Vascular Endothelial Cadherin Promotes Breast Cancer Progression via Transforming Growth Factor β Signaling

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Abstract

Epithelial-to-mesenchymal transition (EMT) is an important event during carcinoma progression and leads to increased tumor cell malignancy. Here, we show that vascular endothelial (VE)-cadherin is induced during EMT in mammary tumor cells and is aberrantly expressed in invasive human breast carcinomas. VE-cadherin enhanced the capacity of fibroblastoid tumor cells to proliferate, form cord-like invasive structures, and adhere to endothelial cells, characteristics that are key contributors to their increased malignancy and metastatic potential. Consistently, VE-cadherin expression in malignant fibroblastoid tumor cells promoted the growth of experimental mammary carcinomas *in vivo*. Analysis of the signaling mechanisms involved revealed that VE-cadherin expression influences the levels of Smad2 phosphorylation and expression of target genes of transforming growth factor-β (TGF-β), a major mediator of advanced tumor progression and malignant tumor cell proliferation. VE-cadherin might thus promote tumor progression not only by contributing to tumor angiogenesis but also by enhancing tumor cell proliferation via the TGF-β signaling pathway. This article provides evidence for a novel function of VE-cadherin in tumor progression and reveals a previously unknown molecular link between VE-cadherin expression and TGF-β signaling. Our findings may have important implications for the clinical application of anti–VE-cadherin strategies. [Cancer Res 2008;68(5):1388–97]

Introduction

Carcinomas account for the majority of tumors and arise from epithelial cells that present an aberrant control of proliferation. Epithelial-to-mesenchymal transition (EMT), a process by which tumor cells lose their epithelial polarity and acquire a fibroblastoid phenotype, is an important event during tumor progression. Key characteristics of EMT are the loss or redistribution of some tight and adherens junction proteins, and the gain of a highly motile and invasive phenotype, which lead to an increased metastatic potential (1, 2).

The role played by transforming growth factor-β (TGF-β) in initiating and maintaining EMT is well-established (3). Although TGF-β acts as a tumor suppressor by inhibiting epithelial cell growth, it also promotes carcinoma progression and metastasis by inducing EMT, invasiveness, and blood vessel intravasation by carcinoma cells (4, 5). TGF-β binds to receptor serine/threonine kinases, which activate intracellular Smads and other pathways that regulate gene expression (6). For example, the TGF-β/Smad pathway can promote the transcription of three known regulators of EMT, the zinc-finger proteins Snail and Slug, and the basic helix-loop-helix protein Twist (7). These transcription factors repress *epithelial-cadherin* (*E-cadherin*) gene expression (5).

The down-regulation of E-cadherin is considered as a key event in EMT (1, 2). Cadherins are transmembrane glycoproteins localized at adherens junctions and mediate calcium-dependent homotypic cell-cell adhesion. Different cell types express a specific panel of cadherins: E-cadherin is preferentially expressed in cells of epithelial origin, mesenchymal and neuronal cells express neuronal-cadherin (N-cadherin), whereas vascular endothelial (VE)-cadherin is specific to endothelial cells (8–10).

VE-cadherin plays a pivotal role in the control of vascular integrity and permeability, and contributes to endothelial cell assembly in tubular structures (11–13). VE-cadherin can associate with vascular endothelial growth factor receptor (VEGFR)-2 and modulate its signaling pathways to promote cell survival and reduce proliferation (14, 15). Inactivation of the *VE-cadherin* gene is lethal in mouse embryos and results in the impairment of vascular remodeling and maturation (15, 16). VE-cadherin is also important for tumor angiogenesis, and its expression is up-regulated in the vasculature of breast carcinoma (17). Blocking VE-cadherin function with monoclonal antibodies in mouse tumor models leads to the inhibition of tumor angiogenesis and growth (18, 19).

Because of the important role cadherins play in cell recognition, adhesion, and signaling, modulation of their function and expression has significant implications for the progression of tumors. Indeed, a switch from E-cadherin to N-cadherin expression has been correlated with increased tumor growth and metastasis in a mouse mammary tumor model (20, 21). Studies on cancer cell lines also indicate that N-cadherin–mediated adhesion is linked to a more malignant and invasive behavior (21). Furthermore, aberrant expression of cadherins has been observed in many tumor cell types: placental-cadherin has been detected in some cases of ductal carcinoma as well as in ~30% of mammary carcinoma cell lines (20, 22), whereas VE-cadherin has been observed in some cases of sarcoma and in highly aggressive melanoma cells (23–25). However, very little is known about the mechanisms leading to the induction of aberrantly expressed VE-cadherin and the possible contributions it may have on tumor cell morphology and behavior.

To address this issue in the context of tumor progression and EMT, we used a mouse mammary carcinoma model, based on epithelial cells transformed by the v-Ha-Ras oncoprotein. These

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cells form rapidly growing tumors in mice and undergo EMT during tumor growth (26). By using cell lines representing different stages of tumor progression, we show that VE-cadherin is present at cell-cell contacts in aggressive fibroblastoid breast cancer cells, and that its expression is induced during TGF-β1–mediated EMT *in vitro*. VE-cadherin expression in fibroblastoid mammary carcinoma cells promotes cell proliferation and tumor growth, and is associated with increased TGF-β signaling. Taken together, our results show that VE-cadherin expression in certain cancer cells promotes tumor progression by enhancing TGF-β signaling.

Materials and Methods

Cell culture. EpH4, Ep5, Ep5ExTu, and 293T cells were cultured in DMEM, 4.5g/mL glucose, and 10% FCS as described (26, 27). MS1 murine endothelial cells (28) were kept in DMEM, 4.5 g/mL glucose, and 5% FCS. Human umbilical vein endothelial cells (HUVEC) were grown in EGM-2 (Cambrex). To induce EMT *in vitro*, Ep5 cells were cultured with 8 ng/mL TGF-β1 (R&D Systems). For TGF-β receptor (TGFβR) kinase inhibition, Ep5ExTu cells were treated with 10 μmol/L SB431542 every other day in fresh culture medium.

Viral vector production and infection of target cells. Small interfering RNA (siRNA)-coding oligos against VE-cadherin (5′-GTCTCTGAGTAC-TTCCTTA-3′) were designed and verified to be specific to VE-cadherin by Blast search against the mouse genome. A scramble sequence without significant homology to murine sequences was used as control (5′-AGTCGC-TTAGAAACGAGAA-3′). The siRNA sequences in sense and antisense interrupted by a 9bp loop sequence (5′-TTCAAGAGA-3′) were cloned in the lentiviral vector pLVTHM. The packaging of the vector was obtained as described (27) by cotransfection of 293T cells with pLVTHM, psPAX2, and pMD2G (vectors kindly provided by D. Trono, University of Lausanne, Lausanne, Switzerland). The Ep5ExTu cells were infected with the lentiviral vector and selected on the basis of green fluorescent protein expression by fluorescence-activated cell sorting (FACS) sorting. Clones were expanded, and the expression of VE-cadherin was monitored by immunoblot.

RNA isolation and reverse transcription-PCR analysis. RNA was isolated from total cell lysates using RNeasy Mini kit (Qiagen). Aliquots of 3 μg RNA were reverse transcribed using Superscript II (Invitrogen) and random hexameric primers (Roche). PCR primers are described in Supplementary Table S1.

