SMOC-2 is a novel member of the SPARC family of matricellular proteins. The purpose of this study was to determine whether SMOC-2 can modulate angiogenic growth factor activity and angiogenesis. SMOC-2 was localized in the extracellular periphery of cultured human umbilical vein endothelial cells (HUVECs). Ectopically expressed SMOC-2 was also secreted into the tissue culture medium. In microarray profiling experiments, a recombinant SMOC-2 adenovirus induced the expression of transcripts required for cell cycle progression in HUVECs. Consistent with a growth-stimulatory role for SMOC-2, its overexpression stimulated DNA synthesis in a dosedependent manner. Overexpressed SMOC-2 also synergized with vascular endothelial growth factor or with basic fibroblast growth factor to stimulate DNA synthesis. Ectopically expressed SMOC-2 stimulated formation of network-like structures as determined by in vitro matrigel angiogenesis assays. Fetal calf serum enhanced the stimulatory effect of overexpressed SMOC-2 in this assay. Conversely, small interference RNA directed toward SMOC-2 inhibited network formation and proliferation. The angiogenic activity of SMOC-2 was also examined in experimental mice by subdermal implantation of Matrigel plugs containing SMOC-2 adenovirus. SMOC-2 adenovirus induced a 3-fold increase in the number of cells invading Matrigel plugs when compared with a control adenoviral vector. Basic fibroblast growth factor and SMOC-2 elicited a synergistic effect on cell invasion. Taken together, our results demonstrate that SMOC-2 is a novel angiogenic factor that potentiates angiogenic effects of growth factors.

Angiogenesis, the formation of new capillaries from existing vasculature, is a vital process in development, tumor formation, and in the restoration of the blood supply to ischemic tissues. Numerous factors are involved in the regulation of this process such as growth factors, oxygen levels, proteases, and extracellular matrix components. Over the past decade, matricellular proteins have gained more attention in their role in regulating cellular functions and angiogenesis. Matricellular proteins are extracellular proteins that do not contribute structurally to the extracellular milieu but regulate interactions between cells and the extracellular matrix (1). Proteins that have been grouped as matricellular proteins include the thrombospondins, tenascins, osteopontin, and the SPARC (secreted protein acidic and rich in cysteine/osteonectin/BM-40) family of proteins. These proteins are expressed in many cell types and are highly expressed during embryogenesis, wound healing, and other instances where there is extensive tissue remodeling.

SPARC is highly expressed during embryogenesis, and its expression becomes more restricted in adult tissues (2). It is highly expressed in adult bone tissues and during processes involving tissue remodeling such as tumorigenesis and wound repair. Mice that are homozygous-null for SPARC are able to develop a relatively normal phenotype but soon develop cataracts (3), have impaired bone formation (4), and show severe deficiencies in wound repair (5, 6). In vitro experiments have shown the influence of SPARC on several cell processes. It has been shown to inhibit cell adhesion to the extracellular matrix and alter cell shape (7). SPARC inhibits cell cycle progression (8), and it binds to collagens and other extracellular matrix proteins and possibly plays a role in the organization of these components (9). SPARC has been shown to bind several growth factors and alter their activity. These include platelet-derived growth factor (PDGF) (10) and vascular endothelial growth factor (VEGF) (11). SPARC indirectly influences the effects of basic fibroblast growth factor (bFGF) (12), and transforming growth factor β (13).

There is evidence that SPARC is inhibitory for angiogenesis. This is based on the observation that in SPARC-null mice there is increased cell invasion into implanted sponges compared with wild-type mice (14). This is also suggested by the binding of VEGF by SPARC and reduced VEGF receptor activation in the presence of SPARC (11). However, it has been observed that

The Novel SPARC Family Member SMOC-2 Potentiates Angiogenic Growth Factor Activity*

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The abbreviations used are: SPARC, secreted protein acidic and rich in cysteine/osteonectin/BM-40; PDGF, platelet-derived growth factor; PDGFβ receptor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; SMOC-2, secreted modular calcium-binding protein-2; Ad-SMOC-2, SMOC-2 adenovirus; Ad-Myc-SMOC-2, Myc-tagged SMOC-2; siCTR, non-targeting control siRNA; siSMOC-2-1 and siSMOC-2-2, two siRNA molecules targeted to different regions of human SMOC-2; GFP, green fluorescent protein; EGM-2, endothelial cell growth medium-2; HUVEC, human umbilical vein endothelial cell; EB-2, endothelial basal medium-2; FBS, fetal bovine serum; siRNA, small interfering RNA; CMV, cytomegalovirus; DAPI, 4’,6-diamidino-2-phenylindole; BrdUrd, bromodeoxyuridine.
cleavage of SPARC by matrix metalloproteinases and plasmin releases peptides that are pro-angiogenic (15, 16). These peptides are found within the follistatin-like domain of SPARC (8, 15). Furthermore, follistatin can induce angiogenesis in rabbit cornea assays, and it can synergize with bFGF in this assay (17).

