

The role of the TGF- β co-receptor endoglin in cancer

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ABSTRACT

Endoglin (CD105) is an auxiliary membrane receptor of transforming growth factor- β (TGF- β) that interacts with type I and type II TGF- β receptors and modulates TGF- β signalling. Mutations in endoglin are involved in Hereditary Hemorrhagic Telangiectasia type I, a disorder characterized by cutaneous telangiectasias, epistaxis (nosebleeds) and major arteriovenous shunts, mainly in liver and lung. Endoglin is overexpressed in the tumor-associated vascular endothelium where it modulates angiogenesis. This feature makes endoglin a promising target for antiangiogenic cancer therapy. Recent studies on human and experimental models of carcinogenesis point to an important tumor cell-autonomous role of endoglin by regulating proliferation, migration, invasion and metastasis. These studies suggest that endoglin behaves as a suppressor of malignancy in experimental and human carcinogenesis. In this review, we evaluate the implication of endoglin in tumor development underlying studies developed in our laboratories in recent years.

KEY WORDS: endoglin (CD105), TGF- β , HHT, angiogenesis, cancer, migration, invasion, malignant progression

INTRODUCTION

The human transforming growth factor- β (TGF- β) family comprises more than 40 structurally related factors including the classical isoforms: TGF- β 1, - β 2 and - β 3, activins, and bone morphogenetic proteins (BMPs), among others. These growth factors play crucial roles in embryonic development, adult tissue homeostasis and the pathogenesis of a number of diseases[1,2]. TGF- β signals through heteromeric cell-surface type I and II Ser/Thr kinase receptors (T β RI and T β RII) that activate the canonical Smad pathway as well as Smad-independent signaling pathways to regulate fundamental cellular processes, such as proliferation, differentiation, migration, programmed cell death, adhesion, cytoskeletal organization, extracellular matrix remodeling and phenotypic plasticity[3-5]. Cancer is a multistep process which involves the evolution of a clonal cell population that has escaped from the control of regulatory circuits that govern normal tissue homeostasis. Cancer cells accumulate gain-of-function mutations in oncogenes, that make them independent of growth factor stimulated proliferation, and recessive loss-of-function mutations in tumor suppressors, that allow evasion of growth inhibitory signals[6]. TGF- β is a potent growth inhibitor of normal epithelial cells and accumulated evidence indicates that it plays a tumor suppressive role in epithelial cancer[7]. Thus, tumor cells evade TGF- β growth inhibition either by inactivating components of the TGF- β signaling machinery, including receptors and Smad proteins, or by selectively preventing the TGF- β cytostatic responses[8]. Paradoxically, TGF- β is overexpressed in many human cancers and becomes a promoter of malignancy during tumor progression[9-11]. TGF- β drives tumorigenesis by a variety of mechanisms acting on tumor and stromal cells. TGF- β stimulates tumor growth by inducing the production of autocrine mitogenic factors. It

also promotes tumor cell migration and invasion by inducing an epithelial-mesenchymal transition (EMT). EMT is a profound phenotypic conversion by which epithelial cells lose their polarity and cohesiveness acquiring motile and invasive properties. EMT plays a crucial role in morphogenetic processes during organogenesis, but also contributes to pathological situations such as fibrosis and cancer[12,13]. In the stroma, TGF- β is involved in the generation of activated myofibroblasts from mesenchymal precursors, the stimulation of angiogenesis, the suppression of the immune surveillance and the promotion of metastasis.

Endoglin is a TGF- β coreceptor that modulates TGF- β -dependent cellular responses. So far, most of research studies on endoglin have focused on its role in angiogenesis and vascular remodelling. However, recent data have emerged suggesting that inactivation or silencing of endoglin in tumor cells contributes to the deregulation of TGF- β signaling and malignant progression.

ENDOGLIN AND TGF- β SIGNALING

Endoglin structure. Isoforms

Endoglin is a type I membrane glycoprotein with a large extracellular domain containing several O- and N-glycans, followed by a hydrophobic membrane-spanning segment and a short cytoplasmic tail. It is expressed at the cell surface as a disulfide-linked homodimer[14-17]. The extracellular region contains at the N-terminus an orphan domain as well as a zona pellucida (ZP) consensus domain in the yuxtamembrane region (Fig. 1a), which is potentially involved in receptor oligomerization[18-20]. Within the ZP domain, human endoglin contains an Arg-Gly-Asp (RGD) motif which is a recognition sequence for integrins[14]. In contrast to the

functional T β RI and T β RII receptors, the cytoplasmic domain of endoglin lacks any obvious enzymatic motif, but contains Ser and Thr residues that can be phosphorylated by the TGF- β receptor kinases (see below and Fig. 1c). In addition, the endoglin cytoplasmic tail holds a PDZ-binding motif (Ser-Ser-Met-Ala) at the C-terminus that seems to modulate phosphorylation of the neighbouring Ser and Thr residues[21].

At least two distinct alternatively spliced isoforms, long (L)-endoglin and short (S)-endoglin, are expressed in human and mouse tissues[17,22] (see Fig. 1b). L- and S-endoglin only differ from each other in the composition of their cytoplasmic domain. The human L isoform has a cytoplasmic domain of 47 residues whereas the cytoplasmic tail of the S isoform contains only 14 residues (Fig. 1c). Both endoglin isoforms share the first 7 juxtamembrane residues of their cytoplasmic tails, but S-endoglin lacks the PDZ-binding motif[17,22]. Although significant expression of S-endoglin transcripts has been found in some tissues, such as lung and liver[22], L-endoglin seems to be the predominant isoform expressed in most of the tissues, particularly in the endothelium, and is the focus of most studies on endoglin biology. Therefore, when the general term of endoglin is used in this review, it is referred to L-endoglin.

Endoglin in the TGF- β receptor complex

TGF- β growth factors exert their biological effects by binding to a heteromeric complex containing the transmembrane Ser/Thr kinase T β RI and T β RII signaling receptors, although it is T β RI which determines the signaling specificity within the receptor complex[23,24]. The human genome encodes seven T β RI (activin-like kinases or ALKs 1–7) and five T β RII receptors. After binding of the ligand to T β RII, it activates and transphosphorylates T β RI, which subsequently transduces the signal by phosphorylating the receptor-regulated Smads (R-Smads). Activated R-Smads associate

with a common homolog named Co-Smad (Smad4 in mammals) and these complexes, then, translocate into the nucleus where they regulate the transcriptional activity of target genes (Fig. 2). TGF- β has been found to activate other signalling mediators in a Smad-independent manner, including several branches of mitogen-activated protein kinase (MAPK) pathways, Rho GTPase signaling pathways and the phosphatidylinositol-3-kinase (PI3K)/AKT pathway[3,5].

