# Cell-cell interactions in the gastrointestinal tumour-microenvironment

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*Chapter 10*

# Endoglin shedding in colorectal cancer

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## *Abstract*

Endoglin is a TGF-β co-receptor, mainly expressed on angiogenic endothelial cells. Besides membrane-bound also a soluble form exists in the circulation. However, the role and shedding mechanism of this soluble receptor has not been established yet. Determination of Endoglin in colorectal neoplasia patients by ELISA revealed enhanced levels in carcinomas, but not in pre-malignant adenomas. Immunohistochemically, Endoglin was mainly observed in angiogenic endothelial cells, but also in fibroblasts in the normal-to-tumour transition zone. Pre-operative soluble Endoglin levels of colorectal cancer patients were not significantly different from plasmas of healthy controls, although mean levels were lower in these patients. We observed that low expression of Endoglin on endothelial cells was accompanied by high expression of MMP-14. *In vitro* experiments revealed high expression of MMP-14 in HUVEC endothelial cells, but treatment of these cells with endogenous MMP-14 did not increase soluble Endoglin levels in the medium. In contrast, co-transfection of membrane bound MMP-14 and Endoglin led to strongly enhanced soluble Endoglin levels. Furthermore, addition of MMP inhibitors inhibited the release of sEndoglin from HUVEC cells, in contrast to other proteinases inhibitors. In conclusion, we have shown that Endoglin expression is enhanced in colorectal cancer and we propose membrane bound MMP-14 to be a major candidate in generating soluble Endoglin.

## *Introduction*

Endoglin (CD105) is a 180 kDa integral membrane-bound glycoprotein, which serves as a high affinity co-receptor for Transforming Growth Factor (TGF)-β1 and -β3[1]. Two Endoglin isoforms are known, L- and S-Endoglin, which differ in their cytoplasmic tail. L-Endoglin is mainly expressed on endothelial cells[2]. Mutations in the gene encoding Endoglin have been linked to hereditary haemorrhagic telangiectasia Type I (HHT-1), a multisystemic vascular disease characterised by bleeding from small vascular lesions in the mucocutaneous tissues and the presence of arteriovenous malformations[2,3]. Endoglin has a crucial role in angiogenesis, as Endoglin knock-out animals die *in utero* because of defects in the vasculature system[4]. Expression of Endoglin is mostly restricted to angiogenic endothelial cells, but has also been shown in tumour cells[5], especially those with high invasive potential[6], cardiac fibroblasts[7], hepatocytes[5,7] and several other cells[5]. Expression of Endoglin can be induced by hypoxia[3,8], TGF-β1[1,9,10] and/or TGF-β3[11]. Endoglin expression was shown to be upregulated in various cancers[1] and correlated with development of metastatic disease in colorectal cancer (CRC)[8]. Together these data indicate a crucial role for Endoglin in tumour angiogenesis.

Besides membrane-bound Endoglin, also a soluble form (sEndoglin) exists in the circulation[3]. Elevated levels of sEndoglin have been reported in pregnant women suffering from pre-eclampsia[12,13], patients with liver cirrhosis and hepatocellular carcinoma[14], and colorectal- and breast cancer patients[15-17]. The mechanism by which Endoglin is released and the role of soluble Endoglin in the circulation has not been revealed yet. Betaglycan (TGF-β RIII), another TGF-β co-receptor, also has a soluble form in the circulation[18]. Shedding of membrane bound betaglycan has been found to be mediated by membrane type (MT)-1 matrix metalloproteinase (MMP-14)[19]. MMP-14 is a membrane bound MMP which is expressed by epithelial cells, endothelial cells and stromal fibroblasts in the colon and is upregulated in cancer[20]. After intracellular activation by furin, MMP-14 is expressed on the cell-membrane, where it has a role in degradation of collagens and heparin sulphate proteoglycans (HSPGs)[21] and is important in the activation of other MMPs[22].

In this study we evaluated if MMPs, in particular MMP-14, could play a role in the generation of soluble Endoglin in CRC. First, we examined tissue Endoglin levels in normal colonic mucosa, adenomas and carcinomas. Next, we determined pre- and postoperative sEndoglin levels in a group of CRC patients. Immunohistochemistry was used to determine cellular localisation and expression of Endoglin and MMP-14. Next, we analysed the Endoglin

shedding mechanism *in vitro*. The data indicate that MMP-14 is a major candidate for Endoglin shedding from endothelial cells.

## *Materials and methods*

*Patient material*

Pre- and three months postoperative citrate plasma samples (n=14) and tissue specimens (n=191) from patients undergoing resection for primary colorectal carcinoma at the Department of Oncologic Surgery, Leiden University Medical Centre, were collected as described before[23,24]. Colorectal adenomas (n=82) were removed endoscopically at the Department of Gastroenterology-Hepatology. Tissue were homogenised and protein concentrations were determined as previously[23]. 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.