SPARC-related proteins are characterized by the presence of a follistatin-like domain and a C-terminal extracellular calcium-binding domain with two EF-hand calcium-binding motifs (18, 19). However, the proteins have highly variable N-terminal regions (20). These SPARC-related proteins include SC1/Hevin/QR1 (21–23), Testican (24), tsc36/FRP (25), and the recently-described SMOC-2 (secreted modular calcium-binding protein)/SMAP2 (26). SMOC-2 is expressed in nearly all tissues, with the highest expression found in the heart, muscle tissue, spleen, and ovary (27), but no function has been assigned to this protein. To date, the only clinically relevant role described for SMOC-2 is in the rat carotid artery of accelerated atherosclerotic lesion formation, which demonstrated an up-regulation of SMOC-2 mRNA in response to injury (26).

In a microarray screen for transcripts that are differentially regulated by the environmental carcinogen benzo[a]pyrene, we identified SMOC-2 as a cell cycle-regulated and benzo[a]pyrene-suppressed transcript. Previous studies from other laboratories have suggested a role for the SMOC-2-related protein SPARC in regulation of angiogenesis (14–16). Therefore, in studies presented here we have examined the effect of SMOC-2 on growth factor-induced mitogenesis and angiogenesis of endothelial cells in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials—Myc tag antibody was purchased from Cell Signaling. CD31 antibody was purchased from BD Pharmingen. α-Tubulin antibody was purchased from Oncogene (Cambridge, MA). Recombinant human VEGF-165 and bFGF were purchased from R&D Systems. Matrigel (Cytoskeleton) was purchased from Cambrex. Small inhibitor RNAs (siRNAs) were purchased from Dharmacon Inc. for a control non-targeting sequence (siCTR, cat. # D-001210-01), and two SMOC-2 targeting sequences, siSMOC-2–1 (sense: 5′-GAGAGUGGAUCAAGAUAADtTdT′-3′; antisense: 5′-UUUA-UUCUGAUAUCCACUCUdTdT′-3′) and siSMOC-2–2 (sense: 5′-CAAUUCCAUCUCGUAACAAdTdT′-3′; antisense: 5′-UGU-UACGGGAUGGAUUGdTdT′-3′) and used according to the manufacturer’s specifications.

Adenovirus Construction and Cell Culture—Replication-deficient adenovirus vectors were produced using a method originally established by Becker et al. (28). Briefly, SMOC-2 cDNA was cloned into the multicloning site of pACCMVpLPa plasmid. pACCMVpLPa-SMOC-2 was co-transfected with pM17 plasmid into 293 cells to allow for homologous recombination to produce the SMOC-2 adenovirus (Ad-SMOC-2). The Myc-tagged SMOC-2 (Ad-Myc-SMOC-2) was constructed by the same protocol but using a vector that contained a Myc tag upstream from the SMOC-2 insert. Control adenoviruses were used that expressed either green fluorescent protein (Ad-GFP) or β-galactosidase (Ad-βgal) under the control of a CMV promoter. Human umbilical vein endothelial cell (HUVEC) cultures infected for 24 h with Ad-GFP at a multiplicity of infection of 10 achieved 90% transduction efficiency (data not shown).

Pooled HUVECs were purchased from BD Bioscience Inc. and maintained according to the manufacturer’s instructions in endothelial cell growth medium-2 (EGM-2). Cells were used in the fourth to fifth cell passages.

Western Blot Analysis—HUVECs grown to 70% confluency on 100-mm dishes were infected with Ad-GFP or Ad-Myc-SMOC-2 and incubated for 24 h. Medium was removed and replaced with 3 ml of fresh EGM-2, and cultures were harvested 24 h later. Proteins were separated by SDS-PAGE as described previously (29). Membranes were incubated with the designated primary antibody (1:1000 for all antibodies) overnight at 4 °C. Bound primary antibody was reacted with anti-rabbit peroxidase-conjugated IgG or anti-mouse peroxidase-conjugated Fab fragments for 1 h and detected by chemiluminescence, according to the manufacturer’s recommendations (Roche Applied Science). Densitometric analyses were made by using the Scion Image computer program (Scion Corp., Frederick, MD).

