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The level of caveolin-1 expression closely correlates with the oncogenic transformation of NIH 3T3 cells, the proliferation of human cancer cells, and the differentiation of adipocytes and muscle cells. However, the role of caveolin-1 in endothelial cell proliferation and differentiation remains unknown. Here, we have shown that angiogenic growth factors that stimulate endothelial cell proliferation lead to dramatic reductions in caveolin-1 expression. In addition, using an *in vitro* Matrigel assay system, we studied the potential role of caveolin-1 in capillary-like tubule formation (*i.e.* endothelial cell differentiation) using human microvascular endothelial cells (HMEC-1). We showed that the level of endogenous caveolin-1 expression increased in a time-dependent manner when endothelial cells underwent differentiation and that the maximum level of caveolin-1 expression occurred just prior to the formation of capillary-like tubules. Interestingly, overexpression of caveolin-1, via an adenoviral gene delivery system, clearly accelerated endothelial cell differentiation/tubule formation and led to a dramatic ~3-fold increase in the number of capillary-like tubular structures. Conversely, down-regulation of caveolin-1 expression, via an antisense adenoviral approach, reduced the number of capillary-like tubules formed by >10-fold. Consistent with the unique function of caveolin-1 in interacting with key signaling molecules, delivery of the caveolin-1 scaffolding domain into the cytoplasm of living endothelial cells was also sufficient to enhance capillary-like tubule formation. Taken together, these results clearly demonstrate that caveolin-1 and the caveolin-1 scaffolding domain play an important positive role in the regulation of endothelial cell differentiation, a prerequisite step in the process of angiogenesis.

In the adult, endothelial cells normally remain quiescent, show a low turnover rate, and rarely proliferate (1). But once they receive an appropriate stimulus, endothelial cells rapidly proliferate and form new blood vessels by a process called angiogenesis. How do endothelial cells determine whether to remain quiescent or to become proliferative? In this regard, our greatest insights into the regulation of the angiogenic process are derived from the study of embryonic development and tumorigenesis. Several growth factors responsible for endothelial cell growth and differentiation have been identified and characterized. These activators include vascular endothelial growth factor (VEGF),[1] also termed vascular permeability factor (2–4); placenta growth factor (5); platelet-derived growth factor (PDGF) (6); fibroblast growth factor (7); and hepatocyte growth factor (HGF), also termed scatter factor (8).

In addition to these activators, negative regulators of angiogenesis are also involved in controlling the formation of new blood vessels (9). These include angiostatin (10), endostatin (11), transforming growth factor-β (12), thrombospondin-1 (13), fumagillin (14), 2-methoxyestradiol (15), and thalidomide (16). These inhibitors block angiogenic activator-induced endothelial cell migration, proliferation, and blood vessel formation (11, 17). Although a variety of angiogenesis activators and inhibitors have been identified and characterized, there is very little information on the mechanisms by which angiogenesis activators and inhibitors regulate the state of endothelial cells. Recently, we demonstrated that angiogenesis activators and inhibitors reciprocally regulate caveolin-1 expression and caveolae formation (18). Overexpression of caveolin-1 inhibits the VEGF receptor (Flk-1)-mediated mitogenic signaling cascade, suggesting that caveolin-1 might be a cellular target for angiogenesis regulators.

Three mammalian caveolin genes encoding four proteins have been identified and characterized (19). These include caveolin-1, caveolin-2, and caveolin-3. Caveolin-1α and caveolin-1β are derived via alternative initiation during translation (20). Caveolin-1 and caveolin-2 are coexpressed and form a hetero-oligomeric complex (21) in many cell types, with particularly high levels in adipocytes, endothelia, epithelia, and fibroblasts, whereas caveolin-3 expression is muscle-specific (22, 23). A large body of evidence supports the idea that caveolins interacts with a number of signaling molecules, including receptor and non-receptor tyrosine kinases (epidermal growth factor, Neu, and Src family tyrosine kinases), protein kinase C, heterotrimeric G-protein α-subunits, and endothelial nitric-oxide synthase (24). Domain mapping studies revealed that interaction of caveolins with these signaling molecules is me-

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[1] The abbreviations used are: VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; HGF, hepatocyte growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; BAECs, bovine aortic endothelial cells; pfu, plaque-forming units.

diated by a membrane-proximal region of the caveolins termed the caveolin scaffolding domain (residues 82–101 in caveolin-1). The caveolin scaffolding domain recognizes a specific caveolin-binding motif (*i.e.* consisting of closely spaced aromatic amino acids) located within the catalytic domain of a given signaling molecule (24, 25). In general, interaction with caveolins or the caveolin scaffolding domain inhibits the activity of caveolin-binding signaling molecules.

Although expressed in many cell types, caveolin-1 is down-regulated or absent in oncogene (v-*abl*, *bcr-abl*, Ha-*ras*)-transformed NIH 3T3 cells (26) as well as in human cancer cells (27, 28). Overexpression of caveolin-1 in Ha-*ras*- and v-*abl*-transformed NIH 3T3 cells abrogates their anchorage-independent growth by directly inhibiting the Ras-MAPK (p42/44) signaling cascade (29). Similarly, overexpression of caveolin-1 in human breast cancer cells (possessing no detectable caveolin-1) inhibits cell proliferation and colony formation (27). Conversely, targeted down-regulation of caveolin-1 by the expression of antisense caveolin-1 is sufficient to induce cell transformation and tumor formation apparently by the activation of MAPK (ERK1/2) (30). Furthermore, caveolin-1 expression is down-regulated in rapidly dividing cells and dramatically up-regulated in confluent cells (30). These results suggest that caveolin may function as a transformation suppressor gene (19). Recently, we demonstrated that caveolin-3 expression is elevated during the differentiation of C2C12 skeletal myoblasts (31), and this differentiation process is prevented by down-regulation of caveolin-3 expression. Consistent with a role for caveolin-1 in cell differentiation, Gumbleton and co-workers (32) observed up-regulation of caveolin-1 expression with concomitant formation of caveolae as alveolar epithelial type II lung cells *trans*-differentiate into alveolar epithelial type I cells. These results imply a potential role for caveolins in cellular differentiation.