Immunoblot analysis. Immunoblot analysis was performed as described previously (29). The primary antibodies were the anti-mouse VE-cadherin monoclonal antibody 11D4.1 (30), anti–β-actin, anti–E-cadherin (Sigma), anti–N-cadherin, anti-Smad2/3, and anti-phosphoSmad2 (Santa Cruz Biotechnology). The secondary antibodies were horseradish peroxidase–conjugated anti-rabbit IgG (Cell Signaling Technology) and anti-rat IgG (Jackson Immunoresearch).

Immunofluorescence. Forty-eight hours after plating, cells were fixed and stained using the same primary antibodies as for immunoblot. The secondary antibodies were goat anti-rat Alexa 488 and chicken anti-rabbit Alexa 594 (Molecular Probes).

Proliferation assay. Cells were plated in 24-well plates in complete culture medium. After 24 h, 100 pg/mL of 11D4.1 VE-cadherin antibody was added. One hundred picograms per mL of Mec13.3 platelet/endothelial cell adhesion molecule 1 (PECAM-1) antibody was used as an isotype control. After 3 days, cells were detached with trypsin and counted. Proliferation was also evaluated after treatment with three independent siRNA sequences directed against murine VE-cadherin [5′-GUCUCUGA-GUACUUCCUUAdTdT-3′ and 5′-GAGCAAGGAUAGCCUCUAAdTdT-3′ (Eurogentec) and siRNA sc-36813 (Santa Cruz Biotechnology)] or Smad2 [5′-CCAAGAAUUUGCUGCUCUUdTdT3′, 5′CCAAGCACUUGCUCU-GAAAdTdT-3′ and 5′-GGCUUGCUCUCCAACGUUAdTdT-3′ (Eurogentec)]. A scramble sequence was used as negative control (5′-AGUCGCUUA-GAAACGAGAAdTdT-3′; Eurogentec). Ep5ExTu cells were transfected with 250 nmol/L siRNA in Lipofectamine2000 diluted in Opti-MEM I (Invitrogen). Cells were detached and counted 2 days after transfection.

Cord-like structure formation. A collagen solution was prepared by mixing acidic type I collagen from rat tail (2 mg/mL; Sigma) with 12.5% v/v 10× HBSS. The pH was adjusted to 7.4 with 0.1 N NaOH. Five hundred microliters of collagen solution was then rapidly added to 500 μL of cell suspension (10^5 cells/mL in DMEM 10% FCS) containing 1 μg/mL of 11D4.1 VE-cadherin antibody and transferred into a 24-well plate. Pictures were taken 6 days after seeding.

Adhesion assay. Tumor cells were stained with 2.5 μg/mL DiI (Invitrogen) and detached as described (31). Cells (10^5) were preincubated with 1 μg/mL of 11D4.1 VE-cadherin antibody, washed, and added to a confluent HUVEC monolayer in a 6-well plate. In some conditions, the antibody was added directly with the tumor cells onto the HUVEC monolayer. The cells were incubated for 30 min at 37°C and washed thrice with PBS. The number of adherent cells was counted in five random fields per condition.

Tumor experiments. Cells (10^5 cells per tumor) were injected in BALB/c mice (Taconic) as previously described (26). Tumors were collected 15 days after injection. VE-cadherin and PECAM-1 expression were visualized on frozen sections by immunohistochemistry using the Vectastain avidin-biotin complex method and AEC kits according to manufacturer's instructions (Vector Laboratories). Sections were counterstained with a hematoxylin solution. Primary antibodies were 11D4.1 anti–VE-cadherin and Mec13.3 anti–PECAM-1. Double-immunofluorescent staining was performed using 11D4.1 anti–VE-cadherin and anti–VEGFR-1 (Sigma). The secondary antibodies were goat anti-rat Alexa 594 and rabbit anti-goat Alexa 488 (Molecular Probes). VEGFR-1 was used instead of PECAM-1 as an endothelial cell marker for double immunofluorescence experiments because both the 11D4.1 anti–VE-cadherin and the Mec13.3 anti–PECAM-1 are of the same isotype. All experiments were approved by and performed according to the guidelines of the animal ethical committee of the Medical Faculty, University of Dresden, Dresden, Germany.

Immunohistochemistry on human tumor sections. Slides were dewaxed, and immunohistochemical staining was performed using an automated immunostainer (Benchmark Ventana) according to the manufacturer's protocols. The primary antibodies were anti-human VE-cadherin F-8 (Santa Cruz Biotechnology) and anti-human VE-cadherin BV6 (Chemicon). The signal was amplified using the VENTANA amplification kit and visualized using avidin-biotin labeling and 3,3′-diaminobenzidine. Slides were counterstained with H&E. Staining with isotype control antibodies was performed to confirm the staining specificity.

Results

VE-cadherin is expressed in aggressive tumor cells *in vitro*. The spontaneously immortalized mouse mammary epithelial cell line, EpH4, exhibits a polarized phenotype (26). These cells, transformed by the v-Ha-Ras oncogene and designated Ep5 cells, retain the epithelial phenotype but form rapidly growing and highly vascularized tumors in BALB/c mice. During tumor progression, they undergo a dramatic conversion from an epithelial to a fibroblastoid, invasive phenotype (26, 29). Ep5ExTu cells, derived from Ep5 tumors grown in mice, have undergone EMT and present a fibroblastoid phenotype. Tumor progression in this experimental mouse mammary carcinoma is associated with a Ras-dependent switch to the angiogenic state (29).

To investigate the effect of EMT on gene expression, we performed an Affymetrix microarray analysis comparing Ep5ExTu to EpH4 cells. The expression of several genes coding for proteins of the cadherin family was modulated by >2-fold (data not shown). Among the up-regulated genes, we identified *N-cadherin* and, unexpectedly, *VE-cadherin*, whereas *E-cadherin* was down-regulated, reflecting the nonepithelial phenotype of the Ep5ExTu cell line.

To confirm these observations, the mRNA and protein expression levels of these cadherins in EpH4, Ep5, and Ep5ExTu cells were determined. We found that VE-cadherin, which is widely

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considered as an endothelial-specific cadherin, is aberrantly expressed in Ep5ExTu cells but not in EpH4 or Ep5 cells (Fig. 1*A* and *B*). N-cadherin was absent from EpH4 cells but strongly expressed in (ras transformed) Ep5 and Ep5ExTu cells. On the other hand, E-cadherin could be detected in (epithelial) EpH4 and Ep5, but not in (fibroblastoid) Ep5ExTu cells, in accordance with the well-established role of E-cadherin in the maintenance of the epithelial phenotype. Immunofluorescence staining revealed

