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# Chapter 5

## **Matrix Metalloproteinase-14 (MT1-MMP)-mediated endoglin shedding inhibits tumour angiogenesis**

Lukas J.A.C. Hawinkels<sup>1,2</sup>, Patricia Kuiper<sup>2</sup>, Eliza Wiercinska<sup>1</sup>, Hein W. Verspaget<sup>2</sup>, Zhen Liu<sup>1</sup>, Evangelia Pardali<sup>1</sup>, Cornelis F.M. Sier<sup>2,3</sup>, and Peter ten Dijke<sup>1</sup>

*Departments of <sup>1</sup>Molecular Cell Biology and Centre for Biomedical Genetics, <sup>2</sup>Gastroenterology-Hepatology, and <sup>3</sup>Surgery, Leiden University Medical Centre, Leiden, the Netherlands*

## **Abstract**

Endoglin is a transforming growth factor- $\beta$  coreceptor with a crucial role in angiogenesis. A soluble form of endoglin is present in the circulation, but the role of soluble endoglin (sEndoglin) is poorly understood. In addition, the endoglin shedding mechanism is not known. Therefore, we examined the role of sEndoglin in tumor angiogenesis and the mechanism by which the extracellular domain of endoglin is released from the membrane. In colorectal cancer specimens, we observed high endothelial endoglin protein expression, accompanied with slightly lower sEndoglin levels in the circulation, compared with healthy controls. *In vitro* analysis using endothelial sprouting assays revealed that sEndoglin reduced spontaneous and vascular endothelial growth factor–induced endothelial sprouting. Human umbilical vascular endothelial cells were found to secrete high levels of sEndoglin. Endoglin shedding was inhibited by matrix metalloproteinase (MMP) inhibitors and MMP-14 short hairpin RNA, indicating MMP-14 as the major endoglin shedding protease. Coexpression of endoglin and membrane-bound MMP-14 led to a strong increase in sEndoglin levels. Endoglin shedding required a direct interaction between endoglin and membrane-localized MMP-14. Using cleavage site mutants, we determined that MMP-14 cleaved endoglin at a site in close proximity to the transmembrane domain. Taken together, this study shows that MMP-14 mediates endoglin shedding, which may regulate the angiogenic potential of endothelial cells in the (colorectal) tumor microenvironment.

**Keywords:** MMP14, Angiogenesis, sEndoglin, MMP inhibitors, VEGF

## Introduction

Endoglin (CD105) is a 180-kDa integral membrane-bound glycoprotein, which serves as a high-affinity coreceptor for transforming growth factor (TGF)- $\beta$ 1 and TGF- $\beta$ 3, in the presence of the TGF- $\beta$  type II receptor (1, 2). Mutations in the gene encoding endoglin lead to hereditary haemorrhagic telangiectasia type I, a multisystemic vascular disease characterized by bleeding from small vascular lesions in the mucocutaneous tissues and the presence of arteriovenous malformations (3, 4). Endoglin is highly expressed by activated endothelial cells, in which it has a crucial role in angiogenesis, shown by the fact that endoglin knockout animals die in utero because of defects in the vascular system (5). Expression of endoglin can be induced by hypoxia (3, 6), TGF- $\beta$ 1 (1, 7, 8), and/or TGF- $\beta$ 3 (9). Endoglin expression was shown to be upregulated in various cancers (1) and correlated with the development of metastatic disease in colorectal cancer (CRC; ref. 6). Together, these data indicate a crucial role for endoglin in tumor angiogenesis. Besides membrane-bound endoglin, in the circulation, a soluble form (sEndoglin) exists (3). Elevated levels of sEndoglin have been reported in pregnant women suffering from preeclampsia (10, 11) and colorectal and breast cancer patients (12–14). However, the studies on sEndoglin in cancer are few and not conclusive.

Receptor shedding is important in regulating cellular homeostasis by influencing cytokine and growth factor signalling. Ectodomain shedding of receptors is mainly regulated through the proteolytic cleavage of the extracellular part of the receptor. The TGF- $\beta$  type I receptor (T $\beta$ RI) is released through tumor necrosis converting enzyme (TACE)-mediated cleavage, which decreases cell surface localized receptors and therefore inhibits TGF- $\beta$  signalling (15). The TGF- $\beta$  coreceptor betaglycan is released through proteolytic cleavage by membrane-type 1 matrix metalloproteinase (MMP-14). Soluble betaglycan can compete with membrane-bound betaglycan for TGF- $\beta$  binding and thereby inhibit TGF- $\beta$  effects on cells (16). sEndoglin has been shown to contribute to endothelial cell dysfunction (11, 17), but the mechanism of shedding and the consecutive effects of sEndoglin on tumor angiogenesis remain to be identified.

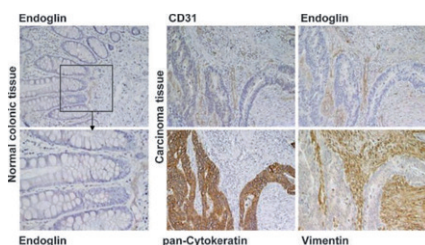
The aim of this study was to analyze the role of sEndoglin in regulating tumor angiogenesis and to identify the mechanism by which the extracellular part of endoglin is cleaved from cells. First, endoglin protein expression was determined in CRC tissue and sEndoglin levels in the circulation of CRC patients. Next, we evaluated the effect of sEndoglin on angiogenesis and the endoglin shedding mechanism in endothelial cells. The data revealed that MMP-14 sheds endoglin at a cleavage site close to the transmembrane domain.

## Results

### Endoglin expression and sEndoglin levels in CRC patients

We determined the expression and cellular localization of endoglin in CRC specimens, corresponding normal mucosa, and premalignant adenomatous polyps using immunohistochemistry. In normal colonic tissue, endoglin protein expression was low and only present in submucosal endothelial cells ( $n = 7$ ; Fig. 1, left). In contrast, in tumors, strongly increased endoglin protein expression in angiogenic endothelial cells was observed, confirmed by staining for the endothelial marker CD31 and vimentin, and absence of pan-cytokeratin staining ( $n = 7$ ; Fig. 1, right). ELISA analysis (Supplementary Fig. S1A) revealed that endoglin levels were significantly increased in carcinoma as compared with normal mucosa, (5.4 ng/mg protein versus 2.7 ng/mg protein, respectively;  $n = 191$ ;  $P < 0.0001$ ) or premalignant adenomas (2.5 ng/mg protein;  $n = 82$ ).

