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**Targeting stromal interactions in the pro-metastatic tumor
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Paauwe, M.

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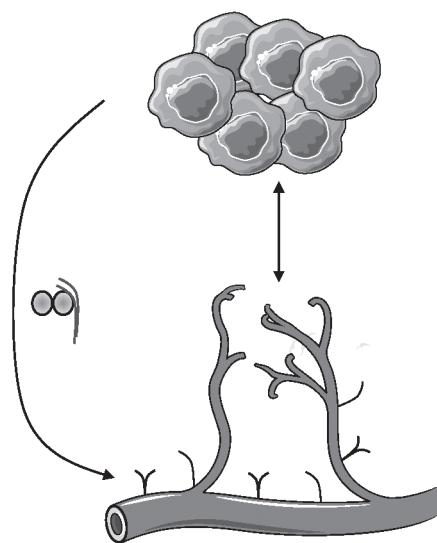


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Author: Paauwe, M.

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Chapter 3

Endoglin for tumor imaging and targeted cancer therapy

Madelon Paauwe, Peter ten Dijke and Lukas J.A.C. Hawinkels

*Department of Molecular Cell Biology, Cancer Genomics Centre Netherlands and Centre for
BioMedical Genetics, Leiden University Medical Center, the Netherlands*

Abstract

Introduction: Although cancer treatment has evolved substantially in the past decades, cancer-related mortality rates are still increasing. Therapies targeting tumor-angiogenesis, crucial for the growth of solid tumors, mainly target vascular endothelial growth factor (VEGF) and have been clinically applied during the last decade. However, these therapies have not met high expectations, which were based on therapeutic efficacy in animal models. This can partly be explained by the upregulation of alternative angiogenic pathways. Therefore, additional therapies targeting other pro-angiogenic pathways are needed.

Areas covered: The transforming growth factor (TGF)- β signaling pathway plays an important role in (tumor-) angiogenesis. Therefore, components of this pathway are interesting candidates for anti-angiogenic therapy. Endoglin, a co-receptor for various TGF- β family members, is specifically overexpressed in tumor vessels and endoglin expression is associated with metastasis and patient survival. Therefore, endoglin might be a good candidate for anti-angiogenic therapy. In this review we discuss the potential of using endoglin to target the tumor vasculature for imaging and therapeutic purposes.

Expert opinion: Considering the promising results from various *in vitro* studies, *in vivo* animal models and the first clinical trial targeting endoglin, we are convinced that endoglin is a valuable tool for the diagnosis, visualization and ultimately treatment of solid cancers.

1. Introduction

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths in 2008 and although multiple new (targeted) therapies have been approved, cancer mortality is estimated to continue to rise to 13.1 million deaths in 2030 [1]. Therefore, novel therapeutic strategies are needed, accompanied by advanced imaging techniques to visualize tumors at an early stage.

Tumor growth beyond a diameter of 3-4 mm is critically dependent on angiogenesis to deliver oxygen and nutrients to, and remove CO₂ and waste products from tumor cells [2]. Besides delivery of nutrients and oxygen, a vascular network provides a route for disseminating tumor cells [3]. Tumor angiogenesis seems to be an attractive target for both imaging and therapeutic strategies. In 1971, vascular endothelial growth factor (VEGF) was identified as a potent factor in inducing tumor angiogenesis [4] and was explored as the first target for anti-angiogenic therapy. Antibody-based anti-VEGF therapies were developed, which showed strong anti-tumor efficacy in various *in vivo* mouse models for cancer [5]. However, the effectiveness of anti-VEGF therapy in the clinic proved to be strongly tumor type dependent and effects were mostly transient, due to resistance. Therefore, these therapies did not meet the high expectations of increasing patient survival in many tumor types and even led to the withdrawal of FDA approval for anti-VEGF therapy in breast cancer [6].

One of the major mechanisms of resistance to anti-VEGF therapy is the upregulation of alternative pro-angiogenic pathways [7], opening the possibility to explore these pathways to serve as novel anti-angiogenesis targets. Among several others, the transforming growth factor- β (TGF- β) pathway plays a prominent role in tumor-angiogenesis. TGF- β is the prototype of a large family of structurally and functionally related cytokines, which include the TGF- β s, bone morphogenetic proteins (BMPs) and activins. The role of the multifunctional cytokine TGF- β seems to be dual and dependent on the cancer stage [8]. TGF- β suppresses tumor growth during the premalignant and early malignant stages, whereas at later stages this inhibitory function is lost. Moreover, in late stages TGF- β acts as a tumor promoter by stimulating cancer cell invasion, immune suppression and inducing angiogenesis [9]. TGF- β induces tumor angiogenesis by affecting both endothelial cells (ECs) and perivascular cells [10, 11]. Therefore, TGF- β seems to be an attractive target for cancer therapy and clinical trials targeting TGF- β pathway ligands or receptors are ongoing [12]. The direct pro-angiogenic effects of TGF- β on ECs are mediated via the TGF- β co-receptor endoglin. Endoglin is highly expressed on proliferating ECs [13-15]. Furthermore, high microvessel density, determined by endoglin expression, has been correlated with poor prognosis in many tumor types [16-18]. Therefore, endoglin appears to be an attractive target for anti-angiogenic therapy in cancer patients. Additionally, the specific expression of endoglin in angiogenic vessels [11, 19, 20] could also render it a suitable candidate for tumor imaging. In this review we focus on the prognostic value of endoglin in cancer, the potential role of targeting endoglin for imaging the tumor vasculature and targeting endoglin for cancer therapy.