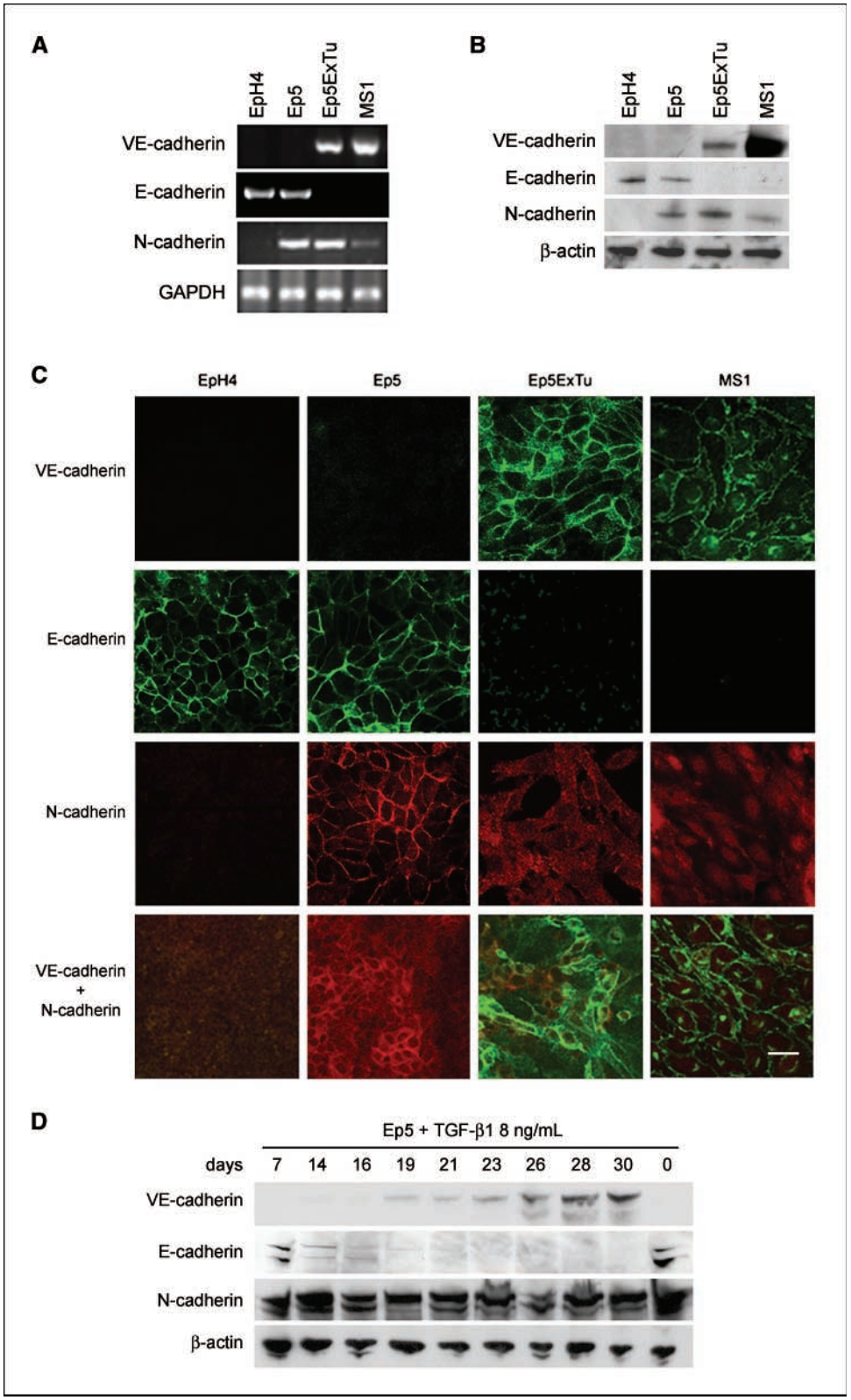

Figure 1. VE-cadherin is expressed in Ep5ExTu cells and is induced by TGF-β1–mediated EMT. *A* and *B*, mRNA (*A*) and protein (*B*) expression levels of VE-cadherin, E-cadherin, and N-cadherin in EpH4, Ep5, and Ep5ExTu cell lines. MS1 endothelial cells were used as a positive control for VE-cadherin expression. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin were used as loading controls. *C,* immunolocalization of VE-cadherin, E-cadherin, and N-cadherin by immunofluorescence microscopy. *Bar,* 50 μm. *D,* Ep5 cells were cultivated in the presence of 8 ng/mL TGF-β1 for the times indicated to induce EMT. VE-cadherin, E-cadherin, and N-cadherin protein expression levels were analyzed by immunoblot. β-actin was used as loading control.

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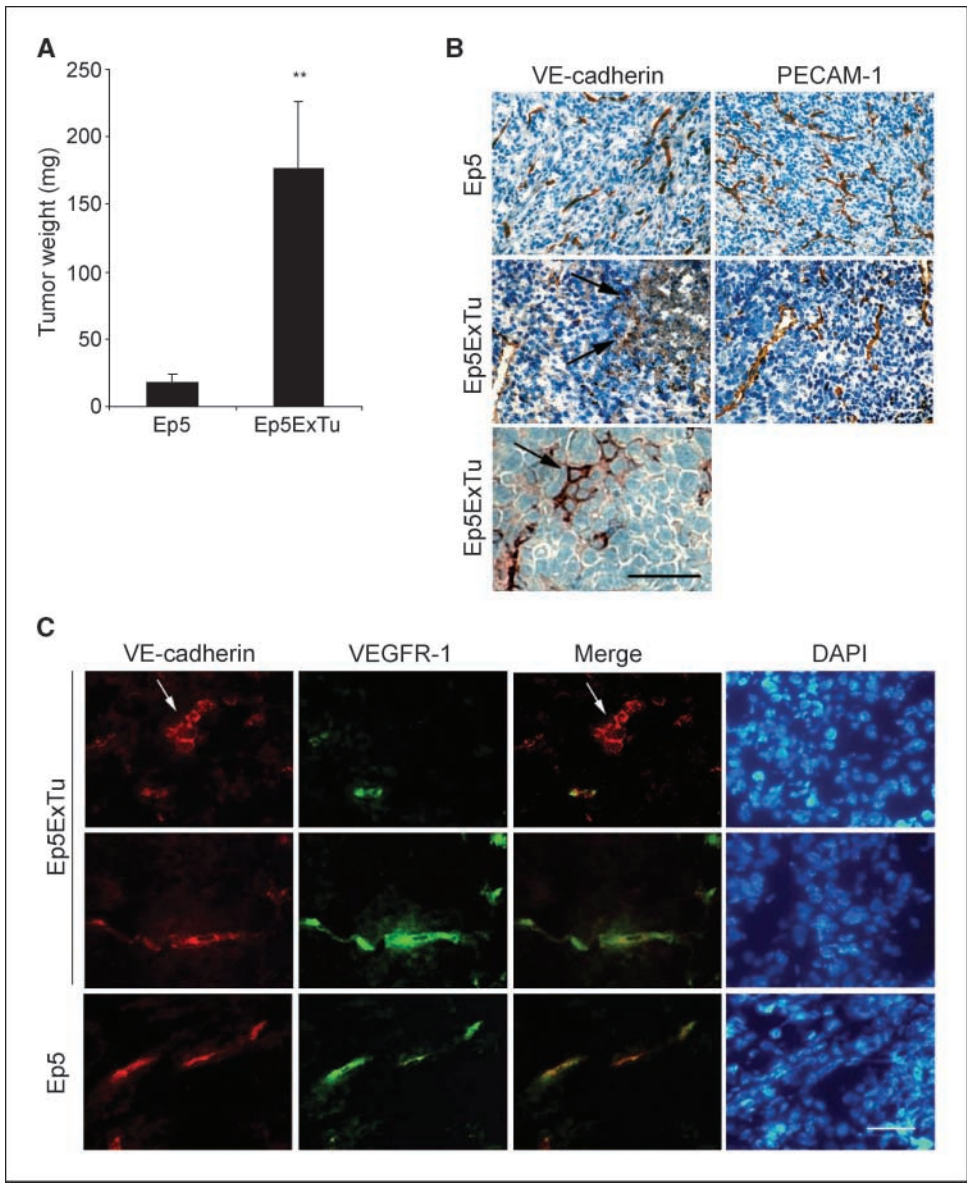

Figure 2. VE-cadherin is expressed in Ep5ExTu tumor cells *in vivo. A,* tumor weight of Ep5 and Ep5ExTu s.c. tumors harvested 15 d after injection. *Columns,* mean (n = 10); *bars,* SE. **, P = 0.007. Statistical significance versus Ep5 tumors was determined by unpaired Student's t test. *B,* immunohistochemical staining for VE-cadherin and PECAM-1 on Ep5 and Ep5ExTu tumor sections. *Arrows,* examples of tumor cells expressing VE-cadherin. *Bars,* 50 μm. *C,* double-immunofluorescent staining for VE-cadherin and VEGFR-1 on Ep5 and Ep5ExTu tumor sections. The overlay confirms that VE-cadherin is expressed in Ep5ExTu tumor cells (*arrows*) but not in Ep5 tumor cells. *Bar,* 25 μm.

