From Department of Medical Biochemistry and Biophysics
Karolinska Institutet, Stockholm, Sweden

EXPLORING THE POTENTIAL OF ANTIANGIOGENIC STRATEGIES TARGETING THE TGF-β FAMILY IN THE TUMOR MICROENVIRONMENT

Sara Cunha

Karolinska Institutet

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Parts of the Introduction will be printed as a chapter in the Springer book entitled "TGF-β and human disease"

To my Dad... I wish you could be still here

ABSTRACT

Members of the transforming growth factor (TGF)-β family exert their effect on virtually all cell types in the body, producing diverse and complex cellular responses. Additionally, TGF-β signaling is deregulated and hyperactive in many malignant conditions, making it an appealing target in the combat of cancer disease. The predominantly endothelial TGF-β receptors, ALK1 and endoglin, which are activated during neoangiogenesis both during development and pathological conditions, pose attractive modulating opportunities to impair tumor vessel formation and cancer progression. However, the precise function of TGF-β family signaling in endothelial cells is difficult to predict, as it appears highly context dependent due to a myriad of ligands and receptors influencing the final outcome. Furthermore, TGF-β is involved in autocrine and intricate dynamic paracrine signaling events in the context of the tumor microenvironment. Pharmacological inhibitors for ALK1 and endoglin have been developed and will facilitate more comprehensive studies on the exact function of the TGF-β family in the tumor-associated endothelium. Here, we summarize the current knowledge on TGF-β signaling in the regulation of the vascular network as alternative targets to VEGF and incorporate our novel and promising findings in the field. Our two studies aiming at dissecting the independent role of ALK1 and endoglin resulted in very distinctive outcomes. While ALK1 suppression results in sustained tumor growth by affecting the tumor neovasculature, endoglin impairment generates a weakened and increasingly lenient vasculature to the passage of malignant cells to and from the bloodstream. Our both seemingly contradictory studies challenge the current view of a strong interconnection between ALK1 and endoglin in the vasculature.

The ongoing clinical trials of inhibitors affecting ALK1 and endoglin involvement in vascular formation during malignant progression will further clarify the valuable potential of targeting alternative pathways to VEGF.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their roman numerals:

I. **Cunha, S.I.,** Pardali, E., Thorikay, M., Anderberg, C., Hawinkels, L., Goumans, M.J., Seehra, J., Heldin, C.H, ten Dijke, P., Pietras, K. (2010). Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. J Exp. Med. Jan. 18; 207(1), 85-100

II. Anderberg, C.*, **Cunha, S.I.*,** Zhai, Z.*, Cortez, E., Pardali, E., Johnson, J., Franco, M., Páez-Ribes, M., Cordiner, R., Fuxe, J., Johansson, B.R., Goumans, M.J., Casanovas, O., ten Dijke, P., Arthur, H.M., Pietras, K.. Deficiency for endoglin in tumor vasculature weakens the endothelial barrier to metastatic dissemination
* These authors contributed equally to this study and are listed by alphabetical order
Manuscript (resubmitted and under revision in Journal of Experimental Medicine)

Other publications not included in this thesis:

Cunha S.I. & Pietras K. ALK1 as an emerging target for antiangiogenic therapy in cancer. Blood. 2011 Jun 30;117(26):6999-7006.

LIST OF ABREVIATIONS

TGF-β	Transforming growth factor β
BMP	Bone morphogenetic protein
GDF	Growth and differentiation factor
ALK	Activin receptor-like linase
FITC	Fluorescein isothiocyanate
HHT	Human hemorrhagic telangiectasia
Id	Inhibitor of DNA binding
PAI-1	Plasminogen activator inhibitor type 1
PDGF	Platelet-derived growth factor
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
LLC	Lewis lung carcinoma
αSMA	Alpha-smooth muscle actin
PECAM1	Platelet endothelial cell adhesion molecule
EMT	Epithelial to mesenchymal transition
EndMT	Endothelial to mesenchymal transition
ECM	Extracellular matrix
IFP	Interstitial fluid pressure
CNS	Central nervous system
PNETs	Pancreatic neuroendocrine tumors
FDA	Food and drug administration
EC	Endothelial cell
GI	Gastrointestinal
HMVEC	Human dermal microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
BAEC	Bovine aortic endothelial cells
MEECs	Mouse embryonic endothelial cells
MTD	Maximum tolerated dose
CAM	Chick chorioallantoic membrane
AMD	Age-related macular degeneration
PAH	Pulmonary arterial hypertension
HNSCC	Head and neck squamous cell carcinoma
RCC	Renal cell carcinoma
CRC	Colorectal cancer
SMC	Smooth muscle cell
VSMC	Vascular smooth muscle cell
AVMs	Arteriovenous malformations

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1. INTRODUCTION

1.1. Cancer therapies targeting the tumor endothelium

The cardiovascular system is the first organ system to form and function during embryogenesis (1). The development of an efficient system to conduct nutrients and oxygen is primordial for supplying the metabolic needs of a rapidly developing embryo. The organization of the vascular tree and the timing of its genesis are highly preserved and tightly regulated among species. The relevance of such preservation is highlighted by the fact that the vascular network does not simply provide metabolic sustenance, but also supplies crucial signaling cues required to orchestrate differentiation and development of other organs during embryogenesis (2). To this respect, as the developing vasculature in the embryo is required for organogenesis, the neovasculature formation in a tumor is preponderant to the onset, maintenance and progression of neoplastic disease.

In adulthood, proangiogenic and antiangiogenic signaling molecules are in homeostatic balance (3, 4). Formation and growth of new vessels is therefore under strict control. The recruitment of the vasculature or activation of angiogenesis, a process termed the angiogenic switch, is a necessary condition for tumor development and progression in most solid tumor types (3, 4). An expanding malignant cell conglomerate, like any other tissue in the body, requires provision of oxygen and nutrients to thrive under hypoxic conditions and ultimately survive. Additionally, further recruitment of cells to the tumor microenvironment mimics to some extent the formation of a multicellular organ (5-7) (Figure 1).
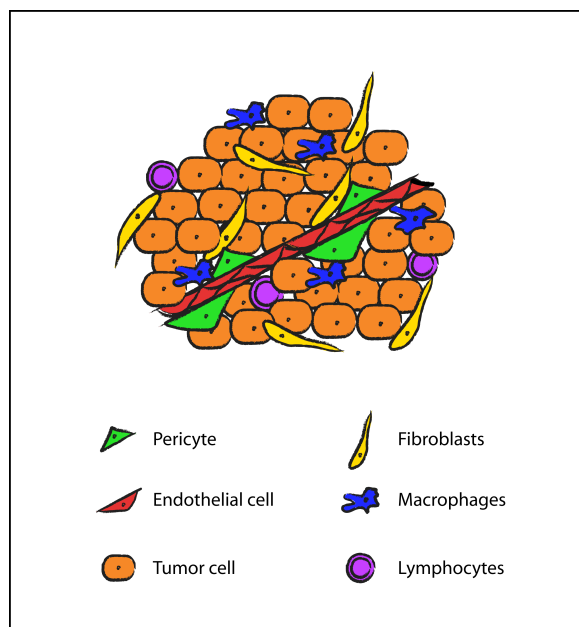

Figure 1. The tumor microenvironment as a multicellular organ with some of its classical components: aberrant tumor cells, endothelial cells, pericytes or mural cells, cancer-associated fibroblasts and immune cells, such as macrophages and lymphocytes.

The formation of new blood vessels in pathological processes, such as cancer, leads to unstable, proliferative and sub-optimally functional vessels in a dynamic state of remodelling (5). Tumor vascular networks support tumor growth, in spite of a range of abnormal features that compromise their physiology. Tumor vessels are commonly tortuous and leaky, causing hemorrhage and increased interstitial fluid pressure (5, 8-10). Inefficient blood flow caused by poor hierarchical anatomy and organization of the tumor vasculature leads to ischemia and necrosis, which are common characteristics of rapidly growing tumors.

These underdeveloped properties of tumor vessels would presumably contribute to increased sensitivity and efficacy of antiangiogenic drugs (11). On one hand, the tumors' critical dependency on a functional actively growing vasculature to support its malignant progression and tumor cell dissemination, and on the other hand, tumor cell instability and aberrant physiology, strongly highlights tumor vasculature targeting approaches as attractive alternative strategies for cancer management. Aiming at a component of the tumor distinct to that contemplated by conventional cytotoxic drugs, further offers the possibility of significant complimentary antitumor efficacy.

Based on several successful preclinical studies, the oncologic clinical field held high expectations on the use of antiangiogenic therapies to combat cancer. Rooted in the belief that vascular endothelial growth factor (VEGF) and its receptors, vascular endothelial growth factor receptors (VEGFRs), played a central role in angiogenesis (12), it was expected that blocking the VEGF pathway would eradicate the tumor vasculature and cancer disease would hence be eliminated. The introduction of VEGF-targeting drugs in cancer treatment generated modest clinical benefit in multiple cancer patients rendering prolonged progression-free-survival and overall survival measured only in months, and mostly in combination with standard care chemotherapy (13). In summary, VEGF-targeting agents are failing to produce enduring clinical responses in most patients (14) even in combination with cytotoxic agents, not conveying the expected persistent cure (15-18).

Recent studies provide evidence for inhibition of primary tumor growth by using VEGF-inhibiting strategies; however they lead to a decrease in mouse survival due to increased invasiveness and metastasis dissemination (19-21). These seemingly paradoxical results may help understand why the clinical efficacy of these drugs is comparatively poor in patients. However, a number of questions were raised based on these new observations. The main concern in the field regards the legitimacy of using VEGF-targeting drugs if they accelerate the disease progression rate into metastatic late stage disease, with metastasis being the eventual cause of death for most cancer patients (22).

The development of resistance and acquisition of a more aggressive and invasive phenotype in tumors that have been treated with VEGF neutralizing approaches thus points towards the need for additional extensive examination of the biology of tumor-nurturing blood vessels, in order to more accurately pinpoint promising and novel antiangiogenic targets. It is though expected that curing cancer is a complex task that requires a combined effort of utilizing various strategic angles targeting different tumor cellular compartments, in addition to aiming at the ample spectrum of signaling networks that are commonly deregulated in malignant cells.

Often altered in neoplasms and involved in multiple cellular functions, the large TGF-β family is, as a result, an obvious candidate for directing such efforts. More specifically,

because TGF-β signaling encompasses a number of vascular restricted receptors, they represent attractive antiangiogenic therapeutic targets in cancer.

1.2. The TGF-β-rich tumor microenvironment

The tumor microenvironment, or stroma, influences the growth of the tumor and its ability to progress and metastasize. The stroma thus constitutes an important aspect to consider when developing therapeutic approaches, as it can change interstitial fluid pressure, limit the access of therapeutics to the tumor, alter drug metabolism or even contribute to the development of drug resistance (7).

Despite the importance of tumor-host stroma interactions, there is limited understanding of the stromal milieu composition, and the complexity and dynamics in the relationship between the tumor malignant cells and the surrounding host cells (23-25). It is, however, nowadays acknowledged that tumor cells and their stroma co-evolve during tumorigenesis and progression (24). Nonetheless, the precise nature of the cells that comprise the normal and tumor stromas, how the cells within the stroma or newly recruited cells are altered during tumor progression, and how they reciprocally exert influence on tumor initiation and progression is still poorly understood (24). Interestingly, stromal elements seem to play a more pronounced supportive role in late stages of cancer disease (7).

The large family of TGF-β extracellular pleiotropic cytokines exerts influence essentially on all cellular strata in the body, namely in epithelial cells, fibroblasts, immune, endothelial, lymphatic and perivascular cells (26). TGF-β is the prototypical element of an extensive ligand group that also includes bone morphogenetic proteins (BMPs), activins, inhibins, Nodal and growth and differentiation factors (GDFs) that elicit signaling activity through a collection of five type II receptors (TGF-βRII, BMPRII, ActRIIa, ActRIIb, AMHRII) and seven type I serine/threonine kinase receptors (ALK1-7) (27). Different ligand-receptor II-receptor I combinations can be assembled, delineating one of the TGF-β family hallmarks of complexity.