*Endoglin and sEndoglin ELISA*

Endoglin levels in tissue homogenates and cell culture media were determined essentially according to the DY1097 human endoglin DuoSet ELISA (R&D Systems, Abingdon, UK. In short, 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. Aspecific binding was blocked with PBS containing 5% Tween-20, 0.05% $NaN_3$ 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% BSA were incubated during 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 protein for tissue samples. The ELISA was validated by western blot analysis and spiking with a fixed amount recombinant human Endoglin. For determination of Endoglin levels in citrate plasma samples a cross reactivity of the capture antibody with human serum albumin was observed. Therefore 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 Humans Serum Albumin (Sigma) to correct for remaining background absorption. The ELISA was performed and validated as described above.

*Immunohistochemistry*

To determine cellular localisation of Endoglin immunohistochemistry was performed as described previously[25]. In short, slides were deparaffinised, 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-pancytokeratin (1:1000), mouse monoclonal anti-vimentin (1:400, both Santa Cruz Biotechnologies, Santa Cruz, USA), mouse monoclonal anti-smooth muscle actin (SMA, 1:800, Progen, Heidelberg, Germany), 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 visualised with diaminobenzidine and $H_2O_2$. Representative photomicrographs were taken with a Nikon Eclipse E900 microscope equipped with a Nikon DXM1200 digital camera.

*In vitro experiments*

Human umbilican vein endothelial cells (HUVECs) were isolated[26] and cultured as described before[27]. To examine the Endoglin cleavage mechanism HUVECs were seeded in fibronectin coated 24 well plates (Greiner Bio-one, 60,000 cells/well). After o/n attachment, cells were treated with 0-160 ng/ml recombinant human MMP-14 (Chemicon, Temacula, CA, USA) or with proteinase inhibitors: 20 μM E64 (cystein protease inhibitor), 10 μg/ml Aprotinin (serine protease inhibitor, both Sigma-Aldrich, Darmstadt, Germany), 1 μM GM6001 (broad spectrum MMP inhibitor, 10 μM Marimastat (kindly provided by British Biotech Pharmaceuticals), 100 nM MMP-2/MMP-9 inhibitor, 100 nm MMP-13 inhibitor or 1 μM specific MMP-3 inhibitor (all Calbiochem, La jolla, CA, USA). Serum Free (SF)-M199 medium and 0.1% DMSO were included as controls. The percentage inhibition versus the appropriate control was calculated. For transfection experiments COS cells were transfected with plasmids (pcDNA3.1) encoding full length Endoglin, (kindly provided by Dr. E. Pardali, LUMC, Dept. of Molecular Cell Biology), full length MMP-14 or MMP-14 lacking the transmembrane domain (MMP-14ΔTM, both kindly provided by Dr. R. Hanemaaijer, TNO Quality of life BioSciences, Leiden, the Netherlands) and empty vector. COS cells were seeded in 24 well plates (Greiner Bio-one, 10.000 cells per well in DMEM containing 10%

FCS and penicillin/streptomycin) and after 48 hours cells were transfected with in total 0.4 μg plasmid DNA using Lipofectamin as a tranfection reagent, according to the manufacturers protocol (Invitrogen). 24 hours after transfection cells were incubated with SF-DMEM for 16 hours and sEndoglin levels were determined by ELISA as described above. All experiments were performed in triplicate.

*Statistical analysis*

Differences were calculated using the Mann-Whitney U-test, or the Wilcoxon signed rank test using SPSS 14.0 statistical package. Correlation analysis was performed using Spearman's correlation. P values <0.05 were considered statistically significant.

## *Results*

*Tissue Endoglin levels*

Before analysing the tissue and cell culture samples the Endoglin ELISA was validated. Standard curves of recombinant human Endoglin were linear in a 0-4 ng/ml range, with a detection limit was 0.125 ng/ml Endoglin. Spiking of tissue samples with a fixed amount recombinant Endoglin revealed an equal increase in signal (not shown). Figure 1A shows decreasing absorbance values with increasing dilution of a colorectal cancer tissue homogenate. Western blot analysis of the same sample revealed a similar pattern as observed by ELISA (figure 1B).

Tissue Endoglin levels were determined in homogenates of 191 CRC homogenates, corresponding normal mucosa and 82 colorectal adenomas. Endoglin levels were significantly increased in carcinomas compared to mucosa, 2.72 ng/mg versus 5.36 ng/mg, $p< 0.0001$) or adenomas (2.45 ng/mg). Adenoma Endoglin levels were lower than normal mucosa (Figure 1C). Analysis of clinico-pathological parameters revealed no associations between presence of dysplasia in adenomas and Endoglin levels, whereas in carcinomas the highest Endoglin levels were detected in Dukes stage C carcinomas (not shown). There were no significant associations between carcinoma tissue Endoglin levels and other clinicopathological parameters of the tumours.

