MFAP4 Promotes Vascular Smooth Muscle Migration, Proliferation and Accelerates Neointima Formation


Objective—Arterial injury stimulates remodeling responses that, when excessive, lead to stenosis. These responses are influenced by integrin signaling in vascular smooth muscle cells (VSMCs). Microfibrillar-associated protein 4 (MFAP4) is an integrin ligand localized to extracellular matrix fibers in the vascular wall. The role of MFAP4 in vascular biology is unknown. We aimed to test the hypothesis that MFAP4 would enhance integrin-dependent VSMC activation.

Approach and Results—We produced Mfap4−/− deficient (Mfap4−/−) mice and performed carotid artery ligation to explore the role of MFAP4 in vascular biology in vivo. Furthermore, we investigated the effects of MFAP4 in neointimal formation ex vivo and in primary VSMC and monocyte cultures in vitro. When challenged with carotid artery ligation, Mfap4−/− mice exhibited delayed neointimal formation, accompanied by early reduction in the number of proliferating medial and neointimal cells, as well as infiltrating leukocytes. Delayed neointimal formation was associated with decreased cross-sectional area of ligated Mfap4−/− carotid arteries resulting in lumen narrowing 28 days after ligation. MFAP4 blockade prohibited the formation of neointimal hyperplasia ex vivo. Moreover, we demonstrated that MFAP4 is a ligand for integrin α3β3 and mediates VSMC phosphorylation of focal adhesion kinase, migration, and proliferation in vitro. MFAP4-dependent VSMC activation was reversible by treatment with MFAP4-blocking antibodies and inhibitors of focal adhesion kinase and downstream kinases. In addition, we showed that MFAP4 promotes monocyte chemotaxis in integrin α3β3-dependent manner.

Conclusions—MFAP4 regulates integrin α3β3-induced VSMC proliferation and migration, as well as monocyte chemotaxis, and accelerates neointimal hyperplasia after vascular injury. (Arterioscler Thromb Vase Biol. 2016;36:122-133. DOI: 10.1161/ATVBAHA.115.306672.)

Key Words: carotid stenosis ◼ extracellular matrix proteins ◼ hyperplasia ◼ integrin alphaVbeta3 ◼ MFAP4 protein, mouse ◼ muscle, smooth, vascular

Vascular smooth muscle cell (VSMC) activation and phenotypic switching are critical for remodeling processes in vascular proliferative disorders, including intimal hyperplasia. Both the migratory and proliferative activities of VSMCs, as well as the interplay between the extracellular matrix (ECM) and integrin receptors essentially, contribute to neointimal hyperplasia and restrictive remodeling processes in the vessels.1 Among integrins, the particular role of integrin α3β3 in the induction of VSMC responses has been shown both in vivo and in vitro.2,3 Microfibrillar-associated protein 4 (MFAP4) is a 36-kDa glycoprotein composed of a short N-terminal region that contains a

Received on: October 2, 2015; final version accepted on: October 29, 2015.
From the Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark (A.S., B.P., L.E.H., K.K., G.B.K., H.W.-J., J.B.M., K.K.-M., L.K.D., P.B.L.H., J.S., E.H., G.I.S.); Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark (E.-M.F.); Department of Pathology, Odense University Hospital, Odense, Denmark (O.N.); Institute of Functional and Applied Anatomy, Hannover Medical School, Hannover, Germany (C.W., J.H., M.O.); Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATHE), Member of the German Center for Lung Research (DZL), Hannover, Germany (C.W., J.H., M.O.); REBIRTH Cluster of Excellence, Hannover, Germany (C.W., J.H., M.O.); German Mouse Clinic, Institute of Experimental Genetics, Helmholtz Zentrum München, German Research Center for Environmental Health, Munich, Germany (B.R., E.W.); Division of Cardiology, Department of Medicine III, University of Heidelberg, Heidelberg, Germany (A.S., R.B.); Chair of Experimental Genetics, Center of Life and Food Sciences Weihenstephan, Technische Universität München, Freising-Weihenstephan, Munich, Germany (M.H.d.A.); Cardiovascular Research Unit, Viborg Hospital, Viborg, Denmark (J.S.L.); and Department of Cardiothoracic and Vascular Surgery, Center of Individualized Medicine in Arterial Diseases (CIMA), Ódense University Hospital, Odense, Denmark (J.S.L.).

*These authors contributed equally to this article.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.115.306672/-/DC1.

Correspondence to Grith L. Sorensen, PhD, Department of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, JB Winslows Vej 253, 5000 Odense, Denmark. E-mail glsorensen@health.sdu.dk

© 2015 American Heart Association, Inc.

Arterioscler Thromb Vase Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.115.306672

122
nonstandard abbreviations and acronyms:

CCL2: chemokine (C-C motif) ligand 2
ECM: extracellular matrix
FAK: focal adhesion kinase
HAoS: human aortic smooth muscle cell
HR: heart rate
MAP: mean arterial blood pressure
MFAP4: microfibrillar-associated protein 4
MAGP36: microfibril-associated glycoprotein
MMP: matrix metalloproteinase-9
PDGF-BB: platelet-derived growth factor-BB
PI3K: phosphoinositide 3-kinase
RGD: arginine-glycine-aspartic acid
VSMC: vascular smooth muscle cell

Results

Characterization of the Vascular Phenotype in Mfap4−/−-Deficient Mice

Initially, we set out to characterize the resting vascular phenotype related to MFAP4 deficiency. Mfap4−/− mice were generated from 129S1/Sv embryonic stem cells and backcrossed on C57BL/6 N background for >10 generations (Figure 1A–ID in the online-only Data Supplement). Homozygous Mfap4−/− mice were viable, bred with normal Mendelian frequency, and seemed indistinguishable from wild-type littermates. MFAP4 was absent from the circulation in Mfap4−/− mice, and immunohistochemical analysis demonstrated a clear lack of detectable signals from Mfap4−/− mouse tissue (Figure IE and IF in the online-only Data Supplement).

A role for MFAP4 in the assembly of microfibrils has previously been proposed, although the elastic laminae in the arteries had the appearance of smooth organized lamellar sheets in both Mfap4+/− and Mfap4−/− mice when investigated by transmission electron microscopy (Figure II in the online-only Data Supplement). In contrast, collagen fibrils in the carotid arterial tunica adventitia in Mfap4−/− mice had significantly increased thickness relative to Mfap4+/− mice (57.1±11 versus 49.4±9 nm, respectively; P=0.038; Figure II in the online-only Data Supplement). There was no significant difference in collagen fibril thickness in the tunica media (38.8±9 versus 35.9±7 nm; Figure II in the online-only Data Supplement). Unchallenged Mfap4−/− mice presented with normal heart rate (HR), blood pressure, circulating cell number, and blood lipid levels (Tables I and II in the online-only Data Supplement). Resting mean arterial blood pressure (MAP), obtained using chronic indwelling catheters placed in the femoral artery in wild-type animals, was stable and averaged 98.8±2.7 mm Hg with a mean HR of 664±18 bpm. In Mfap4−/− mice, MAP averaged 105.5±3.6 mm Hg and HR averaged 661±13 bpm. Phenylephrine caused a significant increase in MAP (149.5±5.1 and 153.1±4.2 mm Hg) and a corresponding decrease in HR (442±40 and 428±27 bpm) in Mfap4+/− and Mfap4−/− mice, respectively (Figure III in the online-only Data Supplement). There were no significant differences found between genotypes in MAP and HR.

Mfap4−/− Mice Exhibit Delayed Neointima Formation After Carotid Artery Ligation Associated With SMC Proliferation and Leukocyte Infiltration

After the basal characterization of Mfap4−/− mice, we investigated the in vivo role of MFAP4 in VSMC activation in flow cessation–induced injury and atherosclerosis.11 Furthermore, recent in silico analysis predicted that Mfap4 deficiency can result in abnormal vascular physiology.11 These observations suggest that MFAP4 potentially plays an important role in VSMC biology and vascular remodeling.

To study the role of MFAP4 in neointimal formation, we generated Mfap4-deficient (Mfap4−/−) mice and evaluated their propensity for neointimal hyperplasia in flow cessation–induced injury; in which, after an early phase of inflammatory cell recruitment, medial VSMCs rapidly proliferate and migrate toward the lumen leading to extensive neointima formation. We also explored the effects of MFAP4 inhibition on neointima formation in ex vivo organ cultures. Furthermore, to gain more insight on the cellular mechanisms of MFAP4 action, we investigated MFAP4-mediated effects on VSMC phenotype, as well as monocyte chemotaxis in vitro.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

potential integrin-binding arginine-glycine-aspartic acid (RGD) motif followed by a fibrinogen-related domain found in a diverse group of human proteins involved in various functions, including coagulation, angiogenesis, tissue growth and remodeling, and innate immunity. MFAP4, known as 36-kDa microfibril-associated glycoprotein (MAGP36) in other species, was first identified as a protein with a resemblance to tenascin in its amino acid composition and localization to the ECM in arteries. MAGP36 is known to interact directly with ECM fibers, including elastin, collagen, and calvasculin. Moreover, MAGP36 was demonstrated to bind integrin receptors on human aortic SMCs in an RGD-dependent manner. MFAP4 shares some characteristics with matricellular proteins because of its localization to matrix fibrils, especially elastic fibers, and its interactions with SMCs. One notable characteristic of matricellular proteins is that they are nonessential for tissue homeostasis, but their loss is associated with a wide range of changes in remodeling tissues.

