Cartilage Oligomeric Matrix Protein Maintains the Contractile Phenotype of Vascular Smooth Muscle Cells by Interacting With $\alpha_7\beta_1$ Integrin

Li Wang, Jingang Zheng, Yaoyao Du, Yaqian Huang, Jing Li, Bo Liu, Chuan-ju Liu, Yi Zhu, Yuansheng Gao, Qingbo Xu, Wei Kong, Xian Wang

Rational: Vascular smooth muscle cells (VSMCs) switching from a contractile/differentiated to a synthetic/dedifferentiated phenotype has an essential role in atherosclerosis, postangioplastic restenosis and hypertension. However, how normal VSMCs maintain the differentiated state is less understood.

Objective: We aimed to identify the factors affecting the expression and integrity of COMP may provide a novel therapeutic target for vascular disorders. (Circ Res. 2010;106:00-00.)

Methods and Results: We demonstrated that COMP was associated positively with the expression of VSMC differentiation marker genes during phenotype transition. Knockdown of COMP by small interfering (si)RNA favored dedifferentiation. Conversely, adenoviral overexpression of COMP markedly suppressed platelet-derived growth factor-BB–elicited VSMC dedifferentiation, characterized by altered VSMC morphology, actin fiber organization, focal adhesion assembly, and the expression of phenotype-dependent markers. Whereas $\alpha_7$ integrin coimmunoprecipitated with COMP in normal rat VSMCs and vessels, neutralizing antibody or siRNA against $\alpha_7$ integrin inhibited VSMC adhesion to COMP, which indicated that $\alpha_7\beta_1$ integrin is a potential receptor for COMP. As well, blocking or interference by siRNA of $\alpha_7$ integrin completely abolished the effect of COMP on conserving the contractile phenotype. In accordance, ectopic adenoviral overexpression of COMP greatly retarded VSMC phenotype switching, rescued contractility of carotid artery ring, and inhibited neointima formation in balloon-injured rats.

Conclusions: Our data suggest that COMP is essential for maintaining a VSMC contractile phenotype and the protective effects of COMP are mainly mediated through interaction with $\alpha_7\beta_1$ integrin. Investigations to identify the factors affecting the expression and integrity of COMP may provide a novel therapeutic target for vascular disorders.

Key Words: smooth muscle cells $\bullet$ phenotype $\bullet$ COMP $\bullet$ integrin $\bullet$ neointima

Unlike cardiac or skeletal muscle cells, vascular smooth muscle cells (VSMCs) have a unique property of plasticity and can undergo reversible changes in phenotype. Normally, they are mainly restricted to the media of adult blood vessels, express a repertoire of contractile proteins such as smooth muscle (SM) myosin heavy chain, SM $\alpha$-actin, SM-22$\alpha$ and calponin, and have low rate of replication. However, on various environmental cues, VSMCs can undergo transition from a quiescent, contractile/differentiated phenotype to a synthetic/dedifferentiated phenotype, with a high rate of migration/proliferation and a concomitant reduction in expression of VSMC marker proteins. Phenotypic switching of VSMCs plays an essential role in the development of cardiovascular diseases such as atherosclerosis, postangioplastic restenosis and hypertension. Studies have demonstrated the contribution to cell dedifferentiation of growth factors, mitogenic cytokines, reactive oxygen species, stretch or injury. Nevertheless, how normal VSMCs maintain the differentiated state is much less understood and has been largely ignored. Understanding the mechanisms that conserve a differentiated phenotype is critical to interfere with the development of cardiovascular diseases.

From studies of VSMCs in vitro and a rat balloon injury model, we recently demonstrated that ADAMTS-7 mediated...
degradation of cartilage oligomeric matrix protein (COMP), an extracellular matrix (ECM) protein in response to injury promoted VSMC migration and intima hyperplasia, which indicates that COMP may act as a negative regulator of VSMC activation. During the process of restenosis, VSMCs dedifferentiate from a contractile to a synthetic phenotype after vascular wall injury, which led us to hypothesize that COMP may act to conserve the contractile phenotype of VSMCs. COMP is a 524-kDa pentameric glycoprotein, the fifth member of the thrombospondin (TSP) gene family. Once thought to be localized only in the musculoskeletal system, COMP is now recognized as a normal component of vascular ECM in humans. Our present results show that COMP indeed negatively regulates VSMC dedifferentiation and maintains VSMCs in a more quiescent stage in vitro and in vivo in rat. We also demonstrate for the first time that the protective effect of COMP on maintaining contractile properties is mainly mediated through interaction with \( \alpha_5\beta_1 \) integrin.

Methods

Animal care and use of carotid-artery injury model in male Sprague–Dawley rats were in accordance with institutional guidelines. VSMCs were isolated from the thoracic aortic arteries of rats by collagenase digestion. Small interfering (si)RNA against COMP, \( \alpha_5 \) integrin, and \( \alpha_6 \) integrin were transfected in vitro by use of Oligofectamine (Invitrogen). The adenovirus for COMP was constructed and a single exposure of 5 plaque forming units was used as described previously.

![Figure 1](https://circres.ahajournals.org)  
**Figure 1.** COMP is degraded in balloon-injured rat carotid arteries. A, Representative Western blot of COMP protein in injured carotid arteries and sham-treated vessels at 4, 7, and 14 days after injury. B, Quantitative analysis of full-length COMP from injured arteries and sham arteries at 4, 7, and 14 days. Values are means±SEM percentage of sham control (n=6 to 10 per group at each time point). *P<0.05 vs sham.

