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

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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 $\alpha_V\beta_3$ 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 $\alpha_V\beta_3$–dependent manner.

Conclusions—MFAP4 regulates integrin $\alpha_V\beta_3$–induced VSMC proliferation and migration, as well as monocyte chemotaxis, and accelerates neointimal hyperplasia after vascular injury. (***Arterioscler Thromb Vasc 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 $\alpha_V\beta_3$ 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

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Nonstandard Abbreviations and Acronyms	
CCL2	chemokine (C-C motif) ligand 2
ECM	extracellular matrix
FAK	focal adhesion kinase
fHAoSMC	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

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.[4] MFAP4, known as 36-kDa microfibril-associated glycoprotein (MAGP36)[5] 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.[6–10] MAGP36 is known to interact directly with ECM fibers, including elastin, collagen, and calvasculin.[6–8,11] Moreover, MAGP36 was demonstrated to bind integrin receptors on human aortic SMCs in an RGD-dependent manner.[7] 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.[12,13]

MFAP4 has been associated with remodeling-related disorders, including liver fibrosis,[14] pulmonary airspace enlargement,[15] and atherosclerosis.[16] Furthermore, we recently showed that MFAP4 promotes airway SMC proliferation in experimental asthma,[17] 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.[18] 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[19]; 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.

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 IA–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,[20] but 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 model of arterial injury. After carotid artery ligation, the resulting neointimal growth seemed to be delayed in *Mfap4*$^{-/-}$ mice, with limited or no formation after 14 days (Figure 1A and 1B). The vessel area defined by the external elastic lamina of the ligated *Mfap4*$^{-/-}$ vessels was significantly decreased compared with *Mfap4*$^{+/+}$ vessels (Figure 1B). At day 28, the lumen of *Mfap4*$^{-/-}$ vessels was, therefore, significantly decreased.

MFAP4 localized to elastic fibers and in addition to medial (most intensely) and neointimal cells (Figure 2A). There was no significant difference in MFAP4 mRNA expression

