ADAM12 is expressed in the tumour vasculature and mediates ectodomain shedding of several membrane-anchored endothelial proteins

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ADAM12 (a disintegrin and metalloprotease) 12 is a metalloprotease implicated in cancer progression. ADAM12 can activate membrane-anchored proteins, such as sonic hedgehog, Delta-like 1 and certain epidermal growth factor receptor ligands, through a process called ectodomain shedding. We screened several membrane-anchored proteins to further dissect the substrate profile of ADAM12-mediated ectodomain shedding, and found shedding of five previously unreported substrates [KitL1, VE-cadherin (vascular endothelial cadherin), Flk-1 (fetal liver kinase 1), Tie-2, and VCAM-1 (vascular cell adhesion molecule 1)], of which the latter four are specifically expressed by endothelial cells. We also observed that ADAM12 expression was increased in the tumour vasculature of infiltrating ductal carcinoma of the human breast as compared with little to no expression in normal breast tissue vasculature, suggesting a role for ADAM12 in tumour vessels. These results prompted us to further evaluate ADAM12-mediated shedding of two endothelial cell proteins, VE-cadherin and Tie-2. Endogenous ADAM12 expression was very low in cultured endothelial cells, but was significantly increased by cytokine stimulation. In parallel, the shed form of VE-cadherin was elevated in such cytokine-stimulated endothelial cells, and ADAM12 siRNA (small interfering RNA) knockdown reduced cytokine-induced shedding of VE-cadherin. In conclusion, the results of the present study demonstrate a role for ADAM12 in ectodomain shedding of several membrane-anchored endothelial proteins. We speculate that this process may have importance in tumour neovascularization or/and tumour cell extravasation.

Key words: a disintegrin and metalloproteinase 12 (ADAM12), breast cancer, ectodomain shedding, endothelial cell, tumour vasculature, vascular endothelial cadherin (VE-cadherin).

INTRODUCTION

Tumour–stroma interactions play critical roles in tumour cell progression, invasion and metastasis. Indeed, the action of cell adhesion molecules and receptors on both tumour and stromal cells, including the blood vessels, are constantly changing, with great significance for tumour progression [1]. Cellular processes on the cell surface, such as protein ectodomain shedding, serve important roles in converting membrane-anchored precursors of receptor ligands, cytokines and cell adhesion molecules into soluble factors [2–4]. Recently, shedding of cell adhesion proteins and receptors on the endothelium has emerged as an important consequence of neovascularization, and elevated levels of soluble forms of these proteins have been found in serum from cancer patients [5]. Cell-surface proteases, such as the ADAMs (a disintegrin and metalloproteinases), are major mediators of ectodomain shedding, and thus play important roles in development and disease, including cancer [5,6]. At present, nine members of the ADAM family have been identified as being involved in ectodomain shedding and, of these, ADAM12 has been particularly implicated in cancer [6,7]. ADAM12 exists in two isoforms resulting from alternative splicing: a membrane-anchored form (termed ADAM12-L) and a secreted form (termed ADAM12-S) [8]. Elevated expression levels of both forms of ADAM12 have been found in several cancer types [7]. Indeed, the expression of ADAM12 is markedly increased in many human carcinomas, including breast, bladder, laryngeal and lung carcinomas, and in glioblastomas [7]. We and others have demonstrated that the level of ADAM12 in urine from breast and bladder cancer patients correlates with disease stage, indicating ADAM12 as a potential biomarker for tumour progression [9–11]. Furthermore, previous findings using mouse models have shown that ADAM12 promotes tumour progression by regulating tumour cell proliferation and survival [12–15].

The ADAM12 molecule is involved in cell adhesion via binding to integrins and syndecan-4, as well as proteolytic cleavage of several substrates [7]. Importantly, it has also been demonstrated that ADAM12 participates in ectodomain shedding, thereby releasing active EGF (epidermal growth factor), HB-EGF (heparin-binding EGF), betacellulin, sonic hedgehog and Delta-like 1 from the cell surface [7,16]. ADAM12 was found to be highly up-regulated in human glioblastomas, as well as positively correlated with the proliferative index and the ability to shed proHB-EGF [17]. Recently, the membrane-anchored

Abbreviations used: ADAM, a disintegrin and metalloproteinase; ADAM12-L, membrane-anchored form of ADAM12; ADAM12-S, secreted form of ADAM12; AP, alkaline phosphatase; bFGF, basic fibroblast growth factor; CTF, C-terminal fragment; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; EGFR, EGF receptor; Flk-1, fetal liver kinase 1; GFP, green fluorescent protein; HB-EGF, heparin-binding EGF; HUVEC, human umbilical vein endothelial cell; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; MEF, mouse embryonic fibroblast; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 MMP; N-TIMP-2, N-terminal domain of human TIMP (tissue inhibitor of metalloproteinase)-2; qPCR, quantitative PCR; rPO, ribosomal phosphoprotein; RT, reverse transcription; siRNA, small interfering RNA; sTie-2, soluble Tie-2; sVE-cadherin, 95 kDa N-terminal ectodomain of VE-cadherin; TDLU, terminal ductal lobular unit; TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumour necrosis factor; VCAM-1, vascular cell adhesion molecule 1; VE-cadherin, vascular endothelial cadherin.

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form of ADAM12 was found to positively correlate with HB-EGF and EGFR (EGF receptor) expression in triple-negative breast cancer, a subtype of breast cancer that lack expression of oestrogen receptor, progesterone receptor and EGFR2/HER2 [18]. Moreover, ectopic ADAM12 expression was found to increase EGFR phosphorylation in a mouse model of early breast cancer [18]. However, ADAM12 seems to not be responsible for PMA-induced HB-EGF shedding [19], and a recent study using a mouse model of invasive breast cancer showed that ADAM12-induced tumour cell proliferation was independent of its proteolytic activity and did not entail phosphorylation of EGFR [12]. Thus, although ADAM12 has been shown to regulate tumour cell proliferation and survival, little is known of the molecular mechanisms whereby ADAM12 modulates tumour progression, invasion and dissemination in human cancers.

