14 See the Supplementary Materials and Methods section for details.

Genotyping of Mutant Mice
Genomic southern hybridization and polymerase chain reaction were used to determine the CCN3 genotype (Supplementary Figure IVA, IVB available online at http://atvb.ahajournals.org) using genomic DNA extracted from embryo stem cells and tails, according to methods previously described.14 See the Supplementary Materials and Methods section for details.

Induction of Diabetes
Diabetes was induced by the injection of streptozotocin. See the Supplementary Materials and Methods section for details.

Immunohistochemistry and Immunocytochemistry
Immunohistochemistry and immunocytochemistry are described in the Supplementary Materials and Methods section.

Femoral Artery Injury
Mice femoral arteries were injured by means of a photochemically induced thrombosis method.15 See the Supplementary Materials and Methods section for details.

Statistical Analysis
All values are expressed as the mean±SD. The statistical significance was evaluated using the unpaired Student t test. P<0.05 was considered to be significant.

Results

Expression of CCN3 Protein in Mouse Aorta and Distribution of CCN3 Gene Expression in Mouse Tissues
First, we determined the expression of the CCN3 protein in the mouse aortas by immunohistochemistry using the anti-CCN3-specific antibody. The anti-alpha smooth muscle actin antibody was used as a marker for VSMC and anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) antibody was used as a marker for endothelial cells. The CCN3 protein was localized in the medial layer of the mouse aorta and colocalized with anti-alpha smooth muscle actin-positive cells but not with anti-PECAM-1-positive cells (Figure 1A). Next, we examined the expression of CCN3 in different mouse tissues by using reverse-transcription polymerase chain reaction. As shown in Figure 1B, the expression of CCN3 mRNA was the highest in the heart, weak in the brain, lung, and muscle tissues, and absent in the spleen and intestine.

CCN3 Inhibits VSMC Proliferation Independent of TGF-β Signaling and VSMC Migration
The expression of CCN3 mRNA was examined in cultured rat VSMC. CCN3 mRNA was detected in rat VSMC and increased with time in culture (Supplementary Figure IA).
Next, the biological functions of CCN3 were examined in cultured VSMC. Because CCN3 has antiproliferative activities in several tumor cell lines, we used human recombinant CCN3 proteins to determine whether CCN3 also inhibited VSMC proliferation in vitro. VSMC were plated in culture dishes in the presence or absence of 100 ng/mL CCN3 and the number of cells was counted every day. The number of VSMC was significantly lower in the presence of CCN3 from day 2 until day 4 after the start of the culture. However, this difference disappeared once the VSMC reached confluence (Supplementary Figure IB). The antiproliferative activity of CCN3 was also evaluated using BrdU incorporation. CCN3 alone had no effect on VSMC proliferation. However, CCN3 significantly inhibited VSMC proliferation induced by 10% fetal bovine serum (FBS), as well as that by platelet-derived growth factor-BB (PDGF-BB), in a dose-dependent manner (Figure 2A). Further, we also examined the effects of CCN3 on the cell cycle by flow cytometric analysis. The VSMC were treated with FBS in the presence of CCN3 or TGF-b and labeled with propidium iodide (PI). The ratio of PI-labeled smooth muscle cells in the G2/M phase to those in the G0/G1 phase was significantly decreased after both CCN3 and TGF-b treatment (Supplementary Figure IC, ID). In addition, the Trypan blue exclusion test was used to confirm that CCN3 was not toxic to cells (data not shown).

TGF-β inhibits the proliferation of a variety of cell types. Therefore, we examined whether TGF-β is involved in the inhibition of VSMC proliferation by CCN3. A TGF-β–specific neutralizing antibody was used to inhibit TGF-β. Both CCN3 and TGF-β inhibited the VSMC proliferation induced by 10% fetal bovine serum. An anti-TGF-β–specific neutralizing antibody could reverse the antiproliferative effects induced by TGF-β, but not those induced by CCN3. The anti-TGF-β–specific neutralizing antibody alone had no effects on VSMC proliferation (Figure 2B). Moreover, CCN3 also inhibited the VSMC migration induced by FBS in a dose-dependent manner (Figure 2C).

**CCN3 Increases the Expression of the CDK Inhibitors p15 and p21 Through the Notch Signaling Pathway**

We examined the expression of cell cycle regulators to determine the molecular mechanism underlying the antiproliferative activity of CCN3. Among the factors studied, both CCN3 and TGF-β upregulated the cyclin-dependent kinase inhibitors p15 and p21 within 12 hours (Figure 3A). A time-course study revealed that CCN3 treatment increased the expression of p21 mRNA after 12 hours, but the expression returned to the baseline within 24 hours, whereas TGF-β treatment increased the expression of p21 mRNA in a time-dependent manner (Supplementary Figure IE). This result supported the idea that CCN3 increases the expression of cell-cycle regulators independently of TGF-β signaling. The effects of CCN3 on TGF-β signaling were also examined. CCN3 had little effect on either the phosphorylation of Smad2 (Figure 3B) or the activation of plasminogen activator inhibitor-1 (PAI-1) promoter, which contains the TGF-β–responsive element (Supplementary Figure IF). Collectively, these data indicated that CCN3 inhibited VSMC proliferation independent of TGF-β signaling.