2. Endoglin structure and function

2.1 Endoglin structure

The endoglin gene is located on the long arm of human chromosome 9 [21] and is composed of exons 1 to 8, 9A and 9B and 11 to 14 [22-24]. The promoter showed strong and selective activity in ECs, when compared to other cell types [25-28]. Human endoglin is a 658 amino acid long protein containing a large extracellular, a single transmembrane and a short cytoplasmic domain [29] (Fig.1). High sequence homology is found in the cytoplasmic and transmembrane regions of human, porcine and murine endoglin proteins. The extracellular domains are mostly divergent, but do contain highly conserved regions [30, 31]. Endoglin is expressed on the cell surface as a disulfide-linked 180 kDa dimer [29, 32-34] and functions as a co-receptor for TGF- β 1 and - β 3 [35]. Endoglin is also able to interact with BMP-2, -7 and -9 [36, 37].

Betaglycan, the other TGF- β co-receptor, is structurally related to endoglin, mainly in the transmembrane and intracellular domains [38, 39], while the extracellular domain demonstrates less than 30% homology [38]. Endoglin can bind BMP-9 in the absence of the type-II TGF- β receptor (T β RII) [40], but requires the oligomerization with T β RII to bind TGF- β 1 and - β 3. Betaglycan, on the other hand, is able to bind all isoforms of TGF- β independent of T β RII [41, 42]. In general, endoglin and betaglycan form homodimers, but betaglycan/endoglin heterodimers have also been reported, although their function remains unclear [43].

The extracellular domain of endoglin contains an orphan and zona pellucida (ZP) domain [29, 44, 45] (Fig.1). The latter domain comprises an arginine-glycine-aspartic acid (RGD) motif [29, 44, 45], which may function as an interaction site for integrins and was recently suggested to be crucial for leukocyte adhesion and transmigration [46]. The RGD motif is present in human but not in mouse endoglin [29]. The ZP domain is involved in endoglin oligomerization and the interaction with T β RII and the type-I TGF- β receptor (T β RI) ALK-5 [47]. The endoglin cytoplasmic domain contains many serine and threonine residues, of which specific residues are phosphorylated by T β RII, ALK-5 and ALK-1 [48-50]. Endoglin expression is upregulated by both TGF- β and hypoxia [51]. A hypoxia-responsive element (HRE) was identified downstream of the endoglin promoter, which can bind the hypoxia-inducible factor (HIF)-1 α . Upon addition of TGF- β in hypoxic conditions, endoglin transcription increased markedly [51]. Endoglin expression on the cell surface can further be regulated by receptor shedding [52]. Membrane-type-1 matrix metalloprotease 1 (MT1-MMP or MMP14) cleaves endoglin from the membrane, releasing a soluble receptor, sEndoglin [53].

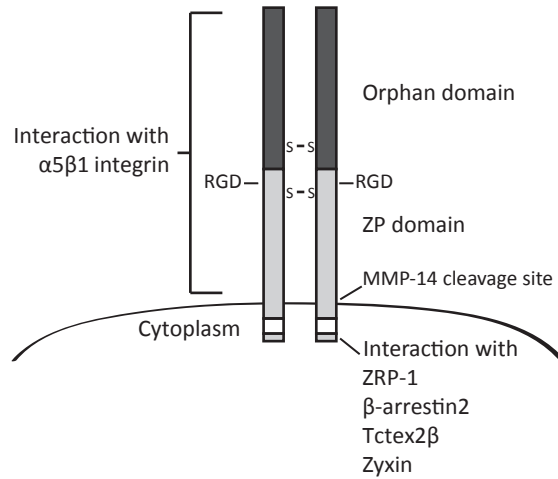


Figure 1 Schematic representation of endoglin structure. Endoglin is expressed at the cell surface as a disulfide-linked dimeric protein, consisting of a large extracellular, a single transmembrane and a short cytoplasmic domain. The extracellular region contains an orphan and a ZP domain, containing a RGD motif. ZP; zona pellucida, RGD; arginine-glycine-aspartic acid, MMP; matrix metalloproteinase.

2.2 Endoglin expression and function in endothelial cells and other cell types

High endoglin expression on vascular ECs has been reported at sites of active angiogenesis (e.g. during embryogenesis, in inflamed tissues and in solid tumors) [13-15]. In addition to ECs, endoglin is also expressed by other cell types. For example, endoglin is expressed by monocytes and its expression is further enhanced upon differentiation into macrophages [54]. During development, endoglin is required for myogenic differentiation [55], while during adulthood endoglin is expressed by hematopoietic stem cells [56], bone marrow stromal fibroblasts [20], melanocytes [57] and on the cell surface of syncytiotrophoblasts in term placenta [58]. Endoglin is also expressed by bone marrow-derived mesenchymal stem cells in which it plays a role in osteogenic differentiation [59]. Low endoglin expressing cells demonstrated increased osteogenic potential compared to high endoglin expressing cells [60]. Mancini and colleagues showed that in order for neural crest stem cells to undergo myogenic differentiation, endoglin is required [55]. Finally, during cardiac repair upon epithelial-to-mesenchymal transition of epicardium-derived stem cells endoglin expression is upregulated [61]. Endoglin is also expressed by a subpopulation of hematopoietic stem cells and can be used as a marker to define long-term repopulating hematopoietic stem cells [56, 62]. These studies indicate a potential role for endoglin in stem cell maintenance or differentiation of stem cells into different lineages. Further studies will be required to establish what the exact function of endoglin is during stem cell growth and differentiation. In pathological conditions, vascular smooth muscle cells inside atherosclerotic plaques were found to display high endoglin expression [63, 64]. After myocardial infarction, endoglin is highly expressed on mononuclear cells, where it has an indispensable role in leukocyte-mediated vascular

repair [65, 66]. In addition to cardiovascular diseases, sEndoglin levels are strongly increased during pre-eclampsia, a serious disease in pregnant women characterized by hypertension and proteinuria [63, 67]. Finally in cancer, besides very high expression on the vasculature, endoglin is also expressed by certain tumor cells including sarcoma and melanoma cells [68, 69]. Ovarian and prostate cancer cell lines were reported to express endoglin *in vitro*, but these results were not confirmed *in vivo* [70, 71]. In prostate cancer cells, endoglin seems to inhibit the migratory potential *in vitro* [72]. The differences in expression *in vitro* and *in vivo* could partly explain the contradictory findings which were reported on the endoglin in cancer.