The TGF- β receptor complex also contains two auxiliary receptors (or type III receptors), endoglin and betaglycan, that bind different members of the TGF- β family[1,25]. Endoglin and betaglycan share a high degree of amino acid sequence homology in the transmembrane and cytoplasmic domains[26]. At variance with endoglin, no alternatively spliced isoforms have so far been described for betaglycan. Betaglycan has been shown to promote high-affinity binding of TGF- β_2 to T β RII, since TGF- β_2 binds T β RII with low affinity in the absence of betaglycan[27]. Endoglin binds TGF- β_1 , TGF- β_3 (but not TGF- β_2), activin-A, BMP-2 and BMP-7 in the presence of the signaling T β RI and T β RII receptors[17,28-30]. In contrast, membrane associated endoglin can bind BMP9 in the absence of signaling receptors[31]. Activated ALK5 and T β RII interact with the extracellular and cytoplasmic domains of endoglin (Fig. 2). However, unphosphorylated ALK5 only interacts with the endoglin cytoplasmic domain. Upon binding, both ALK5 and T β RII phosphorylate Ser and Thr in the endoglin cytoplasmic domain and, then, ALK5 but not T β RII dissociates from the receptor complex[32]. On the other hand, Koleva and coworkers have shown that endoglin is a direct substrate of ALK1 in endothelial cells. ALK1 is an endothelial-specific T β RI which preferentially phosphorylates endoglin in Thr residues, although this requires previous Ser phosphorylation by T β RII[21]. Endoglin phosphorylation

seems to play a regulatory role for ALK1-dependent endothelial cell growth and adhesion. For a summary of endoglin-binding proteins see Table 1.

These studies indicate that endoglin is part of the ligand-T β RI/T β RII complex where it likely plays a role in the dynamics of T β RI-T β RII interactions regulating downstream TGF- β signaling. Indeed, a large number of laboratories have reported that endoglin modulates TGF- β -dependent responses in different cell types. Accordingly, endoglin dysfunction may lead to uncontrolled TGF- β signaling activity often resulting in human disease.

ENDOGLIN AND THE VASCULAR SYSTEM

Regulation of endoglin expression in endothelial cells

Endoglin is expressed at low levels in resting endothelial cells, but its expression strongly increases in active vascular endothelial cells of inflamed tissues during wound healing, in infected tissues, synovial arthritis and psoriatic skin, as well as upon vascular injury and tumor angiogenesis[33,34]. In fact, in cultured tumor endothelial cells, endoglin expression correlates with the rate of cellular proliferation[35]. TGF- β potently stimulates endoglin expression in endothelial cells by a Smad-dependent mechanism[36,37], but the most suitable stimulus upregulating endoglin expression in vessels seems to be hypoxia. The human *ENDOGLIN* (*ENG*) gene promoter contains a functional consensus hypoxia responsive element for the hypoxia inducible factor HIF-1[38]. Whereas hypoxia alone stimulates only moderately endoglin transcription, TGF- β 1 and hypoxia cooperates to induce a strong stimulation of endoglin expression which is mediated by the formation of a transcriptional Smad3/Smad4/Sp1/HIF-1 multimolecular complex[38]. Also, the cooperative interaction between Sp1 and KLF6

transcription factors appears to mediate endoglin transcriptional activation upon vascular injury[39]. Interestingly, the functional and physical cooperation between Sp1 and KLF6 results in upregulation of other injury-related genes such as TGF- β 1 and components of the TGF- β signaling complex. In the placenta, it is TGF- β 3 which mediates stimulation of endoglin expression by hypoxia[40].

Endoglin is a pro-angiogenic molecule

Endoglin in the blood vessel endothelium is involved in the control of cell proliferation, migration and capillary tube formation[41]. Lebrin and coworkers have proposed an elegant mechanism for endoglin to regulate angiogenesis by controlling TGF- β signaling[42]. TGF- β can activate two distinct type I receptors ALK1 and ALK5 in endothelial cells which have opposite effects on cell behaviour. ALK1 activation leads to stimulation of endothelial cell proliferation and migration through phosphorylation of Smad1/Smad5/Smad8, while ALK5 activation results in inhibition of proliferation and migration via phosphorylation of Smad2/Smad3[43,44] (Fig. 2). Thus, TGF- β controls both the active and maturation phases of angiogenesis[45]. In the former phase, endothelial cells degrade the perivascular membrane and migrate into the extracellular space where they proliferate and form capillary lumen, while in the maturation phase cells stop proliferating and migrating, a reconstituted basal membrane surrounds capillary vessels and mesenchymal cells are recruited to the new vessels where they differentiate into vascular smooth muscle cells and pericytes[46,47]. Endoglin is required for TGF- β /ALK1 signaling which indirectly inhibits signalling through TGF- β /ALK5[42,48]. Therefore, in endothelial cells expressing low levels of endoglin, TGF- β signals through ALK5 maintaining a resting state in the endothelium. However, under hypoxia or increased TGF- β levels, endoglin expression is upregulated

and TGF- β switches to ALK1 signaling promoting angiogenesis. Nevertheless, there are conflicting results derived from studies with endothelial cells isolated from *Endoglin* null (*Eng*^{-/-}) embryos. In contrast to the results of Lebrin and colleagues[42], Letarte and colleagues have reported that *Eng*^{-/-} cells display increased TGF- β anti-proliferative response and TGF- β /ALK1 signaling[49].

On the other hand, based on studies using a transgenic mouse model in which the expression of the short S-endoglin isoform was targeted to the blood vascular endothelium (*S-Eng*⁺), we have suggested an anti-angiogenic function for S-endoglin[22]. Both *S-Eng*⁺ and *Endoglin* heterozygous mice (*Eng*^{+/-}) show an angiogenic deficient phenotype[41], suggesting that S-endoglin acts as an antagonist of the predominant L-endoglin form. Moreover, *S-Eng*⁺ and *Eng*^{+/-} mice show dysregulated vascular tone and decreased endothelial nitric oxide synthase (eNOS) expression[50-53]. Interestingly, the ratio of S/L endoglin expression increases in cultured human endothelial cells and vascularized mouse tissues during senescence/aging, indicating that S-endoglin may contribute to age-dependent vascular pathology[53]. L- and S-endoglin differ in their ability to regulate TGF- β cellular responses[54] and in modulating TGF- β signaling[17,55]. Of note, L- and S-endoglin can heterodimerize through their common extracellular domain[22] and, therefore, in cells in which both isoforms are coexpressed, S-endoglin may compete for L-L homodimer formation.

Recently, increased endoglin expression has also been found in tumor lymphatic endothelial cells with respect to lymphatic endothelial cells of normal tissues[56,57]. Interestingly, endoglin expression was upregulated in lymphatic tissue associated with lymphangiectasia in a mutant mouse deficient for the regulatory subunits of PI3K[58],

suggesting that endoglin expression is inhibited by PI3K and that endoglin may play a role in lymph vessel homeostasis.

Endoglin and Hereditary Hemorrhagic Telangiectasia

The critical role of endoglin in the vascular system is demonstrated by the identification of *ENG* as the gene mutated in the vascular syndrome Hereditary Hemorrhagic Telangiectasia type I (HHT1)[59]. Interestingly, a second gene: *ACTIVIN RECEPTOR LIKE KINASE (ACVRL1)*, encoding the ALK1 receptor, was discovered as responsible for HHT2[60]. HHT is an autosomal dominant disease with a prevalence of about 1 in 10,000 individuals. HHT patients are characterized by arteriovenous malformations (AVMs) which range from small capillary dilatations or telangiectases in the skin, nasal epithelium, oral mucosa and gastrointestinal tract to large AVMs in lung, brain and liver that can lead to internal hemorrhage and stroke[61]. The *ENG* and *ACVRL1* loci account for more than 90% of HHT cases. Nevertheless, HHT is linked to two other loci for which the genes remain to be identified. In a small proportion of the HHT population (about 2%), the pathogenic mutations occur in the *MADH4* gene, encoding Smad4. The *MADH4* gene mutations lead to a combined syndrome of Juvenile polyposis (JP) and HHT (see below). Interestingly, all *ENG* mutations to date are found in exons 1 to 12 coding for the endoglin extracellular domain. Most *ENG* and *ACVRL1* mutations lead to unstable or non-functional proteins, supporting haploinsufficiency as the predominant cause of HHT pathogenicity[62].