SMOC-2 Antibody Production and Immunofluorescence—Genemed Synthesis, Inc. (San Francisco, CA) was contacted to produce a SMOC-2 antibody. A synthetic peptide sequence (C-YPTLWTEQVKSRQNK) corresponding to the SMOC-2-specific domain (27) was conjugated to KLH: keyhole limpet hemocyanin and used for immunizations.

Immunofluorescence was performed by culturing HUVECs in 4-well chamber slides to confluence. Cells were transduced with adenovirus made with an empty vector (Ad-Con) or Ad-Myc-SMOC-2 for 48 h. Cells were fixed with 4% paraformaldehyde for 10 min and then permeabilized with 0.2% Triton X-100 for 5 min. Following incubation with anti-SMOC-2 antibody, bound primary antibody was detected with fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody. After washing, the slides were DAPI-stained and mounted with Vectashield solution (Vector Laboratories). Slides were imaged and analyzed using a Delta Vision Image Restoration Microscopy System (dv1301421, Applied Precision).

Microarray Analysis—HUVECs were transduced with Ad-SMOC-2 or Ad-GFP for 48 h. RNA was purified from the HUVECs using a TRIzol kit (Invitrogen). RNA samples were submitted to the microarray core facility at The Department of Genetics and Genomics, Boston University School of Medicine for labeling, hybridization to Affymetrix chips, and data analysis.

Cell Cycle Analysis—Cell cycle distribution of DNA content was determined by flow cytometry as described previously (30). Serum-starved cells pulsed with 10 μM BrdUrd (Roche Applied Science) for 1 h were harvested with trypsin-EDTA, fixed in 65% Dulbecco’s modified Eagle’s medium/35% ethanol for 1 h at 4 °C, and resuspended in 1 ml of 2 M HCl for 20 min at room temperature. Cells were labeled with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (BD Pharmingen #33284X) using standard laboratory procedures. The labeled cells were analyzed for propidium iodide and BrdUrd staining on a BD Biosciences flow cytometer using CellQuest software.

DNA Synthesis Assays—HUVECs were grown in 6-well plates until cells reached 70–80% confluency. Controls and experimental conditions were done in triplicate. The medium was changed to endothelial basal medium-2 (EBM-2), and the
cells were infected with adenovirus using multiplicity of infection (m.o.i.) values of 10 and 50. 24 h later, the medium was changed to fresh EBM-2 and either VEGF or bFGF was added at the indicated doses. After 20 h, the medium was changed to 750 μl of EBM-2 containing 4 μCi/ml [3H]thymidine (PerkinElmer Life Sciences). The HUVECs were labeled for 4 h at 37 °C. Following washing and precipitation with ice-cold 10% trichloroacetic acid, the cells were dissolved in 500 μl of 0.1 M NaOH overnight at 4 °C. The amount of radioactivity in each sample was counted in duplicate using 100 μl of the dissolved cell sample and Ecolite scintillation fluid (ICN). Data were presented relative to Ad-GFP-transduced cells at an m.o.i. of 10 with no growth factors.

Cell Proliferation Studies—HUVECs were plated at 10,000 cells/cm² on 24-well plates. Culture medium was changed every day during the time course. Cells were allowed to adhere; the medium was changed and then transduced with adenoviruses for 24 h. For experiments involving siRNAs, subconfluent HUVECs were transfected with siRNAs according to the manufacturer’s instructions. After 24 h, cultures were trypsinized and plated at 10,000 cells/cm² on 24-well plates. This was designated as day 0. A hemocytometer was utilized to count the number of cells per well after adding 100 μl of trypsin-EDTA per well, and cell viability was determined by trypan blue exclusion.

Migration Assays—Cell migration was assayed using a modified Boyden chamber (ChemoTx® plate, Neuroprobe, Inc., Gaithersburg, MD) according to the manufacturer’s instructions. HUVECs were infected with adenoviral vectors overnight in EGM-2 and then labeled with Dil-labeled acetylated low density lipoprotein (10 μg/ml) for 4 h in EBM-2. Cells were then trypsinized and resuspended in phenol red-free EBM at 10,000 cells/25 μl. VEGF or bFGF in phenol red-free EBM was added into the lower chamber. Each experiment was performed in triplicate, and three separate experiments were performed in each experimental group.