N-cadherin expression at cell-cell junctions in Ep5 cells (Fig. 1*C*). However, in Ep5ExTu cells, which displayed VE-cadherin expression at cell-cell contacts, N-cadherin staining was uniform throughout the cell. The localization of VE-cadherin at the cell surface was confirmed by FACS analysis (Supplementary Fig. S1). No colocalization between N- and VE-cadherin staining could be observed in Ep5ExTu cells, suggesting that these two cadherins are not simultaneously localized at the cell-cell junctions. We therefore tested the possibility that N-cadherin localization could be influenced by VE-cadherin as previously suggested (32, 33). The knock-down of VE-cadherin expression by siRNA in Ep5ExTu cells led to an enrichment of N-cadherin at cell-cell contacts, whereas no differences in N-cadherin mRNA or protein levels were observed (Supplementary Fig. S2). The knock-down of VE-cadherin expression in Ep5ExTu cells or VE-cadherin overexpression in Ep5 cells did not result in changes in N-cadherin processing (Supplementary Fig. S3). VE-cadherin is thus influencing N-cadherin localization without affecting its expression level and processing.

VE-cadherin expression is induced during TGF-β1–mediated EMT *in vitro*. The fact that Ep5ExTu cells, in contrast to Ep5 cells, express VE-cadherin but not E-cadherin suggested that the loss of E-cadherin and the up-regulation of VE-cadherin occur during EMT. To test this hypothesis, we induced EMT in Ep5 cells by TGF-β treatment (26, 34). Morphologic changes from an epithelial to a fibroblastoid appearance could be observed in >50% of the cells 8 days after the start of the treatment (Supplementary Fig. S4; ref. 34). From day 19 onward, VE-cadherin protein expression could be detected, and its concentration gradually increased until the end of the treatment (day 30), demonstrating that VE-cadherin expression is induced by EMT *in vitro* (Fig. 1*D*). Conversely, the expression of E-cadherin was progressively down-regulated. The levels of N-cadherin protein expression were not altered by the treatment. The expression of VE-cadherin could not be induced in Ep5 cells treated with similar or higher doses of TGF-β1 for up to 3 days, suggesting that VE-cadherin expression is not directly regulated by TGF-β1

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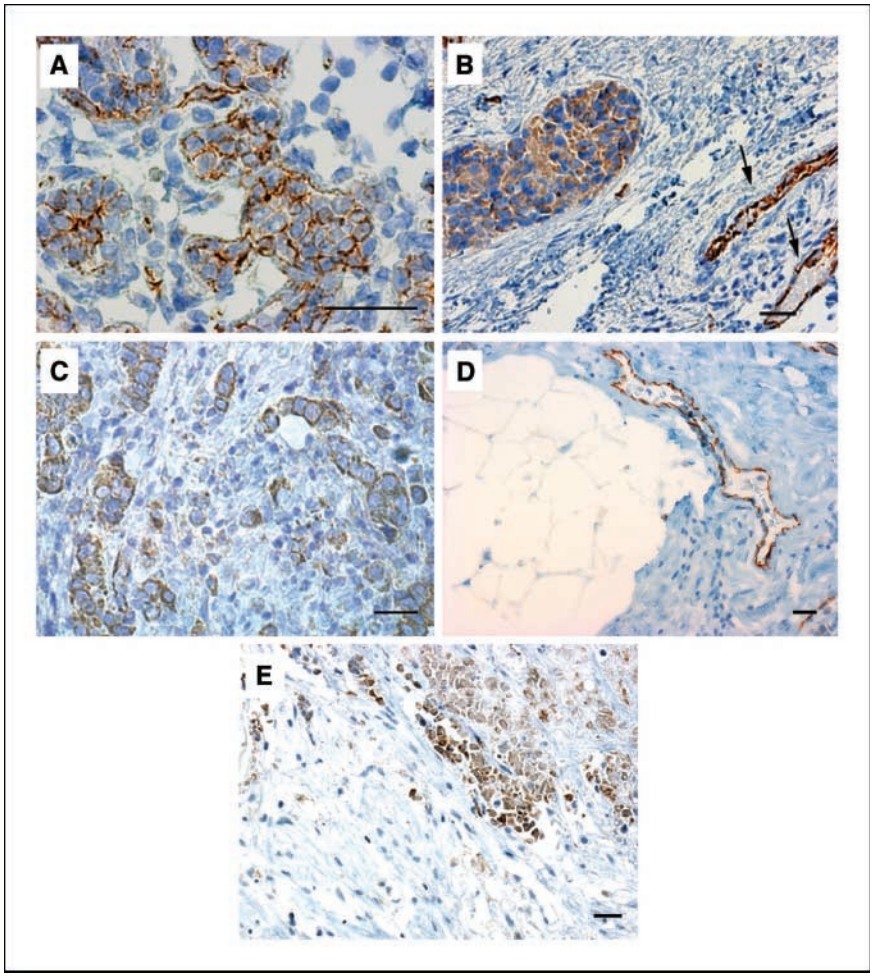

Figure 3. VE-cadherin is expressed in human mammary carcinoma cells. Immunohistochemical staining for VE-cadherin (*A*) in a moderately differentiated lobular invasive breast cancer. *B* and *C*, VE-cadherin expression in two different moderately differentiated ductal invasive breast cancers. *Arrows*, examples of blood vessels within the tumor. *D*, VE-cadherin expression in blood vessels present in the normal tissue surrounding a moderately differentiated ductal invasive breast cancer. *E*, VE-cadherin expression at the invading front of a poorly differentiated ductal invasive breast cancer. Tumor sections were stained with the VE-cadherin F-8 antibody (*A, B, D*, and *E*) or the BV6 antibody (*C*). *Bars*, 25 μm.

but by other molecular mechanisms activated during EMT (Supplementary Fig. S5).

VE-cadherin is expressed in aggressive tumors *in vivo*. To analyze if VE-cadherin is also expressed when Ep5ExTu form tumors *in vivo*, Ep5 or Ep5ExTu cells were injected s.c. in BALB/c mice. Fifteen days after injection, Ep5ExTu tumors were significantly bigger than Ep5 tumors ($P = 0.007$), reflecting the more aggressive phenotype of the former (Fig. 2*A*). In Ep5ExTu tumors, VE-cadherin could be detected by immunohistochemistry in endothelial cells as well as in tumor cells (Fig. 2*B*). In contrast, in Ep5 tumors, the staining patterns for VE-cadherin and PECAM-1 were similar, showing expression in endothelial cells only. These results were confirmed by double immunofluorescence staining for VE-cadherin and VEGFR-1, used as an endothelial cell marker (Fig. 2*C*).