MFAP4 has been associated with remodeling-related disorders, including liver fibrosis, pulmonary airspace enlargement, and atherosclerosis. Furthermore, we recently showed that MFAP4 promotes airway SMC proliferation in experimental asthma, suggesting that similar effects could be relevant for VSMC biology under pathological conditions. Moreover, recent in silico analysis predicted that Mfap4 deficiency can result in abnormal vascular physiology. These observations suggest that MFAP4 potentially plays an important role in VSMC biology and vascular remodeling.
or protein content between ligated and unligated vessels (Figure 2A and 2B). The analysis of matrix metalloproteinase (Mmp)9 mRNA expression indicated that arterial ligation results in a significant transient induction of the Mmp9 gene product 2 days after the procedure. Mfap4 deficiency significantly reduced ligation-induced MMP9 expression (Figure 2C, left). However, the relative MMP9 activity in ligated artery tissue lysates was significantly higher in MFAP4−/− mice compared with MFAP4+/+ mice (Figure 2C, right). Eight days after ligation, Mmp9 expression normalized to low levels and was not different between the genotypes (data not shown). MMP2 activity was unchanged between genotypes (data not shown).

There were no or few detectable Ki-67-, caspase 3-, or CD45-positive cells in the Mfap4−/−-ligated vessels at day
14 after ligation relative to the Mfap4−/−-ligated vessels (Figure 2A and 2D). In addition, we observed a tendency for Mfap4 deficiency reducing the levels of phosphorylated focal adhesion kinase (pFAK) in the neointimal area 28 but not 14 days after ligation (Figure 2D).

**MFAP4 Is Present in Healthy and Diseased Vessel Wall and Promotes Ex Vivo Neointima Formation**

Next, we set out to translate our in vivo observations using clinically relevant tissue in an ex vivo organ culture model. MFAP4 immunoreactivity was found in human vein sections with intimal hyperplasia from a patient with peripheral artery disease in the lower extremities after surgical reconstitution following bypass surgery–induced restenosis. MFAP4 staining was most intense in elastic fibers. MFAP4 was present in the vessel wall, and its deposition was induced in areas directly underneath growing neointima.
but not within neointimal cells themselves (Figure 3B). The formation of neointimal myofibroblast layers was attenuated in tissues cultured in the presence of anti-MFAP4 antibody HG-HYB 7-1 but not isotype control antibody (Figure 3C).

**MFAP4 Is Secreted From VSMCs In Vitro and Binds Integrin αVβ3.**

Next, we explored VSMC synthesis of MFAP4 and VSMC integrin-mediated binding to immobilized MFAP4 to identify the cellular receptors for MFAP4 and the MFAP4-mediated mode of cellular activation. MFAP4 synthesis in fetal human aortic SMCs (fHAoSMCs) was prominent in differentiating culture conditions and regulated in parallel with α-smooth muscle actin production (Figure 3D and 3E).

Flow cytometry verified the presence of RGD-dependent integrins β1, αV, αVβ3, and αVβ5 on the fHAoSMC surface (Figure 4A). The integrins were detectable in all tested cell culture conditions (Figure IV in the online-only Data Supplement). We subsequently demonstrated that fHAoSMCs attached to immobilized recombinant MFAP4 (rMFAP4), laminin, and vitronectin within 1 hour in a concentration-dependent manner (Figure 4B). The synthetic peptide GRGDS, but not control SDGRG peptide, completely abolished the attachment of fHAoSMCs to rMFAP4 (Figure 4C) in line with previous observations for MAGP3.8 Anti-integrin αV and integrin αVβ3 antibodies completely blocked cellular attachment to rMFAP4 after 1 hour, whereas anti-integrin αVβ1 antibody did not cause a significant reduction in attachment (Figure 4D).

Monoclonal anti-MFAP4 antibodies HG-HYB 7-5 and HG-HYB 7 to 14 abrogated fHAoSMC attachment to rMFAP4 (Figure 4E). Integrin-mediated increase in phosphorylated FAK levels was significantly influenced in cells attaching to rMFAP4 relative to poly-D-lysine, suggesting the activation of integrin signaling pathway (Figure 4F). Cellular spreading, focal adhesion points, and cellular stress fibers (detected by vinculin and F-actin staining, respectively) formed within 20 hours on MFAP4 coating and were inhibited when fHAoSMCs were coincubated with MFAP4-blocking antibody.

---

**Figure 3.** Microfibrillar-associated protein 4 (MFAP4) is produced by vascular smooth muscle cells and mediates ex vivo neointima formation. **A**, Immunohistochemical detection of MFAP4 in sections from human vein with neointimal hyperplasia. MFAP4 was visualized by immunostaining, and elastin was visualized using Weigert elastin stain. Top, Scale bar, 1000 µm; original magnification, ×25. Bottom, scale bar, 50 µm; original magnification, ×1000. **B** and **C**, Formation of myofibroblast layers in cultured human saphenous veins is inhibited by MFAP4-blocking antibody. Ex vivo neointimal growth (delineated by black arrows) is observed after 14 and 21 days of culture and can be inhibited by the presence of HG-HYB 7-1 antibody. Data are presented as means±SEM and represent the average number of layers in vessels from n=3 patients. Scale bar, 20 µm. **D** and **E**, MFAP4 production in fetal human aortic SMCs (fHAoSMCs) during differentiation from the synthesizing/proliferating phenotype to the α-smooth muscle actin (α-SMA) positive phenotype. Western blot signal density (D) was quantified by densitometric analysis (E). **F**, MFAP4 concentration in the culture medium from differentiating fHAoSMCs measured by ELISA. Data are presented as means±SEM (C, E, and F) or representative (D) of 3 independent experiments. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001, calculated by 1-way or 2-way ANOVA. H&E indicates hematoxylin and eosin; and IC, isotype control.
Figure 4. Microfibrillar-associated protein 4 (MFAP4) interacts with vascular smooth muscle cells through RGD-dependent integrins. A, Flow cytometry analysis of cell surface integrin expression on fetal human aortic SMCs (fHAoSMCs). The attachment of fHAoSMCs onto immobilized bovine serum albumin (BSA), recombinant MFAP4 (rMFAP4), or vitronectin (B); onto rMFAP4 in competition with integrin inhibitory RGD-containing peptide or DGR-containing control peptide (C); onto rMFAP4 in competition with integrin-blocking antibodies specifically directed against integrins β1, αv, αvβ3, or αvβ5 (D); and onto rMFAP4 in competition with monoclonal anti-MFAP4 antibodies HG-HYB 7 to 14, or anti-αv antibody (E). F, MFAP4 stimulates activation of focal adhesion kinase (FAK) in fHAoSMCs. The relative phosphorylated focal adhesion kinase (pFAK)/total FAK band density after fHAoSMC adhesion is shown. G and H, MFAP4 is involved in focal adhesion and stress fiber formation. The formation of focal adhesions in fHAoSMCs seeded onto poly-D-lysine (PDL), rMFAP4, or fibronectin was observed after 20 hours. Vinculin was detected using anti-vinculin primary antibody and Alexa Fluor 488-conjugated goat antimouse F(ab′)2 (green). F-actin was detected using TRITC-conjugated phalloidin (red). DAPI was used to visualize cellular nuclei (blue). G, The number of round cells is significantly increased after treatment with HG-HYB 7 to 14 or anti-αv antibody. H, Representative pictures are shown. Scale bar, 50 µm; original magnification, ×1000. Insets highlight the focal adhesion points. Data are presented as mean±SEM (B–G) or representative (A and H) of 3 independent experiments.*P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001, calculated by 2-way ANOVA or Student t test. FN indicates fibronectin; IC, isotype control; and RFU, relative fluorescence units.
MFAP4 Induces VSMC Migration and Proliferation
Depending on Integrin-Associated Signaling
To investigate MFAP4-induced integrin-mediated effects on VSMC phenotype and associated signaling, we analyzed proliferative and migratory VSMC response to MFAP4 stimulation. The 20-hour migration of fHAoSMCs was increased almost 2-fold on rMFAP4-coated surface relative to poly-L-lysine control (Figure 5A and 5B). The number of migrating cells was not dependent on rMFAP4 dose within the used range, suggesting that the lowest coating concentration already had a saturating effect on fHAoSMC migration (Figure 5C). MFAP4 blockade with either HG-HYB 7-5 or 7 to 14 antibody significantly reduced the migration (Figure 5A and 5D). Moreover, MFAP4-induced migration was dependent on FAK and further downstream signaling mediators mitogen-activated protein kinase kinase 1/2 and phosphoinositide 3-kinase (PI3K; Figure 5E). The numbers of cells were 9% higher when seeded on MFAP4 relative to the negative control but similar in the presence and absence of antibody coating when tested after 20 hours of culture (Figure VIA in the online-only Data Supplement). These control data indicate that the culture conditions allowed minimal MFAP4-induced proliferation during the test period.

fHAoSMC proliferation was significantly induced when cells were seeded onto rMFAP4 and allowed to proliferate for 48 hours either in the presence or absence of 5 ng/mL platelet-derived growth factor-BB (PDGF-BB), which in itself further induced proliferation (Figure 5F). The stimulating effect of rMFAP4 on cell proliferation was significantly dose dependent (Figure 5G). HG-HYB 7-5/7 to 14 antibodies caused a significant reduction of fHAoSMC proliferation to non–PDGF-BB–treated levels (Figure 5H). Furthermore, MFAP4-induced proliferation was significantly attenuated by FAK and PI3K inhibitors in PDGF-BB–treated cells, whereas the same tendency was observed for non–PDGF-BB–treated cells (Figure 5I). The numbers of adherent cells were similar on all types of coatings either with or without antibody treatment when analyzed 4 hours after seeding (Figure VIB in the online-only Data Supplement). This control experiment showed that although initial attachment of fHAoSMCs was induced by MFAP4, the number of adherent cells was equal in all tested conditions after 4 hours. Thus, the increased number of cells detected after 48 hours was considered a specific result of mitosis.