Cellular migration was also measured using a wounding assay. A grid pattern was drawn on the underside of 6-well plates before HUVECs were plated on them to serve as landmarks for the start of the migration period. HUVECs were grown to confluence and allowed to remain so for a further 24 h. Cultures were then infected for 24 h with adenovirus or were transfected with siRNAs according to the manufacturer’s instructions for 24 h. A cell scraper was used to wipe away the cell monolayer on one side of the start line that had been drawn on the bottom of the plate. Images were captured with a video graphic system (DEI-750 CE Digital Output Camera, Optronics, Goleta, CA) at 4× magnification, and the same areas were photographed at 24 and 48 h with the assistance of the landmarks drawn on the undersurface of the plate. Several fields of view were captured per well, and experiments were repeated three times. Migration was quantified by measuring the area of the cell migration front as they migrated into the scraped area at each time point. Area was quantified using Scion Image analysis software.

In Vitro Cell-network Formation Assay—The formation of network-like structures by HUVECs on Matrigel® (BD Biosciences) was performed as previously described (31). The 12-well culture plates were coated with Matrigel® according to the manufacturer’s instructions. The adenovirus-transduced or siRNA-transfected HUVECs were seeded on coated plates at 3 x 10⁵ cells/cm² in EGM containing 0%, 0.5%, or 5% fetal bovine serum and incubated at 37 °C for overnight. Cells were observed using an inverted phase-contrast microscope (Nikon). Images were captured with a video graphic system (DEI-750 CE Digital Output Camera, Optronics, Goleta, CA).

The degree of network formation was quantified by capturing five high power field images, and the area of the cells was quantified using Scion Corp. (National Institutes of Health Image) area analysis software.

Mouse Angiogenesis Assay—The formation of new vessels in vivo was evaluated by the Matrigel® plug assay (BD Biosciences) employing a modification of the procedures described previously (32). Prior to injection, 0.5 ml of Matrigel® was mixed with heparin (10 units/ml) in chilled tubes. bFGF (50 ng/ml, R&D Systems) was added to the indicated tubes. Adenoviral vectors encoding GFP or SMOC-2 were added to a final concentration of 2 x 10⁶ plaque forming units of virus. Tissues were sectioned, and immunohistochemistry was performed with an anti-CD31 antibody (platelet endothelial cell adhesion molecule-1, 1:100 dilution, BD Pharmingen). Bound primary was detected with biotinylated rabbit anti-rat IgG antibody (1:200 dilution, Vector) and the ABC Elite kit from Vector Laboratories using DAB. The sections were counterstained with Harris’ hematoxylin. Images were captured using an Olympus BX41 microscope with color digital video camera, and an angiogenic response was quantified by cell counts of CD31-positive cells from 10 high power fields per section.

Statistical Analysis—All data were compared by Student’s t test or analysis of variance with the Scheffé post-hoc test using StatView 4.5 (Abacus Software, Burlington, MA). Data are expressed as mean ± S.E. for the number of independent experiments indicated. A p value of <0.05 was considered to be significant.

RESULTS

Cellular Localization of Overexpressed SMOC-2 in HUVECs—To analyze the effects of SMOC-2 growth regulation of endothelial cells we constructed adenoviral vectors for expressing SMOC-2 (Ad-SMOC-2) and an Myc-tagged SMOC-2 (Ad-Myc-SMOC-2). HUVECs were transduced with the Ad-Myc-SMOC-2 virus or a control adenoviral vector expressing green fluorescent protein (Ad-GFP). Lysates from AdGFP and Ad-Myc-SMOC-2-transduced HUVECs were subjected to Western blot analysis using anti-c-Myc antibody (Fig. 1A). Cell extracts from the Ad-Myc-SMOC-2-transduced cells showed an intense anti-c-Myc-reactive band of 56 kDa, which corresponded to the predicted size of SMOC-2 fusion protein (Fig. 1A, lane 2) (27). SMOC-2 fusion protein was also detected in the culture medium, which was expected, because SMOC-2 does contain a signal peptide for secretion (27) (Fig. 1A, lane 4). Endogenous levels of SMOC-2 transcripts were measured by real-time PCR in serum-deprived HUVECs and HUVECs in complete growth medium. As shown in Fig. 1B, serum deprivation led to an 18-fold increase in SMOC-2 mRNA levels.