In normal, unstressed tissue, sustained basal release of TGF-β by local sources regulates homeostasis. In pathological conditions, TGF-β is abundantly released in the tumor microenvironment, initially as a signal to avoid premalignant progression, but eventually as a cue that cancer cells utilize to their own advantage in later stages of malignancy (28, 29). Most human tumors overproduce TGF-β whose autocrine and paracrine actions promote tumor epithelial cell invasiveness and metastasis, functioning as a differentiation switch required for transient but reversible invasiveness of carcinoma cells through epithelial-to-mesenchymal transition (EMT) mechanisms (30). In addition to eliciting mitogenic signals towards carcinoma cells, TGF-β also affects cancer-associated fibroblast-induction of tumor matrix remodeling and regulates angiogenesis by acting on endothelial cells (ECs) and pericytes (Figure 2). Apart from the preexisting classical stromal components, tumors are also infiltrated by a myriad of cell types: leukocytes, macrophages, and bone marrow-derived endothelial, mesenchymal, and myeloid precursor cells (31). Finally, TGF-β suppresses proliferation and differentiation of lymphocytes including T cells and natural killer cells, thus preventing immune surveillance control over the developing tumor.
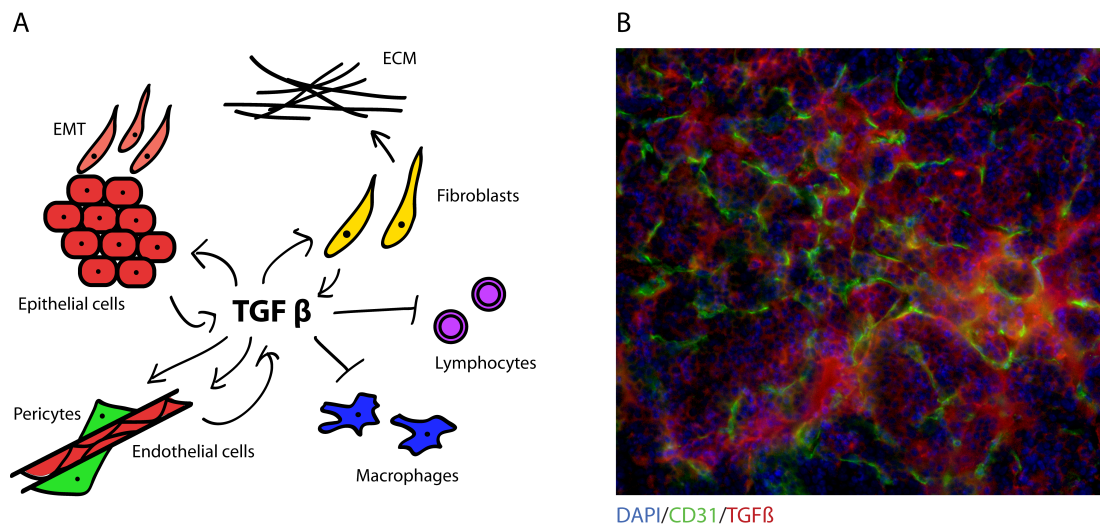
Figure 2. TGF-β in the tumor microenvironment. (A) Illustration of TGF-β affecting multiple cell types in the tumor microenvironment. TGF-β promotes increased epithelial invasiveness triggering, in an autocrine and paracrine fashion, a cell plasticity program called EMT. Tumor cells that have undergone EMT can therefore intravasate into the bloodstream and migrate to distant sites of metastasis. TGF-β also affects tumor extracellular matrix (ECM) remodeling by cancer-associated fibroblasts (CAFs) and regulates angiogenesis by acting on endothelial and mural cells. Tumor infiltration by leukocytes, macrophages, and bone marrow-derived endothelial, mesenchymal, and myeloid precursor cells is also mediated by TGF-β. TGF-β, as an immune suppressor, prevents immune surveillance control over the developing tumor by inhibiting proliferation and differentiation of lymphocytes including T cells and natural killer cells. (B) TGF-β abundant expression in a Rip1-Tag2 tumor (red) by the tumor cells (blue), especially in the vicinity of vessels (green).

In a few words, TGF-β signaling is intimately implicated in tumor development and contributes to all hallmarks of cancer described by Hanahan & Weinberg (32-34). It is, thus, of vital importance to carefully analyze the role of the TGF-β family members in the tumor microenvironment and how its signaling circuits arising from tumor and stromal interactions can be efficiently modulated in cancer therapy.

1.3. Tumor angiogenesis: seeking alternative pathway targets

It is well established that in order for a tumor to grow beyond a certain size, it requires the recruitment of its own blood supply for provision of oxygen and nutrients (4). The conceptual idea of the angiogenic phenomenon as a target in cancer therapy, originally coined by Philippe Shubi (35, 36) and further developed by Folkman (37), has been extensively explored as an additional anticancer strategy in the last decades. Since antiangiogenic drugs aim at the non-malignant and supposedly genetically stable tumor endothelial cells (ECs), it was expected these would be inherently resilient to resistance acquisition (38, 39).

Unfortunately, clinical benefit arising from conventional antiangiogenic therapies, aimed at VEGF, conveyed a modest therapeutic benefit measured in months (15-17). Some apprehension was raised by recent preclinical studies providing evidence for the development of resistance and attainment of a more aggressive and invasive phenotype in tumors that have been treated with anti-VEGF strategies (19-21).

More recently and as a result of the inability to improve overall survival in breast cancer patients with metastatic disease, the United States Food and Drug

Administration (FDA) recently revoked the approval of the VEGF neutralizing antibody, bevacizumab for that indication (40).

The emergence of resistance mechanisms towards VEGF-targeting therapies has been under heated debate. While still in their early stages, preclinical studies strongly suggest caution on the indiscriminate use of antiangiogenic agents, as tumors have been described to become refractory to these drugs after prolonged exposure (14, 19-21, 41).

In short, the angiogenesis field claims for novel antiangiogenic targets and combined targeted strategies aiming at various tumor cellular compartments and at alternative signaling pathways and mechanisms on how these signaling networks impinge upon the angiogenic switch.

The TGF-β signaling circuitry and dynamics may hold central roles in tumor angiogenesis, as suggested by developmental studies genetically targeting its endothelial specific receptors ALK1 and endoglin. Based on such studies, we have investigated the potential of targeting ALK1 and endoglin in tumor angiogenesis. Clinical studies using inhibitors for these receptors are already in place and show great promise in directly interfering with the formation of new tumor vessels, while leaving normal idle vasculature undisturbed.

1.3.1. Involvement of TGF-β in vascular syndromes

In vascular biology, TGF-β has traditionally been seen as a differentiation regulator for vascular smooth muscle cells (VSMCs), ultimately contributing to vessel stabilization and maturation by inducing extracellular matrix (ECM) deposition and inhibiting EC migration and proliferation (42).

The critical relevance of TGF-β signaling in vascular development was, however, recognized by identification of mutations in the endothelial TGF-β receptor genes in a familial vascular syndrome. Germline loss-of-function mutations in *Alk1* or *endoglin* are causal in the development of the human syndrome of hereditary hemorrhagic telangiectasia (HHT) (43). HHT is characterized by cutaneous telangiectases and gastrointestinal (GI) hemorrhage (34, 35). Major arteriovenous malformations (AVMs) occur in lung, liver, or brain, which may ultimately cause severe morbidity and mortality. Although *Alk1* and *endoglin* null mice die at midgestation as a result of severe AVMs (36, 37), mice lacking one copy of the gene for either *Alk1* (*Alk1 +/-*; HHT2) or *endoglin* (*Eng +/-*; HHT1) recapitulate with age the HHT patient phenotype (44, 45).

There is additional strong evidence gathered from *in vivo* studies on genetically manipulated mouse models, for a prominent role of the TGF-β pathway in vasculo- and angiogenesis mechanisms (43). In fact, deletion of TGF-β family ligands (TGF-β1, TGF-β2, BMP2, BMP4) (46-48), receptors (ALK1, ALK2, ALK3, ALK5, Endoglin, TGF-βRII, BMPRII, TGF-βRIII) (49-67), signaling mediators (Smad1, 2, 4, 5, 6, 8) (68-74), and even target genes (*Id1, Id3, Fibronectin1, Thrombospondin1*) (75, 76) results most often, in serious implications on the developing embryonic vasculature leading, ultimately, to lethality in mice (43, 46-52, 54-74, 76-82). Furthermore, many of these genes when mutated in human patients generate a series of vascular syndromes or pathological conditions other than HHT, such as aortic aneurism, pulmonary arterial hypertension (PAH), Loeys-Dietz syndrom, *fibrodysplasia ossificans progressiva* (FOP), Marfan syndrome type 2 (MFS2) and Ehlers-Danlos syndrome.

Increasing attention has been focused on the endothelial-cell specific TGF-β receptors ALK1 and endoglin. Not only do ALK1 and endoglin already have documented involvement in the human syndrome HHT but more importantly, they hold therapeutic promise in pathological conditions, such as cancer, where their upregulated expression in the vascular component indicates a critical role in cancer development.

1.3.2. TGF-β signaling in endothelial cells: ALK1/ALK5 interconnecting crosstalk

Following synthesis, secretion and activation, the mature TGF-β dimeric ligand is released from the ECM to trigger specific serine/threonine type I and type II kinase receptor heterotetrameric complexes (83). In ECs, TGF-β signaling has been described to occur via the ubiquitously and globally expressed type I receptor, ALK5 or alternatively through the predominantly endothelial type I receptor, ALK1. The prevailing recruited type I receptor dictates the activation of a particular Smad transducing cascade. ALK1 activation causes phosphorylation of Smad1, 5 and 8, whereas ALK5 leads to Smad 2 and 3 signaling activation (37). The selected activated Smad subset independently forms a heteromeric complex with a related molecule, Smad4, which translocates the complexes into the nucleus to launch transcription of specific target genes (38, 39), that are eventually involved in distinct angiogenic responses (Figure 3).
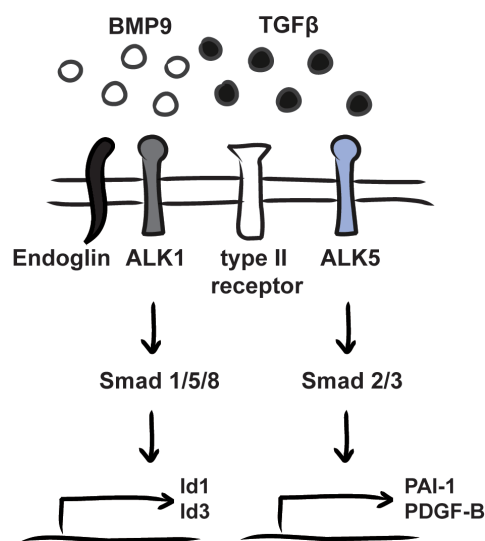
Figure 3. Schematic illustration of signaling events, triggered by the TGF-β family, in endothelial cells. TGF-β activates signaling via the EC-restricted ALK1, and the ubiquitously expressed ALK5, type I receptors, whereas BMP9 only binds ALK1. The affinity of BMP9 for ALK1 is ten-fold higher than that of TGF-β, making it likely that ALK1 predominantly interacts with BMP9 when both ligands are available. Endoglin represents a coreceptor that modulates signaling through ALK1.

TGF-β ligands also interact with coreceptors or type III receptors, represented by betaglycan (TGF-βRIII) and the predominatly endothelial endoglin. However, because these type III receptors lack the kinase domain, they essentially hold a mediation role in ligand binding and signaling activation, adding yet another level of regulation to the TGF-β complex signaling web.
Far from consensual, the TGF-β contribution to vascular biology knowledge has been

constantly under debate due to numerous paradoxical reports (84-86)

A crucial role in angiogenesis for ALK1, was first described in a study reporting ALK1 as pivotal for smooth muscle cell (SMC) recruitment, implying a vital role for the TGF-β/ALK1 signaling axis in the maturation or resolution phase of angiogenesis (49, 84). On the other hand, signaling derived from ALK5 was more pronounced during the activation phase of angiogenesis, when ECs degrade the perivascular basement membrane, invade and migrate into the newly available space, through active proliferation and lumen formation. The balance theory was then hypothesized for the first time, speculating that different levels of TGF-β ligand availability would determine the sequential angiogenic fate and control the properties of the endothelium during angiogenesis (49). In parallel studies Urness *et al.* (2000) presented evidence for development of shunts between arteries and veins and severe AVMs, due to fusion of major arteries and veins in mice lacking ALK1 (50).

The balance-working model was quickly challenged when Goumans and colleagues proposed that TGF-β engages in the activation of ALK1 signaling via Smad1/5, which concomitantly inhibits ALK5 signaling through Smad2/3 (85) ALK5, while critical for ALK1 signaling, as demonstrated by studies on ALK5 deficient mouse embryonic ECs (MEECs), commits to an antiangiogenic cascade of events, while ALK1 mediates proangiogenic activation (87) These studies indicated that TGF-β stimulatory effects on either ALK5 or ALK1 are mutually exclusive, inducing differential transcriptional activation of *Pai-1* and *Id1*, respectively, and ultimately eliciting alternative sets of physiological responses.

Motivated by the fact that both ALK1 and ALK5 null mice render an embryonic lethal phenotype due to extensive vascular abnormalities (49-51). ALK1 dependency on ALK5, by means of signaling or by mere anchoring, has been questioned and addressed with reservation by multiple laboratories.

Transcriptional profiling of human umbilical vein ECs (HUVECs) expressing constitutively active adenoviral constructs of ALK1 or ALK5 demonstrated substantial differences in the transcriptional output from either signaling pathway (88), validating previously described downstream gene regulation. Interestingly, the non-overlapping expression patterns of ALK1 and ALK5 *in vivo* (89) by thorough analysis of a knockin mouse line carrying a lacZ reporter in the *Alk5* gene locus (*Alk5*[lacZ]), also lends support to divergent roles in vascular development for each of the two type I receptors expressed by ECs.

ALK5 suppression, by genetic silencing or small molecule inhibition, was shown not to interfere with BMP9/ALK1-induced phosphorylation of Smad1/5/8 in bovine aortic ECs (BAECs) (90). Instead, silencing of *Alk1* or any of its downstream molecular effectors, by means of siRNA transfection, rather induces a potent upregulation of ALK5 signaling. In agreement with the ALK5-independent action of ALK1 is the notion that the former is present in ECs *in vivo* either at low levels, or only expressed by the neighboring VSMCs, suggesting that ALK5 may only participate in ALK1-dependent angiogenesis in a paracrine fashion (62, 90). Congruent with these results, EC-specific ablation of *Alk5* does not inflict vascular abnormalities in mice or zebrafish (62). However, embryos from knockin mice carrying a mutation on L45 loop in ALK5 rescued to some extent the earliest vascular defects observed in ALK5 complete knockouts (91). It is feasible that this mutation while interfering with ALK5 kinase ability to phosphorylate Smad2 it inherently preserves ALK5 competence to mediate non-Smad signaling and lateral signaling to ALK1.