Discussion
In this study, we found that Mfap4 deficiency inhibited SMC hyperplasia in vivo, which resulted in delayed neointima formation after flow cessation–induced vascular injury in mice. Our observations were supported by inhibition of ex vivo formation of neointimal hyperplasia using anti-MFAP4 antibodies and by in vitro experiments showing that MFAP4 induces integrin α5β1-mediated human VSMC migration and proliferation, as well as monocyte chemotaxis. Our findings are the first to characterize a mechanistic role of MFAP4 in pathological responses of the arterial wall.

To investigate the biological effects of MFAP4 we have generated Mfap4 mice. Histological examinations of Mfap4 mice showed a normal appearance until 3 months of age. The structure of vascular elastic membranes appeared undisturbed, whereas significantly increased collagen fibril thickness was observed in the arterial adventitia in Mfap4 mice. The increase in fibril thickness did not seem to affect the basal vascular phenotype, and the MAP did not differ between Mfap4 mice and Mfap4 mice. These observations demonstrate that MFAP4 is redundant for survival and normal cardiovascular development. Likewise, this study indicates a redundant role for MFAP4 in normal arterial elastin assembly.

MGAP4 Mediates VSMC Dedifferentiation and Protection From Apoptosis and Monocyte Chemotaxis
To gain more mechanistic insight about the mode of MFAP4 actions, we analyzed the expression of selected genes in fHAoSMCs stimulated with MFAP4. MFAP4 had no effect on fHAoSMC expression of adhesion molecules intercellular adhesion molecule 1 and vascular cell adhesion protein 1, canonical antiapoptotic protein B-cell lymphoma 2, or monocyte-attracting chemokine (C-C motif) ligand 2 (CCL2; Figure VII in the online-only Data Supplement). However, the expression of SMC differentiation markers α-smooth muscle actin and, to the lesser extent, SM22 was downregulated in MFAP4-stimulated fHAoSMCs compared with fHAoSMCs seeded on poly-D-lysine (Figure 6A and 6B). This difference was not present between poly-D-lysine– and MFAP4-stimulated cells without concomitant PDGF-BB administration (data not shown). Furthermore, we assessed the effects of MFAP4 on fHAoSMC apoptosis and found that MFAP4 stimulation resulted in significantly lower quantities of early apoptotic cells, with the same tendency observed for fHAoSMCs concomitantly treated with PDGF-BB (Figure 6C).

Finally, we investigated the effects of MFAP4 on monocyte chemotactic properties. MFAP4 significantly and dose dependently promoted THP-1 human monocyctic cell migration toward CCL2 in a transwell-based assay similar to fibronectin used as a positive control (Figure 6D). MFAP4-induced THP-1 cell CCL2-driven chemotaxis was inhibited by anti-integrin α5β1 but not anti–integrin α5β3 antibody (Figure 6E).

To characterize the binding sites of HG-HYB antibodies to MFAP4, we produced MFAP4 mutants with point mutations introduced within an RGD sequence. Anti-MFAP4 antibodies HG-HYB 7 to 14 and to 18 clearly bound both double (AGA) and triple (AAA) RGD-mutated rMFAP4 (Figure V A–V C in the online-only Data Supplement). In contrast, MFAP4 detection signals were reduced for the point-mutated proteins when HG-HYB 7-5 was used as a capture antibody, suggesting that MFAP4-blocking monoclonal antibody HG-HYB 7-5 binds to an epitope covering the RGD sequence in rMFAP4. The latter observation suggests that HG-HYB 7 to 14 may bind near the RGD site. Inhibition experiments showed that HG-HYB 7-1 recognizes the same epitope as HG-HYB 7 to 14 (Figure VD and VE in the online-only Data Supplement).
An experimentally ligated carotid artery adjusts the luminal area in response to the loss of net flow that occurs because of both a decrease in vessel diameter and neointima formation.\(^1\) Fourteen days after ligation, the neointimal SMC mass, the number of proliferating neointimal cells, and the cross-sectional area of the ligated vessels in Mfap4\(^-/-\) mice were all decreased relative to Mfap4\(^+/-\) mice. These early effects are in accord with the fact that MFAP4 is constitutively expressed by the predominating vascular cell type and the contractile VSMCs, and therefore, it is present in the normal mouse vessel before the onset of injury.

As Mfap4\(^-/-\) mice ultimately developed neointimal lesions at day 28 after ligation, delayed neointima formation together with decreased vessel area resulted in decreased luminal patency compared with Mfap4\(^+/-\) mice. Thus, although early MFAP4-mediated induction of vascular smooth muscle...
proliferation and migration may be regarded as deleterious, MFAP4-mediated effects are ultimately redundant or even protective in the used model of flow cessation–induced vascular hyperplasia because of increased arterial shrinkage observed for MFAP4-deficient vessels. As integrin $\alpha$V$\beta$3 ligation by RGD peptides has been previously shown to mediate vessel vasodilation caused by reduction in intracellular calcium,$^{21,22}$ it is possible that the lack of MFAP4 signaling leads to exaggerated vessel constriction. Alternatively, increased adventitial collagen fiber thickness and less uniform fiber size distribution in $\textit{Mfap4}^{-/-}$ mice might compromise the external support normally provided by dense collagen scaffold known to prevent the loss of vessel area.$^{23}$ Further investigations are needed to clarify the precise role of MFAP4 in these phenomena.

As mentioned above, MFAP4 is expressed by contractile VSMCs that constitute the predominant cell type within the normal vessel wall. In the injured tissue, an increasing fraction of proliferating cells arises, but the numbers of proliferating cells with reduced MFAP4 synthesis are still low relative to the numbers of nonproliferating cells. Therefore, the total vascular MFAP4 synthesis may not change significantly during disease development, which is supported by the lack of significant difference in MFAP4 expression between ligated and unligated vessels in vivo and lack of MFAP4-specific staining in neointimal cells within newly formed neointimal hyperplasia ex vivo.

MMPs are proteolytic enzymes that contribute to VSMC migration toward the intima by degrading the ECM of a vessel wall.$^{24,25}$ We observed that MFAP4 deficiency resulted in transient reduction of $\textit{Mmp9}$ expression but increased MMP9 activity 2 days after ligation. The difference in activity was not pronounced and did not seem to explain the observed phenotype, and no alterations in MMP2 activity were found. Future studies are warranted to elucidate the complex mechanisms of MFAP4-mediated MMP regulation.

CCL2 is a chemokine critically involved in monocyte recruitment and subsequent formation of neointimal lesions.$^{26-28}$ We found that MFAP4 promoted CCL2-induced monocyte chemotaxis in an integrin $\alpha$V$\beta$3-dependent manner. As monocytes and monocyte-derived macrophages constitute the majority of leukocytes infiltrating neointimal lesions,$^{29,30}$ our experiments suggest that reduction in leukocyte influx observed in MFAP4-deficient mice can be explained by impaired monocyte recruitment toward the injury site.

The synthesis of MFAP4 by contractile VSMCs in the steady state combined with the observation that MFAP4 directly interacts with ECM fibers supports the previously proposed role for MAGP3/MFAP4 in maintaining homeostatic functions in the vessel wall, as observed for other integrin $\alpha$V$\beta$3 ligands osteopontin and vitronectin.$^{12,31,32}$ However, the lack of detectable naive vascular phenotype in MFAP4-deficient mice demonstrated that the possible role of MFAP4 in normal vascular physiology is redundant. Nevertheless, MFAP4 may be essential in nonhomeostatic conditions, as shown by MFAP4-dependent ex vivo neointima formation. We used an established and widely used model of human saphenous vein organ culture$^{33-35}$ and showed that MFAP4 inhibition resulted in almost complete prohibition of neointimal growth in clinically relevant tissue ex vivo. These data support the essential role of MFAP4 in pathological VSMC responses under reduced flow/no flow conditions.
Reduced carotid neointima formation has been observed in integrin β3-deficient mice, in experiments performed using a gene knockout model for the integrin αβ3 ligand vitronectin and in osteopontin inhibition experiments, all underlining an important role of integrin αβ3 in neointimal hyperplasia. Although basic integrin αβ3 expression on VSMCs is relatively low, it is highly upregulated after vascular injury.  

Primary fetal VSMC cells used in our in vitro studies have a relatively high expression of integrin αβ3, and therefore may represent a partly dedifferentiated cell subset commonly observed in ligated or otherwise injured arteries. The nearly complete disruption of cellular attachment to immobilized MFAP4 caused by integrin-blocking antibodies shows that integrin αβ3 is the dominant MFAP4 receptor on the fibroblast surface.