**Results**

**COMP Level Is Decreased in Balloon-Injured Rat Carotid Arteries**

Using a rat balloon-injury model, we first examined the expression of COMP in carotid arteries after injury. COMP protein level was markedly lower in injured vessels than in sham-operated vessels 4 to 14 days after injury and was paralleled by an increase of COMP degradation fragment (Figure 1). Combined with our previous observation that degradation of COMP promoted VSMC migration and neointima formation, COMP may negatively regulate VSMC activation in response to injury.

**Association of COMP and VSMC Markers In Vitro**

VSMCs change from a contractile to a synthetic phenotype in response to injury, with a concomitant reduced expression of SMC differentiation markers. To gain insight into the relation of COMP and the VSMC phenotype, we analyzed COMP protein expression in differentiated and dedifferentiated VSMCs. As compared with early-passage cells (passage 1 [P1]), late-passage VSMCs (passage 5 [P5]) showed greatly reduced level of differentiation markers, including SM \( \alpha \)-actin, calponin, and SM22\( \alpha \) (Figure 2A), which well represents a cell transition from a differentiated to dedifferentiated phenotype. Of importance, COMP protein level was reduced concurrently, by 64%, from P1 to P5, so COMP may be involved during the process of phenotype transition. In accordance, COMP mRNA level was decreased from passage P1 to P5 (Online Figure I). Furthermore, stimulation of VSMCs with platelet-derived growth factor (PDGF)-BB, a major growth factor stimulating VSMC proliferation and migration revealed greatly inhibited COMP protein expression along with that of SM \( \alpha \)-actin, calponin and SM22\( \alpha \) (Figure 2B). In contrast, transforming growth factor (TGF)-\( \beta \), a potent VSMC differentiation factor, significantly enhanced

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ad</td>
<td>adenovirus</td>
</tr>
<tr>
<td>ADAMTS</td>
<td>a disintegrin and metalloproteinase with thrombospondin motifs</td>
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<tr>
<td>COMP</td>
<td>cartilage oligomeric matrix protein</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>P</td>
<td>passage</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>siRNA</td>
<td>small interfering RNA</td>
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<td>SM</td>
<td>smooth muscle</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>SRF</td>
<td>serum response factor</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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COMP protein expression (Figure 2C). Moreover, serum starvation upregulated both COMP and VSMC markers (Figure 2D). Thus, COMP is associated with expression of differentiation markers during VSMC phenotype switching.

**COMP Is Required for Maintenance of the VSMC Differentiated Phenotype**

After observing an association between COMP expression and differentiation in VSMCs, we next investigated the potential effect of COMP on VSMC phenotype transition by siRNA knockdown. COMP mRNA level was inhibited by 71%, with siRNA knockdown (Figure 3A). The mRNA level of SM α-actin, calponin, and SM22α was downregulated in parallel (Figure 3B). Similarly, the protein level of SM α-actin, calponin and SM22α was markedly reduced by siRNA treatment as compared with scramble siRNA treatment (Figure 3C). These data indicate a causal role of COMP in altering the mRNA and protein level of differentiation marker genes. Another hallmark of the VSMC differentiated state is the assembly of actin cytoskeleton into parallel and elongated stress fibers. As shown by fluorescence staining, COMP siRNA silencing disintegrated actin fibers into short and disorganized fibers in parallel with polygonal shaped VSMCs (Figure 3D). Thus, COMP is necessary for differentiation marker expression and stress fiber assembly, which renders the differentiated VSMC phenotype. Additionally, we investigated the effect of COMP silencing on TGF-β induced VSMC differentiation. In the presence of COMP, TGF-β significantly upregulated the expression of SM α-actin, calponin and SM22α (Figure 3E and 3F). However, COMP knockdown by siRNA markedly blunted the response of differentiation markers to TGF-β. These data further support COMP as being required for maintenance of the VSMC differentiated phenotype.
COMP Overexpression Retains the Characteristics of the Contractile Phenotype in PDGF-BB–Challenged VSMCs

Motivated by the heightened VSMC dedifferentiation with COMP knockdown, we next asked whether adenoviral overexpression of COMP could retain the VSMC differentiated state challenged by PDGF-BB, which plays a critical role in several vascular pathologies. Western blot analysis revealed COMP protein level higher in VSMCs with adenovirus (Ad)-COMP than with Ad-GFP infection (Figure 4B). Interestingly, Ad-COMP overexpression significantly circumvented PDGF-BB–induced suppression of VSMC marker gene expression at both the mRNA and protein level (Figure 4A through 4C). In accordance, enhanced dedifferentiation markers osteopontin and cellular retinol-binding protein-1 were reciprocally downregulated by COMP (Online Figure II). Furthermore, we examined the effect of COMP overexpression on morphological and cytoskeletal changes. Actin stress fibers were disassembled and aggregated around the peri-nuclear region of VSMCs with PDGF-BB treatment, and VSMCs changed shape from spindle-like to polygonal. In contrast, Ad-COMP–infected VSMCs adopted a spindle-like shape and reorganization of the actin network from randomly oriented filaments to thicker and well-oriented fibers (Figure 4D and 4E). The actin cytoskeleton is assembled into parallel stress fibers by a complex of interacting focal adhesion components, including paxillin, vinculin and talin. Recruitment of those components to the adhesion sites leading to focal adhesion assembly is another feature of differentiated VSMCs. In control differentiated (serum-starved) VSMCs, paxillin was localized at the tip and edge of elongated cells, whereas in the presence of PDGF-BB, paxillin was observed more concentrated in the peri-nuclear region. Ad-COMP overexpression led to relocation of paxillin at the tips of the cells along with actin fibers to induce focal adhesion assembly (Figure 4D and 4E). Thus, COMP overexpression could restore PDGF-BB–induced VSMC dedifferentiation and render differentiation in cells. Additionally, COMP overexpression also partially reversed the repression of VSMC markers by alternative dedifferentiation factors (10% serum or basic fibroblast growth factor), which strongly suggests that the protective effect of COMP on maintaining contractile phenotype of SMC is a general phenomena (Online Figure IV).
COMP Inhibits PDGF-BB Signaling in VSMC Dedifferentiation

