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Transforming growth factor-β in the pathogenesis of breast cancer metastasis and fibrosis

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PhD thesis

Transforming Growth Factor-β in Pathogenesis of Breast Cancer Metastasis and Fibrosis

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Transforming Growth Factor-β in Pathogenesis of Breast Cancer Metastasis and Fibrosis

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Preface

This dissertation is the result of four years of dedicated and intense research on the pathobiology of transforming growth factor-β. Particularly focused on breast cancer and skeletal metastasis and the therapeutic intervention with various sites of TGF-β signaling. Through the study of the role of this growth factor in in vitro and in vivo models of pathology and particularly breast cancer metastasis it is my hope that we have contributed to the unraveling of the cancer code and progressed a small step in the direction of improved cancer treatment.

The study was commenced in March 2005 and finished in March 2009 under the supervision of Prof. Dr. Peter ten Dijke in the department of Molecular Cell Biology and later in close collaboration with Dr. Gabri van der Pluijm at the department of Urology and Endocrinology at the Leiden University Medical Center in The Netherlands. The study was part of a European Union Marie Curie Research Training Network "EpiPlastCarcinoma" (project 005428). A fantastic group of European senior scientists whom through their encouraging support not only functioned as great mentors and teachers but also inspired and broadened our scientific view. The consortium has provided a platform for stimulating cross-boarder fertilization of results and a forum for free discussion of results and future directions.

It has been some hectic years of great challenges and an eye-opening adventure to both the greatly rewarding and at times cruel world of academic scientific research.

The Hague, December 2009
Maj Petersen

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Chapter 1

Introduction

This chapter attempts to explain the paradoxical dichotomy of transforming growth factor (TGF)-β in a physiological setting and in pathologies of cancer and fibrosis. TGF-β control tissue homeostasis in normal cells and in primary tumors and later in pathogenesis of cancer and fibrosis acts, in part, as a promoter of malignancy in advanced diseases. These latter responses depend on autocrine and paracrine effects of TGF-β on the cells and the surrounding microenviroment. This two-sided pleiotropic nature of TGF-β in disease pathogenesis, present both a challenge and an opportunity in the development of new therapeutic intervention.

1.1 Transforming growth factor-β superfamily

TGF-β superfamily members include a large group of cytokines which control a myriad of functions both in development and in adults. Thus far, 34 family members have been identified including TGF-βs, bone morphogenetic proteins (BMPs)/growth differentiation factors (GDFs), activins, inhibins, nodal, and anti-müllerian hormone (AMH). TGF-β has a conserved motif of 9 cysteine residues. Eight of these form a tight cysteine knot and the ninth is crucial for dimerization (reviewed in [1]). The ligands are encoded as large secreted precursor proteins which require proteolytic cleavage for activation. This gives rise to a mature 25 kDa homodimeric signaling peptide [2]. TGF-β was discovered nearly three decades ago as a factor capable of inducing a transformed cellular morphology and growth in soft agar [2, 3, 4, 5] and conversely as an inducer of growth arrest [5, 6]. We now know that three highly homologous isoforms of TGF-β exists in humans, TGF-β1, TGF-β2 and TGF-β3. They signal in very similar ways but their expression levels varies depending on the tissue [7, 1].
BMPs were identified as factors capable of stimulating ectopic cartilage and bone formation [8]. More than twenty BMP family members have been identified to-date [9, 1]. These are less homologous compared to the TGF-β isoforms and each serve different physiological functions. Clustering analysis have revealed that BMP-2 and BMP-4 are closely related as are BMP-5, BMP-6, and BMP-7 (reviewed in [9]). Activin was discovered as a gonadal protein highly expressed in reproductive organs [10]. However, it

is produced in a wide rage of cell types at nearly all stages of development [11]. Recent findings suggest that activin may play important roles in diseases (reviewed in [11]). This chapter will primarily focus on TGF-βs and BMPs and their mechanisms of action in normal tissue homeostasis and tumorigenesis.

1.1.1 TGF-β signaling

TGF-β superfamily members bind to type II and type I serine/threonine kinase receptors. In humans, seven different type I receptors, also termed activin receptor-like kinases (ALKs)1 to ALK7, and five type II receptors have been identified. The type II receptors are TGF-β receptor type II (TβRII), BMP receptor type II receptor (BMPRII), activin receptor type II (ActRII), ActRIIB, and AMH receptor type II. A third group of receptors, the type III receptors include endoglin (CD105) and β-glycan. These are not directly involved in signal transduction but are believed to act as accessory receptors which present ligands to the type II receptors (reviewed in [7, 12, 13, 14]). The possible cytokine-receptor combinations are exemplified in table 1.1.

Table 1.1: Nomenclature of TGF-β superfamily receptors and possible type I and type II receptor-ligand combinations. (adapted from [15]). Abbreviations: ActRII, Activin type II receptor; ALK, activin receptor-like kinase; BMPRII, BMP type II receptor; TβRII, TGF-β type II receptor [15, 16].

Ligand	**Type II Receptor**	**Type I Receptor**
TGF-β	TβRII	ALK5,1,2
Activin, Nodal	ActRII, ActRIIB	ALK4,7
BMP	BMPRII, ActRII, ActRIIB	ALK1,2,3,6

The TGF-β superfamily members bind to specific sets of heteromeric receptor complexes. Solely, TGF-β signals via TβRII and it does so in a complex with ALK5 [17] (see figure 1.1). Except in endothelial cells where TGF-β in addition signals via ALK1 [18, 19]. Activin and nodal signal via ActRII or ActRIIB and either ALK4 or ALK7 [17, 1]. The combinatorial nature of type I and type II receptors for BMP signaling is more complex (see table 1.1). BMPs can use BMPRII, ActRII and ActRIIB as type II receptor in association with either ALK1, ALK2, ALK3, or ALK6 in a cell-type specific and contextual manner. BMP-2 and BMP-4 preferentially bind to ALK3 or ALK6 in complex with BMPRII whereas BMP-6 and BMP-7 most readily bind to ALK2 or ALK6 in combination with either ActRII, ActRIIB or BMPRII (reviewed in [20, 21, 22, 17, 23, 24, 25, 26, 27, 28]). Furthermore, BMP-9 and BMP-10 were shown to signal via ALK1 and BMPRII in endothelial cells [29, 30]. The ligand-driven assembly of the TGF-β and BMP receptors is schematically illustrated in figure 1.1. When BMP is present at the cell surface the heteromeric receptor complex is simultaneous assembled. Type I and type II receptors bind independently to different sites on the BMP ligand

[15, 16]. TGF-β, on the other hand, bind to the type II receptors and this results in an induced-fit conformational change in the receptor that enhances its affinity for ALK5 resulting in a six-piece puzzle of ligand and receptors [15, 16].

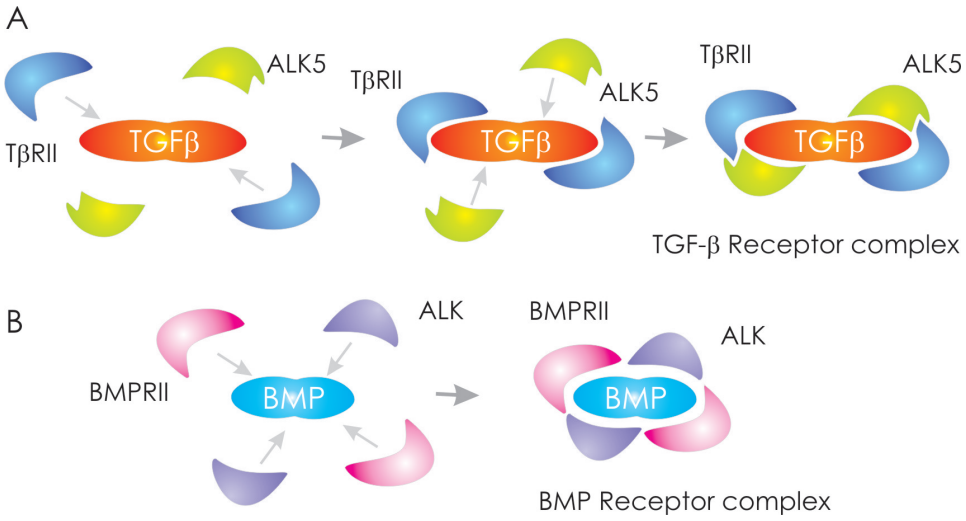


Figure 1.1:
Schematic illustration of ligand-receptor binding of TGF-β and BMP. (A) Active dimeric TGF-β bind to TβRIIs which leads to an induced-fit conformational change in TβRII. This result in recruitment of ALK5 and the type I receptor directly dock into the ligand-type II receptor complex. (B) On the contrary, BMP cell surface avidity leads to simultaneous recruitment of type I and type II receptors. The BMP ligand bridge the heteromeric receptor complex but there is no interaction between the receptors. As an example is shown the binding of BMP to BMPRII, however ActRII and ActRIIB are thought to bind BMPs in a similar manner [15, 16]. Abbreviations: ActRII, Activin type II receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; BMPRII, BMP type II receptor; TGF-β, transforming growth factor β; TβRII, TGF-β type II receptor (reviewed in 285,273).

The activation and bridging of two constitutively active type II receptors with two ALKs results in trans-phosphorylation of the type I receptor kinases. The signal is then transduced through phosphorylation of, the intracellular mediators, the Smads (see figure 1.2). Humans express eight Smad proteins, which can be categorized into three groups; receptor regulated Smads (R-Smads), a common Smad, Smad4, and inhibitory Smads (I-Smads) (reviewed in [12, 13, 14]. The activated ALKs phosphorylate the signal transducing R-Smads, which associate in heteromeric complexes with Smad4 and translocate to the nucleus [31]. Here they regulate transcriptional activation or repression of a diverse array of target genes involved in angiogenesis, immune suppression, growth inhibition, and epithelial-mesenchymal transition (EMT), just to name a few (reviewed in [7, 13, 32]).

Specific R-Smads are used for a given growth factor, whereas Smad4 is a common mediator for all superfamily signaling pathways (see figure 1.2). TGF-β signals via the R-

Smads Smad2 and Smad3, as does activin and nodal. BMPs on the other hand signal via the R-Smads Smad1, Smad5, and Smad8 (reviewed in [7, 32]). However, in endothelial cells TGF-β can also signal via the Smad1/5/8 pathway [19, 18]. Structurally, R-Smads and Smad4 share two homologous proteins domains, the N-terminal MH1 domain and the C-terminal MH2 domain bridged by a less conserved linker region [7, 33]. DNA binding is facilitated through an 11-residue β-hairpin in the MH1 domain. In Smad2 an insert of 30 amino acids upstream of the β-hairpin motif prevents Smad2 from binding directly to DNA [33, 34]. A nuclear localization-like sequence is also present in the MH1 domain. The proline-rich linker region is less conserved and phosphorylated at multiple residues, a mechanism thought to integrate signaling from other kinase pathways. The MH2 domain contains a SXS kinase motif at the extreme C-terminus and mediates Smad-receptor, Smad-Smad and Smad-transcription factor interactions. In order for Smads to bind DNA they must interact with DNA binding co-activators or co-repressors since the affinity of MH1 domain for DNA is low (reviewed in [7, 34]).

Recently, the canonical signal transduction pathway of TGF-β was challenged by the findings that TGF-β activate Smad1 and Smad5 in various tumorigenic cell lines, albeit at much lower levels than Smad2 and Smad3. Phosphorylation of Smad1 and Smad5 occurs through an ALK5-dependent mechanism where the L45 loop in this receptor plays a critical role. Also, ALK2 and ALK3 are needed for this kinase activation [35, 36, 37]. This mechanism of activation was not observed in immortal breast cancer cells and is suggested to be involved in the switch of TGF-β signaling from being pro-apoptotic to pro-invasive and metastatic [36].