Next, we determined plasma sEndoglin levels in healthy volunteers and CRC patients pre-operation and 3 months post-operation. Mean preoperative sEndoglin levels in CRC patients were slightly but significantly lower (mean, 4.9 ng/ mL;  $n = 48$ ) compared with healthy controls (mean, 7.6 ng/ mL;  $n = 96$ ) and increased postoperatively (mean, 5.2 ng/mL;  $n = 25$ ; Supplementary Fig. S1B). In this cohort of CRC patients, sEndoglin levels were not related to the Dukes stage of the tumor (Supplementary Fig. S1C). Together, these data indicate that endoglin expression is not increased in benign adenomas, but strongly increased in CRC with slightly lower sEndoglin levels in the circulation of these patients.



**Fig. 1.** Endoglin expression in CRC. Localization of endoglin in normal colonic tissue (left) and in CRC (right). Staining was performed for endoglin, the pan-endothelial marker CD31, pan-cytokeratin (epithelial marker), and vimentin (mesenchymal marker) on sequential sections. Magnification  $\times 200$ ; bottom left,  $\times 400$ .

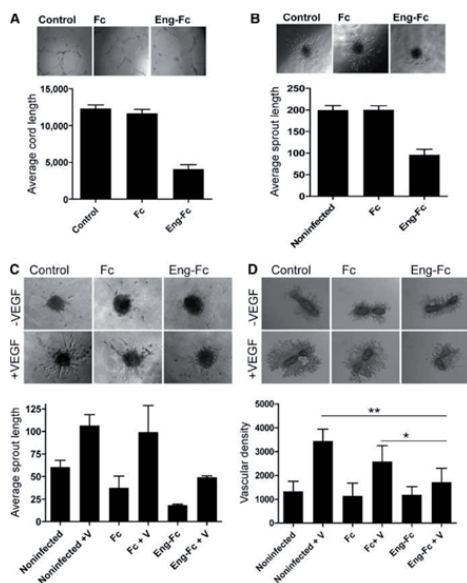
## sEndoglin inhibits angiogenesis

Endoglin plays an important role in endothelial cell function and angiogenesis. Therefore, we evaluated the role of sEndoglin in angiogenesis. HUVEC cells were subjected to cord formation assays on Matrigel and three-dimensional endothelial sprouting assays. HUVEC cells form tubes on Matrigel, which was efficiently inhibited (~65%) by the addition of 1 µg/mL recombinant endoglin-Fc chimera (Eng-Fc), but not by control Fc protein (Fig. 2A). In addition, endoglin-Fc also inhibited VEGF-induced endothelial sprouts in three-dimensional collagen matrices (~55% inhibition; Supplementary Fig. S2). To further investigate the inhibiting role of sEndoglin in angiogenesis, we used ECRF (immortalized HUVEC endothelial cells) and HUVEC cells, which were transduced with lentiviral vectors expressing endoglin-Fc or Fc only as a control. Expression of the Fc constructs was confirmed by Western blot analysis (anti-Fc and anti-endoglin antibodies; data not shown) and did not influence the proliferation of the cells (Supplementary Fig. S3A and B). Spheroids of ECRF cells formed spontaneous sprouts once embedded in collagen, which were left unaffected by the lentiviral-induced expression of the Fc protein. In contrast, sprouting was reduced ~50% when cells were transduced with an endoglin-Fc construct (Fig. 2B).

Furthermore, endoglin-Fc also efficiently inhibited VEGF-induced and basal sprouting of HUVEC spheroids (Fig. 2C). Together, these data indicate that sEndoglin reduces spontaneous sprout formation in ECRF cells, cord formation in HUVECs, and VEGF-induced sprouting of HUVEC cells. To confirm these data, we used a metatarsal assay. This model provides a combination of a quantitative *in vitro* assay combined with the complexity of a multicellular system. Metatarsals from mice were transduced with Fc- or endoglin-Fc expressing adenoviruses and capillary outgrowth was determined. The data revealed that endoglin-Fc significantly reduced VEGF-induced capillary outgrowth from fetal bones compared with Fc or non-infected cells (Fig. 2D).

## Identification of MMP-14 as the endothelial endoglin shedding protease

MMPs have been identified as important regulators of tumor invasion and metastasis, by influencing extracellular matrix remodeling, growth factor activation, and receptor shedding. To analyze the potential role of MMPs in regulating endoglin shedding, we used HUVEC endothelial cells that secrete high levels of sEndoglin into the medium (~1 ng/mL, ELISA). Western blot analysis revealed that the sEndoglin cleavage product has a similar size as the recombinant endoglin extracellular domain (Fig. 3A), which implies a cleavage site close to the transmembrane domain. To investigate the role of proteases in mediating endoglin shedding, HUVEC cells were treated with various protease inhibitors and sEndoglin levels were determined by ELISA.



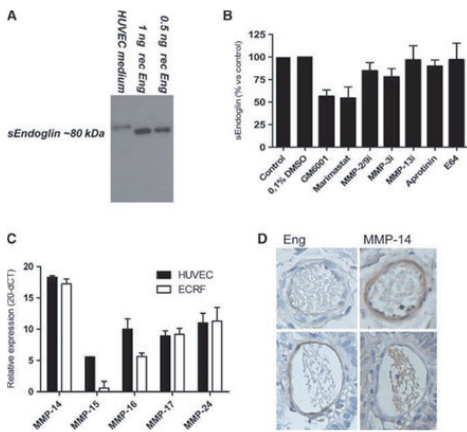
**Fig.2** Effect of sEndoglin on the angiogenic potential of endothelial cells. A, HUVEC cord formation assay. The average cord length in the presence of endoglin-Fc (Eng-Fc) or a control Fc protein (both  $1 \mu\text{g}/\text{mL}$ ) was analyzed by phase-contrast microscopy. Columns, mean from a representative experiment; bars, SD. B and C, ECRF (B) and HUVEC (C) endothelial sprouting assay with endoglin-Fc or Fc-expressing cells (+V, VEGF stimulated). Data represent mean of at least three independent experiment performed in quadruplicate. D, metatarsal assay. Endoglin-Fc expression reduces vascular density compared with Fc (\*,  $P = 0.047$ ) and control (\*\*,  $P = 0.0005$ ).

The broad-spectrum MMP inhibitors GM6001 and Marimastat strongly inhibited sEndoglin levels in contrast to inhibitors of cysteine proteases (E64) and serine proteases (Aprotinin), including cathepsins and plasmin, which had no inhibitory effect. Inhibition of sEndoglin release was 50% by both GM6001 and Marimastat (Fig. 3B). Specific inhibitors of gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3), had only a mild effect on sEndoglin release, whereas a collagenase inhibitor (MMP-13) did not affect sEndoglin levels at all. This indicated that these classes of MMPs were probably not involved in the shedding process and suggested membrane-type MMPs to be the primary endoglin shedding candidates because they are inhibited by these broad-spectrum inhibitors (29).