In the present study, we investigated the catalytic fingerprint of the transmembrane form of ADAM12. We identified five novel ADAM12 substrates [Kitl1, VE-cadherin (vascular endothelial cadherin), Flk-1 (fetal liver kinase 1), Tie-2 and VCAM-1 (vascular cell adhesion molecule 1)], of which endothelial cells specifically express the last four. We detected little or no ADAM12 expression in normal endothelial cells or the vasculature of normal breast tissue; however, we found ADAM12 to be expressed by the tumour vasculature of IDC (infiltrating ductal carcinoma) of the breast. We showed that expression of ADAM12 was induced in cytokine-stimulated endothelial cells and that the shed forms of VE-cadherin were correspondingly elevated in these cells compared with unstimulated cells. Moreover, siRNA (small interfering RNA)-mediated knockdown of ADAM12 reduced the cytokine-induced shedding of VE-cadherin. Also, deletion of ADAM12 in MEFs (mouse embryonic fibroblasts) abolished cytokine-stimulated shedding of VE-cadherin. Taken together, the results of the present study indicate that the increased shedding of VE-cadherin in cytokine-treated endothelial cells and fibroblasts is at least partly mediated by ADAM12.

**MATERIALS AND METHODS**

**Reagents and antibodies**

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich. The broad-spectrum metalloprotease inhibitors batimatstat and marimastat were obtained from Toceis Bioscience, and TAPI-2, GM6001, MMP (matrix metalloproteinase) inhibitor III, MMP-3 inhibitor VIII, TAPI-1, TAPI-0 and MMP inhibitor V were from Calbiochem. N-TIMP-2 [N-terminal domain of human TIMP (tissue inhibitor of metalloproteinase)-2] was expressed and purified as described previously [20], and TIMP-3 was obtained from R&D Systems. TGF (transforming growth factor) β1 was purchased from Sigma–Aldrich, TNF (tumour necrosis factor) α was from Peprotech and bFGF (basic fibroblast growth factor) was from R&D Systems. Antibodies against ADAM12 (mouse monoclonal 6E6, 8F8 and 6C10, and rabbit polyclonal rb122) were generated as described previously [21,22], or purchased from ProteinTech (the rabbit polyclonal antibody). The following antibodies were from the suppliers indicated: rabbit polyclonal anti-β1-integrin, goat polyclonal anti-VE-cadherin and goat polyclonal anti-PLAP [placental AP (alkaline phosphatase)] (all from Santa Cruz Biotechnology), mouse monoclonal anti-AP (Sigma–Aldrich), mouse anti-GFP (green fluorescent protein; Clontech), mouse monoclonal anti-actin (Millipore Chemicon) and mouse monoclonal anti-human CD31 (Dako).

**ADAM12 fluorescent peptide cleavage assay**

Mature human ADAM12-S (Uniprot ID O43184) was purified from HEK (human embryonic kidney)-293 EBNA cells as described previously [23]. The quenched fluorescent peptide harbouring a TNFα-like sequence (Enzo Life Sciences) used in this assay has been reported previously [24]. Inhibitor dilution series were produced by dilution with reaction buffer [20 mM Tris (pH 8) and 0.0005% Brij-35]. Inhibitors were tested as a log2 dilution series with an inhibitor concentration range from 125 μM to 6 nM used in the final assay. Wells containing 2.5% DMSO served as controls. For negative controls without enzyme, reaction buffer was added. A 15 μM working solution of the quenched fluorescent peptide in reaction buffer and a 2 ng/μl (26 nM) working solution of ADAM12-S in reaction buffer was prepared and dispersed into a 384-well plate and incubated at 37°C for 30 min. The reactions were stopped by adding 5 nM EDTA and the fluorescence measured in a Varioskan Flash fluorimeter (Thermo Scientific), using excitation at 485 nm and emission at 530 nm. Experimental data from triplicate measurements were converted into relative activities using the mean fluorescence of wells with enzyme (set to 100% activity) and without enzyme (no activity) as a reference. Relative activities were then fitted against a four-parameter logistic model:

\[
\text{f}(x) = \frac{A + B}{1 + [((x/C)/D)]} \\
\]

using XLFit (ID Business Solutions), yielding the IC50 value and S.E.M. as stated.

**Cell culture**

Human epithelial 293-VnR cells and immortalized MEFs from wild-type, ADAM9,12,15−/− and ADAM9,12,15−/− mice (designated wild-type MEFs, MEF9A,15−/− cells and MEF9A12,15−/− cells) have been described previously [25,26]. HUVECs (human umbilical vein endothelial cells) were obtained from PromoCell. The sEND.1 endothelioma cell line, which originally was derived from the subcutaneous tissue of mice [27], was kindly provided by Dr James R. Whiteford (William Harvey Research Institute, Queen Mary University of London, London, U.K.). 293-VnR, MEFs and sEND.1 cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% FBS (fetal bovine serum), 50 units/ml penicillin (Gibco) and 50 μg/ml streptomycin (Gibco). HUVECs were cultured in endothelial cell growth medium from PromoCell and all experiments were performed using passage 2–6 cells. When indicated, cells were incubated for 24 h with serum-free medium with or without TGFβ and TNFα. The cells were kept in a humidified incubator at 37°C, 5% CO2.

**Constructs and transfection**

Wild-type and catalytically inactive (E351Q point mutation) full-length human ADAM12-L constructs in the pEGFP-N1 vector have been described previously [28,29]. Expression constructs encoding AP-tagged substrates [HB-EGF, EGF, CD40, P-selectin, ICAM-1 (intercellular adhesion molecule 1), Ephrin B2, Ephrin B4, VCAM-1, Flk-1, VE-cadherin, Tie-2, neuropilin 1/β1, neuropilin 1/β2, Kitl1, Kitl2 and EMMPRIN] have been described previously [30–34]. Cells were transfected with constructs using FuGENE® 6 transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions. Lipofectamine™ 2000 reagent (Invitrogen) was used for siRNA transfection into sEND.1 cells and HUVECs, and was added at the same time as TGFβ or TNFα.
siRNA against mouse ADAM12 (J-043494-05, J-043494-06, J-043494-07 and J-043494-08) or siRNA against human ADAM12 (D-005118-01, D-005118-02, D-005118-17 and D-005118-18) was obtained from Dharmacon and a mission siRNA universal negative control was purchased from Sigma–Aldrich. A mixture of four different siRNAs against ADAM12 was used with a final concentration of 25 nM.