We also performed immunofluorescence microscopy to determine the subcellular distribution of SMOC-2. HUVECs
were infected with a control "empty" vector adenovirus (Ad-Con) or Ad-Myc-SMOC-2. The resulting cells were fixed and stained with a c-Myc monoclonal antibody or with rabbit anti-SMOC-2 antisera. The specificity of the anti-SMOC-2 antibody was tested in Rat1 cells, which express low endogenous SMOC-2. Antibody binding to the adenovirus-encoded protein could be inhibited by addition of excess immunizing peptide (data not shown). As shown in Fig. 2A, the SMOC-2 antisera detected a weak signal in the cellular periphery of Ad-Con-infected HUVECs. In contrast, in Ad-Myc-SMOC-2-infected cells we detected very strong staining in the cell periphery and some diffuse staining within the cells. We also performed co-staining experiments in which Ad-Myc-SMOC-2-infected cells were stained with anti-SMOC-2 and anti-c-Myc antisera. As shown in Fig. 2B, the merged images showed good overlap between Myc (red) and SMOC-2 (green) signals indicating that the staining pattern was specific for SMOC-2. Taken together, our immunoblotting and immunofluorescence experiments demonstrate that SMOC-2 is predominantly localized to the cellular periphery and that a fraction is secreted from cells. This expression pattern is similar to that reported by other workers for the SMOC-2-related factor SPARC (33) in that SMOC-2 appears to associate with edges of the cell membrane. This would be consistent with a putative role for SMOC-2 as a regulator of extracellular matrix interactions and/or growth factor receptor signaling.

**Regulation of Cell Cycle-related Transcript by SMOC-2**—Because SMOC-2 has similarity to the angiogenic regulator and growth factor-binding protein SPARC, we performed a microarray screen to identify mRNAs that are differentially expressed in HUVECs transduced with Ad-SMOC-2 and Ad-GFP control virus. HUVECs were serum-starved for 24 h in EBM-2 with 0.5% FBS and then transduced with adenovirus for 24 h. RNA samples from transduced cells were subjected to labeling and hybridization to Affymetrix chips containing arrayed oligonucleotides corresponding to the human "transcriptome." A partial list of the mRNAs that were differentially expressed in Ad-GFP and Ad-SMOC-2-infected cells is presented in Table 1. From the full list it was apparent that numerous cell cycle-related mRNAs were up-regulated in SMOC-2-expressing cells. These include transcripts encoding MCM4 and MCM10 (DNA replication factors required for "licensing" and "initiation" steps of DNA synthesis, respectively), Aurora B kinase (required for G2/M progression), the centromeric protein CENP-F (required for kinetochore assembly and anaphase progression), and Cdc2 cyclin-dependent kinase band its activating partner Cyclin B (required for entry into mitosis). In addition, Rap1a, an inhibitor of proliferation and a regulator of cell adhesion, was down-regulated. Histone-encoding mRNAs (involved in chromatin assembly during DNA synthesis) were also among the most highly induced transcripts. Therefore, SMOC-2 expression increased expression of genes involved in progression through all phases of the cell cycle. Table 1 lists selected transcripts of interest that are involved with promoting cell cycle progression and cell growth.

Interestingly, thrombospondin 1, a matricellular protein known to inhibit angiogenesis and cell proliferation, was down-regulated by SMOC-2 overexpression. Considering that SMOC-2 is also a matricellular protein, but it increased expression of numerous transcripts that promote cell cycle progression, we sought to determine its effect on proliferation and angiogenesis of HUVECs.
Overexpressed SMOC-2 Stimulates Mitogenesis in HUVECs—

Mitogenesis is an important and necessary component of the angiogenic response. To test whether SMOC-2 promotes cell cycle progression, cell cycle analysis was performed by measuring BrdUrd incorporation using fluorescence-activated cell sorting. Serum-deprived HUVECs were infected with Ad-GFP control virus or Ad-SMOC-2 for 48 h in medium containing 0.5% serum. As shown in Fig. 3A, Ad-SMOC-2-transduced cells displayed a 13-fold increase in the S-phase population, consistent with the data obtained from microarray analysis.