Endoglin haploinsufficiency, as the pathogenic mechanism of HHT1, is also supported by experiments with animal models. *Eng*^{+/-} mice develop signs of HHT, such as telangiectasias, epistaxis and even cerebral arteriovenous malformations[63-65]. The strength of these clinical manifestations depends on the animal strain suggesting the

existence of modifier genes in HHT[66]. *Eng*^{+/-} mice also show a reduced angiogenic response as well as increased and reduced levels of cyclooxygenase-2 (COX-2) and eNOS, respectively[41,51,67]. eNOS and COX-2 produce the vasorelaxant substances nitric oxide and prostaglandin E2, respectively and, therefore, endoglin is also involved in the regulation of vascular tone[50-52].

Eng^{-/-} null embryos, on the other hand, die at mid-gestation (E10.5) due to circulatory arrest and haemorrhage. These embryos display important alterations in cardiovascular development, including incomplete yolk sac vessel branching and remodelling, vessel dilatation and cardiac morphogenesis[63,68,69-71]. These and other studies suggest a key role for endoglin in early patterning of the heart[72].

Endoglin as a target for cancer imaging and antiangiogenic therapy

The finding that endoglin is overexpressed in vascular endothelial cells of tissues undergoing angiogenesis suggested that endoglin detection could be used as a marker to analyze angiogenesis and microvessel density (MVD) in tumors. Increased MVD is associated with bad prognosis in cancer patients, and the use of endoglin antibodies has been found to be the most reliable for MVD measurements when compared to other endothelial protein markers[73,74]. Moreover, its association with actively proliferating endothelial cells, while it is weakly expressed in quiescent endothelium has focused the interest on endoglin as a potential target for cancer imaging in vivo[34]. Studies carried out on animal models[75,76] and patients with renal carcinoma[77] clearly show that anti-endoglin monoclonal antibodies specifically target the tumor vasculature. In this respect, endoglin is a more specific marker for new, immature vessels, unlike other endothelial cell-surface markers, such as CD31 and CD34 that are expressed in both mature and immature vessels[78]. The anti-endoglin antibodies are useful to detect

metastatic tumors that conventional imaging techniques cannot visualize. Also, microbubbles conjugated with an anti-endoglin antibody have been successfully utilized to image (by ultrasounds) the effect of gemcitabine on the vasculature in mouse xenografts of human pancreatic tumors[79].

Blocking the tumor blood supply by disrupting or preventing the formation of new blood vessels has been thought a general strategy to treat cancer[80]. The potential of anti-endoglin monoclonal antibodies to be used as a therapeutic anti-angiogenic strategy in human cancer has received considerable support from preclinical studies. There are excellent reviews addressing this topic[34,74] and, therefore, this matter will not be discussed herein. Nevertheless, it is worth to mention that a multicenter phase I clinical trial using the anti-endoglin antibody TRC105 is ongoing in patients with advanced refractory cancer. The preliminary results support good tolerability and early clinical activity of TRC105[81].

ENDOGLIN IN HUMAN CANCER

Most studies about the involvement of endoglin in cancer have focused on its role as a pro-angiogenic molecule and its usefulness as a marker of MVD in tumors. However, there is evidence that endoglin modulates cell proliferation, adhesion and migration of neoplastic cells suggesting a direct involvement of endoglin in cancer either by modulating the response of tumor cells to TGF- β or by as yet not completely understood TGF- β -independent mechanisms. In the next sections, we review the current data implicating endoglin in tumor progression, mainly in carcinomas. Endoglin has also been associated with a hereditary cancer disorder known as juvenile polyposis.

Juvenile polyposis syndrome

Juvenile polyposis (JP) is an autosomal dominant syndrome characterized by multiple hamartomatous polyps occurring throughout the gastrointestinal tract. Patients are usually diagnosed within the first decade, but polyps can appear at any age[82,83]. The term “hamartomatous” was introduced based on the following histological features: dilated cystic glands with retention of mucus and lined by tall columnar epithelium, a markedly expanded lamina propria and diffuse chronic infiltration of inflammatory cells, to be distinguished from adenomatous polyps[84]. Individuals with JP are at risk of developing gastric, colorectal and pancreatic malignancies[85,86]. Mutations in either the *MADH4* or *BMPRIA* genes, the latter encoding a transmembrane type I receptor for BMPs, are responsible for the majority of JP cases[82,83]. The overall prevalence of germline mutations is 20% for *MADH4* and 20% for *BMPRIA* in JP probands[87]. *SMAD4* mutations are correlated to a high incidence of upper gastrointestinal polyps[88,89]. Some JP patients were observed to develop symptoms of HHT, such as pulmonary AVMs, that led to the recognition of a combined syndrome JP-HHT which is caused by mutations in *MADH4* but not in the main genes involved in HHT: *ENG* and *ACVRL1*[90,91]. JP-HHT patients commonly exhibit AVMs, telangiectases and epistaxis as well as gastrointestinal polyps. A recent report has shown that *MADH4* mutations in JP and JP-HHT overlap indicating that there is no apparent molecular distinction between JP and JP-HHT[92].

Also, germline mutations in *ENG* were found in 2 out of 14 JP patients who did not contain *MADH4* or *BMPRIA* mutations[93]. Both JP patients had an early onset of the disease (at ages of 3 and 5 years) compared to those without *ENG* mutations and they did not exhibit clinical manifestations or a family history of HHT. The low frequency of *ENG* mutations found in JP patients raised concerns about whether *ENG* is

a susceptibility gene for JP. Thus, a subsequent report aimed to examine the prevalence of *ENG* mutations in JP patients who did not have germline mutations in *MADH4* and *BMPRIA* failed to confirm the role of *ENG* as a gene predisposing to JP[94].

Endoglin alterations in human sporadic cancer

Only a handful of studies have reported the expression of endoglin in neoplastic cells of primary tumors. In prostate cancer, endoglin was detected in epithelial cells of both prostatic intraepithelial neoplasia and malignant areas, while cells in normal and benign acini were negative[95]. In this study, endoglin staining in prostate cancer cells did not correlate with patient/tumor characteristics, including the levels of the prostatic specific antigen PSA in serum, Gleason score, tumor stage, and periprostatic invasion. In contrast, downregulation of endoglin expression was found in prostate carcinoma cell lines associated with malignant progression. Suppression of endoglin expression by antisense oligonucleotides enhanced migration and invasion of nontumorigenic prostate cell lines, while overexpression of endoglin had the opposite effect[96]. These results suggested a role for endoglin as a modulator of migration and invasiveness in prostate cancer cells, and were in line with the striking observation that only the *ENG* gene among 4,000 genes was found to be downregulated during detachment of metastatic prostate cancer cells[97].