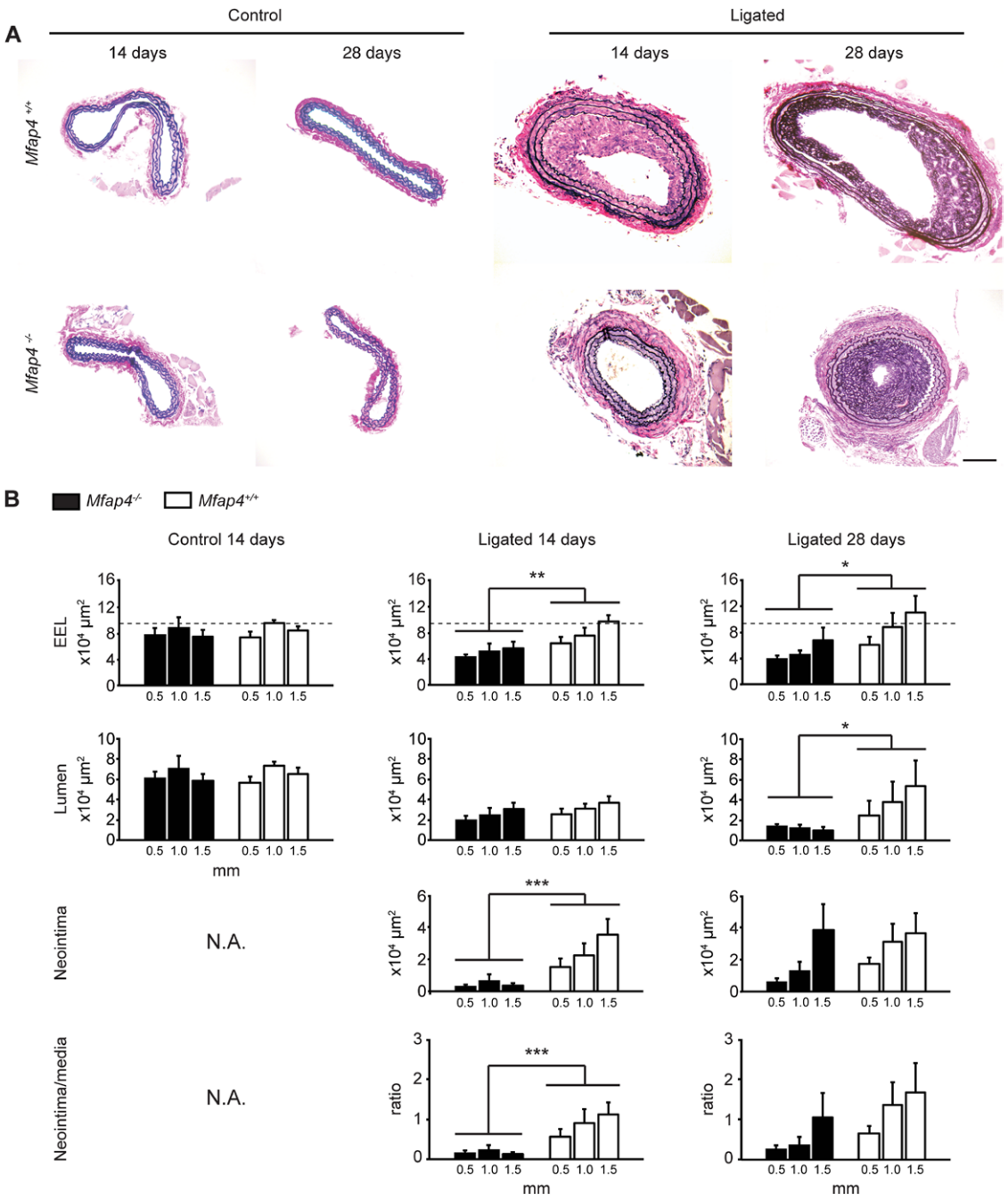

Figure 1. Microfibrillar-associated protein 4 (MFAP4) accelerates neointima formation and restrictive remodeling of the arterial wall. **A**, The unligated right common carotid artery served as a control, and the left common carotid artery was ligated at the bifurcation in *Mfap4*$^{+/+}$ and *Mfap4*$^{-/-}$ mice. Elastin-stained sections were obtained 1.5 mm distal to the bifurcation at day 14 or day 28 after ligation. Representative pictures are shown. Scale bar, 100 µm; original magnification, ×200. **B**, Morphometric analysis of cross-sectional vessel area was performed in unligated control carotid arteries and ligated carotid arteries 0.5, 1.0, and 1.5 mm distal to the bifurcation 14 days (n=6 mice per group) or 28 days (n=3–6 mice per group) after ligation. **Bottom**, Ratios between the neointimal areas and the medial areas. Dashed lines indicate the EEL value of unligated control *Mfap4*$^{+/+}$ artery after 14 days. Data are presented as mean±SEM. **P*<0.05, ***P*<0.01, and ****P*<0.001, calculated by 2-way ANOVA. EEL indicates external elastic lamina; and N.A., not available.

