Altered Angiogenesis in Caveolin-1 Gene–Deficient Mice Is Restored by Ablation of Endothelial Nitric Oxide Synthase

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Caveolin-1 is an essential structural protein of caveolae, specialized plasma membrane organelles highly abundant in endothelial cells, where they regulate multiple functions including angiogenesis. Caveolin-1 exerts a tonic inhibition of endothelial nitric oxide synthase (eNOS) activity. Accordingly, caveolin-1 gene–disrupted mice have enhanced eNOS activity as well as increased systemic nitric oxide (NO) levels. We hypothesized that excess eNOS activity, secondary to caveolin deficiency, would mediate the decreased angiogenesis observed in caveolin-1 gene–disrupted mice. We tested tumor angiogenesis in mice lacking either one or both proteins, using *in vitro*, *ex vivo*, and *in vivo* assays. We show that endothelial cell migration, tube formation, cell sprouting from aortic rings, tumor growth, and angiogenesis are all significantly impaired in both caveolin-1–null and eNOS–null mice. We further show that these parameters were either partially or fully restored in double knockout mice that lack both caveolin-1 and eNOS. Furthermore, the effects of genetic ablation of eNOS are mimicked by the administration of the NOS inhibitor *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME), including the reversal of the caveolin-1–null mouse angiogenic phenotype. This study is the first to demonstrate the detrimental effects of unregulated eNOS activity on angiogenesis, and shows that impaired tumor angiogenesis in caveolin-1–null mice is, at least in part, the result of enhanced eNOS activity. (Am J Pathol 2012, 180:1702–1714; DOI: 10.1016/j.ajpath.2011.12.018)

Caveolae are specialized plasma membrane subdomains that are highly abundant in terminally differentiated cells including endothelial cell (EC).¹ The formation and functions of these organelles require caveolin-1, a protein that structurally supports caveola biogenesis and interacts with multiple signaling molecules, directly regulating their activity. In the endothelium, one such signaling protein is endothelial nitric oxide (eNOS), and the role of caveolin-1 in regulating eNOS activity has been abundantly studied *in vitro*^{2–6} and *in vivo*.^{7,8} In addition, since the development of caveolin-1 gene–disrupted mice 10 years ago, much attention has focused on the ability of caveolin-1 and caveolae to regulate endothelial cell physiology, as various aspects of the phenotype of these mice were related to alteration of endothelial-dependent functions.^{9,10} Indeed, by performing trafficking and signaling functions, caveolae regulate endothelial barrier function, vasomotor activity, vascular homeostasis, and angiogenesis.

The literature documenting the role of caveolin-1 in angiogenesis shows a picture more complex than initially thought (reviewed by Parat¹¹). On one hand, caveolin-1 appears to restrain angiogenesis, as shown by *in vitro* and *in vivo* experiments. Overexpressing caveolin-1 in cultured EC decreased their migration and *in vitro* capillary-like tube formation.¹² *In vivo* caveolin-1 gene delivery to angiogenic EC resulted in EC caveolin-1 overexpression and inhibition of angiogenesis in wild-type mice.¹² In mouse models where angiogenesis was stimulated by ischemia or an adenovirus encoding vascular endothelium growth factor (VEGF), endothelial-specific transgenesis of the caveolin-1 gene decreased neovascularization.¹³ In agreement with these results, siRNA-mediated down-regulation of caveolin-1 increased EC transmigration *in vitro*,¹⁴ and caveolin-1 expression was down-regulated *in vitro* and *in vivo* in EC

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subjected to angiogenic conditions.^{15,16} Similarly, the number of caveolae in the EC of capillaries regressing on anti-VEGF treatment increases dramatically.¹⁷ Finally, increased tumor angiogenesis has been documented in caveolin-1 gene-disrupted mice in two studies.^{18,19}

On the other hand, caveolin-1 seems to be necessary for angiogenesis. Caveolin-1-null mice show impaired angiogenesis elicited by different stimuli^{20–23} and cells derived from these mice exhibit reduced capillary-like tube formation *in vitro*.^{20,21} Adenoviral-mediated caveolin-1 overexpression increased *in vitro* tube formation.¹⁵ Accordingly, down-regulation of caveolin-1 reduced EC transmigration to serum,²⁴ capillary like tube formation^{15,25} and angiogenesis in the chorioallantoic membrane assay.²⁵ These discrepant results suggest an ambiguous role for caveolin-1. It has been proposed that an optimal, physiological amount of caveolin-1 promotes normal angiogenesis, whereas excess or lack of caveolin-1 impairs angiogenesis.²¹