Transcriptional activation of VSMC marker genes was markedly suppressed by PDGF-BB, as supported by the results of transient transfection of SM α-actin-luc or SM22α-luc reporter into VSMCs (Figure 5A). Deletion of the CArG box in the SM22α promoter (SM22α mutant) abolished PDGF-BB–induced suppression of SM22α reporter gene expression, which reinforced that expression of VSMC-specific genes depends on the cis-acting DNA sequence CArG box (CC[A/G]-rich sequences).

Figure 4. COMP overexpression retains the characteristics of the contractile phenotype in the presence of PDGF-BB. (A) Quantitative real-time PCR analysis of expression of VSMC differentiation markers normalized to that of β-actin. VSMCs were infected with Ad-GFP or Ad-COMP for 24 hours before challenge with PDGF-BB (25 μg/L) for additional 24 hours. Results are expressed as means ± SEM from 3 independent experiments performed in duplicate. *P<0.05 compared with Ad-GFP, # P<0.05 compared with Ad-GFP + PDGF-BB. (B) Representative Western blot and (C) quantification of protein expression 48 hours after PDGF-BB stimulation. *P<0.05 compared with Ad-GFP, # P<0.05 compared with Ad-GFP + PDGF-BB. (D) Representative images of F-actin (red) and paxillin (green) distribution of Ad-GFP- or Ad-COMP–infected cells in the presence or absence of PDGF-BB (25 μg/L). Blue indicated nuclei by Hoechst staining. The values are expressed as percentage (spindle- or polygonal-like cells/total cells per group) is indicated in E (left). The paxillin distribution is indicated by percentage value (cells of perinuclear paxillin localization/total cell number per group) in E (right). The χ² test was conducted. The experiment was repeated at least 3 times independently, and χ² test yielded similar results for each experiment. *P<0.05 compared with Ad-GFP + PDGF-BB.
T[G6GG], which serves as the binding site for serum response factor (SRF), a key transcription factor for VSMC differentiation. Conversely, ectopic overexpression of COMP reversed PDGF-BB–modulated transcription of VSMC-specific genes (Figure 5A). Furthermore, chromatin immunoprecipitation assay revealed that COMP circumvented PDGF-BB–suppressed SRF binding to both SMα-actin and calponin gene promoter regions (Figure 5B). In addition, COMP negatively regulated PDGF-BB–elicited SRF subcellular distribution between cytoplasm and nucleus, as seen on laser scanning confocal microscopy (Figure 5C and 5D). Previous studies have demonstrated that PDGF-BB represses VSMC marker gene expression mainly through an extracellular signal-regulated kinase 1/2–mitogen activated protein kinase (ERK1/2-MAPK) pathway by displacing myocardin from SRF with Elk-1 (Ets-like transcription factor-1). Therefore, we further analyzed whether COMP affected PDGF-BB–promoted ERK phosphorylation during VSMC dedifferentiation. Intriguingly, COMP greatly repressed ERK phosphorylation from 5 to 120 minutes after PDGF-BB treatment (Figure 5E). These results strongly suggest that COMP rescues PDGF-BB promoted VSMC dedifferentiation.
by negatively regulating PDGF-BB–induced ERK phosphorylation, SRF nuclear translocation, SRF recruitment to VSMC marker gene promoters and, subsequently, marker gene transcription.

COMP Facilitates Contractile VSMC Phenotype Through α7β1 Integrin

Cells within the vascular wall connect their cytoskeleton to the ECM through a family of cell surface receptors known as integrins. Of the approximately 24 known integrins, only α7β1 and α8β1 integrin promote the contractile phenotype of VSMCs. Therefore, COMP might transmit the differentiated signal to the VSMCs through a bridge of integrin α7β1 or α8β1. To identify the specific interaction between COMP and integrin, we coimmunoprecipitated proteins from detergent extracts of normal VSMCs and rat carotid arteries using specific α7 integrin antibodies. Western blot analysis of immunoprecipitates with anti-COMP antibodies revealed a specific band corresponding to COMP. In accordance, treatment with anti-COMP but not control IgG antibodies revealed a specific α7 integrin band in the immunoprecipitates (Figure 6A and 6B). In contrast, α8 integrin showed no interaction with COMP (data not shown), so COMP specifically binds to α7 integrin but not α8 integrin in VSMCs.