We also examined the effect of Ad-Myc-SMOC-2 on HUVECs mitogenesis by analyzing \(^{3}H\)thymidine incorporation. HUVECs were transduced with Ad-GFP or Ad-SMOC-2, then serum-starved for 24 h to induce cell cycle exit. The growth-arrested cultures of control or SMOC-2-expressing HUVECs were then treated with or without VEGF or bFGF for 24 h in the presence of \(^{3}H\)thymidine to measure relative rates of DNA synthesis under different experimental conditions. HUVECs transduced with Ad-SMOC-2 showed a dose-dependent increase in \(^{3}H\)thymidine incorporation in response to VEGF and bFGF without any significant effect from increased adenovirus load (Fig. 3, B and C, white bars). Interestingly, without growth factor treatment, Ad-SMOC-2-transduced cells showed a 2- to 3-fold increase in \(^{3}H\)thymidine incorporation compared with Ad-GFP at an m.o.i. of 10 (Fig. 3, B and C, gray bars). At a higher m.o.i. of 50, an even greater increase was observed ranging from 9- to 28-fold as shown in the representative experiments in Fig. 3. Experiments were repeated three times with similar results. Treatment of the Ad-SMOC-2 cultures with VEGF and bFGF produced even greater \(^{3}H\)thymidine incorporation that was further stimulated with higher doses of growth factor. With Ad-GFP at an m.o.i. of 10 the -fold change in \(^{3}H\)thymidine incorporation between 0 and 50 ng/ml VEGF was 5, whereas with Ad-SMOC-2 under the same m.o.i. and growth factor concentrations the change was 3-fold. However, \(^{3}H\)thymidine incorporation is at a much higher level at baseline with Ad-SMOC-2. With bFGF, Ad-GFP at an m.o.i. of 10 there was a 3.5-fold increase between 0 and 10 ng/ml bFGF, whereas with Ad-SMOC-2 at the same m.o.i. and growth factor concentrations the change was 2-fold. However, again, \(^{3}H\)thymidine incorporation is at a much higher level at baseline with Ad-SMOC-2. The effect of Ad-SMOC-2 on HUVEC proliferation was confirmed by cell counts, which are shown in Fig. 3D. HUVEC cultures that were transduced with Ad-SMOC-2 displayed a higher proliferation rate as indicated by the higher number of cells present during culture over 4 days in the presence of 0.5% FBS. This difference between Ad-SMOC-2- and Ad-GFP-transduced cells was more pronounced in the presence of 5% FBS.

The role of endogenous SMOC-2 in DNA synthesis was examined using siRNA. Our SMOC-2 antibody did not detect the low endogenous levels of SMOC-2 in HUVECs (data not shown). Therefore, the effectiveness of our siRNA strategy was determined by Western blotting of lysates from HUVECs expressing ectopic hSMOC-2 under the transcriptional control of a CMV promoter. As shown in Fig. 3E, compared with siCTR, siRNAs targeting SMOC-2 elicited a 13-fold decrease in SMOC-2 protein levels with siSMOC-2-1 and a 33-fold decrease with siSMOC-2-2. Therefore, our siRNA strategy was effective for ablating SMOC-2 protein expression.

Fig. 3F shows the effect of siRNA-mediated SMOC-2 ablation on DNA synthesis. SMOC-2 siRNA displayed a trend toward decreased basal rates of DNA synthesis, but this was not statistically significant. However, the mitogenic response to 10 ng/ml bFGF showed a statistically significant decrease (1.6-fold
decrease, \( n = 3, p < 0.01 \) in siSMOC-2-transfected cells. siRNA duplexes targeting different sequences in the SMOC-2 mRNA elicited similar inhibitory effects on DNA synthesis (data not shown).

To determine if the effect of SMOC-2 ablation on DNA synthesis translated into significant effects on cell proliferation we performed cell counts. As shown in Fig. 3G, siSMOC-2-transfected HUVECs showed modestly reduced rates of growth compared with siCTR-transfected cells.

Taken together, these data demonstrate that SMOC-2 elicits a strong mitogenic response in growth-arrested HUVECs and potentiates the mitogenic response to angiogenic growth factors (bFGF and VEGF). Because mitogenesis is a critical component of the angiogenic response in HUVECs, these data suggested a possible role for SMOC-2 as a novel angiogenic regulator. Additional experiments described below were performed to test for potential angiogenic activity of SMOC-2.