One major mechanism by which caveolin-1 and caveolae regulate angiogenesis involves endothelial production of nitric oxide (NO), a signaling molecule key to the angiogenic process.²⁶ The regulation by caveolin-1 and caveolae of endothelial nitric oxide production occurs at multiple levels; while in unstimulated conditions, eNOS is maintained in an inactive state by interaction with the scaffolding domain of caveolin-1, activation requires the compartmentalization in caveolae of eNOS as well as upstream and downstream players of the pathway leading to nitric oxide production.^{27–29} In addition, caveola internalization has been shown to further regulate eNOS activation.^{30,31} Therefore, caveolin-1 is a tonic inhibitor of eNOS but is required for caveola formation, which in turn is necessary for signaling cascades leading to eNOS activation and NO production. The direct inhibitory effect of caveolin-1 on eNOS has been taken advantage of by two *in vivo* approaches, both successful, to inhibit tumor angiogenesis: overexpression of caveolin-1 in angiogenic EC,¹² and delivery of a peptide comprising the domain of caveolin-1 responsible for eNOS inhibition.⁸ In agreement with caveolin-1 being a tonic inhibitor of eNOS, caveolin-1-null mice display excessive NO production as a result of persistent eNOS activation.^{9,10,32,33}

In the present study, we hypothesized that the angiogenic phenotype of caveolin-1 gene-disrupted mice was imputable at least in part to excess basic NO production. To test this hypothesis, we quantified tumor angiogenesis in double knock-out mice lacking both eNOS and caveolin-1, compared with caveolin-1 single knock-out, eNOS single knock-out, and wild-type mice as controls. We validated our findings from this genetic approach by using a pharmacological inhibitor of NO production in wild-type and caveolin-1 gene-disrupted mice.

Materials and Methods

Materials

The following reagents were purchased from Gibco (Invitrogen, Carlsbad, CA): DMEM/F12, fetal bovine serum (FBS), penicillin/streptomycin, and type II collagenase.

The following items were obtained from Sigma-Aldrich (St. Louis, MO): hematoxylin, alcoholic eosin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), heparin, collagenase, fibronectin, collagen from rat tail, *N*^ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), Drabkin's reagent, hemoglobin, Brij's reagent, and dimethyl sulfoxide (DMSO). Human vascular endothelial growth factor (VEGF) was obtained from R&D Systems (Minneapolis, MN).

Animals

Caveolin-1-deficient (stock no. 004585) and eNOS-deficient (stock no. 002684) mice were obtained from Jackson Laboratories (Bar Harbor, ME). The experimental animals of the four genotypes of interest (Cav^{+/+}eNOS^{+/+}, Cav^{-/-}eNOS^{+/+}, Cav^{+/+}eNOS^{-/-}, Cav^{-/-}eNOS^{-/-}) were obtained through continuously renewed heterozygous matings to avoid selection of a background trait and to overcome poor breeding performance of the double knock-out mice. The Cav^{-/-}eNOS^{-/-} mice presented no obvious phenotypic alteration but were obtained at lower than Mendelian ratios. Experiments involving only caveolin-1 and wild-type mice were performed using littermates generated through simple heterozygous matings between Cav^{+/+}eNOS^{+/+} mice. All animal experiments were approved by the Animal Ethics Committee of the University of Queensland or the Animal Care and Use Committee guidelines of the Cleveland Clinic.

Isolation and Culture of Mouse Lung Endothelial Cells

Lungs were harvested in ice-cold DMEM/F12 medium supplemented with FBS (10%), penicillin 50 U/mL, and streptomycin 50 μg/mL. The lungs were cut into small pieces, suspended in medium, and centrifuged at 1200 rpm for 5 minutes at room temperature. The tissue fragments were cut into smaller pieces, suspended in medium containing 0.5 mg/mL of type II collagenase and incubated for 30 minutes at 37°C with frequent agitation. The digest was passed through a 100-μm-mesh sieve and then through a 70-μm-mesh sieve (Becton Dickinson, North Ryde, NSW, Australia). The cells were suspended in medium supplemented with VEGF (2 ng/mL) and heparin (2 μg/mL). Cells from each mouse were seeded onto a fibronectin-coated dish (2 μg/mL in phosphate buffered saline [PBS]). The cells were cultured in a humidified atmosphere of 95% air and 5% CO₂. Endothelial origin of the isolated cells was verified by uptake of 1,1'-dioctadecyl-3,3,3'-tetramethyl-indocarbocyanine perchlorate acetylated low-density lipoprotein (Dil-Ac-LDL), CD31 and von Willebrand factor (vWF) immunoreactivity. Cells from passage 2 were used for experiments.