Specific integrin-mediated FAK phosphorylation is required for the dynamic regulation of focal adhesion turnover, and it is, thus, an integral part of cellular activation occurring for instance in migrating cells. Our data demonstrate that MFAP4 induced phosphorylation of FAK, focal adhesion site formation, and cellular migration and proliferation, in line with studies of known integrin αβ3 ligands osteopontin and vitronectin.  

FAK can act through mitogen-activated protein kinase and PI3K pathways, both with an established role in VSMC migration and proliferation. We showed that MFAP4-induced VSMC migration and proliferation were attenuated by FAK, PI3K, and extracellular signal-regulated kinase inhibitors, thus identifying the potential mechanism underlining MFAP4-mediated VSMC activation. However, we did not address the potential involvement of other pathways, such as p38 and c-Jun NH2-terminal kinase, and cannot exclude their partial contribution in regulation of MFAP4-dependent cellular effects.

Adult VSMCs are not terminally differentiated and exhibit remarkable phenotypic plasticity. After vascular injury, VSMCs undergo phenotypic modulation toward a proliferative, synthetic state associated with downregulation of multiple differentiation markers. We found that MFAP4 stimulation results in lowered expression of canonical smooth muscle markers α-smooth muscle actin and SM22, strengthening our notion that MFAP4 helps to establish a VSMC phenotype optimal for efficient response to local injury. Importantly, we also observed that MFAP4 confers an antiapoptotic effect on VSMCs in vitro, which is consistent with other MFAP4-related actions described here. Although we observed slightly decreased numbers of apoptotic cells in Mfap4−/− mice, we associate this difference with overall decreased cell turnover attributable to attenuation of both VSMC proliferation and leukocyte infiltration.

MFAP4-induced effects on VSMC migration and proliferation could be reversed by MFAP4-blocking antibodies interfering with integrin binding. These inhibition experiments can partly explain that MFAP4-mediated cellular activation was caused by rMFAP4 binding small amounts of growth factors from the cell culture medium, inducing the observed effects. Migration and proliferation assays were not performed for periods exceeding 96 hours, the time point when integrin β3 expression was observed to disappear. No or low MFAP4 expression, low integrin αβ3, expression, and a stable integrin β3 expression was thus expected using these culture conditions.

The constitutive MFAP4 expression in both normal and diseased arteries sets the expressional regulation of MFAP4 apart from the common transient upregulation of other integrin αβ3 ligands and suggests that MFAP4 is permissive for pathology and that MFAP4-mediated cellular effects are regulated by fluctuations in cellular integrin expression or growth factor-mediated activation. Cross talk between integrin and growth factor signaling pathways is important for providing specific responses during vascular pathology. Integrin αβ3 is known to coprecipitate with PDGF receptors. Association between FAK and PI3K is enhanced after PDGF stimulation. Growth factor stimulation potentiates integrin-dependent cellular responses and vice versa, and this joint signaling is required to promote cell proliferation, as well as optimal survival and migration. As MFAP4-dependent effects were most pronounced with accompanying PDGF-BB treatment, we suggest growth factor-mediated activation and disease-induced upregulation of integrin expression to be prerequisites for MFAP4-dependent effects to take place.

In conclusion, the results of this study show that MFAP4 is a novel integrin αβ3 agonist promoting VSMC growth and migration, as well as monocyte chemotaxis, resulting in accelerated neointimal hyperplasia in flow cessation–induced injury.

Acknowledgments

We thank Annette Fuchtbauer for embryonic stem cell work, Peter Kragh for blastocyst injection, and Reinhard Seeliger, Ralf Fischer, Sandra Geißler, and Elif Holupirek, as well as the GMC animal caretaker team of Manuela Huber, Boris Schön, Heidi Marr, Amnica Miedl, Tina Reichelt, Michael Gerslauer, Renate Huber and Horst Wenig, for their expert technical help.

Sources of Funding

This work was supported by Fonden til Lægevidenskabens Fremme (10-344/12-341), the Lundbeck Foundation (R13-A1235/R67-A6409), the Danish Heart Association (12-04-R90-A3936-2271/R75-Rp3701), Oda og Hans Svensningsens Fond (9400), Aase og Ejnar Danielsens Fond, Steen Olsens Fond, the German Federal Ministry of Education and Research to the GMC (NGFN-Plus grant no. 01GS0850, 01GS0851, and 01GS0853; Infrafrontier grant 01KK1012), and EU grants (EUROMIDIC, LSHG-2006-037188, and InfraFrontier Contract no. 211404).

Disclosures

A. Schlosser, U. Holmskov, and G.L. Sorensen have issued a patent: microfibrillar-associated protein 4 (MFAP4) binding antibodies blocking the interaction between MFAP4 and integrin receptors (P1183DK00). The other authors report no conflicts.

References

4. Thomsen T, Schlosser A, Holmskov U, Sorensen GL. Ficolins and FIBCD1: soluble and membrane bound pattern recognition molecules


**Significance**

Integrin ligands are known to affect arterial remodeling responses after injury. Microfibrillar-associated protein 4 (MFAP4) is an integrin ligand with high expression in the vasculature and a yet unexplored role in vascular biology. This study demonstrates that MFAP4 is an important regulator of pathological vascular smooth muscle cell responses. MFAP4-deficient mice exhibited a normal vascular phenotype when unchallenged. However, in a model for pathological growth of vascular smooth muscle cells after arterial injury, the lack of MFAP4 delayed the formation of neointimal hyperplasia, which was accompanied by a reduction in leukocyte infiltration and cell proliferation in the vessel wall and decreased cross-sectional arterial diameter. Moreover, it was shown that MFAP4 is a ligand for integrin αvβ3 and mediates vascular smooth muscle cell migration and proliferation, as well as monocyte chemotaxis. The study identifies MFAP4 with redundant functions in normal vascular biology but as a novel regulator of vascular remodeling responses after arterial injury.
MFAP4 Promotes Vascular Smooth Muscle Migration, Proliferation and Accelerates Neointima Formation


doi: 10.1161/ATVBAHA.115.306672

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/36/1/122

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Materials and methods

**Standard buffers**
TBS: 140 mM NaCl, 10 mM Tris-HCl; in some experiments 0.02% (w/v) NaN₃ was added, and the pH was either 7.2 or 7.4 according to individual protocols. TBS/Tw: TBS with 0.05% (v/v) TWEEN 20 (Merck). PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, pH 7.4.

**Production of anti-MFAP4 monoclonal antibodies**
C57BL/6/N Mfap4-deficient mice were immunized for the production of monoclonal antibodies (HG HYB 7-1, 7-5, 7-14, and 7-18) against rMFAP4 as previously described¹.

**Generation of Mfap4-deficient mice**
Total RNA was purified from the frozen lung tissue from a BALB/c mouse using the RNeasy kit (Qiagen, CA, USA). First-strand synthesis was performed using the SuperScript™ III RNase H- Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) with oligo-dT primers and 1 µg of total mouse lung RNA. mMFAP4 transcripts were amplified using the primers 5’-GCCTCTGGGATCCGGGGAGA-3’ and 5’-TCAGGGCCGACGAATTTTCAT-3’. The sequence corresponds to the coding sequence of Mfap4 not including the signal peptide. Amplification was performed with an initial denaturation for 2 min at 94°C; 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; and finally 5 min at 72°C. The amplified 705 bp fragment was extracted from the gel using a gel extraction kit (NucleoSpin, Macherey-Nagel, Germany). The identity of the amplified fragment was confirmed by sequencing. The 705 bp fragment was labeled using ALL-IN-ONE™ Random Prime DNA labeling Mix (dCTP) (Sigma, Saint Louis, Missouri USA). [α-³²P]dCTP was from Amersham Pharmacia Biotech (UK). A 129Sv mouse genomic library in Lambda FIX II vector (Cat. No 946305, Stratagene) was then screened, and a region encompassing a region from 10 kbp upstream to 700 bp downstream of the Mfap4 locus (3.6 kbp) with a total of 14.1 kbp was cloned. A targeting vector was constructed to delete genomic regions encompassing the core promoter region as well as exons 1, 2, and part of exon 3 for elimination of Mfap4 transcription.

The short arm of the targeting vector was PCR-amplified using the primers 5’-GTACATGTGACGACCCCTGTTGAGAT-3’ and 5’-GTACTAGTAAACGTCTGCT-3’. Amplification was performed with an initial denaturation for 2 min at 96°C; 25 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 2 min; and finally 72°C for 5 min. The proofreading Pwo polymerase (Roche, Manheim, Germany) was used for the reactions according manufacturer’s recommendations. The primers were designed to allow SpeI and PciI digestion of the amplified fragment and to omit the 5’ EcoRI site of EcoRI fragment 4. The 2 kbp-amplified fragment was purified and digested by PciI and SpeI and ligated into the PCR 2.1 cloning vector (Invitrogen), which was previously digested using the same enzymes. A 2 kbp-neomycin expression cassette was obtained from the plloxPneo-1 plasmid (kindly provided by Susanne Mandrup, Department of Molecular Biology, University of Southern Denmark). The ploxPneo-1 was digested with Xhol, blunt ended using Klenow fragment, purified, digested with EcoRI, separated from the parent plasmid on an agarose gel, and purified. The neomycin expression cassette was ligated into the targeting vector, which had been digested with SpeI, blunt ended using Klenow fragment, purified, EcoRI-digested, and purified. To obtain the long arm of the targeting vector, the 7.6 kbp EcoRI fragment 2 was excised from the PCR2.1 cloning vector and gel-purified. The purified EcoRI fragment 2 was ligated into the targeting vector that was previously EcoRI-digested and purified. The identity and orientation of the cloned fragments were confirmed by sequencing. All purification steps were performed using NucleoSpin- (Macherey-Nagel, Germany).

