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ADAM-17 regulates endothelial cell morphology, proliferation, and in vitro angiogenesis

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ABSTRACT

Modulation of angiogenesis is a promising approach for treating a wide variety of human diseases including ischemic heart disease and cancer. In this study, we show that ADAM-17 is an important regulator of several key steps during angiogenesis. Knocking down ADAM-17 expression using lentivirus-delivered siRNA in HUVECs inhibited cell proliferation and the ability of cells to form close contact in two-dimensional cultures. Similarly, ADAM-17 depletion inhibited the ability of HUVECs to form capillary-like networks on top of three-dimensional Matrigel as well as in co-culture with fibroblasts within a three-dimensional scaffold. In mechanistic studies, both baseline and VEGF-induced MMP-2 activation and Matrigel invasion were inhibited by ADAM-17 depletion. Based on our findings we propose that ADAM-17 is part of a novel pro-angiogenic pathway leading to MMP-2 activation and vessel formation.
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Angiogenesis is an indispensable element of normal development and an integral part of a number of pathological processes including cancer or ischemic heart disease. Angiogenesis is initiated by local signals such as the secretion of vascular endothelial growth factor (VEGF) [1]. This pro-angiogenic signal stimulates preexisting endothelial cells (ECs) and endothelial progenitors to proliferate and differentiate. Induced ECs then migrate into the surrounding extracellular matrix (ECM) and assemble into cord-like structures. These EC sprouts develop lumens and fuse together to form an interconnected network. The angiogenic process ends with the recruitment of accessory cells which leads to the maturation and stabilization of forming blood vessels. The disruption of any of these processes leads to functionally and morphologically defective blood vessels.

Angiogenesis is tightly regulated by a complex system of pro- and anti-angiogenic factors including growth factors, cytokines, ECM molecules, and metalloproteinases. Metalloproteinases have attracted significant interest not only because they degrade the ECM molecules that comprise the environment that ECs invade and remodel, but because they also regulate growth factor signaling, cell adhesion, and release pro- and anti-angiogenic fragments of ECM proteins [2]. Inhibitors of metalloproteinase expression and function block angiogenesis in various in vitro and in vivo models. These experiments identified MMP-2, MT1-MMP, and ADAMs-10,-15, and ADAM-17 as being of particular importance [3].

The functions of ADAM-17 in vivo and in vitro make it a particularly interesting metalloproteinase. It was originally identified as the TNF-α activating enzyme [4], but later it was discovered that ADAM-17 processed many cytokines, growth factors and their receptors and had non-enzymatic functions such as integrin binding [5]. The importance of ADAM-17 in angiogenesis is clearly illustrated by the pulmonary hypovascularization and heart valve malformations found in transgenic mice that express catalytically inactive ADAM-17 [6,7]. Unfortunately it is not practical to study the effects of ADAM-17 knock down on specific angiogenic processes in these mice because of the severe dismorphogenesis and perinatal mortality.

The current studies on the roles of ADAM-17 in regulating endothelial cell behavior are particularly novel and significant because, to the best of our knowledge, no previous studies have methodically analyzed the direct consequences of knocking down ADAM-17 expression on angiogenic processes. Specifically, we find that ADAM-17 regulates HUVEC proliferation, cell–cell contact, formation of blood vessel-like structures in three-dimensional culture models, and invasion of Matrigel. Our data also suggest that ADAM-17 is an important intermediate in VEGF signaling in HUVECs.

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Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVEC2) cells (BD Biosciences) were grown on tissue culture-treated plastic in EGM-2 medium [i.e. EBM2 medium supplemented with EGM-2 bullet kit (Clonetics)]. For studies on the effects of ADAM-17 silencing on HUVEC morphology, proliferation, apoptosis and MMP expression, the cells were plated on Matrigel (BD Biosciences) coated plates one day before the experiment.