Characterization of Mouse Lung Endothelial Cells

To test Dil-Ac-LDL uptake, mouse lung endothelial cells (MLEC) were seeded in eight-well chamber slides (Becton Dickinson). After overnight incubation, the culture

medium was replaced with fresh medium containing 10 $\mu\text{g/mL}$ of Dil- Ac-LDL (Invitrogen) and incubated for 6 hours. The cells were rinsed in PBS, fixed in 4% formaldehyde for 20 minutes, rinsed in PBS, and mounted in fluorescent mounting medium containing DAPI (Merck, Kilsyth, Australia). Immunostaining for CD31 and vWF were performed on cells similarly seeded in eight-well chamber slides. After overnight incubation, the cells were rinsed in PBS and fixed in 4% formaldehyde for 20 minutes. The cells were washed in PBS, permeabilized with 0.1% Triton X-100 for 10 minutes, washed in PBS, and blocked with 3% goat serum in PBS for 20 minutes. The cells were then incubated with monoclonal anti-mouse CD31 antibody (Sapphire Bioscience, Waterloo, Australia), or polyclonal anti-rabbit vWF, or rabbit or mouse nonimmune IgGs for 60 minutes. The antibodies were used at a concentration of 1 $\mu\text{g/mL}$ in PBS containing 1.5% goat serum. After washing in PBS, the cells were incubated for 45 minutes with respective secondary antibodies (1:1000) labeled with Alexa Fluor 488 (Invitrogen). Cells were washed in PBS, and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

Cell Proliferation Assay

The DNA content of cells was measured using the CyQUANT cell proliferation assay following the protocol of the manufacturer (Invitrogen). Briefly, 5×10^3 cells were seeded in 96-well black culture plates with optical bottom (Nalge Nunc International, NY) and grown for 48 hours. The culture medium was removed and the cells were incubated in 100 μL of serum-free medium or medium with 0.5% FBS for 24 hours. A 100- μL of CyQUANT reagent, prepared in culture medium, was added to the wells and incubated for a further 60 minutes. The fluorescence was measured using a microplate reader (FLUOstar Omega, BMG Laboratories, Victoria, Australia) with excitation at 480 nm and emission detection at 520 nm.

Transmigration Assay

The transmigration assay was performed using 48-well Boyden chambers as previously described.³⁴ Briefly, polycarbonate membranes with 8- μm pores (Neuro Probe, Gaithersburg, MD) were precoated overnight with rat tail collagen (100 $\mu\text{g/mL}$ in 0.2 N acetic acid), air dried, and used to separate the chambers. The bottom wells were prepared with serum-free medium or medium containing 0.5% FBS. After pH equilibration of the chambers in a humidified atmosphere of 95% air and 5% CO_2 , a suspension of 2×10^4 MLEC in 50 μL of serum-free medium was added to the upper wells. Migration was allowed to proceed for 4 hours. The membranes were washed in PBS, and the cells remaining in the upper surface of the membrane were scraped. The membranes were fixed in 10% buffered formalin for 20 minutes, stained with hematoxylin overnight, rinsed with water, and mounted in Permount mounting medium (Fisher Scientific, PA). Cells on the lower face of the membranes were counted under a microscope by an observer blinded to experimental conditions.

Tube Formation Assay

Wells of a 96-well plates were coated with 50 μL of Matrigel (BD Biosciences, North Ryde, NSW, Australia), and the matrigel was allowed to solidify at 37°C for 60 minutes. MLEC suspended in serum-free medium or in medium containing 0.5% FBS were seeded at a density of 2.5×10^4 cells onto the Matrigel. The organization of cells in tubes after 7 hours was documented by phase contrast micrographs. The number of tubes and the average tube length per field were determined.

Aortic Ring Assay

Aortas were harvested from mice, and the surrounding tissues were dissected out. Any residual blood clot was removed, and the aortas were sliced into approximately 1 mm-thick rings. The rings were embedded in a drop of Matrigel. Serum-free medium containing VEGF (2 or 20 ng/mL), or control serum-free medium was added to the dishes, to surround the Matrigel. The endothelial-driven cell sprouting from the rings is confirmed by lack of response in the absence of VEGF and the absence of outward cell migration after endothelial denudation of the rings. Radial outgrowth of cells from the rings was monitored by phase contrast microscopy and documented over a period of 7 days. The number of cells aligned in tubes and the number of tube-like structures were calculated using ImageJ software (National Institutes of Health, Bethesda, MD).

Mouse Corneal Micropocket Assay

Angiogenesis was tested *in vivo* using the corneal micropocket assay as previously described.³⁵ Hydron pellets containing 50 ng recombinant human VEGF were inserted into corneal micropockets (1 mm from limbus) of $\text{Cav}^{-/-}$ ($n = 6$) and control WT littermates ($n = 7$). Control buffer-containing pellets were similarly inserted in corneas of $\text{Cav}^{+/+}$ and control WT mice ($n = 4$ per group). Corneas were examined with a surgical microscope to monitor angiogenic responses to VEGF. Mice were euthanized 7 days after pellet insertion. To document the neovascular response, animals were perfused with India ink to label the vessels. After enucleation and fixation, the corneas were excised, flattened, and photographed. Angiogenic response was analyzed for mean vessel area using image processing software (Image-Pro Plus 6.1; Media Cybernetics).