CCN3 activates the Notch signaling pathway through its carboxyl-terminal cysteine-rich domain and inhibits osteogenic differentiation and cell proliferation in Kusa-A1 cells. Therefore, the effects of CCN3 on the Notch pathway in VSMC were examined. First, we examined the effect of both TGF-β and CCN3 on the expression of the intracellular domain of Notch1 (ICN1). This domain is proteolytically cleaved from the plasma membrane in conjunction with the activation of the Notch receptor. The
targeted by the Notch signal, increased in the presence of CCN3. The overexpression of RPMS-1 in the VSMC inhibited the CCN3-induced expression of Hey1. The increase in the expression of p21 mRNA induced by CCN3 was suppressed by the overexpression of RPMS-1. These results indicated that CCN3/Notch is a signaling pathway that increases the expression of the cell-cycle regulators. Recently it has been reported that PDGF receptor-β is a direct target of the activated Notch1 and Notch3 receptors. Therefore, we examined the effects of CCN3 on the expression of PDGF receptor-β and its tyrosine phosphorylation and ERK activation in VSMC. None of these responded to CCN3, as shown in Supplementary Figure IIA and IIB.

**Mice Lacking CCN3 Show Enhanced Neointimal Hyperplasia in Response to Injury**

**CCN3**−/− mice were generated by conventional homologous recombination to investigate the physiological and pathological roles of CCN3 in vivo. Bacterial artificial chromosome-based targeting vectors were used to specifically replace exons 1, 2, and a part of exon 3 of the CCN3 gene with a neomycin selection cassette (Figure 4A). Chimeric mice derived from embryonic stem cell clones were bred to C57BL/6 males to produce F1 offspring and CCN3+/− mice were intercrossed to produce homozygous offspring (Supplementary Figure IV A, IVB). The deletion in the mRNA corresponding to the genomic deletion of the CCN3 locus was confirmed using reverse-transcription polymerase chain reaction with cDNA generated from heart RNA that had been extracted from 28-day-old mice (Supplementary Figure IV C). CCN3 protein was not detected in the aortas from CCN3−/− mice by immunohistochemistry (Figure 4B). Intercrosses between CCN3+/− animals produced viable offspring at the expected Mendelian frequency (data not shown). CCN3−/− animals developed normally to adulthood and both the males and females were fertile. The Table shows the background data for the 2-month-old CCN3−/− mice in comparison to their littermate controls. There were no obvious differences in body weight, systolic blood pressure, plasma glucose concentrations, and HbA1c levels between the 2 groups. Hematoxylin and eosin staining and Masson trichrome staining of the aortas dissected from the 2-month-old mice showed that the vasculature appeared to have developed normally (Figure 5A). The amount of type III collagen, which is one of the main components of the extracellular matrix in the aorta, also showed that there were no obvious changes in the amount of extracellular matrix because of the lack of CCN3 (Figure 5A). Next, to evaluate the role of CCN3 in the pathogenesis of neointimal hyperplasia, we injured the femoral arteries of the wild-type (n=6) and CCN3-null (n=6) male mice, using photochemically induced thrombosis. Histopathologic examination of the arteries 21 days after the injury revealed markedly enhanced neointimal thickening in the CCN3-null mice in comparison to the wild-type mice (Figure 5B). The mean intima-to-media ratios at 21 days after the injury were significantly higher in the arteries of the CCN3-null

**Figure 3.** CCN3 upregulated the expression of cell cycle regulators, p15 and p21, through the Notch signaling pathway. A, Cells were serum-starved for 24 hours and then incubated with 1 ng/mL TGF-β1 or 100 ng/mL CCN3 for 12 hours at 37°C. Then, the expressions of p21 and p15 were examined and compared with the expression of GAPDH by reverse-transcription polymerase chain reaction. B, The expressions of phospho-Smad2 and total Smad2/3 were determined by immunoblotting. C, The expression of ICN1 was examined by immunohistochemistry. Bars=20 μm. D, The cells were incubated with 1 ng/mL TGF-β1 or 100 ng/mL CCN3 in the presence or absence of 5 μmol/L γ-secretase inhibitor for 12 hours. The expression of p21 was examined and compared with the expression of GAPDH by reverse-transcription polymerase chain reaction. One representative experiment out of 2 is shown.
mice (1.193 ± 0.071) than those of the wild-type mice (0.205 ± 0.011; P < 0.01; Figure 5C).

To understand how intimal thickening is enhanced in the CCN3-null mice, we studied cell proliferation and endothelialization after the vascular injury (Supplementary Figure VA, VB). We investigated the BrdU incorporated into the vascular wall 1 week after photochemically induced thrombosis. The number of BrdU-positive cells was higher in the aortas of the CCN3-null mice than in those of the control mice, indicating that the VSMC proliferated even in the absence of CCN3. We also investigated the endothelialization after antplatelet endothelial cell adhesion molecule staining and found that, compared to the controls, endothelialization was reduced in the aortas of the CCN3-null mice.

We also examined platelet function by measuring the tail bleeding time (data not shown) and adventitial neovascularization by anti-PECAM-1 staining (Supplementary Figure VB). However, there were no differences in either the tail bleeding time or the adventitial neovascularization.