For protein expression analysis, 1.5×10^5 siAD-17 or siCo were plated in each well of 24-well tissue culture plates and incubated in EGM-2 medium. The next day, cells were incubated in EBM2 medium for 4 h, then incubated in fresh EBM2 medium in the presence or absence of VEGF (10 ng/ml) for an additional 3 h. To detect HB-EGF shedding, the level of HB-EGF in conditioned medium was determined by Western blotting. For use in zymographic and Western blotting analyses the cell layer was dissolved in SDS–PAGE sample buffer.

Fibroblasts (human dermal fibroblasts, Clonetics) were grown on tissue culture-treated plastic in FBM medium supplemented with FGM bullet kit (Clonetics). 293FT cells were cultured in DMEM supplemented with 10% FBS, 100 nM non-essential amino acids, and 500 μg/ml G418 (Invitrogen). All cell culture experiments were conducted at 37 °C in a humidified 95% air/5% CO_2 atmosphere.

ADAM-17 gene silencing in HUVECs. A lentiviral vector that silences ADAM-17 expression was constructed as described [8]. Briefly, oligodeoxyribonucleotides (Sigma Genosys) encoding short hairpin RNAs (shRNA), were designed to target the following 19-nt sequence in human *ADAM-17 splice variant 2* (NCBI Accession No. NM_021832): 5′ggatcttggcaagtgtaag (position 1867–1885) and subsequently cloned into the pLentiLox 3.7 lentiviral vector (a gift from Luk van Parijs, MIT). As a control we used scrambled shRNA. Infectious viruses were produced by cotransfecting the lentiviral vector and packaging constructs into 293FT cells (Invitrogen) using Transfectin reagent (BioRad). Infectious lentivirus particles were harvested 48 h after transfection. HUVECs at passage 2 were infected with each virus at an MOI of 40, then cultured for 5 days. Cells in which ADAM-17 expression was down-regulated by specific siRNA were designated siAD-17; control cells expressing scrambled siRNA were designated siCo. The LentiLox3.7 vector confers enhanced green fluorescent protein (EGFP) expression to the transduced cells. All procedures involving lentivirus were conducted under biosafety level II containment and were approved by the Institutional Biosafety Committee of the Medical University of South Carolina. In an alternative approach, we used Morpholino oligos (Gene Tools LLC) to silence ADAM-17 expression. The following sequences were used for ADAM-17 silencing and control, respectively: 5′-cctcttacctcagttacaatttata (MoAD-17) and 5′-cctcctacctcagttacaatttata (MoCo). Morpholinos were transfected into HUVECs at 4 μM final concentration in 6-well plates at 70% confluency in EGM-2 in the presence of 1 ml/ml EndoPorter (Gene Tools LLC) transfection reagent. Two hours after transfection the medium was changed to fresh EGM-2 and the cells were incubated for 2 days before use in experiments.

Proliferation and apoptosis assays. 1.5×10^6 HUVECs were plated in 150 cm^2 Matrigel-coated tissue culture dishes and incubated in EGM-2 medium for 4 days. The total number of live cells that could be harvested from each dish by trypsinization was determined using a hemocytometer. From the same experiment 1×10^6 cells were used to determine the level of apoptosis in siCo and siAD-17 by measuring the activity of caspases-3 and -8 using the ApoTarget assay (Invitrogen) according to the manufacturers' instructions.

Zymography. Protein samples were separated on 8% SDS–polyacrylamide gels containing 0.1% gelatin under nonreducing conditions. Gels were washed with 2.5% Triton X-100 and incubated overnight at 37 °C in 50 mM Tris (pH 8.0)/ 5 mM $CaCl_2$. The gel was then stained with Coomassie blue G-250 (Bio-Rad).

Western blot analysis. Protein samples were separated on 4–12% NuPAGE minigels (Invitrogen) under reducing conditions. Proteins were transferred to PVDF membranes (Millipore), and probed with one of the following antibodies: 1 μg/ml HB-EGF (EMD Biosciences PC319L), 1:2000 ADAM-17 (R&D Systems MAB2129), 1:1000 MT1-MMP (Chemicon MAB3317), 1:1000 MT3-MMP (Chemicon AB856) or 1:1000 β-actin (Sigma A2228) antibodies. Immunoreactive protein bands were visualized by enhanced chemiluminescence using SuperSignal West Dura Substrate (Pierce).