2.3 Endoglin in developmental angiogenesis and hereditary hemorrhagic telangiectasia

The role of endoglin in developmental angiogenesis has been studied in genetically modified mice. Mice deficient in endoglin die around embryonic day E10.5 due to cardiovascular defects [73-75]. The phenotype observed in endoglin deficient embryos is comparable to that of knock-out animals for other components of the TGF- β signaling pathway, including TGF- β 1, T β RII, ALK-1 and ALK-5 [76]. This indicates an indispensable role for the TGF- β signaling pathway in developmental angiogenesis. In humans, mutations in the endoglin gene have been described. It is generally accepted that endoglin haploinsufficiency results in the autosomal dominant syndrome hereditary hemorrhagic telangiectasia type 1 (HHT1) [22, 77-79]. HHT1 is characterized by telangiectasias, resulting primarily in epistaxis. Arterial venous malformations may also occur that may result in stroke, heart failure or fatal hemorrhage [77, 78]. In order to study HHT1 in an animal model, endoglin haploinsufficient (Eng^{+/-}) mice were generated, which are viable and manifest a phenotype similar to HHT1 patients, including vascular malformations and epistaxis [75]. Current treatment of HHT1 mainly focuses on reducing clinical symptoms. For example, Lebrin and coworkers showed that thalidomide treatment increases perivascular cell coverage in endoglin haploinsufficient mice. In HHT patients, thalidomide treatment reduced nose bleeds and nasal mucosal biopsies also suggested increased perivascular cell coverage [80], although fatigue and transient peripheral neuropathy were reported as side effects [81]. An alternative treatment to relieve HHT symptoms was evaluated by Albiñana and colleagues. This study showed that treatment of ECs with the estrogen receptor modulator raloxifene induced increases in both endoglin mRNA and protein accompanied by increased angiogenesis and EC migration. Following raloxifene treatment, HHT1 patients experienced less frequent epistaxis and did not experience serious side effects [82]. Currently, seven different clinical trials on HHT1 are ongoing. The majority of these trials focus on the use of the anti-VEGF antibody bevacizumab (National Clinical Trial number (NCT) NCT01397695, NCT01402531, NCT01314274, NCT01408030, NCT01507480) for inhibiting recurrent epistaxis. The other two trials, using thalidomide (NCT01485224) or sotradecol (NCT01408732), also aim to decrease the frequency and severity of epistaxis in HHT1 patients by increasing mural cell coverage.

2.4 TGF- β signaling and endoglin

TGF- β is synthesized as a latent precursor and can be activated via proteolytic cleavage or non-proteolytically through conformational changes [76]. Active TGF- β can bind to the T β RII, which recruits and transphosphorylates the T β RI, also called activin-receptor like kinase (ALK5, Fig.2). T β RI transduces the signal by phosphorylating the receptor-regulated Smads (R-Smads), which associate with the co-Smad (Smad-4 in mammals). This complex translocates to the nucleus, where it regulates transcriptional activity of target genes [83] (Fig. 2). Specific target genes are regulated, depending on which T β RI and intracellular Smads are involved. To date, 7 ALKs have been described, of which ALK-1 and ALK-5 are the type-I receptors expressed on ECs, where they play important roles during angiogenesis [84].

The role of TGF- β and ALK-1 and ALK-5 signaling in regulating EC function is complex. The apparent contradictory findings that have been reported for ALK-1 may be explained by the cellular context dependency of ALK-1 signaling, including the type of extracellular matrix surrounding cells [85-87]. In ECs, TGF- β can signal directly through T β RII and ALK-1 [88]. Expression of endoglin on ECs appears to be essential for efficient signaling [35] and induction of EC proliferation [88]. Furthermore, ALK-1 signaling stimulates angiogenesis, both directly and by inhibiting TGF- β /ALK-5 signaling, which is anti-angiogenic [87, 89, 90]. ALK-5 signaling was reported to be important for recruitment of perivascular cells and maturation of newly formed vessels [91]. Until recently, it was accepted that ALK-5 was indispensable for TGF- β /ALK-1 signaling [90], but Tian and colleagues showed that the presence of fibronectin can circumvent the necessity of ALK-5 in this signaling pathway [87].

In addition to being important in ECs, TGF- β signaling via the T β RII/ALK-1/endoglin pathway was also shown to be important in cardiac fibroblasts [92] and in tumor cells [83]. In addition to signaling via the canonical Smad pathway, endoglin is also able to induce downstream signaling in a Smad-independent manner. These pathways include MAP kinase signaling and the Rho-like GTPase and PI3K/AKT pathways [93]. The interaction with β -arrestin2 (see below) results in inhibition of MAPK signaling. In turn, this regulates vascular tone and permeability through interactions with eNOS [94].