Shedding assay
ADAM12-mediated shedding of AP-tagged substrates was determined as described previously [19]. Briefly, 293-VnR cells were co-transfected with either full-length transmembrane ADAM12 or catalytically inactive transmembrane ADAM12-E351Q together with an AP-tagged substrate. Subsequently, cells were washed and incubated for 90 min with serum-free medium. When indicated, cells were treated with inhibitors or vehicle controls for an initial 15 min, followed by incubation for 90 min with inhibitors or vehicle controls. For photometric quantification of shedding, cell-conditioned medium and lysate were harvested and mixed with a solution of the AP substrate 4-nitrophenyl. All experiments were performed at least three times in triplicate. ADAM12-mediated shedding was calculated as AP activity in the conditioned medium divided by AP activity in the medium and corresponding cell lysate after subtracting background signal from non-transfected cells. The value obtained from wild-type ADAM12-transfected cells was divided by the value obtained from ADAM12-E351Q-transfected cells, and the ADAM12-mediated shedding calculated as the fold change compared with that observed in the control cells. Inhibitors were tested as a 10-fold dilution series with a final concentration range 0.01–100 μM inhibitor. Equal amounts of DMSO served as controls. IC₅₀ and S.E.M. values were calculated by fitting the relative activities to a four-parameter logistic model:

\[ f(x) = \frac{A}{x/(C^b) + 1} \]

using XLiFit (ID Business Solutions) and fixing parameters A or B to 0 % and 100 % respectively, as required.

Wild-type MEFs, MEFA9, 15/+/−cells and MEFA9, 12, 15/−/− cells were transfected with AP-tagged substrates and incubated for 24 h. To enrich for MEFs transfected with AP-tagged substrates, cells were sorted with FACS according to a previously published protocol [35] using antibodies against the AP tag (mouse monoclonal anti-AP) using FACS Aria (BD Biosciences). Subsequently, MEFs were treated for 24 h in serum-free DMEM with or without TGFβ. Conditioned medium and cell lysates were then isolated and the AP activity measured using the AP substrate 4-nitrophenyl. The amount of product was quantified by measuring the absorbance at 405 nm. Substrate-shedding activity in TGFβ-treated cells was calculated as AP activity in the conditioned medium divided by AP activity in the medium and corresponding cell lysate after subtracting background signal from non-transfected cells. The value from untreated cells was set to 1. Western blot analysis was performed to ensure equal expression of ADAM12, ADAM12-E351Q and AP-tagged substrate.

qPCR (quantitative PCR)
Total RNA was extracted and isolated from mouse and human cells using TRIZol® (Invitrogen). RNA (1 μg) was treated with DNase I and reverse-transcribed using the High-Capacity cDNA RT (reverse transcription) kit from Applied Biosystems. qPCR was performed using SYBR Green PCR Mastermix from Applied Biosystems and a program of: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Primers used for qPCR have been described previously [12,36]. rPO (ribosomal phosphoprotein) was used as a reference gene, and qPCR data were analysed using the 2(−ΔΔCt) method.

Western blotting and immunoprecipitation
Western blots of cell lysates and 10× concentrated conditioned media were performed as described previously [12]. To enrich for glycoproteins, in particular for ADAM12, cell lysates were subjected to concanaval A–agarose beads (Sigma–Aldrich) and eluted as described previously [12] before electrophoresis and blotting. For immunoprecipitation studies, cells were extracted in lysis buffer [3% SDS, 10% glycerol, 10 mM Na₂HPO₄, and protease and phosphatase inhibitors (Sigma)], incubated with a mixture of antibodies against ADAM12 and precipitated as described previously [22] before electrophoresis and blotting. Following protein separation by SDS/PAGE and transfer to Immobilon-P membranes (Millipore), the antibodies indicated were added and incubated for 16 h at 4°C. HRP (horseradish peroxidase)-conjugated anti-(rabbit IgG) and anti-(mouse IgG) antibodies (Dako) and the chemiluminescence EZ-ECL reagent (Biological Industries) were used for detection.

Immunohistochemistry
Specimens from breast resections of 30 patients [20 with IDC and ten with ILC (infiltrating lobular carcinoma)] who underwent conservation surgery for a breast lump at Turku University Hospital (Turku, Finland) were analysed as described previously [12]. Normal breast tissue was obtained from nine patients at the same hospital who underwent aesthetic breast-reduction surgery. ADAM12 immunostaining was performed as described previously [13], using either polyclonal antibodies against human ADAM12 (rb122; 1:500 dilution) or as a control normal rabbit IgG antibodies (Dako) and the chemiluminescence EZ-ECL reagent (Biological Industries) were used for detection.

Statistical analysis
All experiments were performed at least three times. Statistical analysis was done using Student’s t test for comparing two independent groups. P < 0.05 was considered statistically significant.

RESULTS
ADAM12-mediated ectodomain shedding of Tie-2, Kit1, VE-cadherin, Fik-1 and VCAM-1
We analysed the ability of ADAM12 to shed a panel of biologically important transmembrane proteins, including most EGFR ligands, proteins involved in angiogenesis, cell adhesion molecules and some cytokines. ADAM12 or as a control its catalytically inactive form (ADAM12-E351Q) were co-transfected into 293-VnR cells with individual cDNAs encoding the substrates N-terminally fused to AP to allow detection of released ectodomain molecules into the cell culture medium.
Figure 1  Identification of five new substrates for ADAM12

293-VnR cells were co-transfected with ADAM12 or catalytically inactive ADAM12 (ADAM12-E351Q) together with individual substrates fused to AP (substrate-AP). At 48 h after transfection, cells were incubated for 90 min with serum-free medium. Conditioned media and cell lysates were isolated and mixed with the AP substrate 4-nitrophenyl, and the AP activity was measured as the absorbance at 405 nm. Substrate-shedding activity was calculated as AP activity in medium divided by the total activity in medium and cell lysate, relative to cells transfected with substrate-AP and the ADAM12-E351Q control. Values are means ± S.D., for at least three independent experiments.

Screening of 16 different AP-tagged substrates revealed increased ectodomain shedding of five previously unreported substrates upon overexpression of ADAM12: Tie-2 more than 4-fold, VE-cadherin and Flk-1 more than 3-fold, and Kitl1 and VCAM-1 more than 2-fold, as compared with cells overexpressing ADAM12-E351Q. Importantly, ADAM12 ectodomain shedding of all five of these new substrates, except VCAM-1 and Kitl1, was greater than that of the previously characterized substrate EGF (Figure 1) [19]. Interestingly, endothelial cells specifically express four of these suggested new substrates (Tie-2, VE-cadherin, Flk-1 and VCAM-1), implying a hitherto not considered role for ADAM12 in endothelial cell function.