In Vivo Tumor Angiogenesis

B16-F0 melanoma cells were suspended in ice-cold PBS at a density of 5×10^6 cells/mL. Five mice of each genotype were injected subcutaneously onto the right flank with 100 μL of the cell suspension. Food and water intake, body weight, and tumor growth of the animals were monitored throughout the experiment. For pharmacological NO inhibition experiments, the NOS inhibitor L-NAME was provided in the drinking water 48 hours before the injection of the cells at a concentration of 1 mg/mL for 24 hours, followed by 0.5 mg/mL for the remainder of the experiments. On day 13 after the injection of the cells, the animals were euthanized and the tumors were

excised and weighed. Tumor tissue was fixed in ice-cold 4% buffered formalin or frozen and stored at -80°C .

Hematoxylin and Eosin Staining

Fixed tumors, were washed in PBS, and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek, CA). Cryosections (5 μm) (Leica CM1850 cryostat) were stained with hematoxylin and eosin using standard procedures. The number of blood vessels per area (defined by the graticule of the eye piece, Leica 11527001) in each section, as a measure of neovascularization, was counted under a microscope.

Hemoglobin Measurements

To determine the hemoglobin (Hb) concentration of the tumor explants, equal amounts of tissue were suspended in distilled water and sonicated using a Sonics Vibra Cell

sonicator (Sonics & Materials, CT) for 10 seconds. The suspension was centrifuged at 13,500 rpm at 4°C for 15 minutes. To quantify the hemoglobin content, 50 μL of the tumor suspension was mixed with 950 μL of Drabkin's reagent and incubated in the dark at room temperature for 15 minutes. The absorbance was read at 450 nm in an iMark micro plate reader (Bio-Rad, Hercules, CA). Human hemoglobin was used as a standard. Results were expressed as milligrams of hemoglobin per milligram of tissue.

Statistical Analyses

Results are expressed as mean $\pm$ SEM. Comparisons between groups were performed by analysis of variance with Tukey's *post hoc* test using Prism software (Graph-Pad, San Diego, CA). $P < 0.05$ was considered significant. Where Barlett's test for equal variances showed the variances differed significantly between groups, the data were log transformed before statistical analysis, as indicated where appropriate.

Figure 1. Effect of loss of Cav-1 on *in vitro*, *ex vivo*, and *in vivo* angiogenesis. **A:** EC were tested for their ability to migrate in a modified Boyden chamber assay using serum-free medium or 0.5% serum containing medium. Results are expressed as percentage of the migration of Cav^{+/+} cells toward no serum medium. **B:** Tube formation was tested when EC isolated from Cav^{+/+} and Cav^{-/-} mice were plated on Matrigel. Representative pictures, number of tubes per field, and average tube length are shown and expressed as percentage of Cav^{+/+} cells. Scale bar = 400 μm . **C:** Representative micrographs of aortic ring explants placed in Matrigel surrounded by VEGF-containing, serum-free medium, and quantification of the number of cells aligned in tube-like structures and the number of tubes. Scale bar = 400 μm . **D:** Angiogenic response to VEGF in the corneal micropocket assay. Representative micrographs of angiogenic response to implants releasing buffer or VEGF in Cav^{+/+} and Cav^{-/-} mice. Scale bar = 600 μm . Bar graph shows quantification of vessel area per sample. * $P < 0.05$ and *** $P < 0.001$.

Results

VEGF-Induced Corneal Neovascularization Is Reduced in Caveolin-1-Null Mice

The migration of activated endothelial cells across the degraded basement membrane toward the tumor site is an essential step in angiogenesis. We subjected endothelial cells isolated from *Cav*^{+/+} or *Cav*^{-/-} mice to chemotactic transmigration, and showed that the ability of the *Cav*^{-/-} cells to respond to serum was greatly reduced compared with cells from *Cav*^{+/+} mice (Figure

1A). When seeded on Matrigel, the *Cav*^{-/-} cells also had a reduced ability to form capillary-like structures (Figure 1B). The ability of cells from endothelial-intact, freshly isolated vessels to proliferate and to migrate radially toward a gradient of VEGF is tested with the aortic ring assay. The number of outgrowing tube-like structures and the number of cells aligned in tube-like structures were significantly lower in rings explanted from *Cav*^{-/-} mice (Figure 1C). To test angiogenesis *in vivo*, hydron micropellets containing VEGF or buffer were implanted into the corneas of *Cav*^{+/+} and *Cav*^{-/-} mice, and neo-

Figure 2. Effect of loss of eNOS, Cav-1, or both eNOS and Cav-1 on *in vitro* angiogenesis. **A:** EC were tested for their ability to migrate in a modified Boyden chamber assay using serum-free medium or 0.5% serum-containing medium. Results are expressed as percentage of the migration of *Cav*^{+/+} *eNOS*^{+/+} cells toward no serum medium ($n = 3$). **B:** EC proliferation was tested using the CyQUANT DNA content assay, in serum-free medium and in medium containing 0.5% FBS ($n = 4$). **C:** Micrographs of EC tube formation on matrigel tested in serum-free medium and in medium containing 0.5% FBS. Scale bar = 400 μ m. Tube length (**D**) and tube numbers (**E**) were calculated from printed micrographs. ns, Not statistically significant. $**P < 0.01$ and $***P < 0.001$.

vascularization was assessed after 7 days. The response to VEGF was reduced in the Cav^{-/-} mice compared with their wild-type littermates, and this was confirmed by quantification of the vessel area (Figure 1D).