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

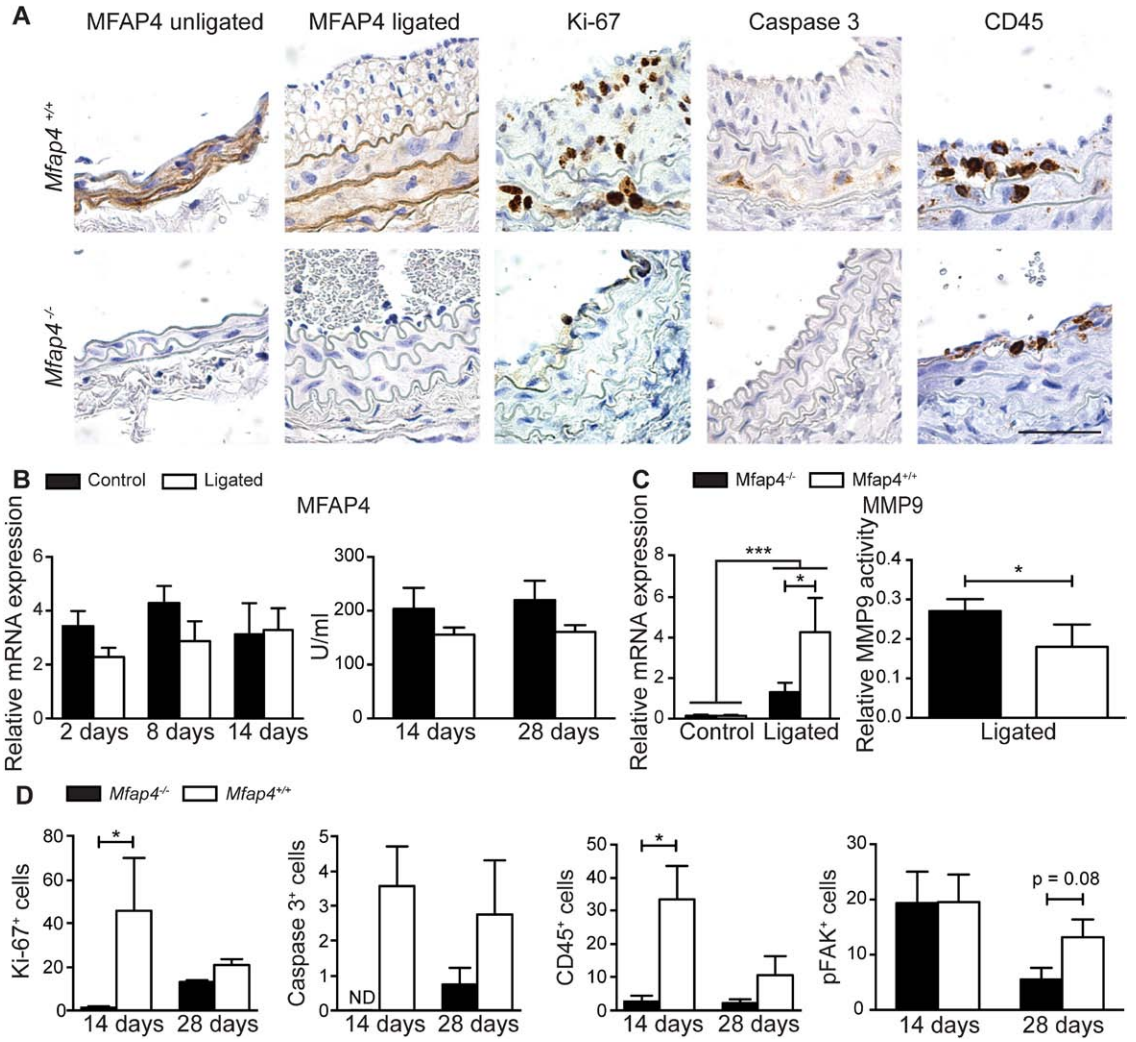

Figure 2. Proliferation of vascular cells and the infiltration of inflammatory cells are decreased in *Mfap4*$^{-/-}$ mice relative to *Mfap4*$^{+/+}$ mice in parallel with decreased neointima formation. **A**, Protein expression of microfibrillar-associated protein 4 (MFAP4), the proliferative marker Ki-67, the apoptotic marker caspase 3, and the leukocyte marker CD45 was analyzed in carotid arterial sections from *Mfap4*$^{-/-}$ and *Mfap4*$^{+/+}$ mice. Displayed sections were obtained 1.5 mm distal to the bifurcation/ligation in mice terminated 14 days after ligation. The MFAP4-stained unligated sections were obtained from the contralateral control carotid artery. Representative pictures are shown. Scale bar, 50 μm; original magnification, ×1000. **B**, Relative *Mfap4* mRNA expression was quantified in carotid artery samples obtained 2 days, 8 days, or 14 days after ligation from both control vessels and ligated *Mfap4*$^{+/+}$ vessels (**left**), and MFAP4 protein content was measured in tissue lysates from ligated and control arteries obtained 14 days or 28 days after ligation (**right**). **C**, Relative *Mmp9* mRNA expression (**left**) and relative MMP9 activity (**right**) in control or ligated carotid arteries obtained 2 days after ligation. **D**, Proliferating cells (Ki-67^{+}), apoptotic cells (caspase 3^{+}), and inflammatory cells (CD45^{+}) were quantified by counting the total number of stained cells in the media and in the neointima from sections obtained 1.5 mm distal to the bifurcation/ligation in mice euthanized 14 or 28 days after ligation. Phosphorylated focal adhesion kinase (pFAK) was quantified by counting the mean number of stained cells in the neointima from sections obtained 0.5 and 1 mm distal to the bifurcation/ligation. Data are presented as mean±SEM from n=3 to 7 mice per group. *P<0.05 and ***P<0.001, calculated by 1-way ANOVA or Student t test. MMP indicates matrix metalloproteinase; and ND, not detected.