The physiological functions of TβRIIIs, β-glycan and endoglin, are not entirely clear. These receptors lack the cytoplasmic domains and are believed to present ligands to the type II receptor [38]. β-glycan is crucial for the binding of TGF-β2 to TβRII, since by itself TGF-β2 has low affinity for the receptors [39, 40]. Endoglin interact with TGF-β1, TGF-β3, activin-A, BMP-7 and BMP-2 solely in the presence of type I or type II receptors [41].

It seems intriguing that such a large range of ligands signal via such a small subset of intracellular mediators. However, the growth factors, receptors, Smads and Smad co-factors are expressed in a cell-type specific and contextual manner and hereby determine the specificity and duration of the signal.

1.1.2 Non-canonical Smad signaling

TGF-β and BMPs can also signal via Smad-independent pathways. These pathways include the rapid activation of mitogen activated protein kinases ERK1/2, JNK and p38, the phosphatidylinositol 3-kinase (PI3K), and Rho-like small GTPases (reviewed in [42, 43, 44]). The kinase kinetics induced by TGF-β or BMPs are cell-type and context dependent. Signals are propagated through auto-phosphorylation of TβRII at tyrosine residues, which leads to trans-tyrosine phosphorylation of the ALKs (reviewed in [45, 43, 44]). Non-canonical Smad signaling pathways have been extensively reviewed in

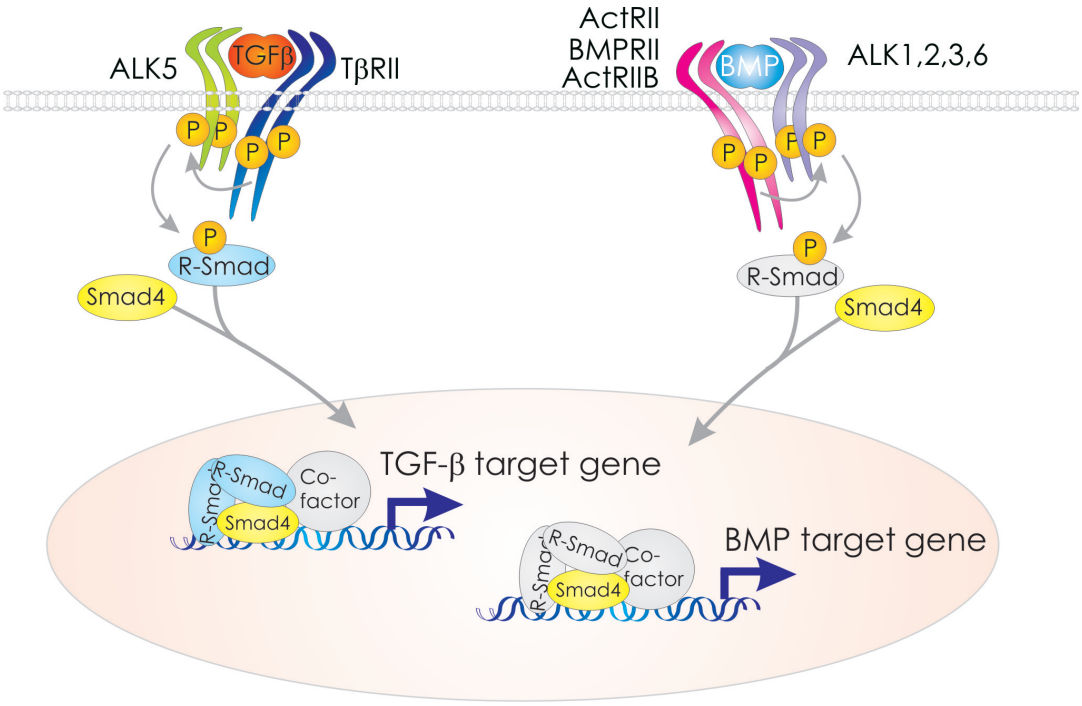


Figure 1.2:
Schematic illustration of TGF-β and BMP signaling. *(Left)*, TGF-β binds to the TβRII which leads to recruitment and activation of ALK5. Smad2 and Smad3 are phosphorylated by type I receptor kinases and form heteromeric complexes with Smad4. R-Smad/Smad4 translocate to the nucleus and regulate TGF-β target genes in conjunction with various co-factors. *(Right)*. BMP simultaneously binds type I and type II receptors on the cell surface. This triggers phosphorylation of the R-Smads, Smad1, Smad5, and Smad8. These form complexes with Smad4 and translocate to the nucleus and regulate specific BMP target genes. Abbreviations: ActRII, activin receptor type II; ALK, activin receptor-like kinase; BMPRII, BMP type II receptor, R-Smad, Receptor-regulated Smad; TβRII, TGF-β type II receptor.

[42, 43, 44] and will only be briefly discussed here. However, Smad-independent signaling regulates, among others, ubiquitination and proteasomal degradation of Smads, as will be introduced next.

1.1.3 Controlling TGF-β signaling

Due to the wide expression of TGF-β family members and their receptors tight regulation is essential. Several regulatory mechanisms have been identified for the signaling pathways. These include extra-cellular ligand encapsulation by natural binding proteins, inhibition of activation factors of latent TGF-β, receptor-interacting partners (BAMBI, SARA and FKBP12), I-Smads, post-translational modification by intracellular E3 ubiquitin ligases, co-repressors, and phosphatases (reviewed in [46]).

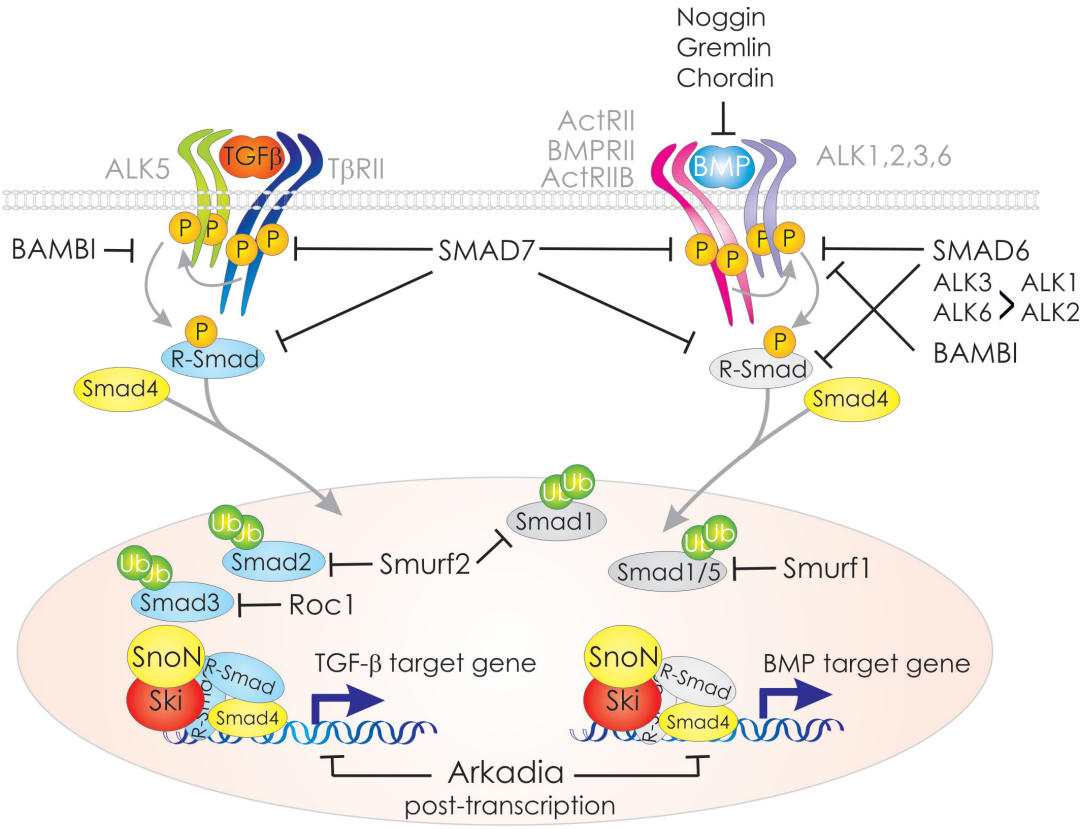


Figure 1.3:
Illustration of negative regulators of TGF-β and BMP signaling. TGF-β and BMP signaling is controlled at various levels in the signaling cascade. The pseudoreceptor, BAMBI, antagonize signaling by all receptors, except ALK2. Smad7 inhibit both TGF-β and BMP signaling at type I receptor and R-Smad level. Smad6 dephosphorylates and target Smad1 and Smad5 and inhibits ALK3/6 in preference to ALK1/2. Smurf1, Smurf2 and Roc1 target phosphorylated R-Smads for ubiquitination in the nucleus. SnoN, Ski and Arkadia target signaling at a transcriptional level. SnoN and Ski are transcriptional repressors which bind R-Smads and Smad4. Arkadia, on the other hand, is an E3 ubiquitin ligase that enhance signaling by terminating Smad transcription through uncoupling the Smad heteromeric complexes from DNA at post-transcriptional level hereby allowing new Smad complexes to bind the promoter and start transcription (reviewed in [43, 46]). Abbreviations: BAMBI, BMP and activin membrane-bound inhibitor; Ski, Sloan-Kettering virus; SnoN, Ski-related novel protein N; Smurf, Smad ubiquitin regulatory factor

Natural occurring antagonists limit the access of TGF-β superfamily members to their cognate receptors. Moreover, TGF-β family members are secreted as inactive complexes with latency-associated peptide (LAP) which sequesters TGF-β in the ECM [47]. BMPs signaling can be antagonized by proteins such as Noggin, Chordin, Gremlin, folistatin and others (reviewed in [9, 48]). The BMP and activin membrane-bound inhibitor (BAMBI) is an ALK-like receptor lacking the intracellular kinase domain. BAMBI antagonizes

heteromeric receptor complex formation by binding to either type I or type II receptors. BAMBI can form receptor complexes with all receptors of the TGF-β and BMP signaling cascade with the exception of ALK2 [49, 50].

I-Smads, Smad6 and Smad7, negatively regulate TGF-β and BMP signaling. This occurs through; directed ubiquitination or dephosphorylation of type I receptors, via degradation of R-Smads, and through disruption of R-Smad-type I receptor interactions and R-Smad-Smad4 complex formation [51, 52, 53]. Smad7 inhibits signaling from all branches of the TGF-β superfamily whereas Smad6 strictly inhibits BMP signaling from ALK3 and ALK6 in preference to ALK1 and ALK2 [54, 53, 51].

Another level of regulation occurs through Smad ubiquitin regulatory factor (Smurf) 1 and Smurf2. Smurfs are E3 ligases which bind Smads at specific PPYX motifs in the linker region. This interaction results in cytoplasmic retention of Smads followed by poly-ubiquitination. Smurf1 interacts with phosphorylated Smad1 and Smad5 and Smurf2 with activated Smad1 and Smad2. Smad3 is not directly targeted by Smurfs instead, the ring finger protein, ROC1 controls the degradation of activated Smad3 (reviewed in [46, 55, 56]). Several ubiquitin ligases and de-ubiquitinating enzymes regulate the degradation of Smad4, including; Smurf1, Smurf2, ectodermin/TIF1γ and FAM/USP9x (reviewed in [7, 46, 57, 58, 59]). In contrast, the E3 ubiquitin ligase Arkadia was recently described to manipulate TGF-β signaling by controlling proteasomal degradation of phosphorylated R-Smads on target genes [56, 46, 60]. This ubiquitination occurs after Smad transcriptional activation and provide a mechanism for rapid signal termination after gene transcription. This is speculated to potentiate the binding of more Smad complexes the promoter and further induce transcription [60].

The co-repressors Ski and SnoN also negatively regulate TGF-β and BMP signaling at transcriptional level by simultaneous binding to R-Smads and Smad4 or interacting with co-factors such as p300/CBP. This inactivates the DNA bound Smad complex and inhibit transcription of target genes and possibly prevent further binding of active Smad complexes to a given promoter (reviewed in [57, 61, 56]).