A report on esophageal squamous cell carcinomas (SCCs) showed that endoglin expression is downregulated in both primary tumors and cell lines, and that overexpression of endoglin in esophageal SCC cells led to reduced invasiveness and tumorigenicity[98]. The mechanisms for endoglin downregulation in esophageal tumors include epigenetic silencing by gene methylation and loss of heterozygosity (LOH). Previous studies from the same authors had identified that the 9q33-34 region, where

the *ENG* gene was mapped[99], is frequently lost in esophageal cancer[100,101], pointing to the existence of a tumor suppressor gene therein.

Recently, strong evidence pointing to endoglin as a tumor suppressor has been obtained in breast cancer[102]. This report shows that lack of endoglin expression in primary tumors correlates with *ENG* gene methylation and poor clinical outcome. Furthermore, loss of endoglin expression seems to cooperate with the activated ErbB2 oncogene to promote migration and invasion of nontumorigenic breast epithelial cells, while endoglin overexpression reduces the metastatic ability of the aggressive MDA-MB-231 carcinoma cell line. These observations indicate that endoglin acts as a suppressor of invasion and metastasis in breast cancer, and argue against the report of Oxmann and colleagues[103] pointing to a pro-invasive rather than suppressor role for endoglin in breast cancer. This latter report shows a direct correlation between enhanced endoglin expression and the metastatic abilities of MDA-MB-231 derived cell lines; however, no study on primary tumors is presented. A summary of endoglin alterations found in sporadic human cancer and their correlation with tumor progression is presented in Table 2.

Endoglin expression has also been found by indirect immunofluorescence in hematopoietic malignancies; i.e., 47% of acute myelogenous leukemias (AML), 82% of B-cell acute lymphoblastic leukemias (B-ALL) and 7% of T-cell ALL (T-ALL). Apparently, endoglin is always expressed by the most immature subtypes of acute leukemias and absent in more differentiated ones[73].

On the other hand, a study in a panel of human tumor cell lines showed that endoglin expression is low in carcinoma cells while it is high in sarcoma cells. Interestingly, in some carcinoma, melanoma and osteosarcoma cell lines the subcellular

location of endoglin was the cytoplasm rather than the plasma membrane[104]. The functional significance of this observation remains to be investigated.

Endoglin modulates cell migration and invasiveness

It is clear from the above mentioned studies that endoglin modulates adhesion and migration of cancer cells. This regulatory function of endoglin was also described in different types of nontumorigenic cells, including myoblasts, fibroblasts and endothelial cells[105-110]. Regulation of cell migration by endoglin seems to involve TGF- β -dependent as well as TGF- β -independent mechanisms. Thus, it has been reported that endoglin reduces migration of prostate carcinoma cells by activating the T β RI ALK2, which in turn activates Smad1[111]. Moreover, in prostate carcinoma cells expressing low levels of endoglin, the soy isoflavone genistein inhibited migration and invasiveness by activating ALK2/Smad1, which suggests a high therapeutic benefit for patients with prostate carcinomas expressing low levels of endoglin by treating them with genistein[112]. A recent study suggests, however, that ALK2-mediated phosphorylation of the endoglin cytoplasmic tail also contributes to prostate cancer cell migration through a Smad-independent mechanism[113].

Endoglin is thought to regulate endothelial cell migration by modulating the balance between the ALK5/Smad2/3 and ALK1/Smad1/5/8 TGF- β pathways (see above). Blobe and colleagues, on the other hand, found that by associating with the scaffolding protein GAIP-interacting protein, C-terminus (GIPC) through the cytoplasmic PDZ binding motif, endoglin stimulates TGF- β -induced Smad1/5/8 signaling and inhibits endothelial cell migration[109]. The same laboratory found that endoglin decreases endothelial cell migration by antagonizing TGF- β -mediated extracellular signal regulated kinase (ERK) activation in a manner dependent on its

ability to interact with β -arrestin2, which results in the internalization of endoglin and β -arrestin2 in endocytic vesicles[108]. In addition, a recent report point to ALK5-mediated endoglin phosphorylation on serines 646 and 649 present in the cytoplasmic tail as an important mechanism for regulating TGF- β 1/Smad1/5/8 signaling and migration of endothelial cells[110].

Other studies aimed to identify intracellular proteins interacting with the endoglin cytoplasmic domain have reported zyxin and zyxin-related protein 1 (see Table 1), two components of the cytoskeleton located at focal adhesions, as the first cytosolic proteins that bind endoglin[106,107]. Through these protein-protein interactions that could be regulated by phosphorylation[21], endoglin may modulate the organization of the actin cytoskeleton and the adhesive properties of the cells by a TGF- β -independent mechanism. The cytoplasmic domain of endoglin (as well as that of T β RII) also binds Tctex2b, a novel t-complex testis-expressed protein 1/2 (Tctex1/2)-like protein, which is a member of the dynein light chain protein family involved in the transport of cargo, in a retrograde fashion, along the microtubules towards the nucleus[114].

Soluble endoglin in cancer and other diseases

The extracellular domain of membrane-bound endoglin can be proteolytically cleaved releasing a circulating form of endoglin, named soluble endoglin (Sol-Eng). Increased levels of Sol-Eng are linked to the pathogenesis of severe vascular diseases, including systemic sclerosis, a condition characterized by early generalized microangiopathy with disturbed angiogenesis[115], sporadic brain arteriovenous malformations[116], and the pregnancy-specific hypertensive syndrome known as preeclampsia[117-119]. High circulating Sol-Eng levels have also been associated with

attenuation of the anti-inflammatory response in patients with plasmodium falciparum malaria[120], and with poor clinical outcome of biliary atresia patients[121]. Biliary atresia is a chronic progressive inflammatory disorder of the extrahepatic and intrahepatic biliary system that commonly occurs in children.

Also, a number of laboratories have found elevated levels of Sol-Eng in body fluids from cancer patients with respect to healthy donors (Table 2 and Fig. 3). Increased Sol-Eng levels correlate with metastasis in breast cancer and colorectal cancer[122,123]. Sol-Eng levels were decreased in patients receiving chemotherapy, which reduces the utility of Sol-Eng as a prognostic marker to the long-term follow-up of cancer survivors who are not treated with chemotherapy[123]. Elevated levels of plasma Sol-Eng also predict decreased response and survival to hormone therapy of women with metastatic breast cancer[124]. In prostate cancer, high levels of serum Sol-Eng correlate with advanced stage of tumor progression, and urinary Sol-Eng seems to be a useful marker for diagnosis[125]. In addition, recent reports have shown that determination of plasma Sol-Eng in prostate cancer has a predictive value for metastasis to the pelvic lymph nodes[126] as well as for increased risk of prostate-specific antigen (PSA) recurrence in patients treated with radical prostatectomy and bilateral pelvic lymphadenectomy[127]. High levels of Sol-Eng are also present in myeloid malignancies that are characterized by a high cellular proliferation rate, such as acute myeloid leukaemia and chronic myeloproliferative disorders[128]. Nonetheless, other reports on gastric, esophageal and ovarian tumors failed to find Sol-Eng as a valuable marker in the assessment of cancer spread[129-133].