Besides interactions with TGF- β ligands, endoglin also enables protein-protein interaction via its intracellular domain in a TGF- β -independent manner (Fig.1). For example, endoglin interacts with zyxin and ZRP-1, which are localized in focal adhesions and therefore could play a role in endoglin dependent cell migration [95, 96]. Additionally, interaction between β -arrestin2 and the intracellular domain of endoglin results in endosomal internalization of endoglin (Fig.1), decreased EC migration and inhibition of TGF- β -mediated ERK activation [97]. Moreover, the intracellular domain of endoglin interacts with Tctex2 β , a protein involved in retrograde protein transport, thereby inhibiting transcriptional activity induced by TGF- β [90]. In ECs, endoglin has been shown to inhibit hypoxia mediated apoptosis. HIF-1 α binds to the hypoxia responsive domain downstream of the endoglin promoter, resulting in upregulated endoglin expression [98]. Recently, the extracellular domain

of endoglin has also been shown to interact with $\alpha 5$ - and $\beta 1$ - integrins, which induces endoglin/ALK-1 complex formation in the presence of fibronectin [87].

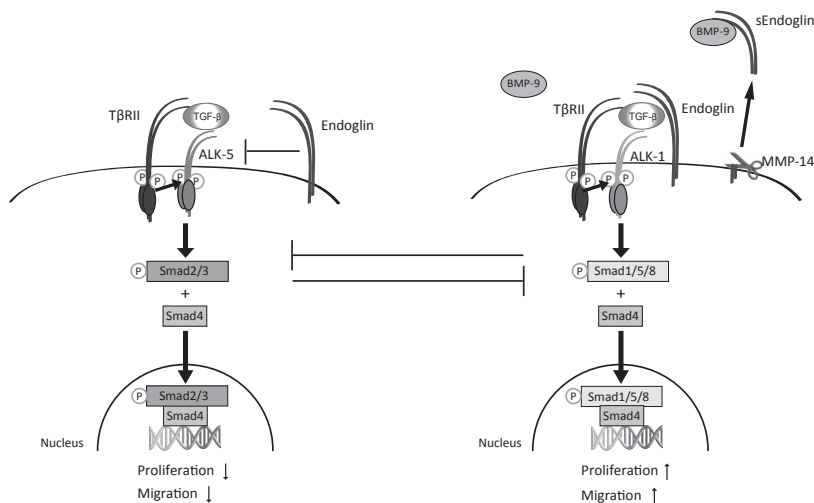


Figure 2 TGF- β signaling pathways in endothelial cells. In endothelial cells, TGF- β family members signal through different T β RII and T β RI combinations. TGF- β binds to T β RII and upon ALK-5 recruitment Smad-2/3 is phosphorylated. Activated Smad-2/3 proteins can form heteromeric complexes with Smad-4, followed by translocation and accumulation in the nucleus. When endoglin is present, TGF- β /ALK-5 signaling is inhibited and TGF- β signaling through ALK-1 is favored resulting in Smad-1/5/8 phosphorylation. Additionally, BMP-9 can bind to endoglin, also inducing Smad-1/5/8 phosphorylation. After complexation with Smad-4 these complexes accumulate in the nucleus, where pro-angiogenic transcriptional responses are initiated. Endoglin can be cleaved from the membrane causing membrane endoglin levels to decrease and soluble endoglin, which can function as a ligand trap, to increase.

3. The role of endoglin in disease

3.1 Fibrosis

TGF- β is known to play an important pro-fibrogenic role, for example, by increasing collagen deposition by fibroblasts in lung and liver fibrosis, Crohn's disease, diabetic nephropathy and pulmonary hypertension [99-101]. TGF- β 1 induces fibroblast differentiation into myofibroblasts resulting in increased deposition of extracellular matrix and collagen type-I, leading to decreased function of the affected organ [102]. Endoglin is overexpressed in liver- and renal fibrosis and scleroderma tissues, indicating a role for endoglin in fibrosis [100]. However, contradictory findings on the pro- or anti-fibrotic role of endoglin in TGF- β -induced fibrosis have been reported (reviewed in [101]).

Recently, Kapur and coworkers performed a study on the role of endoglin in cardiac fibrosis. This group showed that reduced endoglin expression results in better left ventricular function, reduced collagen deposition and increased survival rates in a mouse model of heart failure, compared to mice with wild type endoglin levels [92]. To further explore the

therapeutic potential, these mice were treated with sEndoglin, which acts as a ligand trap for cytokines that normally bind to membrane-localized endoglin (Fig.2). These data showed decreased TGF- β signaling and improved heart function following treatment. In conclusion, endoglin seems to be involved in fibrosis, although its exact function is not known.

Tumors are, in addition to the epithelial tumor cells, composed of cancer-associated fibroblasts (CAFs), which show similarities to the activated fibroblasts observed in fibrotic tissues. TGF- β signaling is known to play an important role in the activation of these CAFs [103, 104]. Moreover, our recent work has shown hyperactivation of TGF- β signaling after interaction with epithelial cancer cells [105]. Based on the similarities between fibroblasts and CAFs and their activation by TGF- β , this opens the interesting possibility of a potential role for endoglin signaling in CAFs during cancer progression. Recently, Vary and colleagues have shown that endoglin expression on CAFs influences tumor growth and metastatic potential of cancer cells in a mouse model of prostate cancer [106]. However, since endoglin was reduced in all cell types, this effect could also be caused by decreased tumor vascularization in endoglin haploinsufficient (Eng^{+/-}) mice [106]. These Eng^{+/-} mice develop smaller and less metastatic prostate cancers and these tumors lack CAFs when compared to Eng^{+/+} mice. Yet, this effect was not observed in endoglin haploinsufficient xenografted lung carcinomas [107], suggesting a prostate cancer-specific mechanism. Additional studies will reveal the involvement of endoglin dependent signaling in CAF activation and their contribution to cancer progression and metastasis.