Moreover, additional feed-back mechanisms have been described of these negative regulators. Smad7 was shown to catalytically activate Smurfs which then evade from the nucleus to the cytoplasm and start ubiquitination. In addition, Smurfs and Arkadia target Smad7 for proteasomal degradation along with associated receptors (reviewed in [46, 60]). Also, TGF-β directly up-regulate expression of *SnoN*, *Smurf1*, *Smurf2*, and *Arkadia* which can results in targeted degradation of SnoN and Ski (reviewed in [57, 46]). The exact sequence of events of these numerous regulatory mechanisms are only beginning to be revealed. Later, we will discuss the roles of negative and positive regulators in pathophysiological settings.

1.2 TGF-β superfamily in development and developmental EMT

Having described the basic concepts of TGF-β superfamily signaling we will now discuss the role of TGF-β and BMPs in a physiological context. Gene ablation studies targeting TGF-β and BMP ligands, receptors, and Smads have identified crucial and differential roles for these growth factors in embryonic development. Such findings have provided new insights to the signalling pathways and led scientist on the track of potential malfunctioning of TGF-β and BMP signaling in human diseases. In this section we will provide a short insight into some important developmental discoveries. Table 1.2 below summarizes the findings from gene ablation studies of players in TGF-β superfamily signaling.

1.2.1 Ligands, receptors, and Smads in development

Homozygous deletion of *tgfβ1* results in embryonic lethality (E9.5-11.5) in 50% of conceptuses due to defects in vasculogenesis [62, 63, 64]. *Tgfβ2* embryos are perinatally lethal [65], and *tgfβ3* knockout mice die shortly after birth [66, 67]. *Bmp3*, *Bmp5*, *Bmp6*, and *Bmp7* null mice are viable, though *Bmp7* $^{-/-}$ mice die within the first 24 hours due to renal failure (reviewed in [68, 32]). Also, *Bmp7* knockout mice have a smaller skeleton and decreased bone mineralization whereas *Bmp3* $^{-/-}$ mice displayed increased bone density and trabecular volume [68]. BMP-3 is, therefore, speculated to antagonize the bone morphogenetic properties of BMP-7. In contrast, homozygous deletion of *Bmp2*, *Bmp4*, and the BMP type I receptors, *Alk2*, *Alk3*, and *Alk6* all gave rise to embryonic lethality (E7.5-10.5) (reviewed in [68]). Strikingly similar phenotypes were observed when TGFβ receptors of either type I or II were targeted with mice showing severe vascular disorders and exhibiting lethality at E10.5. A similar phenotype was observed in *Alk1*$^{-/-}$ and endoglin (*Eng*$^{-/-}$) homozygous embryos (reviewed in [68]).

Selective disruption of individual Smads have determined the specific and overlapping function of these in development. Homozygous deletion of *Smad1, 2, 4*, or *5* result in embryonic lethal phenotypes (E7.5-E10.5) (reviewed in [68]) while mice homozygously deleted for *Smad3, 6, 7*, or *8* are viable though displaying distinct phenotypic abnormalities (reviewed in [68, 69]) (see table 1.2). Embryos deficient in *Smad4* present with the most severe phenotype compared to any other Smad knock out mouse and die shortly after implantation in the uterus [70, 71]. Also, *Smad2* knock out mice fail to undergo gastrulation and mesoderm formation [72]. Double mutants of *Smad2*$^{-/-}$ and *Smad3*$^{-/-}$ exhibit an even more severe phenotype compared to *Smad4*$^{-/-}$ embryos [71], suggesting that Smad4-independent mechanisms through either Smad2 or Smad3 play important roles in development. *Smad5*$^{-/-}$ mice lack blood vessels in the yolk sac and have abnormal vessel formation suggesting severe defects in angiogenesis. Also, apoptosis in the embryonic mesenchyme was observed resulting in lack of stromal cells in much of the embryo, possibly suggesting a role for Smad5 in cell survival and in developmental EMT [73, 71]. *Smad3*$^{-/-}$ mice are viable though they eventually die of impaired immunity [74]

and are resistant to TGF-β-induced fibrosis (reviewed in [68]). Both *Smad6* and *Smad7*

Table 1.2:
Knockout studies of TGF-β superfamily signaling components in mouse models

Gene	Phenotype	Lethality	Ref.
Tgfβ1	Hematopoiesis and vasculogenesis defects	E10.5	[62, 63, 64]
Tgfβ2	Cardiac, lung, craniofacial, limb, urogenital defects	Perinatal lethal	[65]
Tgfβ3	Cleft palate	Post-natal lethal	[66, 67]
Bmp2	Vascular disorders, heart malformation	E7.5-E10.5	[68, 1]
Bmp3	Increased bone density	Viable	[1]
Bmp4	Gastrulation & mesoderm retarded, vascular disorders	E7.5-E10.5	[68, 1]
Bmp5	Viable	Viable	[68]
Bmp6	Viable	Viable	[1]
Bmp7	Small skeleton and renal failure	Perinatal lethal	[75, 76]
TβrII	Severe vascular disorders defects in hematopoiesis	E10.5	[77]
BmprII	Gastrulation defects lack mesoderm (as ALK3)	E9.5	[78]
Alk1	Abnormal yolk sac and vascular defects	E10.5-E11.5	[79]
Alk2	Primitive streak elongation failure, mesoderm malformation	E7.5-E9.5	[80]
Alk3	Mesoderm formation & epiblast proliferation failure	E7.5-E9.5	[81]
Alk5	Severe vascular disorders	E10.5	[82]
Alk6	Normal HSC function	E7.5-E10.5	[68]
Smad1	Embryonic lethal	E9.5	[83]
Smad2	Gastrulation and mesoderm formation failure	E7.5-E8.5	[72]
Smad3	Impaired immunity, protected from fibrosis	Viable	[74]
Smad4	No mesoderm, growth retardation	E7.5-E8.5	[70, 71]
Smad5	Yolk sac & vascular disorders, mesenchymal apoptosis	E9.5-E11.5	[73]
Smad6	Defects in self-renewal, aortic ossification	Viable	[84]
Smad7	Cardiac defects, increased self-renewal	Viable	[69]

mutant mice, were postnatally lethal due to cardiovascular abnormalities [84] and 90% *Smad7*$^{-/-}$ embryos died *in utero* [69]. Thus, highlight the necessity of inhibitory Smads in tissue specific modulation of TGF-β and BMP signaling.

Most of the phenotypes observed in genetically modified animals give rise to severe defects in vasculogenesis. Similar pathophysiological characteristics are observed in patients which carry mutation in *ALK1* or *endoglin*. Such patients present in the clinic with hereditary hemorrhagic telangiectasia (HHT) and display vascular dysplasia and hemorrhages (reviewed in [85, 86]). Other vascular disorders include Marfan and Loeys-Dietz syndrome which are caused by mutations in the *TGFBR1* and *TGFBR2* genes [87]. Also, mutations in *BMPR2* and *ALK1* were identified in patients with pulmonary arterial hypertension [87].

Other genetic alterations have been identified in the BMP and TGF-β pathway. An activation mutation was recently described in *ALK2* giving rise to fibrodysplasia ossificans progressiva (FOP) which causes endochondral bone formation at extra-skeletal sites in response to tissue injury. Finally, patients with mutations in *ALK5*, *TGFBR2*, *Smad4* or *Smad2* are predisposed to some cancers (reviewed in [88, 86]).

1.2.2 TGF-β and BMPs in developmental EMT

Epithelial to mesenchymal transition (EMT) is a fundamental and reversible mechanism governing morphogenesis during embryonic development. It takes place during mesoderm and neural crest formation, palatogenesis, cardiogenesis and organ morphogenesis (reviewed in [89, 90]). EMT is a process whereby epithelial cells acquire a mesenchymal invasive phenotype and become migratory. The normal epithelium is polarized having basal, lateral, and apical membrane domains and cells are laterally attached to one another by tight cell-cell adherence junctions [90]. EMT results in dissolution of cell-cell and cell-matrix adherence through transcriptional repression of cadherins, occludin, and claudin. The cell loose apical-basal polarity followed by shifts in the cytoskeletal dynamics and acquire a spindle-like morphology which facilitates cell migration (reviewed in [89, 91, 90, 92]). Key players in this process include E-Cadherin, vimentin and other adherence junction proteins. These are regulated by various transcription factors including LEF-1/TCFs, SNAIL1, SNAIL2 (also referred to as Slug) [93, 94, 92, 95], the zinc-finger E-box binding homeobox (ZEB1, also known as δ-EF1), ZEB2 (also referred to as SIP1), E12, E47 and TWIST [96, 97, 98, 99].

TGF-β is a major inducer of EMT and is expressed in many embryonic tissues undergoing EMT such as palate, lung, heart, perichondrium [90, 38, 100]. Direct mapping of the three TGF-β isoforms during cardiogenesis in the mouse embryo, led to the discovery of a unique and overlapping pattern of expression which might explain the functional redundancy between the TGF-β isoforms in some settings [101]. Though, in palatogenesis the three TGF-β isoforms play distinct roles when compared head to head [102, 38]. Here, TGF-β1 and TGF-β2 regulates growth and TGF-β3 regulate the transformation of epithelium to mesenchyme [102]. Genetic ablation studies of TGF-β3 in this model system led to complete halt of developmental EMT thus, highlighting the crucial role of this isoform in embryogenesis [103]. The downstream mediators of TGF-β signaling Smad2, Smad3, and Smad4 were also found to differentially regulate EMT i.e. in palatogenesis, only Smad2 was expressed and necessary for TGF-β-induced EMT [94].

The reverse process of EMT, termed mesenchymal-epithelial transition (MET), direct the development of polarized epithelial organs by mesodermal cells during embryogenesis (reviewed in [89, 90]). The MET program is speculated to be controlled by BMP-7 which counteract TGF-β-induced EMT processes [104, 105, 106]. Studying the role of TGF-βs and BMPs in EMT and MET in development have provided great mechanistic insights to the field and into potential levels of dysregulation of TGF-β superfamily signaling in pathological conditions such as fibrosis, tumorigenesis and metastasis. The crucial role of EMT in tumorigenesis will be reviewed in greater detail later in section 1.3.2. In the next sections, we will closely examine the characteristic of TGF-β and BMP signaling in pathobiology of breast cancer and fibrosis.

1.3 Transforming growth factor-β in breast cancer: Angel or devil

Cancer is pathologically defined as a set of diseases characterized by abnormal cell growth leading to invasion of the surrounding tissue which ultimately can lead to metastasis. Carcinogenesis is a result of genetic insults leading to accumulation of errors in vital regulatory pathways or disruptive alterations in the extracellular matrix (ECM). The result being progressive genomic alterations and loss of tissue architecture (reviewed in [7, 107, 108]). Epithelial tissues are the most common site of cancers giving rise to carcinomas [109]. Other types of cancer include, among others, sarcomas, blastomas, lymphomas and leukemia [109]. The human breast is a mass of adipose and fibrous tissue that consists of branching ductal networks which terminates in ductal lobular units (see figure 1.4). Three epithelial cell types are found in these structures, an inner layer of luminal epithelial cells, an outer layer of myoepithelial cells and tubular epithelial cells lining the terminal lobular ducts. The epithelium consist of thin sheets of cells separated from deep complex layers of stroma by the basement membrane [109, 107]. Breast cancer can occur in the lobules but is most common in the ducts and invasive ductal carcinoma (IDC) accounts for (80%) of breast cancer cases diagnoses. IDC is characterized by abnormal growth of ductal epithelial cells which have penetrated the walls of the duct and invaded through the basement membrane into the stroma and adipose tissue of the breast as illustrated in figure 1.4 (reviewed in [7, 107, 108, 109]).

When the primary tumor mass expand beyond 1-2 mm^3 there is a limited diffusion of nutrients and oxygen to the cancer tissue. Solid tumors therefore stimulate the formation of a new blood supply from pre-existing vessels through angiogenesis. Angiogenesis is regulated by a plethora of factors, with hypoxia-inducible factor (HIF)-1 being a key player (reviewed in [113, 114, 115, 116, 85]). The establishment of a new vascular network allows the tumor to further expand and eventually escape the primary site of origin. Cells acquire an invasive and motile mesenchymal phenotype through induction of EMT [89, 117, 118]. Metastasis results as invasive cancerous cells escape from the primary tumor mass. These cells evade the tissue and travel via blood or lymphatics to seek out sites to establish micrometastatic colonies (reviewed in [110, 119, 112]).