Angiogenesis is a complex process that involves a series of discrete stages or phases, including (i) an initial proliferative phase and (ii) a terminal differentiation phase. In this work, we show that caveolin-1 is down-regulated by endothelial growth factors that stimulate the initial proliferative phase; this is consistent with previous evidence demonstrating that caveolin-1 is a negative regulator of cell proliferation and cell cycle progression. Conversely, we evaluated the effect of caveolin-1 expression on endothelial cell differentiation. This was experimentally achieved by plating the endothelial cells at confluency to avoid the known negative effects of caveolin-1 on cell proliferation. Under these conditions of complete confluency, we show that caveolin-1 expression stimulates endothelial cell differentiation and tubule formation. This is also consistent with the previous observations that caveolin-1 and caveolin-3 are most highly expressed in terminally differentiated cell types and that caveolin gene expression is up-regulated during a variety of differentiation processes. Consistent with this interpretation, we also show that caveolin-1 levels are normally transiently up-regulated during the endothelial cell differentiation process. Thus, caveolin-1 expression is down-regulated during the proliferative phase and up-regulated during the differentiation phase of angiogenesis. Furthermore, our current findings are the first demonstration that caveolin-1 expression can stimulate a differentiation process. Similarly, we have shown previously that caveolin-1 can reverse the oncogenic behavior of transformed NIH 3T3 fibroblasts in culture (29).

EXPERIMENTAL PROCEDURES

Materials—A monoclonal antibody directed against caveolin-1 (clone 2297, recognizing residues 61–71) (20) was kindly provided by Dr. Roberto Campos-Gonzalez (BD-Transduction Laboratories, Lexington, KY). Reagents and other supplies were obtained from the following commercial sources. VEGF, PDGF, and basic fibroblast growth factor were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). HGF and a monoclonal antibody directed against β-actin were purchased from Sigma. Antibodies against PECAM-1, VEGF receptor-2 (Flk-1), GFP, and c-Myc were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rhodamine-conjugated streptavidin was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The angiogenesis assay kit was purchased from Chemicon International, Inc. (Temecula, CA). Penetratin-caveolin-1 peptides were synthesized by the NIH-designated Peptide Core Facility of Tufts Medical School (Boston, MA). The ProLong Anti-fade reagent was purchased from Molecular Probes, Inc. (Eugene, OR). The BCA protein assay kit was purchased from Pierce.

Cell Culture and Growth Factor Treatment—Bovine aortic endothelial cells (BAECs) were grown in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum (normal growth medium). Human microvascular endothelial cells (HMEC-1) were grown in MCDB 131 with 10% heat-inactivated fetal bovine serum (normal growth medium), as described previously (33). Western blot analysis using antibodies against PECAM-1 and VEGF receptor-2 (Flk-1) confirmed the endothelial origin of HMEC-1 cells.[2] To study the effect of growth factors on caveolin-1 expression, BAECs (1×10^4 cells/well) or HMEC-1 cells (2×10^4 cells/well) were treated with VEGF, PDGF, or HGF in medium containing 0.5% fetal bovine serum for 24 h. After treatment, the cells were washed with phosphate-buffered saline, lysed, and prepared for Western blot analysis as described below.

Infection of HMEC-1 Cells with an Adenoviral Vector Containing Either Sense or Antisense Caveolin-1—The construction of recombinant Myc epitope-tagged caveolin-1 adenovirus (Ad-Cav-1), green fluorescent protein adenovirus (Ad-GFP), and tetracycline transactivator adenovirus (Ad-tTA) were as we described previously (34). This adenoviral vector system is inducible and requires a coactivator for expression (Ad-tTA) as previously described (34). The adenovirus harboring antisense caveolin-1 (Ad-Cav-1-AS) is not inducible and was constructed and characterized as we previously described (35). HMEC-1 cells were seeded at a density of 8×10^4 cells/ml in six-well plates and co-incubated with Ad-Cav-1 and Ad-tTA in serum-free medium for 1 h, followed by incubation in growth medium for 24 h. The level of caveolin-1 expression was assessed by Western blot analysis using a specific monoclonal antibody directed against either caveolin-1 or the c-Myc epitope tag. A combination of various titers of Ad-Cav-1 and Ad-tTA was employed to achieve optimum levels of caveolin-1 expression. The efficiency of the infection was determined by immunofluorescent staining with antibodies against caveolin-1. As a control, HMEC-1 cells were co-infected with Ad-GFP and Ad-tTA or infected with Ad-Cav-1 alone. For antisense caveolin-1 studies, HMEC-1 cells seeded at a density of 8×10^4 cells/ml in six-well plates were incubated with Ad-Cav-1-AS in serum-free medium for 1 h, followed by incubation in growth medium for 24 h. The level of caveolin-1 down-regulation was assessed by Western blot analysis using a specific monoclonal antibody directed against caveolin-1.

Capillary-like Tubule Formation Assay—The formation of capillary-like tubular structures was assessed in Matrigel-coated multiwell plates essentially as described previously (36). HMEC-1 cells were infected with or without adenovirus harboring sense or antisense caveolin-1 for 24 h as described above. Cells were then seeded at a density of 30×10^4 cells/ml in growth medium in Matrigel-coated multiwell plates and incubated at 37 °C for a period of 0–24 h. To study the effect of caveolin-1 peptides on tubule formation, HMEC-1 cells (30×10^4 cells/ml) were incubated in Matrigel-coated multiwell plates at 37 °C for 1 h to allow cells to attach to the Matrigel, followed by treatment with penetratin-caveolin-1 fusion peptides (5 μM) for 20 h. The formation of tubular structures was examined using an Olympus IX70 microscope.

Immunoblot Analysis—The expression levels of caveolin-1 were determined by Western blot analysis. Briefly, cells were washed with phosphate-buffered saline and solubilized with lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 1% Triton X-100, 60 mM octyl glucoside, and protease inhibitors. Total cellular proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes for immunoblotting using enhanced chemiluminescence. Prior to loading, the protein concentration of the samples was measured by the BCA method using bovine serum albumin as the standard.

Internalization of Penetratin-Caveolin Scaffolding Domain Peptides by HMEC-1 Cells—After incubation with biotin-penetratin (B-Pen) or

[2] J. Liu, X. Wang, D. S. Park, and M. P. Lisanti, unpublished data.

biotin-penetratin-caveolin-1 scaffolding domain (B-Pen-C1-SD) fusion peptides (at a concentration of 2 μM) in growth medium at 37 °C for 2 h, HMEC-1 cells were washed with phosphate-buffered saline and fixed with 3% paraformaldehyde. After fixation, cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline containing 0.2% bovine serum albumin. Internalized peptides were visualized by incubation of the cells with rhodamine-conjugated streptavidin in the permeabilization buffer. The internalized peptides were visualized using the Olympus IX70 fluorescent microscope equipped with a photometric CCD camera under the rhodamine channel.

RESULTS

Angiogenic Growth Factors Down-regulate Caveolin-1 Expression in Subconfluent Endothelial Cells—We have previously shown that angiogenic growth factors down-regulate caveolin-1 expression in ECV 304 cells (18), suggesting that caveolin-1 may be important in angiogenesis. After publication of our results, the endothelial origin of ECV 304 cells was recently questioned (37). To re-evaluate the possible effects of angiogenic factors on caveolin-1 expression in endothelial cells, we next treated primary BAECs and a well established human endothelial cell line (HMEC-1) (33) with a panel of different angiogenic growth factors such as VEGF, PDGF, and HGF and examined the level of caveolin-1 expression in these cells.