3.2 Cancer

3.2.1 Endoglin and tumor angiogenesis

Anti-angiogenic therapies, mainly targeting the VEGF pathway, have been approved for patients with various solid tumors including renal, lung and gastrointestinal tumors [108]. Anti-angiogenic therapy can be applied both as monotherapy and in combination with conventional chemotherapy. Both approaches have been effective in tumor regression and inhibition of metastasis in preclinical and clinical settings [109, 110]. However, clinical anti-tumor effects have been temporary and are followed by tumor progression. Resistance is caused by normalization of the tumor vasculature (i.e., improved vessel functionality) and the ability of the ECs to overcome anti-angiogenic treatment by upregulating alternative anti-angiogenic pathways [7].

Therefore, targeting other angiogenic pathways, including proteins highly expressed on proliferating vessels, might be a promising approach. Endoglin is such a candidate, as it directs TGF- β signaling towards a proliferative and migratory phenotype, is essential for angiogenesis and is highly overexpressed in proliferating rather than quiescent vasculature. Therapy directed at endoglin is therefore expected to manifest anti-angiogenic effects specifically in the cancer vasculature.

Since endoglin is highly and specifically expressed on tumor ECs, it could also be used as a marker to assess vascularization of a tumor. The number of microvessels per high power

field in the areas of most intensive neovascularization, also called microvessel density (MVD), is a very useful marker for the prognosis of many cancer types [111]. MVD can be assessed using a variety of endothelial cell markers, including CD34, CD31 (PECAM) or von Willebrand factor, all of which have different predictive values [112]. With regard to endoglin, several studies have shown an inverse correlation between endoglin MVD and overall and disease-free survival in breast cancer patients [112-114]. In endometrial cancer, low endoglin MVD was associated with increased overall survival and was shown to be superior to CD34 and VEGF staining [115]. Additionally, endoglin MVD in early stage cervical cancer was the best predictor for the presence of lymph node metastases [116] and was shown to be an independent predictor of survival in ovarian cancer [117]. A similar observation was made for oral squamous cell carcinoma [118]. Endoglin MVD was also related to metastasis, tumor aggressiveness and survival in esophageal, oral and head and neck cancer as well as tumor recurrence and disease-free survival in laryngeal carcinomas [119-122]. In colorectal cancer, endoglin MVD correlated with both lymph node and liver metastases [123] and correlated with the risk of progression from dysplasia to carcinoma in colorectal mucosa [124]. Endoglin MVD was correlated with survival in non-small cell lung cancer [125] and hepatocellular carcinomas [126], and with tumor stage and metastasis in prostate cancer [127] and breast cancer [113]. Recently, Lin and colleagues showed that endoglin MVD is an independent prognostic marker in predicting survival of cervical cancer patients after receiving chemoradiation therapy [128]. These studies indicate that endoglin MVD has a higher prognostic value compared to other endothelial markers, including CD34, CD31 and von Willebrand factor.

3.2.2 sEndoglin in cancer

In addition to endoglin MVD, the soluble form of the receptor generated via MMP-14-dependent cleavage of membrane bound endoglin [53], has been evaluated for prognostic purposes. Although ECs are the most probable source, a role for other cells (e.g. tumor cells or fibroblasts) in contributing to sEndoglin levels in the plasma of patients cannot be excluded [129]. Several researchers have reported increased levels of sEndoglin in serum of pregnant women diagnosed with pre-eclampsia [67, 130]. sEndoglin has been correlated with metastatic disease in breast and colorectal cancer patients [131, 132]. However, other groups reported no changes in sEndoglin plasma levels in breast, gastrointestinal and esophageal cancer patients [133-136]. Differences in methods to quantify sEndoglin may explain variations between these studies. In patients suffering from both liver cirrhosis and hepatocellular carcinoma (HCC), sEndoglin serum levels were reported to be about two times higher than in patients suffering from either one of those conditions alone [137]. These data suggest that increased sEndoglin levels could indicate the presence of HCC in cirrhotic patients. sEndoglin levels in the urine of prostate cancer patients were found to be correlated with disease stage, lymph node metastases, tumor aggressiveness and recurrence [138-140]. Taken together, although there are indications that sEndoglin could have some diagnostic or prognostic value,

studies are not conclusive and more studies are required to evaluate the prognostic value of sEndoglin in cancer.

3.2.3 Endoglin as tumor vasculature imaging tool

Non-invasive molecular imaging plays an important role during cancer diagnosis and treatment, and potentially could lead to personalized treatment because of improved tumor characterization [141]. The luminal localization on angiogenic vessels makes endoglin easily accessible by antibodies and therefore renders it an interesting candidate for tumor imaging (Fig. 3A). Among different imaging techniques, ultrasound imaging is commonly used in the clinic because of its safety, low costs and ease of use, but tissue penetration is low. Anti-endoglin antibodies were used to visualize tumors by ultrasound to monitor anti-angiogenic treatment response in mouse models. Avidin-coated microbubbles were used to bind biotinylated MJ7/18 anti-endoglin antibodies. The data revealed highly specific binding to endoglin expressing cells in the tumor vasculature in mice bearing orthotopic human pancreatic tumors [142]. The ultrasound signal intensity of specific endoglin-conjugated microbubbles correlated with the MVD during treatment. Although the sensitivity of ultrasound is limited, improvements are possible through the use of advanced contrast agents and antibody-contrast agent conjugation methods [143].