VE-cadherin is expressed in human mammary carcinomas. The expression of VE-cadherin was also investigated in human mammary carcinomas. Using the F-8 VE-cadherin antibody, VE-cadherin was detected in a subset of tumor cells in 27 of the 37 (73%) invasive carcinomas examined, as well as in the tumor vasculature (Fig. 3; Supplementary Table S2). The immunoreactivity pattern in tumor cells ranged from absent, to weak in 38% of the cases, to moderate or strong staining in 35% of the cases (Supplementary Table S2). In some tumor cells, VE-cadherin staining was enriched at the cell membrane (Fig. 3*A* and *C*), whereas in other subsets of tumor cells, positive staining could also be observed in the cytoplasm (Fig. 3*B*). VE-cadherin–positive tumor cells were present in the tumor center as well as at invading fronts (Fig. 3*E*). Adjacent normal breast tissues were negative for VE-cadherin (Fig. 3*D*; Supplementary Fig. S6). Similar results were also obtained with a second VE-cadherin antibody (BV6), which recognizes a different VE-cadherin epitope (Fig. 3*C*).

VE-cadherin expression by malignant breast cancer cells promotes their proliferation, cord-like structure formation, and adhesion to endothelial cells. To investigate the functional role played by VE-cadherin in Ep5ExTu cells, we performed various *in vitro* assays indicative of tumor growth and metastasis potential. The influence of VE-cadherin on cell proliferation was assayed by treating the EpH4, Ep5, Ep5ExTu mammary cells, and MS1 endothelial cells with 100 pg/mL of VE-cadherin blocking antibody. This treatment reduced Ep5ExTu cell proliferation by ~40% (Fig. 4*A*), whereas the proliferation rate of the other cell lines was not affected. When Ep5ExTu cells were treated with similar amounts of an anti–PECAM-1 antibody, used as an isotype control [PECAM-1 is not expressed by Ep5ExTu cells based on FACS analysis (Supplementary Fig. S1) and immunoblot analysis (data not shown)], no change in the proliferation rate was observed, showing that the inhibition of cell proliferation is specific to VE-cadherin blocking. The inhibitory effect of the anti–VE-cadherin antibody was dose dependent at concentrations ranging from 50 to 100 pg/mL (Supplementary Fig. S7). The function of VE-cadherin in tumor cell proliferation was confirmed by RNA

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interference experiments. The treatment with VE-cadherin siRNA resulted in the down-regulation of VE-cadherin protein levels and reduced the proliferation of Ep5ExTu cells by ~45% (Fig. 4*B*). Additionally, BrdUrd incorporation in VE-cadherin siRNA–treated cells was reduced to 59% ± 13% of that of control cells (Supplementary Fig. S8). Similar results were obtained with two other independent mouse VE-cadherin siRNA (data not shown). No increase in cell death was observed in samples treated with siRNA or VE-cadherin antibody, as measured by trypan blue exclusion (data not shown) or by the detection of cleaved caspase-3 (Supplementary Fig. S9). These results show that VE-cadherin expression promotes the proliferation of Ep5ExTu cells without influencing cell survival.

We also tested if the growth and invasion of Ep5ExTu cells in collagen I gels was affected by a VE-cadherin blocking antibody. Ep5ExTu cells easily invaded collagen I gels and organized themselves in networks of cord-like structures, whereas Ep5 cells were unable to form such networks (Fig. 4*C*). However, when Ep5ExTu cells were treated with VE-cadherin blocking antibody, the formation of the cord-like structures was impaired (Fig. 4*C*; quantification in Supplementary Fig. S10), indicating that VE-cadherin expression is required for this function.

Because VE-cadherin is involved in homotypic cell-cell interactions, we tested if the expression of VE-cadherin by Ep5ExTu cells could promote their adhesion to endothelial cells. Ep5ExTu cells had a better capacity to bind to a HUVEC monolayer than Ep5 cells (Fig. 4*D*). Furthermore, when the 11D4.1 VE-cadherin blocking antibody was added, the number of Ep5ExTu cells bound to the HUVEC monolayer was reduced. This was also observed with cells that were preincubated with the VE-cadherin antibody before their addition to the HUVEC monolayer. Because the 11D4.1 VE-cadherin antibody does not bind to human VE-cadherin, based on immunoblot analysis (Fig. 4*D, inset*), this excludes the possibility that the reduced cell adhesion depends on blocking of VE-cadherin expressed by HUVEC.

VE-cadherin promotes tumor growth *in vivo*. To study the effect of VE-cadherin on tumor growth, Ep5ExTu cell lines stably expressing a VE-cadherin short hairpin RNA (shRNA) were generated. Two independent clones (VE-cad shRNA.1 and VE-cad shRNA.2) that showed strongly reduced VE-cadherin expression compared with control cell lines stably expressing a scrambled shRNA or the empty vector were selected (Fig. 5*A*). Consistent with the results obtained with the transient knock-down of VE-cadherin (Fig. 4*B*), the control cell lines grew faster than the Ep5ExTu cells expressing VE-cadherin shRNA (Supplementary Fig. S11). When injected in mice, both VE-cad shRNA.1 and VE-cad shRNA.2 cell lines formed tumors that grew significantly slower than the controls (Fig. 5*B*). Furthermore, the weights of tumors, collected 15 days after injection, were significantly reduced for the VE-cad shRNA.1 and VE-cad shRNA.2 clones compared with the Scramble shRNA.1 and Empty vector.1 clones (Fig. 5*C*). The down-regulation of VE-cadherin expression in the VE-cad shRNA.1 and VE-cad