Fig. 1*A* shows that treatment of BAECs with either VEGF or PDGF at a concentration of 10 ng/ml for 24 h dramatically down-regulated caveolin-1 expression. Similarly, treatment of rat capillary endothelial cells with an angiogenic factor (basic fibroblast growth factor) led to down-regulation of caveolin-1 expression (data not shown). Furthermore, treatment of HMEC-1 cells with VEGF, PDGF, or HGF caused the dose-dependent down-regulation of caveolin-1 expression (Fig. 1*B*). Our current results are consistent with our previous observations, indicating that caveolin-1 may be a *bona fide* cellular target for angiogenesis regulators in endothelial cells. Importantly, however, angiogenic growth factors did not affect caveolin-1 expression in confluent endothelial cells (data not shown).

Up-regulation of Caveolin-1 Expression Occurs Just Prior to Endothelial Tubule Formation—Angiogenesis involves the migration, proliferation, and differentiation of endothelial cells. To test the hypothesis that caveolin-1 plays an important role in angiogenesis, we next employed an *in vitro* angiogenesis assay system. To focus on the possible effects of caveolin-1 on endothelial cell differentiation, we seeded HMEC-1 cells at super-confluency (see "Experimental Procedures") to minimize the effects of caveolin-1 on cell proliferation.

We have recently shown that in nontransformed NIH 3T3 cells, caveolin-1 levels are down-regulated in rapidly dividing cells, but dramatically increased in confluent cells; in these confluent cells, caveolin-1 was specifically concentrated at the areas of cell-cell contact (30, 38). These results suggest a potential role for caveolin-1 in regulating contact inhibition and perhaps cellular differentiation. Indeed, we (31) and others (32) have demonstrated that caveolins are critical for the differentiation of muscle cells as well as epithelial cells. Based on these observations, we would predict that caveolin-1 should increase during the process of endothelial cell differentiation/tubule formation.

Fig. 2*A* shows that incubation of HMEC-1 cells on Matrigel from 0 to 24 h caused the time-dependent formation of tubular structures. These tubular structures first appeared after 12 h of incubation and developed into a well organized capillary-like network after 24 h of incubation. Interestingly, Western blot analysis revealed that caveolin-1 levels steadily increased in a time-dependent manner and reached their maximum after 8 h of incubation (Fig. 2*B*), ~4 h prior to the beginning of tubule formation (Fig. 2*A*). Conversely, caveolin-1 levels then decreased from 12 to 24 h and returned to basal levels after 24 h

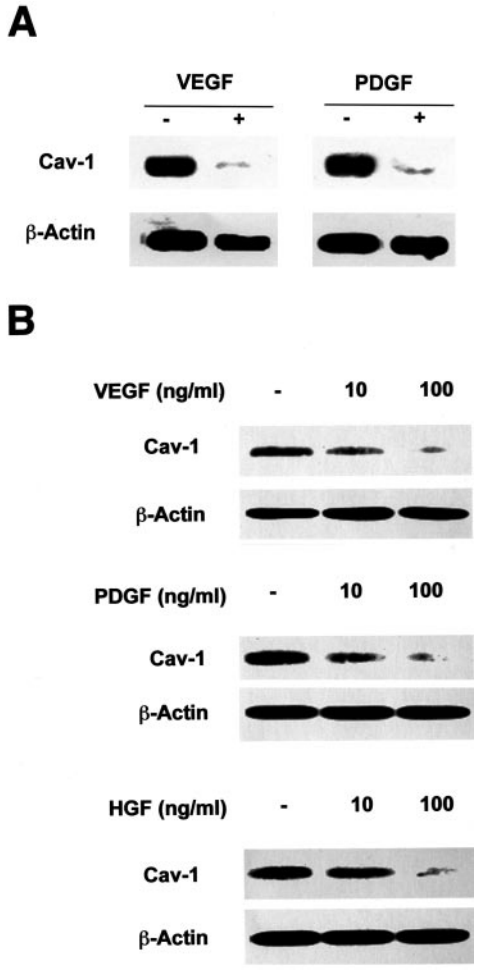

FIG. 1. **Angiogenesis activators down-regulate caveolin-1 expression in endothelial cells.** *A*, down-regulation of caveolin-1 expression in BAECs. BAECs (seeded at a density of 1×10^4 cells/well in 24-well plates; see "Experimental Procedures") were treated with or without 10 ng/ml VEGF or PDGF in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum for 24 h. The level of caveolin-1 was determined by Western blot analysis using a specific antibody directed against caveolin-1 (*Cav-1*) (*upper panels*). The same membrane was reprobed with an antibody directed against β-actin as a control for equal protein loading (*lower panels*). *B*, down-regulation of caveolin-1 expression in HMEC-1 cells. Human microvascular endothelial cells (seeded at a density of 2×10^4 cells/well in 24-well plates) were treated with or without 0–100 ng/ml VEGF, PDGF, or HGF in MCDB 131 medium containing 0.5% fetal bovine serum for 24 h. The level of caveolin-1 was determined by Western blot analysis using a specific antibody directed against caveolin-1. The same membranes were reprobed with a monoclonal antibody directed against β-actin as a control for equal protein loading.

of incubation (Fig. 2*B*). These results suggest that a transient increase in caveolin-1 levels may be functionally important in the regulation of endothelial cell differentiation/tubule formation.

Recombinant Expression of Caveolin-1 Enhances the Formation of Endothelial Tubular Structures—To better assess the potential role of caveolin-1 in the regulation of endothelial cell differentiation, we next transiently up-regulated or down-regulated the levels of caveolin-1 in confluent HMEC-1 cells. For this purpose, we employed a series of well characterized adenoviral vectors that harbor the caveolin-1 cDNA either in the sense or antisense orientation.