An endotoxin-free plasmid preparation of the targeting construct for electroporation of embryonic stem cells was obtained using the NucleoBond® PC 500 EF plasmid purification kit (Macherey-Nagel, Easton, USA). Approximately 100 µg of the targeting vector was linearized by NotI digestion. Restriction enzymes, T4 ligase, and Klenow fragment were obtained from Invitrogen.
CJ7 embryonic stem cells\(^2\) were electroporated with 25 \(\mu\)g of the NotI-linearized targeting vector using the Gene Pulser (Bio-Rad). Electroporation conditions were as follows: 240 V and 500 \(\mu\)F. Cells were selected using 350 mg/ml G418 and 0.5 \(\mu\)M FIAU, and homologous recombinants were identified by Southern blotting and PCR. Chimeric mice were generated by injection of targeted ES cell clones into B6D2F2 blastocysts\(^3\) to obtain mice transmitting the \(Mfap4\)-knockout mutation. The mutation was backcrossed to C57Bl/6N mice (Charles River Laboratories International) for 11 generations and maintained as heterozygotes. The heterozygous mice were inter-crossed to produce homozygous offspring.

**Genotyping of mutant mice by Southern blotting and PCR**

Genomic DNA was isolated, and approximately 5 \(\mu\)g was digested with 50 units of EcoRI, HindIII, or BamHI. Reaction products were separated by electrophoresis on a 0.7% (w/v) agarose gel. Alkaline Southern blotting was performed with 0.4 M NaOH capillary transfer overnight onto a nylon membrane (BioTrace® HP, Gelman Sciences). All hybridization and washing procedures were performed at 65°C. After washing, the membranes were placed on plastic-coated paper, covered with plastic wrap, and exposed to double-coated Hyperfilm (Hyperfilm MS, Amersham) with an intensifying screen for 24 h at -80°C. The probes used for Southern blotting (P1 and P2) were generated by digestion of the cloned fragment of the mouse genome using \(EcoR1\) (probe a) or \(EcoR1\) and \(PvuI\) (probe b) and labeled using ALL-IN-ONE™ Random Prime DNA labeling Mix (dCTP) (Sigma, St. Louis, Missouri USA). \(^{\alpha\text{-}}^{32}\text{P}\) dCTP was from Amersham Pharmacia Biotech (UK). For multiplex PCR genotyping, DNA was extracted from tail biopsies using the RedExtract kit (Sigma) according to the manufacturer’s recommendations. Multiplex PCR was performed using 3 specific oligonucleotide primers (a: 5´-GGCGGCTCTGAAACCAATTA-3´; b: 5´-CCCGGCGGCGGAGGAATCTCT-3´; and c: 5´-GTCAGGACAGCGTGGGAA-3´) for amplifying the wild-type and knock-in alleles. A 300 bp band corresponded to the mutant allele, whereas a 224 bp band corresponded to the wild-type allele. Amplification was performed using an Applied Biosystems 9700 PCR machine with an initial denaturation for 5 min at 95°C; 33 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and finally 72°C for 2 min.

**RT-PCR for confirmation of MFAP4 deletion**

Total RNA was purified from snap-frozen lung tissue from \(Mfap4\)-deficient and wild-type C57BL/6N mice using the RNeasy kit (Qiagen, CA, USA). First-strand synthesis was performed using the SuperScript™ III RNase H- Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) with oligo-dT primers and 1 \(\mu\)g of total mouse lung RNA. \(Mfap4\) transcripts were amplified using the primers 5´-GCCCTGAGATCAGGGAGGATG-3´ and 5´-TCAGGCCGACCAGTGGATG-3´. GAPDH transcripts were amplified using the primers 5´-AGGCCGGTGTGAGATGATGTC-3´ and 5´-GTCCTGCTCCACCACTTCTCT-3´. Amplification was performed with an initial denaturation for 2 min at 94°C; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and finally 5 min at 72°C.

**Carotid artery ligation model**

All mouse experiments were performed under a license obtained from The National Animal Experiments Inspectorate, who also approved the study (ref. no. 2012-15-2934-00095). Generated \(Mfap4\)-deficient mice were crossbred with C57BL/6N mice for 11 generations before they were used for experiments. The arterial ligation model was essentially performed as described in Kumar and Lindner (1997).\(^4\) For all experiments, 16-week-old male \(Mfap4^{+/+}\) and \(Mfap4^{--}\) littermates were used. The animals were anesthetized by intraperitoneal injection of a solution of 1.26 \(\mu\)g body weight Hypnorm (Veta Pharma) and 20 \(\mu\)g Dormicum (HameIn). The left common carotid artery was dissected and ligated at the carotid bifurcation at 48 h, 8 days, 14 days, or 28 days after ligation. The mice were fixed by perfusion with 4% (w/v) formaldehyde in PBS (pH 7.0) under physiological pressure and left for 24 h before excision of the left and right carotid arteries. The vessels were embedded in...
paraffin before further immunohistochemical preparation and morphometric analysis. Digitized images of the vessels were analyzed with the ImageTool version 3.0 (http://compdent.uthscsa.edu/dig/itdesc.html). The circumferences of the lumen, internal elastic lamina, and EEL were determined by tracing along these structures under the assumption that the structures were circular. The areas were measured 0.5, 1.0, and 1.5 mm distal to the bifurcation/ligation or at the corresponding positions in the unligated control vessels.

**qRT-PCR analysis of arterial gene expression**

Total RNA from ligated and unligated carotid arteries was processed using standard methods. Relative gene expression was assessed using the following TaqMan® assays (Applied Biosystems by Life Technologies): Mtap4, Mm00840681_m1; Mmp9, Mm00442991_m1; TATA-binding protein (Tbp) (endogenous control 1), Mm00446973_m1; Gapdh (endogenous control 2), Mm99999915_g1.

**Detection of soluble MFAP4**

MFAP4 levels in carotid arteries from MFAP4+/+ mice, lysed in TBS with 0.1% Triton (Sigma), complete mini protease inhibitor cocktail tablet (Roche) and PhosSTOP phosphatase inhibitor Tablet (Roche), were measured by AlphaLISA as described previously and shown as U/ml. 1 U/ml corresponds to 38 ng/ml in human serum.

**Zymography**

Carotid arterial lysates from Mfap4−/− and Mfap4+/+ mice were prepared as described above. Lysate samples and MMP2/MMP9 markers (Proenzyme, Calbiochem, Merck Millipore) were standardized for protein concentration and loaded on 10% Novex Zymogram Gelatin Gels (Novex, Life Technologies). The gels were developed according to the manufacturer’s instructions. The MMP activity was visualized using Fusion x7 reader (Vilber Lourmat, France) and is shown as band intensity relative to an inter-gel marker control.

**Phenotyping studies of MFAP4-deficient mice**

A cohort of Mfap4−/− mice and Mfap4+/+ littermates were phenotypically analyzed at the German Mouse Clinic in a two-pipeline systematic primary phenotyping screen as previously described, and 10 mice per group were utilized for each test. In the present study, we evaluated cardiovascular parameters, hematological parameters, and clinical chemistry parameters.

**Cardiovascular parameters:**

During the primary screen, various cardiovascular parameters were measured. Blood pressure analysis was performed in unanesthetized mice with a non-invasive tail-cuff method using the MC4000 Blood Pressure Analysis System (Hat-teras Instruments Inc., Cary, North Carolina, USA). Animals were restrained on a pre-warmed metal platform in metal boxes. Their tails were looped through a tail-cuff and fixed in a notch containing an optical path with a LED light and a photosensor. After 5 initial inflation runs for habituation, 12 measurement runs were performed for each animal in one session. Blood pressure was measured in 19 Mfap4+/+ and 16 Mfap4−/− mice.

To assess functional cardiac parameters, echocardiography was performed in anesthetized mice using a 30 MHz transducer and 30 Hz frame rate (Vevo 660; VisualSonics, Toronto, Ontario). The shaved and anesthetized mice (1% isoflurane inhalation, Baxter, Munich, Germany) were fixed in the supine position on a heated platform equipped with ECG electrodes for heart rate monitoring. Body temperature was maintained at 36–38°C and monitored via a rectal thermometer (Indus Instruments, Houston, Texas, USA). Left ventricular parasternal short-axis views were obtained at the papillary muscle level by recording 2-dimensional B-mode images and time-motion M-mode images. In M-mode imaging, we performed 3 recordings per animal and averaged measurements from 4 cardiac cycles. Left ventricular end-diastolic and end-systolic diameters, heart rate, and
fractional shortening were measured. Echocardiography was performed in 17 Mfap4+/+ and 16 Mfap4−/− mice.

During the final examination in the pathology screen, both heart and total body weights were determined. The heart weight was obtained wet after the organ was blotted on paper towels. Heart weight analysis was performed in 17 Mfap4+/+ and 17 Mfap4−/− mice.

**Hematological parameters:**
For the analysis of hematological parameters, 50 µl blood was collected from the retrobulbar vein plexus of mice under isoflurane anesthesia into K-EDTA-coated sample tubes (Kabe Laborteknik, Germany). After being mixed carefully, samples were placed on an overhead rotary agitator until the analysis. An abc-Vet hematology analyzer (Scil-Animal care company, Germany) was used to determine the complete peripheral blood cell count, including the following parameters: total red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), total white blood cell count (WBC), and platelet count (PLT).