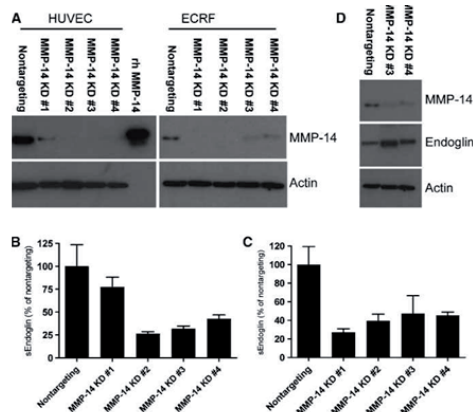
Therefore, we analyzed expression of MT-MMPs in HUVEC and ECRF cells by real-time PCR, which revealed that MMP-14 is the most abundantly expressed MT-MMP in endothelial cells (Fig. 3C). MMP-15, MMP-16, MMP-17, and MMP-24 are also expressed but to a lower extent, whereas MMP-25 was undetectable. MMP-14 plays an important role in cancer progression by influencing angiogenesis, invasion, and receptor shedding (30). Therefore, we analyzed the tissue distribution of MMP-14 in relation to endoglin in CRC cancer ( $n = 7$ ). MMP-14 expression was observed in malignant epithelial cells and endothelial cells. Interestingly, staining of sequential sections for MMP-14 and endoglin revealed that strong MMP-14 staining was accompanied by lower or absent endoglin staining (Fig. 3D, top). Accordingly, we observed that strong endoglin staining was accompanied by low or absent MMP-14 staining (Fig. 3D, bottom). These data indicate MMP-14 as the primary candidate to mediate endothelial endoglin shedding and was therefore further analyzed.

### MMP-14 knockdown strongly reduces endoglin shedding

To confirm the role of MMP-14 on endoglin shedding, we performed shRNA-mediated knockdown experiments. MMP-14 shRNA efficiently reduced MMP-14 expression in HUVEC and ECRF cells (Fig. 4A). Levels of sEndoglin were reduced up to 75% in the conditioned media of HUVEC cells (constructs #2, #3, and #4; Fig. 4B). A milder MMP-14 knockdown (construct #1) resulted in a minor decrease of sEndoglin, indicating that MMP-14-mediated endoglin shedding is very efficient and low levels of MMP-14 are already sufficient to mediate endoglin shedding. In ECRF cells, sEndoglin levels were reduced by 60% to 75% (Fig. 4C). Interestingly, a reduction of MMP-14 and sEndoglin was accompanied by a higher membrane-bound endoglin expression (Fig. 4D). These data indicate that in endothelial cells, MMP-14 is the main endoglin shedding protease.



**Fig. 3** MMP-14-mediated endoglin shedding in endothelial cells. *A*, Western blot analysis HUVEC conditioned medium. A band at similar height as recombinant human endoglin extracellular domain (Rec Eng) was observed. *B*, sEndoglin levels in HUVEC conditioned medium after additional protease inhibitors. GM6001 and Marimastat: broad-spectrum MMP inhibitors, Aprotinin and E64, and serine and cysteine protease inhibitors, respectively. % versus appropriate control, *n* = 4 to 6 independent experiments performed in triplicate; columns, mean; bars, SEM. *C*, real-time quantitative PCR for membrane-type MMPs in HUVEC and ECRF cells. Columns, mean from representative experiment performed in duplicate; bars, SD. *D*, Endoglin (Eng; left) and MMP-14 (right) staining on sequential colorectal carcinoma sections. Magnification,  $\times 400$ .

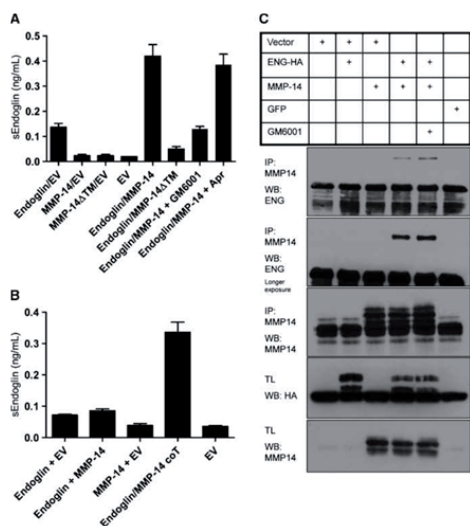


**Fig. 4** MMP-14 knockdown in endothelial cells. *A*, MMP-14 knockdown efficiency in HUVEC and ECRF cells analyzed by Western blot. Rh MMP-14, recombinant human MMP-14. sEndoglin levels in HUVEC (*B*) or ECRF (*C*) conditioned medium after MMP-14 knockdown. *D*, Western blot analysis of ECRF cells revealing increased membrane localized endoglin upon MMP-14 knockdown. All experiments were performed at least thrice in triplicate. Data shown are from a representative experiment.



## Membrane localization of MMP-14 is required for efficient endoglin shedding

To examine the endoglin shedding mechanism, we used COS-7 cells transiently transfected with endoglin and MMP-14 expression plasmids. After O/N starvation, sEndoglin levels were determined in the conditioned media. Co-expression of endoglin with membrane localized MMP-14 led to increased sEndoglin levels in the medium. This increase was not observed with the MMP-14 mutant lacking the transmembrane domain MMP-14 $\Delta$ TM (Fig. 5A). Accordingly, treatment with recombinant soluble MMP-14 did not increase sEndoglin levels (data not shown). The increase in endoglin shedding could be inhibited by the addition of the MMP inhibitor GM6001, but not by the addition of the serine protease inhibitor aprotinin. Finally, experiments showed that co-transfection with MMP-13, which is activated by MMP-14, did not increase endoglin shedding (data not shown).



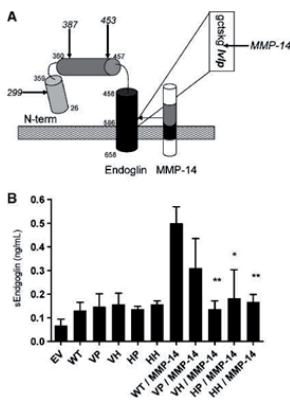
**Fig. 5** Endoglin and MMP-14 transfection in COS cells. *A*, COS cells were transfected with endoglin, and MMP-14 expression plasmids and sEndoglin levels were determined in the medium. EV, empty vector (pcDNA3.1); MMP-14  $\Delta$ TM, MMP-14 lacking the transmembrane domain; GM6001, broad-spectrum MMP inhibitor; Apr, aprotinin. *B*, COS cells were transfected with endoglin, MMP-14, or cotransfected (cot) expression plasmids and were reseeded. sEndoglin levels were determined. Columns, mean of three independent experiments performed in triplicate; bars, SEM. *C*, COS cells were cotransfected with endoglin and MMP-14. Next, immunoprecipitation of MMP-14 followed by endoglin Western blotting (WB) was performed. IP, immunoprecipitate; TL, total lysates before immunoprecipitation; HA, hemagglutinin tag. Green fluorescent protein was used as a transfection control.