Moreover, VSMC adhesion assays on purified COMP were performed to verify that COMP bound to α7β1 integrin. Our data revealed VSMCs adhering to COMP or fibronectin faster than to nontreated plates. The adhesion of VSMCs to COMP was inhibited by functional blockage with anti-α7 integrin antibody (Figure 6C and 6D) or α7 integrin siRNA knockdown, whereas α8 integrin siRNA interference had no effect (Figure 6E and 6F). In contrast, the adhesion of VSMCs to fibronectin was not affected by either blocking or knocking down α8.
integrin. Therefore, \( \alpha_7 \beta_1 \) integrin associates with COMP in VSMCs and the vessel wall.

To further explore COMP facilitating a contractile VSMC phenotype through \( \alpha_7 \beta_1 \) integrin, VSMCs were seeded on COMP- or fibronectin-coated plates and serum starved for 48 hours. Cells grown on both polymerized collagen I and COMP show a spindle-like morphology (differentiated), whereas cells on fibronectin show a rhomboid appearance (dedifferentiated). Incubation of neutralizing \( \alpha_7 \) integrin antibody with cells plated on COMP switched the phenotype to dedifferentiation. Three independent experiments were performed.

**Figure 7.** COMP facilitates contractile VSMC phenotype through \( \alpha_7 \beta_1 \) integrin. A, Representative confocal images revealed F-actin (red) and paxillin (green) distribution in VSMCs plated on polymerized collagen I (Col I), COMP, or fibronectin. VSMCs were serum-starved for 48 hours. Cells grown on both polymerized collagen I and COMP show a spindle-like morphology (differentiated), whereas cells on fibronectin show a rhomboid appearance (dedifferentiated). Incubation of neutralizing \( \alpha_7 \) integrin antibody with cells plated on COMP switched the phenotype to dedifferentiation. Three independent experiments were performed. B, Effect of Ad-COMP overexpression and \( \alpha_7 \) integrin silencing by siRNA on PDGF-BB-induced repression of VSMC marker gene expression. Representative Western blot of 3 independent experiments and quantitative analysis was shown in B and C. *P<0.05. D, Effect of neutralizing \( \alpha_7 \) integrin antibody on COMP protein expression with PDGF-BB treatment. VSMCs were incubated with neutralizing \( \alpha_7 \) integrin antibody (10 mg/L) for 1 hour before administration of PDGF-BB (25 \( \mu \)g/L) and 3 \( \mu \)g of purified COMP. After 24 hours, cells were extracted and resolved for Western blot analysis and quantitative analysis was shown in E. F, Western blot analysis of ERK1/2 phosphorylation in Ad-GFP- or Ad-COMP-infected VSMCs exposed to PDGF-BB (25 \( \mu \)g/L) for various time points in the presence or absence of \( \alpha_7 \) integrin silencing.
moted VSMCs shifting toward the synthetic state. Application of neutralizing α7 integrin mAb abolished the effect of COMP, which indicates the importance of α7β1 integrin during COMP-facilitated VSMC differentiation. Alternatively, functional blockage of integrin α7 by specific siRNA or mAb completely abolished the protective effect of COMP on PDGF-BB–induced dedifferentiation, as evidenced by the expression of the VSMC marker genes SM α-actin, calponin, and SM22α (Figure 7B through 7E). These results strongly support COMP exerting its beneficial effect on the VSMC contractile state mainly by interacting with α7β1 integrin. Finally, knockdown α7β2 integrin by RNA interference abolished the inhibitory effect of COMP on PDGF-BB evoked ERK phosphorylation as early as 5 or 15 minutes stimulation (Figure 7F). These data further demonstrate that COMP modulates PDGF-BB signaling mainly through membrane receptor α7β1 integrin.

**COMP Suppresses VSMC Dedifferentiation in Response to Injury In Vivo**

To further test our hypothesis in vivo that COMP acts as a negative regulator of cell dedifferentiation, we used a rat carotid artery balloon-injury model to mimic the process of VSMC phenotypic switching. COMP protein level was markedly decreased in response to balloon injury (Figures 1A and 8A). Therefore, we infected injured arteries with Ad-COMP (Online Figure II) and examined differentiation markers. Indeed, Ad-COMP overexpression rescued the injury-induced repression of SM α-actin, calponin and SM22α expression as early as 3 days after injury, as revealed on both
Western blot analysis and immunohistochemical staining (Figure 8A through 8C). Furthermore, we compared the contractility of VSMCs among freshly isolated cells (P1), late-passage cells (P5, passage 5) grown in the absence or presence of purified COMP (Figure 8D and 8E). Our results indicate that COMP overexpression restores the culture-decreased contractility of VSMCs in vitro. In addition, Ad-GFP or Ad-COMP infected carotid artery were dissected and cut into rings and the contractility was compared 3 days after injury (Figure 8F). Our data clearly demonstrate that COMP restores injury-repressed vascular contractility in vivo. Therefore, COMP acts as negative modulator of VSMC dedifferentiation both in vitro and in vivo. COMP repression after injury may lose its inhibitory effect on phenotypic transition and subsequently facilitate neointima formation. This notion was further supported by Ad-COMP overexpression inhibiting neointima hyperplasia.9