Table. Basic Characteristic of CCN3-Null Mice Compared to Littermate Controls

<table>
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<th>CCN3+/+</th>
<th>Systolic blood pressure, mm Hg</th>
<th>Plasma glucose, mg/dL</th>
<th>HbA1c, %</th>
<th>N</th>
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<tr>
<td>Body weight, grams</td>
<td>22.9±4.0</td>
<td>102.1±10.5</td>
<td>227.9±95.5</td>
<td>3.8±0.35</td>
<td>14</td>
</tr>
<tr>
<td>CCN3−/−</td>
<td>23.2±3.6</td>
<td>113.6±19.4</td>
<td>198.1±104.0</td>
<td>3.5±0.38</td>
<td>17</td>
</tr>
</tbody>
</table>

Figure 4. Generation of CCN3−/− mice. A, Genomic organization of the CCN3 gene (top), targeting vector (middle), and targeted allele (bottom) were indicated. The targeting vector was constructed by replacement of the exon 1 to a part of 3 in CCN3 genomic fragment with the PGK-neo cassette (NEO) and attachment of the HSV-tk cassette (TK) to its flank for the negative selection marker. B, Immunohistochemical staining of wild-type and mutant 2-month-old aorta with antibodies against CCN3. Bars=50 μm.

**CCN3 Expression Decreases in the Aorta of Streptozotocin-Induced Diabetic Mice**

Finally, to determine the relevance of CCN3 expression to disease conditions, we examined the CCN3 expression in diabetic mice and found that it was significantly lower in the aorta of diabetic rats in comparison to control rats (Supplementary Figure VIA, VIB). Insulin treatment for reducing the
blood glucose level increased the CCN3 expression in vessels, excluding the possibility that CCN3 expression decreased because of the toxic effects of streptozotocin (Supplementary Figure VIC).

Discussion

This article reports 5 novel findings. First, the CCN3 protein was expressed in the medial layer of the mouse aorta, especially in VSMC in vivo. Second, CCN3 inhibited VSMC proliferation and migration. Third, CCN3 inhibited VSMC proliferation independent of TGF-β signaling. Fourth, CCN3 increased the expression of the cyclin-dependent kinase inhibitor, p21, at least partly through Notch signaling in VSMC. Fifth, CCN3−/− mice had a normal vascular phenotype under normal conditions; however, CCN3 enhanced the neointimal hyperplasia in femoral arteries induced by endothelial injury.

Atherosclerosis is a leading cause of cardiovascular diseases around the world. VSMC migration and proliferation in the subendothelial space are pivotal steps in atherosclerosis development and in restenosis formation after vascular intervention. The recent development of drug-eluting stents, which release small amounts of anti-proliferative agents locally, is an effective strategy for reducing restenosis, which is primarily caused by the proliferation of VSMC. However, reducing the incidence of restenosis remains a considerable burden for the medical community. Therefore, the biological stimuli that inhibit VSMC proliferation and migration must be completely understood to address various clinical problems.

CCN3 plays potent roles in tumorigenesis, chondrogenesis, skeletal and cardiac development, and hematopoietic stem cell regulation. However, the roles of CCN3 in vessels are not yet fully understood. Ellis et al. precisely examined the expression of CCN3 in the rat aorta and in the carotid artery. They localized CCN3 mRNA expression to the smooth muscle cells in both the aorta and carotid artery. Further, they showed 7 days after injury to the carotid artery CCN3 expression in the media is substantially reduced but that at the edge of the intima increases. At 14 days after the injury, the CCN3 expression in the media remains low, but it is substantially increased throughout the intima in the VSMC. These observations strongly indicate that CCN3 is involved in vascular ho-
meostasis, especially after vascular injury. Using immuno-
histology, we established that the CCN3 protein is ex-
pressed in the smooth muscle cell layer in the mouse aorta.
To our knowledge, this is the first in vivo report to confirm
CCN3 protein expression in aortic smooth muscle cells.

In agreement with the antiproliferative effects of CCN3
in a large variety of cell types, we could also show that
CCN3 inhibited VSMC proliferation in vitro (Figure 2A,
2B). However, Ellis et al\(^8\) reported that CCN3 has no effect
on VSMC proliferation. However, there were some signif-
icant differences in the experimental procedures used. The
current study used human recombinant CCN3, which
shares 80% amino acid identity with that of mouse CCN3.
In contrast, Ellis et al used recombinant CCN3 prepared
using a baculovirus system. They also used a higher
concentration of PDGF-BB and a different kind of fetal
bovine serum, which induced higher proliferation rates
than those seen in the current study. Therefore, the
antiproliferative effects of CCN3 may have been masked
by the potent mitogenic stimulation. Furthermore, the
antiproliferative effects of CCN3 seemed transient (Sup-
plementary Figure IB). Therefore, it might be difficult to
determine these antiproliferative effects if the incubation
time is longer than that used in this experiment.