Next, we studied if the expression of endoglin and MMP-14 on the same cell is required for efficient shedding. Cells were transfected with endoglin or MMP-14 plasmids or co-transfected with both. After reseeding, the cells in a 1:1 ratio sEndoglin levels were determined in the medium. Figure 5B shows that only cotransfection of endoglin and MMP-14 leads to endoglin shedding, indicating that expression of endoglin and MMP-14 on the same cell is required. To further evaluate if endoglin directly interacts with MMP-14, we performed coimmunoprecipitation experiments. Lysates from endoglin and MMP-14 co-transfected cells were immunoprecipitated with MMP-14 antibodies and subjected to Western blot analysis for endoglin. Figure 5C shows that endoglin coprecipitates with MMP-14 and that addition of GM6001 increased interaction between these proteins. Together, these data revealed that membrane localization of MMP-14 is required for efficient endoglin shedding and that the shedding process involves a direct interaction between endoglin and MMP-14.

## Determination of the MMP-14 cleavage site in endoglin

Because we observed that MMP-14 cleaves endoglin, releasing a fragment close to the size of the complete extracellular domain, we evaluated potential cleavage sites in proximity to the transmembrane domain. Analysis of 79 MMP-14 substrates with known cleavage site revealed that the majority of the cleavage sites have a flanking G-L amino acid sequence (Supplementary Fig. S4A and B). The endoglin sequence contains four G-L sequences located in different domains of the protein (Fig. 6A). One of these cleavage sites at position 586 to 587 is in close proximity to the transmembrane domain and would therefore lead to a cleavage product consisting of the complete extracellular domain. Therefore, we hypothesized that MMP-14 cleaves endoglin at position 586-587.

To investigate this possibility, we mutated amino acids 586 and 587 using site-directed mutagenesis. Four mutants were created, based on non-occurring amino acids in the natural MMP-14 substrates and keeping as much as possible the amino acid characteristics intact. The wild-type (WT) endoglin G-L sequence was mutated to V-P, V-H, H-P, and H-H. WT and mutant plasmids were cotransfected with MMP-14 in COS cells and sEndoglin levels in the medium were determined after O/N starvation. Mutation of these amino acids did not change the membrane localization of endoglin, as confirmed by FACS analysis (data not shown). Decreased sEndoglin levels were observed especially for the V-H, H-P, and H-H mutants and to a lesser extent with the V-P mutant (Fig. 6B). Western blot analysis confirmed the decrease in 80 kDa sEndoglin in the medium (data not shown). Taken together, these data indicate that MMP-14 cleaves endoglin at the G-L bond at position 586 to 587, releasing nearly full-length endoglin extracellular domain into the circulation.



**Fig. 6** Mutation of MMP-14 cleavage site in endoglin. **A**, endoglin contains four potential cleavage sites located in the extracellular domain at positions 299, 387, 453, and 586. **B**, the proposed MMP-14 cleavage site was mutated using site-directed mutagenesis and sEndoglin levels were determined in the conditioned media. EV, empty vector transfected cells; WT, WT endoglin. Columns, mean of representative experiment from three independent experiments; bars, SD. \*,  $P = 0.02$  versus WT; \*\*,  $P < 0.002$  versus WT.

## **Discussion**

In this study, we identify MMP-14 as the main endoglin shedding protease. We reveal that coexpression of endoglin with MMP-14 on the cell membrane leads to the cleavage of endoglin at the G-L bond at position 586, releasing the nearly complete endoglin extracellular domain. In addition we show that sEndoglin has antiangiogenic properties; it is capable of reducing spontaneous and VEGF- induced angiogenesis.

Our work shows that MMP-14 is the most abundantly expressed MT-MMP in endothelial cells and that knockdown of MMP-14 strongly reduces sEndoglin levels in the conditioned media of these cells. This process is very efficient because milder shRNA-mediated knockdown of MMP-14 still results in high sEndoglin levels. MMP-14 belongs to the subclass of the membrane-type MMPs and is highly expressed by (malignant) epithelial cells, endothelial cells, and several other cell types. MMP-14 can directly degrade extracellular matrix components, but also cleaves cell surface molecules such as EMMPRIN (CD147, an inducer of MMP expression), LRP, CD44, and cadherins (30–33). In addition, MMP-14 is the major activator of MMP-2 and MMP-13 (34) and it has an important role in tube formation during angiogenesis (35). This study shows that the coexpression of membrane-localized MMP-14 and endoglin results in increased endoglin shedding and this effect depends on a direct interaction between MMP-14 and endoglin. Cotransfection with MMP-13, which is downstream of MMP-14 in the MMP activation cascade, did not show this increase. Moreover, we identified the cleavage sequence used by MMP-14 to shed endoglin. Analysis of natural MMP-14 substrates revealed broad substrate specificity, but in the majority (40%) of the cases, the cleavage site was flanked by a glycine and leucine residue. Endoglin contains four G-L sequences in the extracellular domain, of which one is very close to the transmembrane domain. Mutation of the G-L sequence at position 586 to 587 resulted in a dramatic decrease in sEndoglin levels and therefore indicates this site to be the natural cleavage site.