In agreement with the latter findings, in pathological conditions, ALK1 signal inhibition proved to interfere, not only with its own target genes but ALK5 signal transduction also suffered suppressive modulation, in a model of pancreatic neuroendocrine tumors (PNETs) (92).

More recently, it has been demonstrated that selective deletion of *Alk5* in ECs, using an Alk1[GFPCre] mouse line results in embryonic lethality due to brain vessel pathological morphology and intracerebral hemorrhage (93). Independent observations of EC-specific deletion of Smad2/3 using Tie2-Cre transgenic mice revealed critical hemorrhaging and embryonic lethality around E12.5. In this study, vascular maturation was incomplete owing to inadequate assembly of mural cells in the vasculature, most likely because of impaired expression of PDGF-B by the Smad2/3 ablated endothelium (94).

Collectively, these reports substantiate that ALK5 signaling is indeed relevant for endothelial homeostasis either as a signaling anchor to ALK1 or by actively participating in the vasculogenic process. Further studies aiming at dichotomizing ALK1 *versus* ALK5 signaling in endothelial and in perivascular cells during development and in the tumor microenvironment are thus required to clarify and conciliate previous paradoxical observations and infer about the benefit or risk of clinically targeting such pathways without proper amendments. It is plausible that the relative stoichiometry of ALK1 and ALK5 signaling may be crucial for proper regulation of gene expression (95).

1.3.3. Bone morphogenetic proteins (BMPs)

The BMPs, a subcategory of the TGF-β superfamily, were first identified in extracts from bone matrix and characterized by their ability to induce ectopic bone formation when implanted subcutaneously in rats (96). It soon became clear that BMPs play a key role in vertebrate organogenesis as well as in embryonic vascularization (97-99).

The BMP family, including the growth and differentiation factors (GDFs), comprises a group of 20 ligands that activate a classical BMP pathway in vertebrates (100, 101). In the canonical BMP signaling pathway, three type II receptors (BMPRII, ActRIIa and ActRII2b) and four type I receptors (ALK1, ALK2, ALK3 and ALK6) can be activated (59, 60). In addition to primarily triggering Smad1, 5 and 8, BMP cues may also activate Smad2 (61) and Smad-independent signaling (102).

Making an analogy with embryonic development where proper ontogenic patterning requires the concerted action of extracellular modulators, receptors, coreceptors and cytosolic proteins orchestrating the specificity and accuracy of signals on spatiotemporal control, it is predictable that similar mechanisms may also affect homeostasis (101, 102) and angiogenesis, in particular (99). Indeed, BMP expression is also detected in adulthood, for which reason, one can anticipate that its deregulation may be intimately connected to pathological conditions.

1.3.4. BMP9 signaling in endothelial cells

BMP9 has been implicated in hematopoiesis, hepato-, osteo-, chondro- and adipogenesis (102-106). It has also been described as a regulator of glucose metabolism (107) and as a differentiation factor for cholinergic neurons in the CNS (108).

More recently, BMP9 was pinpointed as the physiologically functional high affinity ligand for the predominantly endothelial receptor, ALK1, highlighting BMP9 as a critical modulator of angiogenesis (109-111).

BMP9 was originally cloned from a rodent cDNA library obtained from mouse liver, where it was shown to be highly expressed (112). Accordingly, the liver was characterized as the major source of human and mouse BMP9, expressed by hepatocytes and intrahepatic biliary epithelial cells, while it is present in the brain and lung at much lower levels (113). In line with these observations, the Human Protein Atlas profile for BMP9 in normal tissues indicates that it is highly expressed in liver, pancreas, placenta, lung, epididimus, GI tract, gall bladder and thyroid, but also in hematopoietic cells (www.proteinatlas.org).

Interestingly, it is now clearly described that pulmonary and cerebral AVMs occur more often in HHT1 (caused by endoglin mutation), while hepatic AVMs are more frequent in HHT2 (caused by ALK1 mutation) (114). In fact, in HHT2, the frequency of hepatic AVMs is between 38-41%, while in HHT1 it only ranges between 2.5-8% (115). The specific expression of the ALK1 ligand, BMP9, predominantly in the liver reflects a seemingly tissue-specific manifestation in HHT2 (110).

The *modus operandus* of BMP ligand interaction with their receptors differs from that of TGF-β: while TGF-β exhibits higher affinity for type II receptors and does not stably interact with type I receptors alone, BMPs bind independently to both type I and type II receptors (116, 117). The BMP ligands also display affinity to the coreceptors endoglin and betaglycan (101). In fact, BMP9 can also directly bind endoglin (111).

BMP9 is synthesized as a precursor protein, which is then cleaved by furin, a serine-endoprotease, forming a short dimeric mature form to which the prodomain can remain non-covalently associated (113). Until recently neither BMP9, nor its closely related family member, BMP10 were found to be negatively regulated by common BMP pathway antagonists (109, 118). However, recent studies showed that ALK1 activation by BMP9 induces expression of matrix Gla protein and crossveinless 2 (CV2), both known as antagonists of BMP4-induced angiogenesis (119-121). CV2, a member of the Chordin family, preferentially binds and inhibits BMP9 thereby providing strong feedback inhibition on ALK1 (119), which suggests a critical mutual regulation by BMP9 and CV2 in the vasculature.

Analogous to TGF-β signaling mediated by ALK1, BMP9 has also been reported to have incongruent effects on ECs. For example, BMP9 exhibits antiangiogenic effects counteracting fibroblast growth factor (FGF)-induced angiogenesis in *ex vivo* metatarsal models (110, 111) and acts as a circulating vascular quiescent factor (109). Nevertheless, multiple types of ECs activate their proliferative status *in vitro* in response to BMP9, which proangiogenic properties also activate matrigel plug vascularization and tumor angiogenesis in a pancreatic cancer xenograft model (122).

In order to unmistakably clarify the effects on the endothelium by BMP9 stimulation and its specific downstream mediators, an extensive analysis of BMP9 downstream activation in comparison to other ligands on ECs is mandatory for the field. According to the present knowledge, BMP-induced responses have as common denominators the Smad1/5/8 pathway and *Id1, 2* and *3* as target genes, suggesting that other differentially responsive genes may exist, more specifically induced by each BMP. In a recent study such efforts were initiated where EC-specific Smad1/5 target genes were characterized and upregulation of Notch signaling-related genes were identified upon BMP9 stimulation (123). Moreover, a novel embryonic endothelium-enriched gene

activated by BMP9 and BMP10 through the ALK1 receptor, *Tmem100*, has been described (124). Interestingly, *Tmem100* null mice exhibit embryonic lethality due to impaired differentiation of arterial endothelium and defects of vascular morphogenesis, phenocopying the *Alk1* complete knockout (49, 50, 124).

Despite the paucity of detailed studies on the involvement of BMP9 in cancer pathology, the Human Protein Atlas profile in cancer disease indicates that BMP9 expression is increased in colorectal cancer, head and neck squamous cell carcinoma, pancreatic and liver cancers (www.proteinatalas.com). Interestingly, BMP9 is primarily expressed in the *islets of Langerhans* in the pancreas, by the tumor cellular component, in mouse PNETs and is increasingly expressed in the malignant lesions as the tumorigenic pathway progresses (92). There is therefore strong evidence for an important functional role of BMP9/ALK1 signaling in cancer progression.

1.3.5. Other proangiogenic observations in the TGF-β signaling pathway

Both BMP9 and BMP10 have been identified as functional activators of ALK1 in ECs, inducing comparable cellular effects. In agreement with a 65% amino acid sequence homology between both ligands, BMP10, much like BMP9, exhibits angiostatic properties on human dermal microvascular ECs (HMVECs) (110). BMP10, however, binds to ALK1 with lower affinity than BMP9 (110) and is mainly expressed in the murine embryonic and postnatal heart. The lethally impaired cardiac growth and physiology in the BMP10 knockout mouse, coupled to normal vascular development of embryo and yolk sac propose a critical role for BMP10 in cardiogenesis (125). Interestingly, it has also been demonstrated that BMP10 can additionally bind to ALK3 (126). Of note, ALK3 targeted deletion in neural crest cells generates embryonic heart failure (127). Comprehensively, these observations suggest the cardiac-specific nature of BMP10 signaling most likely through ALK3, rather than ALK1.

However, the direct effect of BMP10 on the vasculature should not be overlooked, as it has the potential to cooperate or even compensate for BMP9 signaling through ALK1. In fact, very recently Ricard *et al.* (2012) revealed that BMP9 knockout (KO) mice do not exhibit defective vascularization in the retina (128). However, injection of the extracellular domain of ALK1 or a neutralizing anti-BMP10 antibody impaired retinal vascularization in BMP9-KO neonates, reducing retinal vascular expansion and exacerbating vascular density (128). These data thus sustain a cooperative or compensatory role for BMP9 and BMP10 in postnatal vascular remodeling of the retina. It remains to be determined if this cooperative role occurs also in the context of cancer. Of note is the peak of expression of BMP10 only during the angiogenic stage of tumorigenesis in the Rip1-Tag2 model (50), which may suggest a role during that phase of tumor development in PNETs, especially in the absence of BMP9, which is more abundantly expressed.

1.3.6. ALK1 interplay with other type I receptors

The complexity of TGF-β and BMP9 signaling circuitry in ECs is far from being completely represented by the usual simplistic two-route mechanism between ALK1 and ALK5 as illustrated in Figure 3. As mentioned earlier, ALK1 shares similar properties in terms of BMP-dependent activation of Smad1/5/8 signaling with the related BMP type I receptors ALK2, ALK3 and ALK6. Ligand specificity has not been

carefully elucidated, and many ligands, including BMP2, BMP4, BMP6, BMP7, BMP9 and BMP10, exhibit a multitude of effects on ECs, ranging from metabolism, endothelial-to-mesenchymal transition (EndMT) and tumor angiogenesis (129-135). While described as the physiological ligand for ALK1, BMP9 has also documented binding ability towards ALK2 in non-EC, such as myoblasts and breast tumor cells (111) with the BMP9/ALK2 signaling axis also being linked to promotion of proliferation of ovarian cancer cells (136). In support of the need for substantial analysis of the signaling arising from these receptors in the endothelium, vascular ECs have been shown to transform into multipotent stem-like cells in an ALK2-dependent fashion in lesions from individuals with *fibrodysplasia ossificans progressiva* (FOP) (131). This disabling disorder occurs as a result of gain-of-function mutations in ALK2 in humans or mirrored in mice by constitutive activation of ALK2 signaling on chondrocytes and osteoblasts. Lineage tracing of heterotopic ossification in mice using a Tie2-Cre construct disclosed the endothelial origin of these cell types (131). In agreement with this finding, ECs conditionally deficient for ALK2 do not succeed to undergo EndMT during endocardial cushion formation in embryogenesis (137). Of note, ALK2 has been demonstrated to upregulate ALK1 in ECs in response to high-density lipoproteins; after which ALK1 in turn promotes survival by inducing expression of VEGF-A (129). Glucose level augmentation co-regulates ALK1 and ALK2 expression in human aortic ECs (HAECs) (130). Also, BMP/TGF-β receptors appear to be activated and function sequentially: ALK3, ALK2, ALK1 and ALK5, where each receptor can possibly entail a distinct function and correlate to a specific stage in vascular growth and development (90, 129, 130).

Since it is not completely clear whether ALK2 is exclusively expressed in non-ECs, more insight should be gathered as ALK1 and ALK2 can either compensate for each other absence or be simultaneously targeted due to interaction with the same ligands.
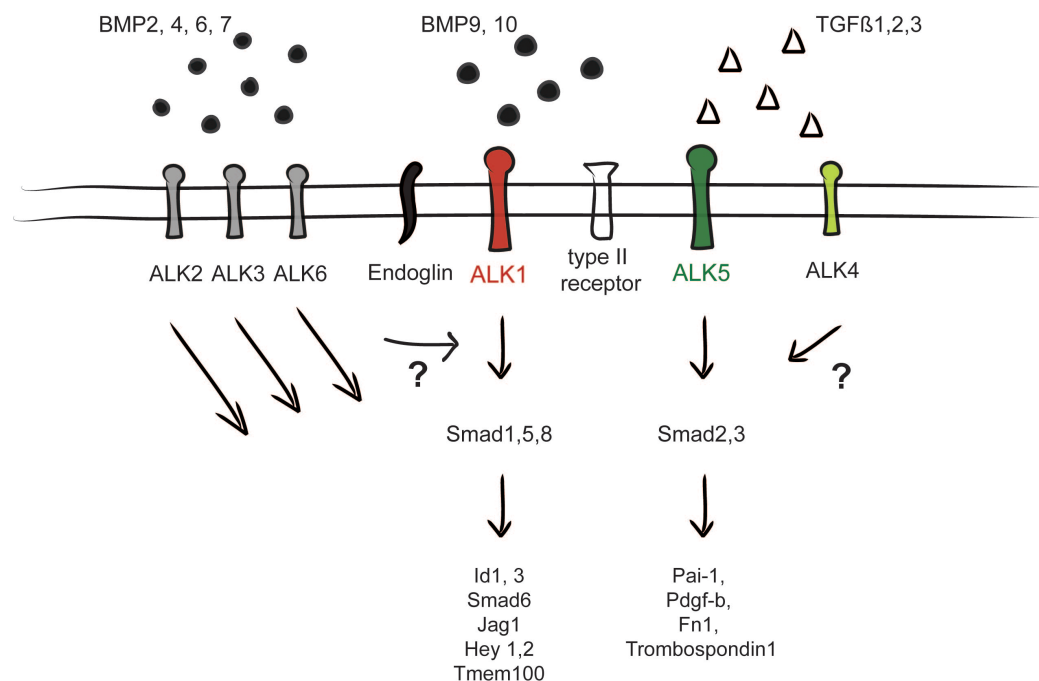

Figure 4. Possible network crosstalk within the TGF-β family affecting endothelial cells. TGF-β activates both ALK1 and ALK5 type I receptors expressed by endothelial cells, whereas BMP9 only binds ALK1. The affinity of BMP9 for ALK1 is higher than that of TGF-β, making it likely that ALK1 will predominantly bind

BMP9 when both ligands are available. In addition, BMP10 also has the ability to bind ALK1 and compensate for BMP9 absence. Endoglin acts as a coreceptor modulating signaling through ALK1. Smad1, 5 and 8 are preferentially phosphorylated and activated by ALK1, while Smad2 and 3 are predominantly activated downstream from ALK5. Subsequently, Smads are translocated to the nucleus where they regulate specific gene expression. ALK2, ALK3 and ALK6 may compensate or crosstalk with ALK1 signaling cascade while ALK4 can compensate for ALK5 signaling.