Discussion

The extensive plasticity of VSMCs not only evolutionarily confers blood vessel repair and regeneration but also makes cells susceptible to various stimuli inducing changes in phenotype that contribute to the etiology of cardiovascular diseases.20 So far, studies have explored how environmental stimuli such as growth factors, injury or reactive oxidative species stimulate a proliferative/synthetic state in VSMCs. Conversely, little is known about the mechanisms promoting VSMC differentiation/maturation. This work describes a novel underlying mechanism for such differentiation process. We show that COMP, a macromolecular ECM protein, is necessary for maintaining the VSMC differentiated phenotype. Moreover, overexpression of COMP could rescue the PDGF-BB-induced cell phenotypic transition to a synthetic state in vitro. In addition, COMP circumvented the injury-elicted suppression of VSMC marker gene expression, injury-reduced vessel tension, and inhibited subsequent neointima formation in vivo. These protective effects are mainly mediated through binding to αvβ3 integrin. Our results reveal intrinsic ECM-associated mechanisms regulating the plasticity of the VSMC phenotype.

For more than 3 decades, VSMC phenotype has been shown to be modulated when cells were cultured on various ECM components. Substrates of fibronectin, collagen I and collagen III induced shifts toward the synthetic phenotype after vascular wall injury. Processes such as endothelial denudation, direct VSMC trauma, and subsequent release of multiple growth factors such as PDGF-BB all play a role in promoting a dedifferentiated VSMC phenotype.9 We therefore tested the expression of COMP during balloon injury and found decreased COMP level in response to injury as compared with a sham control from 4 to 14 days. The net effect of COMP decrease may be attributable to excessive degradation of COMP by ADAMTS-7, as we demonstrated recently.6 Adenoviral overexpression of COMP after balloon injury revealed successful inhibition of VSMCs switching to a synthetic state, which reinforced our hypothesis that neointima formation after injury was attributable to ablation of the protective ECM factor COMP. Consistent with this finding, neointima formation was greatly reduced by COMP at 7 and 14 days after injury, which further demonstrates the inhibitory effect of COMP on VSMC phenotype transition. The uniqueness of our study lies in providing a systemic and dynamic evaluation of the alteration in level of the ECM protein COMP during VSMC phenotype switching and in intima hyperplasia postinjury. Also, our results underscore the importance of the integrity of the ECM in phenotype-related vascular remodeling such as in atherosclerosis and restenosis.

Although ECM constituents such as COMP are important biological cues regulating the phenotype plasticity of VSMCs, relatively little is known about the repertoire of cell surface receptors needed to mediate their effects. Integrins are a large family of cell surface receptors that provide for adhesion of cells to the ECM. The ability of integrins to act as a bridge between the extracellular environmental and cytoskeleton/signal protein kinases enables them to mediate both bidirectional force transmission and signal transduction.15,16,22,23 Of the approximately 24 known integrins, VSMCs have been reported to have α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, α10β1, α11β1, and α12β1.14 The role for these integrins in the regulation of VSMC phenotype has not been fully investigated. Furthermore, the extent of involvement of the different ECM proteins is incompletely defined. In the present study, we identified αvβ3 integrin as a potential cell surface receptor of COMP that mediates the effect of COMP on VSMC maturation. In contrast, αvβ1 integrin, shown to maintain the VSMC differentiated phenotype, did not interact with COMP. Although the binding motif of COMP-αvβ3 integrin interaction needs to be further confirmed, recent disclosure of COMP crystal structure have indicated potential interactive domain for integrin.24 The αvβ3 integrin is a major laminin-binding receptor in VSMCs and is increased in level after differentiation. Previous studies have shown αv integrin was modulated by chemical-induced injury and PDGF-BB in cultured rat VSMCs.25,26 αv integrin–null mice show VSMC defects, including hyperplasia and hypertrophy.17 As well, VSMC-specific αv integrin–null mice show reduced expression of VSMC contractile proteins in response to injury, with pronounced neointima formation and reduced vascular compliance.18 These data indicate that αvβ3 integrin promotes the contractile phenotype of VSMCs, which is in agreement with our studies that COMP retains the contractile phenotype of VSMCs mainly through αvβ1 integrin. Similarly, laminin-binding αv integrin was reported to be required for contractile phenotype expression by human airway myocytes.27 COMP was shown previously to mediate chondrocyte attachment through αvβ3 integrin but not β1 integrin, which suggests a
pleiotropic effect of COMP on cell behavior through different integrin receptors.28

To date, compelling evidence has indicated that PDGF-BB can induce a profound suppression of VSMC marker gene expression through multiple complementary pathways and play a pivotal role during restenosis.11,12,29 We found that COMP inhibited PDGF-BB–induced suppression of VSMC-specific gene transcription, binding of SRF to the CarG element in the promoters of VSMC marker genes, as well as SRF nuclear translocation. Furthermore, COMP overexpression markedly suppressed PDGF-BB–evoked ERK1/2 activation. This finding agrees with PDGF-BB promoting cell dedifferentiation, at least in part, through ERK-dependent suppression of the formation of a myocardin–SRF–CarG complex of VSMC-specific genes.12 Moreover, these data reinforce our hypothesis that the ECM protein COMP modulates the VSMC phenotype mainly through an outside-in signal pathway. Recently, α5β1 integrin was found to promote the differentiated phenotype of VSMCs through suppression of ERK1/2-MAPK activation, which agrees with our observation.18 Interestingly, laminin, another α5β1 integrin ligand, has been demonstrated to maintain the differentiated state of VSMCs through activation of the p38–MAPK signaling pathway, whereas fibronectin promotes the synthetic phenotype through activation of the ERK–MAPK pathway.30 Whether other mechanisms are involved in COMP-mediated cell maturation and differentiation needs further exploration.