Sol-Eng contains the binding site for different members of the TGF- β superfamily, and may act as a scavenger of circulating ligands preventing their binding to the functional receptors. Thus, Sol-Eng impairs TGF- β signaling interfering with

vascular function and inhibiting angiogenesis[117,134]. An intriguing question is the source of Sol-Eng detected in cancer patients. Since endoglin levels are higher in tumor vessels than in tumor cells, it is thought that Sol-Eng derives from endoglin shedding in vascular endothelial cells and that it may represent a surrogate marker of angiogenic activity[135]. However, our studies in an experimental model of carcinogenesis (see below) suggest that endoglin shedding in tumor cells can also contribute to increase the levels of Sol-Eng (Fig. 3).

On the other hand, the correlation between elevated Sol-Eng levels and poor clinical outcome of cancer patients is paradoxical, as Sol-Eng seems to be an anti-angiogenic agent. In this respect, recent epidemiological studies report that formerly preeclamptic women have a reduced incidence of breast cancer. The mechanism underlying this observation is unknown, but as preeclampsia is linked to an imbalance between pro-angiogenic (such as VEGF) and anti-angiogenic factors, such as Sol-Eng and soluble fms-like tyrosine kinase-1 (sFlt-1), persistent mild elevations of anti-angiogenic Sol-Eng and sFlt-1 may contribute to the inhibition of tumor development later in life[136].

Recently, it has been reported that the membrane-type matrix metalloproteinase-14 (MMP14, also called MT1-MMP) is involved in Sol-Eng production[134]. This study shows that efficient endoglin shedding requires a direct interaction of endoglin and MMP14, suggesting that the origin of Sol-Eng in cancer resides in those sites where endoglin and MMP14 are colocalized (Fig. 3).

ENDOGLIN IN EXPERIMENTAL CARCINOGENESIS

The two-stage mouse skin carcinogenesis model

Two-stage mouse skin chemical carcinogenesis has become one of the best established *in vivo* models to study the sequential development of tumors[137-139]. Concepts such as tumor initiation, promotion and progression emerged from this model[140], that has been classically utilized in environmental cancer studies to test chemicals that may cause, prevent or cure cancer[141]. Nowadays, the mouse skin chemical carcinogenesis system is also used to evaluate the impact of genetic background and genetic manipulation on tumorigenesis. Tumor induction in two-stage carcinogenesis involves a single sub-carcinogenic dose of a carcinogen initiator, such as 7,12-dimethylbenz(a)anthracene (DMBA). This event alone does not give rise to tumors unless followed by repeated application of a tumor promoter, such as 12-O-tetradecanoylphorbol-13-acetate (TPA). This protocol gives rise to the appearance of multiple benign papillomas representing clonal outgrowths of epidermal keratinocytes with initiating mutations in the *Hras1* gene[142]. Papillomas can progress to malignant SCCs; however, there is evidence that papillomas are heterogeneous in their potential for malignant conversion. Thus, two entities named as low-risk and high-risk benign papillomas with differing rates of premalignant progression have been defined[143]. SCCs are highly vascularized inward invasive lesions that can be classified into well differentiated (grade I), moderately differentiated (grade II) and poorly differentiated (grade III/IV) according to the degree of squamous differentiation[144]. This latter group includes the highly aggressive spindle cell carcinoma (SpCC) type of tumor formed by elongated or spindle-shaped cells that have elicited an irreversible epithelial-mesenchymal transition[145] (Fig. 4). Progression from papillomas to SCCs is associated with trisomies of chromosomes 6 and 7 (the *Hras1* gene is located on chromosome 7), as well as inactivating mutations in *Trp53* and increasing aneuploidy and dysplasia[137,145]. The SCC-SpCC transition correlates with increased ratio of

oncogenic versus normal *Hras1* expression and loss or misregulation of the *Ink4* locus encoding the cell cycle inhibitors p16Ink4a, p15Ink4b and p19Arf[145]. Interestingly, p15Ink4b mediates the growth inhibitory response induced by TGF- β 1 in epithelial cells[8], and SpCC cells do not respond to TGF- β -induced growth inhibition.

TGF- β is an important regulator of epidermal homeostasis. TGF- β was documented to inhibit proliferation but promote differentiation of keratinocytes, suggesting that it primarily acts as a tumor suppressor in skin carcinogenesis[146]. Indeed, a biphasic role for TGF- β 1 as a suppressor and a promoter of carcinogenesis (see Fig. 4) was demonstrated in transgenic mice overexpressing the growth factor in differentiating epidermal keratinocytes[147]. TGF- β 1 is thought to inhibit proliferation and induce apoptosis during initiation and early stages of carcinogenesis. At later stages, elevated levels of TGF- β 1 stimulate malignant progression by promoting tumor cell migration, invasion and metastasis in a cell-autonomous manner, and by influencing the tumor microenvironment facilitating angiogenesis and evasion of the immune system[9,10]. TGF- β 1 seems to be the physiological agent involved in pushing the SCC-SpCC transition during mouse skin carcinogenesis, likely in cooperation with the *Hras1* oncogene[145].

Endoglin studies on mouse skin carcinogenesis

When *Eng*^{+/-} heterozygous mice were subjected to two-stage skin carcinogenesis with DMBA and TPA, these animals developed a lower number of tumors than control *Eng*^{+/+} mice, but the frequency of papilloma-carcinoma conversion and the incidence of poorly differentiated SCCs was vastly increased[22,148]. Accelerated malignant progression in *Eng*^{+/-} mice occurred in adverse conditions, as these animals exhibit a deficient angiogenic switch[41]. The carcinogenic phenotype of *Eng*^{+/-} mice was

identical to that of mice with targeted expression of TGF- β 1 to the epidermis[147], mirroring the dual role of TGF- β 1 in carcinogenesis as a tumor suppressor and promoter. These results suggested that endoglin contributes to epidermal homeostasis by attenuating the growth inhibitory response elicited by TGF- β in basal keratinocytes, which is the compartment expressing endoglin in normal epidermis[148]. This hypothesis was confirmed by demonstrating that indeed endoglin inhibits the TGF- β /ALK5/Smad2/3 signaling pathway in keratinocytes[149]. In addition, the tumor phenotype of *Eng*^{+/-} mice pointed to a role for endoglin as a suppressor of malignancy at late stages of skin carcinogenesis.

The analysis of endoglin expression in chemically-induced mouse skin tumors and cell lines revealed a novel mechanism for inactivation of endoglin in tumor cells by shedding. The truncation of membrane-associated endoglin releasing Sol-Eng into the stroma and blood stream was associated with the SCC-SpCC transition[149] (Fig. 4). Endoglin expression studies also showed that the S-endoglin isoform is neither expressed in normal skin nor tumors and consequently does not play any role in skin carcinogenesis.