In this study, we observed strongly increased tissue endoglin levels in colorectal carcinoma, but not in premalignant adenomas, corresponding to what has previously been shown for benign gastric lesions (36). High endoglin expression on tumor-associated endothelial cells has been associated with poor survival in various types of cancers (1, 6, 13), even being prognostically superior to other angiogenic markers such as CD31 or CD34 (13, 36, 37). Disturbance in the balance between membrane-localized and soluble receptors can result in pathologic conditions. For example, in pregnant women suffering from preeclampsia (a severe complication during pregnancies characterized by hypertension and proteinuria), soluble VEGF receptor (sFlt-1) and sEndoglin levels are strongly increased (10). Interestingly, gene expression analysis recently showed that in preeclampsia patients, both endoglin and MMP-14 gene expression is upregulated (38). Soluble receptors are capable of scavenging circulating ligands. VEGF can be bound to sFlt,

whereas it has been described that endoglin can bind different TGF- $\beta$  superfamily members (11, 39), which could influence angiogenesis. Our data show that sEndoglin inhibits spontaneous cord formation in HUVECs, sprout formation in ECRF cells, and VEGF- induced HUVEC sprouting. In addition, we show that sEndoglin inhibits capillary outgrowth from fetal mouse metatarsals. Our data are consistent with previous findings showing that adenoviral overexpression of sEndoglin interferes with vascular function by decreasing perfusion of the vessels and increasing their permeability (17). Preliminary data from our group show that endoglin-Fc strongly reduces microvessel density in a mouse model of invasive ductal breast carcinoma<sup>4</sup>. The exact role of sEndoglin in malignancies remains unclear. Several studies revealed increased levels in cancer patients compared with controls (12, 14, 40), whereas other showed no increased levels (41–44). On the other hand, our results from CRC patients support an anti-angiogenic role of sEndoglin because we observed lower circulating sEndoglin levels in these patients and would therefore suggest higher angiogenic activity in these patients. In line with our results, changes of sEndoglin levels in the circulation reported previously are generally low. This indicates that sEndoglin in the circulation cannot be used as a powerful marker of angiogenic activity in cancer patients. However, local regulation of endoglin shedding could result in dramatic changes in sEndoglin levels in the tumour microenvironment and therefore have stronger effects on the angiogenic potential of tumor-associated endothelial cells. Local up-regulation of endothelial MMP-14 expression will increase sEndoglin, decrease membrane localized endoglin, and transform the endothelium to a quiescent state. This notion is supported by our immune-staining results, showing the absence of endoglin expression with high MMP-14 staining on endothelial cells and vice versa.

Recent data showed a cross-talk between the TGF- $\beta$  signalling pathway and the VEGF-induced angiogenesis (45). Furthermore, we have shown that combined TGF- $\beta$  and BMP-9 treatment synergistically induces angiogenesis (46). Although the exact mechanism is subject of ongoing research, we speculate that scavenging of ligands such as BMP-9 and/or TGF- $\beta$  by sEndoglin can disturb this delicate balance required for efficient angiogenesis and the normalization of blood vessels. Together, these data indicate an important role for sEndoglin in regulating (tumour) angiogenesis and highlights the importance of the identification of the endoglin shedding protease.

## **Materials and Methods**

### **Patient material**

Preoperative and 3 months postoperative citrate plasma samples (n = 48 and n = 25, respectively) and tissue specimens (n = 191) from patients undergoing resection for primary CRC at the Department of Oncologic Surgery, Leiden University Medical Centre, were collected as described before (18, 19). In addition, plasma samples were collected from 22 healthy volunteers and an additional group of 74 healthy controls previously described (20). Colorectal adenomas (n = 82) were removed endoscopically at the Department of Gastroenterology-Hepatology. Tissue was homogenized and protein concentrations were determined as previously described (18). For immunohistochemistry, tumour tissue and adjacent normal mucosa were collected, fixated, dehydrated, and embedded in paraffin. Human samples were used according to guidelines of the Medical Ethics Committee of the Leiden University Medical Centre.

### **Cell culture**

COS cells were purchased from the American Type Culture Collection and maintained in DMEM, supplemented with 10% fetal bovine serum (FBS) and penicillin/ streptomycin. Human umbilical vein endothelial (HUVEC) cells were isolated from umbilical cords as described before (21). Cells were maintained in M199 medium supplemented with 20% FBS, penicillin/streptomycin, heparin (Leo Pharma), and bovine pituitary extract. ECRF cells (an immortalized HUVEC cell line; ref. 22) were a gift from Dr. R. Fontijn, Amsterdam Medical Center (Amsterdam, the Netherlands) and were cultured in HUVEC medium in fibronectin-coated cell culture flasks.

### **Endoglin and sEndoglin ELISA**

Endoglin levels in tissue homogenates (5  $\mu$ L) and cell culture media (50  $\mu$ L) were determined essentially according to the DY1097 human endoglin ELISA (R&D Systems). For sEndoglin determination in plasma samples, the capture antibody was replaced by mouse monoclonal anti-endoglin (clone E9, Cell Sciences). Detailed information can be found in Supplementary Materials and Methods.

### **Immunohistochemistry**

To determine tissue localization of endoglin, immunohistochemistry was performed as previously described (23). Representative photomicrographs were taken with a Nikon Eclipse E900 microscope equipped with a Nikon DXM1200 digital camera. Detailed information can be found in Supplementary Materials and Methods.

## Cord formation assay

HUVEC cells ( $3 \times 10^3$  cells/well) were resuspended in Medium 199 containing 4% FBS, 1  $\mu\text{g}/\text{mL}$  endoglin extracellular domain/Fc chimera (endoglin-Fc), or 1  $\mu\text{g}/\text{mL}$  Fc protein and seeded on growth factor reduced Matrigel coated (BD Biosciences) 96-well plates. After 16 hours, photographs were acquired with a phase contrast microscope in four different fields. The length of branches was quantified by automated image analysis using the Olympus analysis software.

## Endothelial sprouting assay

Endothelial sprouting assays were performed in the presence or absence of endoglin-Fc (1  $\mu\text{g}/\text{mL}$ ; R&D Systems) or Fc protein (1  $\mu\text{g}/\text{mL}$ ) as described before (24, 25). In addition, ECRF and HUVEC cells were infected with lentiviral vectors encoding endoglin-Fc or Fc only. Expression of the proteins was examined by Western blot using anti-Fc antibodies (dilution, 1:1,000; R&D Systems). Angiogenic activity was analyzed as described above. ECRF spheroids form spontaneous sprouts in collagen and were therefore not vascular endothelial growth factor (VEGF) stimulated.

## Metatarsal assay

Metatarsals were dissected from 17-day-old albino fetal mice and the assay was performed as described before (26). Bones were transduced with endoglin-Fc or endoglin-Fc adenovirus at  $1 \times 10^6$  plaque-forming unit at day 4. After stimulation with 50 ng/mL VEGF for 7 days, vessel formation was visualized by anti-CD31 staining. Vascular density was quantified by automated image analysis with Image J. Animal experiments were approved by the Dutch animal ethics committee.

## Protease inhibitor experiments

HUVEC cells were seeded in 24-well plates and treated with 0 to 160 ng/mL recombinant human MMP-14 (Chemicon) or with proteinase inhibitors: 20  $\mu\text{mol}/\text{L}$  E64 (cystein protease inhibitor), 10  $\mu\text{g}/\text{mL}$  Aprotinin (serine protease inhibitor, both Sigma- Aldrich), 1  $\mu\text{mol}/\text{L}$  GM6001 (broad-spectrum MMP inhibitor), 10  $\mu\text{mol}/\text{L}$  Marimastat (kindly provided by British Biotech Pharmaceuticals), 100 nmol/L MMP-2/MMP-9 inhibitor, 100 nm MMP-13 inhibitor, or 1  $\mu\text{mol}/\text{L}$  specific MMP-3 inhibitor (all Calbiochem). Serum-free M199 medium and 0.1% DMSO were included as controls. The percentage inhibition versus the appropriate control was calculated.