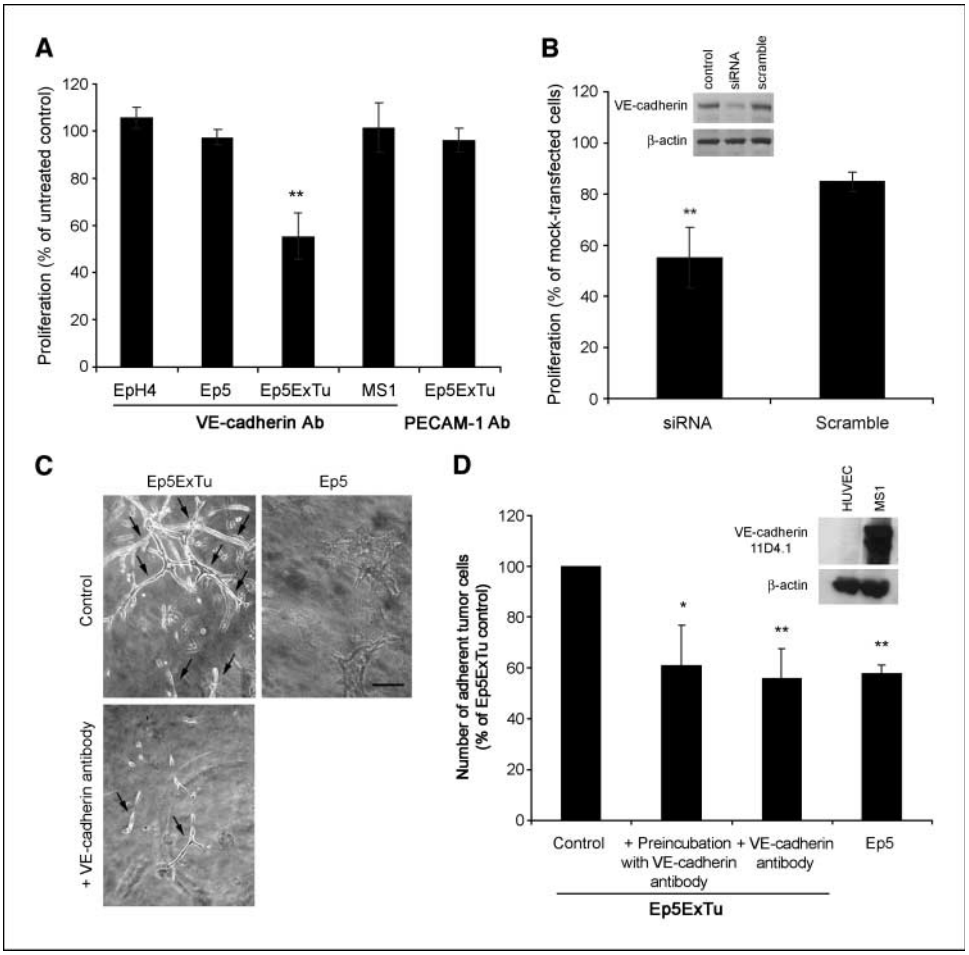

Figure 4. VE-cadherin expression by malignant breast cancer cells promotes their proliferation, cord-like structure formation, and adhesion to endothelial cells. *A,* cells were cultured in complete culture medium ± 100 pg/mL VE-cadherin antibody (*VE-cadherin Ab*) and counted after 3 d. Ep5ExTu were also treated with a PECAM-1 antibody used as an isotype control (*PECAM-1 Ab*). *B,* Ep5ExTu cells were transfected either with Lipofectamine2000 only (*control*), VE-cadherin siRNA (*siRNA*) or a scrambled siRNA sequence (*scramble*) and counted 2 d after transfection. The efficiency of VE-cadherin protein expression knock-down was analyzed by immunoblot (*inset*). *C,* Ep5ExTu cells were cultivated in collagen I gel in complete culture medium ± 1 μg/mL VE-cadherin antibody. Pictures were taken 6 d after seeding. Ep5 cells grown in complete culture medium are also shown. *Arrows,* cord-like structures. *Bar,* 100 μm. *D,* Ep5ExTu cells were stained with DiI, incubated with or without 1 μg/mL of VE-cadherin antibody, and added to HUVEC monolayers as described in Materials and Methods. Fluorescent cells from five different fields were counted (magnification, ×100). The specificity of the 11D4.1 VE-cadherin antibody to mouse VE-cadherin is shown by immunoblot with endothelial cells of human (*HUVEC*) and mouse (*MS1*) origin (*inset*). β-actin was used as loading control. The results are expressed as a percentage of the number of cells in untreated or mock-transfected controls. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$. Statistical significance versus untreated or mock-transfected controls was determined by unpaired Student's *t* test.

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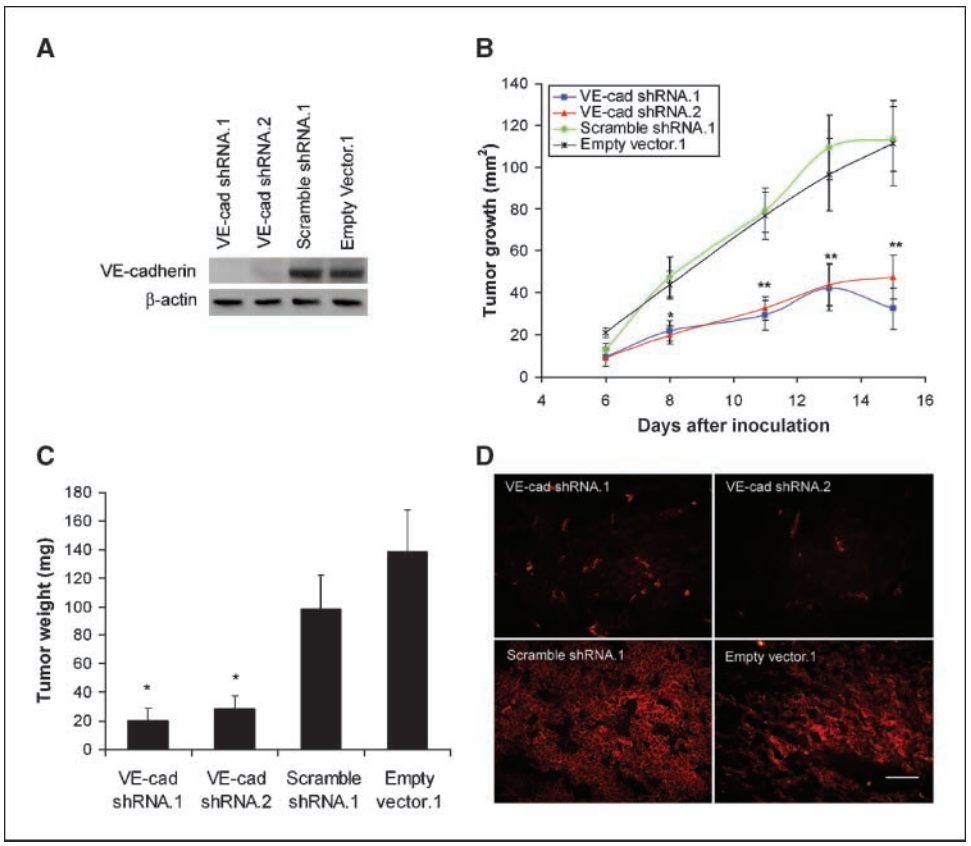

Figure 5. Stable suppression of VE-cadherin expression by shRNA reduces Ep5ExTu tumor growth *in vivo*. *A,* VE-cadherin protein expression was examined by immunoblot in Ep5ExTu clones stably expressing scrambled shRNA (*scramble shRNA.1*), empty vector (*empty vector.1*), or VE-cadherin shRNA. Two independent clones expressing VE-cadherin shRNA are shown (*VE-cad shRNA.1* and *VE-cad shRNA.2*). β-actin was used as loading control. *B,* growth curve of mammary tumors formed by Ep5ExTu clones. *C,* weight of mammary tumors formed by Ep5ExTu clones 15 d after s.c. injection. *D,* immunofluorescent staining for VE-cadherin on tumor sections. *Bar,* 25 μm. *Columns,* mean of one of three independent experiments ($n = 10$); *bars,* SE. *, $P < 0.05$; **, $P < 0.01$. Statistical significance versus Scramble shRNA.1 was determined by unpaired Student's *t* test.

shRNA.2 tumors was confirmed by immunofluorescence (Fig. 5*D*). Scramble shRNA.1 and Empty vector.1 tumors displayed areas where tumor cells were VE-cadherin positive, whereas VE-cadherin was only detectable in blood vessels in VE-cad shRNA.1 and VE-cad shRNA.2 tumors.

Suppression of VE-cadherin expression affects Ep5ExTu cell morphology and expression of EMT-related genes. VE-cad shRNA.1 and VE-cad shRNA.2 cell lines adopted a more epithelial phenotype than the control cell lines, which displayed the characteristic fibroblastoid phenotype of Ep5ExTu cells (Fig. 6*A*). To test if this change in morphology could be associated with a reversion to an epithelial phenotype, we investigated the expression of EMT-related genes. The expression of the transcription factors Snail and Slug was down-regulated in Ep5ExTu treated with VE-cadherin shRNA. However, Twist and E-cadherin mRNA levels were not affected (Fig. 6*B*).