The functional consequences of inactivating membrane endoglin in tumor cells were approached by using short hairpin RNA (shRNA) interfering technology. Downregulation of endoglin expression in cultured SCC cells induced the constitutive activation of the ALK5/Smad2/3 signaling pathway independently of ligand binding leading to EMT and a SCC-SpCC conversion in vivo[149]. In this respect, it should be mentioned that a role for endoglin in embryonic EMT during cardiac valve formation was also simultaneously reported[150]. These results unequivocally demonstrated that endoglin acts as a suppressor of malignancy during mouse skin carcinogenesis. Likewise, since endoglin knockdown in SCC cells was also linked to reduced cell

growth and delayed tumor latencies, we postulated that inactivation of membrane endoglin in tumor cells exerts a strong selective pressure during SCC-SpCC progression by priming cells that have lost the TGF- β anti-proliferative response. This could be the reason why loss of the TGF- β growth inhibitory response co-segregates with the spindle tumor phenotype[151].

Recent results suggest a novel regulatory function for endoglin on mouse skin carcinogenesis. TGF- β 1 stimulates *Hras1* gene expression in transformed keratinocytes by a Smad-independent mechanism involving the Ras/ERK signaling pathway. Interestingly, endoglin not only inhibits basal and TGF- β -mediated ERK signalling activity but protect cells from *Hras* oncogene transformation (Santibáñez et al., manuscript submitted for publication). This finding unveil a new aspect for the suppressor role of endoglin during mouse skin carcinogenesis, a model in which *Hras1* activation drives not only tumor initiation but also malignant progression[138,145].

CONCLUSIONS AND FUTURE DIRECTIONS

Endoglin by virtue of its pro-angiogenic activity has become a well established marker to analyze angiogenesis and MVD in tumors. It also has revealed its potential as a target for cancer imaging and anti-angiogenic therapy. New insights have emerged recently on the role of endoglin as a tumor suppressor during progression to malignancy. However, a greater effort is needed to unravel the clinical significance of endoglin expression in human primary tumors by studying larger cohorts of patients and using a better classification of tumors. Whereas some reports suggest that the basis for the involvement of endoglin in tumor cell migration and invasiveness resides on its ability to modulate TGF- β signaling, in most cases the precise mechanism for which

endoglin regulate these processes is unknown. In particular, studies on the interaction of endoglin with components of the cytoskeleton and on its regulation of TGF- β -independent cell responses should be continued.

The assigned role of endoglin as a suppressor of malignancy in epithelial cancer raises important questions for HHT patients, which inherit an inactivated *ENG* allele by the germline. The studies on *Eng*^{+/-} mice predict that HHT1 patients might be protected for tumor formation, but tumors arising in these individuals will be prone to progress rapidly to malignant carcinomas. Nonetheless, epidemiological data for cancer incidence in HHT1 patients is lacking at the present time.

Further investigations are also required to solve the contradiction of Sol-Eng being a bad prognostic factor in some types of human cancer while having an anti-angiogenic function in preeclampsia and other vascular diseases. An explanation for this paradox might be that production of Sol-Eng is a late event during carcinogenesis (see Fig. 4) and, therefore, its anti-angiogenic activity may be irrelevant for tumor growth at this stage. Nevertheless, it should be of interest to study the effects of Sol-Eng on tumor cell behaviour and on the competence of stromal cells (cancer-associated fibroblasts or CAFs, inflammatory cells) to sustain tumor growth (Fig. 3). On the other hand, there are no studies addressing the role of membrane-associated endoglin expressed in the stromal compartment. In particular, whether endoglin regulates the activity of CAFs to promote tumor growth and progression should be investigated in the next years.

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TABLE 1
Endoglin interacting proteins

Endoglin domain	TGF-β superfamily ligands	TβRI	TβRII	Other proteins
Extracellular domain	TGF- β 1	ALK1	T β RII	
	TGF- β 3	ALK2	ActRII	
	activin A	ALK3	BMPRII	
	BMP-2	ALK5		
	BMP-7	ALK6		
Cytoplasmic domain	BMP-9			
		ALK1		Zyxin
		ALK5		ZRP-1
				β -arrestin2
				Tctex2 β

TABLE 2
Changes in endoglin expression found in sporadic human cancer

Tumor type	Endoglin expression	Biological source	Correlation with/ comments	References
Leukemia	High levels of Sol-Eng	Serum	AML, CML	[128]
CRC	High levels of Sol-Eng	Serum	Metastasis	[123]
Breast carcinoma	High levels of Sol-Eng	Serum/plasma	Metastasis/reduced clinical benefit rate to hormone therapy	[122-124]
	Downregulated	Primary tumors	Poor clinical outcome	[102]
	Upregulated	MDA-MB-231 derived cell lines	Metastasis	[103]
Prostate carcinoma	Positive in PIN and cancer cells	Primary tumors	No correlation with clinical parameters	[95]
	High levels of Sol-Eng	Plasma	Metastasis/PSA recurrence after radical prostatectomy	[126,127]
		Serum/urine	Tumor progression/higher tumor volume	[125]
	Downregulated	Cell lines	Metastasis	[96]
Esophageal SCC	Downregulated	Primary tumors and cell lines	Tumor progression	[98]

AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; CRC, colorectal cancer; Sol-Eng, soluble endoglin; PIN, prostatic intraepithelial neoplasia