## Quantitative real-time PCR

To analyze the expression of membrane-type MMPs, total RNA was isolated from HUVEC and ECRF cells using Nucleospin RNA columns (Macherey-Nagel) followed by cDNA synthesis using Revertaid first strand cDNA synthesis kit (Fermentas). Quantitative PCR was performed as

previously described (27), according to the manufacturers' protocol, using MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25 primers ordered at the supplier (Applied Biosystems).

## Knockdown experiments

Lentiviral constructs expressing short hairpin RNAs (shRNA) targeting human MMP-14 were obtained from the Sigma Mission shRNA library. HUVEC and ECRF cells were infected with the lentiviral knockdown vectors, and 1 week after infection, the cells were plated in 24-well plates. Knockdown was confirmed by MMP-14 Western blot (Abcam; dilution, 1:2,000). After O/N starvation, serum-free medium was collected and subjected to sEndoglin ELISA.

## Transfections

COS cells were transfected with plasmids encoding HA-tagged endoglin (28), full-length MMP-14, MMP-14 lacking the transmembrane domain (MMP-14 $\Delta$ TM, both kindly provided by Dr. R. Hanemaaijer, TNO Quality of life BioSciences, Leiden, the Netherlands), or empty vector in triplicate. DNA (0.4  $\mu$ g) was transfected using Lipofectamin, according to the manufacturers' protocol (Invitrogen). Twenty-four hours after transfection, cells were serum starved for 16 hours and sEndoglin levels were determined by ELISA. To examine the interaction between endoglin and MMP-14, COS cells were transfected with endoglin and MMP-14 plasmids as described above in six-well plates. After 48 hours, cells were lysed and incubated with rabbit monoclonal MMP-14 antibodies (Abcam; 1:100) for 3 hours at 4°C, followed by protein-A Sepharose beads for another 30 minutes at 4°C. After extensive washing, samples were subjected to SDS-PAGE and Western blot detection with MMP-14 (Abcam; dilution, 1:2,000) and endoglin (R&D; dilution, 1:500) antibodies.

## Mutation of MMP-14 cleavage sequence in endoglin

The predicted MMP-14 cleavage site in endoglin [glycine-leucine (G-L) amino acid 586-7] was mutated into V-P, V-H, H-P, and H-H variants. PCR-mediated overlap extension method was used for site-directed mutagenesis using the pDisplay-endoglin plasmid as a template (28). Detailed information can be found in Supplementary Materials and Methods. The obtained constructs were sequenced and transfected into COS cells. Transmembrane localization of endoglin mutants was confirmed by fluorescence-activated cell sorting (FACS). After O/N starvation, medium was collected and subjected to sEndoglin ELISA.

## Statistical analysis

Differences were calculated using the t test, Mann-Whitney U test, or the Wilcoxon signed-rank test using the SPSS 14.0 statistical package. P values of <0.05 were considered statistically significant.

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## Supplementary Information

### Supplementary materials and methods

#### Endoglin and sEndoglin ELISA

96 well plates (MaxiSorp, Nunc, Glostrup, Denmark) were coated with mouse monoclonal anti human endoglin antibodies (R&D Systems, 2 µg/ml in PBS) overnight at 4°C. Plates were washed with PBS containing 0.05% Tween-20 (Merck, Darmstadt, Germany) after each step. Nonspecific binding was blocked with PBS containing 5% Tween-20, 0.05% Na<sub>3</sub>N for 2 h at room temperature (RT). Samples (5 µl tissue homogenates or 50 µl cell culture media) or standard (0-4 ng/ml recombinant human endoglin, R&D Systems) diluted in PBS with 1% bovine serum albumin (BSA, Sigma, Germany) were incubated for 2h at RT. Immunodetection was performed with biotinylated goat anti-human endoglin antibodies (2 µg/ml in PBS/1% BSA, R&D systems) for 2h at RT, and a substrate reagent pack according to the manufacturers' protocol (R&D Systems). Values were calculated in ng/ml for cell-culture media and in ng/mg total protein for tissue samples. The ELISA was validated by western blot analysis and spiking with a fixed amount of recombinant human endoglin. For determination of endoglin levels in citrate plasma samples the capture antibody was replaced by mouse monoclonal anti-endoglin (clone E9, Cell Sciences, Canton, MA, USA). The standard curve was diluted in PBS containing 40 mg/ml human serum albumin (Sigma) to correct for background absorption. The ELISA was validated using a serially diluted plasma samples and spiking of these samples with a fixed amount recombinant human endoglin, which accordingly increased the ELISA signal (data not shown). The ELISA was performed and validated as described above.

#### Immunohistochemistry

For immunohistochemical staining slides were deparaffinized, rehydrated and antigen retrieval was performed by boiling in 0.01M sodium citrate buffer pH 6.0. Slides were incubated overnight (O/N) at RT with unlabeled primary antibodies: mouse monoclonal anti-pan-cytokeratin (1:1000), mouse monoclonal anti-vimentin (1:400, both Santa Cruz Biotechnologies, Santa Cruz, USA), mouse monoclonal anti-CD31 (1:400, Dako, Glostrup, Denmark), mouse monoclonal anti-MMP-14 (1:1600), or biotinylated goat-anti human endoglin (1:200, both R&D systems). Immunodetection was performed with goat anti-mouse antibodies and streptavidin-biotin complex (all Dako). Staining was visualized with diaminobenzidine and H<sub>2</sub>O<sub>2</sub>.

#### Endothelial sprouting assays

To analyze the angiogenic potential, endothelial sprouting assays were performed. In short, endothelial spheroids were prepared (750 cells/spheroid) and after overnight incubation embedded

in a collagen type-1 matrix. HUVEC spheroids were stimulated with 50 ng/ml vascular endothelial growth factor (VEGF, R&D systems), which stimulates endothelial sprouting, in the presence or absence of endoglin-Fc (1 µg/ml, R&D systems) or Fc protein (1 µg/ml) as a control. After overnight incubation sprout length was determined as described before (1, 2). In addition ECRF and HUVEC cells were infected with lentiviral vectors encoding Endoglin-Fc or Fc only. Expression of the constructs was examined by western blot using anti-Fc antibodies (dilution 1:1000, R&D systems). Angiogenic activity was analyzed as described above. ECRF spheroids form spontaneous sprouts once embedded in collagen, and were therefore not VEGF stimulated.