Decreased Endothelial Cell Migration and in Vitro Tube Formation in Caveolin-1 Gene-Disrupted Mice Are Restored by eNOS Genetic Ablation

We used a modified Boyden chamber assay to study the role of caveolin-1 and eNOS in endothelial cell transmigration. No significant difference in random migration (toward serum-free medium) was observed among the four genotypes (Figure 2A). Wild-type endothelial cells (Cav^{+/+}eNOS^{+/+}) were capable of chemotactic stimulation ($P < 0.001$, 0% FBS versus 0.5% FBS). This chemotactic response was lost in caveolin-1-null (Cav^{-/-}eNOS^{+/+}) and in eNOS-null (Cav^{+/+}eNOS^{-/-}) MLEC. However, when both caveolin-1 and eNOS were absent, transmigration was partially restored although this was not statistically significant (Figure 2A). To rule out the possibility that the observed differences in transmigration were the reflection of differential proliferation rates, we measured proliferation of all four genotypes. No significant differ-

ence in proliferation was observed, in unstimulated or serum-stimulated conditions, between cells from the four genotypes (Figure 2B), confirming that the observed difference in transmigration was independent of proliferation. Next we examined the effect of caveolin-1 and eNOS genetic ablation on the ability of MLEC to form capillary-like tubes when seeded onto Matrigel. In serum-free and serum-stimulated conditions, tube formation, as measured by tube length or tube number, was significantly impaired in Cav^{-/-}eNOS^{+/+} and Cav^{+/+}eNOS^{-/-} when compared to wild-type cells (Figure 2, C–E). In contrast, in Cav^{-/-}eNOS^{-/-} MLEC, tube formation was rescued, and increased when compared with either of the single knockouts, (Figure 2, C–E) and this effect was statistically significant in the presence of serum. In summary, the *in vitro* angiogenic potential of MLEC is impaired by genetic ablation of either caveolin-1 or eNOS, but partially or totally recovered when both proteins are absent.

Decreased ex Vivo Angiogenesis in Caveolin-1 Gene-Disrupted Mice Is Rescued by eNOS Genetic Ablation

The migration of cells and the sprouting of tube-like structures from the aortic rings in response to VEGF was

Figure 3. Effect of loss of eNOS, Cav-1, or both eNOS and Cav-1 on *ex vivo* angiogenesis. **A:** Representative micrographs of aortic ring explants placed in matrigel surrounded by serum-free medium or medium containing 2 or 20 ng/mL VEGF for 5 days. Scale bar = 400 μ m. **B:** Quantification of the number of cells aligned in tube-like structures sprouting in response to 20 ng/mL of VEGF. **C:** Quantification of the number of tube-like structures sprouting in response to 20 ng/mL of VEGF. * $P < 0.05$ and ** $P < 0.01$, Cav^{-/-}eNOS^{+/+} and Cav^{+/+}eNOS^{-/-} versus Cav^{+/+}eNOS^{+/+}. † $P < 0.05$ and †† $P < 0.01$, Cav^{-/-}eNOS^{+/+} and Cav^{+/+}eNOS^{-/-} versus Cav^{-/-}eNOS^{-/-}.

dose-dependent in Cav^{+/+}eNOS^{+/+} explants (Figure 3A). Quantification of the number of cells aligned in tubes (Figure 3B) and of the tube-like structures (Figure 3C) in response to 20 ng/mL VEGF at daily time points, showed a significant decrease of both parameters in Cav^{-/-}eNOS^{+/+} and Cav^{+/+}eNOS^{-/-} compared to Cav^{+/+}eNOS^{+/+} aortic rings. In Cav^{-/-}eNOS^{-/-} aortic rings, sprouting was restored in a statistically significant fashion, returning to the levels of outmigration seen with Cav^{+/+}eNOS^{+/+} rings (Figure 3, A–C). These *ex vivo* results therefore confirmed our *in vitro* data and indicate that genetic ablation of eNOS can compensate the angiogenic phenotype observed in caveolin-1-null mice.