ADAM12 localizes to tumour-associated vessels in human breast cancer

Most studies on ADAM12 expression patterns have not reported a distinct presence of ADAM12 in the vasculature in human tissue [7]. ADAM12 is highly expressed in tumour cells of a variety of different cancer types [7], but most recently ADAM12 has also been shown to be expressed by the tumour vasculature of human ovarian cancer, but not in the normal vasculature [37]. As demonstrated in Figure 2, ADAM12 immunohistochemistry confirmed multiple previous reports that ADAM12 is highly expressed by the tumour cells of human breast cancer, and was only weakly expressed in the normal breast [10,12,38]. Semi-quantitative immunohistochemical analyses (Figure 2A) demonstrated detectable ADAM12 immunoreactivity in a mean of approximately 6% of the vessels in IDC samples (Figures 2B and 2C). Consecutive serial sections demonstrate ADAM12 staining in CD31-positive vessels (Figures 2D and 2E). In comparison, the ILC samples (Figure 2F) revealed significantly less ADAM12 immunostaining in the vasculature, with a mean of less than 0.2% of the tumour-related vessels counted staining positive (Figure 2A) (P = 0.012). In comparison, no vessels in the normal tissue examined stained positive for ADAM12 (Figures 2A and 2G). This result indicates that ADAM12 expression is specifically associated with the tumour vasculature of IDC of the breast, albeit in only a few vessels.

Inhibitor profiling of ADAM12-mediated ectodomain shedding

The fact that ADAM12 can release several vascular proteins (Tie-2, VE-cadherin, Flk-1 and VCAM-1) from the cell surface by ectodomain shedding (Figure 1) and further that ADAM12 is present in tumour vasculature (Figure 2) prompted us to characterize the shedding process in more detail. First, we determined the inhibitor profile of the protease activity of ADAM12-S, using an in vitro quenched fluorescent peptide assay with a TNF-α sequence as the substrate [39]. Using the in vitro assay, batimastat and MMP inhibitor V were the most effective inhibitors, with an IC50 of 0.018 ± 0.002 μM (± S.E.M.) and 0.071 ± 0.011 μM respectively. In contrast, TAPI-1, GM6001, MMP-3 inhibitor VIII and TAPI-2 were the weakest ADAM12 inhibitors, with estimated IC50 values of approximately 2 μM (Table 1).

Next, these inhibitors against ADAM12 were used to test their inhibitory effect in the cell-based shedding assay, using AP-tagged VE-cadherin, Tie-2 and Flk-1 as substrates for ADAM12. We treated cell cultures with increasing concentrations (0.01, 0.1, 1, 10 and 100 μM) of batimastat, MMP inhibitor V, GM6001 and TAPI-2. The inhibition of VE-cadherin, Tie-2 and Flk-1 shedding by batimastat and MMP inhibitor V was dose-dependent (Figure 3A), whereas GM6001 and TAPI-2 were unable to inhibit ADAM12-mediated shedding (Figure 3A). IC50 values of batimastat and MMP inhibitor V were determined for each substrate (Table 2). Notably, IC50 values of batimastat and MMP inhibitor V were approximately 10-fold higher in the cell-based shedding assay compared with the in vitro assay.

Western blot analysis demonstrated less sTie-2 (soluble Tie-2) in the conditioned medium from batimastat- and MMP inhibitor
Figure 2  ADAM12 localizes to tumour-associated vessels

Immunohistochemical staining of ADAM12 in breast cancer tissue using an ADAM12 polyclonal antiserum (rb122) and counterstained with haematoxylin. (A) Dot plot illustrating the percentage of vessels staining positive for ADAM12 in non-tumour cases (n = 9), IDC (n = 20) and ILC (n = 10). A range of 20–200 vessels was counted in each tissue sample, and a total of >600 vessels was counted in non-tumour cases, >1600 vessels was counted in IDC cases and >500 vessels was counted in ILC cases. (B and C) Two cases of IDC (T, tumour) with neovessel staining positive for ADAM12 (black arrows). (D and E) Consecutive serial sections of IDC with immunohistochemical staining of ADAM12 (D) and CD31 (E). (F) ILC with single tumour cells positive for ADAM12 (red arrow). The adjacent vessels are unstained (black arrow). (G) TDLUs from normal breast tissue obtained from breast reduction surgery. The vessels are unstained. (B, C and G) Scale bar = 40μm. (D and E) Scale bar = 20μm. (F) Scale bar = 60μm. *P < 0.05.

Table 1: Inhibitor profile of ADAM12-S

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC50 (μM)</th>
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<tbody>
<tr>
<td>Batimastat</td>
<td>0.018 ± 0.002</td>
</tr>
<tr>
<td>MMP inhibitor V</td>
<td>0.071 ± 0.011</td>
</tr>
<tr>
<td>TAPI-0</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>Marimastat</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>MMP inhibitor III</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>TAPI-1</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>GM6001</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>MMP-3 inhibitor VIII</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>TAPI-2</td>
<td>6.4 ± 0.9</td>
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</table>

V-treated ADAM12 and Tie2 co-transfected cells compared with DMSO control-treated cells (Supplementary Figure S1A at http://www.biochemj.org/bj/452/bj4520097add.htm). Similar results were observed for VE-cadherin and Flk-1 (results not shown). However, as shown by Western blotting, although sTie-2 was still present in conditioned medium from cells overexpressing wild-type ADAM12 and treated with GM6001 and TAPI-2, both inhibitors completely blocked the constitutive ectodomain shedding observed in cells overexpressing ADAM12-E351Q (Supplementary Figure S1B). Similar results were observed for VE-cadherin, Kitl1 and Flk-1 (results not shown). These results indicate that constitutive shedding of VE-cadherin, Tie-2, Kitl1 and Flk-1 substrates in 293-VnR cells is mediated by metalloproteinases other than ADAM12. No difference in ectodomain shedding was observed when ADAM12-E351Q was exchanged with empty vector, suggesting that in this cell system catalytically inactive ADAM12 does not induce shedding by other proteases (Supplementary Figure S2 at http://www.biochemj.org/bj/452/bj4520097add.htm). Thus, among the tested commercially available inhibitors, batimastat and MMP inhibitor V were the most efficient inhibitors against ADAM12-mediated shedding of VE-cadherin, Tie-2 and Flk-1, whereas GM6001 and TAPI-2 were the weakest, or were not at all efficient, for inhibiting the catalytic activity of ADAM12 in these assays.