Capillary network formation on three-dimensional Matrigel. Seventy microliters of Matrigel was allowed to polymerize at 37 °C for 30 min in each well of a 96-well plate. 4×10^4 HUVECs (siCo or siAD-17) in 200 μl EGM-2 were seeded on top of the Matrigel. TAPI-1 and GM6001 negative control compound (EMD Biosciences) were dissolved in DMSO and added to the cell suspension prior to plating. The final DMSO concentration was adjusted to 0.1% in each well. At 5, 24, and 48 h fluorescent images were taken using a Zeiss Axiovert 35 microscope equipped with a CCD camera (Diagnostic Instruments) using a 2.5× objective.

Capillary formation in chitosan-GAG-collagen scaffolds. Scaffolds were prepared as described previously [9]. Briefly, 95% deacetylated chitosan (Biosyntec) dissolved in 0.1% acetic acid and Chondroitin 4–6 sulfates (Xenos) dissolved in distilled H_2O were mixed together with high concentration rat tail collagen type I (BD Biosciences) to give final concentrations of 9, 2.5, and 1 mg/ml, respectively. The solution was aliquoted into plastic Petri dishes and lyophilized. Fibroblasts and HUVECs were seeded on top of the scaffold at $2.4 \times 10^5/cm^2$. The co-cultures were incubated for 21 days and fixed for 15 min with 4% formaldehyde. Z-series images were obtained with a Zeiss LSM 510 META laser-scanning microscope (Molecular Imaging Facility, Hollings Cancer Center, Medical University of South Carolina), and were reconstructed into the images shown in Fig. 3 using Volocity 3 software (Improvision).

HUVEC invasion through three-dimensional Matrigel. HUVECs (siCo or siAD-17) were harvested by trypsinization, resuspended in EGM-2, and 5×10^4 cells were seeded into the upper chamber of Matrigel-coated Fluoroblok inserts (BD BioCoat™ Angiogenesis System: Endothelial Cell Invasion, BD Biosciences). The bottom chamber was filled with EGM-2 with or without 10 ng/ml VEGF (R&D systems). After 12-h incubation, the cells were stained with Calcein AM (Invitrogen) and the rate of invasion was quantified by fluorometry using a VICTOR 2 fluorescent plate reader (Perkin–Elmer).

Statistical analysis. Data are presented as mean ± SEM. Two way analysis of variance (ANOVA) was performed using the GraphPad Prism software (GraphPad Software) to analyze the results of the experiments with two independent variables. Bonferroni multiple comparison tests were performed for post hoc pairwise comparisons. The effect of ADAM-17 silencing on cell proliferation, ADAM-17 protein expression, and HB-EGF shedding was analyzed using the two tailed *t*-test. Tests in which *p* values were less than 0.05 were considered to be statistically significant. Data from at least three independent experiments were used for statistical analysis.

Results and discussion

ADAM-17 silencing inhibits proliferation and cell–cell contact formation by human umbilical vein endothelial cells (HUVECs)

We inhibited the expression of ADAM-17 using ADAM-17-specific Morpholinos and lentivirus-delivered shRNA [8]. Western

blotting of HUVECs 5 days after infection showed that ADAM-17 protein expression was inhibited by approximately 80% in ADAM-17 silenced cells (siAD-17, MoAD-17) compared to control HUVECs (siCo, MoCo) (Fig. 1A). ADAM-17 activity was also decreased in siAD-17 cells as evidenced by the decreased level of HB-EGF released into the medium by these cells (Fig. 1A). We noticed a striking difference in the morphology of subconfluent ADAM-17 silenced and control HUVEC's. While siCo and MoCo showed extensive cell–cell contact, siAD-17 and MoAD-17 were irregular in shape and avoided cell–cell contact (Fig. 1B). The prominent morphological changes in ADAM-17-deficient HUVECs may involve changes in the expression or the activity of adhesion molecules [10].