Suppression of VE-cadherin down-regulates Smad2 phosphorylation and TGF-β target gene expression in Ep5ExTu cells. Because TGF-β is a key inducer of EMT and was recently reported to regulate Snail, Slug, and Twist expression via Smad signaling (7), we investigated if VE-cadherin expression could interfere with this pathway. The expression level of the regulatory Smads, Smad2 and Smad3, remained unaffected by the down-regulation of VE-cadherin expression. However, the phosphorylation levels of Smad2 were reduced in VE-cad shRNA.1 and VE-cad shRNA.2 cell lines compared with the controls (Fig. 6*C*). Moreover, the mRNA levels of plasminogen activator inhibitor (PAI-1) and matrix metalloproteinase-9 (MMP-9), two known target genes of the Smad signaling pathway (Supplementary Figs. S12 and S13; refs. 35, 36), were down-regulated in the VE-cadherin knock-down cell lines, whereas the mRNA levels of TGF-β receptors I (Alk-5) and II (Tgfbr2) were unaffected (Fig. 6*B*). Reduced MMP-9 activity in the conditioned medium from VE-cad shRNA.1 and VE-cad shRNA.2 cell lines compared with the controls was also observed by zymography (Supplementary Fig. S14).

Inhibition of the TGF-β pathway reduces Ep5ExTu cell proliferation. To test whether the inhibition of TGF-β signaling observed after VE-cadherin suppression could account for the reduction in proliferation, we treated the cell lines with the TGF-β-receptor antagonist SB431542. This compound has no effect on components of the extracellular signal-regulated kinase, c-Jun-NH_2-kinase, or p38 mitogen-activated protein kinase (MAPK) pathways, or on components of the signaling pathways activated in response to serum (37). The Smad2 phosphorylation level as well as PAI-1 and MMP-9 mRNA levels were reduced in SB431542-treated Ep5ExTu cells, whereas the phosphorylation of p42/p44 MAPK and the VE-cadherin protein levels were not affected (Supplementary Fig. S12). The blockade of the TGF-β pathway led to a reduction in proliferation of ~50% for the Ep5ExTu, Scramble shRNA.1, and Empty vector.1 cell lines. However, the proliferation of the VE-cad shRNA.1 and VE-cad shRNA.2 cell lines was not further reduced, suggesting that VE-cadherin knock-down and TGF-β signaling inhibition influence the same pathway (Fig. 6*D, left*). Treating the Ep5ExTu cells with Smad2 siRNA also led to a reduction in cell proliferation that correlated with diminished Smad2 expression and phosphorylation levels, and with impaired expression of TGF-β target genes. The Smad2 siRNA treatment did not affect VE-cadherin expression (Fig. 6*D, right*; Supplementary Fig. S13).

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Discussion

Local invasion and the formation of tumor metastases are clinically among the most relevant processes involved in tumor progression but still only partially understood at the molecular level. The characterization of the process leading from carcinoma *in situ* to invasive tumors is therefore of great importance. In this study, we provide the first direct evidence that VE-cadherin is induced in mammary tumor cells during EMT and promotes tumor growth and progression by enhancing TGF-β signaling. This interplay between VE-cadherin and TGF-β signaling represents a novel mechanism involved in tumor progression and shows that, in certain tumor cells, VE-cadherin can induce cellular responses that are in contrast to its role in cell-cell contact growth inhibition in endothelial cells. Our results thus reveal a new function for VE-cadherin in tumor cells that has additional important implications for the clinical application of anti–VE-cadherin strategies.

VE-cadherin is crucial for vessel assembly and integrity during angiogenesis, and blocking its function with antibodies has been previously proposed as a promising therapeutic approach against tumor angiogenesis (11, 13, 18, 19). As VE-cadherin is widely considered as an endothelial-specific cadherin, its expression in human mammary tumor cells and in an aggressive mouse mammary tumor cell line of nonendothelial origin was unexpected (9, 10). However, aberrant expression of VE-cadherin has been reported in a few tumor cases, for example in sarcomas and melanomas (23–25). VE-cadherin was also previously detected in aggressive cell lines isolated from human melanomas but not in poorly aggressive cells isolated from the same tumors (25). Although these observations raised the possibility that aberrant VE-cadherin expression in cancer cells could be relevant for tumor progression, direct evidence supporting this hypothesis was lacking.

In endothelial cells, VE-cadherin expression induces cell-cell contact growth inhibition (38). It was thus unexpected to observe a decrease in Ep5ExTu cell proliferation in the presence of antibodies or siRNA against VE-cadherin. However, in endothelial cells, the contact inhibition of cell growth is linked to a reduction of the response to VEGF via clustering of VEGFR-2 and VE-cadherin (38). In Ep5ExTu cells, this mechanism is not likely to occur because VEGFR-2 is not expressed (data not shown). The role played by VE-cadherin in Ep5ExTu cell proliferation seems thus to be