Previous studies examining the natural TIMPs demonstrated a unique inhibitor profile for ADAM12 [19,39]. Although most membrane-bound ADAMs are inhibited by TIMP-3 [5], it has been shown that TIMP-2 can also inhibit the catalytic activity of both ADAM12-S and ADAM12-L, although higher doses were required [19,39]. Hence we tested the effect of N-TIMP-2 and TIMP-3 on ADAM12-mediated shedding of VE-cadherin and Tie-2. We found that 1 μM N-TIMP-2 inhibited ADAM12-mediated shedding of VE-cadherin and Tie-2 by 39 and 47% respectively, whereas 0.1 μM did not have any inhibitory effect (Figure 3B). Both 0.01 and 0.1 μM TIMP-3 inhibited ADAM12-mediated shedding of VE-cadherin by 35%, whereas
Batimastat and MMP inhibitor V, as well as N-TIMP2 and TIMP-3, inhibit ADAM12-mediated shedding

293-VnR cells were co-transfected with ADAM12 or catalytically inactive ADAM12 (ADAM12-E351Q) together with the indicated substrates fused to AP. At 48 h after transfection, cells were incubated for 90 min with serum-free medium with or without the inhibitors indicated: (A) batimastat, MMP inhibitor V, GM6001 and TAPI-2; (B) N-TIMP-2; and (C) TIMP-3. DMSO served as control in (A). For all, conditioned media and cell lysates were isolated and mixed with the AP substrate 4-nitrophenyl, and the AP activity was measured as the absorbance at 405 nm. Substrate-shedding activity was calculated as AP activity in medium divided by the total activity in medium and cell lysate, relative to cells transfected with substrate-AP and the ADAM12-E351Q control. The values obtained for controls were set to 100 %.

Table 2 Inhibitor profile of the transmembrane form of ADAM12

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>VE-cadherin IC50 (μM)</th>
<th>Tie-2 IC50 (μM)</th>
<th>Flk-1 IC50 (μM)</th>
</tr>
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<tbody>
<tr>
<td>Batimastat</td>
<td>0.12 ± 0.07</td>
<td>0.33 ± 0.11</td>
<td>12.43 ± 4.25</td>
</tr>
<tr>
<td>MMP inhibitor V</td>
<td>1.23 ± 5.49</td>
<td>14.50 ± 6.07</td>
<td>26.09 ± 6.45</td>
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TIMP-3 inhibited Tie-2 in a dose-dependent manner, with 0.01 μM inhibiting shedding by 35 % and 0.1 μM inhibiting shedding by 50 % (Figure 3C). Because the inhibition was not complete, it cannot be ruled out that other metalloproteinases could be involved.

Taken together, these data support the notion that ADAM12 mediates ectodomain shedding of the endothelial-specific substrates VE-cadherin, Tie-2 and Flk-1.

TGFβ regulates endogenous ADAM12-mediated shedding of VE-cadherin and Tie-2 in MEFs

To test the specificity of endogenous ADAM12 in ectodomain shedding of VE-cadherin and Tie-2, we took advantage of a ‘loss-of-function’ approach in which MEFs from wild-type mice were compared with MEFs from MEF9,15−/− cells and MEF9,12,15−/− cells respectively. It has been previously reported that wild-type MEFs express ADAM12 [26], and that expression is up-regulated after TGFβ stimulation [40]. We tested the effect of TGFβ on wild-type MEFs, MEF9,15−/− cells and MEF9,12,15−/− cells and found an increased level of endogenous mature ADAM12 protein upon TGFβ stimulation in wild-type MEFs and MEF9,15−/− cells, and a lack of ADAM12 expression in MEF9,12,15−/− cells (Figure 4A). To test whether the TGFβ-induced increased levels of ADAM12 in wild-type MEFs and MEF9,15−/− cells were associated with enhanced ectodomain shedding, we expressed AP-tagged VE-cadherin and Tie-2 in all three MEF cell lines. In wild-type MEFs, treatment with TGFβ increased levels of soluble VE-cadherin by 2.8 ± 0.76-fold (P = 0.024) and Tie-2 by 2.5 ± 0.7-fold (P = 0.069) compared with untreated cells (Figures 4B and 4C). Shedding of VE-cadherin and Tie-2 was increased...
and cell lysates were isolated and mixed with the AP substrate 4-nitrophenyl, and the AP activity was measured as the absorbance at 405 nm. Substrate-shedding activity in TGFβ was calculated as AP activity in the medium divided by the total activity in media and cell lysate, relative to untreated cells. Values are means ± S.D. for at least three independent experiments. *P < 0.05.

Importantly, ectodomain shedding of VE-cadherin and Tie-2 [31,41]. Thus to exclude the possibility that the increased level of ADAM12 protein in sEND.1 cells and HUVECs after TGFβ treatment is a consequence of increased expression of related ADAMs (Supplementary Figure S3). Previous reports have shown that various ADAMs facilitate ectodomain shedding of VE-cadherin and Tie-2 [31,41]. Thus to exclude the possibility that the increased levels of shedding observed after TGFβ treatment is a consequence of increased expression of related ADAM proteins with known proteolytic activity, we investigated whether cytokine-stimulated endothelial cells with these factors for 24 h, TGFβ induced the expression of ADAM12 mRNA in sEND.1 cells (Figure 5A), whereas in HUVECs, ADAM12 expression was up-regulated upon TNFα stimulation (Figure 5B). In both sEND.1 cells and HUVECs, the expression of ADAM12 mRNA increased in a dose-dependent manner (Figures 5A and 5B). Western blotting confirmed an increased level of ADAM12 protein in sEND.1 cells and HUVECs after TGFβ and TNFα treatment respectively (Figures 5C and 5D). Next, we asked whether stimulation of endothelial cells with these cytokines would enhance ectodomain shedding of endogenous VE-cadherin. VE-cadherin shedding not only releases a 95 kDa N-terminal ectodomain into the culture media (termed sVE-cadherin), but also generated a CTF (C-terminal fragment) in the cytoplasm [45,46]. We analysed cell lysates from sEND.1 cells treated with TGFβ by Western blotting, using an antibody against the C-terminal part of VE-cadherin, and found increased generation of the CTF compared with non-treated sEND.1 cells (Figure 5E). The level of ectodomain shedding was determined by quantifying band intensities and demonstrated a significant approximately 2-fold induction of VE-cadherin in TGFβ-stimulated sEND.1 cells (Figure 5E). The level of ectodomain shedding was determined by quantifying band intensities and demonstrated a significant approximately 2-fold induction of VE-cadherin in TGFβ-stimulated sEND.1 cells compared with control cells (5 ng/ml TGFβ, P = 0.0005; 20 ng/ml TGFβ, P = 0.03). Likewise, cell lysates from HUVECs treated with or without TNFα for 24 h were analysed for generation of CTF. In addition, a human-specific antibody against VE-cadherin allowed detection of sVE-cadherin in the conditioned medium from HUVECs. In both cases we found a 2-fold induction in shedding of VE-cadherin in TNFα-treated HUVECs compared with control-treated cells (CTF, P = 0.0471; sVE-cadherin, P = 0.029) (Figure 5F).