Proliferation of endothelial cells is an absolute requirement for angiogenesis. The depletion of ADAM-17 resulted in approximately a 30% decrease in the proliferation rate of siAD-17 and MoAD-17 compared to siCo or MoCo (Fig. 1C), while it did not affect apoptosis in these cells (Fig. 1D). The substantially decreased proliferation rate of ADAM-17 depleted cells is not surprising, since ADAM-17 activates several growth factor and cytokine signaling pathways that are important in EC proliferation [8,11]. Therefore the decreased HB-EGF shedding in siAD-17 (Fig. 1A) may contribute to the decreased proliferation rate in these cells (Fig. 1C). It is also possible that the negative effect of ADAM-17 depletion on cell contact/adhesion contributes to the observed inhibition of proliferation. For example, EC proliferation can be affected by changes in αvβ3 integrin expression [12]. Since ADAM-17 is known to interact with integrins (most likely via its disintegrin domain) [13], the lack of ADAM-17 may lead to disruption in integrin signaling in ECs. The finding that overexpression of the recombinant disintegrin domain of ADAM-15 inhibits EC proliferation, migration, and capillary formation (presumably by interfering with endogenous ADAM-15 function [14]) lends strong support for this hypothesis.

ADAM-17 silencing results in destabilization of capillary networks on three-dimensional Matrigel

Another important step during angiogenesis is the formation of interconnecting networks by the endothelial tubes. We used two different in vitro models to study different aspects of EC network formation. First, we tested the network-forming ability of siAD-17 on three-dimensional Matrigel. Six hours after plating, siCo cells form a capillary-like network, which is stable for up to 48 h. Although network formation by siAD-17 initially proceeds much as in control cells, siAD-17 networks soon begin to deteriorate. At 24 h siAD-17 networks contain fewer branch points and larger enclosed spaces than siCo and by 48 h degenerate into intermittent clumps of cells (Fig. 2). The addition of a synthetic inhibitor of ADAM-17 (TAPI-1), but not an inactive control compound (GMneg), to the control siCo cells also resulted in early destabilization of the network. While TAPI-1 inhibits other ADAMs in addition to ADAM-17, the fact that TAPI-1 treatment and ADAM-17 depletion have similar effect supports the idea that the accelerated destabilization of EC networks in siAD-17 is a direct effect of the depletion of ADAM-17 activity. It will require further investigation to determine the contributions of decreased proliferation and altered cell–cell and cell–matrix interaction to the instability of EC networks of siAD-17 cells.