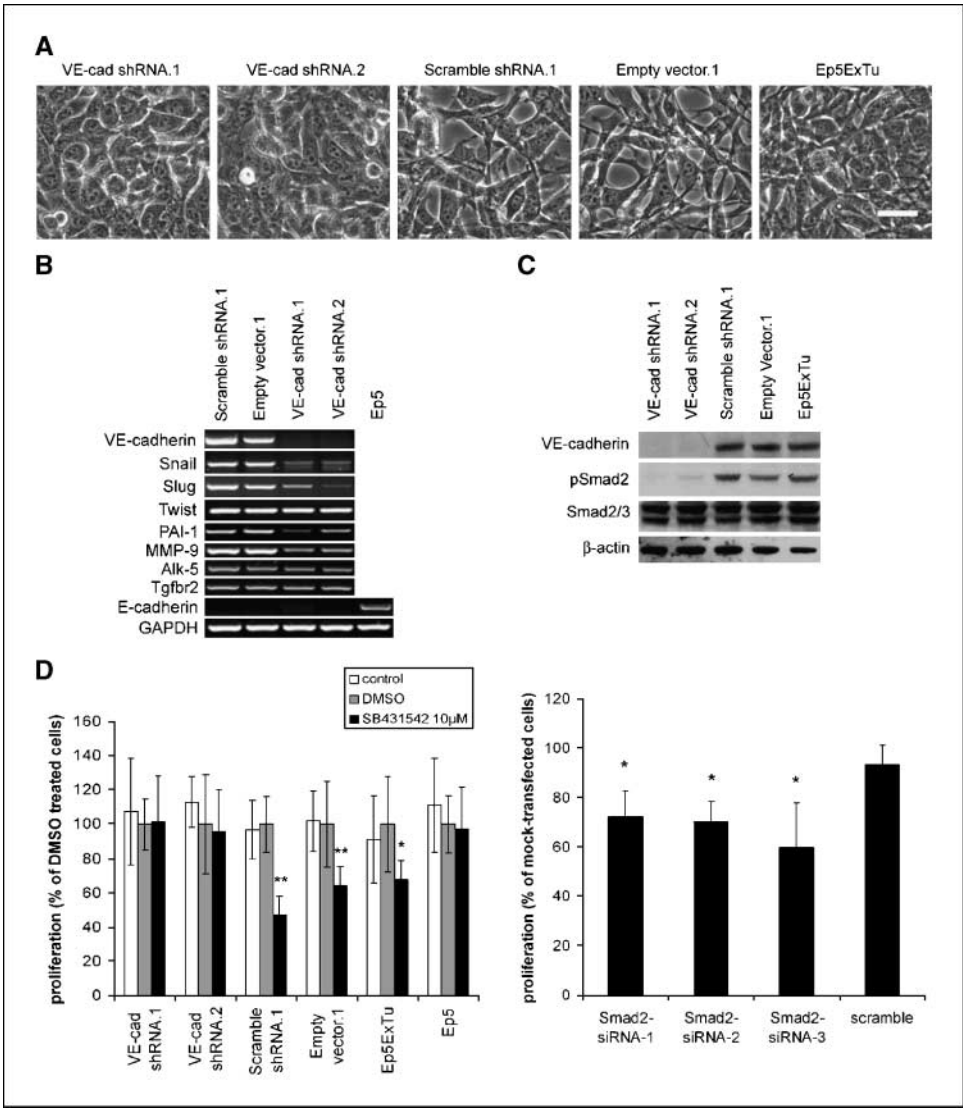

Figure 6. Stable suppression of VE-cadherin expression affects the morphology of Ep5ExTu cells and down-regulates the TGF-β signaling pathway. *A,* phase contrast microscopy of Ep5ExTu clones expressing either scrambled shRNA, empty vector, or VE-cadherin shRNA. *Bar,* 25 μm. *B,* VE-cadherin, Snail, Slug, Twist, PAI-1, MMP-9, Alk-5, Tgfbr2, and E-cadherin mRNA expression levels in Ep5ExTu clones. GAPDH was used as a loading control. *C,* protein expression of VE-cadherin, phospho-Smad2, and total Smad2/3 was examined by immunoblot in Ep5ExTu clones. β-actin was used as loading control. *D,* Ep5ExTu clones were cultured in complete culture medium supplemented with 10 μmol/L of SB431542 or DMSO alone and counted after 3 d (*left*). Ep5ExTu cells were transfected either with Lipofectamine2000 only (mock-transfected cells), three independent Smad2 siRNA (*Smad2-siRNA-1,-2*, and *-3*) or a scrambled siRNA sequence (*scramble*) and counted 2 d after transfection (*right*). *Columns,* mean of one of three independent experiments; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$. Statistical significance versus the DMSO or mock-transfected control was determined by unpaired Student's *t* test.

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different than in endothelial cells because, as shown in our experiments, MS1 endothelial cell proliferation was not affected by the treatment with the antibody. The contrasting effects of VE-cadherin in tumor cells versus endothelial cells are thus due to the interaction of VE-cadherin with distinct signaling pathways. Nevertheless, under pathologic conditions, VE-cadherin has been associated with a positive effect on proliferation even in endothelial cells. VE-cadherin is expressed at higher levels in breast tumor endothelium than in normal endothelium (17), and dominant-negative mutants of VE-cadherin inhibit endothelial cell growth (39).

Our results show that aggressive Ep5ExTu breast carcinoma cells that have undergone EMT mimic endothelial cells with regard to VE-, E-, and N-cadherin expression and localization (31, 32, 40). Moreover, Ep5ExTu cells displayed increased adhesion to endothelial cells and could form cord-like structure in collagen, similarly to endothelial cells (19), characteristics that we showed to be dependent on VE-cadherin. VE-cadherin expression by malignant tumor cells may thus have profound consequences on tumor-vessel interactions. It is tempting to speculate that the aggressive Ep5ExTu tumor cells might be more prone to incorporate in the vasculature than tumor cells that do not express VE-cadherin, and that their metastatic spread might be facilitated by their enhanced interaction with endothelial cells either at the site of the primary tumor, or at distant sites of metastasis formation. However, further experiments are necessary to address these questions.

Importantly, by suppressing VE-cadherin expression by shRNA in Ep5ExTu cells, we show that VE-cadherin expression promotes tumor growth *in vivo*. This result correlates well with the diminution in cell proliferation observed *in vitro* after VE-cadherin blockade or suppression and underscores the potential role of VE-cadherin in advanced tumors. Furthermore, the morphology of VE-cad shRNA.1 and VE-cad shRNA.2 clones suggested their reversion to a more epithelial phenotype, a characteristic that has been associated with reduced malignancy (2). This reversion was however only partial because the levels of *Twist* remained unaffected, and no induction of *E-cadherin* could be observed despite the down-regulation of *Snail* and *Slug*. Interestingly, a similar partial mesenchymal-epithelial transition was observed after TGF-β signaling inhibition in mesenchymal FosER cells (41).

How can VE-cadherin influence tumor cell behavior? The answer may well lie in its capacity to promote TGF-β signaling in fibroblastoid tumor cells. Although TGF-β inhibits the growth of early carcinomas, later in carcinogenesis, it can induce EMT and promote an invasive metastatic tumor phenotype (4, 5, 26, 42, 43). We provide here several lines of evidence to support a role for VE-cadherin in the enhancement of TGF-β signaling in Ep5ExTu cells. First, the knock-down of VE-cadherin inhibited Smad2 phosphorylation and correlated with a reduced expression of the TGF-β target genes *PAI-1, MMP-9, Snail*, and *Slug*, indicating that VE-cadherin expression enhances the activation of the TGF-β/Smad signaling pathway. Second, the effects of VE-cadherin suppression on tumor growth and cell morphology are consistent with an inhibition of the TGF-β pathway. As previously reported, introducing a dominant-negative form of TGFβRII in Ep5 cells prevents EMT and retards primary tumor growth, whereas in mesenchymal tumor cells, it induces mesenchymal-epithelial transition, retards tumor formation, and prevents metastasis (43). Third, inhibiting the TGF-β/Smad signaling pathway in Ep5ExTu cells with the TGFβRI kinase inhibitor SB431542 or with Smad2 siRNA both resulted in a reduction in cell proliferation similar to the one obtained by knocking-down VE-cadherin expression. This result is also consistent with the previously reported effect of TGF-β inhibition in several mesenchymal or dedifferentiated tumor cell lines (44–46). Fourth, inhibiting the TGF-β/Smad signaling pathway in the VE-cadherin knock-down cell lines did not lead to a further reduction in cell proliferation, suggesting that the same pathway, which promotes cell proliferation, is affected in these two cases.

We also have investigated the possibility that VE-cadherin might interfere with other signal transduction pathways leading to cell proliferation. However, no difference could be found in the levels of cyclin D1, β-catenin, phospho-p42/p44 MAPK, phospho-p53, phospho-p38 MAPK, and phospho-S6 when VE-cadherin was knocked-down in Ep5ExTu cells (Supplementary Fig. S15). These data, together with the fact that reduced Smad2 phosphorylation correlates with decreased cell proliferation, further support the hypothesis that VE-cadherin affects cell proliferation by enhancing the TGF-β/Smad signaling pathway.

In conclusion, our studies show for the first time that VE-cadherin is induced by EMT in malignant breast cancer cells and promotes tumor growth. VE-cadherin is enhancing the capacity of Ep5ExTu cells to proliferate, form cord-like invasive structures, and adhere to endothelial cells, characteristics that may be key contributors to their increased malignancy and metastatic potential. Importantly, we show that VE-cadherin expression in these tumor cells enhances the TGF-β signaling pathway, a major mediator of tumor progression. Taken together, these data provide evidence for a hitherto unknown function of VE-cadherin in tumor progression that is independent from its function in tumor angiogenesis and may contribute to the antitumor activity of anti–VE-cadherin strategies. VE-cadherin could thus be considered as a potential target for pharmacologic intervention to reduce the metastatic potential of breast cancer.

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