Thus, the interplay and/or compensatory crosstalk primarily between ALK1 and ALK2, but also with ALK3 and ALK6, which is of critical importance in a therapeutic context, promptly begs for more detailed studies (Figure 4), especially in the context of tumors.

1.3.7. TGF-β and BMP signaling pathways: competitive or synergistic?

Closely connected to receptor interplay is the role specifically played by the ligands. Classically, BMPs and TGF-βs have long been described to exert parallel antagonistic effect on the other pathway in a variety of biological contexts (138).

Moreover, in physiological conditions, cells in the body are exposed to multiple ligands simultaneously, which may trigger alternative responses *in vivo,* to what is customarily studied *in vitro* analyzing the effects of each ligand in isolation.

In an attempt to clarify the role of ALK1 signaling in EC, we recently described an unanticipated synergistic effect of TGF-β with BMP9 on tumor angiogenesis. We demonstrated *in vitro* and *in vivo* in various systems, that while either cytokine on its own exerted suppressive action on the endothelium, when in combination, both ligands boosted the EC response towards other proangiogenic stimuli (92). On a molecular level, simultaneous ECs induction with TGF-β and BMP9 induces a synergistic response on ALK5 signaling arm, demonstrated by increased Smad2 phosphorylation and its downstream target gene expression (eg. *Pai-1* and *Pdgf-b*) (92).

Another publication demonstrated that BMP2 synergistically enhances TGF-β3-induced initial phenotypic changes associated with EndMT occurring during endocardial cushion formation (139). In fact, BMPs and their receptors are expressed at many sites in which EMT or EndMT occurs during developmental organogenesis (140-142).

TGF-β and BMP7 also coadjuvantly stimulate angiogenesis in the chick chorioallantoic membrane (CAM) assay (135) and collaboratively activate prostate cancer cells (143) and osteoblast differentiation (144). In contrast, BMP7 counteracts TGF-β-induced EndMT in a model of cardiac fibrosis, rendering the ECs capable to preserve their endothelial identity (132). These observations suggest context-dependency of the synergy between TGF-βs and BMPs.

The numerous BMP ligands and type I receptors exert a variety of effects on ECs, yet the fact that different ligands utilize common pathway components raises important questions, which may have been neglected until recently: how cells respond specifically to individual ligands and how cells integrate and interpret signals received from multiple ligands. Concerning this context, worthy of note are studies suggesting that pre-formed BMP receptor complexes or BMP-induced oligomerization of type I and type II receptors, predominantly activate Smad-dependent or –independent signaling, respectively (145, 146). Also, the choice of type II receptor influences the

signaling outcome of BMP stimulation as downstream specific binding of Limk1 to the BMP type II receptor, but not to TGF-β or Activin type II receptors, have been reported (147, 148). More recently, different R-Smad complex formation Smad1/5-Smad2 *versus* Smad1/5-Smad3 were described (138), opening the possibility that the novel complexes may be the source of antagonistic *versus* synergistic responses in different studies.

Evidently, signaling through non-Smad effectors, the recruitment of distinct type II receptors and perhaps more importantly the variability created by alternative Smad complex formation should be further examined, as the explanations for the diverse effects may rely on these factors.

1.4. TGF-β endothelial signaling pathways as cancer pharmacological targets

1.4.1. Physiological role of ALK1 in the vasculature

The importance of this receptor became obvious, when *Alk1* loss of function studies revealed that its complete loss causes embryonic lethality at midgestation, due to severe vascular abnormalities including vessel hyperdilation, AVMs resulting from fusion of major arteries and veins, and impaired recruitment of VSMC (49) (50). Mutations in the *Alk1* gene have been identified as the underlying cause for development of HHT, a rare, human autosomal dominant disease characterized by the presence of recurrent epistaxis and small characteristic malformations of the peripheral blood vessels near the surface of the skin or GI mucosal linings (149). AVMs of the lung, liver, and CNS are also known clinical findings. Interestingly, EC-specific deletion of the *Alk1* gene in the mouse results in neonatal lethality at P5, with the pups exhibiting hemorrhaging in the brain, lung and GI tract (150). In attempts to evaluate the contribution of ALK1 to vascular homeostasis in adult mice, Park *et al.* (2009) deleted *Alk1* in 2 months old $R26^+/^{CreER}$ $Acvrl1^{2loxP/loxP}$ mice (150). Tamoxifen-induced *Alk1* deletion resulted in severe internal hemorrhage in lung, small intestine, uterine vessels, and ultimately in death.

Strong expression of ALK1 has been reported during developmental and neonatal stages, while it is suppressed during adulthood, except in certain organs, i.e., the lungs (62). Supportive of that is the observation that ALK1 is fundamental for umbilical and placental blood vessel formation (151) and its expression is induced in feeding arteries and newly formed blood vessels during wound healing in adult subdermal blood vessels (150). AVMs appearing only in subdermal blood vessels where a wound is inflicted provide *in vivo* experimental evidence that genetic predisposition by *endoglin* or *Alk1* mutations is not enough for development of *de novo* AVMs in HHT (62). Interestingly, only selected vascular beds in HHT patients develop telangiectasis or AVM lesions, while other areas (>99.9%) remain normal (152).

Three independent studies demonstrated that inhibition of ALK1 by systemic injection of an ALK1 soluble extracellular domain efficiently impaired retinal neonatal angiogenesis, inducing retinal hypervascularization and appearance of AVMs in neonatal mice (128, 153, 154). Incidentally, the most recent studies also report a cooperative effect of ALK1 and Notch signaling pathways (123, 128, 154). The reported synergy between ALK1 and Notch pathways generated exacerbation of the hypervascularization phenotype, inducing potentiated expression of Notch target

genes in the stalk cells, which concomitantly suppress VEGF signaling to the endothelial tip-cell (154). In parallel studies, it was shown that endothelial-specific inactivation of Smad1/5 in mouse embryonic development yields impaired Dll4/Notch signaling and augments tip-cell number in detriment to stalk cell number (155). These studies put forward a regulatory crosstalk loop amongst BMP9/ALK1/Smad1/5 and Notch signaling coordinating tip *versus* stalk cell specification.

Additionally to being expressed by blood ECs, ALK1 is also expressed by lymphatic ECs (LECs) (153). *In vitro* stimulation of LECs by BMP9 generates downstream target gene transactivation. Furthermore, inhibition of ALK1 signaling by means of an ALK1-Fc soluble fusion protein diminishes neonatal retinal lymphangiogenesis, while the use of an ALK1 targeting monoclonal antibody also impairs Lyve-1-positive lymphangiogenesis in mammary fat pad–implanted MDA-MB-231 breast carcinoma xenografts (156). Lymphatic vessel development seems to comprise coordinate and synergistic ALK1 and VEGFR3 signaling regulation (153), reminescent of the crosstalk between ALK1/ALK5, ALK1/ALK2 and VEGF-receptor signaling in blood vessel angiogenesis (92, 157).

1.4.1.1.   Clinical relevance of ALK1 in cancer

ALK1 expression in the early developing mouse embryo coincides with sites of vasculo- and angiogenesis (158), with prevailing expression in developing arterial endothelium, while nearly absent from small capillaries (89). During early mouse development ALK1 is strongly expressed, whilst it tends to be concealed in the adult quiescent vasculature. The expression of ALK1 is reversibly turned on during neo-angiogenesis events in wound healing or in tumors (89).

Information about pattern and intensity of ALK1 expression in human normal and cancerous tissues is unfortunately still scarce. The public Human Protein Atlas program (159) has characterized ALK1 immunostaining of both normal and neoplastic tissues. In this study, ALK1 exhibited frequent strong expression most notably in neuronal cells of the cerebral cortex, hippocampus, ventricle and cerebellum, in the gall bladder, GI tract, and in tubular cells in the kidney, in line with the murine ALK1 expression profile (160, 161). The same organization identified ALK1 prevailing expression in neoplasms in the colorectal tract, pancreas, stomach, and thyroid, as well in malignant lymphomas and melanomas (www.proteinatlas.com). A preliminary descriptive study reported a weak but widespread pattern of expression in the vasculature of normal tissues, including positive staining in lymphatic tissues, lung, intestine and pancreas (162). In a follow-up study by the same entity, ALK1 was found to be extensively present on tumor blood vessels, especially in lymphomas, and malignant tissues of the prostate, skin, thyroid, kidney, ovary, lung, pancreas and liver (156) Thorough studies analyzing the prognostic strength and diagnostic significance of ALK1 are highly warranted.

1.4.1.2.   Inhibitory molecules targeting ALK1

Given the extensive literature describing paradoxical effects of signaling stemming from ALK1 in ECs, predicting the net outcome for acute inhibition of ALK1 in cancer is an intricate task. Furthermore, the complex ligand-receptor binding specificity and/or redundancy within the TGF-β family adds multiple hurdles in estimating therapeutic

efficacy, benefit and side effects for the various ALK1 inhibitors currently under development.

Small molecules comprising a broad inhibitory spectrum against BMP type I receptor kinases, including ALK1, have been generated. Smad-dependent and -independent signaling, induced by BMPs can be blocked by compounds such as dorsomorphin and its analogue LDN-193189 (163, 164). These compounds can be useful and potent in inhibiting BMP type I receptor signaling in a range of diseases and familial syndromes (165). However, their effect on BMP-induced tumor angiogenesis remains to be determined. Non-selective inhibitors also raise the concern of off-target effects, and dorsomorphin was in fact recently reported to potently inhibit multiple kinases (166, 167). Further development of small molecule with a more specific inhibition profile aiming primarily at BMP type I receptors, and specifically towards ALK1, should be considered.

Antibodies or soluble extracellular receptor domain traps may alternatively provide a tighter inhibition of specific ALK1 activity. ALK1 inhibitors are currently under development for cancer treatment as antiangiogenic drugs. Up to now, several biological inhibitors against ALK1 have been generated for *in vivo* use. Firstly, Pfizer is currently conducting phase I trials with PF-03446962, a fully human monoclonal antibody against ALK1 (168). Secondly, Genentech reported the use of an ALK1-Fc fusion protein (amino acids 23-119 of mouse ALK1), on mouse hematogenous and lymphatic vessel development studies (153). Another ALK1-targeting agent is Dalantercept/ACE-041 (mouse counterpart RAP-041, amino acids 22-117 of mouse ALK1) (169), a human ALK1-Fc fusion protein, currently undergoing a phase II clinical trials coordinated by Acceleron Pharma.

PF-03446692

Preclinical tumor studies using PF-03446962 have recently been reported by Pfizer (156). ALK1 blockade exhibited attenuation of VEGF-induced EC proliferation and tube formation *in vitro*. In addition, therapeutic treatment with the ALK1-neutralizing antibody delays tumor growth *in vivo* in MDA-MB-231 breast carcinoma and M24met/R melanoma mouse models (156).

The anti-hALK1 antibody from Pfizer is currently in phase I clinical trials for patients with advanced solid tumors (168). The primary endpoint for the study is to determine a maximum tolerated dose (MTD) for phase II trials, whereas secondary outcomes include the record of preliminary signs of antitumor activity and tumor blood flow. Preliminary evidence from the trial indicates that the anti-hALK1 antibody reduced the amount of ALK1-positive circulating ECs (170), which were found to be present in increased levels in advanced stage cancer patients (171). The PF-03446962 antibody was well tolerated at doses up to 10 mg/kg and did not present significant adverse events in all 44 patients participating in the phase I trial. The most common side effects included transient thrombocytopenia and asymptomatic elevation of pancreatic enzymes. Hence, preliminary observations evoke encouraging clinical activity where three partial responses were observed in patients who have previously received fruitless VEGF-targeting antiangiogenic regimens (168). A phase II clinical trial on relapsed or refractory urothelial cancer is already planned (www.clinicaltrials.gov).

The mechanism of action of this antibody has recently been described (172). The anti-hALK1 antibody selectively recognizes human ALK1 and interferes with BMP9-induced signaling in ECs. Moreover, the anti-hALK1 antibody competitively obstructs BMP9

and TGF-β binding to ALK1 receptor and prevents BMP9-dependent recruitment of endoglin into the angiogenesis-mediating signaling complex, which may eventually further hinder the BMP9/ALK1 proangiogenic effects.