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 (FAK) 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 (Figure 3A), in line with previous observations from normal vessels.[16]

To evaluate the effects of endogenous MFAP4 on proliferative vessel responses, we cultured freshly isolated human saphenous veins under growth-promoting conditions. We observed neointimal growth on the luminal side of the vessels after 14 and 21 days of serum-rich culture (Figure 3B). 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 $\alpha_v\beta_3$

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 β_1, α_v, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ 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 MAGP36.[7] Anti–integrin α_v and integrin $\alpha_v\beta_3$ antibodies completely blocked cellular attachment to rMFAP4 after 1 hour, whereas anti–integrin $\alpha_v\beta_5$ 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–ᴅ-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

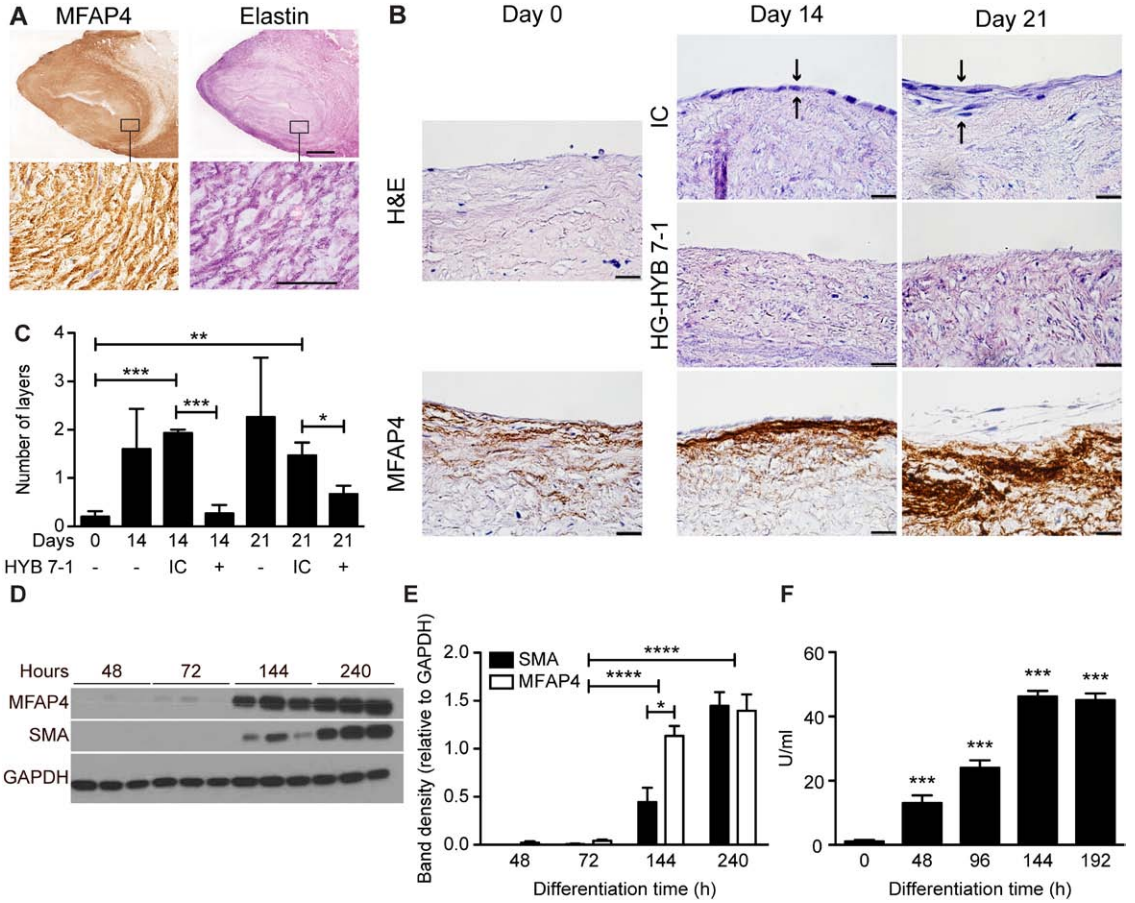

Figure 3. Microfibrillar-associated protein 4 (MFAP4) is produced by vascular smooth muscle cells and mediates ex vivo neointima formation. **A**, Immunohistochemical detection of MFAP4 in sections from human vein with neointimal hyperplasia. MFAP4 was visualized by immunostaining, and elastin was visualized 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 mean±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 mean±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.