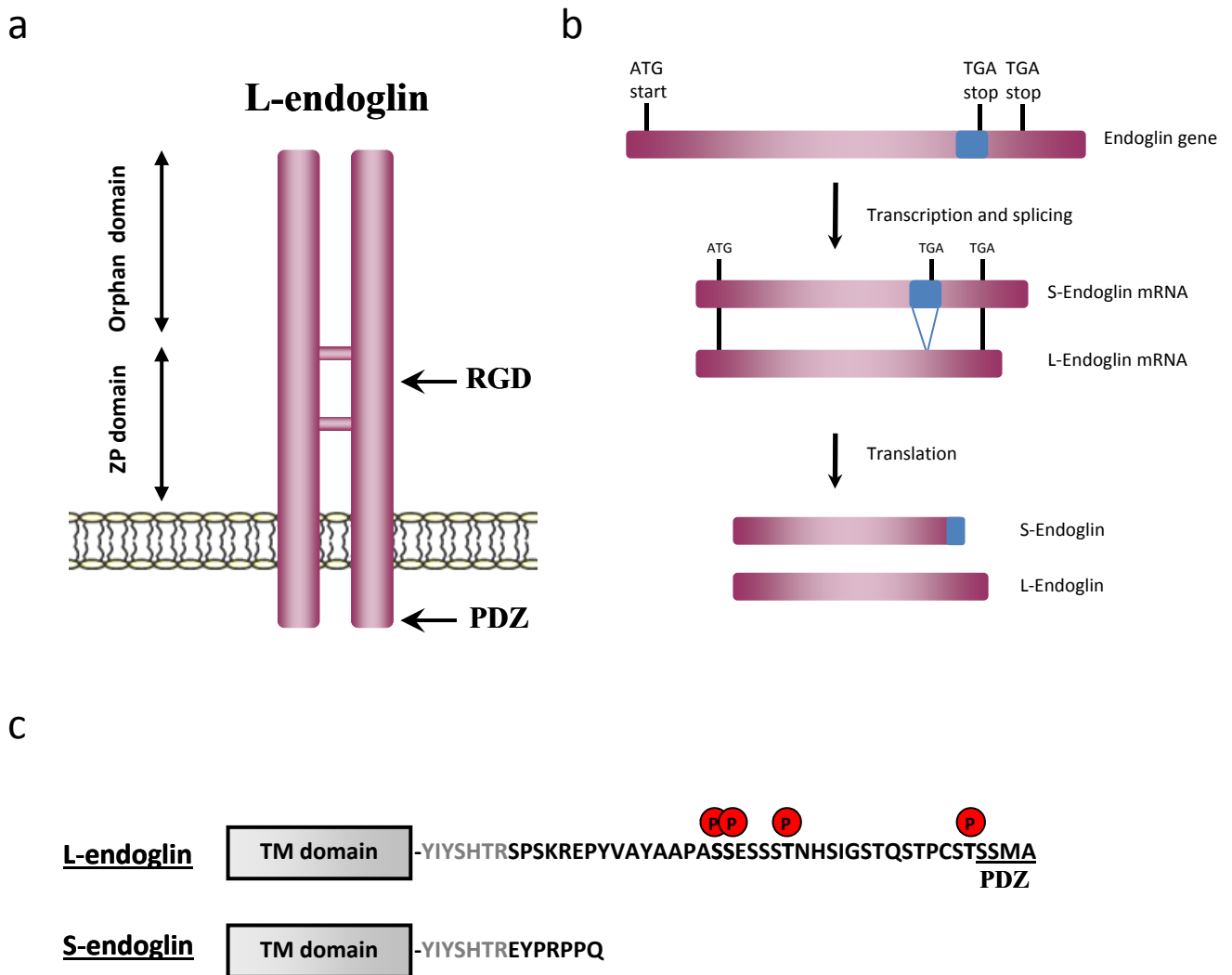


FIGURE 1. Endoglin structure and isoforms. (a) Schematic representation of endoglin structure. The location of the integrin-binding RGD motif and PDZ-binding motif are indicated. ZP, zona pellucida (b) Diagram representing the alternate splicing mechanism originating L- and S-endoglin. The intron located between exons 14 and 15 (in blue), which contains a TAG stop codon, is transcribed into the S-endoglin mRNA, but is spliced out to yield the L-endoglin transcript. (c) Comparison of human L- and S- endoglin cytoplasmic domains. Identical amino acids are in grey. L-endoglin is phosphorylated at Ser/Thr residues by T β R2, ALK1 and ALK5. TM, transmembrane.

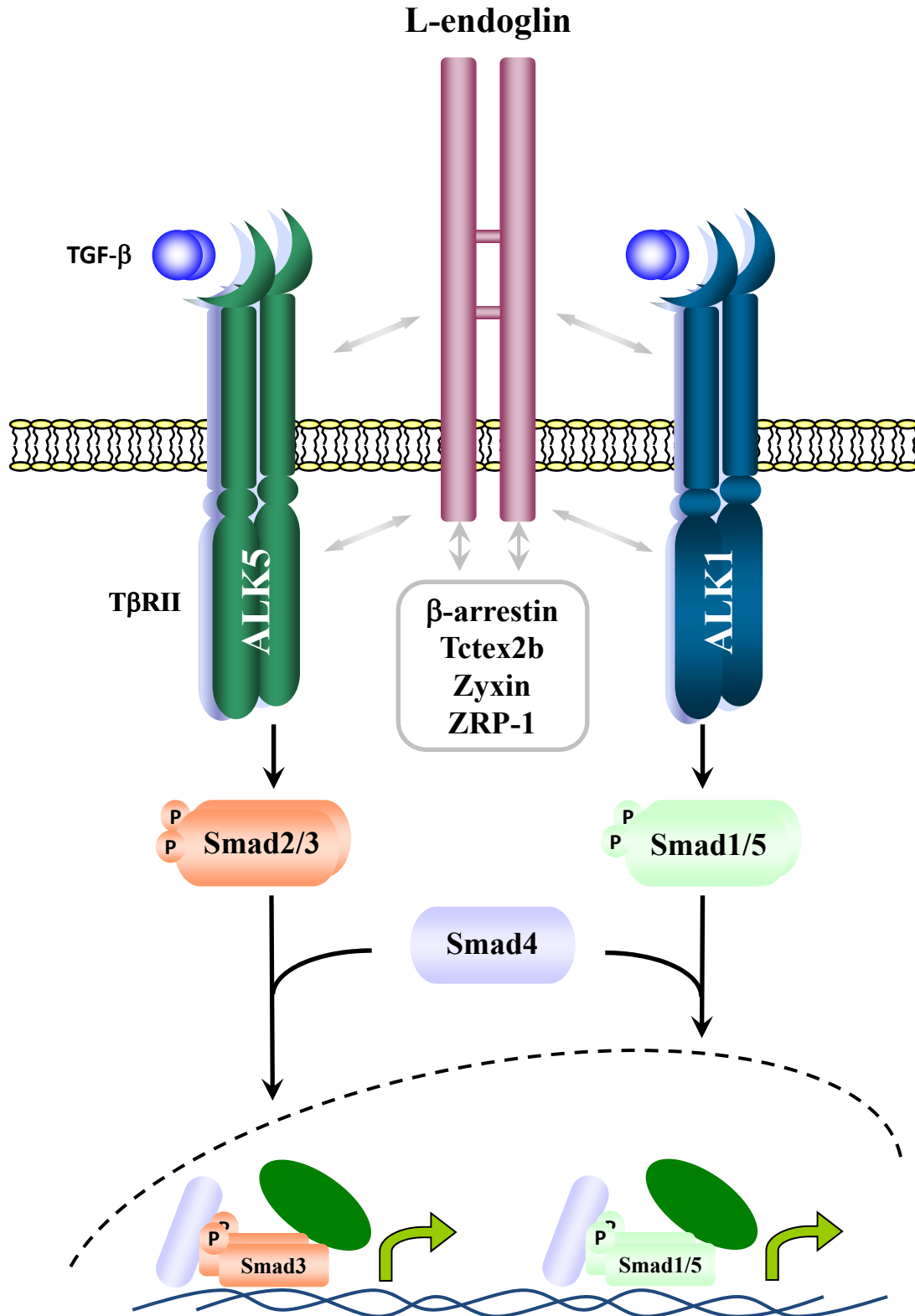


FIGURE 2. Hypothetical model for endoglin regulation of TGF- β dependent and independent cell responses. Endoglin modulates TGF- β signaling by interacting with T β RI (ALK5 and ALK1) and T β RII through its extracellular and cytoplasmic domains. The endoglin cytoplasmic domain interacts with β -arrestin, Tctex2b, zyxin and ZRP-1 proteins. Through these interactions endoglin could mediate F-actin rearrangements, cell adhesion and migration, as well as protein transport via endocytic vesicles.