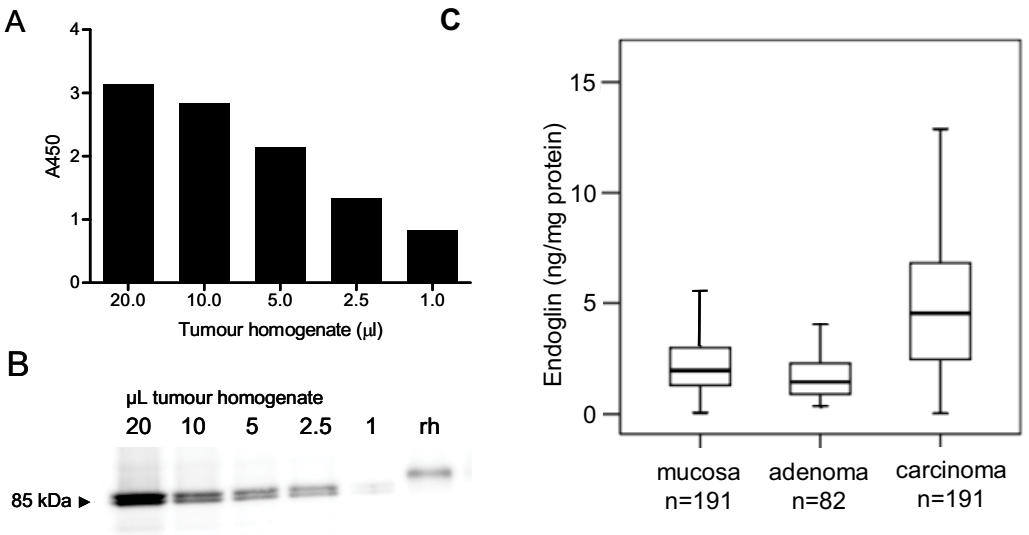


*Figure 1. Validation Endoglin ELISA and CRC tissue Endoglin levels. A) Endoglin ELISA absorbance values for a increasing dilution series of a CRC homogenate. B) Endoglin western blot on the same CRC homogenate rh= recombinant human Endoglin. C) Tissue Endoglin levels in normal colon mucosa, adenomas and carcinomas*

*Immunohistochemistry*

Immunohistochemistry on CRCs and normal mucosa revealed that Endoglin expression was low in normal mucosa except for some staining in the submucosal area (Figure 2, left panel, arrowhead). In tumours strongly increased Endoglin expression in angiogenic endothelial was observed in the proximity of tumour cells. Furthermore, expression of Endoglin was occasionally observed in fibroblasts, especially in the normal to tumour transition zone (Figure 2, right panel, arrowhead). CD31 stained all blood vessels in normal and tumour tissue (not shown). MMP-14 expression was observed in epithelial cells and endothelial cells. Interestingly, tumour endothelial cells strongly expressing MMP-14 were negative for Endoglin (Figure 2, bottom panel).

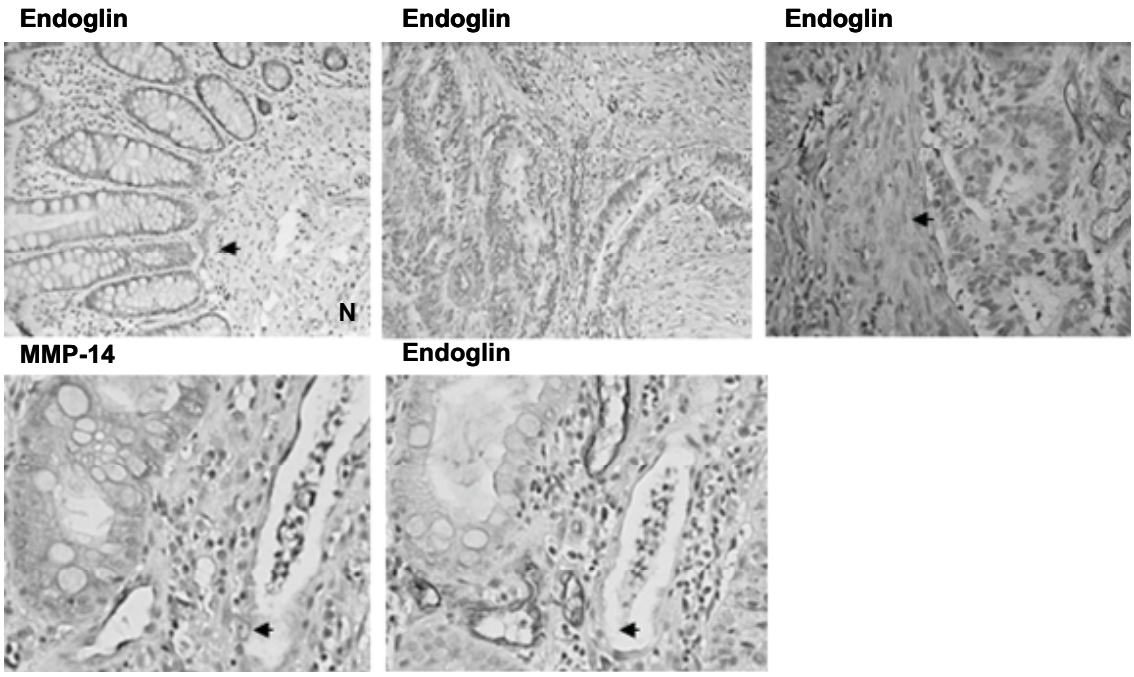


*Figure 2. Endoglin (CD105)immunohistochemistry on normal mucosa (N, left panel) and colorectal cancer specimens. The lower panels show staining on sequential sections for Endoglin and MMP-14. Full-colour illustration at page 208*