Dalantercept/ACE-041/RAP-041

The effects of RAP-041 were recently analyzed in the Rip1-Tag2 transgenic mouse model of pancreatic neuroendocrine tumorigenesis (92). Rip1-Tag2 tumors readily express ALK1 exclusively on ECs, mirroring the expression profile of common vascular markers during tumor progression. Rip1-Tag2 mice treated with two weekly doses between 1-12 mg/kg of RAP-041 results in a dose-dependent retardation of tumor growth and the highest dose of 12 mg/kg effectively prevents further tumor expansion. Incidentally, *Alk1* heterozygous mice under the Rip1-Tag2 context recapitulated the effects obtained by systemic inhibition by RAP-041. Specificity of the ALK1-targeting treatment was validated by decreased expression of ALK1 downstream target genes in tumors from mice treated with RAP-041. Alternative studies with the same inhibitor demonstrated that RAP-041 also possesses growth-inhibitory effect in orthotopic MCF-7 breast carcinomas (157) and in the breast cancer transgenic mouse model MMTV-PyMT (personal observation).

Acceleron Pharma recently concluded a phase I trial for the human ALK1-Fc fusion protein Dalantercept/ACE-041 (169), which binds and neutralizes BMP9 and BMP10, but does not interact with any of the TGF-β isoforms (92, 157). Thirty seven patients with solid tumors or refractory multiple myeloma were recruited to assess safety and tolerability of Dalantercept/ACE-041, as well as changes in tumor metabolism evaluated by ^{18}F deoxyglucose positron emission tomography (FDG-PET). The Dalantercept/ACE-041 regimen included an administration every three weeks and was well tolerated at doses up to 1.6 mg/kg. Common collateral effects comprised peripheral edema, fatigue, nausea, headache, anorexia and anemia. Interestingly, toxicities usually associated with VEGF inhibition, such as hypertension, proteinuria, GI tract perforation and hemorrhaging were not observed. The milder side effects caused by ALK1 inhibition are probably due to ALK1 predominant expression in actively cycling endothelium within the tumor milieu and preferentially localizing in arterial endothelium, whereas VEGFRs present a more global distribution (173).

In this clinical study, one patient with refractory head and neck squamous cell carcinoma (HNSCC) exhibited a partial response (>30weeks) and six other patients exhibited stable disease. Rapid reduction in tumor metabolic activity (>20%) was observed in ten patients, measured by FDG-PET scanning (174). Of note, many of the patients included in this trial had been previously inefficiently treated with other therapeutic regimens, including VEGF-neutralizing drugs.

After such an encouraging phase I clinical trial, the ALK1 inhibitor Dalantercept/ACE-041 is undergoing phase II clinical studies with expanded cohorts on HNSCC, renal cell carcinoma (RCC) and recurrent or persistent endometrial cancer patients (http://clinicaltrials.gov). Furthermore, BMP9 has been shown to be elevated and may feasibly function as a biomarker for selecting patients who can possibly benefit from Dalantercept/ACE-041. In fact, in an analysis of archived tumor samples from patients with HNSCC, 79% of the samples presented with moderate to high expression of BMP9 (communication from Acceleron Pharma). Apart from its anticancer specification, Dalantercept/ACE-041 is also being developed for testing in age-related macular degeneration (AMD) treatment.

1.4.1.3. Possible side effects from ALK1 inhibition

Antiangiogenic therapies, mainly in the form of inhibitors of VEGF signaling, have been in routine clinical use for several years for various malignancies. Side effects from inhibiting angiogenesis are in general milder than those arising from conventional chemotherapeutic treatment and include most frequently bleeding, hypertension, fatigue, and nausea. Specifically, given the causal relationship between impaired ALK1 signaling and HHT-related symptoms, inhibition of ALK1 signaling in the vasculature may induce *de novo* AVMs and hemorrhaging. In fact, skin telangiectasis were observed in a patient treated with Dalantercept/ACE-041 (personal communication by Acceleron Pharma), validating the on-target effect of this inhibitory agent, additionally indicating that the appearance of telangiectases may be useful as a surrogate marker for efficacy. Loss-of-function mutations in ALK1 linked to hereditary pulmonary arterial hypertension (PAH) (175) highlight the risk for pulmonary circulation hemodynamic perturbations with ALK1 inhibitors. Importantly, complete blockade of ALK1 signaling triggered by both BMP9 and BMP10 resulted in lung hemorrhaging (128), an organ that should thus be primarily supervised. Furthermore, as HHT2 symptoms seem to be more prone to be manifested by the liver (110) and given this organ´s high expression levels of BMP9, the liver should also be carefully monitored (113). Finally, as ALK1 is reported to be expressed by, and possibly important for lymphatic ECs, cells of the pituitary gland, chondrocytes and pancreatic ductal cells, special care should be taken to record adverse events from the use of ALK1 inhibitors related to processes regulated by these particular tissues (153, 176-179).

1.4.2. Physiological role of endoglin in the vasculature

Endoglin, an auxiliary receptor for TGF-β, is required for angiogenesis during development (180). It is expressed primarily in ECs and its expression is substantially incremented during EC activation, inflammation, ischemia, and tumor angiogenesis (181-184). The mechanisms involved in endoglin upregulation are presumably multifactorial, but hypoxia is a probable inducer as it prevails in most pathophysiological environments where endoglin is enhanced (185).

Endoglin associates with type II receptors of the TGF-β family in the presence of ligand and with the type I signaling receptors, ALK1 and ALK5, even in the absence of exogenous ligand (186). Despite possessing no enzymatic activity, endoglin has been reported to be necessary to modulate ligand-receptor interaction in ALK1, but not in ALK5 signaling (111, 187-189). More recently, ALK5 was shown to phosphorylate the cytoplasmic domain of endoglin in ECs (190). Depending on the phosphorilation status of only serine 646 or both 646 and 649 serine residues, response to TGF-β/BMP9 signaling results in loss of endoglin-mediated inhibition or activation of Smad1/5/8 signaling, respectively (190). Taken together, these results indicate that endoglin phosphorylation by ALK5 is an important mechanism for regulating TGF-β and BMP signaling in ECs.

Even though endoglin has an undeniably well documented connection to ALK1 and its signaling enhancement (189, 191), it is imperative to mention that it interacts with ligands other than TGF-β and BMP9 (TGF-β3, Activin A, BMP2 and BMP7) but also with several different type I and type II receptors involved in BMP and TGF-β signaling (186). A crosstalk has been described between endoglin-mediated

fibronectin/α5β1 integrin complex and the TGF-β pathway. This complex alters the responses of ECs to TGF-β, switching TGF-β from promoter to suppressor of migration, supporting capillary stability, and partially mediating developmental angiogenesis *in vivo* (192).

Alternatively, endoglin has also been implicated in interactions with cytoplasmic proteins such as Zyxin, ZRP-1, β-Arrestin and Tctex2β, which may further generate additional cellular outcomes (185). The unrestrictive signaling of endoglin consequently adds an extra degree of intricacy to the elaborate signaling networks deriving from the TGF-β family in the angiogenesis field. Moreover, the fact that endoglin is positively associated with EC proliferation (while weakly expressed in quiescent endothelium) has focused the interest on endoglin as a potential target for cancer *in vivo*.

1.4.2.1. Clinical relevance of endoglin in cancer

Endoglin positive intratumoral microvascular density strongly correlates with poor prognosis in cancer, being associated with shorter survival and relapse-free survival rates (185, 193). Moreover, subcutaneous tumor neovascularization and growth are impaired in endoglin heterozygous mice, reiterating the relevance of endoglin in tumor angiogenesis (194). An extensive body of literature highlights the potential of endoglin as a tumor vessel marker in preclinical and clinical studies (193, 195-197). To this respect, endoglin can be a more specific marker for new, immature tumor vessels, unlike other EC markers, widely expressed in both mature and immature vessels (198). High levels of endoglin expression have, therefore, been confirmed in several experimental models of breast, prostate and colorectal cancer (CRC) (198-200). Accordingly, endoglin is moderately expressed in most malignant tissues, with strong staining in liver cancers and in several cases of colorectal, stomach and pancreatic cancers (www.proteinatlas.org).

Endoglin expression has also been associated with predisposition of colorectal mucosa dysplastic tissue evolution into fully developed carcinomas (200). In prostate cancer, endoglin positive microvessel density correlates with Gleason score, metastasis and tumor stage (199). Paradoxically, the same study indicated that endoglin-positive vessels were more poorly covered by α-smooth muscle actin (αSMA)-positive cells and correlated with survival. This observation strongly corroborates our studies, where endoglin depletion triggers EndMT with loss of the vascular marker CD31 and concurrent gain of the mesenchymal marker αSMA (Anderberg, Cunha, Zhai *et al.,* under revision). Another study on CRC patients reported that microvessel density evaluated by endoglin staining is significantly associated with survival. Additionally, other reports connect loss of endoglin with prostate cancer progression and aggressiveness (201) and endoglin presence with decrease in prostate tumor cell motility (202). Our own work strengthens the vascular role of endoglin as protective against tumor cell metastatic seeding (Anderberg, Cunha, Zhai *et al.,* under revision).

Endoglin exists in the body in two different forms: membrane-bound and circulating (185). Levels of soluble endoglin have been reported in plasma of pregnant women suffering from preeclampsia (203) and also in patients suffering from colorectal, breast, prostate and leukemic cancers, correlating positively with metastatic disease (204-208). However, the role of soluble endoglin in cancer is poorly understood. Since soluble endoglin contains a binding site for different ligands of the TGF-β family, it

may act as a scavenger of circulating ligands, preventing their binding to the functional receptors, hence interfering with vascular function and angiogenesis (209). An intriguing question concerns the source of soluble endoglin detected in cancer patients. Since endoglin levels are higher in tumor vessels, soluble endoglin may conceivably derive from shedding by tumor ECs and, importantly, it may represent a surrogate marker of angiogenic activity (210).

1.4.2.2. Inhibitory biological agents targeting endoglin

TRC105

The potential of endoglin-targeting monoclonal antibodies to be used as a therapeutic antiangiogenic strategy in human cancer has received considerable support from preclinical studies.

Intravenous systemic administration of anti-endoglin monoclonal antibody TRC105 was shown to suppress angiogenesis, tumor growth and metastasis without overt toxicity in mice (207, 211-213). This antibody is robustly taken up by the tumor, as compared to other organs, in the 4T1 breast cancer model (214). The combination of an endoglin-targeting antibody with cyclophosphamide and doxorubicin was reported to exhibit synergistic antitumor efficacy in human skin/SCID mouse chimeras, including in metronomic regimens (215, 216).

A total of 50 patients with advanced refractory solid tumors were included in a TRC105 phase I clinical trial (212). Doses up to 10 or 15 mg/kg were administered every week or every other week, respectively, to assess efficacy, toxicity and tolerability of TRC105. Common adverse events included anemia, telangiectasis, and infusion reactions, which reflect the mechanism of action of the antibody. Stable disease or response was observed in 21 out of 45 evaluated patients (47%), including two ongoing responses after 48 and 18 months (217).

A phase I clinical trial has been initiated with breast metastatic cancer patients to determine MTD of TRC105 in combination with capecitabine, a DNA synthesis blocking agent, approved by the FDA as adjuvant treatment for colon cancer, as well as, for CRC and metastatic breast cancer (www.clinicaltrials.gov).

Endoglin-Fc

More recently, Acceleron Pharma characterized a soluble mouse and human endoglin extracellular domain fused to an immunoglobulin Fc domain (human endoglin amino acid sequence 26–359). This endoglin ligand trap binds specifically and with high affinity to BMP9 and BMP10 *in vitro*. This agent significantly impaired VEGF-induced CAM assay *in vivo*. Finally, murine soluble endoglin extracellular domain acted as an antiangiogenic factor decreasing blood vessel sprouting in VEGF/FGF-induced angiogenesis in *in vivo* angioreactors and tumor burden in the colon-26 mouse tumor model (218). Together these findings indicate an important role for soluble endoglin in the regulation of angiogenesis and evoke the prospective efficacy of endoglin-Fc as an antiangiogenic therapeutic agent. However, since this inhibitor binds to BMP9 and BMP10, rather then interfering directly with endoglin physiology, it may be difficult to discern between the benefit of using an ALK1-Fc or an Eng-Fc soluble inhibitors.

1.4.2.3.   Possible side effects of targeting endoglin

Similarly to ALK1 inhibition, the appearance of telangiectasis upon endoglin inhibition represents a surrogate marker for assessing on-target effect. Interestingly, TRC105 induced telangiectasis in one patient during phase I clinical trial (217).

Based on a recent study, a careful follow-up of the patients treated with endoglin targeting molecules should be carried out. Our preclinical studies using mouse models where endoglin was genetically suppressed indicated the emergence of a refractory behavior and worsened phenotypes leading ultimately to increased metastatic seeding (Anderberg, Cunha, Zhai *et al.,* under revision).

How the novel results associating endoglin inhibition with a poorer prognosis and the beneficial effect of using a monoclonal antibody targeting endoglin for cancer treatment relate to one another, warrants urgent insight.

2. AIMS

Given the moderate therapeutic benefit in the clinic and reports of resistance acquisition by the use of VEGFR signaling inhibitors in preclinical studies, the overall aim of this thesis was to investigate the TGF-β pathway for possible novel and alternative antiangiogenic targets for anticancer use.