The next series of experiments examined how CCN3
inhibits VSMC proliferation. CCN3 physically interacts
with fibulin C,\(^25\) integrins,\(^5\) and connexin 43.\(^26\) In addition,
CCN3 inhibits proliferation and differentiation through the
Notch pathway.\(^17,27\) Notch signaling dictates the cell fate
and critically influences cell proliferation, differentiation,
and apoptosis. It is observed in various tissues, including
vessels. For instance, Notch ligands,\(^28\) receptors,\(^29\) and
effectors\(^30\) are expressed in VSMC in vivo. Furthermore,
Notch signaling also plays a critical role in the correct
architecture of vascular systems.\(^31\) Therefore, we inves-
tigated the involvement of the Notch signaling pathway in
the antiproliferative activity of CCN3. CCN3 increased the
expression of ICN1, whereas \(\gamma\)-secretase inhibitor reversed
the antiproliferative effects of CCN3 and partially inhib-
ited the CCN3-induced upregulation of p21 (Figure 3C,
3D, and Supplementary Figure II); this indicated that the
Notch signaling pathway was at least partially involved in
the antiproliferative activity of CCN3. In addition, we
examined the participation of TGF-\(\beta\) signaling in the
CCN3-induced antiproliferative activity of VSMC, be-
cause TGF-\(\beta\) inhibits cell growth in a variety of cell types.
Moreover, TGF-\(\beta\) and the Notch pathway cooperatively
suppress epithelial cell growth.\(^32\) However, anti-TGF-\(\beta\)
neutralizing antibody could not reverse the CCN3-induced
antiproliferative activity in VSMC (Figure 2B). Further-
more, CCN3 had no effect on the phosphorylation of
Smad2, which mediates TGF-\(\beta\) signaling and the activity of
the plasminogen activator inhibitor-1 promoter, which
contains the TGF-\(\beta\)-responsive element. CCN3 also did
not have any effect on the expression of the mRNA of
either TGF-\(\beta\) or its receptors (data not shown). These
results indicate that the inhibition of VSMC proliferation
by CCN3 is partially dependent on the Notch pathway but
independent of TGF-\(\beta\) signaling.

Despite the growth inhibitory effects of CCN3 in a wide
variety of cell types, CCN3\(^-/-\) mice were surprisingly
viable and fertile. CCN3\(^-/-\) mice could not be distin-
guished from their littermate controls by their gross
appearance or vascular structure (Figure 5A).

CCN3 knockout mice were recently generated by another
exon 3 of the CCN3 gene was targeted (NOV\(^\text{del3/-}\)) and some, but not all, of the NOV\(^\text{del3/-}\) mice were embryonically lethal and exhibited defects in
both skeletal and cardiovascular development. However,
NOV\(^\text{del3/-}\) mice produce a mutant form of the CCN3
protein, which can be secreted and lacks only the von
Willebrand factor (VWC) domain but contains 3 other
intact functional domains. Furthermore, this phenotype is
partly transmitted by dominant inheritance. Therefore, this
protein is strongly suspected to result in the phenotypes
observed in NOV\(^\text{del3/-}\) mice. In contrast, the mRNA in
CCN3\(^-/-\) mice lacked a region encoding 127 amino acids
from the N-terminal end. This may result in a peptide that
is translated from the fourth Methionine in complete CCN3
mRNA and is not secreted because of the absence of the
presequence required for secretion. Therefore, we believe
that the presence of the mutant CCN3 peptide only in
NOV\(^\text{del3/-}\) leads to phenotypic differences between both
types of mutant mice.

Although the vascular phenotype was normal under
normal conditions, the enhancement of the intimal thick-
ening in the CCN3-null mice after endothelial injury suggested that CCN3 plays important roles in pathological
situations and also indicated that CCN3 inhibited VSMC
proliferation and migration. Among the several methods
that could induce the intimal thickening, such as flexible
wire, balloon catheter, and laser injury, we chose the
photochemically induced thrombosis method, which could
induce intimal thickening constantly and relatively milder
than the others.

It has also been reported that CCN3 induces neovascular-
ization. Further, we observed reduced endothelialization in
CCN3-null mice. Therefore, increased VSMC proliferation
and reduced endothelialization can affect the increased inti-
mal thickening seen in the CCN3-null mice.

Finally, we studied CCN3 expression in diabetic condi-
tions and found that this expression was reduced in
diabetic aortas as compared to the control aortas. Diabetic
patients are prone to atherosclerotic vascular disease\(^33\)
and diabetic vascular lesions are prone to restenosis after
angioplasty.\(^34\) However, the mechanism by which ather-
genesis is accelerated in patients with diabetes mellitus has
not yet been determined. Because CCN3 inhibited VSMC
proliferation and migration, the reduced expression of
CCN3 might be linked with the accelerated atherogenesis
in diabetes. So far, there have been few reports describing
CCN3 gene regulation. For instance, 1 report\(^35\) describes
that Wilms tumor 1, a transcriptional factor, binds to the
CCN3 promoter region and regulates its expression. In
addition, the expression of the tumor suppressor gene \(p53\)
depends on CCN3 expression.\(^36\) It therefore would be
useful to investigate the precise transcriptional regulation
of CCN3 and seek pharmacological agents to prevent the
downregulation of CCN3 in diabetic vessels to reduce the risk of diabetic vascular complications in the future.

In summary, this study demonstrated that CCN3 is a regulator of VSMC proliferation and migration and of neointimal hyperplasia. Therefore, these results indicate the potential usefulness of this molecule in the modulation of atherosclerotic vascular disease.

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Disclosures

None

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Supplementary Figure II

A

**BrdU uptake (%) (relative to untreated)**

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</table>

**p** < 0.05

**p** < 0.01

B

**p21**

- - + + - - + + - - + +

**Hey1**

- - + + - - + + - - + +

**RPMS-1**

- - + + - - + + - - + +

GAPDH

**p21/GAPDH**

\[ p < 0.01 \]

\[ p < 0.01 \]

**Hey1/GAPDH**

\[ p < 0.05 \]

\[ p < 0.05 \]

**RPMS-1/GAPDH**

- - + + - - + + - - + +
Supplementary Figure III
Supplementary Figure IV

A

- Genotype +/+ +/
  - Probe a: 20 → 3.5

B

- Genotype +/+ -/
  - 1.5 kb

C

- Genotype -/- +/+:
  - 561 bp

Probe b: 20 → 16
Supplementary Figure V
**Supplementary Figure VI**

**A**

Control mouse  

STZ-induced diabetic mouse

**B**

![Bar graph showing CCN3 positive area comparison between control and STZ-induced diabetic mice](image)

Control mouse  
STZ-induced diabetic mouse

P < 0.05

**C**

Control  
STZ  
STZ+insulin

![Images showing CCN3 expression in different conditions](image)
On Line Supplement

Expanded Material and Methods

Reagents

Recombinant human CCN3, platelet-derived growth factor (PDGF)-BB and TGF-β were purchased from R&D Systems. Gamma secretase inhibitor was purchased from Calbiochem.