An imaging technique offering higher contrast, spatial resolution and tissue penetration than ultrasound, is molecular MRI (mMRI). mMRI combines the acquisition of anatomical structures of MRI with the detection of biological processes at the molecular level [144]. In a recent report, the potential use of endoglin as an imaging agent in combination with mMRI was analyzed. Radioactive iron (^{59}Fe) coupled endoglin MJ7/18 antibodies increased the sensitivity of detecting subcutaneous tumors in mice by mMRI, compared to control Gd-coupled antibodies [144].

The most promising methods for tumor imaging are radionuclide-based, including positron emission tomography (PET) and single photon emission computed tomography (SPECT), due to their high sensitivity and tissue penetration [15, 145, 146]. Several animal studies were performed to analyze the potential of endoglin as an imaging tool for the tumor vasculature. One group showed efficient imaging of melanomas upon intravenous injections of ^{111}In dium Chloride-labeled MJ7/18 anti-endoglin antibodies in a mouse model [145]. Autoradiography and immunohistology showed the highest signal at the tumor edges, where vascular density appeared to be highest. Additionally, in a spontaneous breast cancer model in dogs, ^{125}I odine-labeled MAEND3 anti-human endoglin antibodies were rapidly taken up by the tumors and produced an intense signal without systemic side effects [15]. Furthermore, Cai and coworkers investigated the future clinical applicability of using PET scans to image the tumor vasculature in a mouse model. Injection of graphene oxide-conjugated TRC105 anti-endoglin antibodies resulted in specific imaging of the tumor vasculature in mice by PET imaging [147]. Specific tumor uptake of the antibodies was confirmed by immunohistochemistry. Although promising, improvements are possible

concerning the rate of antibody accumulation in the tumor and the background level in the clearing organs (e.g., the liver).

PET combined with near-infrared fluorescence (NIRF) imaging, known for its high resolution, was also explored for tumor imaging. Zhang and coworkers labeled TRC105 with both a NIRF dye and radioactive copper (^{64}Cu) and demonstrated specific tumor imaging in an *in vivo* breast cancer model using NIRF and PET imaging [148]. Although NIRF has low tissue penetration, it can be very useful during endoscopy and image-guided surgery.

The potential clinical use of endoglin-based imaging in cancer diagnosis was underlined when fresh nephrectomy specimens from patients with renal cell carcinoma were perfused with a ^{99}Tc -labeled E9 anti-endoglin antibody. The observed radiographic hot spots corresponded with tumors identified in preoperative MRI scans. Remarkably, another tumor was identified, which was not observed in preoperative scans using the labeled anti-endoglin antibody [146].

These data suggest that endoglin-based tumor imaging is promising, although there is still room for improvement before these techniques can be translated to the clinic. Combining different imaging techniques, like PET and NIRF imaging, will likely increase both sensitivity and specificity of endoglin-based tumor imaging and could result in valuable tools for cancer treatment.

4. Endoglin for targeted cancer therapy

Considering the specific overexpression of endoglin on tumor vasculature, it seems to be a promising target for anti-angiogenic therapy. Several approaches targeting endoglin have been evaluated, including endoglin-Fc constructs, endoglin vaccines and endoglin neutralizing antibodies (Fig.3B).

4.1 Endoglin-Fc

Endoglin-Fc is a chimeric protein, consisting of the extracellular part of endoglin fused to the Fc tail of human IgG [44]. This molecule functions as a ligand trap for endoglin ligands including BMP-9 and BMP-10 (Fig. 3B, left panel). Two groups showed that endoglin-Fc inhibits angiogenesis in *in vitro* assays [37, 53] and in an orthotopic breast cancer model (Hawinkels et al., unpublished observations). Furthermore, Castonguay and coworkers showed that endoglin-Fc significantly reduced tumor angiogenesis, and therefore tumor growth, in BALB/c mice subcutaneously inoculated with colon adenocarcinoma cells [37]. Further studies on the therapeutic potential of endoglin-Fc are ongoing.

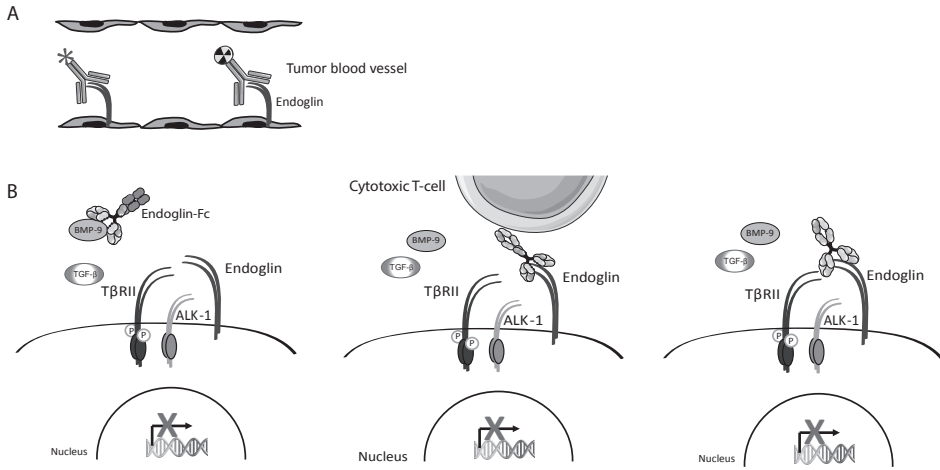


Figure 3 Endoglin antibodies in imaging and targeted treatment. (A) Luminal localization of endoglin on endothelial cells facilitates access of endoglin antibodies. Antibodies labeled with fluorescent (left) or radioactive epitopes (right) can be used in imaging the tumors' vasculature. (B) Left panel; Endoglin-Fc functions as a ligand trap for endoglin ligands, such as BMP-9, thereby inhibiting signaling. Middle panel; Vaccines against endoglin elicit an immune response which results in a cytotoxic T-cell response. Right panel: Endoglin neutralizing antibodies bind to the extracellular domain of endoglin, thereby inhibiting downstream signaling. These strategies result in specific inhibition of tumor-angiogenesis by either inducing cell death (vaccine) or blocking endoglin signaling (endoglin-Fc and neutralizing antibodies).