*Soluble Endoglin levels in CRC patients*

To detect soluble Endoglin in plasma samples, we developed an ELISA which was validated using a serially diluted plasma sample series and spiking of these samples with a fixed amount recombinant Endoglin (Figure 3A). To examine the presence of sEndoglin in the circulation of CRC patients we determined sEndoglin in pre- and three months postoperative plasma samples (Figure 3B). Table 1 shows median pre- and postoperative sEndoglin levels in patients (n=19) and in a group of healthy volunteers (n=15). Mean pre-operative sEndoglin levels were lower than the controls and increased post-operatively (Table 1), although these differences were not statistically significant. Interestingly, individual changes in sEndoglin levels pre- versus post-operation were apparent. There was no relation between sEndoglin levels and stage of the tumour (figure 3B).

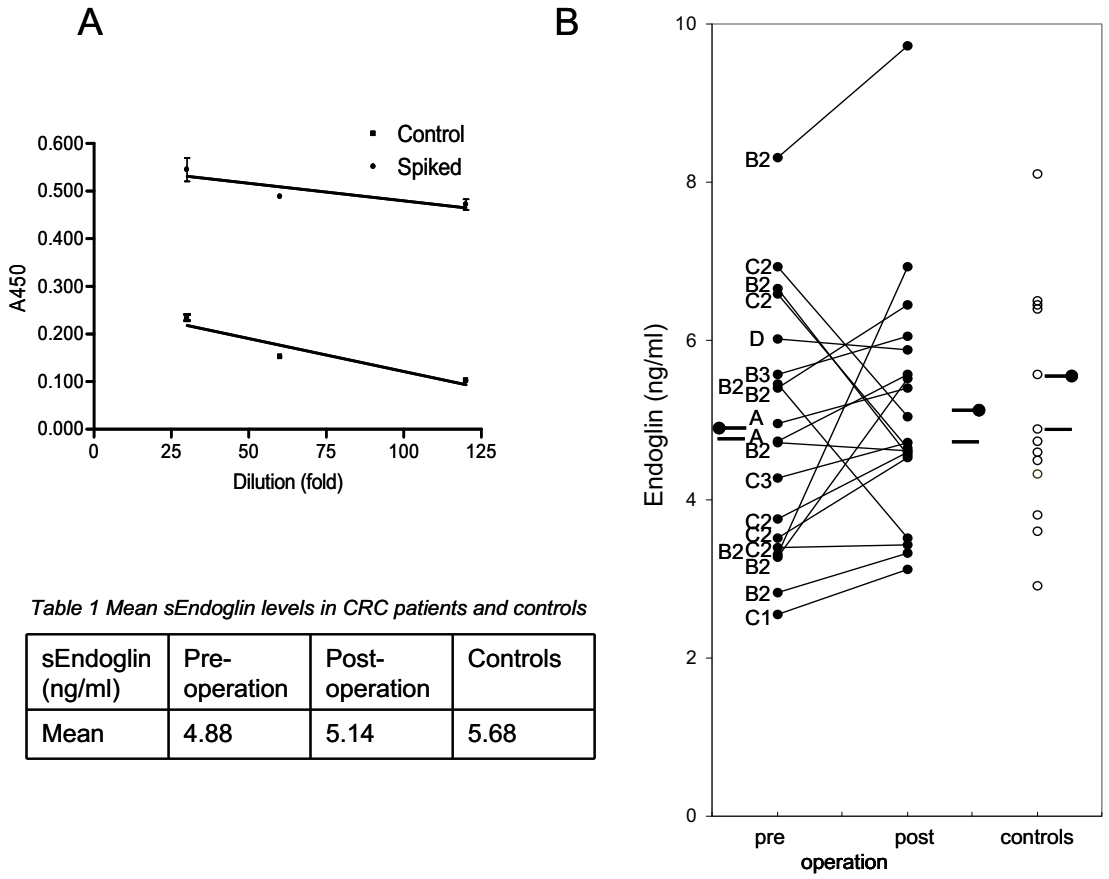


*Table 1 Mean sEndoglin levels in CRC patients and controls*

| sEndoglin (ng/ml) | Pre-operation | Post-operation | Controls |
|---|---|---|---|
| Mean | 4.88 | 5.14 | 5.68 |

*Figure 3. sEndoglin in CRC. A) Validation of sEndoglin plasma ELISA. A control serum sample was spiked with a fixed amount of recombinant human Endoglin. B) sEndoglin levels in 19 CRC patients pre-and post-operation and 15 healthy controls. Lines indicate median values, whereas lines with dots represent mean values. Dukes stage of the tumours is indicated (A, B2, C1, C2 and D).*

*The role of MMPs in Endoglin shedding*

Since soluble Endoglin was detectable in CRC patients, where it might have an anti-angiogenic role as has been described for other pathological conditions[12], we analysed if MMPs could play a role in Endoglin shedding from the endothelial cell membrane. Analysis of the Endoglin amino acid sequence revealed that it contains at least two MMP sensitive cleavage sites indicated by arrows (Figure 4), of which one is located in the transmembrane domain.