Cell Culture

Primary cultures of rat aortic SMC were isolated from 250-300 g Wister Rats. In brief, the tunica media was separated from the adventitia and endothelium by collagenase treatment. The cells were dispersed in collagenase and elastase and maintained in DMEM supplemented with 10% (vol/vol) FBS.

Semi-quantitative reverse transcription-PCR

Total RNA was isolated from cultured rat aortic VSMCs, as well as from several mouse tissues, using the RNeasy Mini Kit (Qiagen). One to 5 µg of total RNA was primed with Oligo dT primer and reversely transcribed using SuperScript III reverse transcriptase (Invitrogen). The polymerase chain reaction was performed in a 25 µl PCR reaction
containing 1 µl cDNA, Taq reaction buffer (Go Taq, Promega) and 10 µM dNTPs and the following primer sequence was used: p21 (Accession number: NM_080782)

5´-TCCACACAGGAGCAAAGTAT-3´ and 5´-CGCTTGGAGTGATAGAAATC-3´,
p15 (NM_130812): 5´-GAGACGAGGGCTGTAATAATC-3´ and
5´-CCTGCTCTTCAGCTAAGTCTCT-3´, CCN3 (NM_002514):
5´-CTATAAGCCAGGTTCTGTTAATGAACAGC-3´,
TGF-β1: (XM_0010777389) 5´-TTTGGAGGAGGCGAGCTTTTA-3´ and
5´-ACCAGTGAGGAGACCCCAATG-3´, Hey1 (NP_034553):
5´-GGGAAAGGGATGGTTGAGTTA-3´ and 5´-ATGCTCAGATAACGAGGAC-3´.
GAPDH (NM_017008): 5´-GGCACAGTCAAGGCTGAGAAT-3´ and
5´-TCTCGCTCTGGAAGATGGAAT-3´. The PCR was performed for 30-35 cycles, with each cycle consisting of 2 min denaturation at 94ºC, 30 s of annealing at 55ºC and 30 s of elongation at 72ºC. The PCR amplification was followed by a 7 min final extension at 72ºC. The size of the PCR amplification products were as follows: p21: 435 bp, p15: 413 bp, CCN3: 460 bp, TGF-β: 407 bp, Hey1: 567 bp, GAPDH: 483 bp. GAPDH was used as an internal control. Ten µl of the PCR product were separated by electrophoresis on a 2% agarose gel (Ronza), which was then stained with an ethidium bromide solution (Sigma). The signals were photographed by a CCD camera system (Printograph,
ATTO) and densitometric analyses of fluorograms were performed using an image scanner (EPSON GT-X900) with the Scion image software program (Scion Corporation).

**Proliferation Assay**

For the BrdU incorporation, the VSMCs were plated at a density of $1 \times 10^4$ cells/well in a 96 well tissue culture plate. After 24 h serum starvation, the cells were treated with various concentrations of FBS or PDGF-BB in the presence of BrdU at 37°C in a humidified incubator containing 5% CO₂ for 24 hr. In the case of treatment with CCN3 and/or the neutralizing anti-TGF-β₁-β₂-β₃ antibody (R&D Systems), the cells were incubated with CCN3 and/or the anti-TGF-β antibody prior to adding the FBS or PDGF-BB. The incorporation of the BrdU into the DNA was detected using a monoclonal antibody against BrdU, followed by a fluorochrome-conjugated second antibody. The absorbance values were measured at 405 nm (reference 490 nm) using a microplate reader (SUNRISE remote, WAKO).

**Migration Assay**
The cell migration ability of SMC toward CCN3 was examined using a 96-well chemotaxis chamber. (AB96, Neuro Probe). A polyvinylpyrrolidone-free polycarbonate membrane with an 8.0 µm pore size (PFD8, Neuro Probe) was coated with 100 mg/ml of type I collagen (Vitrogen 100, Collagen Corp.) before use. For each set of experiments, any migration in the absence of CCN3 (considered to be random migration) served as the control and was referred to as 100% migration. Cell counts were made in a blinded manner. The experiments were performed in triplicate for every concentration of CCN3 and were repeated five times.

**Western blotting**

The cells were serum-starved for 24 hr and then incubated with 1 ng/ml TGF-β1 or 100 ng/ml CCN3 for 30 min at 37°C. Next, the cells were lysed in boiling Laemmli’s sample buffer and then subjected to SDS-10% polyacrylamide gel electrophoresis (PAGE). Immunoblotting was performed essentially as described before. Briefly, the samples were blotted to nitrocellulose membrane (Hybond ECL, GE healthcare), the blots were blocked with 3% BSA and probed with the anti phospho-Smad 2 antibody (1:1000 dilution, Millipore), anti Smad 2/3 antibody (1:500 dilution, BD biosciences) overnight. Then the blots were washed and incubated either with peroxidase-conjugated anti rabbit
(1:2500 dilution, GE healthcare) or anti mouse (1:5000 dilution, Jackson Immunolaboratory) immunoglobulins. After washing, the sites of antibody binding were visualized using an ECL Western blotting detection system (GE healthcare).