Fig. 3 shows the dose-dependent overexpression of caveolin-1 in HMEC-1 cells co-infected with Ad-Cav-1 and Ad-tTA. A maximum level of caveolin-1 expression was obtained by infecting HMEC-1 cells with both Ad-Cav-1 and Ad-tTA (200 pfu/cell

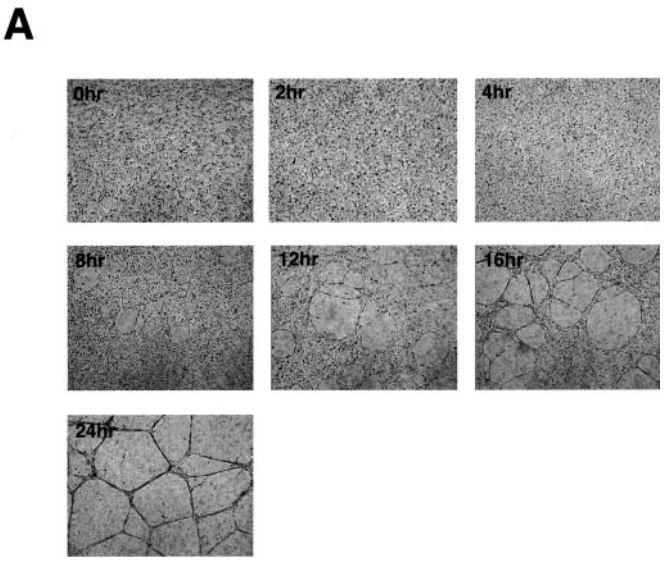

FIG. 2. **Up-regulation of caveolin-1 expression occurs just prior to endothelial tubule formation.** HMEC-1 cells were seeded at a density of 30×10^4 cells/ml in 24-well plates precoated with Matrigel (as suggested by Chemicon International, Inc.) and incubated from 0 to 24 h as shown in *A*. At the indicated times, cells were lysed, and cell lysates were subjected to SDS-PAGE and Western blot analysis using a specific antibody probe directed against caveolin-1 (*Cav-1*) (*B*). Note that the level of caveolin-1 increased in a time-dependent manner and reached maximum levels after 8 h of incubation (see *arrow* in *B*), a time at which few or no capillary-like tubular structures were observed (*A*). Tubules first became apparent after 12 h of incubation. In *B*, the same membrane was reprobed with a monoclonal antibody directed against β-actin as a control for equal protein loading.

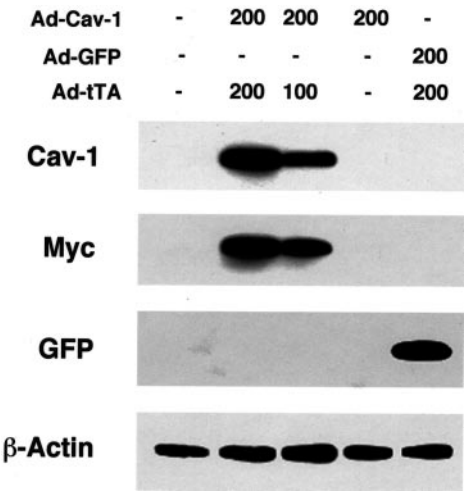

FIG. 3. **Adenoviral vector-mediated expression of caveolin-1 and GFP in HMEC-1 cells.** HMEC-1 cells were transduced with the following three adenoviral vectors alone or in combination: Ad-Cav-1, Ad-GFP, or Ad-tTA. Note that co-infection with Ad-tTA was required for the expression of either caveolin-1 or GFP, as expected (see "Experimental Procedures"). Twenty-four hours post-infection, the cells were lysed and subjected to SDS-PAGE and Western blot analysis using a specific monoclonal antibody probe directed against caveolin-1 (*Cav-1*), c-Myc, or GFP. The same membrane was reprobed with a monoclonal antibody directed against β-actin as a control for equal protein loading.

each) (Figs. 3 and 5*B*). Longer exposures were required to reveal endogenous caveolin-1 expression in the HMEC-1 cells (see Fig. 5*B*). Importantly, there was no transduced expression of caveolin-1 either in cells infected with Ad-Cav-1 alone or in cells co-infected with Ad-GFP and Ad-tTA, although co-infection of Ad-GFP and Ad-tTA led to GFP expression (Figs. 3 and 5*B*).

If caveolin-1 expression is important for the regulation of endothelial cell differentiation, we would predict that premature up-regulation of caveolin-1 might lead to early endothelial tubule formation. Fig. 2*A* shows that the formation of mature tubules required ~24 h in non-infected HMEC-1 cells. However, overexpression of caveolin-1 resulted in the initial formation of tubular structures as early as 6 h of incubation on Matrigel (data not shown). By 12 h, caveolin-1 overexpression resulted in the formation of 17 ± 2.5 capillary-like tubules/well, whereas only 5 ± 0.9 tubules were found in non-infected cells (Fig. 4).

After 20 h of incubation, ~23 ± 2.5 tubules/well were observed in non-infected cells. In striking contrast, the tubules increased to 62 ± 5.5/well in caveolin-1-overexpressing cells, representing a ~2.7-fold increase in tubule formation ($p < 0.001$) (Fig. 5).

As a critical negative control, we also assessed the effects of expressing an irrelevant protein, *i.e.* GFP. Importantly, infection of HMEC-1 cells with Ad-Cav-1 alone or co-infection with Ad-GFP and Ad-tTA did not affect tubule formation (Fig. 5).

Down-regulation of Caveolin-1 Dramatically Inhibits Endothelial Tubule Formation—Our previous results demonstrated that down-regulation of caveolin-1 expression causes hyperactivation of the p42/44 MAPK cascade and oncogenic transformation of NIH 3T3 cells (30), indicating that down-regulation of caveolin-1 can negatively affect cell differentiation. To further examine the effect of caveolin-1 on endothelial cell differentiation, we next used an antisense approach. HMEC-1 cells were first infected with the antisense caveolin-1 adenoviral vector for 1 h, followed by incubation in growth medium for 24 h to significantly down-regulate the levels of caveolin-1 (Fig. 6, *A* and *B*). The transduced cells were then plated onto Matrigel and incubated for an additional 24 h. In non-infected control cells, tubular structures appeared as early as 12 h of incubation; and after 24 h of incubation, 27 ± 2.08 tubules/well developed (Fig. 6). In striking contrast, cells expressing antisense caveolin-1 showed few or no tubular structures even after 24 h of incubation (Fig. 6). Thus, down-regulation of caveolin-1 apparently prevents endothelial cell differentiation/tubule formation.

Internalization of the Caveolin-1 Scaffolding Domain Peptide Stimulates Endothelial Tubule Formation—To examine the possible role of the caveolin-1 scaffolding domain in mediating the ability of caveolin-1 to stimulate endothelial cell differentiation, we next synthesized a panel of caveolin-1 scaffolding domain peptides as C-terminal fusion proteins with penetratin (Pen-C1-SD) (Fig. 7). Penetratin is a 16-amino acid peptide that corresponds to amino acids 43–58 of the homeodomain of antennapedia, a *Drosophila* transcription factor. Attachment of penetratin to peptides or oligonucleotides allows their efficient delivery to the cytoplasm and the nucleus of a wide variety of living cells via a non-endocytic and non-degradative pathway (39, 40). Because a mutant form of penetratin with three introduced prolines (3Pro-penetratin) is delivered preferentially to the cytoplasm of living cells (39), we also synthesized another set of fusion peptides with this form of penetratin attached to