Specifically, the aims of this thesis were:

- to study the effect of ALK1 impairment in the angiogenic switch and in tumor development
- to investigate the potential of ALK1 pharmacological inhibition as an alternative to other antiangiogenic therapies
- to study how genetic suppression of endoglin affects the tumor vasculature and tumor burden
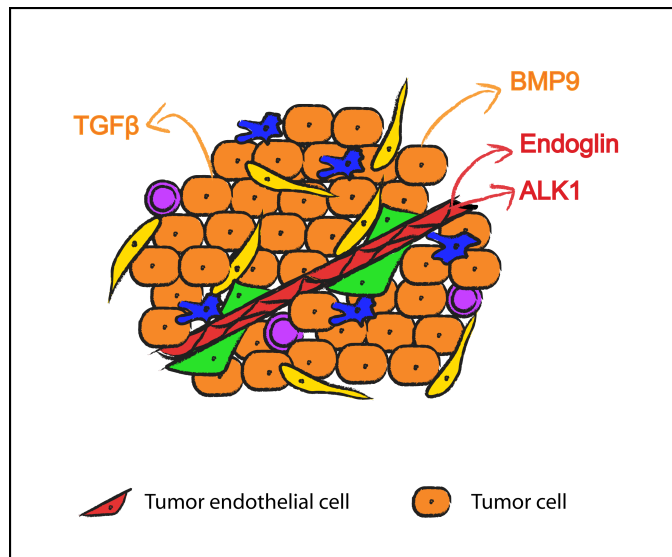- to evaluate the effect of concurrent combinatorial targeting of endoglin and VEGFR signaling

Figure 5. Paracrine signaling circuitry between the TGF-β family receptors predominantly expressed by the tumor endothelial cells (ALK1 and endoglin) and the ligands (BMP9 and TGF-β) secreted by the aberrant tumor cells in the tumor microenvironment.

3. RESULTS

3.1. Article I – Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis

Taking into account that most solid cancers rely on a functioning vascular network to supply oxygen and nutrients, the therapeutic strategy of interfering with the tumor vasculature held great promise in the cancer field.

VEGF and its receptors have emerged as the most relevant angiogenesis-regulating pathways, providing a solid base for the development of antiangiogenic strategies aiming at VEGF-driven angiogenesis. Significant developments in this field, in recent years, have resulted in the identification of a variety of potential targets and an extensive collection of drugs, some of which: the anti-VEGF antibody bevacizumab, and the tyrosine kinase inhibitors sorafenib and sunitinib, attracted attention. Most of these compounds have presented with promising preclinical tumor responses and were approved by the FDA for clinical use in metastatic renal and breast cancers, CRC, glioblastoma, non-small cell lung carcinoma (NSCLC) and hepatocellular carcinoma (219, 220).

The inherent instability of tumor vascular beds distinct from normal vessels combined with the fact that antiangiogenic drugs do not directly target the genetically unstable malignant cells fueled the expectations on the effect of such drugs in tumor progression. On the one hand, tumor ECs would be more prone to be exquisitely affected by selective targeting drugs and on the other hand as host–derived "normal" cells, the development of resistance by these ECs was thought to be a remote possibility (11, 221).

Despite all favorable indications, the scientific community has witnessed a suboptimal outcome from antiangiogenic treatment of human cancer during the past few years, even in combinatorial regimens with chemotherapeutic agents. Minimal success in extending overall survival thus strongly suggests the need for emergence and improvement of alternative therapeutic paths to be explored. Moreover, bevacizumab use for metastatic breast cancer indication has recently been revoked, due to its inability to improve therapeutic benefit in late stage disease.

The need for alternative routes to hinder tumorigenesis has prompted us to evaluate the potential of targeting an alternative proangiogenic pathway, the TGF-β/BMP9/ALK1 pathway. This signaling web has been long neglected until recently despite all evidence for an important role in vascular development.

Interestingly, examination of the ALK1 messenger RNA expression profile during the Rip1-Tag2 tumorigenic pathway demonstrated that ALK1 presents with a peak of expression during the onset of the angiogenic switch, a pattern reminiscent of that of other vascular markers during malignant development. We also demonstrated that ALK1 is essentially expressed by the tumor vessels, while its ligands TGF-β and BMP9 are primarily expressed by the malignant cell compartment in the mouse model of PNETs, Rip1-Tag2 (222).

Since the complete ALK1 knockout mouse is embryonic lethal at midgestation, to assess the relevance of ALK1 signaling in tumor angiogenesis, we have analyzed heterozygous mice for *Alk1* under the Rip1-Tag2 tumor model context (Rip1-Tag2;

Alk1 +/-) and their littermate controls. This analysis unveiled an important role for ALK1 during the angiogenic switch, concomitant with the formation of fewer mature malignant lesions and diminished overall tumor burden. The impairment of the tumor vascular network was additionally confirmed by a significant reduction in total CD31-positive vessel density and decreased vessel perfusion (FITC-lectin), translating into a weakened endothelium and poorer physiology of the ALK1-attenuated tumor vessels.

Trying to assess the possibility of modulating ALK1 signaling as a therapeutic tool in the combat of cancer, we made use of an ALK1-Fc fusion protein, RAP-041 (Acceleron Pharma). The effect of targeting ALK1 systemically by injecting this soluble ligand trap intraperitoneally was analyzed. As previously observed with partial ALK1 genetic dosage attenuation, treatment of Rip1-Tag2 tumor-bearing mice with RAP-041 promptly suppressed tumor growth and progression in a dose-dependent manner. Intervention and regression trials with RAP-041 for two weeks at 10 or 12 weeks of age, respectively, were performed to infer about the efficacy of RAP-041 interfering with angiogenesis in different disease stages (223) (Figure 6). An early stage trial (typically 10-12 weeks of age in the Rip1-Tag2 model) allows assessment on how the tested drug with interferes the angiogenic switch and how it affects further tumor development. On the other hand, a late stage trial (typically 12-14 weeks of age in the Rip1-Tag2 model) applied when the tumor disease is effectively in place with fully grown hemorrhaging, encapsulated and/or locally invasive tumors permits assessment of the drugs capability to not only hinder tumor progression, but to further diminish tumor size and ultimately improve survival. Both therapeutic trials generated a debilitated vasculature and hindered tumor formation.
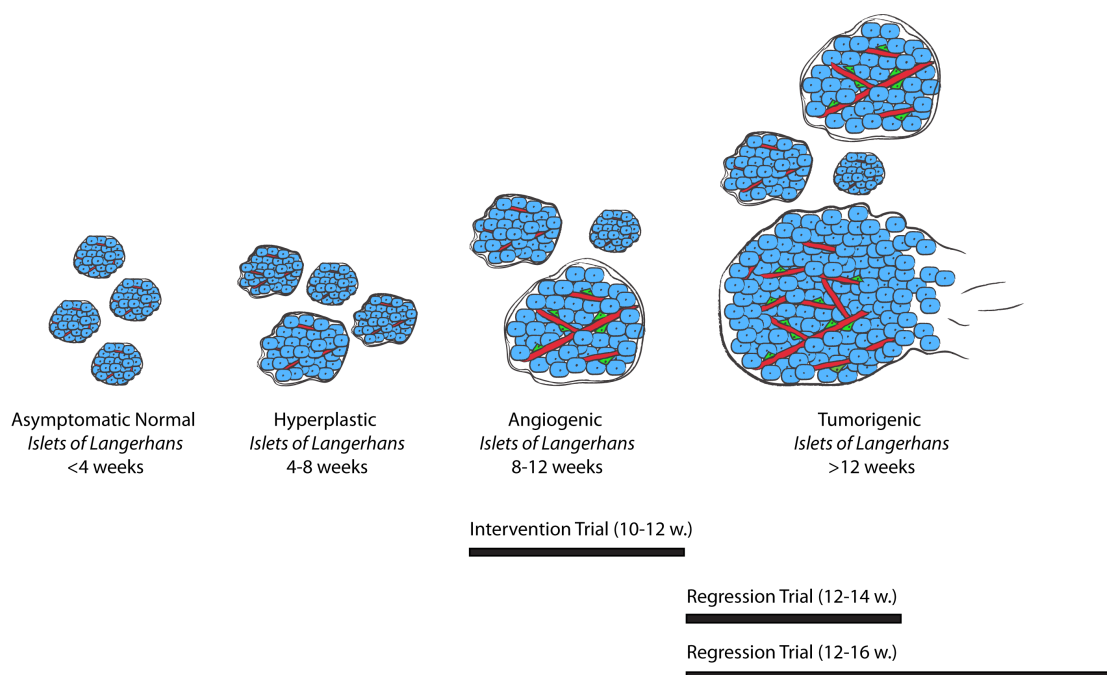
Figure 6. Rip1-Tag2 pancreatic neuroendocrine tumor model. In this model, the rat insulin promoter drives the expression of the SV40 oncogene in the pancreatic β cells, which leads to the development of PNETs in a stepwise and synchronized fashion, from normal asymptomatic *islets of Langerhans*, to hyperplastic, angiogenic and fully developed tumors.

For both genetic and pharmacological targeting analysis we confirmed that ALK1 target genes (*Id1* and *Id3*) messenger RNA levels were correspondingly reduced, as

well as, ALK5 target gene *Pai-1*. Such results suggested that not only our therapeutic strategy had an on-target effect, but it also implies that ALK1 and ALK5 are intimately connected in the tumor angiogenic process mediated by ALK1.

Seeking the mechanism to explain the observed effect, and based on progressively increased expression of both TGF-β and BMP9 during the Rip1-Tag2 tumorigenesis, we hypothesized that both these ligands were likely to hold a key role in tumor angiogenesis. A highly unanticipated and controversial synergy between TGF-β and BMP9 was disclosed. We provided evidence supporting that TGF-β and BMP9 collective action synergistically augmented the EC-response to proangiogenic stimuli, such as VEGF and FGF. We, therefore, provide relevant data for a decisive role for signaling in the tumor microenvironment by TGF-β family members in fueling angiogenesis. Our preclinical studies on the role of ALK1 in tumor angiogenesis thus sustain a mechanistic insight and a biological rationale for clinical development of ALK1 targeting drugs for cancer indications. Further studies, relying on differential specific target gene activation by each ligand alone and in combination will reveal important molecular information to more clearly understand the underlying molecular mechanisms of this synergy favouring the angiogenic switch (Ongoing studies).

As discussed earlier, BMP9 also binds to ALK2 in non-EC cells. In the event that ALK2 is also expressed by the PNETs, the effect we observed in terms of tumor burden may not be totally attributed to impairment of ALK1 signaling in the tumor vessels triggered by BMP9. Since we analyzed total mRNA levels of ALK1 target genes to evaluate on-target effects in this study, a more detailed investigation on tumor ECs should be more informative. In fact, inhibitory treatment of Rip1-Tag2 tumor-bearing mice with RAP-041 also diminished Id1 messenger RNA expression in the EC compartment, comparatively to control treated mice with isotype matched IgG (personal observation). However, since we have not inferred about ALK2 expression in PNETs nor in the isolated vascular fragments, we cannot totally exclude that ALK2 signaling, triggered by BMP9 and/or BMP10, may be also affected by the soluble ALK1-Fc, that predominantly sequesters these ligands. Such studies on ALK2 should therefore be performed, even though ALK2 has been described to preferentially bind to BMP7 (224).

Another important aspect to further consider is whether RAP-041 is effectively binding primarily to BMP9 during the Rip1-Tag2 tumor progression, stunting angiogenic ALK1-derived activity. To answer that question we have analyzed BMP9 knockout mice in the Rip1-Tag2 tumor context, in order to evaluate the role of BMP9 in the PNET model tumorigenic pathway. Alternatively to the BMP10 KO mice, BMP9-KO mice are viable and fertile (128). Validating our previous results, BMP9 indeed plays a direct critical role in tumor development in a dose-dependent manner (personal observation).

Last but not least, other antiangiogenic targeting approaches while conveying promising initial tumor growth suppression, tumors eventually become refractory and dissemination of metastatic lesions becomes dramatically successful (14, 19-21, 219). ALK1 inhibition studies should carefully assess the metastatic dissemination index, in order to prevail as more advantageous alternative therapeutics. Analysis of hepatic material from PNET-tumor bearing mice treated with RAP-041 for two weeks (between 10 and 12 weeks of age) did not alter the number of metastatic *foci* (personal observation). These results corroborate previous studies associating *Id1*

suppression in endothelial progenitor cells and reduction of pulmonary macrometastasis but maintenance of micrometastatic *foci* in a breast cancer model (225).

Because tumors most frequently become refractory to antiangiogenic agents in long-term trials, it is mandatory to evaluate such scenario with the RAP-041. Four week long treatment of Rip1-Tag2 mice with the ALK1 inhibitor RAP-041 in a regression trial from 12 to 16 weeks of age renders sustained inhibition of tumor growth and vessel formation (personal observation). However, metastatic dissemination under these conditions is yet to be analyzed (ongoing studies). Moreover, a four-week treatment (8-12 weeks of age) of a transgenic mouse model of breast cancer (MMTV-PyMT) has generated a 60% reduction in tumor burden and 80% decrease in the number of metastatic *foci* in the lungs (personal observation). These results strongly suggest that ALK1 inhibition is beneficial in two different tumor models, both by modulating primary tumor growth but perhaps more importantly, by additionally counteracting metastatic scattering.

ALK1 targeting agents as monotherapies have been incredibly successful so far. In phase I clinical trials, Dalantercept/ACE-041, the human counterpart of RAP-041, elicited one response in a HNSCC patient and 6 other patients presented with stable disease. Extended cohorts of HNSCC, RCC and endometrial cancer are presently being recruited for Phase II clinical evaluation.