Impaired Tumor Growth and Angiogenesis in Vivo in Caveolin-1 Gene-Disrupted Mice Are Partially Recovered in Cav^{-/-}eNOS^{-/-} Mice

To determine whether caveolin-1 and eNOS were similarly controlling *in vivo* angiogenesis, we performed a tumor angiogenesis assay with mice from each genotype. Tumor growth was markedly reduced in either Cav^{-/-}eNOS^{+/+} or Cav^{+/+}eNOS^{-/-} mice compared to Cav^{+/+}eNOS^{+/+} mice (Figure 4, A and B). However, tumor growth was significantly higher in Cav^{-/-}eNOS^{-/-} mice than in Cav^{-/-}eNOS^{+/+} or Cav^{+/+}eNOS^{-/-} mice (Figure 4, A and B). H&E-stained sections of tumor tissue

Figure 4. Effect of loss of eNOS, Cav-1, or both eNOS and Cav-1 on tumor growth and angiogenesis. **A:** Micrographs of representative tumors. Scale bar = 1 cm. **B:** Tumor weight 13 days after B16 cell implantation; *n* = 5. **C:** Representative H&E-stained sections of the tumors. Scale bar = 200 μ m. **D:** Vessel count of the tumor samples in five randomly selected areas, expressed as number of blood vessels per field. **E:** Hemoglobin content of the tumors. *P < 0.05, **P < 0.01, ***P < 0.001. ns, Not significant.

(Figure 4C) displayed a marked reduction in number of blood-containing vessels in tumors implanted either in Cav^{-/-}eNOS^{+/+} or in Cav^{+/+}eNOS^{-/-} mice (Figure 4D). Confirming our *in vitro* and *ex vivo* experiments, the reduction in blood vessel counts was rescued in tumors from Cav^{-/-}eNOS^{-/-} mice, where the number of vessels was increased compared to Cav^{-/-}eNOS^{+/+} or Cav^{+/+}eNOS^{-/-} (Figure 4D). Furthermore, the rescue as measured by the hemoglobin content of the tumors was also highly significant (Figure 4E).

Pharmacological NOS Inhibition Recapitulates Effects of eNOS Genetic Ablation on *in Vitro* and *ex Vivo* Angiogenesis Alterations Induced by Loss of Caveolin-1

To generate further evidence that excess NO mediates impaired angiogenesis in Caveolin-1-null mice, we per-

formed experiments using the NOS inhibitor L-NAME. MLEC were exposed to increasing concentrations of L-NAME for 24 hours and cell viability assessed. Concentrations of up to 5 μ mol/L L-NAME did not induce any changes in cell viability of MLEC (data not shown) and therefore, this concentration was used for further *in vitro* and *ex vivo* experiments. L-NAME did not induce any significant changes in random transmigration (toward serum-free medium) of Cav^{+/+}eNOS^{+/+} or Cav^{-/-}eNOS^{+/+} MLEC (Figure 5, A and B). However, L-NAME-treated Cav^{+/+}eNOS^{+/+} MLEC displayed a dramatic decrease in chemotactic migration (toward 0.5% FBS) compared to untreated Cav^{+/+}eNOS^{+/+} cells (Figure 4A). This confirms the key role played by nitric oxide production in EC chemotaxis. In contrast, L-NAME increased the chemotactic migration of Cav^{-/-}eNOS^{+/+} EC ($P < 0.001$, Cav^{-/-}eNOS^{+/+} – L-NAME versus Cav^{-/-}eNOS^{+/+} + L-NAME) (Figure 5B), in agreement with the data obtained with genetic ablation of eNOS.

Figure 5. Effect of the NOS inhibitor L-NAME on EC migration and *ex vivo* angiogenesis from wild-type and caveolin-1-null mice. **A:** Effect of L-NAME on transmigration of Cav^{+/+}eNOS^{+/+} MLEC. **B:** Effect of L-NAME on transmigration of Cav^{-/-}eNOS^{+/+} MLEC. ** $P < 0.01$; *** $P < 0.001$. ns, Not significant. **C:** Representative micrographs of aortic rings showing endothelial cell in response to 20 ng/mL of VEGF at day 7. Scale bar = 400 μ m. **D:** Quantification of the number of cells aligned in tube-like structures sprouting in response to 20 ng/mL of VEGF. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, cav^{-/-}-L-NAME versus cav^{+/+}-L-NAME. † $P < 0.05$, cav^{-/-}+L-NAME versus cav^{+/+}-L-NAME.

To study the ability of L-NAME to rescue the *ex vivo* angiogenesis impairment induced by loss of caveolin-1, aortic rings from Cav^{+/+}eNOS^{+/+} or Cav^{-/-}eNOS^{+/+} mice were embedded in Matrigel and subjected to serum-free medium containing 20 ng/mL VEGF, in the presence or absence of 5 μ mol/L L-NAME for up to 7 days. Representative micrographs of aortic rings at day 7 are shown in Figure 5C. L-NAME impaired the ability of EC from WT rings to respond to VEGF (Figure 5, D and E). However, L-NAME treatment restored EC sprouting in Cav^{-/-}eNOS^{+/+} aortic rings (Figure 5C). The number of Cav^{-/-}eNOS^{+/+} aligned cells (Figure 5D) and tube-like structures (Figure 5E) were increased at days 5 to 7 in L-NAME-treated Cav^{-/-}eNOS^{+/+} rings when compared

with untreated Cav^{-/-}eNOS^{+/+} rings, reaching statistical significance on day 7.