Overexpression of RPMS-1 in VSMCs
cDNA plasmids containing RPMS-1 ORF were kindly provided by Professor Paul J Farrell (Imperial College Faculty of Medicine, London)\(^2\). Fifty thousand VSMCs were seeded in 12-well dishes. After 16 h of incubation, the cells were transfected with 0.6 \(\mu\)g of RPMS-1 construct by using Lipofectamine 2000 reagents (Invitrogen), according to the manufacturer’s instructions. After transfection, the cells were serum-starved for 48 h. Next, the cells were incubated with or without CCN3 for 12 h. The gene expressions were analyzed using RT-PCR.

Effects of CCN3 on the cell cycle distribution of VSMCs

VSMCs were seeded with 60% confluence. After 16 h of incubation, the cells were starved for 24 h. The cells were then incubated with 100 ng/ml CCN3 or 1 ng/ml TGF-\(\beta\) in the presence or absence of FBS for 24 h. For cell-cycle analysis, the cells were
stained with 50 μg/ml propidium iodide (PI; Sigma Aldrich) as previously described 3, and the DNA content was analyzed by flow cytometry (FACSCalibur; BD Immunocytometry Systems, San Jose, CA)

*Effects of CCN3 on PDGFR-β expression and tyrosine phosphorylation and ERK activation*

Sub-confluent VSMCs in 100-mm dishes were serum-starved for 24 h. The cells were then treated with 100 ng/ml CCN3 or 50 ng/ml PDGF-BB for 5 min at 37°C. After incubation, the cells were washed once with ice-cold PBS; solubilized with 1 ml of lysis buffer (50 mM TrisHCl [pH 7.4], 150 mM EDTA, 1% sodium deoxycholate, 1% Triton X100, and 0.1% SDS), 500 μM sodium orthovanadate, and complete protease inhibitor cocktail (Roche); placed on ice for 15 min; and centrifuged at 14,000g to remove insoluble materials. The supernatant (20 μl) was subjected to 10% SDS in order to examine the activation of ERK. To study PDGFR-β expression and tyrosine phosphorylation, immunoprecipitation was performed as described earlier, using anti-PDGFR-β antibody followed by absorption to protein G-Sepharose 4. Immunoblotting was performed essentially as described above. The following commercial antibodies were used: mouse anti-p44/42 MAPK (1:1000 dilution, #9102) and mouse anti-phospho p44/42 MAPK (1:2000, #9106) (Cell Signaling).
Anti-phosphotyrosine (1:1000, 4G10) was obtained from Millipore. Rabbit anti-PDGFR-β antibody has been described before.

**Generation of CCN3 mutant mice**

A BAC (bacterial artificial chromosome) clone containing the CCN3 was screened from a mouse BAC library (Research Genetics). Using the BAC, a targeting vector was constructed to delete the genomic region encompassing exons 1, 2 and a part of 3 for complete elimination of the CCN3 transcription (Figure 4A). This vector was introduced into R1 ES cells and homologous recombinants were cloned through selection with antibiotics and verification of the genotype. Chimeric mice with the targeted ES cell clones were developed to obtain mice heterozygously transmitting the CCN3-knockout genome. Their descendants were backcrossed to C57Bl/6J (The Jackson Laboratory) for at least 6 generations and maintained as heterozygotes. The heterozygous mice were inter-crossed to produce homozygous offspring. CCN3 null and wild-type male mice that were 10 weeks of age were used for the experiments. All of the experimental procedures were performed in accordance with Chiba institutional guidelines for animal research. The body weight, blood pressure, fasting blood glucose levels and HbA1c were measured at the time of sacrifice. The blood glucose level was
measured by a Medisafe mini system (TERUMO Corporation) and the blood pressure was measured by the tail cuff method using a softron BP-98A (Softron).

*Genotyping of mutant mice*

Genomic southern hybridization (Supplemental Figure 2A) and PCR (Supplemental Figure 2B) using the genomic DNA extracted from ES cells and tails were used to determined the CCN3 genotype. The sequences of the PCR primers for amplifying the CCN3-intact and CCN3-knockout genome indicated in Figure 4A are c: CTTCCCTGCTCTTCCATCTCT, d: AATGCCAGTCTGGTTGTTGG, e: TGCATCTGAGCAGTTCTGAG, f: CTCGTGCTTTACGGTATCG.

PCR amplification of genomic DNA extracted from tail biopsies was used to genotype the CCN3 mutant mice. Briefly, tail samples were incubated in a lysis buffer containing 0.2M NaCl, 0.02M EDTA, 0.05M Tris HCl, 0.5% SDS and 0.5 mg/ml proteinase K overnight at 56°C. The PCR was performed using four specific oligonucleotide primers for amplifying the wild-type and knock-in alleles. A 1.4 kb band corresponds to the mutant allele, while a 381-bp band corresponds to the wild-type allele (Supplementary Figure 2A). The PCRs contained 2 µl 10 × PCR buffer with MgCl₂, 2 µl dNTPs (0.25 mM), 0.4 µl primers (1 mM), 0.2 µl Taq DNA polymerase (5 U/µl, ExTaq, Takera), 1 µl
DNA template and distilled H$_2$O in a final volume of 20 µl. Amplification was
performed using an Applied Biosystem 9700 PCR machine with the following program:
94 ºC for 5 min and then 94ºC for 30 s, 56ºC for 30 s, 72ºC for 30 s for 30 cycles and
finally 72ºC for 2 min.