In the 1880s, Fuchs and Paget postulated, the "Seed and Soil theory" saying that though studying cancer cells (the seed) is important for characterization of metastatic diseases observing the properties of the tissue whereto cancer cell metastasize (the soil) may also reveal valuable insights into metastatic peculiarities in cancer patients [120, 121]. Now 200 years later, this theory is still of high importance as it addresses the organ specificity of metastatic progression and ascribes a key role of the primary and distant microenvironments for directing cell fate in malignant progression [122, 107, 108, 110].

In this section we will discuss the dual roles of TGF-β in mammary carcinogenesis during primary tumor growth and review the role and regulation of epithelial plasticity in cancer.

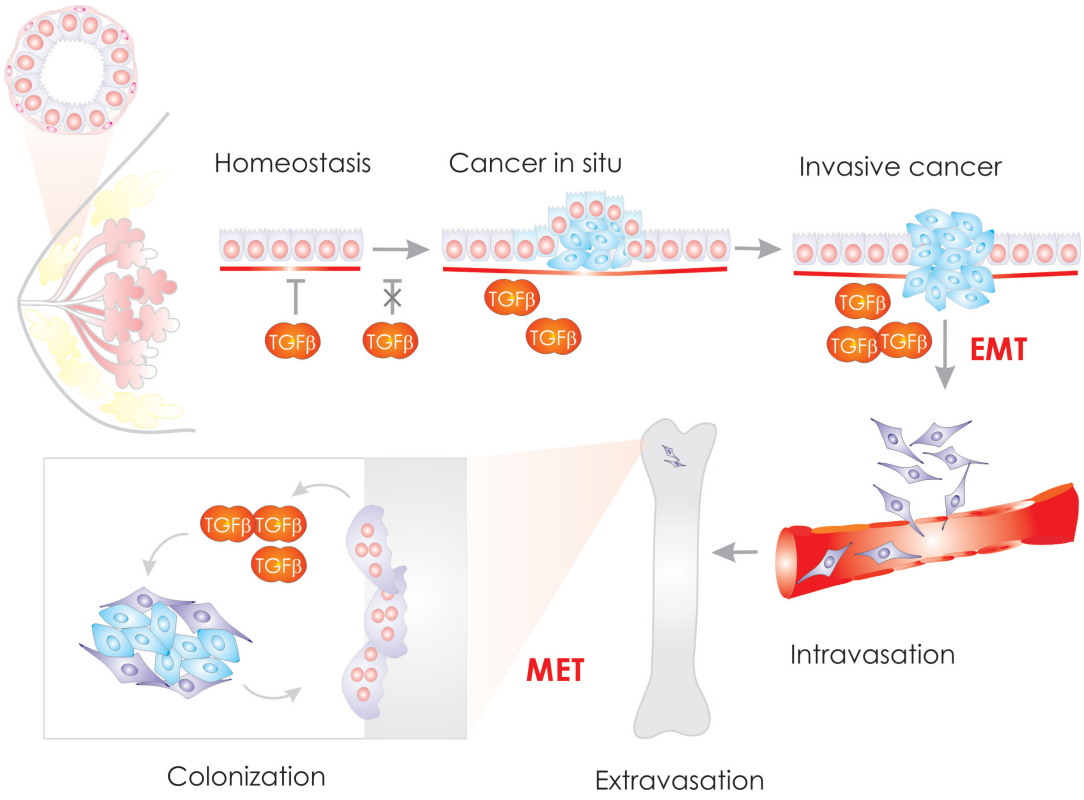


Figure 1.4:
From mammary cancer in situ to breast cancer bone metastasis. The human breast is composed of ductal and lobular acini structures embedded in a fatty collagenous stroma. Polarized epithelial cells line the lobules and ducts and are separated from the stroma by thin layers of myoepithelial cells and the basal layer. In normal tissues, TGF-β controls cellular homeostasis. However, as a result of multiple oncogenic events cells start to hyper proliferate (cancer *in situ*) and the mass eventually becomes invasive and breaks through the basement membrane. At this point in time the tumor-suppressive effect of TGF-β is frequently lost and instead TGF-β promote invasion and metastatic dissemination through induction of EMT. Cancer cells extravasate at secondary sites, such as the vascular bed of the bone, partially de-differentiate through MET, and metastatic progression can proceed. In bone, (*inset*), the tumor cells induce the establishment of a vicious cycle of osteoclastic bone resorption, growth factor release, and cancer cell proliferation. Abbreviations: EMT, epithelial-mesenchymal transition; MET, mesenchymal-epithelial transition. (adapted from [110, 111, 112])

1.3.1 The primary tumor

Sustained basal release of TGF-β controls homeostasis in normal tissues and premalignant lesions. Growth factors of the TGF-β superfamily are essential regulators of apoptosis, cytostasis or survival and angiogenesis depending on the cellular contextual setting and the differentiation status of the target cells. Dysregulation of TGF-β signaling is a critical event in multiple cancers. The pro- and anti-tumorigenic events which takes place in early stages of mammary carcinogenesis will be discussed in this section.

Cytokine expression in mammary carcinomas

Cancers can adopt several mechanisms to promote oncogenesis such as gene amplification, epigenetic inactivation, frame-shift activation and silencing or gain-of-function mutations. Such carcinogenic events can result in altered expression patterns of growth factors such as TGF-βs and BMPs (reviewed in [7, 110, 123]). In breast cancer patients, a functional polymorphism was recently identified in the promoter region of the *TGFB2* gene [124]. This polymorphism enhances the transcriptional activity and expression levels of *TGFB2* and was correlated with lymph node metastasis independent of hormone receptor status [124]. In contrast, a general polymorphism in the *TGFB1* gene has been identified which result in increased serum levels of TGF-β1 and is associated with reduced risk of breast cancer in postmenopausal women [125].

In advanced mammary carcinomas, TGF-β expression can be observed in and around malignant epithelial cells. Intensive staining is observed at the invasive front of tumors and correlates with malignant progression and poor prognosis (reviewed in 11). Samples from breast cancer patients with skeletal metastasis showed elevated TGF-β1 and TGF-β3 protein in plasma, which may in part be derived from bone [126, 127, 128, 129, 130]. Also, intratumoral determination of TGF-β1 protein levels (median of 86.7 pg/mg) were correlated with menopausal status. Premenopausal women displayed increased TGF-β1 levels and high intratumoral TGF-β1 was associated with shorter disease free survival in node-negative patients [126]. Together, these findings suggest that increased systemic levels of TGF-β could predict the severity and stage of invasive breast cancer.

The BMP isoforms are expressed at various levels in carcinoma cell lines [131, 132]. Specifically, BMP-2 was shown to induce tumor growth via ALK3 [133, 131]. On the contrary, increasing aggressiveness of a panel of breast cancer cell lines was associated with loss of BMP-7 expression [134, 135]. Moreover, in clinical samples of primary mammary carcinomas, loss of BMP-7 expression was correlated with poor prognosis [134]. In estrogen receptor (ER) negative breast tumor samples, hyper-methylation and silencing of the BMP-6 promoter was observed [136, 137].

Thus, epigenetic regulation of TGF-β superfamily ligands results in altered expression patterns and genetic dysregulations may confer either a tumor suppressor or tumor promoting effect depending on the stage of malignancy and cytokine affected.

Alterations in TGF-β superfamily receptors or Smads

The type II receptor gene is sensitive to loss of mismatch repair function in tumors with microsatellite instability (MSI). MSI frame-shift mutations in the third exon of *TGFBR2* results in loss of receptor function and have been identified in gastric, colon, glioma, and lung cancers but not observed in breast tumors with MSI (reviewed in [7, 123]). One group have reported the occurrence of somatic point mutations in the *TGFBR2* gene in breast cancer [138]. This was correlated with advanced and recurrent carcinomas. Such mutations were bi-allelic missense mutations in the kinase domain or transmembrane domain of TβRII and thought to mediate receptor conformational changes or inhibit the kinase activity (reviewed in [43]). In line with these findings, loss of TβRII, assessed

immunohistochemically, was observed in 55% of women with DCIS and 68% with invasive mammary carcinomas and directly correlated with a more aggressive phenotype [139]. Together, these two studies suggest that loss of TβRII could define a transition from low- to high-grade neoplasia and that this loss of TGF-β responsiveness is an important event in progression of mammary carcinomas [138, 139].

Alterations in type I receptors have also been identified in cancer. A somatic inactivation mutation was identified in the kinase domain of ALK5, S387Y, in 16% of invasive ductal carcinomas [140]. The occurrence of this mutation was 40% in lymph-node metastasis [140]. Albeit, another study failed to identify this point mutations in breast cancers [141]. Also, epigenetic hypermethylation of the *TGFBRI* promoter was reported in other cancers resulting in suppression of ALK5 expression (reviewed in [123]). For BMP receptors, germline mutations in *ALK3* have been implicated in disease pathogenesis of juvenile polyposis syndrome (reviewed in [43, 123]). Moreover, in ER-positive breast cancers, expression of ALK3 was demonstrated to be a major hallmark of progression, anti-apoptotic activity and poor prognosis [142].

As mentioned above, higher stromal expression of TβRII is associated with poorer prognosis in patients with breast cancer [138]. When the same set of primary breast cancer samples were analyzed for Smad4 expression, 23% of the biopsies were negative for Smad4 and 41% showed a weak staining compared to the surrounding normal epithelia. Loss of Smad4 was correlated with loss of TβRII and ALK5 and a trend for longer 5-year survival was observed in patient with Smad4 negative tumors [143].

In breast cancer patients, homozygous deletion of *SMAD4* was identified in 12% of cases analyzed and in another report 2% had lost *SMAD4* expression (reviewed in [123]). Also, a commonly used human breast cancer cell line, MDA-MB-468, which originates from a pleural effusion of a patient with distant recurrent disease, lacks both *SMAD4* and *SMAD8* as a result of epigenetic silencing [143]. Moreover, the MCF10 series of human mammary cell lines ranging from non-malignant to metastatic display an inverse correlation between Smad4 expression and increased malignancy [143]. Furthermore, germline mutations have been reported in *SMAD4* and can result in familial juvenile polyposis as reported for ALK3 and patients with this syndrome are predisposed to develop intestinal cancers (reviewed in [43]).

SMAD2 mutations have been reported in the MH1 or MH2 domain though these occurs at very low frequency (reviewed in [144, 123]). Strikingly, in patients with stage II breast cancer, reduced P-Smad2 staining was correlated with a shorter overall survival [145]. In line with these findings, in invasive breast carcinomas nuclear localization of P-Smad2 was an indicator of better prognosis and a less invasive aggressive phenotype [146]. In contrast, P-Smad2 staining in the stromal compartment was correlated with an invasive phenotype [146]. Homozygous deletion of Smad2, in a skin cancer model, result in direct induction of EMT and enhanced tumor aggressiveness [147]. In addition, Smad2 heterozygous mice displayed accelerated tumor formation and progression compared to wild type control animals. Epigenetic silencing of *SMAD8* was described in a third of breast cancers (reviewed in [148, 123]). No other genetic alterations have been reported for R-Smads or I-Smads in mammary cancers. Genetic alterations and

epigenetic silencing of TGF-β, its receptors, and intracellular mediators are considered rare events in breast cancers thus highlighting the pivotal role of TGF-β in malignant breast cancer progression.