In summary, the present study identified a critical role of the intrinsic ECM factor COMP on VSMCs retaining a differentiated phenotype. These findings reveal a self-protective mechanism of VSMCs in response to various environmental cues and highlight the importance of ECM integrity in VSMC phenotype modulation during vascular diseases such as atherosclerosis and restenosis. The finding of a COMP–α5β1 integrin interaction broadens our understanding of how ECM affects cell behavior and may lead to novel targets of therapy for cardiovascular disease.

Acknowledgments

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Disclosures

None.

Novelty and Significance

Vascular smooth muscle cells (VSMCs) can undergo transition between a quiescent, contractile phenotype and a proliferative synthetic phenotype in response to physiological and pathological stimuli. This phenotypic plasticity of VSMCs contributes directly to vascular remodeling during atherosclerosis, restenosis following angioplasty, and hypertension. Although mounting evidence has indicated growth factors, mitogenic cytokines, reactive oxygen species or injury promote synthetic phenotype transition, little is known with regard to how VSMCs normally maintain the contractile phenotype and vascular homeostasis. We demonstrate for the first time that cartilage oligomeric matrix protein (COMP), a normal vascular extracellular macromolecule, is essential for maintaining a VSMC contractile phenotype under physiological and pathological stimuli. Once COMP is repressed or degraded, the VSMCs will switch to a synthetic phenotype and therefore contribute to neointima formation in response to injury. The protective effects of COMP are mainly mediated through interaction with its membrane receptor α5β1 integrin. Our study advances present understanding of how intrinsic cellular microenvironmental such as extracellular matrix modulates VSMC phenotype and maintains the vascular homeostasis. Our demonstration that COMP may prevent neointima formation highlights the potential of targeting key factors interfering with the expression/integrity of COMP as an applicable therapeutic tool to ameliorate intimal hyperplasia during atherosclerosis and restenosis.

What Is Known?

- The phenotypic transition of VSMCs plays a critical role in the pathogenesis of atherosclerosis, restenosis, and hypertension.
- Multiple environmental cues actively regulate VSMC phenotypic switching.
- COMP is a normal extracellular matrix protein in human vessels and VSMCs.

What New Information Does This Article Contribute?

- COMP is essential for maintaining a contractile phenotype of VSMCs.
- COMP maintains the contractile phenotype of VSMCs through interacting with α5β1 integrin.
- The expression level or integrity of COMP is important for vascular homeostasis.

References


Cartilage Oligomeric Matrix Protein Maintains the Contractile Phenotype of Vascular Smooth Muscle Cells by Interacting With $\alpha_7\beta_1$ Integrin

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Supplement Material

Materials

Recombinant platelet-derived growth factor-BB (PDGF-BB) was from R&D (Flanders, NJ) and transforming growth factor-β (TGF-β) was from CytoLab Ltd. (Rehovot, Israel). Antibodies against COMP and SM22α were from Abcam plc (Cambridge, UK). Rhodamine phallodin was from Invitrogen (Carlsbad, CA). Rabbit anti-paxillin antibody was from Epitomics (Burlingame, CA). Antibodies against serum response factor (SRF), myocardin, p-ERK, t-ERK and α7 integrin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against SM α-actin and calponin were from Sigma-Aldrich (St. Louis, MO). Neutralizing α7 integrin monoclonal antibody was from MBL (Naka-ku Nagoya, Japan).

Animal Artery Injury

The carotid-artery injury model in male Sprague-Dawley rats, weighing 210 to 230 g, used in all experiments was described previously.\textsuperscript{1} All studies followed the guidelines of the Animal Care and Use Committee of Peking University.

VSMC Culture and Real-time Quantitative PCR Analysis

VSMCs were isolated from the thoracic aortic arteries of 150-180 g, male Sprague-Dawley rats by collagenase digestion as described previously.\textsuperscript{2} The purity of VSMCs was verified by anti-SM α-actin antibody staining. Cells from passages 1 to 5 were used in all experiments. Real-time PCR amplification involved use of an
Mx3000 Multiplex Quantitative PCR System (Stratagene Corp, La Jolla, CA) and SYBR Green I reagent, and expression was normalized to that of β-actin. Primer sequences of target genes are in supplement online Table I.

**Western Blot Analysis**

Rat tissue or VSMC extracts containing equal amounts of total protein were resolved by SDS-PAGE. The membranes were incubated with primary antibody and IRD ye®-conjugated secondary antibody (Rockland Inc., Gilbertsville, PA). The immunofluorescence signal was detected by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

**siRNA Transfection**

Small interfering RNA (siRNA) against COMP, α7 integrin and α8 integrin were designed by use of the Block-iT® RNAi Designer and chemically modified by the manufacturer (Invitrogen). Sequences corresponding to the siRNA of COMP were sense, 5’-AGAAACUUGAGCGUGUUGAUAGCC-3’, and antisense, 5’-GGCUAUCAAGACAGCUAAGUUUCU-3’; for α7 integrin siRNA were sense, 5’-UUCAUGUACACAUACACCGCACCUC-3’, and antisense, 5’-GGCUAUCAAGACAGCUAAGUUUCU-3’; and for α8 integrin siRNA were sense, 5’-AAUAACCGACGUCUUAACCGCUG-3’, and antisense, 5’-CACAGCGGUUAAGACGUCGGUUAUU-3’. Transfection of rat VSMCs with the siRNA *in vitro* was by use of oligofectamine (Invitrogen).
RNAi duplex (Cat. No. 45-2001, Invitrogen) served as a negative control.