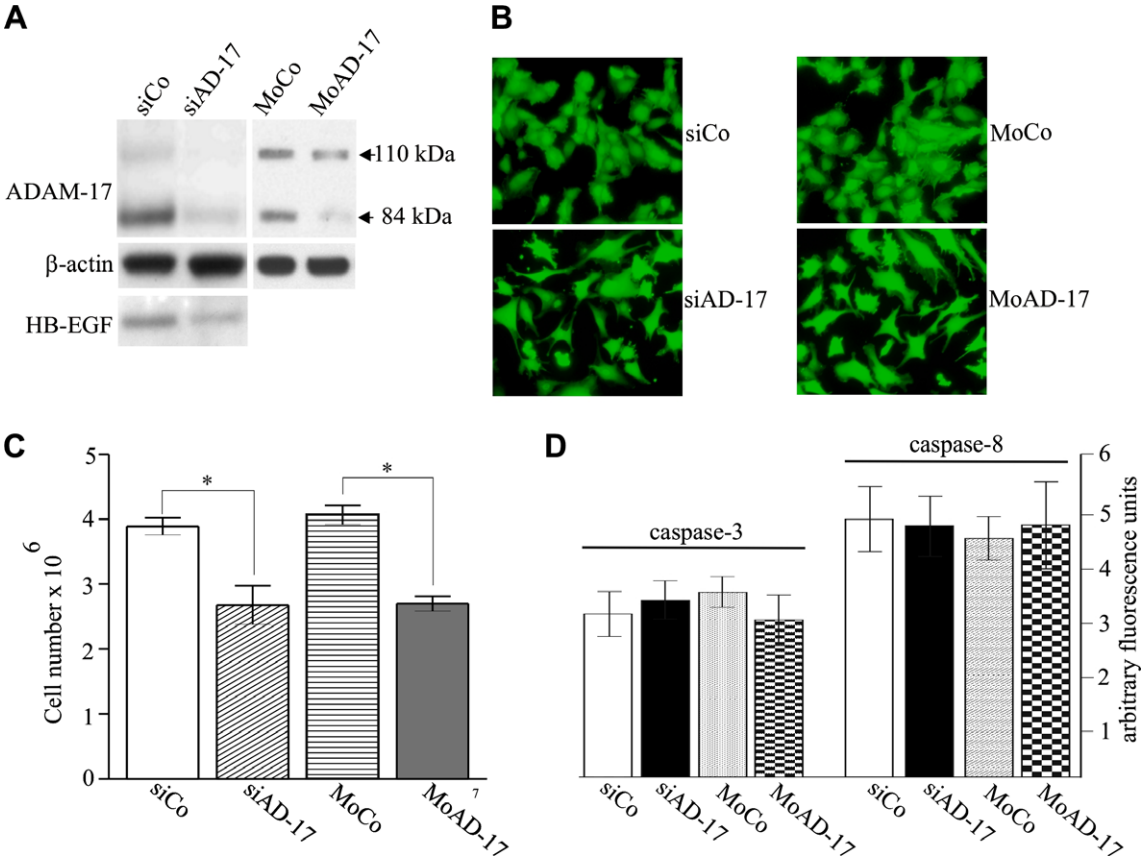

Fig. 1. Characterization of ADAM-17 silencing in HUVECs. (A) Western blot analyses confirmed efficient inhibition of ADAM-17 expression ($p < 0.001$) and function (HB-EGF shedding, $p < 0.01$. (B) Fluorescence microscopic image illustrating the morphological differences between EGFP labeled siAD-17/MoAD-17 and siCo/MoCo. ADAM-17 silencing inhibits HUVEC proliferation (C) ($^{*}p < 0.05$), but not apoptosis (D).