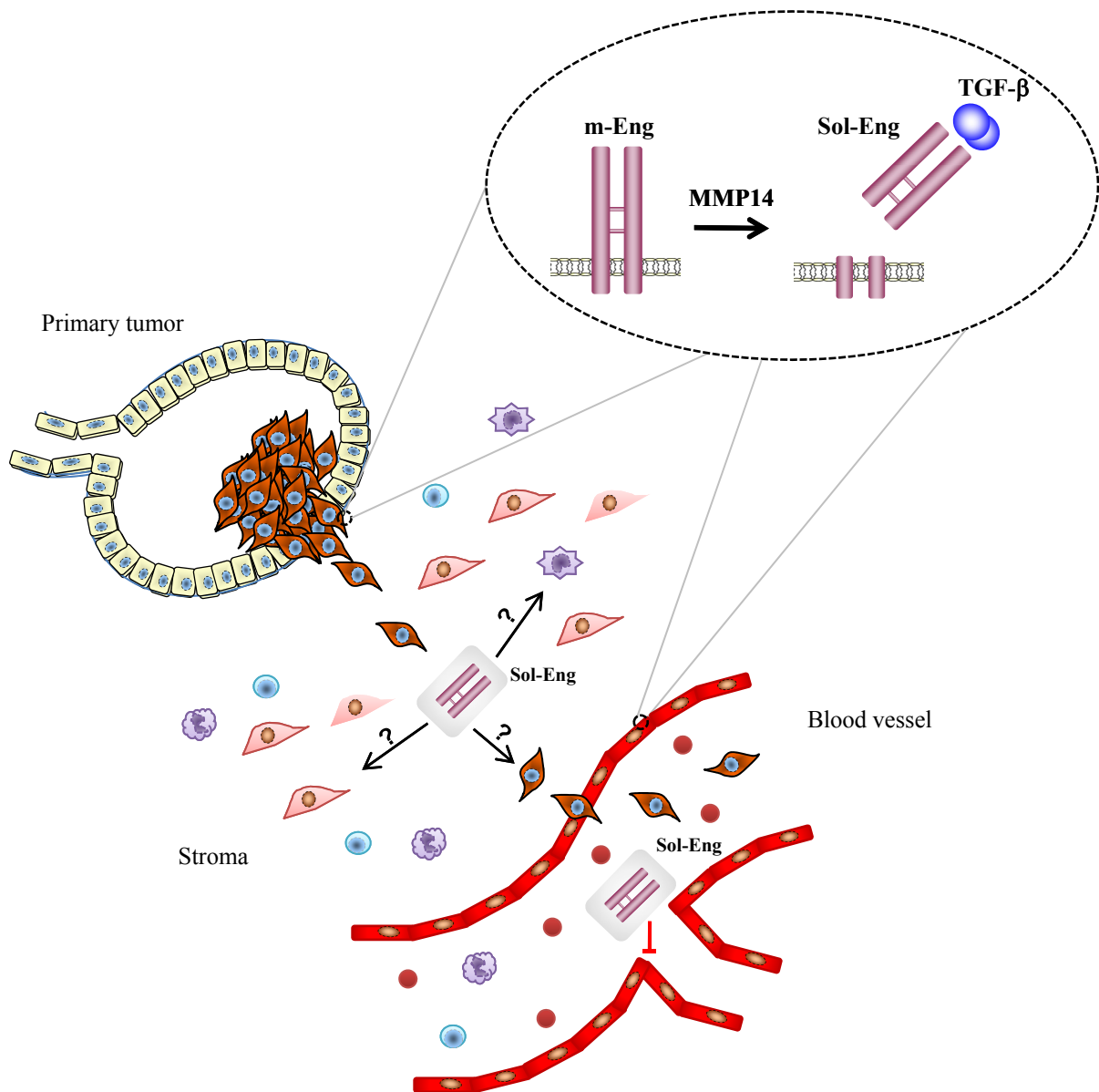


FIGURE 3. Generation of soluble endoglin in tumors and its possible actions. The proteolytic cleavage of membrane endoglin (m-Eng) to release soluble endoglin (Sol-Eng) can occur in the primary tumor as well as in the stroma and blood vessels. It has been shown that Sol-Eng inhibits angiogenesis, but its effects on the behavior of tumor cells, cancer-associated fibroblasts (pink) and inflammatory cells (blue and purple) have not been tested yet.

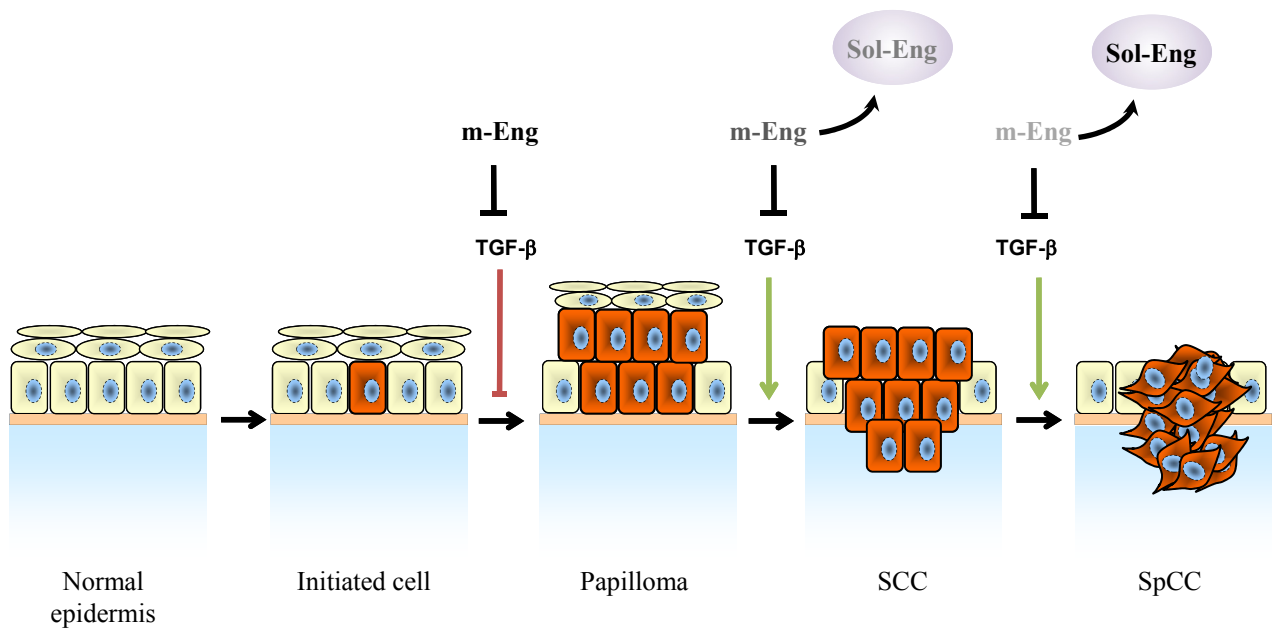


FIGURE 4. Schematic representation of the different stages of chemical mouse skin carcinogenesis. The keratinocyte in the epidermis colored in brown represents a cell with *Hras1* mutation initiated by DMBA. Mutated *Hras1* gene expression increases during tumor progression. TGF- β 1 behaves as a tumor suppressor at early stages of carcinogenesis, but promotes tumor progression at later stages. Proteolysis of m-Eng to produce Sol-Eng mainly occurs during the transition from squamous cell carcinomas (SCCs) to spindle cell carcinomas (SpCCs). m-Eng which inhibits TGF- β signaling in keratinocytes behaves as a suppressor of malignancy at later stages of carcinogenesis.