Cytostatic and proapoptotic function of TGF-β

The tumor suppressor actions of TGF-β in primary tumors occur mainly through cell-cycle arrest at the early G1 phase. This is mediated via several mechanisms; (i) by induced expression of cyclin-dependent kinase (CDK) inhibitors, (ii) through direct suppression of *c-myc* expression and (iii) through repressed expression of CDK-inactivating phosphatase cdc25A. (i) TGF-β and BMP induce expression of the INK4 (inhibitor of CDK4) kinase inhibitor p15^{INK4b} which bind to CKD4 and CDK6 and prevent their interaction with cyclinD (reviewed in [43, 112]). TGF-β further up-regulate the expression of the CDK inhibitors *p21* ^{CIP1} and *p27* ^{KIP1} in epithelial cells [149, 150] and *p57* ^{KIP2} in hematopoietic cells [151] which bind complexes of cyclinE/A-CDK2. The net result being blockage of CDK activity and cell cycle arrest at G1 to S phase transition [149, 150, 151]. (ii) The oncogene *c-myc* is a key transcriptional inducer of cell proliferation. TGF-β suppress *c-myc* transcription by direct binding of Smad3/4 to the *c-myc* promoter (reviewed in [43, 112]). Furthermore, (iii) TGF-β both suppresses expression of the CDK-inactivating phosphatase cdc25A and negatively regulates it through RhoA/p160^{ROCK} mediated phosphorylation, which acts downstream of TGF-β [152]. Phosphorylation of Cdc25A inhibits its phosphatase activity ultimately leading to inhibition of CDK2 kinase activity and G1 cell-cycle arrest [152].

TGF-β has a dual role in apoptosis and can either target a cell for death or survival. In a physiological setting, whether a cell lives or dies is determined by cell-autonomous and environmental factors [153, 89]. Much remains elusive in this field and the search for transcriptional cofactors and target genes which can induce or disrupt the pro-apoptotic signals continues. Critical target genes of TGF-β have been identified in various cell types i.e. the TGF-β-inducible early response gene 1, a transcription factor, is involved in the regulation of apoptotic genes, the signaling protein GADD45β, an upstream activator of p38 kinase, which induces apoptosis via activation of the caspase pathway (reviewed in [43]), Bcl2-homology domain-only factor is an activator of apoptotic factors which mediate mitochondrial activation and apoptosis, PI3K, which promotes survival through phosphorylation of Akt and others (reviewed in [153, 43, 154, 89]).

The inhibitor of TGF-β and BMP signaling, Smad7, additionally promotes TGF-β-induced apoptosis of epithelial cells through transcriptional repression of an inhibitor of the pro-survival factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) (reviewed in [153]). Smad7 might also interact with β-catenin and LEF-1 in a TGF-β-dependent manner and hereby promote nuclear β-catenin translocation and apoptosis [155]. Furthermore, Smad7 was found to activate p38 via TGF-β-activated kinase 1 (TAK1) and MAPK kinase (MKK)3 and hereby induce apoptosis [156]. In line with these findings, overexpression of Smad7 induced apoptosis and downregulation of Smad7 decreased the sensitivity of the cells to TGF-β-induced apoptosis in epithe-

lial cells (reviewed in [153]). Though in the highly metastatic 1205-Lu melanoma cells overexpression of Smad7 did not alter the proliferative capacity or their resistance to TGF-β-induced growth arrest [157].

Recently, the human telomerase reverse transcriptase (hTERT) was found to be a direct downstream target of TGF-β. Smad3 bind the hTERT promoter with the transcription factor E2F-1 and rapidly repress gene transcription resulting in inhibition of telomerase activity and eventually senescence or apoptosis [158]. BMP-7 can also repress telomerase activity through a similar mechanism resulting in programmed cell-death of cervical cancer cells [159, 160]. Also, BMP-2 and BMP-5 were shown to induce apoptosis in a cell contextual manner [161, 162, 163]. BMP signaling via the canonical Smad pathway is thought to promote survival whereas BMP-induced MAPK signaling promote apoptosis. Additional, pro-apoptotic mechanisms of TGF-β through both Smad-dependent and Smad-independent signaling and the crucial cross-talk with other signaling pathways have been extensively reviewed in [43].

TGF-β and BMP can confer their actions through direct regulation of ID (inhibitors of differentiation/DNA binding) proteins. ID proteins, i.e. ID1, ID2, ID3, and ID4 are dominant negative transcriptional repressors belonging to the basic-helix loop helix (bHLH) family of transcription factors. They lack the basic DNA binding basic region but can dimerise with conventional bHLH transcription factors and antagonize their actions [164]. ID1, ID2, and ID3 are abundantly expressed in most tissues and confer critical regulation of angiogenesis both during embryogenesis and cancer [165, 166]. TGF-β inhibits the expression of ID1, ID2, and ID3 which results in inhibition of cell proliferation and mitogenic signals [167] and reviewed in [112]. Paradoxically, *ID1* is an immediate-early TGF-β target gene and later *ID1* expression is repressed by TGF-β [167, 168]. *ID1*, *ID2* and *ID3* are also classical BMP target genes which mediate differentiation of osteoblasts, fibroblasts, epithelial cells, and endothelial cells. (reviewed in [167]). Though BMP enhance $p21^{CIP1}$ and suppress *c-myc* expression, the BMP-induced ID1 response limit the cytostatic response in epithelial cells [169]. The antagonistic regulation of *ID* genes by TGF-β and BMPs is thought to partly explain the dichotomous roles of these cytokines in disease pathogenesis [167]. The regulation of ID genes by TGF-β family members are cell-contextual and the exact mechanisms remains to be elucidated.

Thus, in a primary tumor setting, TGF-β and BMPs efficiently activates pro-apoptotic or cytostatic responses either through inhibition of CDK activity, suppression of critical cell-cycle genes, through inhibition of telomerase activity or by induction or repression of ID proteins.

Tumor angiogenesis

The primary driving force of angiogenesis is low oxygen tension. Tissues respond to hypoxia by producing soluble pro-angiogenic factors which induce the remodeling of the local microenvironment, stimulate endothelial cell proliferation and migration, and form new capillary networks. HIF-1 is a key mediator of angiogenesis and its expression is

directly stabilized in response to hypoxia (reviewed in [116, 170, 171]). HIF-1 activates a myriad of hypoxic-responsive genes a crucial target being vascular endothelial growth factor (VEGF) [172].
TGF-β superfamily members play pivotal roles in vasculogenesis and angiogenesis. This was immediately evident from findings in genetically modified mouse models where angiogenesis is dramatically affected by defects in TGF-β signaling components (see table 1.2)(reviewed in [85]). TGF-β has a dual role in vascular remodeling with pro-angiogenic and anti-angiogenic properties [19, 18, 85]. In endothelial cells TGF-β can signal via ALK5/Smad2/3 or through ALK1/Smad1/5/8 [19, 18]. A delicate balance between these two routes of signaling determines the distinct angiogenic response. Signaling via ALK1 stimulates endothelial cell proliferation and invasion via ID1. Signaling via ALK5 mediate maturation resulting in decreased endothelial cell proliferation and migration via primarily plasminogen activator inhibitor (PAI)-1 [18]. Endoglin, the TβRIII, might selectively influence the balance and determine the functional outcome by indirectly inhibiting ALK5 and activating ALK1 signaling [173, 174].

Besides the direct effect of TGF-β on endothelial cells, it can also stimulate secretions of pro-angiogenic factors in tumor and stromal cells such as VEGF-A and connective tissue growth factor (CTGF). Also, a synergistic interplay on target gene expression can be observed between TGF-β and HIF-1 [172, 175, 176, 116, 177]. In addition, both *TGF-β2* and *TGF-β3* are direct target genes of HIF-1 (reviewed in [116].)
Moreover, endothelial cell migration is further facilitated through TGF-β-mediated expression of metalloproteinase such as, MT1-MMP, MMP-9 and MMP-2 and inhibition of tissue inhibitor of MMP (TIMP) 1 in tumor and stromal cells [178, 114]. Blocking TGF-β signaling by administration of a small molecule antagonist to ALK5 or anti-TGF-β antibodies potently inhibit the pro-angiogenic functions of TGF-β *in vivo* [176, 179, 40].

BMP-2, BMP-4, and BMP-6 can also stimulate angiogenesis in various settings through direct up-regulation of VEGF-A secretion in various cell types [180]. Moreover, exogenous addition and ectopic expression of BMP-2 promote tumor vascularization *in vivo* and endothelial cell migration *in vitro* possibly through ID1 and p38 and/or ERK-1/2 MAPK [131, 181]. In addition, BMP-2 stimulates VEGF and placenta growth factor (PlGF) secretion in mesenchymal stem cells (MSCs) and this mechanism is thought to mediate recruitment of nearby hematopoietic stem cells (HSCs) and endothelial cells [182]. Also BMP-6 was identified as a direct stimulator of endothelial cell migration and tube formation through ID1 [165] and ID1 and ID3 are highly expressed in later stages of tumorigenesis [166]. In contrast, BMP-9 and BMP-10 inhibits basal and VEGF-induced angiogenesis *in vitro* [29, 30].

Interestingly, the BMP antagonist Gremlin was identified as a pro-angiogenic factor and shown to be highly expressed in stromal and endothelial cells in tumor xenografts in mice and in the tumor vasculature in tissue samples [183]. Surprisingly, Gremlin did not block BMP-4 but potentiated BMP-4-induced angiogenic responses *in vivo* [183]. In contrast, the antagonist of BMP-2 and BMP-4, Noggin, blocked basal levels and BMP-2-induced angiogenesis of human tumor xenografts in mice [131]. Collectively, evidence from *in vitro* and *in vivo* demonstrates critical roles of TGF-β and BMP in tumor

angiogenesis through direct and indirect angiogenic stimuli on tumor cells and cells in the host microenvironment.

Having introduced the pleiotropic nature of TGF-β in several aspects of primary tumorigenesis, we will in the next section review the tumor promoter functions of TGF-β in EMT, a later stage of malignancy critical for distant metastatic spread.

1.3.2 Epithelial plasticity

Epithelial-mesenchymal transition is an important mechanism during embryogenesis whereby quiescent epithelial cells acquire a motile mesenchymal phenotype, as was briefly introduced in section 1.1.2. In pathogenesis, EMT provides a crucial mechanism for cancer cells to acquire a motile, invasive phenotype with enhanced disseminative capacities [90, 38, 184, 89]. Carcinoma cells displaying an EMT-induced mesenchymal phenotype are often apparent at the invasive border of a tumor mass (see figure 1.4). However, EMT has received skepticism from pathologists who claim that spindle-shaped cell carcinomas is a rare phenomenon in human cancers (reviewed in [185]). EMT is generally thought to explain the reversible plasticity and enhanced motility of immobile epithelial cancer cells. This section will present the functions of EMT in cancer and the recent findings of EMT inducers and inhibitors in breast cancer settings.

The epithelial plasticity program

In a normal quiescent epithelium, cells are tightly packed and attached by cell-cell and cell-matrix interactions in a cobble-stone like pattern (reviewed in [89, 184]). Epithelial cells are polarized with an apical, lateral, and basal surface and separated from the stromal compartment by a monolayer of myoepithelial cells and a highly proteinous basement membrane [107] (figure 1.4). As a result of carcinogenic events cells become hyper-proliferative and loose their apical-basal polarity through shifts in cytoskeletal dynamics [38, 111, 117]. A hallmark of EMT is the functional loss of the adherence junction protein, E-cadherin and the concomitant loss of the tight junctions and desmosome proteins occludin, claudins, zona occludens (ZO)-1, and desmosome cadherins (reviewed in [89, 91, 118, 117, 111]). Simultaneously, the expression of mesenchymal markers, such as the cytoskeletal protein vimentin is up-regulated and there is an increased deposition of ECM components including fibronectin and collagens [111]. Enhanced expression of α-smooth muscle actin (α-SMA) further supports the rearrangement of the actin cytoskeleton, which align into stress fibers. Furthermore, up-regulation of N-cadherin and matrix proteases lead to the degradation of ECM proteins and render tumor cells invasive. Finally, the disassembly of lateral junctions results in complete loss of apical-basal polarity, reorganization of the cytoskeleton and complete phenotypic transitions of cancer cells into a fibroblast-like mesenchymal cells (reviewed in [184, 89, 117, 111]).