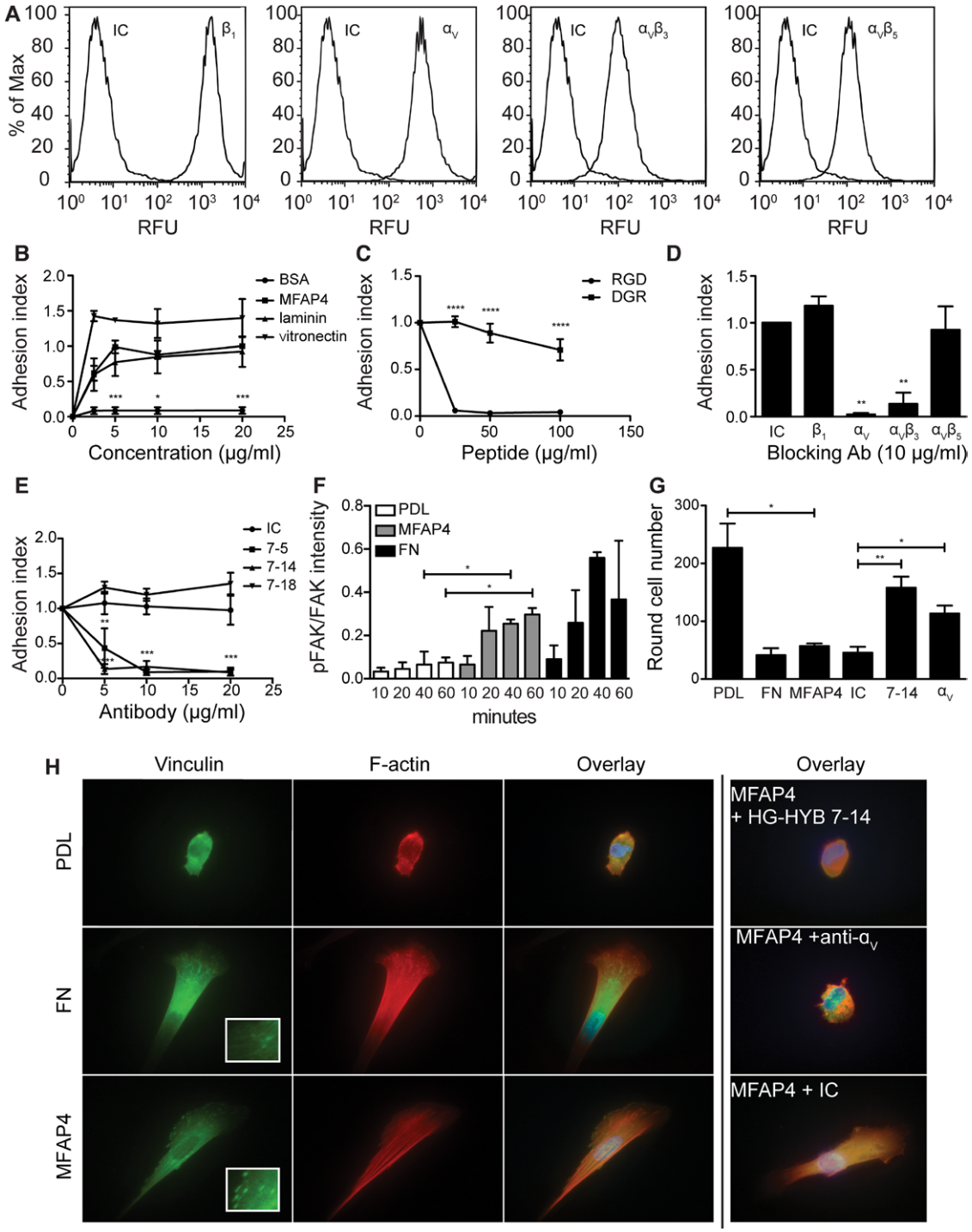

Figure 4. Microfibrillar-associated protein 4 (MFAP4) interacts with vascular smooth muscle cells through RGD-dependent integrins. **A**, Flow cytometry analysis of cell surface integrin expression on fetal human aortic SMCs (fHAoSMCs). The attachment of fHAoSMCs onto immobilized bovine serum albumin (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 β_1, α_V, $\alpha_V\beta_3$, or $\alpha_V\beta_5$ (**D**); and onto rMFAP4 in competition with monoclonal anti-MFAP4 antibodies HG-HYB 7-5, 7 to 14, or 7 to 18 (**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-α_V 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.

HG-HYB 7 to 14 or anti–integrin α_v antibody during seeding (Figure 4G and 4H).

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 7 to 18 clearly bound both double (AGA) and triple (AAA) RGD-mutated rMFAP4 (Figure VA–VC in the online-only Data Supplement). In contrast, rMFAP4 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).

MFAP4 Induces VSMC Migration and Proliferation Dependent 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–D-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.

MFAP4 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 monocytic 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 $\alpha_v\beta_3$ but not anti–integrin $\alpha_v\beta_5$ antibody (Figure 6E).

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 $\alpha_v\beta_3$–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*$^{-/-}$ elastic tissues, including arteries, skin, and lung showed a normal gross 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*$^{+/+}$ 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.