**Ad-Myc-SMOC-2 Stimulates HUVEC Migration**—Angiogenesis requires the migration of endothelial cells to the sites of new capillary formation in ischemic tissues, and cellular migration in vitro is an indicator of the angiogenic potential of a factor. Therefore, we determined the effect of SMOC-2 on migration of HUVECs using two assays; a modified Boyden chamber assay using bFGF as a chemoattractant and a cell monolayer-wounding assay. In the Boyden chamber assays, bFGF increased the number of migratory cells in a dose-dependent manner up to 1.5-fold at 100 ng/ml bFGF in control cells transduced with an adenoarial vector expressing \( \beta \)-galactosidase.
Transduction with Ad-SMOC-2 significantly increased the amount of migratory cells at the higher doses of bFGF (3.6-fold at 50 ng/ml bFGF) but not at lower doses of bFGF or in the absence of bFGF.

The effect of Ad-SMOC-2 on HUVEC migration was also examined in a wounding assay performed in the presence of EGM-2 with and without 5% FBS. Ad-SMOC-2-transduced HUVECs displayed a greater capacity to migrate into the wounded area in the absence of serum compared with control cells at 24 h (1.8-fold higher) and 48 h (1.9-fold higher) following wounding of the cell monolayer (Fig. 4B). With the addition of 5% FBS, Ad-SMOC-2-transduced cells migrated 2.2-fold higher at 24 and 48 h compared with Ad-GFP-transduced control cells.

SMOC-2 Stimulates Angiogenic Activity—In vitro angiogenesis assays and in vivo Matrigel plug invasion assays were used to test the angiogenic potential of SMOC-2. HUVECs grown to 70–80% confluency were placed in EBM-2 and transduced with viruses at 50 m.o.i. After 24 h, the cells were gently trypsinized and plated onto Matrigel-coated plates with the indicated concentration of FBS in EBM-2. Unstimulated HUVECs transduced with Ad-Myc-SMOC-2 showed more projections compared with Ad-GFP-transduced HUVECs (Fig. 5, top two micrographs). The addition of FBS increased the number of projections in Ad-GFP-transduced cells with increasing concentration of FBS (Fig. 5, left-hand micrographs). The addition of FBS to the Ad-SMOC-2-transduced cells also increased the number of projections with increased FBS concentrations (Fig. 5, right-hand micrographs), but there appeared to be more projections in these cells compared with the Ad-GFP-transduced cells at the same FBS concentration. Quantification of angiogenic activity by measuring cell-network area in multiple micrographs showed that Ad-SMOC-2-transduced HUVECs displayed a 1.5-fold increase in cell-network area compared with Ad-GFP-transduced cells in the absence of serum (Fig. 5B). With the addition of 0.5% FBS, Ad-SMOC-2-transduced cells produced 1.6-fold more networks, and with 5% FBS, 1.7-fold more. We also determined the effects of SMOC-2 ablation on network formation. As shown in Fig. 5C, SMOC-2 siRNA did not significantly inhibit network formation in unstimulated HUVECs. However, SMOC-2 ablation inhibited serum-induced network formation by 25% in the presence of 0.5% serum and by 36% in the presence of 5% serum. Thus, HUVECs require SMOC-2 for efficient serum-induced network formation in vitro.

A subcutaneous Matrigel plug assay was employed in mice to directly test whether SMOC-2 can promote angiogenesis in vivo (34). Adenoviral vectors (2 × 10⁸ plaque-forming units) were incorporated in the Matrigel plugs with or without 50 ng/ml bFGF prior to subcutaneous implantation in the abdomens of C57BL/6 mice for 10 days. In this assay, the Matrigel serves as a reservoir for the viral vector and cells that infiltrate the plug become transduced and express the transgene. Endothelial cell infiltration of these plugs was assessed by immunohistochemical analysis of CD31-positive cells. The presence of Ad-SMOC-2 lead to the recruitment of 3-fold more CD31-pos-
positive cells into the Matrigel® plug compared with Ad-GFP in the absence of bFGF (Fig. 6, top two micrographs and bottom graph). The addition of 50 ng/ml bFGF increased the number of CD31-positive cells in the plugs containing Ad-GFP by 1.5-fold (Fig. 6, lower left micrograph and bottom graph). However, the addition of bFGF to the plugs containing Ad-Myc-SMOC-2 increased the number of CD31-positive cells by 3.3-fold compared with Ad-GFP-containing plugs with the same concentration of bFGF. The cells found in the Ad-SMOC-2-containing plugs with bFGF appeared to form large circular clusters that are characteristic of vessels (Fig. 6, lower right micrograph).