Main findings of article I:

- ALK1 signaling impairment affects the angiogenic switch and tumor growth
- Pharmacological modulation of ALK1 physiology exhibits therapeutic benefit in a PNET model
- TGF-β and BMP9 synergize to improve EC response to other proangiogenic stimuli
- ALK1 and ALK5 act coordinately in the PNET vessel formation

3.2. Article II - Deficiency for endoglin in tumor vasculature weakens the endothelial barrier to metastatic dissemination

Experimental preclinical studies have accumulated evidence for the refractoriness of tumors that have been subjected to VEGF-targeting drug treatment (14, 19-21, 226). There are several studies suggesting a number of mechanisms of resistance to antiangiogenic therapies (14, 41), including upregulation of additional proangiogenic stimuli in response to treatment (19, 227), enhanced protective coverage of pericytes (228), mobilization of bone marrow-derived cells (229) and myeloid progenitors (230). Mechanistic insight into evasive or intrinsic resistance to antiangiogenic therapy comes from recent experimental preclinical trials (226) and it is still unclear how these evasion mechanisms arise and whether they are endothelium specific or a commonly induced therapy effect.

Up until now, only cases of acquisition of antiangiogenic resistance towards VEGF-targeting agents have been published. In an attempt to better characterize alternative antiangiogenic targets we evaluated the contribution of endoglin to tumor vessel formation and malignant development. Our study, however, unequivocally shows that resistance to antiangiogenic treatments can also arise as a result of targeting alternative pathways in the tumor-associated vasculature. The broader spectrum of the concept warrants caution and calls for detailed studies on benefits or caveats of using certain drugs indiscriminately.

As previously described, we confirmed that endoglin presents with prevailing expression in the tumor in actively proliferating endothelium. Such an event coupled with a strong enhancement of endoglin expression during the activation of angiogenesis, clearly suggested a key involvement of endoglin in tumor progression. By analyzing the role of endoglin during PNET formation in Rip1-Tag2; *Eng +/-* and wt control littermates, it became clear that endoglin is seemingly needed for the angiogenic switch as manifested by a clear reduction in the premalignant pancreatic angiogenic lesions both at 9 and 12 weeks of age. The initial delay in tumor progression due to endoglin absence was eventually compensated by generation of a comparably functional vasculature and tumor burden in both endoglin deficient and control mice. Even though the angiogenic insult did not affect the primary tumor growth, the metastatic dissemination was significantly enhanced in mice with endoglin depletion. These unexpected observations were also mirrored in breast cancer EO771 tumors and Lewis Lung carcinoma, in the endoglin heterozygous and tamoxifen-inducible EC-specific knockout (*Eng*[iKOe]) mice. Our results are strongly corroborated by the enhanced levels of circulating soluble endoglin in metastatic cancer patients (205-208). The soluble form of endoglin functions, in this case, as a systemically circulating extracellular domain of endoglin that binds to a number of ligands.

In order to more extensively comprehend the events leading to the increased metastatic scattering in the absence of endoglin, we were prompted to evaluate several possibilities: is it a tumor cell autonomous effect translated in enhanced invasive properties or is the endoglin-deficient endothelium more lenient to malignant cell shedding and homing. The tumor cells-derived contribution was ruled out by

analysis of a panel of prometastatic genes delivering no significant differences. Careful analysis of tumor isolated ECs, however, revealed a pronounced increase of expression of the EMT/EndMT-related transcription factor Twist. Also, the tumor vasculature of endoglin-ablated mice displayed suppression and redistribution of the vascular marker CD31 with concomitant enhancement of the mesenchymal marker αSMA. For the first time, the TGF-β/ALK5-induced cell plasticity program of EndMT (231) has been implicated in triggering metastatic dissemination by permeabilizing the tumor-associated vessels from tumors refractory to an antiangiogenic hit. These original results strongly support endoglin as indispensable for endothelial integrity, whilst its absence facilitates tumor aberrant cells intra- and extravasation to and from the cardiovascular system. An option that should be investigated in the future relies on the use of TGF-β inhibitors in cooperation with endoglin suppression. Such a cumulative effect would feasibly help revert the EndMT phenotype, as we showed in transmigration assays, by sealing the "open" endothelial barrier triggered by endoglin attenuation. Furthermore, the use of ALK5 inhibitors would additionally impinge upon other tumor microenvironment layers. Most studies on the role of TGF-β in cancer primarily rely on this cytokine effect on the cancerous epithelial cells and on how it affects their cellular plasticity, more specifically through EMT-mediated metastatic dispersion. Interestingly, the majority of the tumor studies under ALK5 signaling suppressive conditions evaluated primary tumor growth, EMT and the net metastatic index, but despite being, in those studies, only a side observation ALK5 inhibition tends to also negatively affect the tumor vasculature (232-235). Our study, on the contrary, focuses essentially on an endoglin-absence-derived shift of signaling, tipping the balance into an intensified effect of TGF-β through ALK5 on the tumor vasculature. Whether the vasculature leniency to tumor cells' intra- and extravasation mediated by TGF-β can be prevented, by blocking TGF-β/ALK5 signaling *in vivo* warrants further investigation. Interestingly, combinatorial inhibition of VEGFR2 and CXCR4, an important chemokine receptor involved in malignant cell colonization of distant sites of metastasis through EMT, inhibited metastatic dissemination more extensively than either monotherapy alone (236).

To circumvent the emergence of resilience towards an antiangiogenic insult it has been suggested that cooperative efforts of targeting additional pathways (19) or alternative cellular components in the tumor (226), such as pericytes or cancer associated fibroblasts. Collective actions would conceavably contribute to weaken the rich tumor microenvironment and represent an advantage to finally annihilate the malignancy. Given that endoglin ablation exhibits strong resemblance to VEGF inhibition in terms of tumor relapse and adaptation to the absence of their proangiogenic effect (Figure 7), we sought to analyze the effect of combined inhibition of both pathways. Indeed, concurrent impairment of endoglin and VEGF signaling in the tumor-forming endothelium strongly arrested tumor formation and sequential metastatic scattering. Collectively, these results suggest a strategy to bypass the evasion mechanisms of resistance induced independently by VEGF or endoglin impairment. More importantly, these studies also evoke that the cumulative action of multiple targeting the tumor vasculature is of benefit in cancer disease treatment (Figure 8). These results should of course be validated with pharmacological inhibitors for both VEGFR signaling and endoglin.

A pertinent question remains for ALK1 and endoglin cooperative signaling. Despite the clear phenotypic similarities of HHT patients bearing *Alk1* or *endoglin* mutations, the similarities of phenotype between ALK1 and endoglin complete knockouts and strong *in vitro* evidence for physical interaction between ALK1 and endoglin and their ligands, it is also apparent that endoglin and ALK1 may possess alternative signaling traits and differential ways to regulate the endothelium. This is evident at least in our tumor studies, but we cannot confirm that developmental angiogenesis is accordingly regulated.
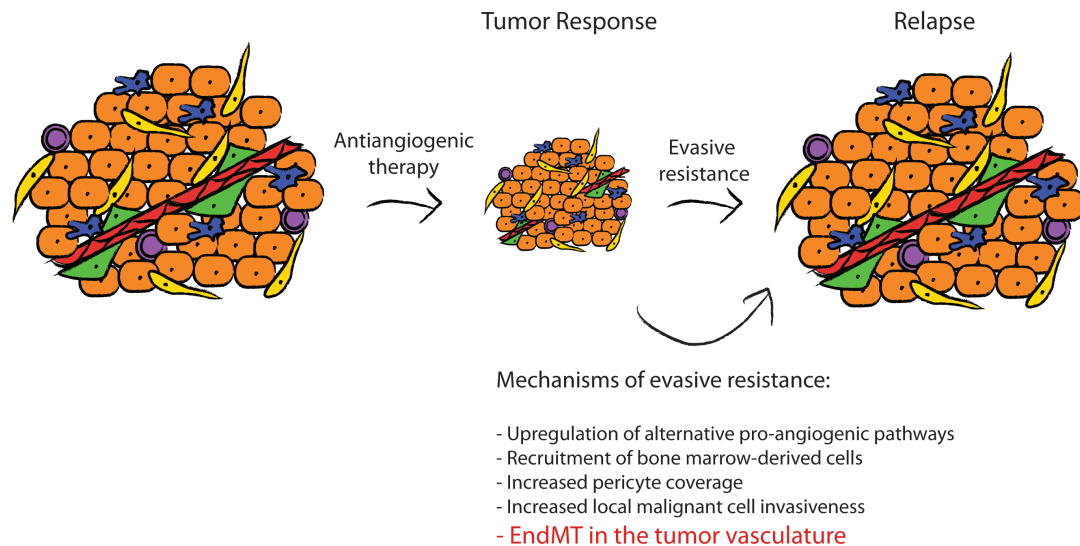

Tumor Response   Relapse

Antiangiogenic therapy   Evasive resistance

Mechanisms of evasive resistance:

- Upregulation of alternative pro-angiogenic pathways
- Recruitment of bone marrow-derived cells
- Increased pericyte coverage
- Increased local malignant cell invasiveness
- EndMT in the tumor vasculature

Figure 7. Mechanisms of evasive or adaptive resistance to antiangiogenic therapies. Upon treatment with antiangiogenic drugs and a transient tumor growth response to the disturbed endothelium, the tumors manifest strategies to adapt to the debilitated vasculature. These strategies often encompass eventual insensitization towards the drug, revascularization by means of upregulation of alternative pathways, increased local invasiveness where the malignant cells strive to find alternative neighboring vessel beds, or even enhanced tumor cells dissemination to distant sites of metastasis. In Article II, we have for the first time added the EC plasticity mechanism of EndMT (that occurs as a result of endoglin impairment) favoring malignant cell systemic scattering.

Main findings of article II:

- Genetic stunted endoglin transiently affects the angiogenic switch
- Similarly to tumors subjected to VEGF-targeting compounds genetic depletion of endoglin renders tumors refractory
- By means of endoglin signaling impairment, the tumor endothelium becomes more permeable to malignant cell intra- and extravasation to distant sites of metastasis
- EndMT is described as an adaptive mechanism to antiangiogenic strategies
- While tumor vessels become resilient to independent targeting of VEGFR or endoglin signaling, combinatorial therapeutic targeting of both pathways primes the endothelium with increased sensitivity

4. DISCUSSION

The complexity of the TGF-β and BMP pathways by means of redundancy, cooperation or by simply having different levels of regulation reflects the uniqueness and tight tissue-specificity of this intriguing pathway. However, despite the complexity of TGF-β signaling, this pathway may hold central roles in tumor angiogenesis, as suggested by studies targeting its endothelial specific receptors ALK1 and endoglin. Inhibitors for these receptors have already been generated and they hold great promise in the impairment of newly formed tumor vessels, while leaving normal quiescent vasculature undisturbed. This prevents additional induction of collateral effects from targeting the whole vasculature within the tumor-bearing organism.

The pleiotropy and intricacy of TGF-β family signaling conveys effects virtually in all cell types in the body. In cancerous disease, it is well established that TGF-β holds a bipolar role in carcinogenesis, acting as tumor suppressor during the initial stages of tumor development, whilst promoting tumor growth and metastatic spread in advanced stages of disease (237). As a consequence, the potential of using to our advantage the knowledge on TGF-β biology is still not fully embraced. The development and use of inhibitors of the TGF-β family activity for the treatment of cancer may result in disparate effects depending on the stage of the disease. Furthermore, in ECs, the overall result of signaling from TGF-β family receptors is manifold and determined by a plethora of factors: ligand specificity or redundancy, engaged type I, type II receptors and coreceptors (95). In spite of the challenges of the elaborated signaling network outcomes from the TGF-β receptors in the various cell types of a tumor, the dividends for modulating the TGF-β critical network in therapeutic regimens may be incredibly rewarding. The current preclinical data and preliminary clinical results readily support the feasibility of using ALK1 and endoglin inhibitors as angiogenesis counteracting agents.

Potential for combinatorial therapeutic studies

Antiangiogenic therapeutics may create a favorable environment for improved drug delivery and priming the tumor microenvironment with increased sensitivity to cytotoxic drugs (238). The greatest utility of antiangiogenic drugs is therefore unlikely to be achieved with single agent strategies. The still scarce preclinical and clinical data currently available advocate for an antiangiogenic and growth inhibitory effect of attenuated ALK1 signaling in cancer, hence sustaining the clinical development of pharmacological agents blocking ALK1. Furthermore, while ALK1 targeting monotherapies have been exceptionally successful both in preclinical and clinical settings, so far, it is reasonable to anticipate that a combined targeted therapy can represent an agonistic effect in fighting cancer disease.

The multitude of cancers and heterogeneity within each malignancy combined with case-to-case unique demands strongly requests for the incorporation of two or three drugs with different targets that have independently been beneficial. Future studies should therefore embark on such endeavors. Predictably, the best results may most likely be accomplished in combinatorial schemes with either alternative antiangiogenic drugs targeting different pathways, conventional chemotherapeutic drugs or even compounds targeting additional cellular compartments within the tumor stroma. Aiming at various niches in the tumor microenvironment is likely to

capitalize on the benefits provided by principles of enhanced antitumor efficacy, non-overlapping toxicities, spatial cooperation of targeting different cellular categories and last, but not least, the notion that the use of reduced dosages will potentially result in minimized toxicity for the patient and cost reduction for the healthcare system.