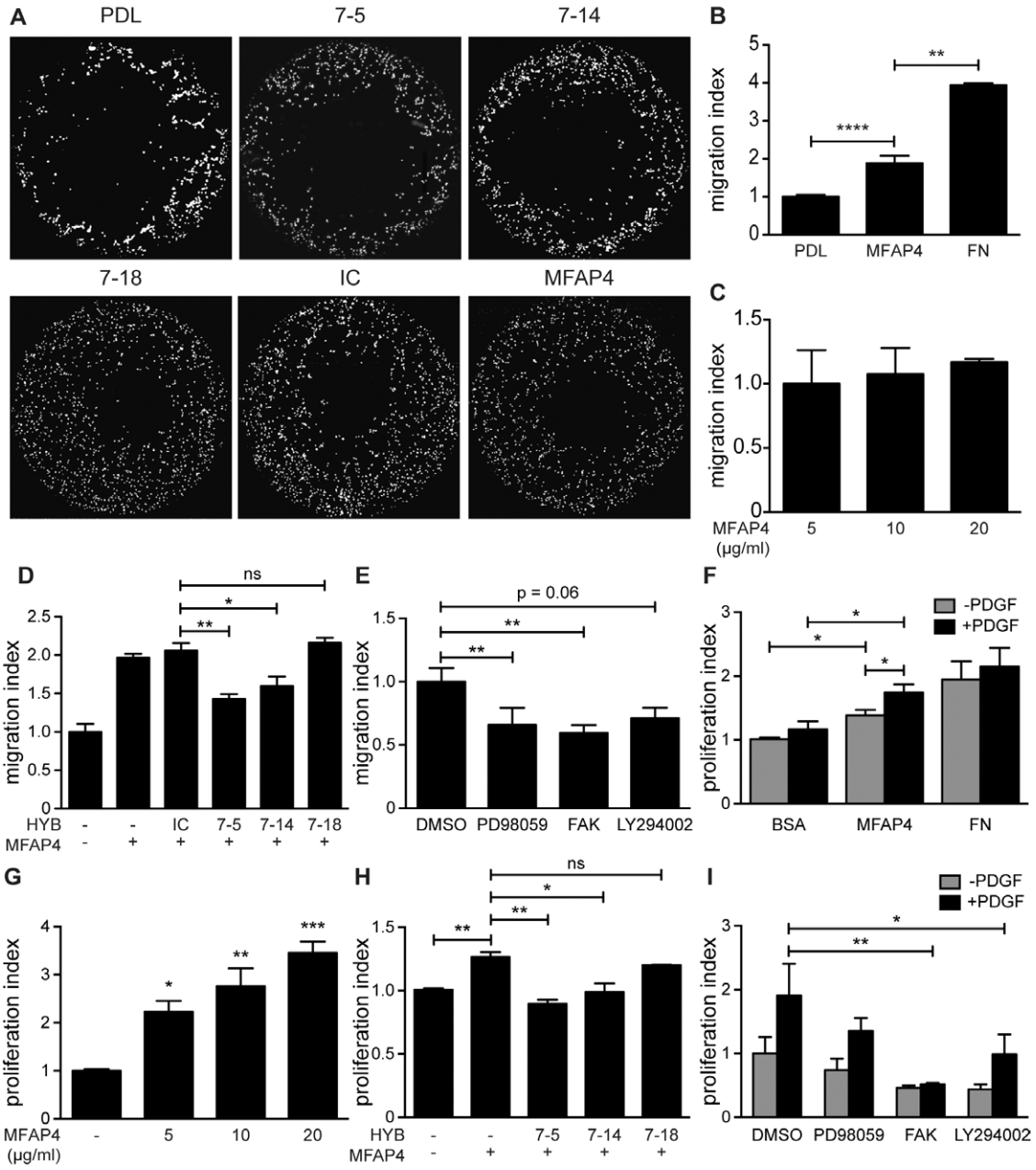

Figure 5. Microfibrillar-associated protein 4 (MFAP4) induces vascular smooth muscle cell migration and proliferation dependent on integrin signaling. **A**, Representative pictures of migrating fetal human aortic SMCs (fHAoSMCs) seeded on poly–D-lysine (PDL; negative control), recombinant MFAP4 (rMFAP4), or rMFAP4 pretreated with anti-MFAP4 antibodies. MFAP4 stimulates fHAoSMC migration (**B**) but without the dose dependency within used concentration range (**C**). **D** and **E**, MFAP4-dependent fHAoSMC migration is attenuated by anti-MFAP4 antibodies HG-HYB 7-5 and 7 to 14 (**D**), as well as focal adhesion kinase (FAK), phosphoinositide 3-kinase, and mitogen-activated protein kinase kinase 1/2 inhibitors (**E**). MFAP4 stimulates fHAoSMC proliferation (**F**) in a dose-dependent manner (**G**). **H** and **I**, MFAP4-dependent fHAoSMC proliferation is blocked by anti-MFAP4 antibodies HG-HYB 7-5 and 7 to 14, as well as FAK and PI3K kinase inhibitors (I). DMSO was used as a vehicle control for kinase inhibitors. FN, fibronectin. IC, isotype control. Data are presented as mean±SEM of at least 3 independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001, calculated by 1-way ANOVA or Student *t* test. DMSO indicates dimethyl sulfoxide; FN, fibronectin; and ns, not significant.