### Mutation of of MMP-14 cleavage sequence in endoglin

The predicted MMP-14 cleavage site in endoglin sequence G-L (aa 586-7) was mutated into V-P, V-H, H-P and H-H variants. PCR-mediated overlap extension method was used for site-directed-mutagenesis.

In brief, two flanking primers: for 5'-ccaagacactttagcccg-3', rev 5'-acgtggcttctctgccaagc-3' and two mutagenic primers per construct:

V-P: for 5'-aagcaaagtccccgtcctgccc-3', rev 5'-caggacggggactttgcttg-3';

V-H: for 5'-aagcaaagtccacgtcctgccc-3', rev 5'-caggacgtggactttgcttg-3';

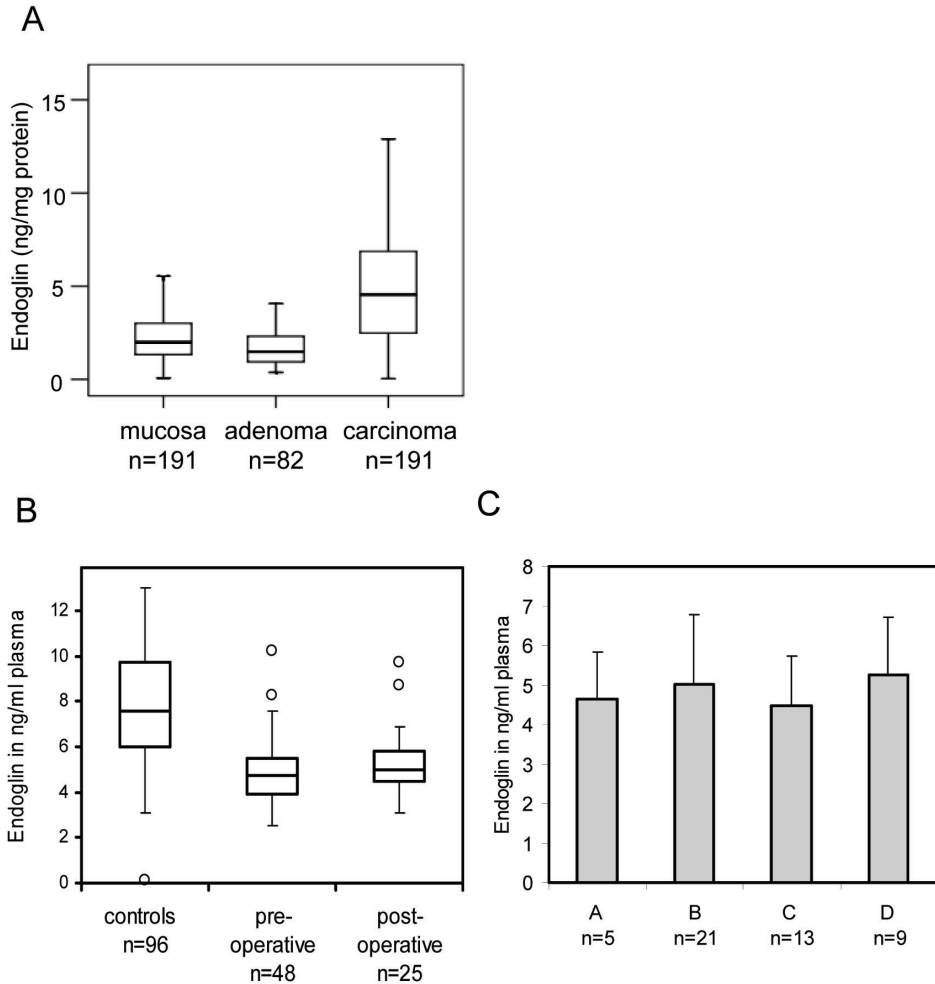
H-P: for 5'-aagcaaacacccctcctgccc-3', rev 5'-caggacgggggtttgcttg-3';

H-H: for 5'-aagcaaacaccacgtcctgccc-3', rev 5'-gcaggacgtgggtttgcttg-3' were used for generation of intermediate PCR products (two per construct) with overlapping regions containing the desired mutations (mutations introduced into nucleotide sequence are underlined). pDisplay-endoglin plasmid was used as a template (3). The two PCR fragments per construct were mixed and used for a second round of PCR with flanking primers. Subsequently the obtained products were digested and cloned into SacI/SalI site in pDisplay-endoglin vector leading to an exchange of wildtype G-L site with the desired mutations. All PCR reactions were performed using Phusion High-Fidelity PCR Master Mix (Finnzymes, Espoo, Finland) and 98°C-30", (98°C-5", 60°C-20", 72°C-15") x 35, 72°C-5' conditions. The obtained constructs were sequenced and used for transfection experiments. Wild type or endoglin mutants were transfected into COS cells in 6 well plates (250.000 cells/well). Transmembrane localization of endoglin mutants was confirmed by flow cytometry (FACS). After O/N starvation medium was collected and sEndoglin levels were determined by ELISA as described above.

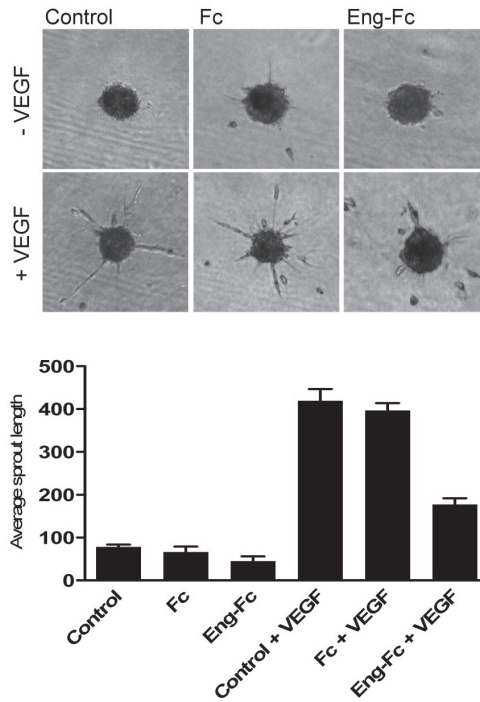
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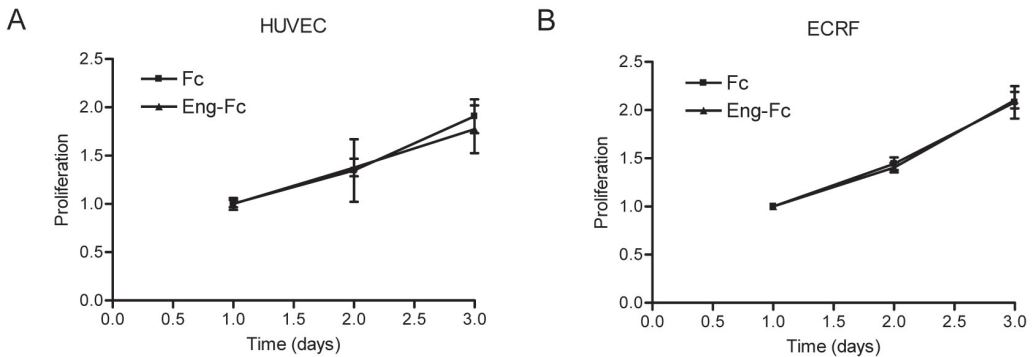
cleavage induces colorectal cancer angiogenesis. *Eur J Cancer* 2008;44:1904-13. (3) Guerrero-Esteo M, Sanchez-Elsner T, Letamendia A, Bernabeu C. Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor- $\beta$  receptors I and II. *J Biol Chem* 2002;277:29197-209