NOS Inhibition Decreases Tumor Growth and Angiogenesis in Cav^{+/+}eNOS^{+/+} Mice but Increases Them in Cav^{-/-}eNOS^{+/+} Mice

To test whether NOS inhibition could rescue the deficit in angiogenesis induced by the loss of caveolin-1 *in vivo*, we performed a tumor angiogenesis assay in wild-type or caveolin-1-null mice exposed to L-NAME. Representative tumors from Cav^{+/+}eNOS^{+/+} and Cav^{-/-}eNOS^{+/+} mice treated or not treated with L-NAME are shown in

Figure 6. Effect of the NOS inhibitor L-NAME on tumor growth and angiogenesis in Cav^{+/+}eNOS^{+/+} and Cav^{-/-}eNOS^{+/+} mice. L-NAME was given in the drinking water 48 hours before B16 cell implantation and for the duration of the assay. **A:** Representative micrographs of tumors from each group. Scale bar = 1 cm. **B:** Tumor weight 13 days after inoculation of the B16 cells ($n = 5$). For statistical analysis data, were log transformed, as Barlett's test showed significantly different variances. **C:** Representative H&E-stained sections. Scale bar = 200 μ m. **D:** Vessel count from H&E-stained sections ($n = 4$). **E:** Quantification of the hemoglobin content in tumor tissue ($n = 5$). Data were log transformed, as Barlett's test showed significantly different variances. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

Figure 6A. In response to L-NAME, tumor growth was significantly decreased in Cav^{+/+}eNOS^{+/+} mice (**Figure 6, A and B**). In contrast, L-NAME increased tumor growth in Cav^{-/-}eNOS^{+/+} mice (**Figure 6, A and B**). H&E-stained sections of tumors from L-NAME-treated Cav^{+/+}eNOS^{+/+} mice displayed a significant decrease in blood vessels when compared with tumors from control Cav^{+/+}eNOS^{+/+} mice (**Figure 6C**). In contrast, L-NAME dramatically increased the number of blood vessels in tumors from Cav^{-/-}eNOS^{+/+} mice (**Figure 6D**). This was apparent from counting the vessels as well as measuring the tumor hemoglobin content (**Figure 6, C–E**), and was statistically significant. In summary, NOS pharmacological inhibition recapitulated the effect of genetic ablation of eNOS in rescuing the angiogenesis defect induced by the loss of caveolin-1 *in vivo* as well as *in vitro*.

Discussion

Our results demonstrate reduced angiogenesis elicited by VEGF in the corneal micropocket assay in caveolin-1 gene-disrupted mice. By *in vitro*, *ex vivo*, and *in vivo* experiments, our results further confirm previous studies showing that the individual loss of eNOS³⁶ or caveolin-1^{20,22} impairs the angiogenic response elicited by B16 melanoma cells inoculated *in vivo*. In addition, we show for the first time that reduced tumor angiogenesis in caveolin-1-null mice is due to unabated endothelial nitric oxide production, as genetic disruption of eNOS in the caveolin-1-null background partially or totally restores angiogenesis quantitatively. Therefore, when combined, the lack of caveolin-1 and the lack of eNOS do not act in an additive or synergistic fashion to decrease angiogenesis but instead counteract each other, and their combination rescues the decrease seen with each individual loss.

Decreased pathological neovascularization in caveolin-1-null mice has not been unanimously reported. Decreased angiogenesis was shown using bFGF-containing matrigel

plugs,²⁰ VEGF-expressing adenoviral vector,²² experimental colitis²³ or cerebral ischemia.³⁷ Reduced ischemic hindlimb reperfusion after femoral artery resection is also apparent in these mice.²¹ Defective mobilization of endothelial progenitors in response to ischemia was further documented in caveolin-1-deficient mice.³⁸ However, using tumor angiogenesis assays, both an increase^{18,19} and a decrease^{20,22} in the number of angiogenic blood vessels have been reported. Studies reporting increased tumor angiogenesis also documented increased tumor growth in caveolin-1-null mice,^{18,19} whereas studies reporting decreased angiogenesis showed decreased tumor growth.^{20,22} The method for numbering the tumor blood vessels has been suggested to explain the difference between these studies.¹⁸ In our experiments, CD31 and α -smooth muscle cell actin staining of tumor sections confirmed the decreased number in vessels seen with the H&E staining in the caveolin-1 gene-deficient mice (not shown). The studies showing increased tumor angiogenesis in caveolin-1-deficient mice both documented higher microvascular permeability.^{18,19} It is interesting to note that the two articles that reported an increase in angiogenesis used a different knockout mouse⁹ than those showing decreased angiogenesis in the absence of caveolin-1,¹⁰ and that angiogenesis is not the only aspect of the phenotype of caveolin-1 gene-disrupted mice that is clearly different between these two murine models.^{39,40} However, most recently, decreased breast tumor growth has been reported with both murine models within the same study.⁴¹ Using the same tumor cells and the same murine model as Woodman et al²⁰ and Chang et al,²² we confirmed decreased tumor growth and decreased tumor angiogenesis in the caveolin-1-deficient mice.