1 mdrgtlplav alllascsls ptslaetvhc dlqpvgperg evtyttsqvs kgcvaqapna
61 ilevhvlfle fptgpsqlel tlqaskqngt wprevllvls vnssvflhlq algiplhlay
121 nsslvtfqep pgvnttelps fpktqilewa aergpitsaa elndpqsill rlgqaqgsls
181 fcmleasqdm grtlewrprt palvrgchle gvaghkeahi lrvlpghsag prtvtvkvel
241 scapgdldav lilqgppyvs wlidanhnmq iwttgeysfk ifpeknirgf klpdtpqgll
301 gearmlnasi vasfvelpla sivslhassc ggrlqtspap iqttppkdtc spellmsliq
361 tkcaddamtl vlkkelvahl kctitgltfw dpsceaedrg dkfvlrsays scgmqvsasm
421 isneavvnil sssspqrkkv hclnmdslsf qlglylsphf lqasntiepg qqsfvqvrvs
481 psvsefllql dschldlgpe ggtveliqgr aakgncvsll spspegdprf sfllhfytvp
541 ipktgtlsct valrpktgsq dqevhrtvfm rlniispdls gctskg**lvlp avlgitfgaf**
601 **ligalltaal w**yiyshtrsp skrepvvava apassessst nhsigstqst pcstssma

*Figure 4. Amino acid sequence of Endoglin with two possible MMP sensitive cleavage sites (arrows) of which one is located in the trans-membrane domain* ***(bold)***

MMP-14 is a membrane-bound MMP, which has been shown to be capable of shedding of membrane bound receptors[19]. To evaluate if MMP-14 is able to shed Endoglin from the endothelium membrane we treated HUVECs, expressing high levels of Endoglin (Figure 5A), with recombinant MMP-14. Figure 5B shows high levels of sEndoglin in control medium, which was not increased by treatment with recombinant human MMP-14. To further analyse if MMP-14 was capable of mediating Endoglin cleavage we co-transfected COS cells with plasmids encoding Endoglin, full length membrane bound MMP-14 or MMP-14 lacking the transmembrane domain (MMP-14 ΔTM). As shown in figure 5C co-transfection of Endoglin only with membrane bound MMP-14, and not with MMP-14ΔTM, led to increased sEndoglin levels in the medium. To further analyse if endothelial MMP-14 could mediate shedding of Endoglin from endothelial cells, HUVEC cells were treated with various proteinases inhibitors. Inhibitors of cystein and serine proteases (including cathepsins and plasmin) did not reduce sEndoglin levels, in contrast to broad spectrum MMP inhibitors GM6001 and Marimastat, which have been described to also inhibit MMP-14[28]. Inhibition of soluble Endoglin release was 50% by both GM6001 and Marimastat (Figure 5D). Specific inhibitors of gelatinases (MMP-2 and MMP-9), Stromelysins (MMP-3) had only a mild effect on sEndoglin release, whereas and an MMP-13 inhibitor did not affect levels at all. This indicates that these classes of MMPs are probably not involved in the shedding process.