ADAM12-mediated shedding of endogenous VE-cadherin in cytokine-stimulated endothelial cells

Next, we tested the expression of endogenous ADAM12 in cultured endothelial cells. RT–PCR detected ADAM12 mRNA in mouse sEND.1 cells and HUVECs; however, as determined by qPCR, the levels were fairly low (results not shown). We and others have shown that humoral factors, such as TGFβ, TNFα and bFGF, up-regulate the expression of ADAM12 in various cell lines [42–44]. When we treated cultured endothelial cells with these factors for 24 h, TGFβ induced the expression of ADAM12 mRNA in sEND.1 cells (Figure 5A), whereas in HUVECs, ADAM12 expression was up-regulated upon TNFα stimulation (Figure 5B). In both sEND.1 cells and HUVECs, the expression of ADAM12 mRNA increased in a dose-dependent manner (Figures 5A and 5B). Western blotting confirmed an increased level of ADAM12 protein in sEND.1 cells and HUVECs after TGFβ and TNFα treatment respectively (Figures 5C and 5D). Next, we asked whether stimulation of endothelial cells with these cytokines would enhance ectodomain shedding of endogenous VE-cadherin. VE-cadherin shedding not only releases a 95 kDa N-terminal ectodomain into the culture media (termed sVE-cadherin), but also generated a CTF (C-terminal fragment) in the cytoplasm [45,46]. We analysed cell lysates from sEND.1 cells treated with TGFβ by Western blotting, using an antibody against the C-terminal part of VE-cadherin, and found increased generation of the CTF compared with non-treated sEND.1 cells (Figure 5E). The level of ectodomain shedding was determined by quantifying band intensities and demonstrated a significant approximately 2-fold induction of VE-cadherin in TGFβ-stimulated sEND.1 cells compared with control cells (5 ng/ml TGFβ, P = 0.0005; 20 ng/ml TGFβ, P = 0.03). Likewise, cell lysates from HUVECs treated with or without TNFα for 24 h were analysed for generation of CTF. In addition, a human-specific antibody against VE-cadherin allowed detection of sVE-cadherin in the conditioned medium from HUVECs. In both cases we found a 2-fold induction in shedding of VE-cadherin in TNFα-treated HUVECs compared with control-treated cells (CTF, P = 0.0471; sVE-cadherin, P = 0.029) (Figure 5F).
To further investigate whether increased ADAM12 expression is responsible for the observed ectodomain shedding of VE-cadherin in cytokine-stimulated endothelial cells, we transfected cells with a mixture of four different siRNAs against ADAM12 or control siRNA. In order to examine the specificity of the ADAM12 siRNAs, the mRNA levels of related ADAMs were analysed by qPCR in HUVECs and sEND.1 cells, showing that none were regulated by the siRNAs against ADAM12 (Supplementary Figure S4 at http://www.biochemj.org/bj/452/bj4520097add.htm). ADAM12 was knocked down by approximately 80% in unstimulated sEND.1 cells compared with control cells, and approximately 60% in sEND.1 cells treated with TGFβ, as compared with TGFβ-stimulated control cells (Figure 6A). Although lack of knockdown in unstimulated HUVECs might reflect the very low levels of mRNA expression of ADAM12 in these cells, we did observe 50% knockdown of ADAM12 mRNA in HUVECs treated with TNFα, as compared with the TNFα-stimulated siRNA control HUVECs (Figure 6B). However, in the knockdown of ADAM12 in TGFβ-treated sEND.1 cells and in TNFα-treated HUVECs, ADAM12 mRNA was still up-regulated by 2–3-fold as compared with untreated cells, indicating that the siRNA-mediated knockdown could not compete entirely with the cytokine-induced expression of ADAM12 (Figures 6A and 6B). To ensure also that protein levels
ADAM12 mediates ectodomain shedding of vascular proteins

Figure 6 Knockdown of ADAM12 inhibits ectodomain shedding

(A, C and E) sEND.1 cells were transfected with siRNAs against mouse ADAM12 or control siRNAs, and at the same time treated with or without TGFβ for 48 h. (B, D and F) HUVECs were transfected with siRNAs against human ADAM12 or control siRNAs, and at the same time treated with or without TNFα for 48 h. (A and B) Conditioned medium was harvested and total RNA was extracted from cell lysates from sEND.1 cells (A) and HUVECs (B). Expression of ADAM12 in cell lysates was examined by qPCR. (C) Total cell lysate from TGFβ-stimulated sEND.1 cells were subjected to immunoprecipitation, using beads bound to a mixture of antibodies against ADAM12 (mouse monoclonal antibody 6C10 and a polyclonal antibody from ProteinTech). Eluates were subjected to Western blotting and analysed for ADAM12 expression using a polyclonal antibody against ADAM12. Total cell lysates were analysed for actin expression to ensure an equal amount of protein. (D) Total cell lysate from TNFα-stimulated HUVECs were subjected to immunoprecipitation, using beads bound to a mixture of mouse monoclonal antibodies against ADAM12 (6C10, 8F8 and 6E6). Eluates were subjected to Western blotting and analysed for ADAM12 expression using a polyclonal rabbit antibody. Total cell lysates were analysed for actin expression to ensure an equal amount of protein. (E and F) Protein from cell lysates (10 μg) was subjected to Western blotting and analysed for the presence of VE-cadherin. The arrow indicates a CTF, which is released when VE-cadherin is ectodomain-shed. Numbers under the Western blot images depict fold changes in CTF/actin, which was determined by quantification of the band intensities (using ImageJ analysis software). VE-cadherin from the conditioned media (F) was analysed by Western blotting as described above. The blots are representative of three independent experiments. (G and H) Expression of ADAM8, ADAM9, ADAM10, ADAM12, ADAM15, ADAM17 and MT1-MMP (MMP14) mRNA in sEND.1 cells treated with or without TGFβ (G) and HUVECs treated with or without TNFα (H) for 24 h and examined by qPCR. (A, B, G and H) rPO was used as a reference gene and untreated control cells were set to a value of 1. The data were analysed using the 2[−ΔΔCT] method. *P < 0.05. For Western blots, the molecular mass in kDa is indicated. IP, immunoprecipitation.

of ADAM12 were reduced in cytokine-stimulated endothelial cells, immunoprecipitation and subsequent Western blot analysis of siRNA- and cytokine-treated sEND.1 cells and HUVECs were performed (Figures 6C and 6D). In agreement with the qPCR data, siRNA against ADAM12 caused a clear reduction in ADAM12 protein levels in both TGFβ- and TNFα-treated HUVECs.

Next, we compared the level of shed VE-cadherin in cytokine-stimulated sEND.1 cells and HUVECs with cells treated with siRNA against ADAM12. Importantly, our data showed that knockdown of ADAM12 decreased the stimulatory effects of TGFβ- and TNFα-induced shedding of VE-cadherin in sEND.1 cells and HUVECs respectively (Figures 6E and 6F).