Our experiments show that ablation of endothelial NO production can increase angiogenesis in caveolin-1-null mice. This may seem counterintuitive, as endothelial NO is clearly pro-angiogenic: NO has been shown to promote increased vascular permeability, endothelial cell

Figure 7. Proposed regulation of angiogenesis by caveolin-1 and eNOS. **A:** When both proteins are expressed, caveolin-1 regulation of eNOS compartmentalization and activation promotes optimal endothelial NO production in response to proangiogenic signals. **B:** In the absence of caveolin-1, excessive endothelial nitric oxide production, together with increased superoxide production, results in impaired angiogenesis. **C:** In eNOS knockout mice, insufficient endothelial nitric oxide production prevents optimal neovascularization. **D:** In mice lacking both eNOS and caveolin-1, endothelial nitric oxide production is reduced, but other angiogenic pathways are activated in the absence of caveolin-1, resulting in eNOS-independent angiogenesis.

migration and proliferation,⁴² mural cell recruitment, morphogenesis, and stabilization of angiogenic vessels.³⁶ In addition, angiogenesis is impaired in eNOS-deficient mice.^{26,43} Indeed, reduced angiogenesis in the absence of eNOS is confirmed in our experiments from the *in vitro*, *ex vivo* and *in vivo* angiogenic phenotype of Cav^{+/+}eNOS^{-/-} mice, and substantiated by the reduced angiogenesis in wild-type mice treated with L-NAME. In agreement with our results, excess NO production has been shown to mediate other aspects of the phenotype of caveolin-1 knock-out mice, namely pulmonary hypertension^{44,45} even though NO per se is a potent vasodilator, reduction of innate immunity⁴⁶ even though NO has direct and indirect antimicrobial activities, and cardiomyopathy.⁴⁷ One mechanism for these consequences of increased eNOS activity in caveolin-1-null mice has been proposed to be the formation of peroxynitrite when NO is combined to superoxide, and the subsequent deleterious nitration reactions affecting functional proteins such as protein kinase G⁴¹ or interleukin-1 receptor-associated kinase.⁴² It is well known that superoxide can be produced by eNOS itself in certain conditions.⁴⁸ Interestingly, increased superoxide levels have been detected in cardiac and vascular tissues from caveolin-1-null mice but returned to normal after prolonged L-NAME treatment.⁴⁷

If increased endothelial NOS activity due to caveolin-1 ablation causes decreased endothelial cell migration, tube formation, and angiogenesis, as indicated by our results, then overexpression of eNOS would be expected to have the same consequences. This is not observed, as transgenic eNOS mice have been generated⁴⁹ and display an increased, rather than decreased, neovascularization in response to hind limb ischemia.⁵⁰ This indicates that neovascularization is not simply proportional to the activity of eNOS or the amount of NO produced. Other factors may include the circumstances in which eNOS is able to produce superoxide, and the subcellular localization of eNOS, which in the absence of caveolae cannot be compartmentalized with upstream and downstream partners of the pathways leading to NO production. Targeting of eNOS to different cellular compartments has been shown to regulate the mechanism and degree of its activation^{51,52}; and, given that NO is short lived, the subcellular localization of its production is known to affect its actions, including protein S-nitrosylation.⁵³

Another way of interpreting our results is that ablation of caveolin-1 rescues the altered angiogenesis seen in the absence of eNOS. This could be hypothesized to be the result of hyperactivation, in the absence of caveolin-1 and caveolae, of pro-angiogenic proteins other than eNOS. Alternatively, antiangiogenic receptors that need caveolae localization or interaction with caveolin-1 may be inactive when caveolin-1 is ablated. Prostacyclin synthase produces the potent vasodilator prostacyclin in response to VEGF⁵⁴ and promotes angiogenesis.^{55,56}

Prostacyclin synthase is located in caveolae where it interacts with caveolin-1.⁵⁷ The angiotensin receptor TEK/Tie2 exerts tight control on angiogenesis (maintaining them quiescent on angiotensin-1 binding, but promoting endothelial cell activation on angiotensin-2 binding) and has also been suggested to localize in endothelial

cell caveolae.⁵⁸ CD36 is a critical anti-angiogenic receptor of endothelial cells⁵⁹ which co-fractionates with caveolae and co-immunoprecipitates with caveolin-1.⁶⁰ This suggests that caveolin-1, and caveolae, may regulate angiogenesis by multiple pathways, including NO-mediated and NO-independent mechanisms (Figure 7).

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