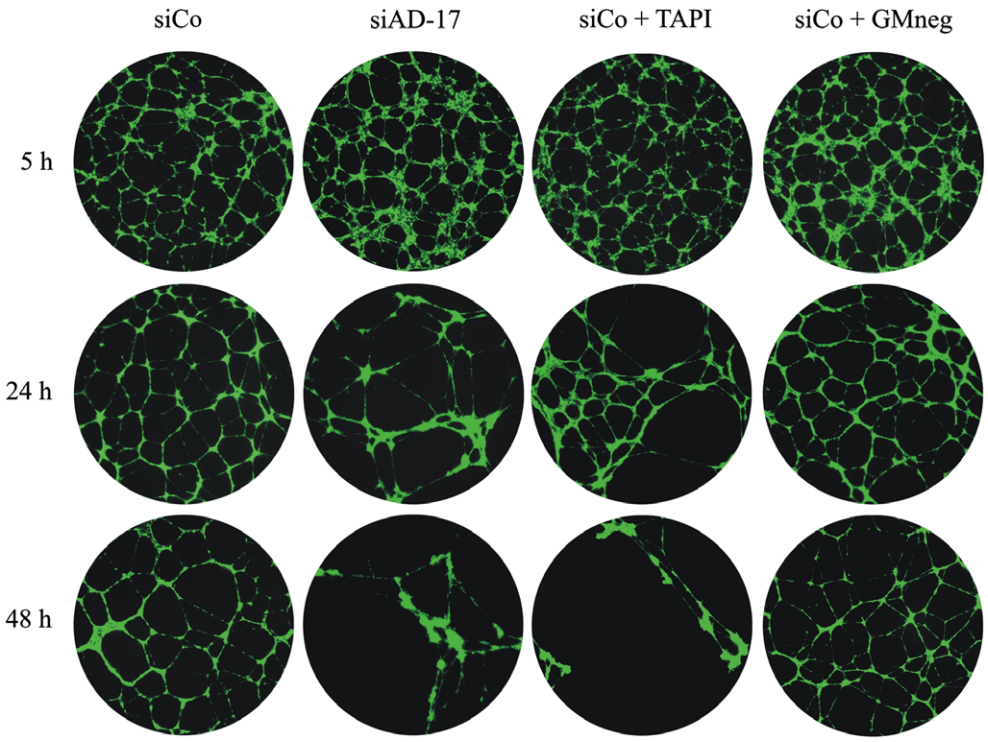

Fig. 2. The effect of the inhibition of ADAM-17 activity and expression on HUVEC network formation. Inhibition of ADAM-17 protein expression by RNAi (siAD-17) and ADAM-17 activity with TAPI-1 (siCo + TAPI) causes early destabilization of HUVEC networks on three-dimensional Matrigel, when compared to their respective controls (siCo and siCo + GMneg).

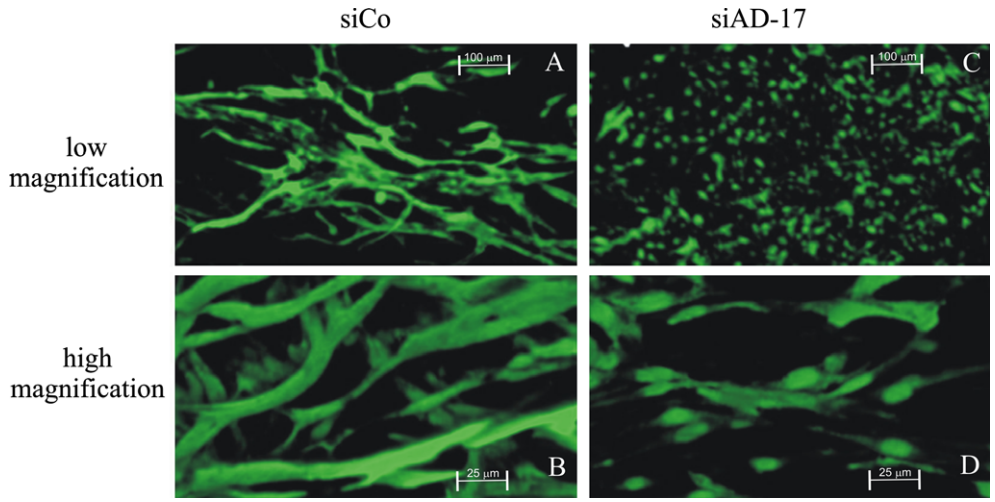

Fig. 3. ADAM-17 silencing blocks capillary formation in HUVEC-fibroblast co-cultures in chitosan-GAG-collagen gels. Capillary like structures are observed in siCo cultures (A, B) but not in siAD-17 cultures (C, D). Because only the HUVECs express EGFP, the fibroblast and the matrix are not visualized.

Capillary formation in chitosan-GAG-collagen (CGC) scaffolds

To test the possibility that the expression of ADAM-17 by supporting cells might compensate for the lack of ADAM-17 expression by ECs and thereby rescue the ability of siAD-17 cells to participate in angiogenesis, we co-cultured siCo or siAD-17 with fibroblasts in CGC scaffolds (Fig. 3). This model mimics in vivo angiogenesis better than other in vitro models because in CGC scaffolds ECs rest on a well-developed basal lamina and form long, interconnecting capillary tubes that are stable for extended time periods. Many of the fibroblasts in this model are arranged around the capillaries like pericytes around capillaries in vivo. Pro- and anti-angiogenic agents modulate the number of capillaries formed within the scaffold. Moreover, the capillaries formed in this model interconnect with the host vasculature when transplanted into mice [9]. Chitosan is considered to be inert as evidenced by its use in human clinical trials [15].