**Immunohistochemistry**

Mice aortas were frozen in OCT compound and then were cut into 10 µm sections. The
sections were air-dried, fixed in ice-cold methanol for 10 min, rinsed in PBT
(phosphate-buffered saline (PBS) with 0.1% Tween 20) and blocked in PBT containing
0.5% BSA for 30 min. Primary antibodies were diluted in 0.5% BSA in PBT and
applied overnight at 4ºC. After rinsing several times with PBT, fluorophore-conjugated
secondary antibodies were applied for 2 hr. The sections were then rinsed in PBT,
nuclear stained with Hoechst 33342 (1:200; Dojindo), mounted (Fluoromount G,
SouthernBiotech) and viewed with a fluorescence microscope (Axio Oberver, Leica) or
with a confocal laser scanning microscope (Leica LSM5 PASCAL). The images were
processed using Adobe Photoshop. The following antibodies were used: rabbit
anti-CCN3 antibody (1:100, ab10888: Abcam), anti-PECAM-1 antibody (1:200: MEC
**Immunocytochemistry**

SMC were cultured on Lab-Tek II chamber slides (Nalge Nunc International), deprived of serum for 24 hr and stimulated either with 1 ng/ml TGF-β or 100 ng/ml CCN3 for 30 min. Next, the cells were fixed in ice-cold methanol for 10 min, rinsed with PBS and incubated in a blocking buffer of 0.5% BSA, 0.25% Tween 20 in PBS for 30 min at room temperature. The slides were then incubated with an anti ICN1 antibody (Val 1774, 1:100 dilution, Cell signaling) overnight at 4°C, washed with PBS, followed by incubation with 1:1000 dilution of fluorescent-conjugated secondary antibody (Alexa Fluor 488 goat anti rabbit IgG, Invitrogen) for 2 hr at room temperature. The slides were then washed with in PBS, nuclear stained with Hoechst 33342 and mounted with a fluorescence mounting medium.

**Femoral Artery Injury**

The femoral arteries of the mice were injured using a photochemically-induced thrombosis method. The mice were anesthetized with the intraperitoneal administration of pentobarbital (50 µg/g body weight). Rose-Bengal dye (10 mg/mL in phosphate-buffered saline) was then injected into the orbital venous plexus (25 µg/g
body weight). Meanwhile, the left femoral artery was carefully exposed and transilluminated with a heat-absorbing filter (Hamamatsu photonics) for 10 min. This procedure resulted in local intraluminal thrombus formation accompanied by endothelial injury. Twenty-one days later, the mice were anesthetized and perfusion fixed in 2% formaldehyde in PBS.

**Luciferase assay**

SMC were plated in 12-well dishes at 50% confluence and transfected with 400 ng per well p3TP-lux construct that contained three consecutive TPA response elements and a portion of the plasminogen activator inhibitor 1 (PAI-1) promoter region by Fugene 6 transfection reagent (Roche Molecular Biochemicals). At 6 hr-post-transfection, cells were deprived of serum for 24 hr. Thereafter, the cells were stimulated either with 1 ng/ml TGF-β or 100 ng/ml CCN3 for 16 hr. The luciferase activities in the cell lysate were measured in a 1420 ARVO SX multilabel counter (Wallac, Inc, Gaithersburg, MD) using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). To correct for potential variation in transfection efficiency, 400 ng per well pRL-TK vector (Promega) was cotransfected in all experiments. All assays were performed in quadruplicate and then were repeated three times.
Induction of diabetes

Diabetes was induced in mice by intraperitoneally injecting them with streptozotocin (STZ; Sigma Chemical, St. Louis, MS, USA). STZ was dissolved in an ice-cold citrate buffer at pH 4.6 and immediately injected into the mice at a dose of 100 mg·kg body weight\(^{-1}\)·day\(^{-1}\) for 3 days. Control mice received only the citrate buffer. The first day of STZ injection was designated as day 0. On day 14, the mice were killed, and their aortas were dissected. The blood glucose levels were significantly higher in the STZ-induced diabetic mice (n = 4) than in the control mice (n = 8) (306.3 (30.9) mg/dl vs. 120.4 (30.0) mg/dl, \(P < 0.01\)). Moreover, the body weight of STZ-induced diabetic mice was significantly lower than that of the control mice (23.3 (1.0) g vs. 25.8 (1.0) g, \(P < 0.01\)).

We also treated some STZ-induced diabetic mice (n = 6) with 0.1 U/body of insulin from day 5 until day 14. The blood glucose levels of insulin-treated mice were significantly lower than those of untreated insulin (234 (60.6) mg/dl vs. 306.3 (30.9) mg/dl, \(P < 0.05\)).

Legends to Supplemental Figures
Supplemental Figure I.

Expression of CCN3 in VSMCs and the effects of CCN3 on the VSMCs.

A. The expression of CCN3 mRNA was examined and compared with the culture time by using RT-PCR. One representative of 2 experiments is shown.

B. The effects on CCN3 on VSMC proliferation. The number of cells was counted in the absence (open bars) or presence (filled bars) of CCN3 at the indicated times.

C. Effects of CCN3 on the cell cycle of VSMCs. Flow cytometric analysis of the cell cycle distribution of VSMCs. The histograms show a representative of 3 experiments.