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.[19] 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

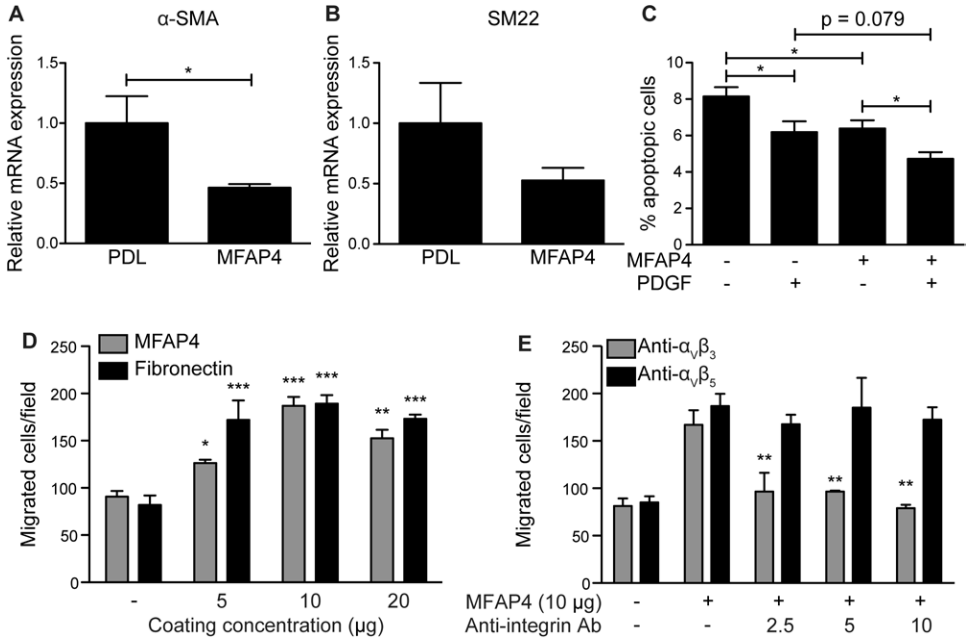

Figure 6. Microfibrillar-associated protein 4 (MFAP4) mediates vascular smooth muscle cell dedifferentiation and survival, as well as monocyte migration. **A** and **B**, Fetal human aortic SMC (fHAoSMC) expression of differentiation markers α-smooth muscle actin (α-SMA; **A**) and SM22 (**B**) is attenuated by MFAP4 stimulation. **C**, MFAP4 has an antiapoptotic effect on fHAoSMCs. **D** and **E**, MFAP4 stimulates chemokine (C-C motif) ligand 2–driven THP-1 cell chemotaxis in a dose-dependent manner (**D**) that can be inhibited by anti–integrin $\alpha_V\beta_3$ antibody but not $\alpha_V\beta_5$ antibody (**E**). Data are presented as mean±SEM of at least 3 independent experiments. **P*<0.05, ***P*<0.01, and ****P*<0.001, calculated by 1-way ANOVA or Student *t* test. PDL indicates poly–D-lysine.

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 *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 *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 MAGP36/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 β_3–deficient mice,[36] in experiments performed using a gene knockout model for the integrin $\alpha_v\beta_3$ ligand vitronectin[37] and in osteopontin inhibition experiments,[38] all underlining an important role of integrin $\alpha_v\beta_3$ in neointimal hyperplasia. Although basic integrin $\alpha_v\beta_3$ expression on VSMCs is relatively low, it is highly upregulated after vascular injury.[39]

Primary fetal VSMC cells used in our in vitro studies have a relatively high expression of integrin $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ is the dominant MFAP4 receptor on the fHA-oSMC 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.[40] 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 $\alpha_v\beta_3$ ligands osteopontin and vitronectin.[41,42] FAK can act through mitogen-activated protein kinase and PI3K pathways, both with an established role in VSMC migration and proliferation.[43–45] 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 underlying 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.[46,47] 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 exclude 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 β_3 expression was observed to disappear. No or low MFAP4 expression, low integrin α_v expression, and a stable integrin β_3 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 $\alpha_v\beta_3$ 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 $\alpha_v\beta_3$ is known to coprecipitate with PDGF receptors.[48] Association between FAK and PI3K is enhanced after PDGF stimulation.[49] 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.[50] 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 $\alpha_v\beta_3$ agonist promoting VSMC growth and migration, as well as monocyte chemotaxis, resulting in accelerated neointimal hyperplasia in flow cessation–induced injury.

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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.

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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 $\alpha_v\beta_3$ 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.