4.2 Endoglin vaccines

Another possible therapeutic approach in solid cancers might be immunotherapy directed against antigens expressed on the surface of the tumor vascular ECs. Such therapies elicit an immune response against cells expressing this antigen (Fig.3B, middle panel). When the antigen is tumor vessel-specific, such as endoglin, it abrogates tumor angiogenesis [149]. One study explored the use of an endoglin protein vaccine with low dose cisplatin. Lung or liver cancer cells were subcutaneously injected in mice treated with the protein vaccine, cisplatin or a combination [150]. Both monotherapies inhibited tumor growth, while combination therapy synergistically decreased tumor volume, induced auto-antibodies against endoglin and induced EC apoptosis *in vivo* [150]. More recently, endoglin-based DNA vaccines were developed and studied in pre-clinical models. Wood and coworkers reported that treatment with *Listeria monocytogenes* containing an endoglin-expressing plasmid inhibited the growth and metastasis of established breast cancers *in vivo* [151]. In another study, attenuated *Salmonella Typhimurium* containing an endoglin-expressing plasmid was orally administered to mice bearing subcutaneous tumors. Treatment inhibited tumor growth, enhanced T-cell response, inhibited tumor angiogenesis and mediated endoglin-specific cytotoxicity [152]. These effects were enhanced when the treatment was combined with either intratumoral administration of IL-12 or systemic cyclophosphamide treatment. Importantly, treatment did not affect physiological angiogenic processes, such as wound healing.

4.3 Endoglin neutralizing antibodies

In addition to targeting endoglin with the use of endoglin-Fc chimeras or endoglin based vaccines, much effort has been devoted to targeting endoglin with neutralizing antibodies (Fig.3B, right panel). Seon and colleagues showed that targeting the tumor vasculature using deglycosylated ricin A-chain-coupled anti-endoglin antibodies suppressed growth of subcutaneously established human breast cancers in immunodeficient mice [153]. Combination therapy of anti-endoglin an antibody with the chemotherapeutic drug cyclophosphamide produced synergistic antitumor effects [154]. Anti-endoglin antibodies, either unconjugated or conjugated to toxins, also suppressed the formation of metastases from mammary and colorectal carcinomas in mouse models [155].

Tsujie *et al.* investigated the working mechanism by which unconjugated endoglin antibodies inhibit tumor growth and suppress angiogenesis. They observed that the anti-endoglin SN6j antibody was more effective in suppressing tumor growth in immunocompetent mice than in severe combined immunodeficiency (SCID) mice [156]. Depletion of CD4⁺ and/or CD8⁺ T-cells abrogated the tumor inhibitory effects of SN6j, indicating that antibody activity is dependent on an immune response. However, SN6j also induced apoptosis in human ECs *in vitro* [156], suggesting an additional mechanism of action, for example loss of inhibition of hypoxia-mediated cell death by endoglin [98].

TRC105 is a chimeric IgG1 monoclonal antibody, based on the SN6j antibody, that binds human endoglin with high affinity and induces antibody-dependent cytotoxicity and apoptosis of endoglin-expressing cells [157]. Recently, results from a first phase-I clinical trial with TRC105 in patients suffering from advanced solid cancers were published [157]. In this phase I trial, 50 patients with advanced solid cancers were included and treated in a dose escalation protocol that explored doses from 0.01 to 15 mg/kg. Nearly half of the refractory cancer patients enrolled in the trial were progression-free after 2 months and 14% were progression-free after 4 months of TRC105 treatment. Two remarkable cases were observed during the trial. One patient with castrate-refractory prostate cancer, diffuse skeletal metastases and increased prostate specific antigen (PSA) demonstrated marked improvement in bone scans and an undetectable PSA two months after initiation of TRC105 treatment. The bone scan improvement and complete PSA response are ongoing for over 4 years (Fig.4). A second patient with metastatic chemotherapy-refractory uterine carcinosarcoma and several lung metastases demonstrated radiographic regression of disease lasting for 18 months. The duration of TRC105 treatment exceeded the total duration of three prior treatments, each of which was discontinued for progressive disease. Adverse effects were manageable and rarely serious and three patients developed HHT-like telangiectases. Overall, TRC105 treatment is well-tolerated at clinically relevant doses that demonstrated anti-tumor activity [157]. Currently, multiple phase-II clinical studies are ongoing (Table 1)[158], evaluating the therapeutic efficacy of TRC105 treatment alone and in combination with bevacizumab and other anti-cancer agents.