**Supplemental Fig 1.** Endoglin levels in colorectal cancer A, Endoglin levels in colon tissue homogenates determined by ELISA. Levels are increased in carcinomas, compared to normal tissue and premalignant adenomas. B, Box-whisker plots showing levels of soluble endoglin in citrate plasma samples in colorectal cancer patients pre- and 3 months post-operation, and healthy controls. Levels were determined by ELISA. Soluble endoglin levels are lower in CRC patients before operation compared to healthy volunteers. (Healthy versus pre-operation,  $P < 0.0001$ ). C, Soluble endoglin levels in CRC patients show no relation with Dukes stage.



**Supplemental Fig 2.** HUVEC endothelial sprouting assay with Endoglin-Fc HUVEC spheroids were prepared, embedded in collagen and stimulated with 50 ng/ml VEGF in the presence of control Fc protein or Endoglin-Fc (both 1 µg/ml). Sprout length was determined from at least 10 spheroids per experimental condition after O/N incubation. Data represent average sprout length ± SD from a representative experiment.



**Supplemental Fig 3.** Influence endoglin-Fc on proliferation endothelial cells. HUVEC and ECRF cells were transduced with Fc- or Endoglin Fc expressing lentiviruses. Proliferation was determined using the MTS assay. Data show no effect of Endoglin-Fc on proliferation of HUVEC (A) or ECRF cells (B).

A  
Supplementary Figure 4

G	12	P	20	A	13	G	26
L	11	L	18	Q	11	A	12
A	8,6	A	9,7	G	9,7	R	11
D	8,6	S	8,6	E	8,6	N	9,7
Q	8,6	V	8,6	S	8,6	D	7,5
V	7,5	R	5,4	L	7,5	P	5,4
N	6,5	Q	4,3	V	6,5	S	5,4
T	6,5	T	3,2	P	6,5	K	4,3
R	6,5	I	3,2	I	4,3	Q	4,3
P	5,4	Y	2,2	R	4,3	T	3,2
E	4,3	E	2,2	Y	3,2	L	3,2
I	3,2	D	2,2	F	3,2	Y	3,2
S	3,2	F	2,2	K	3,2	E	3,2
K	3,2	G	2,2	N	3,2	H	1,1
H	2,2	C	2,2	T	2,2	W	1,1
C	1,1	N	1,1	D	2,2		
M	1,1	K	1,1	C	1,1		
F	1,1	W	1,1	H	1,1		
		H	1,1	M	1,1		
		M	1,1				

L	39	V	12	T	12	E	14	
L	I	11	A	9,7	A	11	A	9,7
E	Y	9,7	E	8,6	L	9,7	S	8,6
A	S	7,5	N	8,6	E	9,7	L	8,6
V	M	5,4	R	7,5	G	8,6	R	8,6
A	D	4,3	Q	6,5	S	8,6	N	7,5
G	T	4,3	S	6,5	K	7,5	Q	6,5
E	Q	3,2	G	5,4	V	6,5	P	6,5
-	V	3,2	L	5,4	P	4,3	T	4,3
S	A	3,2	K	4,3	R	4,3	K	4,3
I	F	3,2	T	4,3	N	3,2	G	4,3
T	G	2,2	F	4,3	H	3,2	F	3,2
E	N	1,1	Y	3,2	Q	3,2	H	3,2
	W	1,1	M	3,2	F	3,2	D	3,2
	R	1,1	P	2,2	D	2,2	V	3,2
	E	1,1	D	2,2	C	1,1	Y	2,2
			I	2,2	I	1,1	W	1,1
			H	2,2	Y	1,1	I	1,1
			C	1,1				
			W	1,1				

B

1 *mdrgtlplav allascsls ptslae* tvhc dlqpvqperg evtyttsqvs kgcvaaqapna  
 61 ilevhvifle fptgpsqlel tqaskqngt wprevlvlv vnssvfihlq algiphlay  
 121 nsslvtfqep pgvnttelps fpktqilewa aergpitsaa eindpqsil rlgqaqgsls  
 81 fcmleasqdm grtlewrprt palvrgchle gvaghkeahi lrvipghsag prtvtvkjel  
 241 scapgdldav lllqpppyvs wldanhnmq iwtgeysfk ifpeknirgf klpdtpggil  
 301 gearlnasi vasfvelpla sivslpasc ggriqtspap iqtppkdtc spellmsliq  
 361 tkcaddamtl vkkkelvahl kctitglfw dpscbaedrg dkfvlsays scgmqvsasm  
 421 isneavnil sssspqrkv hclnmdslsf qlglylspfh lqasntiepg qqsfvqrvs  
 481 psvseflilq dschldgpe ggtveliqgr aakgncvsl spspgedprf sflhfytp  
 541 ipktgtlscet valrpkgtgsq dqevhrtvm rlniispdls gctskg **lvlp avlgitfgaf**  
 601 **ligalltaal** wiyishtrsp skrepvava apassessst nhsigstqst pcstssma

**Supplemental Fig.4 .** Determination MMP-14 cleavage sequence in endoglin A, Analysis of amino acids flanking the MMP-14 cleavage site in natural substrates. 79 natural MMP-14 substrates with known cleavage sites were analyzed. Occurrence of the amino acids flanking the cleavage site is shown in % of total. B, Endoglin amino acid sequence with potential MMP-14 cleavage sites based on the consensus G—L sequence indicated by arrows (G-L sequences). Transmembrane domain is indicated in bold