Besides TGF-β, several oncogenic signaling cascades can trigger EMT, such as receptor tyrosine kinases, Wnt and Notch. However, TGF-β is potent inducer that regulate a plethora of factors involved in transitional epithelial plasticity. Downstream mediators

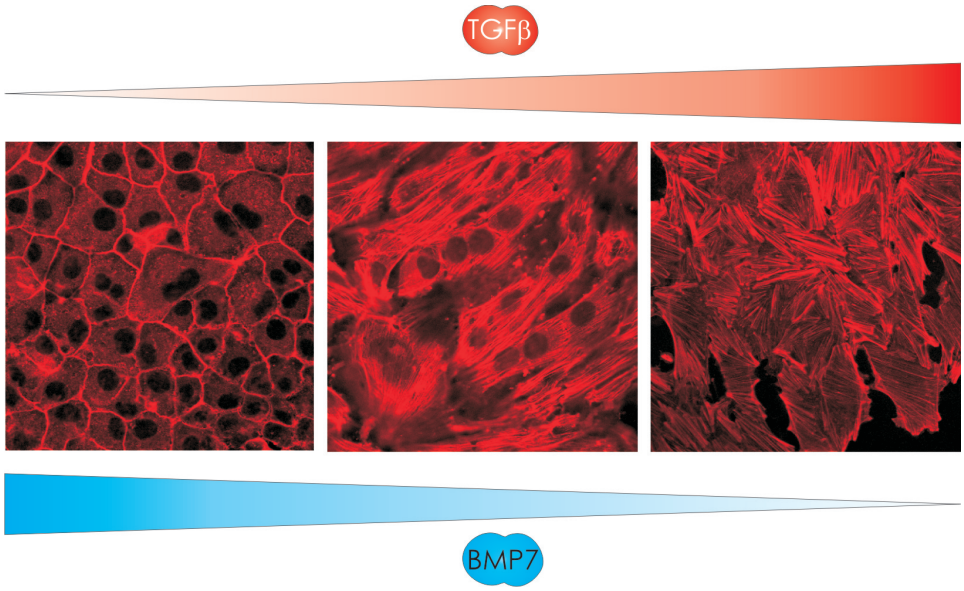


Figure 1.5: EMT induced by TGF-β and reversion by BMP-7-induced MET
The murine mammary epithelial cell line, NMuMG, undergo EMT in response to TGF-β. The cytoskeletal changes can be visualized by staining actin stress fibers with phalloidin. Complete transition from a cuboidal to an elongated fibroblastoid phenotype is observed and extensive mesh of actin stress fibers are formed in response to TGF-β. BMP-7 is suggested to counteract TGF-β-induced EMT and reverse the mesenchymal phenotype through MET and restore the epithelial cobble-stone characteristics though this have not been observed in NMuMG cells [89, 91, 134, 106].

and target genes of TGF-β include transcription factors, matrix protease, proteolytic enzymes, and cytoskeletal molecules (reviewed in [89, 91, 118]).

In vitro EMT models

There are a few *in vitro* models which recapitulate the events of TGF-β-induced EMT [186]. Most of these cellular systems originate from either kidney or mammary epithelium and include, NMuMG (namru murine mammary gland) epithelial cells [186], murine mammary carcinoma cell lines RT3 and 4T1 [187], MDCK (Madin-Darby canine kidney cells), murine MCT proximal tubular epithelial cells, the murine mammary EpH4-EpRas-EpXT cell system [188, 189], the MCF10 human breast cancer cell series consisting of four cells lines ranging from nonmalignant to metastatic [190], human primary mammary epithelial cells (HMECs) [99] and primary keratinocytes [147]. Figure 1.5 illustrates the mesenchymal-like changes in the actin cytoskeleton occurring in NMuMG epithelial cells which undergo EMT in response to TGF-β.

TGF-β superfamily receptors and Smads in EMT

Studies in cellular model systems have provided important cues about the precise sequence of events and mechanisms governing EMT and the role of TGF-β herein. Ectopic expression of a constitutive active ALK5 receptor mediated trans-differentiation in NMuMG cells [186, 191]. In addition to ALK5, the activin and nodal receptors, ALK4 and ALK7 could also induce EMT when constitutive active receptors were over-expressed in NMuMG cells [191]. Direct inhibition of these receptors, with small molecule antagonists, potently blocked and reversed TGF-β-mediated EMT in NMuMG cells [37, 192, 193]. Also, the TGF-β-induced migratory and invasive properties of RT3, 4T1, and MDA-MB-231 cells could be blocked by ALK5 kinase inhibitors [187, 194].

Smad4 was shown to be crucial in this process since depletion of Smad4 by RNAi resulted in a complete halt of TGF-β-induced EMT in NMuMG cells [195]. Smad3 and more weakly Smad2, when over-expressed in combination with Smad4, could induce EMT of NMuMG cells [186, 191]. Furthermore in this cell line, ectopic expression of Smad7 completely inhibited TGF-β-induced EMT [191].
In a model system of embryogenic EMT, the palate system, only Smad2 was expressed and necessary for TGF-β-mediated EMT [102, 38]. Also, in a squamous skin tumorigenesis models over-expression of activated Smad2/3 was shown to increase cell motility [196]. In contrast, a homozygous deletion of Smad2 in keratinocytes triggered EMT in skin tumors in mice [147]. This was observed by down-regulation of E-cadherin expression and induction of vimentin, α-SMA, and SNAIL1 [147]. Together these observations strongly confirm a critical role for TGF-β as a potent inducer of EMT.

Transcription factors in EMT

EMT is driven in part by a set of transcription factors that mediates either gene repression or activation of key EMT players. Many transcriptional regulators are responsive to TGF-β and functionally repress the cell-cell adhesion molecule E-cadherin (reviewed in [89, 91, 98]). These include the zinc-finger transcription factors SNAIL1, SNAIL2, ZEB1, ZEB2, and the bHLH transcription factors TWIST, and E12/E47 [197, 198]. The transcriptional regulator HMGA2 (high mobility group A2) is an additional factor with an important regulatory role in EMT [199, 200]. Recent studies show that TGF-β require HMGA2 to orchestrate the EMT program [200, 201]. HMGA2, Smad3, and Smad4 form a ternary complex on the promoter sequence of *SNAIL1* and mediate gene transcription [201]. Ectopic expression of HMGA2 induce the mesenchymal phenotype in mammary epithelial cells, independent of TGF-β [201]. This occur through direct up-regulation of *SNAIL1*, *SNAIL2*, *ZEB1*, *ZEB2*, and *TWIST* but not *E47* and *E2-2* [201]. SNAIL1 is a major regulator of EMT but cannot complete the transition single handed. Knockdown of SNAIL1 can reverse the HMGA2-induced mesenchymal phenotype but not restore cell membrane expression of E-cadherin [201]. Loss of SNAIL1 results in a concomitant decrease in *SNAIL2*, *ZEB1*, and *ZEB2*. *TWIST* expression levels are not affected by SNAIL1 depletion. Thus, TWIST is required for full blown EMT and can explain the continuous repression of E-cadherin in SNAIL1 knockdown cells [201]. Furthermore,

the transcription factor LEF-1, which plays a crucial role during embryonic EMT was recently identified to suppress E-cadherin gene transcription [38, 100, 95, 202]. *LEF-1* expression is directly up-regulated by Smad2-Smad4 complexes [102] and recently, both SNAIL1 and LEF-1 were shown to be necessary for complete loss of E-cadherin and induction of vimentin by TGF-β1 in MDCK cells [95, 202].

The ID protein family can antagonize signaling by bHLH transcription factors, as previously introduced [91, 200, 167]. At early time points, TGF-β induces *ID* gene expression and can later suppress their transcription, whereas BMPs continuously induce *ID1*, *ID2*, and *ID3* gene expression [167]. These opposite functions of TGF-β and BMP on ID gene regulation is suggested to control the delicate balance of mesenchymal transformation or maintenance of an epithelial phenotype [203, 167]. In the EpH4-EpH-XT cell system, expression of *Id1*, *Id2* and *Id4*, was strongly down-regulated as a result of TGF-β-induced EMT [189]. ID1, ID2, and ID3 specifically inhibit the E-cadherin repressors E12 and E47 [203]. Ectopic expression of ID2 blocks TGF-β-induced EMT and migration in mammary epithelial cells [203] and ID2 was identified as the most potent inhibitor of TGF-β1-induced EMT [167]. Paradoxically, when SNAIL1, SNAIL2, or E47 was stably overexpressed in MDCK cells, *ID1*, *ID2*, *ID3* and *ID4* were highly induced and most strongly in SNAIL2 and E47 expressing cells [204]. Together, these observations suggest the existence of delicate balance of negative self-regulatory feed-back-loops.

BMP-7 and MET

BMP-7 can reverse TGF-β-induced EMT (see figure 1.5) [105, 167, 205]. BMP-7 hereby stimulates the maintenance of an intact epithelial phenotype in non-transformed cells or even induce mesenchymal-epithelial transition [105, 134]. This mechanism is crucial in early development and in various pathogenic settings such as kidney fibrosis and malignancy. In a mouse model of renal fibrosis, BMP-7 was described to reverse the TGF-β-induced tubular injury through induction of MET of renal fibroblasts and hereby repair organ function [205, 105]. In line with these observations, BMP-7 was found to counteract TGF-β-induced up-regulation of the mesenchymal marker vimentin in highly metastatic breast cancer cells [134]. Also, reversion of EMT to MET was also observed when a dominant-negative TβRII was over-expressed in mesenchymal cancer cells [185]. The MET program is hypothesized to be the mechanism whereby metastatic tumor cells, subsequent to intravasation and extravasation, reconcile and initiate metastatic tumor growth at the distant site (see figure 1.4) (reviewed in [89, 91, 117]). Although the exact downstream mediators which control the dichotomous functions of BMP-7 and TGF-β on EMT/MET remain to be elucidated the regulation is thought to be partly mediated by the differential regulation of ID proteins [167].

microRNAs in EMT

MicroRNAs (miRs) are small single stranded RNAs that function as transcriptional and post-transcriptional regulators of gene function acting either as oncogenes or as tumor

suppressors depending on the context (reviewed in [206, 207, 208]). Recently, some miRs have been demonstrated to play critical roles in EMT [209]. MiRs implicated in tumorigenesis include let-7, miR-7, miR-10b, miR-21, miR-29a, miR-122a, miR-126, miR-199a*, miR-205, miR-206, miR-221, miR-222, miR-335, miR-373, miR-489, miR-520c and the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) [210, 211, 206, 209, 208, 212, 213, 214] and the list is rapidly expanding. For example, the oncogenic miR-10b was found to be overexpressed in breast cancer and correlate with clinical progression. Furthermore, miR-10b was shown to be directly induced by TWIST [215]. In contrast, loss of the miR-200 family and miR-205 correlates with metastatic breast cancer and these miRs are down-regulated in response to TGF-β-induced EMT [209]. These miRs target the E-cadherin repressors ZEB1 and ZEB2 and hereby inhibit EMT [206]. Furthermore, ZEB1 was found to directly repress transcription of miR-141 and miR-200c. Thus, ZEB1 promotes an EMT-stabilizing feed-forward loop by down-regulating *miR-141* and *miR-200c* expression which relieve *ZEB1* and *TGF-β2* [206]. When ectopically expressed the miR-200a, miR-200b, and miR-205 synergistically reversed the TGF-β-induced mesenchymal phenotype through MET [209]. MiR-21 is described as an oncogenic EMT-specific miR which is highly over-expressed in breast tumors [216]. TGF-β1, BMP-2, and BMP-4 could induce post-transcriptional expression of mature miR-21 and miR-199a [216, 210]. Smad1,2,3, and 5 were found to interact with the DROSHA microprocessor complex and be recruited to the pre-miR-21 complex [210]. Interestingly, BMP-6 was shown to inhibit miR-21 expression by down-regulating the expression of ZEB1 [212]. Finally, TGF-β was shown to induce the expression of miR-155 which is needed for TGF-β-mediated EMT, cell migration, and invasion of breast epithelial cells [217].