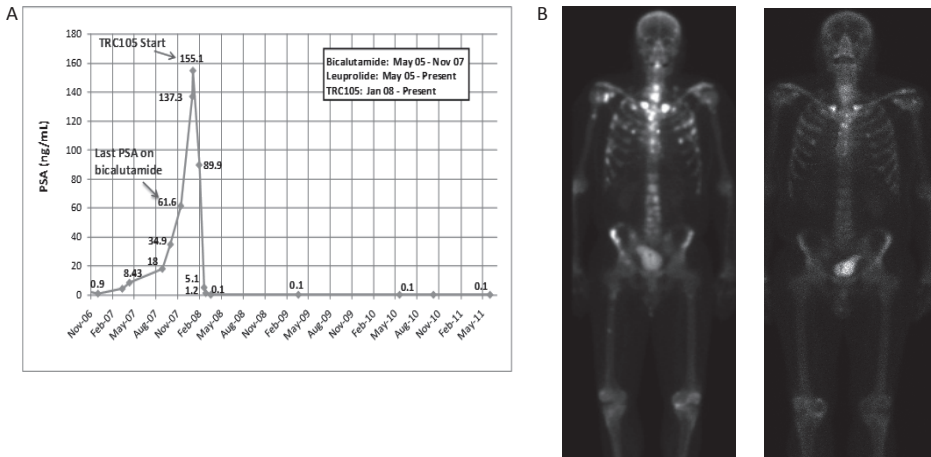


Figure 4 Disease stabilization in prostate cancer after TRC105 treatment. A patient with castrate-refractory prostate cancer showed normalization of PSA levels upon treatment with the anti-endoglin antibody TRC105 (A). Furthermore significant improvement on bone scans (B, right) was observed compared to bone scans before TRC105 treatment (B, left). PSA; prostate-specific antigen. *Reprinted with permission from the American Association for Cancer Research Clinical Cancer Research, 2012; 18 #17; 4820-4829; Rosen et al – figure 2 (A & B) [157].*

5. Conclusion

Because of its selective overexpression and luminal localization on ECs, endoglin is considered to be a promising target for cancer diagnosis and tumor-specific anti-cancer treatment. Endoglin MVD correlates with patient survival and metastatic disease, being superior to several other described markers of angiogenesis. In addition, tumor imaging by endoglin-specific antibody conjugates has been shown to be promising in several pre-clinical *in vivo* studies and awaits clinical translation.

Endoglin targeting for cancer therapy has demonstrated anti-angiogenic responses using either endoglin neutralizing antibodies or endoglin-Fc in several pre-clinical studies. Exciting data were reported from a first-in-human phase-I clinical trial targeting tumor vasculature using anti-endoglin antibodies. Anti-endoglin therapy showed anti-tumor activity at well tolerated doses administered to refractory cancer patients [157]. The results from the ongoing phase II studies, including those of TRC105 in combination with other (anti-angiogenic) therapies will be important to further establish the therapeutic potential of targeting endoglin for cancer therapy.

6. Expert opinion

Anti-angiogenic therapies have been clinically applied for the last decade, but their efficiency in terms of increasing survival of cancer patients has not reached the high expectations indicated by pre-clinical data. Resistance of tumors to therapy and normalization of blood vessels seem to be major problems with the currently approved therapies that primarily target the VEGF pathway. Novel anti-angiogenic therapies are being developed to target unique pathways that may overcome resistance to VEGF therapy, including the TGF- β pathway. Endoglin is a TGF- β co-receptor that is a promising target for tumor imaging and cancer therapy for several reasons:

First, endoglin is highly and specifically expressed on the tumor vasculature, which reduces the risk of side effects. Targeting endoglin will probably induce fewer side effects than targeting other components of the TGF- β signaling pathway, which are expressed in many other cell types and have important roles in tissue homeostasis. Not surprisingly, anti-endoglin antibody treatment was well tolerated in a phase-I clinical trial and demonstrated anti-tumor activity as a single agent [157].

Second, the specific luminal localization of endoglin in vessels opens a window of opportunity to use anti-endoglin therapy for imaging the tumor vasculature. Delivery of antibodies to targets expressed on tumor cells can be hampered by poor tumor penetration. However, endoglin expression on ECs circumvents the necessity of tissue penetration. Labeling anti-endoglin antibodies with radioactive or fluorescent labels could aid in tumor diagnosis and monitor the response during treatment to facilitate personalized medicine. Moreover, the use of a fluorescent labeled anti-endoglin antibodies could aid surgeons during surgery and decrease the risk of incomplete tumor resection.

In addition to imaging, targeting the tumor vasculature with an anti-endoglin antibody conjugated to chemotherapeutic agents, toxins or radioactive compounds could lead to specific and potent targeting of the tumor vasculature. Such therapies, using radioimmunoisotopes (ibritumomab tiuxetan) and immunotoxins (brentuximab vedotin) have been approved for the treatment of non-Hodgkin's lymphoma. Indications of activity by targeting endoglin with TRC105 as monotherapy in a phase-I trial are encouraging and antibody potency could be augmented through conjugation.

Third, most anti-angiogenic therapies targeting a single pathway promote resistance quite rapidly by upregulating alternative angiogenic pathways. While patients responded to TRC105 monotherapy (inhibition of disease progression and reduction in tumor burden) without serious side effects, it is likely that anti-angiogenic treatments will need to be combined to produce more sustainable anti-tumor effects. In this regard, the combination of anti-VEGF therapy with therapy targeting endoglin, a target that is upregulated following anti-VEGF therapy [159] could prove to be effective in the treatment of various solid cancers.

Finally, although ECs very highly express endoglin, several studies have shown endoglin expression on other cell types within the tumor microenvironment. Recently, it was shown that endoglin is important for TGF- β signaling in fibroblasts [92] and that TGF- β is a major player in the transdifferentiation of resident fibroblasts into CAFs [105, 160, 161]. Additionally, endoglin regulates the invasive and migratory potential of CAFs and tumor cells in prostate cancer [106]. Therefore, targeting endoglin on these cells in addition to the endothelium might enhance the anti-tumor effect. Further studies will be required to elucidate the exact role of endoglin on non-ECs and its potential function in tumor cell migration and invasion.

In conclusion, targeting endoglin seems to be a valuable approach for diagnostic purposes and therapy, both as mono anti-angiogenic therapy and in combination with existing anti-angiogenic or chemotherapeutic treatments.

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Conflicts of interest

The authors declare to have no conflicts of interest to disclose.

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