**Immunohistochemistry, Immunofluorescence and Confocal Laser Scanning Microscopy**

Consecutive frozen sections of carotid arteries were immunostained with anti-SM α-actin, calponin or SM22α antibodies. For immunofluorescence staining, cells were first incubated with the rabbit anti-paxillin antibody or rabbit anti-SRF, then secondary Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:300) (Invitrogen). Cells were counterstained for F-actin with rhodamine phalloidin for 30 minutes. The nuclei were visualized by staining with Hoechst for 2 minutes. The fluorescence signal was then monitored by confocal laser scanning microscopy (Olympus, Japan).

**Recombinant Adenovirus Construction**

The adenovirus for COMP (Ad-COMP) was constructed and amplified according to the manufacturer’s protocol (BD Biosciences Clontech, CA). An adenovirus carrying green fluorescence protein (Ad-GFP) was used as a negative control. For *in vivo* studies, immediate after balloon injury, a single exposure of $5 \times 10^8$ plaque forming units (pfu) of Ad-COMP was luminally delivered to balloon-injured carotid segments and kept inside for 30 minutes to allow for sufficient infection. The adenovirus solution was then removed and blood flow was restored.
**Luciferase Reporter Assays**

Promoter sequences were cloned into pGL3-luc basic vector (Promega, Madison, WI) and designated SM α-actin-luc and SM22α-luc. A SM22α reporter construct with deleted CArG-boxes (SM22α-mutant-luc) was generated as described previously. Transient transfection involved use of jet-PEI reagent (Polyplus-transfection Inc., New York, NY) according to the manufacturer’s instructions. Relative luciferase activities were detected by use of a luciferase assay kit (Promega) 24 hours after transfection and PDGF-BB treatment. Relative promoter activities are expressed as luminescence relative units normalized to β-galactosidase expression in the cell extracts.

**Co-Immunoprecipitation (Co-IP)**

Rat carotid arteries or VSMC lysates were incubated with anti-COMP or anti-α7 integrin antibodies prior to immunoprecipitation with protein A/G agarose beads (Vigorous, Beijing, China). Precipitated proteins were resolved by 10% SDS-PAGE gel electrophoresis and then immunoblotted with anti-α7 integrin or anti-COMP antibodies respectively. Rabbit normal IgG served as negative control.

**Chromatin Immunoprecipitation (ChIP) and Real-time PCR Analysis**

Rat VSMCs were infected with Ad-GFP or Ad-COMP. After 24 hours, cells were serum starved for 24 hours and then stimulated with PDGF-BB (25 μg/L) for 2 hours. Cells were fixed, sonicated and immunoprecipitated with 2 μg anti-SRF
antibody or normal IgG, together with single-strand salmon sperm DNA saturated with protein A agarose beads (Vigorous, Beijing). The precipitated chromatin DNA bound to SRF was then purified and amplified by PCR for enrichment of the target sequences. Primers for SM α-actin promoter were sense 5’-TGTAGCTCCTTAGCTTGCATG-3’ and antisense 5’-GTTCACTCCTGACATCCACTT-3’; and for the calponin promoter, sense 5’-AGGCAGAGGTTATGTGTAGACAG-3’ and antisense 5’-ATTGGGAGCTGACGTTGTCATTG-3’.

**Recombinant Expression of COMP in HEK293 cells and COMP Purification**

The cDNA clone coding for full-length COMP was reconstituted as described previously and transfected into the HEK293 cell line by use of Lipofectamine 2000 (Invitrogen). The empty vector pCDNA3.1 was transfected into HEK293 cells as a control. Transfected cells were selected with 750 mg/L G418 for 2 weeks. The cells were then maintained in DMEM medium supplemented with 10% fetal bovine serum and G418 at 37°C in 95% air, 5% CO₂ with 95% humidity. For COMP purification, stable transfected cells were incubated in serum-free opti-MEM medium (GIBCO) without G418 for 24 hours. The conditioned medium was applied to a heparin-agarose column (Sigma) equilibrated with Tris-buffered saline containing 2 mM CaCl₂ for binding overnight at 4°C by gentle agitation. After five washes, COMP was eluted with 0.75 M NaCl (buffered in 10 mM Tris, pH 7.5) containing 2 mM CaCl₂, and the purity was verified by western blot analysis.
**Cell Adhesion Assays**

Non-treated 48-well plates (Corning Costar Corp. Corning, NY) were coated with purified COMP (30 mg/L), fibronectin (200 mg/L) or polymerized collagen I (50 mg/L) at 4°C overnight and blocked with 1% heat-denatured BSA. Cells were trypsinized, washed and incubated with neutralizing anti-α7 integrin monoclonal antibody (mAb; 10 mg/L) or mouse normal IgG for 10 minutes at room temperature, then transferred to the coated plates. About 10,000 cells were added to each well and incubated at 37°C for 30 minutes. Non-adherent cells were removed by gentle washing with PBS three times. Adherent cells were stained and quantified by the average of 4 randomly chosen high-power fields (HPF) per well under a light microscope in three independent duplicate experiments.