To exclude the possibility that increased expression of related ADAMs and MMPs was responsible for the TGFβ- and TNFα-induced shedding of VE-cadherin in sEND.1 cells and HUVECs respectively, we analysed by qPCR the expression level of ADAM8, ADAM9, ADAM10, ADAM15, ADAM17 and
MT1-MMP/MMP14 (membrane-type 1 MMP) in cytokine-stimulated cells. In sEND.1 cells, TGFβ did not regulate the expression of any of these related metalloproteases (Figure 6F); however, in HUVECs, TNFα induced expression of ADAM8 (Figure 6G). Taken together, our data suggest that endogenous ADAM12 is at least partly involved in shedding of VE-cadherin in endothelial cells.

ADAM12-mediated ectodomain shedding occurs in a cell-autonomous fashion

ADAM12 is up-regulated in several cancer types, and the fact that ADAM12 is highly expressed by the tumour cells themselves, as well as in tumour-associated vessels, raised the question as to whether ADAM12 sheds its endothelial-specific substrates in a cell-autonomous (in cis) or a non-cell-autonomous (in trans) fashion. To test whether ADAM12 sheds in a cell-autonomous manner, 293-VnR cells were co-transfected with ADAM12 and the individual AP-tagged substrates (Flik-1 or VE-cadherin). To test for in trans, we co-cultured 293-VnR cells transfected with either ADAM12 or with AP-tagged VE-cadherin or Flik-1. To ensure cell-cell contact, cells were seeded and grown to 100% confluence before detection of AP-tagged substrates in the cell culture medium. ADAM12 only shed Flik-1 and VE-cadherin when co-expressed in the same cells, suggesting that ADAM12 acts in a cell-autonomous manner (Figure 7A). In line with this finding, we showed that ADAM12 co-immunoprecipitates with its substrates and that the interaction is enhanced by inactivating the catalytic site of ADAM12 (ADAM12-E351Q mutant) (Figure 7B). Taken together, these data imply that ADAM12 is an important inducible sheddase in endothelial cells.

DISCUSSION

Ectodomain shedding of surface molecules in the vasculature is of critical importance for vascular biology [5]. Members of the ADAM protease family, including ADAM8, ADAM9, ADAM10, ADAM12, ADAM17, ADAM19, ADAM28 and ADAM33, shed a large variety of these vascular cell-surface proteins, and several ADAMs have overlapping substrate specificities [5]. In the present study we add to the understanding of this complex system, demonstrating that ADAM12 is capable of ectodomain shedding Kitl1, VE-cadherin, Tie-2, Flik-1 and VCAM-1.

ADAM12 has been shown to be highly up-regulated in tumour cells of various different carcinomas [7,12]; however, in the present study, for the first time, we provide evidence that
ADAM12 is furthermore present in the tumour vasculature in breast cancer tissue, but absent in normal breast tissue. A similar finding for ADAM12 in the vasculature of breast tumours was reported at the Annual Meeting of the American Association for Cancer Research, 2012 [47].

Most interestingly, we found ADAM12 expression in vessels associated with IDC, whereas vessels associated with ILC showed very little or no expression of ADAM12. These data corroborate a previous report identifying ADAM12 as a novel tumour-specific vascular marker for ovarian cancer [37]. In light of this, increased serum or plasma levels of the ADAM12 substrates identified (Kitl1, VE-cadherin, Tie-2, Flk-1 and VCAM-1) have been associated with several pathological conditions, including cancer, and therefore have been suggested as potential biomarkers [48–53]. Future studies will be needed to elucidate whether increased levels of these soluble substrates correlate with increased expression of ADAM12 in the vasculature of the primary tumour.

The consequences of ADAM12-mediated ectodomain shedding of vascular cell-surface proteins depend on the type of molecule shed. Thus the potential role of ADAM12 as a sheddase of vascular cell-surface proteins is of great interest, since neovascularization is an essential factor of tumour progression. In fact, ectodomain shedding of receptor tyrosine kinases, such as Flk-1 and Tie-2, may affect neovascularization [54–56]. We and others have previously shown that deletion of ADAM12 delays tumour progression in a mouse model of breast and prostate cancer [12,14]. Although we demonstrated that mouse stroma, which includes the endothelium, was not important for ADAM12-mediated breast tumour progression [12], we cannot exclude the possibility that increased expression of ADAM12 in tumour-associated vessels of human cancers will affect neovascularization or tumour dissemination. Increases in vascular permeability are seen in many pathological conditions, including inflammation and cancer growth and metastasis. Endothelial cell–cell junctions control the vascular permeability and depend on VE-cadherin-mediated homophilic interactions. Previously, ectodomain shedding of VE-cadherin was shown to regulate endothelial permeability [45]. We have previously shown that ADAM12-knockout mice exhibit significantly reduced incidence of lung metastasis [12]; thus future in vivo studies should clarify whether these observations reflect a reduced transmigration of tumour cells across the vessel wall.

All of the new potential substrates for ADAM12 we have identified in the present study are shed by more than one member of the ADAM family or the related MMP family: Kitl1 (ADAM8, 9, 17, 19 and 33), VE-cadherin (ADAM9 and 10), Tie-2 (ADAM8, 9, 17, and MT1-MMP), Flk-1 (ADAM10 and 17) and VCAM-1 (ADAM8 and 17) [5,31,41,45,57]. This redundancy raises the question under which circumstances a specific ADAM or MMP is required. Rapid regulation of shedding activity, such as during acute inflammation, involves cytokines (e.g. TNFα and interferon γ), ligands of GPCRs (G-protein-coupled receptors) and inducers of oxidative stress, as well as thrombin, all post-transcriptional regulators of the major shedding proteases ADAM10 and ADAM17 [58]. On the other hand, tumour tissue may provide a sustained environment for processes such as neovascularization and spreading of tumour cells to distant organs, which may involve induction of enzyme synthesis in the endothelium. The present study revealed little or no expression of ADAM12 in unstimulated endothelial cells. However, 24 h stimulation with TGFβ or TNFα induced the transcription of ADAM12, depending on the cell line used. These results confirm previous work demonstrating that TGFβ and TNFα induce expression of ADAM12 in various cell lines [12,40,42,44]. Interestingly, in the present study neither of these factors regulated transcription of other ADAMs tested (ADAM9, 10, 15 or 17), except for ADAM8, whose expression was also induced by TNFα. Importantly, our observations of TGFβ- or TNFα-induced ADAM12 expression and increased shedding of VE-cadherin, which was partly inhibited by siRNA-mediated knockdown of ADAM12, suggest that ADAM12-mediated ectodomain shedding of the newly identified substrates is under strict transcriptional regulation. These results, taken together with the observation that ADAM12 is specifically localized to tumour-associated vessels in human breast and ovarian cancer, imply that factors within the tumour–stroma compartment, such as TGFβ and TNFα, may switch on ADAM12 expression in the tumour vasculature, where it cleaves important endothelial factors.