**Clinical chemistry parameters:**
Plasma (110 µl) from blood samples collected into tubes coated with Li-heparin (Kabe Laborteknik, Germany) after an overnight fast was used to determine clinical chemistry parameters. Samples collected from the fasted mice were stored on ice and separated by centrifugation (5,000 g, 10 min at 8°C) within an hour. Clinical chemical parameters were determined using an AU400 clinical chemistry analyzer (Olympus, Germany) and adapted reagents supplied by Randox Laboratories (Glycerol), Wako (NEFA HR) or Olympus.

**Blood pressure and heart rate measurements in conscious, freely moving mice**
Blood pressure and heart rate measurements were performed as previously described with the following modifications. Mice were anesthetized (ketamine/xylazine) and chronic indwelling catheters were placed in the femoral artery and vein for measurements of MAP and drug infusions, respectively. The mice were allowed to recover for 5 days before continuous measurements of MAP and heart rate both before and after phenylephrine infusion (50 µg/kg/min).

**Transmission electron microscopy**
Five 16-week-old male Mfap4+/+ mice and five Mfap4−/− littermate male mice were used. The animals were anesthetized by intraperitoneal injection of a solution of 1.26 µg/g body weight Hypnorm (Veta Pharma) and 20 µg/g Dormicum (Hameln). The anesthetized mice were perfused with PBS at physiological pressure and fixed by perfusion for 5 minutes with 1.5% paraformaldehyde, 1.5% glutaraldehyde in 0.15 M HEPES at physiological pressure. Excised left carotid arteries were stored in fixative at 4°C until further processing. Post-fixation was done in 2% OsO4 for 2 h at room temperature and in half-saturated uranyl acetate overnight at 4°C (both solutions in water), followed by stepwise dehydration in acetone and embedding in EPON. Then, 50 nm ultrathin sections were post-stained with uranyl acetate and lead citrate. Samples were observed in a Morgagni TEM (FEI) at 80 kV. Images were taken with a 2K side-mounted Veleta camera.

**Estimation of collagen fibril diameter**
Images for measurements were collected by systematic uniform random sampling on one complete ultrathin cross-section of the artery of each animal. Fields of view were recorded consecutively according to a randomly positioned, regular squarish pattern with the step size of 50 µm, covering the complete section (magnification 8,900x). On each image, the diameters of all collagen fibrils included in 9 regularly positioned squares of 1 µm² were estimated by measuring the shortest diameter of each fibril profile, distinguishing between media and adventitia.

**Immunohistochemical analysis**
Mouse tissues were obtained from Mfap4−/− and Mfap4+/+ mice. Human vein sections with intimal hyperplasia were obtained from the Vascular Research Unit of Viborg Hospital. The local ethical committee in the Mid Region of Denmark approved the use of human tissue
sections (Ref. No. 1-16-02-1-08).

Antibodies used included the following: anti-MFAP4 (HG-HYB 7-14), fluorescein isothiocyanate (FITC)-conjugated anti-MFAP4 (HG-HYB 7-14), anti-α-SMA (Dako #M0851), FITC-conjugated anti-α-SMA (Sigma, clone 1A4), anti-mouse CD45 (BD Pharmingen, clone 30-F11), anti-Ki-67 (Dako, clone MIB-1), anti-caspase-3 (Cell Signaling #9664), anti-phospho-focal adhesion kinase (FAK) (Tyr397) (Abcam, ab4803) and horseradish peroxidase (HRP)-conjugated rabbit polyclonal anti-FITC (Dako, P5100). Proteins were detected using the PowerVision+ HRP detection system (ImmunoVision Technologies) or the Envision+HRP detection system (Dako). For elastin staining, 5 µm-thick murine cross-sections were stained with hematoxylin and eosin (H&E) and Verhoeff-van Gieson’s picrofuchsin counterstaining, and elastic laminae in human sections were enhanced using Weigert’s stain. Collagen stainings were performed using Picosirius Red.

**Ex vivo neointima formation**

Human saphenous vein organ cultures were performed essentially as described previously. Briefly, pieces of human saphenous veins from patients undergoing an in situ bypass operation in the leg were obtained from the Department of Cardiac, Thoracic and Vascular Surgery of Odense University Hospital. The veins were placed in ice-cold Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO), isolated from the surrounding connective tissue and cut open longitudinally. Segments of the vein were cut transversally so that each segment contained the whole circumference of the vein. The segments were pinned down onto a Sylgard-coated petri dish with the intima facing upwards and cultured for 0, 14 or 21(23) days in RPMI 1640 supplemented with 30% heat-inactivated fetal calf serum (Invitrogen) ± 80 µg/ml anti-MFAP4 antibody HG-HYB 7-1 or isotype-matched anti-chicken ovalbumin antibody (The State Serum Institute, Copenhagen). The segments were then fixed in formalin for 24 h, transferred to PBS containing 0.05% NaN₃, embedded in paraffin, cut and stained with H&E. The number of myofibroblast layers at the luminal surface of the segments was counted at 5 independent fields/segment in a blinded manner.

**Production of rMFAP4 and RGD mutants**

Wild-type human rMFAP4 and 3 different genetically modified versions of human rMFAP4 containing mutations of the RGD sequence motif were expressed (RGD -> RAD, AGA, or AAA). Point mutations were introduced into the wild-type MFAP4 coding sequence, cloned into the expression vector pcDNA5/FRT/V5-His TOPO® TA (Invitrogen), and transformed into E. coli according to directions in the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) revision C. The MFAP4-coding plasmid of interest was purified using the GeneJET™ Plasmid Miniprep Kit (Fermentas) according to the manufacturer’s protocol. Flp-In™ CHO cells were co-transfected with a pOG44 plasmid expressing Flp recombinase. Flp-In™ CHO cells were stably transfected in accordance with the in vitro DNA transfection protocol using JetPET™ DNA transfection reagent (Polyplus transfection™) and hygromycin B (Invitrogen) selection. Cells were cultured in Ham’s F-12 medium with L-glutamine (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Production of rMFAP4 in the harvested culture supernatant was determined by ELISA. Purification of rMFAP4 from Flp-In™ CHO cell culture supernatant was performed by HG-HYB 7-5 antibody-affinity chromatography using the fast performance liquid chromatography (FPLC) system (GE healthcare) by standard methods. The monoclonal antibodies were coupled to CNBr-activated Sepharose™ 4B (GE Healthcare) and packed into a XK16 column (GE Healthcare).

**Cell culture**

Cells were grown at 37°C in a 5% CO₂ humidified incubator (Hera cell, Heraeus). Fetal human aortic smooth muscle cells (fHAoSMCs) or adult cells (both from Cell Application, Inc.) derived from normal human tunica intima and tunica media of fetal or adult aortas, respectively, were cultured in Smooth Muscle Cell Growth Medium (Growth Medium; Cell Application, Inc.) or allowed to differentiate in Smooth Muscle Cell Differentiating Medium
(Cell Application, Inc.). Cells from passages 3-7 were used. THP-1 human monocytes (ATCC) were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin and 2 mM L-glutamine (Invitrogen).

**SDS-PAGE and Western blotting**

Trypsinized cells were lysed in RIPA buffer consisting of TBS, 1% (v/v) NP-40 (Sigma-Aldrich), 1% (w/v) sodium deoxycholate (Sigma-Aldrich), 0.1% (w/v) SDS and cOmplete Mini Protease Inhibitor Cocktail Tablet (Roche). Cells were lysed on ice for 20 min, and the lysates were centrifuged at 10,000 g for 10 min before Western blotting. SDS-PAGE and Western blotting were performed using standard methods. Primary antibodies included mouse anti-human integrin αV (CD51), clone 21 (BD Biosciences); mouse anti-human integrin β1, clone BV7 (Abcam); polyclonal goat anti-human integrin β3, C-20 (Santa Cruz Biotechnology); polyclonal rabbit anti-human integrin β5, clone H-96 (Santa Cruz Biotechnology); anti-MFAP4, monoclonal HG-HYB 7-5; mouse anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), clone 6C5 (Santa Cruz Biotechnology); mouse anti-human α-SMA, clone 1A4 (Sigma-Aldrich); polyclonal rabbit anti-FAK, (Cell Signaling Technology); and polyclonal rabbit anti-phospho-FAK (Tyr397), (Cell Signaling Technology). Secondary antibodies included HRP-labeled donkey anti-goat Ig (Santa Cruz Biotechnology), HRP-labeled goat anti-rabbit Ig (Dako), and HRP-labeled rabbit anti-mouse Ig (Dako).

**Detection of MFAP4 by ELISA**

Sandwich ELISA assays were performed in 96-well Maxisorp microplates (Nunc) essentially as previously described. Monoclonal antibodies HG-HYB 7-5, 7-14, and 7-18 were used in different combinations or with a standard setup (detecting both human and mouse MFAP4) with HG-HYB 7-18 as the capture antibody and HG-HYB 7-14 as the detection antibody. In inhibition experiments, plates coated with 250 ng/ml rMFAP4 were incubated with fixed concentration (0.5 µg/ml) of biotinylated HG-HYB 7-1 or biotinylated HG-HYB 7-14 as the detection antibody together with free HG-HYB 7-1, HG-HYB 7-14 or a negative control antibody (concentration 1 ng/ml – 4 µg/ml).