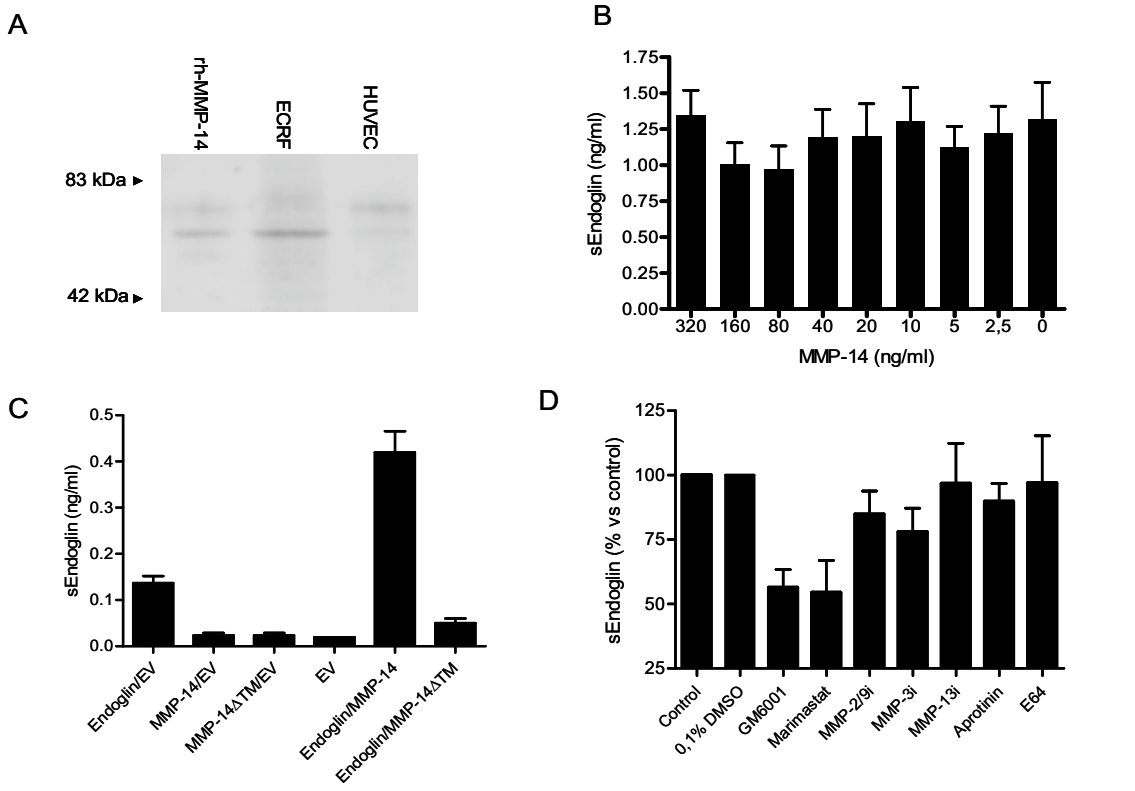


*Figure 5. A) Western blot analysis of MMP-14 expression in HUVEC and ECRF endothelial cell lysates. B) sEndoglin medium levels after treatment of HUVEC cells with recombinant human MMP-14 (Three independent experiment in triplo). C) sEndoglin medium levels in COS cells after transfection with Endoglin, Empty vector (EV), full length MMP-14, or MMP-14 lacking the transmembrane domain (MMP-14 Δ TM) plasmids Data represent mean+ standard deviation of one experiment in triplo. D) Soluble Endoglin levels in HUVEC medium after treatment with various proteinases inhibitors (% versus appropriate control, n=5 independent experiments performed in triplo, mean+SEM).*

## *Discussion*

Of several membrane receptors like the VEGF receptor and Endoglin also soluble variants have been described, which seem to be involved in several pathological conditions. Increased levels of sEndoglin have been reported in pre-eclampsia patients[12,13], whereas studies describing sEndoglin levels in malignancies are not conclusive. Several studies revealed increased levels in cancer patients compared to controls[15,17,29], whereas other showed no increased levels[30,31]. In our study we found that pre-operative sEndoglin in levels in CRC patients appeared to be lower than controls, although differences did not reach statistical significance. This would correspond to a possible anti-angiogenic role by savaging of pro-angiogenic molecules, like TGF-β or VEGF[30,31]. In contrast, sEndoglin might also function as

a circulating TGF-β reservoir[10,32]. The role of sEndoglin might also depend on the cell type it is cleaved from. High expression of the TGF-β co-receptor Endoglin on angiogenic endothelial cells has been associated with poor survival in various types of cancers[1,8,16], even being prognostic superior to other angiogenic markers like CD31 or CD34[16,33,34]. We observed strongly increased tissue Endoglin levels in colorectal carcinoma, but not in pre-malignant adenomas, corresponding to what has previously been shown for benign gastric lesions[34]. Endoglin expression was mainly observed in angiogenic endothelial cells, but also in fibroblasts especially in the normal to tumour transition zone. Expression of Endoglin on non-endothelial cells seems to play a role in invasive processes. Recently it was shown that over-expression of Endoglin in MDA epithelial breast cancer cells increases their invasive behaviour by upregulation of MMP expression and the formation of invadipodia[6]. In contrast, another study showed that shedding of Endoglin from mouse keratinocytes increases their invasive potential[35]. Our immunohistochemical analysis revealed expression of Endoglin specifically in fibroblasts in the normal to tumour transition zone and could therefore be involved in the invasion of fibroblasts into the surrounding normal tissue.

Betaglycan another TGF-β co-receptor can be released into the circulation by proteolytic cleavage by MMP-14[19]. Because Endoglin shows high homology with betaglycan and contains and MMP sensitive cleavage sequence in the transmembrane domain, we examined the role of MMP-14 in Endoglin shedding. Treatment of endothelial cells with recombinant MMP-14 did not increase soluble Endoglin levels. Further analysis using co-transfection of Endoglin with a MMP-14 revealed that the localisation of MMP-14 on the cell membrane is crucial for shedding Endoglin into the medium as MMP-14 lacking the transmembrane domain was not capable of shedding Endoglin. The contribution of MMP-14 to Endoglin shedding from endothelial cells was further supported by the fact that immunohistochemical analysis showed that endothelial cells highly expressing MMP-14 did not stain for Endoglin. Next to that, addition of only broad spectrum MMP inhibitors could reduce sEndoglin release into the medium. Cystein-, or serine-protease inhibitors did not reduce sEndoglin levels, whereas gelatinase, stromelysin-, or collagenase specific inhibitors had only a mild effect. Broad spectrum MMP inhibitors inhibit, besides the specific MMP classes mentioned before, also MMP-14, and showed a 50% reduction in sEndoglin levels. Preliminary data using shRNA mediated knockdown of MMP-14 in endothelial cells revealed a comparable inhibition as observed with the MMP inhibitors. This would further extent the important role MMP-14 has in cancer progression besides its pro-tumerogenic role in the activation of MMP-2, cleavage of ECM component[22,36], and its role in angiogenesis[37]. Shedding of

Endoglin releasing an anti-angiogenic soluble variant could act tumour-suppressive by reduction of angiogenesis.