Using siCo in these experiments, we confirmed that long interconnecting blood vessel-like structures (Fig. 3A and B) with lumens (data not shown) formed within 3 weeks. Despite the presence of ADAM-17 in the fibroblasts, ADAM-17-depleted HUVECs remained primarily as single cells and did not form capillary-like networks (Fig. 3C and D). These observations suggest that the defective angiogenesis that occurs in ADAM-17 knockout mice is primarily due

to the lack of ADAM-17 in ECs and not in other cell types. On the other hand the fact that there is a complete lack of capillary formation by siAD-17 in CGC scaffolds while some blood vessels are present in ADAM-17 knockout mice [6] suggests the presence of mechanisms in vivo that partially compensate for the lack of ADAM-17 activity.

ADAM-17 is required for both VEGF-dependent and VEGF-independent HUVEC invasion of three-dimensional Matrigel

Angiogenesis in vivo involves the invasion of ECs or endothelial precursor cells through the basement membrane into the surrounding ECM. Therefore we examined the role of ADAM-17 in HUVEC invasion through three-dimensional Matrigel in the presence or absence of added VEGF (Fig. 4A). In the absence of VEGF, Matrigel invasion was partially blocked by ADAM-17 depletion (41% less in siAD-17 than in siCo). Strikingly, while VEGF treatment increased the invasion of siCo by 43%, ADAM-17 depletion completely blocked VEGF-induced Matrigel invasion by HUVECs. Two way ANOVA confirmed that siAD-17 responded differently to VEGF treatment than siCo ($p < 0.01$).

ADAM-17 silencing inhibits both VEGF-dependent and VEGF-independent MMP-2 activation

Given the known relationship between MMP-2 and endothelial cell morphology, network formation, invasion and tumor induced angiogenesis [2,3,16], we examined the expression of MMP-2 by siCo and siAD-17 cultured in Matrigel-coated wells in the presence or absence of VEGF (Fig. 4B–E).

These studies revealed a striking relationship between ADAM-17 and the activation of MMP-2. Whereas ADAM-17 depletion led to only a non-significant (10%) inhibition of the expression of the latent form of MMP-2, expression of the intermediate-sized form was inhibited by 47%, and expression of the fully activated form was inhibited by 90%. These results suggest that ADAM-17 is involved in the conversion of MMP-2 from the latent form to the intermediate-sized form and plays a particularly key role in the conversion of the intermediate-sized form to the fully activated form.

Because MT1-MMP and MT3-MMP were reported to activate MMP-2 in ECs [2,3,17] we evaluated the basal and VEGF-induced expression and processing of these enzymes in siCo and siAD-17. The fact that neither the depletion of ADAM-17 nor VEGF treatment altered the level of expression or molecular form of MT1-MMP or MT3-MMP suggests that ADAM-17 and VEGF do not participate in MMP-2 activation simply by altering the expression or activation of these MT-MMPs.

Our finding that MMP-2 activation in HUVECs is dependent on ADAM-17 suggests that: ADAM-17 may regulate MT-MMP-mediated MMP-2 activation, ADAM-17 may participate in an MT-MMP-independent mechanism for MMP-2 activation, or ADAM-17 may act sequentially (after MT1-MMP) to activate MMP-2. There is a precedent for another protease acting after MT1-MMP to activate MMP-2; thrombin is able to convert the intermediate form of MMP-2, but not the proform, into the fully active form in HUVECs [18].

One possible mechanism for ADAM-17 mediated MMP-2 activation and HUVEC invasion into Matrigel involves integrins. ADAM-17 contains a disintegrin domain and has been shown to interact