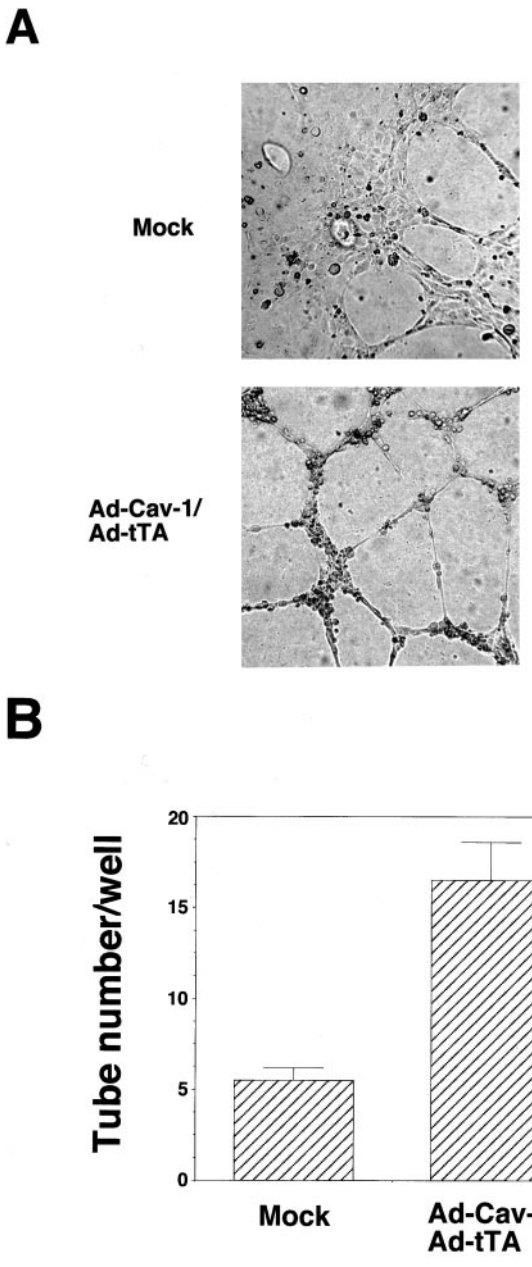

FIG. 4. **Recombinant expression of caveolin-1 dramatically enhances tubule formation by HMEC-1 cells.** Twenty-four hours post-infection with Ad-Cav-1 (200 pfu/cell) and Ad-tTA (200 pfu/ml) (*Ad-Cav-1*/*Ad-tTA*), HMEC-1 cells were seeded at a density of 3×10^4 cells/well in Matrigel-precoated 96-well plates and incubated for 12 h at 37 °C. *A*, bright-field views were taken using an Olympus IX70 fluorescent microscope equipped with a photometric CCD camera. Note that as compared with control cells (mock-transduced; *upper panel*), more capillary-like tubules developed in the cells overexpressing caveolin-1 (*lower panel*). *B*, capillary-like tubular structures were scored by counting the number of tubules in each well, *i.e.* of either control or caveolin-1-overexpressing cells. Data are the means ± S.D. from three independent experiments, each performed in duplicate.

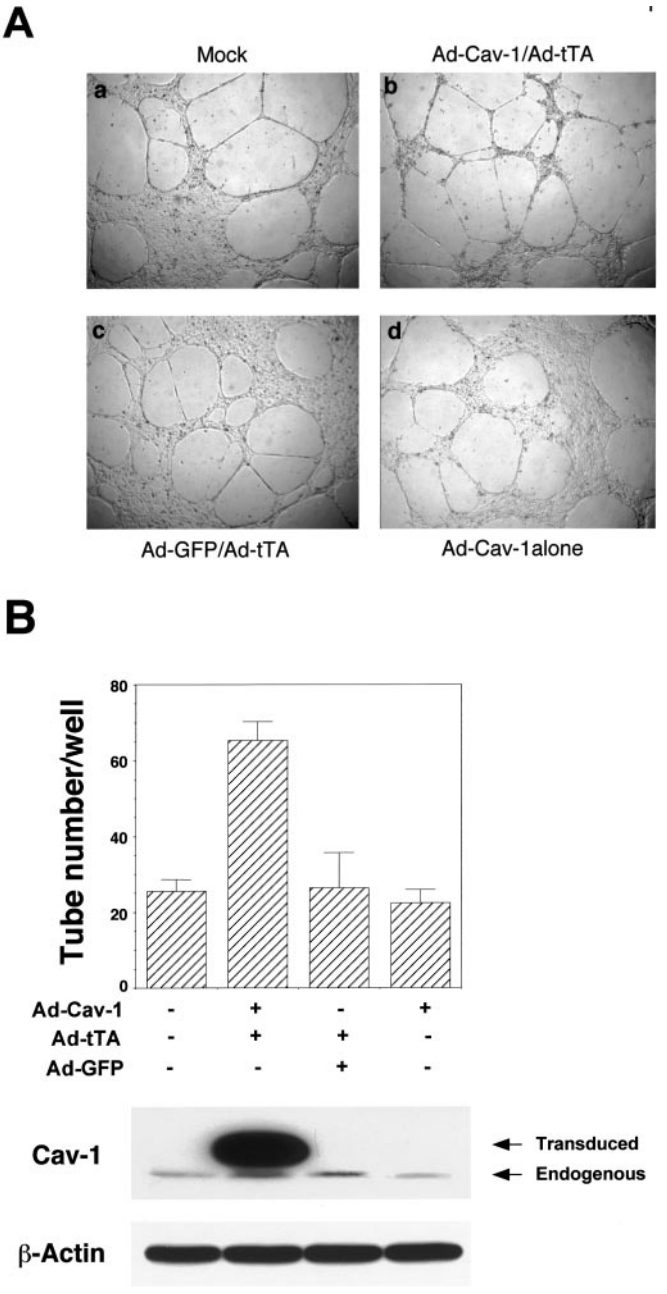

FIG. 5. **Expression of caveolin-1, but not GFP, enhances endothelial tubule formation.** Twenty-four hours post-infection, HMEC-1 cells were seeded at a density of 3×10^4 cells/well in Matrigel-precoated 96-well plates and incubated for 20 h at 37 °C. *A*, bright-field views of non-infected cells (*Mock*; *panel a*), caveolin-1 (*Cav-1*)-overexpressing cells (*panel b*), GFP-overexpressing cells (*panel c*), and cells infected with Ad-Cav-1 alone (*panel d*) are shown after 20 h of incubation on Matrigel. *Panel a*, mock transduction; *panel b*, Ad-Cav-1 (200 pfu/cell) plus Ad-tTA (200 pfu/cell); *panel c*, Ad-GFP (200 pfu/cell) plus Ad-tTA (200 pfu/cell); *panel d*, Ad-Cav-1 alone (200 pfu/cell). *B*, the number of capillary-like tubules formed after 20 h was determined as described in the legend of Fig. 4. Data are the means ± S.D. from three independent experiments, each performed in duplicate. Also, an aliquot of the cells from each group was lysed and subjected to SDS-PAGE and Western blot analysis using a specific antibody probe directed against caveolin-1. Note that the level of transduced caveolin-1 expression was dramatically higher than that of endogenous caveolin-1 expression (see *arrows*). Expression of GFP under these conditions was also confirmed (data not shown; see Fig. 3). The same membrane was reprobed with a monoclonal antibody directed against β-actin as a control for equal protein loading.

the caveolin-1 scaffolding domain (Fig. 7).