**Flow cytometry analysis**

Pelleted fHAoSMCs were resuspended and stained with the relevant primary anti-integrin antibodies described under “Cell attachment assay” beneath or isotype-matched anti-chicken ovalbumin antibody and polyclonal anti-mouse FITC-conjugated goat F(ab')2 (Dako) as a secondary antibody. Cells were analyzed using a Becton Dickinson (BD) Flow Cytometer FACScan™ (BD Biosciences) and BD CellQuest™ Software (BD Biosciences).

**Cell attachment assay for detection of MFAP4-integrin interactions**

Black 96-well Maxisorp FluoroNunc™ microtiter plates (Nunc) were coated at 4°C overnight with vitronectin (Sigma-Aldrich), rMFAP4, laminin (Sigma-Aldrich), or BSA (Sigma-Aldrich) in PBS, washed and blocked with 10 mg/ml BSA. In MFAP4 blocking experiments, immobilized rMFAP4 (10 µg/ml) was incubated with 20 µg/ml HG-HYB 7-5, 7-14, or 7-18 for 1 h at room temperature before seeding cells. In experiments testing the involvement of the RGD site, fHAoSMCs were pre-incubated for 30 min at room temperature with synthetic GRGDS or SDGRG peptides (25-100 µg/ml; Sigma-Aldrich) or of one of the following anti-integrin antibodies (10 µg/ml): mouse anti-human integrin αv, clone L230 (Alexis Biochemicals); mouse anti-human integrin β1, clone P4C10 (Millipore); mouse anti-human integrin αVβ5, clone P1F6 (Santa Cruz Biotechnologies); mouse anti-human integrin αVβ3, clone LM609 (Millipore); monoclonal mouse anti-human FIBCD1, clone 12-5 (control antibody produced in house). A Vybrant™ cell adhesion assay kit (Molecular Probes, Invitrogen) was used to quantify cell adhesion according to the manufacturer’s instructions. fHAoSMCs were grown in Growth Medium before detachment. Cells were detached using alaphzeme (GE Healthcare), washed twice with PBS and resuspended in serum-free Smooth Muscle Cell Basal Medium (Basal Medium; Cell Application Inc.) containing 5 µM calcein AM fluorogenic dye (Molecular Probes, Invitrogen) for 5 × 10^6 cells/ml. After incubation at 37°C for 30 min,
cells were washed, seeded in Basal Medium (300,000 cells/well) and incubated at 37°C for 1 h. After washing, 200 µl PBS was added to each well and the fluorescence was measured using a plate reader (Victor™ 3 Multilabel Plate Reader, Perkin Elmer) with a fluorescence filter set (excitation at 494 nm and emission at 517 nm).

**Focal adhesion assay**
Chamber slides were coated first with poly-D-lysine (PDL, Sigma-Aldrich) and then with either fibronectin (Sigma-Aldrich) or rMFAP4 overnight. When fHAoSMC cultures reached approximately 60% confluence, they were plated on coated chamber slides and allowed to adhere in serum-free DMEM for 20 h at 37°C and 5% CO₂ humidity. The focal adhesion staining kit (Millipore) was then used to monitor induction of focal adhesion according to the manufacturer’s recommendations. In some experiments, cells were seeded in the presence of the MFAP4-blocking antibody HG-HYB 7-14 or the integrin-blocking antibody anti-integrin α₅ monoclonal mouse anti-human antibody clone L230 (Alexis Biochemicals) in the seeding medium. The primary antibody anti-vinculin was diluted 1:400 in blocking solution, applied to each chamber, and incubated for 1 h. An Alexa Fluor® 488-labeled F(ab’)₂ fragment of goat anti-mouse IgG (H+L) (A11017) (Life Technologies™) was diluted in PBS to 5 µg/ml and was added along with TRITC-conjugated phalloidin diluted 1:800 and incubated for 1 h. Prolong® Gold antifade reagent with DAPI (Invitrogen) was applied after extensive washing. A Leica DMLB fluorescent microscope (Leica) was used to visualize the focal adhesions and actin stress fibrils. The number of round cells in each experimental condition was counted in 5 independent fields of vision in a blinded manner.

**Cell migration assay**
A cell migration assay was performed using the Oris™ Migration Assembly Kit (Platypus Technologies Madison, WI). The Oris-compatible 96-well plate was coated as above with human rMFAP4, fibronectin (Sigma-Aldrich) or PDL as a negative control. After blocking and washing, the cell stoppers blocking the central well area were inserted into the wells. The day before the experiment, fHAoSMCs were serum-starved overnight in Basal Medium. Cells were seeded in Growth Medium at the density of 25,000 cells/well and incubated for 4 h at 37°C. Afterwards, the cell stoppers were removed, the wells were gently washed, and the medium was changed to Basal Medium containing 0.5% (v/v) FBS and 5 ng/ml platelet-derived growth factor-BB (PDGF-BB; R&D Systems, Inc.). The plate was incubated for 20 h at 37°C to allow cells to migrate. For blocking experiments, after stopper removal the wells were incubated for 30 min with HG-HYB 7-5, 7-14, or 7-18, or anti-chicken ovalbumin antibody as an isotype control at room temperature. In some experiments, cells were incubated with 25 µM of the MEK1/2 inhibitor PD98059 (Cell Signaling Technology), 50 µM of the PI3K inhibitor LY294002 (Cell Signaling Technology) or 50 µM of the FAK inhibitor 1,2,4,5-benzenetetramine tetrahydrochloride (Sigma-Aldrich) after initial adherence 1 h prior to PDGF stimulation and for the rest of the culture period. The following day, cells were fixed with 4% paraformaldehyde and stained with 0.5 µg/ml 4’,6-diamidino-2-phenylindole (DAPI) solution (Invitrogen) for 5 min. Pictures of each well were taken using the Olympus IX71 fluorescence microscope. The migrating cells were quantified using ImageJ software, version 1.44P (NIH). The numbers of cells were quantified by MTT assay to distinguish the effects of proliferation from the effects of migration in control experiments.

**Proliferation assays**
fHAoSMCs were serum-starved in Basal Medium supplemented with 0.1% (v/v) FBS overnight. A 96-well tissue culture plate was coated with 10 µg/ml rMFAP4 or fibronectin (Sigma-Aldrich) at 4°C overnight, washed and blocked with 10 mg/ml BSA. For blocking experiments, the protein-coated wells were then incubated with 20 µg/ml HG-HYB 7-5, 7-14, or 7-18 for 1 h at room temperature. Then, 6,000 cells/well were seeded in the coated wells and incubated in Basal Medium supplemented with 0.3% (v/v) FBS ± 5 ng/ml recombinant human PDGF-BB. Cells were then allowed to proliferate for 4 or 48 h at 37°C. The number of viable cells was determined by the MTT assay. Briefly, cells were incubated with 1 mg/ml
MTT (Sigma-Aldrich) for 4 h at 37°C. Next, the medium was removed and 150 μl 0.1 M hydrochloric acid in isopropanol was added, and the plate was incubated for 15 min at room temperature with gentle shaking. The absorbance was read using the Sunrise Microplate Reader (Tecan) at 570 nm with a reference wavelength of 690 nm.

In some experiments, 10,000 cells/well were seeded in Basal Medium and allowed to adhere for 3 h. Afterwards, cells were incubated with 50 μM of the MEK1/2 inhibitor PD98059 (Cell Signaling Technology), 50 μM of the PI3K inhibitor LY294002 (Cell Signaling Technology) or 50 μM of the FAK inhibitor 1,2,4,5-benzenetetramine tetrahydrochloride (Sigma-Aldrich) 1 h prior to PDGF stimulation and for the rest of the culture period. Cell proliferation was measured by BrdU Colorimetric Assay Kit (Cell Signaling Technology) according to the manufacturer’s instructions.

*qRT-PCR analysis*

fhAoSMCs were seeded on PDL- or MFAP4-precoated 12-well plates (10 μg/ml) in Growth Medium at the density of 200,000 cells/well and were allowed to attach for 6-8 h. Cells were then serum-starved in Basal Medium overnight. The next day, the medium was replaced with fresh Basal Medium containing 5 ng/ml recombinant human PDGF-BB. After 12 h of stimulation cells were washed and frozen in TRIzol reagent (Life Technologies). RNA extraction was performed according to the manufacturer’s instructions. One μg RNA was used for cDNA synthesis with M-MLV Reverse Transcriptase (Sigma) according to the manufacturer’s instructions. Relative gene expression was assessed using the following TaqMan® assays: GAPDH, Hs99999905_m1; MFAP4, Hs00412974_m1; ICAM1, Hs00164932_m1; VCAM1, Hs01003372_m1; ACTA2, Hs00909449_m1; TAGLN, Hs01038777_g1; BCL2, Hs00608023_m1; CCL2, Hs00234140_m1.

*Apoptosis assay*

fhAoSMCs were seeded on PDL- or MFAP4-precoated 12-well plates (10 μg/ml) in Growth Medium at the density of 100,000 cells/well and were allowed to attach for 6-8 h. Cells were then serum-starved in Basal Medium overnight. The next day the medium was replaced with fresh Basal Medium ± 5 ng/ml recombinant human PDGF-BB. After 24 h of stimulation cells were collected and stained with FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer’s instructions. Samples were collected on LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). The apoptotic cells were defined as Annexin V-positive, propidium iodide-negative.