Cancer stem cells and EMT

A subject of intense investigation is tumor-initiating cells or cancer stem cells. Recent evidence suggest a role for the EMT program in self-renewal in cancer stem cells [218]. A small population of cells in breast cancers express high CD44 and low CD24 cell surface markers. This minority population is postulated to constitute tumor-initiating breast cancer cells with self-renewal or stem cell properties [219]. Mani et al. recently found that human immortalized mammary epithelial cells that had undergone EMT acquired similar tumor initiating properties as mammary cancer stem cells. When these cells were transformed to a mesenchymal-like state by ectopic expression of SNAIL or TWIST or by stimulation with TGF-β1 a significant increase in the $CD44^{high}/CD24^{low}$ population was observed [218]. Albeit, to render the human immortalized epithelial cells tumorigenic *in vivo* oncogenic H-Ras transformation was additionally required [218].

Smad-independent signaling and EMT

Crosstalk of Smad-dependent and Smad-independent signaling is necessary to drive and maintain TGF-β-induced EMT (reviewed in [38, 188, 44]). Oncogenic Ras cooperates with TGF-β to induce EMT through a series of downstream mediators [196]. In brief,

ERK/MAPK signaling promote disassembly of adherence junctions and induction of cell motility through up-regulation of SNAIL1 [93]. JNK/p38/MAPKs are crucial for reorganization of the actin cytoskeleton [220, 221] and Rho-like GTPases play a role in dissolution of tight junctions [222]. Furthermore, TGF-β-induced activation of PI3K-Akt disrupts β-catenin/E-Cadherin complexes, which results in loss of tight junctions and partial loss of E-cadherin [196, 38, 95].

Thus, TGF-β is a central regulator of EMT acting through a complex network of effectors in cooperation with various signaling pathways. Although we have gained a deeper understanding of EMT in the last decades much still remains to be discovered in this rapidly moving field of science. Through dynamic and transient EMT cancer cells can metastasize to distal loci and commence metastatic tumor growth. Mammary carcinoma cells often spread to bone, liver and lung [9, 223, 224].

Having described the role of TGF-β and BMP signaling at the primary neoplastic site we will in the following sections review what makes the bone such an attractive soil for the breast cancer cells and the recent findings on breast cancer bone metastasis.

1.4 Breast cancer bone metastasis

Dissemination of tumors to secondary organs cannot solely be explained by the blood circulation from the primary carcinoma to the site of metastasis [119, 195, 109]. One third of distant recurrent breast cancers display specific tissue- or organotropic characteristics. Some microenvironments represent a fertile soil for metastatic tumor growth and allow expansion of macrometastasis (reviewed in [108, 109, 110, 225]). The vast majority of micrometastases remain in a non-dividing dormant state due to insufficient growth and survival stimuli in the new microenvironment. One third of women diagnosed with breast cancer will have thousands of disseminated tumor cells but only half of these women will suffer from macrometastatic relapse [109]. The colonization in the bone parenchyma is a complex and a rate-limiting step of the metastatic cascade. In this section, we will review the bone microenvironment and the interplay between malignant breast cancer cells and cells in the bone stroma. The recent findings on models for studying these interactions in animal models and the identification of gene signatures of breast cancer bone metastasis will be highlighted.

1.4.1 The bone microenvironment

Bone is a highly dynamic tissue undergoing continuous remodeling. Bone resorption is mediated by osteoclasts whereas osteoblasts are bone-forming cells. Bone remodeling occurs at distinct sites of basic multicellular units [226] (see figure 1.6). Anatomically, bone consists of diaphysial cortical compact bone and epiphysial spongy trabecular bone. The spongy bone houses the red bone marrow whereas yellow marrow, also referred to as the stroma of the bone, is found in the medulla cavity of diaphysis. Red marrow is a

myeloid tissue whereas yellow marrow mostly consists of adipose tissue and fibroblasts, macrophages, osteoblasts, osteoclasts, and endothelial cells (reviewed in [9, 227, 228]). The mineralized bone matrix is a major reservoir for growth factors among others, TGF-βs and BMPs (see table 1.3) [130, 229, 230]. The BMP storage remains stable with age [230] whereas levels of TGF-βs decline. Expression of TGF-β1 and TGF-β2 varies according to the sites of the skeleton suggesting regulation by mechanical stimuli [229, 130]. BMP-2, BMP-4, and BMP-7 are believed to be the most osteo-inductive BMPs [230]. They attract mesenchymal progenitor cells and act as morphogens to direct the conversion to the osteoblastic bone forming lineage [230].

Table 1.3: Physiological levels of TGF-βs and BMPs in mineralized cortical bone.

Cytokine	**ng/g demineralized bone**	**Ref.**
TGF-β1	188	[229]
TGF-β2	14	[229]
TGF-β3	36	[130]
BMP-2	21	[230]
BMP-4	5	[230]
BMP-7	84	[230]

1.4.2 Physiological niches of the bone marrow

Circulating cancer cells extravasate into the red bone marrow in highly permeable fenestrated sinusoidal vessels as depicted in figure 1.6. Endothelial cells in sinusoids have no connective tissue covering and are in direct contact with the parenchyma of the marrow. These thin-walled dilated structures allow efficient exchange with the peripheral blood and vice versa (reviewed in [228, 231]). Hematopoietic stem cell niches are anatomical micro-structures located in the endosteum of the bone, around blood vessels, and in the sinusoid networks. The niche is composed of fibroblastic stromal cells, adipocytes, and osteoclastic and osteoblastic cells [9, 227] (see figure 1.6). These structures both support quiescence and direct progenitor cell differentiation and migration. In the endosteal niche, hematopoietic progenitors (HPCs) are maintained in a hibernating undifferentiated state and are shuttled to an active vascular stem cell niche to undergo differentiation and exchange with the peripheral circulation [227, 231, 228].

It was recently proposed that pre-metastatic niches are primed in response to endocrine factors secreted by the primary neoplasm [232, 228, 233]. Also, bone marrow-derived HPCs are recruited in vast numbers to primary tumor sites and support migration and intravasation of tumor cells. HPCs were shown to significantly increase the metastatic potential of MDA-MB-231 cells when implanted co-orthotopically [233].

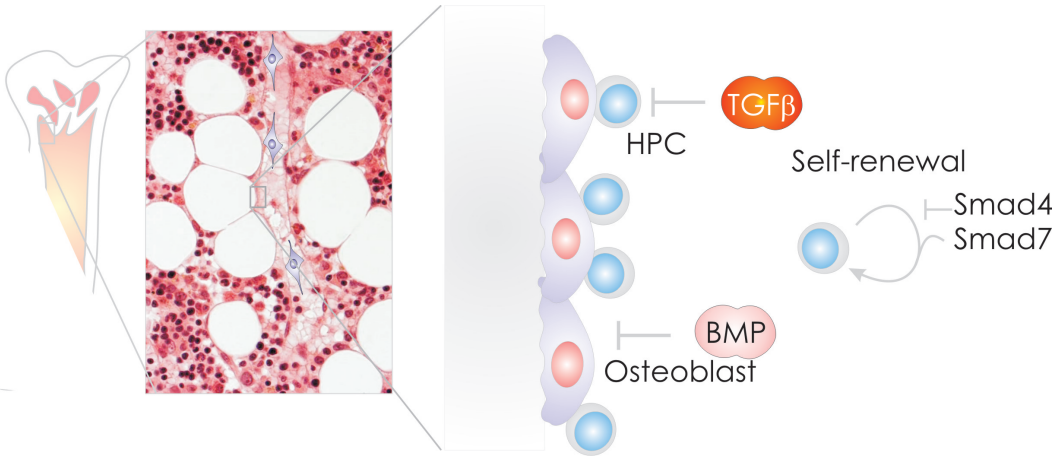


Figure 1.6: The hematopoietic stem cell niche

Hematopoietic stem cell (HSCs) niches are located at the endosteal surface of trabecular bone. These micro structures support quiescence and hematopoiesis. Hematopoietic cells attach to shaped N-cadherin+ CD45- osteoblastic (SNO) cells which line the HSC niche and support long term quiescent HSCs. TGF-β is a potent inhibitor of HSC growth and may maintain HSCs in a hibernating state. BMPs negatively regulate the number of SNO cells and hereby indirectly control HSC numbers. Smad4 inhibits self-renewal whereas Smad7 overexpression was shown to induce this capacity in hematopoietic progenitor cells (reviewed in [228, 231, 227]).

This mechanism is critically dependent on CCL5 expression by HPCs [233]. In addition, platelets transport growth factors and chemokines released from the primary tumor to distant pre-metastatic niches and potentiate activation and mobilization of HPCs [228]. These bone marrow-derived progenitor cells prime the pre-metastatic niche by activating bone fibroblasts and recruiting HSCs and myeloid progenitor cells [232]. (Such myeloid progenitors express markers such as VEGFR-1, c-Kit, Sca-1, and CD11b [232]).

Tightly regulated mechanisms are crucial for the establishment of a pro-metastatic microenvironment [232, 234]. Hypoxic conditions in the primary tumor stimulate secretion of factors such as lysyl oxidase (LOX), via HIF-1-induced transcription, which are crucial for pre-metastatic niche formation and myeloid progenitor recruitment [235, 236]. $CD11b^+$ myeloid cells are speculated to be mobilized from the bone marrow to the tumor via VEGF-A gradients and TGF-β/tumor necrosis factor-α (TNF-α) signaling pathways [235]. Furthermore, the activated bone marrow-derived HPCs induce degradation of the matrix at the pre-metastatic site through induced expression of MMP-9 [234].

Recently, osteopontin and angiopoietin-1 (Ang-1) were found to support tumor cell recruitment to the bone. Physiologically these molecules stimulate osteoclast and HSC adherence to bone [237, 238]. A similar adhesive function is hypothesized for Ang-1 and osteopontin in tumor extravasation and micrometastatic growth. Homing and retention of HSCs is regulated by CXCL12/CXCR4 chemokine gradients [239, 240]. Increased expression of CXCL12 by cancer-associated fibroblasts in the primary tumor induces carcinoma cells to up-regulate CXCR4 and recruit endothelial progenitors [241].

Bones are a major source of CXCL12 and cancer cells metastasize to bone in a CXCR4-dependent manner ([239, 240] and reviewed in [228]).

In vitro, TGF-β is a potent negative regulator of hematopoietic progenitors and stem cells [231] and ALK5 null HSC display increased proliferative capacity [242]. More committed progenitors are less affected by TGF-β growth inhibition and are even stimulated to proliferate in some cases [243, 242]. Recently, TGF-β signaling was found to maintain HSCs in the bone marrow niche in a quiescent/hibernative state and maintain intact stemness properties *in vitro* [227]. On the contrary, *in vivo* ALK5$^{-/-}$ HSCs displayed normal stem cell characteristics suggesting that TGF-β signaling is not a regulator of hibernation [242] (see table 1.4).

BMP was shown to promote stem cell differentiation and exit from stem cell compartments [244] though Smad5 null HPCs display normal stem cell functions *in vivo* [243]. The common mediator of TGF-β and BMP signaling, Smad4, on the other hand, was found to be critical for self-renewal of HSCs [245] (see figure 1.6). Intriguingly, blocking the Smad signaling pathway by forced overexpression of Smad7, potentiates self-renewal capacities of HSCs *in vivo* and modulates cell-fate (reviewed in [231]). Taken together, these findings suggest either an important role for non-canonical Smad signaling or a redundancy between ligands such as TGF-β and activin or receptors such ALK5 and ALK1 in maintenance of intact stemness and quiescence [231].