To directly visualize the uptake of the fusion peptides by endothelial cells, a biotin moiety was attached to the N terminus of these peptides. As shown in Fig. 8 (*c* and *d*), incubation of HMEC-1 cells with the biotin-penetratin peptides (with or without the attached caveolin-1 scaffolding domain) resulted in efficient uptake of the peptides into the cytoplasm and nucleus. Consistent with previous observations (39), the biotin-3Pro-penetratin peptides (with or without the attached caveolin-1 scaffolding domain (B-3Pro-C1-SD and B-3Pro, respectively)) were internalized and concentrated in the cytoplasm (data not shown).

As observed above, incubation of HMEC-1 cells on Matrigel for 20 h resulted in the formation of numerous capillary-like

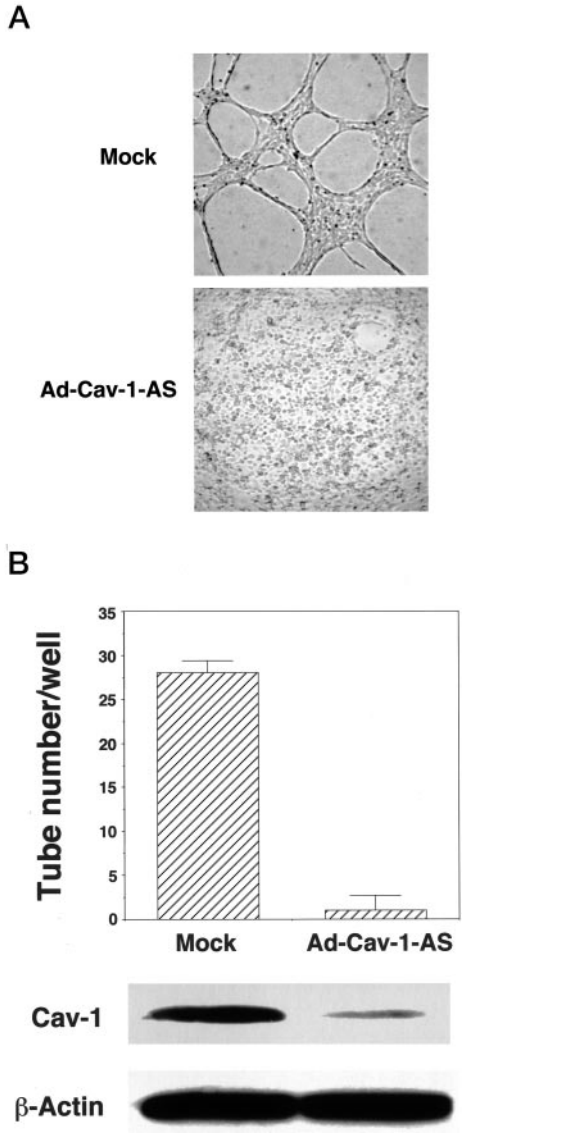

FIG. 6. **Targeted down-regulation of caveolin-1 expression inhibits endothelial tubule formation.** Twenty-four hours post-infection with Ad-Cav-1-AS (300 pfu/cell), HMEC-1 cells were seeded at a density of 3×10^4 cells/well in Matrigel-precoated 96-well plates and incubated for 24 h at 37 °C. *A*, bright-field views of non-infected cells (*Mock*) or cells harboring Ad-Cav-1-AS were taken after 24 h of incubation on Matrigel. *B*, capillary-like tubular structures were scored by counting the number of tubules in each well. Data are the means ± S.D. from three independent experiments, each performed in duplicate. After infection, an aliquot from either non-infected or Ad-Cav-1-AS-infected cells was lysed and subjected to SDS-PAGE and Western blot analysis using a specific antibody probe directed against caveolin-1 (*Cav-1*). The same membrane was reprobed with a monoclonal antibody directed against β-actin as a control for equal protein loading.

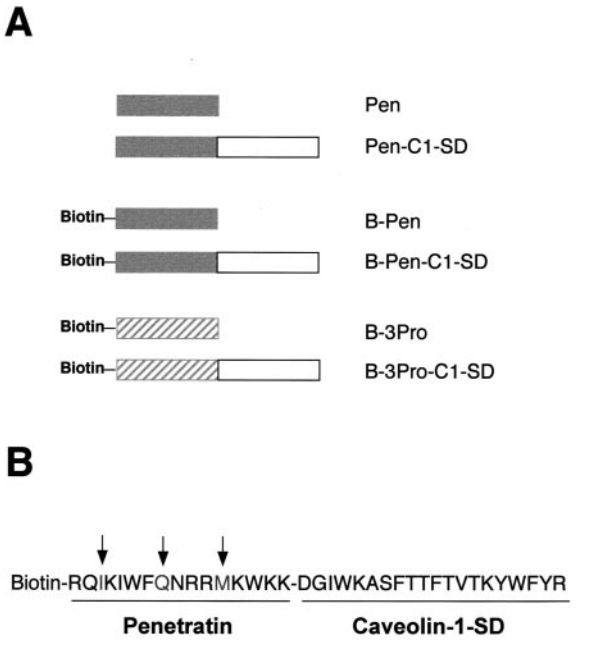

FIG. 7. **Schematic representation of the penetratin-caveolin-1 fusion peptides.** *A*, penetratin (*Pen*; *gray bars*), a 16-amino acid peptide corresponding to amino acids 43–58 of the homeodomain of antennapedia, a *Drosophila* transcription factor, was fused at its C terminus to the caveolin-1 scaffolding domain (*white bars*), yielding Pen-C1-SD. 3Pro-penetratin is a modified form of penetratin that is preferentially targeted to the cytoplasm and excluded from the nucleus. To monitor the uptake of these penetratin peptides, in some cases, we placed a biotin moiety at their extreme N terminus. *B*, shown is the amino acid sequence of the fusion peptides consisting of penetratin and the caveolin-1 scaffolding domain (*SD*). *Arrows* point to the three amino acids (Ile, Gln, and Met) that were changed to proline in 3Pro-penetratin.