Taken together, our results show that SMOC-2 increases basal as well as growth factor-induced angiogenic activity in cultured cells and in vivo.

**DISCUSSION**

Based on the similarity between SMOC-2 and SPARC, a known growth factor-binding protein and angiogenic regulator, we have tested a role for SMOC-2 in regulation of angiogenesis. We show here that overexpressed SMOC-2 is modestly mitogenic in endothelial cells but elicits strong mitogenic response when combined with bFGF or VEGF. Additionally, SMOC-2 overexpression induces migration and cellular network formation by endothelial cells in vitro and invasion of CD31-positive endothelial cells as measured using an in vivo
It is possible that SMOC-2 binds directly to FGF or VEGF, thereby recruiting angiogenic factors to the cell surface and creating locally high concentrations of ligand near receptor sites. Alternatively, it is possible that SMOC-2 facilitates interactions between angiogenic factors and their receptors, perhaps via formation of ternary ligand/SMOC-2/receptor complexes. Another possibility is that there exists a cell surface receptor for SMOC-2 that when activated generates signals that synergize with FGF- or VEGF-induced angiogenic pathways. These putative mechanisms are not necessarily mutually exclusive and are similar to ones proposed for regulation of growth factor signaling by the SMOC-2-related matricellular proteins such as SPARC, which is also secreted and binds to VEGF and other growth factors (10, 11), as well as follistatin, which binds activin (36). Furthermore, other follistatin-related proteins that have one or more follistatin-like domains have been shown to bind members of the transforming growth factor family (37). Therefore, it is possible that SMOC-2 elicits its effects through modulation of growth factor activities via its follistatin-like domains.

Proteins related to SMOC-2 have been shown to modulate angiogenesis. SPARC suppresses angiogenesis as evidenced in the SPARC-null mice that show increased angiogenic activity in sponge cell invasion assays (14). The angiogenic activity of SPARC is complicated, though, by the fact that different proteolytic fragments show differing effects. An epidermal growth factor-like module of the follistatin-like domain in SPARC has been shown to be angiostatic (38). In addition, another fragment of this domain in SPARC consists of a KGHK amino acid sequence that is pro-angiogenic (8, 15). Nevertheless, it is possible that SMOC-2 exerts its effects via the follistatin-like domain, which has been shown to have growth factor binding potential.

There is a strong precedent for down-regulation of components of growth factor signaling pathways following mitogenic stimulation. For example we showed that PDGFβ receptor (PDGFβR) expression is elevated in quiescent cells and is transcriptionally down-regulated concomitantly with cell cycle re-entry (39). It appears that PDGFβR induction during quiescence “primes” growth-arrested cells for stimulation by PDGF, and that PDGFβR down-regulation during cell cycle entry represents a desensitization mechanism to prevent excessive or aberrant PDGF signaling. In our microarray data, we observed that overexpression of SMOC-2 up-regulates the expression of numerous factors that promote cell cycle progression in growth factor-starved cells. Essentially, SMOC-2 may also prime cells during quiescence and may serve to sensitize cells to extracellular mitogens and facilitate proliferation.

Many growth factors and their receptors (including PDGF ligands and receptors) are aberrantly overexpressed and contribute to growth deregulation in proliferative diseases such as cancer. Potentially, aberrant expression of SMOC-2 could confer increased sensitivity to growth factors, thereby promoting hyperproliferation and migration of tumor cells. Therefore, it will be interesting to determine if SMOC-2 is overexpressed in cancers and contributes to tumorigenesis.

In conclusion, we have demonstrated that SMOC-2, a novel SPARC-related matricellular protein, can stimulate endothelial cell proliferation, migration, as well as angiogenic activity in vitro and in vivo. Endothelial cells play a critical role in angiogenesis, a physiological or pathological neovascularization process in response to tissue ischemia and tumor growth or metastasis. Clearly, the interaction between endothelial cells and the extracellular matrix can greatly influence angiogenesis. Therefore, SMOC-2 may serve as a novel target for controlling angiogenesis in clinical scenarios such as tumor growth and myocardial ischemia. Experiments are underway to identify SMOC-2-binding partners that might represent components of growth factor signaling pathways regulated by SMOC-2.

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