In conclusion, we have shown increased Endoglin expression in CRC, but not in pre-malignant adenomas and that mean pre-operative sEndoglin levels in CRC patients seem to be lower compared to controls. Based on *in vitro* experiments we propose MMP-14 to be an important candidate for Endoglin shedding from angiogenic endothelial cells (Figure 6)

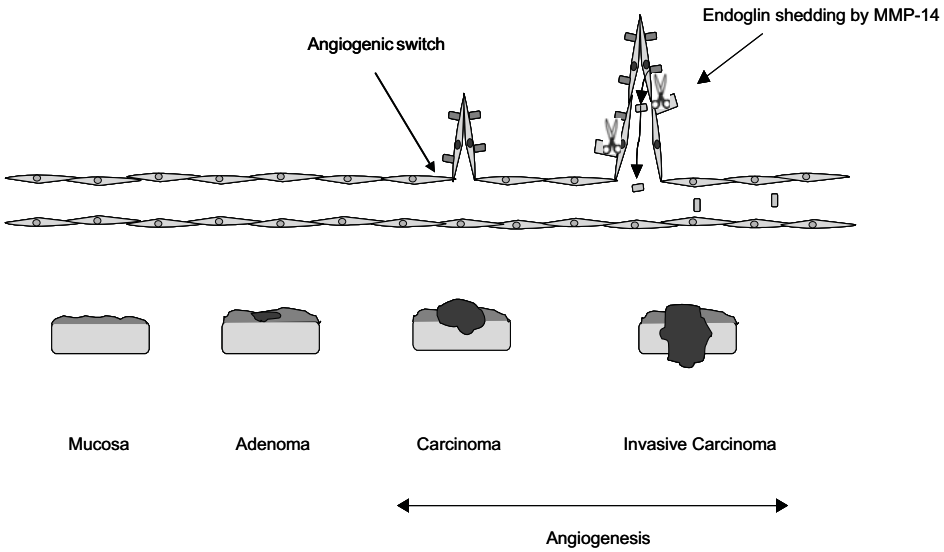


*Figure 6. Proposed mechanism of Endoglin expression and generation of sEndoglin in colorectal cancer.* *Full-colour illustration at page 208.*

## *References*

1. Duff,S.E., Li,C., Garland,J.M., & Kumar,S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J.* **17**, 984-992 (2003).
2. Fonsatti,E., Altomonte,M., Nicotra,M.R., Natali,P.G., & Maio,M. Endoglin (CD105): a powerful therapeutic target on tumor-associated angiogenetic blood vessels. *Oncogene* **22**, 6557-6563 (2003).
3. Li,C., Guo,B., Bernabeu,C., & Kumar,S. Angiogenesis in breast cancer: the role of transforming growth factor beta and CD105. *Microsc. Res. Tech.* **52**, 437-449 (2001).
4. ten Dijke,P. & Arthur,H.M. Extracellular control of TGF-β signalling in vascular development and disease. *Nat. Rev. Mol. Cell Biol.* **8**, 857-869 (2007).
5. Minhajat,R. *et al.* Organ-specific endoglin (CD105) expression in the angiogenesis of human cancers. *Pathol. Int.* **56**, 717-723 (2006).
6. Oxmann,D. *et al.* Endoglin expression in metastatic breast cancer cells enhances their invasive phenotype. *Oncogene*(2008).
7. Chen,K., Mehta,J.L., Li,D., Joseph,L., & Joseph,J. Transforming growth factor-β receptor endoglin is expressed in cardiac fibroblasts and modulates profibrogenic actions of angiotensin II. *Circ. Res.* **95**, 1167-1173 (2004).