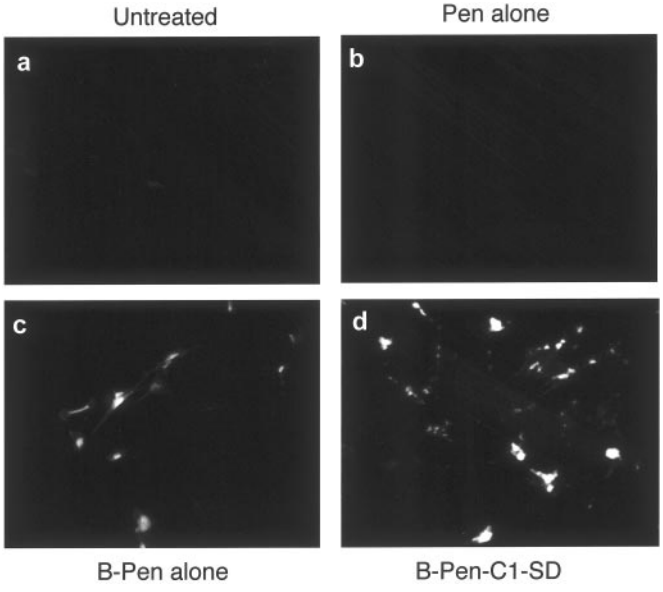

FIG. 8. **Uptake of the penetratin-caveolin-1 fusion peptides by HMEC-1 cells.** HMEC-1 cells were left untreated (*a*) or were treated with penetratin (*Pen*) alone (*b*), B-Pen alone (*c*), or B-Pen-C1-SD (*d*) at a concentration of 2 μM for 2 h at 37 °C. After fixation and permeabilization, the biotinylated peptides (B-Pen alone and B-Pen-C1-SD) were visualized by staining with rhodamine-conjugated streptavidin. Note that B-Pen and B-Pen-C1-SD were detected both in the cytoplasm and the nucleus of most cells. Negative controls shown in *a* and *b* did not reveal any staining, as expected.

tubular structures. Interestingly, treatment of HMEC-1 cells with caveolin-1 scaffolding domain peptides Pen-C1-SD and B-Pen-C1-SD (5 μM) increased the number of capillary-like tubules by ~5- and ~4.5-fold, respectively (Fig. 9, *A*, *panels b* and *d*; and *B*). These results are consistent with our hypothesis that caveolin-1 plays an important role in the regulation of endothelial cell differentiation. Furthermore, these results suggest that this action is mediated through the caveolin-1 scaffolding domain. Similarly, treatment of HMEC-1 cells with B-3Pro-C1-SD (5 μM) caused an ~4.5-fold increase in the formation of tubular structures (Fig. 9, *A*, *panel f*; and *B*), indicating that the effect of the caveolin-1 scaffolding domain on endothelial cell differentiation does not require translocation of the peptide into the nucleus.

As a critical negative control for these studies, we also assessed the potential activity of the penetratin moiety itself.

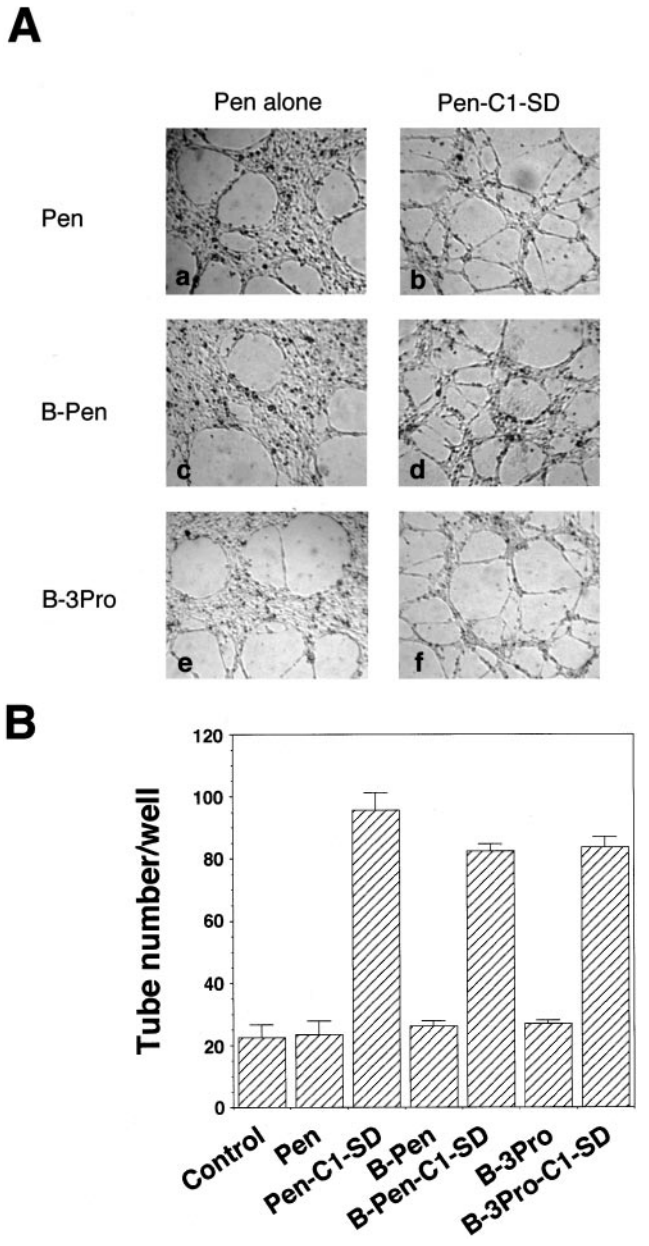

FIG. 9. **The caveolin-1 scaffolding domain is sufficient to enhance endothelial tubule formation.** HMEC-1 cells were seeded at a density of 3×10^4 cells/well in Matrigel-precoated 96-well plates. One hour after plating, the cells were treated with a panel of penetratin-derived peptides (illustrated in Fig. 7) at a concentration of 5 μM for a period of 20 h. *A*, bright-field views were taken after 20 h of incubation on Matrigel. *Panel a*, penetratin (*Pen*) alone; *panel b*, Pen-C1-SD; *panel c*, B-Pen alone; *panel d*, B-Pen-C1-SD; *panel e*, B-3Pro alone; *panel f*, B-3Pro-C1-SD. *B*, capillary-like tubular structures were scored by counting the number of tubules in each well. Data are the means ± S.D. from three independent experiments, each performed in duplicate.

Importantly, all the penetratin-only peptides used (penetratin, B-Pen, or B-3Pro) (5 μM) showed no activity in this assay system (Fig. 9, *A*, *panels a*, *c*, and *e*; and *B*).