D. Percentage of cells in the S+G2/M phase. VSMCs were initially serum-starved for 24 h and then treated with CCN3 or TGF-β for 24 h in the presence or absence of FBS. Cell cycle distribution was determined by flow cytometric analysis of the DNA content in propidium iodide. *P < 0.05, **P < 0.001

E. Cells were serum-starved for 24 h and then incubated with 1 ng/ml TGF-b1 or 100 ng/ml CCN3 at the indicated time points at 37°C. Thereafter, the expression of p21 and p15 was examined and compared with that of GAPDH by RT-PCR.

F. VSMCs were transiently transfected with p3TP-Lux, a TGF-b responsive luciferase reporter construct, deprived of serum for 24 h, and then stimulated either with 1 ng/ml TGF-b or 100 ng/ml CCN3 for 16 h. The luciferase activity in the cell lysates was
measured using the Dual-Luciferase Reporter Assay System. All assays were performed in quadruplicate and were then repeated 3 times.

Supplemental Figure II.

CCN3 inhibited VSMC proliferation by inhibiting the Notch signaling pathway.

(A) We examined the effects of a γ-secretase inhibitor (GSI), which blocks the Notch signaling pathway, on the inhibition of VSMC proliferation by CCN3, using BrdU incorporation as described in the legends to Figure 2. Data are presented as mean (SE); all determinations were performed in triplicate. (B) We examined the effects of RPMS-1, a known blocker of the RBP-J-mediated Notch signaling pathway, on the expression of p21 as well as Hey1 by RT-PCR as described in the legends to Figure 3A.

Supplemental Figure III.

Effects of CCN3 on PDGFR-ß expression and its tyrosine phosphorylation and ERK activation.

SMCs were seeded in 100-mm dishes and serum-starved for 24 h. The cells were then treated with 100 ng/ml CCN3 or 50 ng/ml PDGF-BB for 5 min at 37°C.
After incubation, the cells were lysed. The lysates were either directly separated by 10% SDS-PAGE followed by immunoblotting with anti-phospho ERK or anti-ERK antibody (A) or immunoprecipitated with anti-PDGFR-β antibody and separated with 5% SDS-PAGE, followed by immunoblotting with anti-phospho tyrosine (Tyr) antibody or anti-PDGFR-β antibody (B).

Supplemental Figure IV.

Generation of CCN3<sup>−/−</sup> mice.

A. Genomic Southern analysis of the recombinant ES clone. The genomic DNA of the ES clone was digested with EcoRI and analyzed by Southern hybridization, using genomic fragment a or b, as indicated in Figure 4A. The representative results of the original ES cells (+/+) and homologous recombinant clone (+/−) are shown.

B. PCR analyses of the targeted mouse. The genotype was determined by PCR using the specific primers indicated in Figure 4A for wild type (c and d) and targeted alleles (e and f). Representative results of a homozygote (−/−), heterozygote (+/−), and wild type (+/+) are shown. The 0.4-kb and 1.5-kb bands indicate the wild and targeted genomes, respectively.
C. RT-PCR analysis for \textit{CCN3} mRNA in heart RNA obtained at postnatal day 28.

Homozygous mice did not express a 561-bp band derived from the \textit{CCN3} mRNA.

Supplemental Figure V.

Vascular wall responses to the injury to the femoral arteries induced by photochemically induced thrombosis (Pit).

The femoral arteries of the mice were injured using photochemically induced thrombosis. One week after Pit, BrdU (50 mg/kg) was injected into the peritoneal spaces 6 h before sacrificing the mice. The femoral arteries were excised, fixed with 4% paraformaldehyde, embedded in OCT compound, and cut into 10-\textmu m sections. The sections were stained with anti-BrdU antibody (A) or anti-PECAM antibody and co-stained with anti-alpha smooth muscle cell actin (ASMA) antibody (B).

From each group, 3 animals (CCN3\textsuperscript{-/-} and CCN3\textsuperscript{+/+}) were examined, which yielded essentially similar results, and the representative results are shown in this figure.

Supplemental Figure VI.

\textit{CCN3} expression in the aorta of STZ-induced diabetic mice as compared to the wild-type controls. (A) Diabetes was induced in 8-week-old male mice by
intraperitoneal injections of STZ. After 4 weeks of STZ injections, the aorta was
dissected, snap-frozen in OCT, sectioned, stained using anti-CCN3-specific antibody,
and compared to the non-diabetic controls (n = 3 in each group). (B) The
CCN3-positive areas were measured and compared with the total area of the medial
layer. At least 3 discontinuous aortic sections from each animal were used and
compared statistically. *P < 0.01 compared with wild-type controls. (C) The
STZ-induced diabetic mice were treated with 0.1 U/body of insulin from day 5 until day
28. The aorta was then dissected, snap-frozen in OCT, sectioned, stained using
anti-CCN3-specific antibody, and compared with the non-diabetic control. This figure is
a representative of 4 animals, with each yielding essentially similar results.

References

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**Supplementary Figure I**

(A) CCN3/GAPDH (fold-increase) over 6 days.

(B) Cell number (x10^4/well) over 6 days.

(C) Flow cytometry analysis showing S+G2/M percentages under different conditions.

(D) Bar graph comparing S+G2/M percentages under different conditions.

(E) Graph showing p21/GAPDH (fold-increase) over 24 hours.

(F) Luciferase activity (Arbitrary) comparison between Control, TGF-β, and CCN3 treatments.