Interestingly, VEGF levels are elevated in the aorta, lungs, liver, and intestine of ALK1-deficient mice (90), suggesting that double targeting VEGFR and ALK1 signaling pathways may not only be the route to a more efficacious treatment plan, but may also circumvent the risk of refractoriness to antiangiogenic drugs. Of note, bevacizumab was recently reported to attenuate VEGF-induced angiogenesis in ALK1 deletion-induced vascular malformations in the adult mouse brain (239).

In a human/mouse chimera tumor model, targeting human ALK1 decreased tumor vessel density and improved antitumor efficacy when combined with bevacizumab (156). This study therefore raised the question whether ALK1 signaling may be part of a set of adaptive mechanisms in tumors refractory to VEGF inhibition (156). Unfortunately, no data have been generated addressing such questions. Moreover, Dalantercept/ACE-041 used in combination with sunitinib impaired tumor growth in two xenograft models of VEGF-inhibitor resistant RCC, A498 and 786-O (Acceleron Pharma communication). These two models represent surrogates of RCC tumors that typically display a transitory shrinkage upon VEGF inhibition but quickly restore angiogenesis and resume the tumorigenic program, despite continuation of treatment. In this study, the combined effect of Dalantercept/ACE-041 and sunitinib prevented the restoration of tumor perfusion during the resistant phase of sunitinib-as single agent treatment and decreased tumor perfusion to a greater extent (Acceleron Pharma communication).

None of the ALK1-targeting studies have so far analyzed the potential adaptive effects of ALK1 targeting drugs in prolonged regimens. In order for ALK1 inhibitors to prevail as opposed to VEGF-targeting drugs, such analysis is mandatory.

Given the recent studies suggesting a regulatory crosstalk loop amongst BMP9/ALK1 and Notch signaling coordinating tip *versus* stalk cell specification, one may immediately anticipate possible therapeutic benefit arising from a combinatorial targeting of both pathways in tumor biology.

Neutralization of Dll4-Notch signaling in tumors results in excessive, non-productive angiogenesis with subsequent inhibitory effects on tumor growth, due to poor perfusion-induced hypoxia (240, 241). Furthermore, Dll4 has been reported to mediate tumor resistance to bevacizumab *in vivo* as a compensatory mechanism to VEGF neutralization (242). Pharmacological targeting of Dll4/Notch signaling in preclinical tumor models has been achieved by several different inhibitory strategies. Specific targeting with anti-Dll4 antibodies does not induce overt toxicity and Dll4 has thus emerged as an attractive target for antiangiogenic cancer therapy (243). As Dll4 inhibitors are entering clinical trials for the treatment of solid malignancies, this may pose a novel combinatorial therapeutic opportunity in breast, colon and renal cancer, where Dll4 is selectively expressed by the endothelium of malignant tissues (244-246) and where patients may profit from combinatorial actions.

The acknowledgement that some tumors become refractory to antiangiogenic drugs targeting VEGF signaling (19-21) and our own observation that endoglin suppression

enhances metastatic shedding (Anderberg, Cunha, Zhai *et al.,* under revision) further highlights the urgency of novel combinatorial strategies. Once again, the key for overcoming emergence of mechanisms of resistance to antiangiogenic strategies may possibly be, as we unequivocally show, the combined inhibitory action of targeting endoglin and VEGFR signaling simultaneously. The double intervention seems to enable the tumors to circumvent acquisition of adaptive strategies, previously revealed by targeting each pathway independently (Figure 8).

The phase I clinical trial on TRC105 evokes the combinatorial use of drugs impinging their inhibitory effect on both endoglin and VEGFRs, which have shown to generate an ameliorated therapeutic benefit both in the primary tumor burden but also on metastatic dissemination. In fact, a clinical trial analyzing the effect of TRC105 in cooperation with standard-dose bevacizumab in advanced solid tumors, for which bevacizumab is indicated, has been launched (www.clinicaltrials.gov). Interestingly, a study reported on a patient with HHT1 who had a substantiated response to bevacizumab (247). In this study, the epistaxis episodes became sparser and shorter. In different studies, a patient with HHT1 who received bevacizumab for malignant mesothelioma had a dramatic reduction in GI bleeding from AVMs (248). Also, a HHT patient with severe hepatic disease who received six courses of bevacizumab no longer required liver transplantation and was well 6 months after completing the treatment (249). These studies strongly suggest a strong collective action of double targeting simultaneously endoglin and VEGF signaling to reacquire endothelial homeostasis. Furthermore, studies in our laboratory provide evidence for an agonistic effect by using endoglin and VEGFR targeting strategies to reduce primary tumor burden and metastatic dissemination (Anderberg, Cunha and Zhai *et al.,* under revision).

An interesting question is what would happen in the case of targeting simultaneously ALK1 and endoglin. Would the adaptive phenotype exhibited by endoglin ablation be even further enhanced or would concurrent inhibition of both receptors render a phenotype similar to that observed with multitargeting of endoglin and VEGFR signaling (Anderberg, Cunha, Zhai *et al.*, under revision)? Preliminary observations show that ALK1 and endoglin double partial genetic ablation (Rip1-Tag2;*Alk1*+/-; *Eng*+/-) results in further diminished tumor burden and vascularization compared to Rip1-Tag2;*Alk1*+/- (personal observation), reminiscent of the results obtained by integrating inhibitory insults upon endoglin and VEGFRs signaling. This observation thus suggests once again that ALK1 and endoglin may possess independent alternative routes to impact on the tumor endothelium. Interestingly, attenuation of endoglin signaling did not result in ALK1 signaling attenuation in isolated tumor ECs.

Tumor Response

Relapse

Antiangiogenic therapy

Evasive resistance

Tumor Response

Tumor Response Sustenance

Antiangiogenic therapy

+

- Other antiangiogenic agents
- Anti-EndMT drugs
- Anti-metastatic drugs
…

Evasive resistance bypass
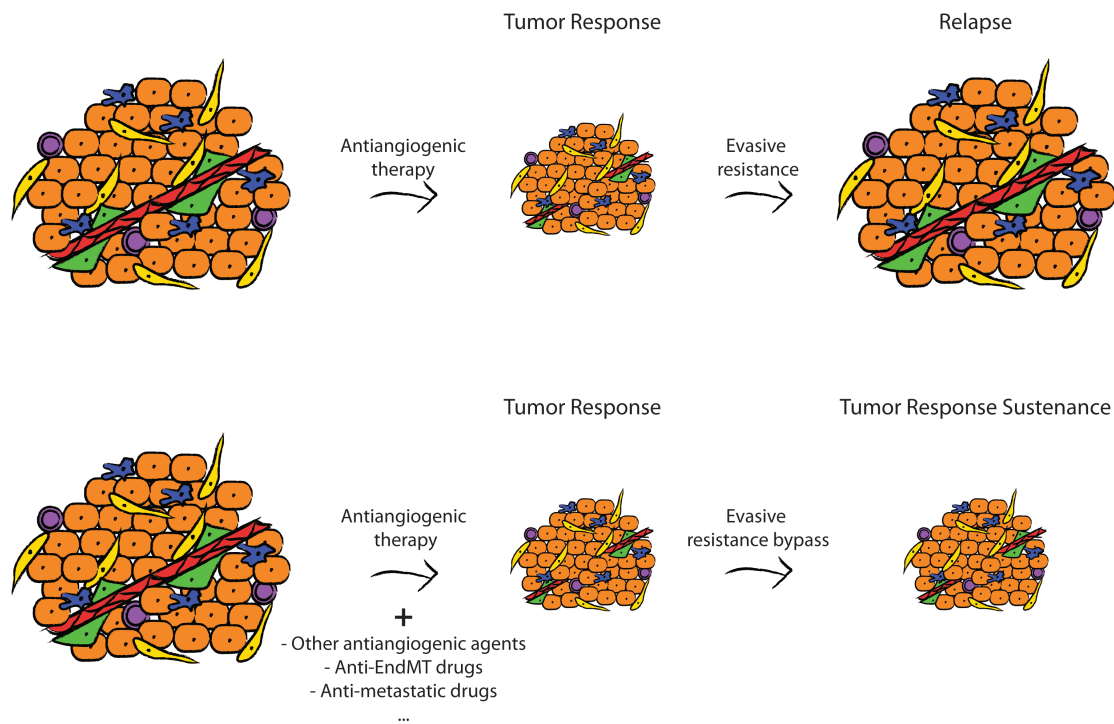
Figure 8. Resistance mechanisms to antiangiogenic therapies are not exclusive of VEGF-targeting approaches. Endoglin impairment also translates in adaptations that render the tumors refractory (Article II). Our studies suggest that antiangiogenic therapies combined with simultaneous targeting the tumor either by using other antiangiogenic drugs, anti-metastatic, anti-EndMT or targeting an alternative cellular compartment in the tumor microenvironment permit to bypass evasive strategies or adaptation to antiangiogenic insults (Article II).

5. PERSPECTIVES

Cancer medical treatment is moving towards personalized diagnostics and therapeutics that aggressively embrace integrative approaches (250). The greatest utility of antiangiogenic drugs is therefore unlikely to be achieved with monotherapeutic regimens. The best outcome may, most conceivably be obtained by combinatorial schemes with either alternative antiangiogenic drugs targeting multiple pathway circuitries, conventional standard of care chemotherapeutic drugs (that perturb proliferative and survival processes) or even additive compounds targeting other non-epithelial cellular compartments and/or interactions within the tumor malignant microenvironment.

Such combinatorial studies should primarily take place in preclinical settings in interdisciplinary teams, in order to close the gap between researchers and clinicians. Collaborative efforts require researchers who can make valuable contributions in terms of treatment plan and predictive benefit, and clinicians who are able to make educated choices of the best drug selection for their patients. As our studies indicate, preclinical studies convey powerful information in terms of efficacy but also warrant caution for treatment plans that may worsen the patients' condition. Optimally, in the future we should better select the patients that are more prone to better response to treatment and thereby preliminarily investigate expression patterns of tumor markers in multiple cancer types. The improvement in patient selection will facilitate the choice of one drug or a combinatorial regimen in detriment of another. Such amended diagnostic and prognostic tools and treatment plan decision strategies in personalized cancer therapy will eventually meet the patient-tailored treatment platforms that can in the future provide significant improvement of patient care, treatment efficacy, drug-dose reduction, toxicity minimization and shrinkage of health care costs (251-253).

In that sense, our studies strongly contributed to bringing the TGF-β superfamily to the antiangiogenic stage. Despite substantial evidence for a preponderant role in angiogenesis, this family has a long neglected therapeutic potential. For the first time, our studies unveiled ALK1 as an encouraging novel target in cancer treatment (Article I), which provide consistent scientific support for clinical trial development. In fact, clinical trials on Dalantercept/ACE-041 have been progressing through a successful phase I clinical trial in patients with solid tumors or refractory multiple myeloma and subsequent phase II trials in expanded cohorts of HNSCC, RCC and endometrial cancer patients. After our pioneering work, a whole new avenue of research has been initiated and conducted to deepen the knowledge on ALK1 and BMP9 roles in vascular biology.

The use of proper preclinical models is also of utmost importance. Initially, monotherapeutic and combinatorial schemes should be tested in preclinical settings with transgenic mouse models that recapitulate the disease in its rightful microenvironment and therefore mimic changes in the multitude of cell types in the tumor. In a follow-up phase, the future of drug testing should rely on human patient-derived tumor xenografts (PDX) (6, 254). The PDX strategy opens a variety of possibilities in terms of therapeutics that can be simultaneously be tested in mice, having the advantage of having the graft mirroring the original tumor pathology and

progression. Moreover, the use of cancer patient surgical resections conveys the possibility to more accurately evaluate disease outcomes and response to various therapeutic cocktails. This strategy prevents the unnecessary subjection of patients to a myriad of drugs, from which they may not benefit from and in addition may worsen their condition by means of increased toxicity levels or cumulative acquisition of drug resistance.

A good example of the risks associated with assuming that a certain therapeutic approach can be beneficial, is the fact that even though according to present knowledge ALK1 and endoglin are tightly interconnected, and targeting them would expectedly generate similar results, our data suggest that endoglin and ALK1 possibly possess alternative signaling routes and mechanisms of EC homeostasis regulation (Article II). In Article II, we have also described for the first time the EC plasticity mechanism of EndMT as an adaptive mechanism to circumvent an antiangiogenic infliction. Our studies show that EndMT occurs in the tumor endothelium as a result of endoglin impairment, which ultimately favors malignant cell systemic scattering. This observation opens a new avenue of research contemplating the future of collaborative effect of antiangiogenic therapies with anti-EndMT drugs.

Furthermore, our study also suggests a strategy to overcome adaptive mechanisms to antiangiogenic therapies by applying integrative approaches of inhibiting two alternative antiangiogenic relevant pathways (Article II). This observation further demonstrates that while antiangiogenic drugs that as single agents induce adaptive resistance, collaborative concurrent action of such drugs can reach impressive results in tumor growth blockade and interfere with systemic disease dissemination.

Further data arising from the clinical trials on ALK1 and endoglin inhibitors will provide additional evidence on the benefit of using alternative routes to affect the tumor endothelium other than primarily impacting VEGF pathways. More insight is necessary to more thoroughly understand the TGF-β pathway and its relevance in vascular biology in order to validate its true potential as an additional, complimentary or even alternative tool in the combat of cancer disease.

Increasing knowledge regarding new therapeutic strategies to extinguish primary and metastatic disease, explanations on how tumors manoeuver to become insensitive to therapy and the use of multitargeting regimens will eventually improve clinical translatability of the experimental preclinical data. As a result, cancer treatment and prognosis will hopefully be dramatically improved.

6. ACKNOWLEDGEMENTS

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