**VSMC Contractility Assay**

The contractility of VSMCs was examined as described previously. Cells of freshly isolated (P1) or late-passage (P5) cultured in the presence or absence of COMP were stimulated with KCL (80mM), and monitored under the microscope up to 10 min. Images of the same field before and after KCL treatment were snapped and compared. Cell contractility was determined by measuring planar cell surface areas using Spot Image software and was expressed as the contraction index, $\frac{\Delta A}{A_0}$, in which a reduction of cell area ($\Delta A = A_0 - A_t$) at various time points after stimulation was normalized for the initial cell area at $t = 0$ ($A_0$).
**Vessel Tension Study**

The detailed procedure of vessel tension measurement was previously described. Briefly, carotid arteries were dissected from rats three days after injury. The vessels were cut into rings 5-mm long and suspended in modified Krebs-Ringer solution (in mM: 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 11.1 glucose) maintained at 37 ±0.5°C and aerated with 20% O₂-5% CO₂ (pH 7.4). At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension by step-wise stretching until the active contraction of the vessel ring to 80 mM/L KCl reached a plateau. The optimal resting tension of carotid arteries was ~1 g. After the vessels were brought to their optimal resting tension, 1 h of equilibration was allowed. Then a dose-response curve to Phenylephrine (0.1nM – 10 μM) in vessels was constructed in a cumulative fashion. Results were expressed as gram (g) of tension per 5 mm length artery.

**Statistical Analysis**

All continuous data are presented as mean±standard error of the mean (SEM). Protein band density was normalized to the corresponding loading control and then to the mean of the corresponding control group. Statistical evaluation of the data was performed by paired t-test (two-sided) for comparison between paired groups. One-way ANOVA is applied when the effects of recombinant COMP adenovirus on VSMC marker genes expression, luciferase activity or contraction index were
compared among groups, followed by Student-Newman-Keuls test for post hoc comparison as appropriate. Two-way ANOVA followed by Bonferroni test for comparisons between groups was performed in analyzing the effect of balloon-injury on COMP expression level over time, the effect of COMP silencing on VSMC marker genes expression in response to various concentration of TGF-β, the effect of α7 integrin knockdown or blocking on PDGF-BB-induced VSMC marker genes expression, and the effect of COMP adenovirus on contractility of carotid artery ring. Log transformation was performed as appropriate to obtain normality and variance homogeneity. The chi-square test was used to compare the percentage of cells among groups. The Kruskal-Wallis test followed by Dunns test was performed for the cell adhesion assay. All statistical analysis were preformed by GraphPad Prism4.0 (GraphPad Software Inc, La Jolla, CA) or a free statistics package R (http://cran.r-project.org/). A $P < 0.05$ was considered statistically significant.
References


Online Figure Legends

**Online Figure I.** Cartilage oligomeric matrix protein (COMP) mRNA level in VSMCs. (A). RT-PCR analysis of COMP mRNA from early- (P1) and late-passage (P5) VSMCs normalized to expression of β-actin. Values represent mean±SEM from three independent experiments. *P < 0.05 versus P1. COMP mRNA level was examined in VSMCs treated with (B) PDGF-BB (25 μg/L) for 24 hours, (C) TGF-β (2.5 μg/L) for 24 hours or (D) serum starvation for 48 hours. *P<0.05 compared with control.

**Online Figure II.** Relative mRNA level of dedifferentiation marker genes osteopontin (OPN) and CRBP-1 in response to PDGF-BB in the presence or absence of COMP. Data are means±SEM from 3 independent experiments. *P<0.05 compared with Ad-GFP+PDGF-BB.

**Online Figure III.** Effect of COMP on VSMCs proliferation by [3H]-TdR incorporation assay. Data are means±SEM from 3 independent experiments. *P<0.05 compared with Ad-GFP or Ad-COMP. ns, not statistically significant.

**Online Figure IV.** COMP overexpression retains the characteristics of the contractile VSMCs phenotype in response to 10% serum (A) or bFGF (B). Representative western blot analysis of VSMC differentiation markers expression normalized to that of eIF5 from three independent experiments. VSMCs were infected with Ad-GFP or
Ad-COMP for 24 hours before challenging with 10% serum (A) or 50 μg/L bFGF (B) for additional 48 hours.

**Online Figure V.** Representative western blot of three independent experiments showing effect of ERK1/2 MAPK inhibitor PD98059 on PDGF-BB repressed VSMC marker genes expression. VSMCs were pretreated with PD98059 (50 μM) for 1 hour prior to PDGF-BB stimulation for 48 hours.

**Online Figure VI.** Expression of integrin α7β1 by VSMCs grown on purified COMP (30 mg/L) for 48h.

**Online Figure VII.** Representative western blot of rat carotid arteries infected with 5 × 10⁸ pfu Ad-GFP or Ad-COMP 3 days after balloon injury.

**Online Figure VIII.** Verification of purified COMP by western blot analysis revealed a specific band (110 KD).
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<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
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<tr>
<td>COMP</td>
<td>5'-GTGACTTCGATGGCTGACAAAGGT-3'</td>
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