*Cell cycle analysis*

fhAoSMCs were prepared and collected as described for the apoptosis assay above and fixed overnight in 75% ethanol. The next day cells were washed twice in PBS, treated with 100 μg/ml RNase A (Sigma) and stained with 50 μg/ml propidium iodide. The number of cells in G0/G1, S and G2/M phases was identified using FlowJo software.

*Monocyte migration assay*

Bottom sides of transwell inserts were coated with BSA, MFAP4 or fibronectin at 4°C overnight, blocked with BSA for 1 h at room temperature and washed. THP-1 cells were differentiated for 48 h with 10 nM phorbol 12-myristate 13-acetate and seeded in the upper chamber of a transwell insert (200,000 cells/well) in RPMI 1640 containing 0.1% BSA, with lower chamber containing 100 ng/ml recombinant human CCL2. In some experiments, cells were pre-incubated with anti-integrin αVβ3 or αVβ5 antibodies before seeding. After 3 h the upper side of the inserts was washed and scraped with a cotton swab to remove residual cells. The inserts were then fixed with 4% formaldehyde and stained with DAPI. The number of cells that have migrated through the transwell was counted in 7 independent fields of vision in a blinded manner.

*Statistical methods*
Statistical significance between groups in *in vitro* and *in vivo* experiments was assessed by Student’s t-test or either one-way or two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparisons test. Data were analyzed using GraphPad Prism 5. *p* < 0.05 was considered statistically significant.
References
Data supplement

Supplemental tables

Table TI. Cardiovascular and clinical chemistry parameters were measured using blood sampling from 12-14-week-old male and female Mfap4+/+ (+/+) and Mfap4−/− (−/−) mice. LV = left ventricular. NEFA = non-esterified fatty acids. n = 5-10. Data are means ± SEM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male</th>
<th>Female</th>
<th>p-value</th>
<th>Genotype</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight [g]</td>
<td>27.4±1.4</td>
<td>21.9±0.4</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Heart weight [g]</td>
<td>0.14±0.00</td>
<td>0.12±0.00</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Systolic pressure [mmHg]</td>
<td>119.8±3.1</td>
<td>116.4±3.3</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Pulse [bpm]</td>
<td>531.1±13.2</td>
<td>523.2±10.4</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Fractional shortening [%]</td>
<td>35.9±2.1</td>
<td>36.5±1.7</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>LV volume diastolic [ml]</td>
<td>71.7±2.2</td>
<td>59.3±6.4</td>
<td>&lt;0.01</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>LV volume systolic [ml]</td>
<td>24.8±2.2</td>
<td>20.2±3.0</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Ejection fraction [%]</td>
<td>65.5±2.8</td>
<td>66.7±2.2</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Cholesterol [mg/dl]</td>
<td>107.5±2.17</td>
<td>82.2±2.85</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Triglycerides [mg/dl]</td>
<td>90±5.9</td>
<td>70±3.5</td>
<td>&lt;0.01</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>NEFA [mmol/l]</td>
<td>1.06±0.071</td>
<td>1.12±0.079</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Glucose [mg/dl]</td>
<td>80±11.4</td>
<td>73.5±10</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Table TII. Hematological parameters evaluated in blood samples from 12-14-week-old male and female *Mfap4*+/+(+/+) and *Mfap4*−/−(−/−) mice. WBC = white blood cells, RBC = red blood cells, PLT = platelets. n = 5-10. Data are means ± SEM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
<th>Sex p-value</th>
<th>Genotype p-value</th>
<th>Interaction p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC [103/µl]</td>
<td>12.6±0.67</td>
<td>6.0±0.38</td>
<td>14.4±0.87</td>
<td>6.2±0.39</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>RBC [106/µl]</td>
<td>10.3±0.08</td>
<td>10.2±0.1</td>
<td>10.1±0.08</td>
<td>10.2±0.1</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>PLT [g/dl]</td>
<td>1516±81</td>
<td>1151±51.9</td>
<td>1413±35.3</td>
<td>1265±61.6</td>
<td>&lt;0.001</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hemoglobin [g/dl]</td>
<td>15.4±0.11</td>
<td>14.9±0.18</td>
<td>15.3±0.13</td>
<td>15.0±0.2</td>
<td>&lt;0.01</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hematocrit [%]</td>
<td>54.7±0.36</td>
<td>53.2±0.78</td>
<td>54.5±0.5</td>
<td>53.4±0.64</td>
<td>&lt;0.05</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Supplemental figures and figure legends

**Figure SI.** Targeted disruption of mouse *Mfap4* gene. (A) The *Mfap4* gene product is transcribed from 6 exons on chromosome 17. The mature protein comprises an N-terminal region including one free sulfhydryl group and an RGD integrin-binding sequence. (B) The wild-type *Mfap4* allele was used to produce the targeting vector, designed to exclude a region of 1.5 kbp of the non-coding 5’ region and the first 3 exons of the *Mfap4* gene by exclusion of an EcoRI fragment and replacement with the PGK-neo expression cassette. Top and bottom panels show the wild-type mouse *Mfap4* allele and mutant allele with PGK-neo expression cassette, respectively. C) Duplex PCR analysis of genomic DNA from wild-type and gene-deleted mouse tails. (D) RT-PCR analysis of *Mfap4* and *Gapdh* gene transcription from wild-type and gene-deleted mouse pulmonary tissue. (E) Quantitative ELISA on 2-fold dilutions of serum from *Mfap4*+/+ and *Mfap4*−/− mice relative to rMFAP4. (F) Immunohistochemical analysis of arterial tissue from *Mfap4*+/+ and *Mfap4*−/− mice developed with HG-HYB 7-14. M, size marker. Scale bar = 50 μm, original magnification 1,000×.
Figure SII. Transmission electron microscopy of cross-sectioned carotid arteries from $Mfap4^{-/-}$ and $Mfap4^{+/+}$ mice. (A-C) Overview of (A) the whole $Mfap4^{-/-}$ artery, (B) $Mfap4^{-/-}$ elastic membranes, and (C) $Mfap4^{-/-}$ tunica adventitia collagen fibrils. Arrowheads indicate large fibrils. (D-F) Overview of (D) the whole $Mfap4^{+/+}$ artery, (E) $Mfap4^{+/+}$ elastic membranes, and (F) $Mfap4^{+/+}$ tunica adventitia collagen fibrils. (G-H) Distribution of collagen fibrils according to thickness in (G) tunica adventitia (3048-6034 fibrils analyzed, n = 5 mice) or (H) tunica media (1764-1910 fibrils analyzed, n = 5 mice). Black = $Mfap4^{-/-}$, gray = $Mfap4^{+/+}$. Scale bar = 100 µm (A, D), 10 µm (B, E), 1 µm (C, F).
Figure SIII. Mean arterial blood pressure (MAP) in unchallenged *Mfap4⁻/⁻* and *Mfap4⁺/⁺* mice. MAP (A) and heart rate (HR) (B) were obtained using chronic indwelling catheters placed in the femoral artery and vein for measurements before and after infusion of phenylephrine (PE). Data are means + SEM from n = 6 mice/group.

Figure SIV. Integrin monomers αᵥ, β₁, β₃, and β₅ are all present in smooth muscle cells at detectable levels under experimental conditions. The figure shows semi-quantitative Western blotting analysis of integrin expression from lysates of adult or fHAoS MCs differentiating from the synthesizing/proliferating phenotype to differentiated/contractile phenotype over time. Data are representative of at least 2 individual experiments.
Figure SV. HG-HYB 7-1, HG-HYB 7-5 and HG-HYB 7-14 are MFAP4 blocking antibodies interfering with integrin binding. (A-C) Anti-MFAP4 antibodies HG-HYB 7-5, HG-HYB 7-14 and HG-HYB 7-18 were used in different combinations as capture antibodies and biotinylated detection antibodies in ELISA for detection of rMFAP4. The capture antibody/detection antibody combinations are shown in the panel titles. Cell culture supernatants containing wild-type rMFAP4 with an intact RGD sequence (RGD (WT)); point-mutated rMFAP4 with 1, 2, or all 3 RGD-amino acids replaced with alanine (RAD, AGA, AAA, respectively); or cell culture supernatant from mock-transfected cells (background) were used as samples and are depicted with the symbols shown in (A). The horizontal axes display the inverse dilution factors of the cell culture supernatants. The vertical axes display the optical density intensity obtained from the colorimetric ELISA. (D-E) HG-HYB 7-1 and HG-HYB 7-14 bind to the same epitope on MFAP4. Both HG-HYB 7-1 and HG-HYB 7-14 inhibited interaction between immobilized MFAP4 and biotinylated detection antibodies HG-HYB 7-1 (D) and HG-HYB 7-14 (E) in a dose-dependent manner. Data are means from experimental triplicates and representative of at least 2 independent experiments.

Figure SVI. MFAP4 effects on fHAoSMC numbers after 4 h and 20 h. fHAoSMC numbers are not profoundly influenced by MFAP4 after 20 h (A) or 4 h (B) of culture. Data are means ± SEM of 3-4 individual experiments. OD, optical density.
Figure SVII. Gene expression profile of fHAoSMCs. Expression levels of ICAM1 (A), VCAM1 (B), BCL2 (C) and CCL2 (D) in fHAoSMCs are not influenced by MFAP4. Data are means ± SEM of at least 3 independent experiments.