8. Romani,A.A., Borghetti,A.F., Del Rio,P., Sianesi,M., & Soliani,P. The risk of developing metastatic disease in colorectal cancer is related to CD105-positive vessel count. *J. Surg. Oncol.* **93**, 446-455 (2006).
9. Sanchez-Elsner,T., Botella,L.M., Velasco,B., Langa,C., & Bernabeu,C. Endoglin expression is regulated by transcriptional cooperation between the hypoxia and transforming growth factor-β pathways. *J. Biol. Chem.* **277**, 43799-43808 (2002).
10. Fonsatti,E. *et al.* Endoglin: An accessory component of the TGF-β-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. *J. Cell Physiol* **188**, 1-7 (2001).
11. Yinon,Y. *et al.* Severe Intrauterine Growth Restriction Pregnancies Have Increased Placental Endoglin Levels: Hypoxic Regulation via Transforming Growth Factor- 3. *Am. J. Pathol.* **172**, 77-85 (2008).
12. Levine,R.J. *et al.* Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N. Engl. J. Med.* **355**, 992-1005 (2006).
13. Venkatesha,S. *et al.* Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat. Med.* **12**, 642-649 (2006).
14. Yagmur,E. *et al.* Elevation of endoglin (CD105) concentrations in serum of patients with liver cirrhosis and carcinoma. *Eur. J. Gastroenterol. Hepatol.* **19**, 755-761 (2007).
15. Li,C. *et al.* Plasma levels of soluble CD105 correlate with metastasis in patients with breast cancer. *Int. J. Cancer* **89**, 122-126 (2000).
16. Li,C. *et al.* Both high intratumoral microvessel density determined using CD105 antibody and elevated plasma levels of CD105 in colorectal cancer patients correlate with poor prognosis. *Br. J. Cancer* **88**, 1424-1431 (2003).
17. Takahashi,N. *et al.* Association of serum endoglin with metastasis in patients with colorectal, breast, and other solid tumors, and suppressive effect of chemotherapy on the serum endoglin. *Clin. Cancer Res.* **7**, 524-532 (2001).
18. Philip,A., Hannah,R., & O'connor-McCourt,M. Ectodomain cleavage and shedding of the type III transforming growth factor-beta receptor in lung membranes effect of temperature, ligand binding and membrane solubilization. *Eur. J. Biochem.* **261**, 618-628 (1999).
19. Velasco-Loyden,G., Arribas,J., & Lopez-Casillas,F. The shedding of betaglycan is regulated by pervanadate and mediated by membrane type matrix metalloprotease-1. *J. Biol. Chem.* **279**, 7721-7733 (2004).
20. Wagenaar-Miller,R.A., Gorden,L., & Matrisian,L.M. Matrix metalloproteinases in colorectal cancer: is it worth talking about? *Cancer Metastasis Rev.* **23**, 119-135 (2004).
21. Chabottaux,V. & Noel,A. Breast cancer progression: insights into multifaceted matrix metalloproteinases. *Clin. Exp. Metastasis* **24**, 647-656 (2007).
22. Chakraborti,S., Mandal,M., Das,S., Mandal,A., & Chakraborti,T. Regulation of matrix metalloproteinases: an overview. *Mol. Cell Biochem.* **253**, 269-285 (2003).
23. Ganesh,S. *et al.* Prognostic relevance of plasminogen activators and their inhibitors in colorectal cancer. *Cancer Res.* **54**, 4065-4071 (1994).
24. Sier,C.F. *et al.* Imbalance of plasminogen activators and their inhibitors in human colorectal neoplasia. Implications of urokinase in colorectal carcinogenesis. *Gastroenterology* **101**, 1522-1528 (1991).

25. Hawinkels,L.J. *et al.* Tissue level, activation and cellular localisation of TGF-beta1 and association with survival in gastric cancer patients. *Br. J. Cancer* **97**, 398-404 (2007).
26. Jaffe,E.A., Nachman,R.L., Becker,C.G., & Minick,C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest* **52**, 2745-2756 (1973).
27. Hawinkels,L.J. *et al.* Efficient degradation-aided selection of protease inhibitors by phage display. *Biochem. Biophys. Res. Commun.* **364**, 549-555 (2007).
28. Zhou,J., Olson,B.L., & Windsor,L.J. Nicotine increases the collagen-degrading ability of human gingival fibroblasts. *J. Periodontal Res.* **42**, 228-235 (2007).
29. Calabro,L. *et al.* Differential levels of soluble endoglin (CD105) in myeloid malignancies. *J. Cell Physiol* **194**, 171-175 (2003).
30. Furstenberger,G. *et al.* Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br. J. Cancer* **94**, 524-531 (2006).
31. Bellone,G. *et al.* Transforming Growth Factor-β Binding Receptor Endoglin (CD105) Expression in Esophageal Cancer and in Adjacent Nontumorous Esophagus as Prognostic Predictor of Recurrence. *Ann. Surg. Oncol.* **143**, 3232-3242 (2007).
32. Gu,Y., Lewis,D.F., & Wang,Y. Placental Productions and Expressions of sEndoglin, sFlt-1, and PlGF in Normal and Preeclamptic Pregnancies. *J. Clin. Endocrinol. Metab*(2007).
33. Tanaka,F. *et al.* Evaluation of angiogenesis in non-small cell lung cancer: comparison between anti-CD34 antibody and anti-CD105 antibody. *Clin. Cancer Res.* **7**, 3410-3415 (2001).
34. Ding,S. *et al.* Comparative evaluation of microvessel density determined by CD34 or CD105 in benign and malignant gastric lesions. *Hum. Pathol.* **37**, 861-866 (2006).
35. Perez-Gomez,E. *et al.* A Role for Endoglin as a Suppressor of Malignancy during Mouse Skin Carcinogenesis. *Cancer Res.* **67**, 10268-10277 (2007).
36. Noel,A., Jost,M., & Maquoi,E. Matrix metalloproteinases at cancer tumor-host interface. *Semin. Cell Dev. Biol.* **19**, 52-60 (2008).
37. Lafleur,M.A., Handsley,M.M., Knauper,V., Murphy,G., & Edwards,D.R. Endothelial tubulogenesis within fibrin gels specifically requires the activity of membrane-type-matrix metalloproteinases (MT-MMPs). *J. Cell Sci.* **115**, 3427-3438 (2002).