Ectodomain shedding events by ADAMs and MMPs are regulated by the natural inhibitors TIMP-1–4 [5]. The results of the present study confirm a unique inhibitor profile for ADAM12 among most ADAM proteins [19], in that ADAM12-mediated shedding of VE-cadherin and Tie-2 is inhibited by N-TIMP-2 (at the 1 μM level) and in particular TIMP-3 (at the 10 nM level). We have previously shown that 0.1 μM TIMP-3 almost completely inhibited ADAM12-mediated shedding of EGF [19]. However, the present study only demonstrates a 40–50% inhibition of ADAM12-mediated shedding of VE-cadherin and Tie-2 using 0.1 μM TIMP3. A possible explanation for the lack of efficacy in the present study may be differences in activity of different batches of purified TIMP-3 used in these experiments. Indeed, the inhibitory activity of TIMP-3 used in our present study was less sufficient for inhibiting ADAM12-mediated shedding of EGF (results not shown) compared with our previous study [19].

Owing to the important impact of metalloproteinases in several pathological processes, a variety of agents targeting proteolytic activities have been developed. Our data confirm that GM6001 and TAPI-2 inadequately inhibit the catalytic activity of ADAM12-S [24] and furthermore, demonstrate that these compounds have no effect on ADAM12-L-mediated ectodomain shedding. In contrast with GM6001 and TAPI-2, we show in the present study that the hydroxamate-based compounds batimastat and MMP inhibitor V efficiently inhibit catalytic activity of both the secreted and transmembrane forms of ADAM12; however, IC50 values for each inhibitor were approximately 10-fold higher for ADAM12-mediated shedding of cell-surface proteins, as compared with IC50 values determined by the in vitro quenched fluorescent peptide assay. The discrepancies can be explained by the assay conditions, which significantly differ in the cell-based and in vitro assays. Although the in vitro assay is performed in a controlled environment, the cell-based shedding assay is undefined given the complex composition of the culture medium and presence of other metalloproteins in 293-VnR cells. The presence of free metal ions and metalloproteins, as well as unspecific binding interactions, may well affect the bioavailability of the hydroxamate inhibitors, which are therefore required at higher concentrations in order to fully inhibit ADAM12. Furthermore, the minor variations in IC50 values suggest that the effect of batimastat and MMP inhibitor V on ADAM12-mediated shedding is dependent on the substrates in question.

Like MMP inhibitor V, batimastat acts as a broad-spectrum inhibitor against MMPs and ADAMs, but failed in clinical trials due to severe toxicity [59]. The development of selective inhibitors is hampered by the high degree of structural similarity in the catalytic sites of ADAM and MMP [58]. Instead, new strategies for inhibitors might involve selectively targeting these metalloproteinases with therapeutic antibodies.

In summary, in the present study we describe five new potential substrates for ADAM12: Kitl1, VE-cadherin, Flk-1, VCAM-1 and...
Tie-2. In addition to being highly up-regulated in tumour cells, we found ADAM12 to be selectively expressed by the tumour-associated vessels in IDC, and that expression of ADAM12 was induced in cytokine-stimulated endothelial cells, where the shed form of VE-cadherin was concurrently elevated. We propose that in cancer settings increased expression of ADAM12 may regulate tumour angiogenesis and/or transmigration of tumour cells across the vessel wall.

AUTHOR CONTRIBUTION
Camilla Fröhlich designed the study, performed the main research, and wrote the paper. Marie Kiliåkjaer, Julie Noer, Alexander Kotshz and Camilla Nehammer performed additional experiments. Reidar Albrechtsen contributed with experiments and interpretation of data, and reviewed/edited the paper before submission. Paulina Kronqvist provided the human tissue material and helped with evaluations. Carl Blobel provided key reagents and, together with Jens Berthelsen, contributed with guidance and expertise. Marie Kveiborg and Ulla Wewer helped to design the study, contributed with interpretation of data and reviewed/edited the paper before submission.

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SUPPLEMENTARY ONLINE DATA

ADAM12 is expressed in the tumour vasculature and mediates ectodomain shedding of several membrane-anchored endothelial proteins

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Figure S1  293-VnR cells were co-transfected with ADAM12 or catalytically inactive ADAM12 (ADAM12-E351Q) fused to GFP together with the indicated substrates fused to AP

At 48 h after transfection, cells were incubated for 2 h with serum-free medium with or without the inhibitors indicated. Conditioned medium and cell lysate from co-transfected cells with or without the inhibitors indicated were analysed by Western blotting using antibodies against AP (for detection of Tie-2 and sTie-2), GFP (for detection of ADAM12) and actin (as a loading control).

Figure S2  293-VnR cells were co-transfected with ADAM12, catalytically inactive ADAM12 (ADAM12-E351Q) or a vector control together with substrates (VE-cadherin) fused to AP

At 48 h after transfection, cells were incubated for 2 h with serum-free medium. Conditioned medium and cell lysates were isolated and mixed with the AP substrate 4-nitrophenyl, and the AP activity was measured as the absorbance at 405 nm. Substrate-shedding activity was calculated as AP activity in medium divided by the total activity in medium and cell lysate, relative to cells transfected with VE-cadherin–AP and ADAM12-E351Q or a vector control.

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Figure S3  Expression of ADAM9, ADAM10, ADAM12, ADAM15 and ADAM17 mRNA in wild-type MEFs, and MEFA9,15−/− and MEFA9,12,15−/− cells treated with or without TGFβ for 24 h was examined by qPCR.

rPO was used as a reference gene, and untreated MEFs were set to a value of 1. The data were analysed using the 2(−ΔΔCt) method.

Figure S4  sEND.1 cells (A) and HUVECs (B) were transfected with siRNAs against mouse (A) or human (B) ADAM12 or control siRNA.

At 48 h after transfection RNA was harvested and expression of ADAM8, ADAM9, ADAM10, ADAM15 and ADAM17 mRNA in sEND.1 cells (A) and HUVECs (B) was examined by qPCR.

rPO was used as a reference gene and untreated control cells were set to a value of 1. The data were analysed using the 2(−ΔΔCt) method.

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