DISCUSSION

Angiogenesis is the process by which new vasculature is derived from pre-existing blood vessels (41–43). The tight regulation of this process is mediated through a complex interplay between the endothelial cell and the surrounding matrix. Some of the mediators regulating this process, such as VEGF and fibroblast growth factor, act through their cognate receptors on endothelial cells to activate signaling pathways that include Ras, phosphatidylinositol 3-kinase, protein kinase C, and c-Src. The endothelial cell responds by secreting proteases and plasminogen activators, resulting in basement membrane degradation, followed by migration into the surrounding matrix, proliferation, and, ultimately, differentiation. However, how these familiar signaling pathways produce this wide variety of effects in endothelial cells remains a mystery. Therefore, the search for downstream effectors and how they translate signaling pathways into a proliferative response *versus* a differentiation response are critical. We present evidence that caveolin-1 is a major downstream effector in endothelial cell development that is down-regulated during cell proliferation, but when up-regulated, is a potent stimulus for endothelial cell differentiation.

One of the major conclusions of this study is that caveolin-1 is down-regulated by angiogenic growth factors in subconfluent endothelial cells (Fig. 1); however, these growth factors do not down-regulate caveolin-1 expression if these endothelial cells are confluent (data not shown). Thus, the effects of angiogenic growth factors on caveolin-1 expression are cell density-dependent. In contrast, recombinant expression of caveolin-1 in confluent endothelial cells stimulates endothelial cell differentiation and tubule formation (Figs. 4 and 5); this effect can be mimicked by cell-permeable caveolin-derived peptides (Figs. 7, 8, and 9). These observations are consistent with the finding that caveolin-1 protein levels are normally up-regulated during endothelial cell differentiation and tubule formation (Fig. 2). Conversely, antisense-mediated ablation of caveolin-1 expression during endothelial cell differentiation prevents tubule formation (Fig. 6). Thus, caveolin-1 is down-regulated during endothelial cell proliferation and up-regulated during endothelial cell differentiation/tubule formation, and caveolin-1 expression facilitates endothelial cell differentiation. As such, caveolin-1 expression serves different roles during the different phases of angiogenesis: (i) the initial proliferative phase *versus* (ii) the terminal differentiation phase. As caveolin-1 is a known inhibitor of cell proliferation and a widely accepted marker of differentiation, it would be predicted that caveolin-1 levels would be down-regulated during endothelial cell proliferation and up-regulated during endothelial differentiation, exactly as we observed here. Furthermore, our current findings are the first demonstration that caveolin-1 expression can stimulate a differentiation process.

Proliferation and differentiation are hallmarks of the developmental process; however, what lies at the interface between these two opposing processes remains to be elucidated. Several features of caveolin-1 make it an attractive candidate to act as a "toggle switch" between proliferation and differentiation in numerous cell types. In NIH 3T3 cells, caveolin-1 expression levels are down-regulated when cells are rapidly dividing and are dramatically up-regulated at confluency (30), suggesting that up-regulation of caveolin-1 expression may be important to mediate normal contact inhibition. In addition, caveolin-1 expression has been demonstrated to negatively regulate the activated state of the p42/44 MAPK cascade in numerous cell types (30). Further evidence has also shown that overexpression of caveolin-1 blocks the oncogenic transformation of NIH 3T3 cells and inhibits the proliferation of human breast cancer cells (27, 29–32).

Indeed, caveolins are most abundantly expressed in terminally differentiated cells such as endothelial cells, adipocytes, and myocytes and are dramatically up-regulated during adipogenesis and myotube formation (21, 23). For example, C2C12 muscle cells dramatically induce caveolin-3 mRNA and protein expression during differentiation, coincident with myotube formation. Interestingly, using an antisense approach, our group has demonstrated that C2C12 cells that fail to express caveo-

lin-3 are unable to undergo myoblast fusion and therefore cannot form myotubes (31). In addition, we found that three human rhabdomyosarcoma cell lines that do not express caveolin-3 fail to undergo myoblast fusion.

Therefore, one hypothesis is that caveolin may serve as a "differentiation sensor" that monitors and responds to changes in the relative balance of positive and negative factors to "tell" a target cell whether to remain quiescent or to become proliferative. It is clear that this hypothesis, which has been supported by adipocytes and muscle cells, is now supported by endothelial cells as well. In addition, Griffoni *et al.* (44) showed that antisense caveolin-1 oligodeoxynucleotides partially inhibit blood vessel development in chick embryo chorioallantoic membranes. However, a lack of critical controls leaves the conclusions of this study open to question. To investigate whether caveolin-1 behaves similarly in endothelial cells as compared with other cell systems, we asked three separate questions: 1) do angiogenic signals given under growth conditions (low confluency) down-regulate caveolin-1 expression; 2) is caveolin-1 up-regulated during endothelial tubule formation; and 3) is caveolin-1 expression necessary for tubule formation (confluent conditions)? To answer the latter question, we employed the Matrigel three-dimensional gel system.

It is now well accepted that the Matrigel system is one of the reliable models to assess angiogenesis *in vitro* (45, 46). Short-term culture on Matrigel does not involve cell proliferation and migration (47, 48). Hence, in this study, we employed the Matrigel assay system to investigate the potential role of caveolin in endothelial cell differentiation. The Matrigel matrix is a mixture of mouse basement membrane extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins (47, 49, 50). It is composed of laminin, collagen type IV, entactin/nidogen, and heparan sulfate proteoglycan. It also contains growth factors, matrix metalloproteinases, and other components. Thus, the Matrigel matrix closely mimics the physical properties and functional characteristics of basement membranes *in vivo* (51).

We seeded endothelial cells at super-confluency and incubated them on Matrigel for a short period of time (not >24 h) to focus primarily on the effect of caveolin-1 on endothelial cell differentiation. Our current results clearly demonstrate that up-regulation of caveolin-1 expression dramatically accelerates endothelial cell differentiation (*i.e.* tubule formation appeared after only 6 h of incubation in caveolin-1-overexpressing cells *versus* 12 h in control cells) and leads to a dramatic increase in the number of capillary-like tubular structures. These observations are further supported by our use of an antisense adenoviral approach that shows that down-regulation of caveolin-1 expression almost completely prevents tubule formation (*i.e.* it reduced the formation of tubular structures by >10-fold). Consistent with the unique function of the caveolin-1 scaffolding domain and its interaction with key signaling molecules, we show that delivery of the caveolin-1 scaffolding domain into the cytoplasm of living endothelial cells is sufficient to enhance capillary-like tubule formation. Given the observations that (i) caveolin-1 functions as an endogenous inhibitor of the p42/44 MAPK signaling cascade and (ii) up-regulation of caveolins is crucial for the differentiation of muscle cells as well as epithelial cells (31, 32, 52), our current results are consistent with the idea that caveolin-1 expression also plays a critical role in the regulation of endothelial cell differentiation and angiogenesis.

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