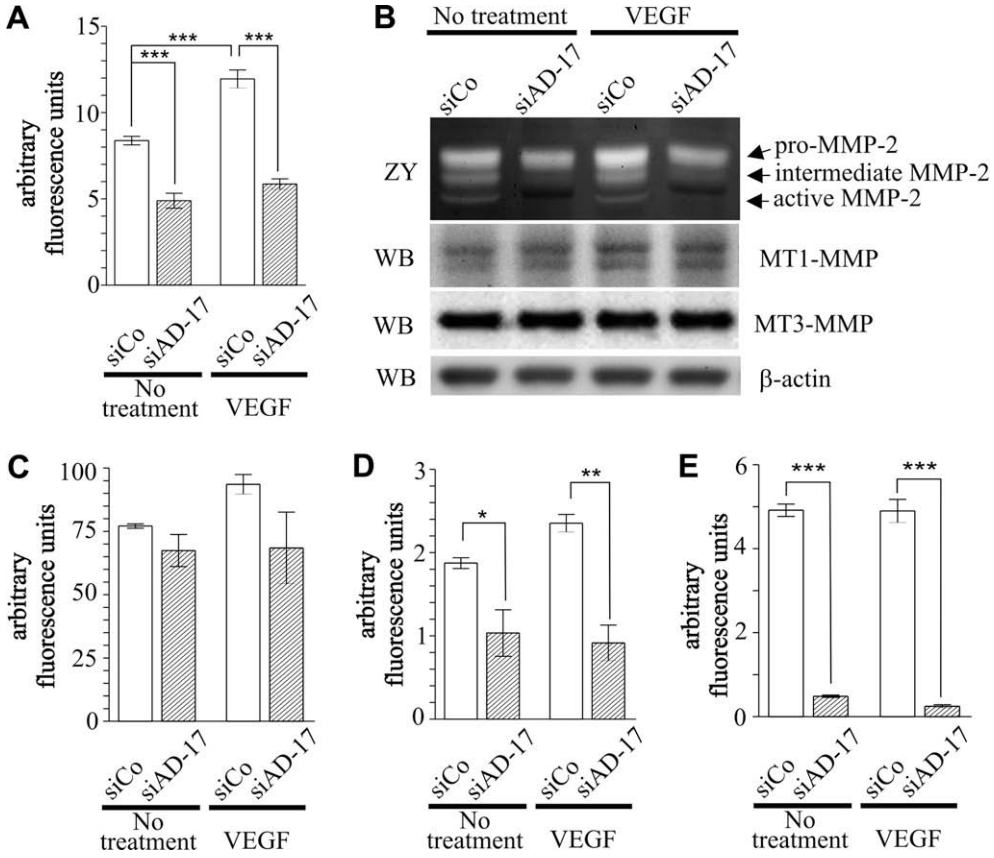

Fig. 4. ADAM-17 in VEGF signaling. (A) ADAM-17 silencing inhibits basal and VEGF-induced HUVEC invasion through three-dimensional Matrigel. Invading cells were detected by fluorescent labeling using Calcein. (B) ADAM-17 silencing inhibits basal and VEGF-induced MMP-2 activation in HUVEC, but does not affect MT1-MMP and MT3-MMP expression. Representative zymogram (ZY) and Western blots (WB) from three independent experiments. Densitometric analyses of the zymogram in (B): (C) latent MMP-2, (D) intermediate size MMP-2, and (E) fully activated MMP-2 ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

with integrins in various cells including ECs [13,19]. Since integrins play an important role in EC migration and MMP-2 activation, it is reasonable to hypothesize that ADAMs and MT-MMPs are brought together by integrins in invadopodia and act in concert [20]. Alternatively ADAM-17 may induce MMP-2 activation and invasion by activating growth factor or cytokine signaling pathways via the release of HB-EGF or TNF-α from their membrane bound precursors [8,11].

Our results clearly indicate that VEGF regulates HUVEC invasion and MMP-2 activation in an ADAM-17 dependent way. A short treatment with VEGF induces a 25% increase in the expression of both the latent and intermediate-sized forms of MMP-2 and this increase is completely blocked in ADAM-17-depleted HUVECs. Although it has previously been shown that G-protein coupled receptors can activate ADAM-17 in other cells [8,11], to our best knowledge VEGF signaling has not previously been connected to ADAM-17 function.

Conclusions

This study demonstrates that ADAM-17 may regulate angiogenesis via its effects on EC proliferation, network formation, invasion and MMP-2 activation. A further novel finding is that VEGF signaling critically relies on the presence of ADAM-17 in HUVECs. Therefore ADAM-17 may serve as a novel therapeutic target for diseases in which the inhibition or stimulation of angiogenesis could be beneficial. Future studies are required to clarify the mechanism of the effects of ADAM-17 on angiogenesis.

Acknowledgments

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