The interaction between HPCs, endosteal osteoblasts and osteoclasts is essential for the

Table 1.4: Conditional knockout models of hematopoietic stem cells

Conditional HSC KO	**Phenotypic changes (Ref)**
ALK3 $^{-/-}$	Increased niche size and increased LT-HSC [246]
ALK5 $^{-/-}$	Normal HSC function [242]
SMAD4 $^{-/-}$	Defects in self-renewal [245]
SMAD5 $^{-/-}$	Normal HSC function [243]
SMAD7 ectopic expression	Increased self-renewal, modulation of cell fate [231]

maintenance of the HSC niche and mobilization of stem cells [247, 246]. An elegant study, by Gillette et al., describe the intimate interactions of HPCs and osteoblasts and suggests that TGF-β signaling in osteoblasts is down-regulated in response to intercellular transfer from HPCs [247]. Simultaneously, there is an elevated secretion of CXCL12 by osteoblasts [247] thus suggesting that migration and adhesion of HPCs is induced. The HPCs and osteoblasts interact through specific uropod protrusions in the HPC which are phagocytozed by the osteoblasts and delivered to endosomes [247]. BMP signaling through ALK3 was shown to control the size of the HSC niche through regulation of shaped N-cadherin^{+}/CD45^{-} osteoblastic (SNO) lining cells. HSCs attach to SNO cells in the niche and BMP signaling, through ALK3, thus regulate the number of HSCs by negatively regulating osteoblast numbers [246] as illustrated in figure 1.6.

Co-cultures of bone marrow stromal cells and various cancer cell lines display decreased endoglin expression and attenuated Smad2/3 and Smad1 signaling in the bone

stromal cells and decreased cell proliferation [248]. Thus, suggesting that cancer cells modulate TGF-β responsiveness of bone marrow stromal cells to facilitate cancer cell growth in the bone [248]. Taken together, these observations suggest inverse functions of BMP and TGF-β on HSC migration and a key role of the osteoblastic lineage in regulating HSC numbers.

That only a minority of micrometastatic clones evolve into overt bone metastatic lesions is a paradigm. However, this can in part be explained by the concept of tumor stem cells or the necessity of a primed pre-metastatic niche. On one hand, thousands of tumor cells escape from the primary tumor and are circulating in the body. They arrest in sinusoid but only those with stem characteristics can survive, self-renew and form metastasis and perhaps cells of the osteoblastic lineage are critical for their survival. Alternatively, only a limited number of pre-metastatic niches are primed for the tumor cells.

1.4.3 The vicious cycle of bone metastasis

Common sites of skeletal breast cancer metastasis include the pelvis, vertebrae, ribs, cranium, and less commonly the appendicular bones such as long bones [119, 225, 110]. Osteolytic mammary metastases are destructive and associated with pathological fractures and severe patient morbidity [119, 113]. Once settled in a pro-metastatic environment, in the bone marrow, the micrometastatic lesions may evolve into overt macrometastasis through a tumor-induced vicious cycle of osteolysis and tumor progression [249] and reviewed in [110]. This vicious cycle is a result of an advanced symbiotic interplay between cancer cells, bone cells and the bone matrix [113] see figure 1.7. Tumor cells secrete PTHrP, RANKL, IL-1, IL-8, IL-6, and IL-11 which further stimulate RANKL expression by osteoblasts and stromal cells. Simultaneously, cancer cells inhibit secretion of ostoprotegerin (OPG) by osteoblasts and stromal cells. OPG is a soluble decoy receptor which can antagonize RANKL binding [250]. RANKL bind to RANK on osteoclast progenitors and trigger osteoclastogenesis and activation of osteoclasts (reviewed in [119, 110]). Mature and activated osteoclasts resorb bone and release bone matrix encapsulated growth factors, such as TGF-β. This increase in locally active TGF-β alters tumor-cell behavior and further promotes growth and bone destruction at the metastatic site. The net result being a highly efficient self-amplifying feed-forward loop of osteolysis and tumor cell progression [249, 113, 119, 110].

1.4.4 Animal models of osteotropic breast cancers and *in vivo* imaging

Human breast cancer cell lines which give rise to bone lesions when inoculated in immunodeficient mice include selected clones of MDA-MB-231, MCF-7, SUM1315, T-47D and ZR-75-1 [195, 226, 251, 252, 253]. Also, murine epithelial breast cancer lines such as 4T1 and cell lines derived from a conditional knockout mouse of E-cadherin and p53 (the *K14cre;Cdh1*$^{F/+}$*;Trp53*$^{F/F}$ model) develop skeletal metastasis when implanted or-

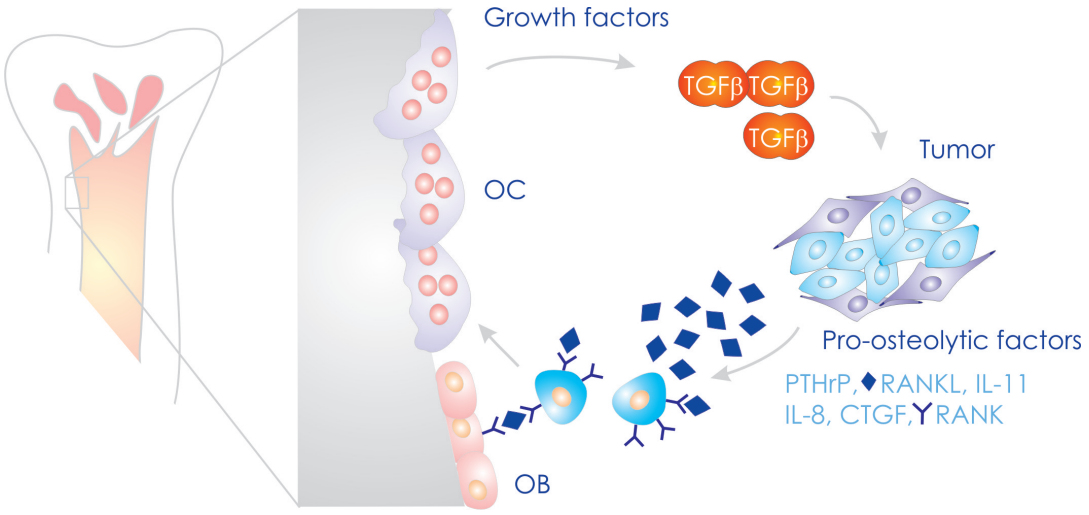


Figure 1.7: The vicious cycle of bone metastasis

Metastatic cancer cells settle in bone marrow along the endosteal bone surface. The cancer cells secrete various osteolytic factors such as PTHrP, RANKL, IL-1, IL-6, IL-8, IL-11, CTGF, and others. PTHrP and RANKL are key stimulaters of osteoclastogenesis. Mature multinucleated osteoclasts resorb the bone and release matrix encapsulated growth factors such as TGF-β, insulin-like growth factor and others. These growth factors act back on the cancer cells and the bone precursors and stimulate further growth of cancer cells and osteolysis and so the cycle goes. The net result being overt osteolytic bone lesions causing severe patient morbidity and pathological fractures (adapted from [249, 119, 113, 110]). Abbreviations: CTGF, connective tissue growth factor; OB, osteoblast; OC, osteoclast; PTHrP, parathyroid hormone related peptide; IL, interleukin; RANKL, receptor activator of nuclear factor-κB ligand.

thotopically in mammary glands [254]. It is important to note that the MDA-MB-435, is a highly metastatic melanoma cell line frequently mistaken for a breast cancer cell line and a commonly used metastasis model [255].

The best described osteolytic breast cancer cell line is the MDA-MB-231 and the vast majority of studies on breast cancer bone metastasis is performed with this model system. Systemic metastasis can be induced with this cell line by intracardiac or tail vein inoculation of approximately 10^5 luciferase positive cancer cells resulting in micrometastatic lesions [226, 134, 256]. These metastases models recapitulate the later stages of tumorigenesis such as survival in the blood circulation, homing and extravasation in a distant parenchyme, establishment and survival in the new stroma, and finally expansion from a micrometastasis to a full blown macrometastatic lesion. Alternatively, cells can be surgically implanted directly in the tibia [134, 256]. Both routes of inoculation give rise to aggressive osteolytic or osteo-inductive lesions [195, 223, 252, 253, 256, 226].

A range of optical imaging techniques are now available for small laboratory animals and include 2D and 3D bioluminescent imaging (BLI), μ-CT and fluorescent imaging. An extensively used modality is BLI which allows quantitative and spatiotemporal analysis of tumor growth of luciferase positive cancer cells [256, 257]. By combining 3D BLI and

μ-CT both the tumor growth and the pathogenic loss of bone microarchitecture can be monitored [257, 258] (see figure 1.8).

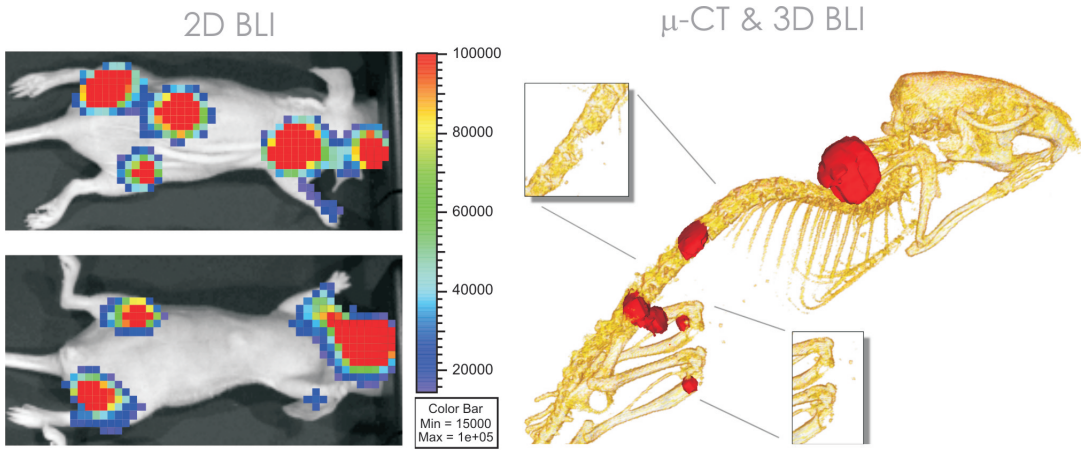


Figure 1.8: Non-invasive 2D and 3D imaging techniques

Two dimensional bioluminescent imaging of an anaesthetized mouse six weeks post intra-cardiac inoculation of osteotropic MDA-MB-231/luc breast cancer cells (*left*). We additionally imaged this mouse by 3D BLI and scanning μ-CT. The two modalities were combined by computer analysis and a three dimensional surface image produced (*right*). Abbreviations: BLI, bioluminescent imaging; μ-CT, micro-computed tomography. [257, 258].

1.4.5 Gene signatures of breast cancer bone metastasis

Transcriptomic profiling of human primary carcinomas have identified gene expression patterns that correlate with poor prognosis and tissue tropism of metastasis (reviewed in [110, 7]). Characterizing such signatures in cancer cells used in metastatic animal models can help to identify clinically relevant and functional mediators of metastatic relapse in breast cancer patients [259, 7, 260, 261, 262]. Systemic administration of parental MDA-MB-231 breast cancer cells result in metastasis of with various tissue tropism such as soft tissues and bone. Re-establishment of cell lines from these metastasis led to the identification of specific gene signatures with lung or bone metastatic activity [260, 261, 262, 259, 263]. Genes which are up-regulated include molecules which mediate survival advantages of the cancer cells in the circulation, in the new microenvironment and which assist in extravasation at specific secondary sites (see table 1.5)[263, 261, 262, 260].

MDA-MB-231 subclones with a specific lung metastatic phenotype were unable to survive and progress when implanted in bone and vice versa for osteotropic clones used in lung metastatic animal models [261]. Thus, highly organ-specific properties of the respective subclones are observed [261]. The lung metastatic clones displayed enhanced expression of *ID1* and knockdown of this gene inhibited orthotopic growth and lung metastatic activity of the breast cancer cells [260]. Except for ID1, no other gene could

Table 1.5: Metastatic gene signatures of MDA-MB-231 cells with specific tissue tropism for lung or bone. [261, 262, 260, 263, 259, 264, 265].

Gene	**Bone clone**	**Lung clone**
CXCR4; chemokine receptor	up	down
ID1; transcriptional inhibitor	down	up
MMP1; matrix metalloproteinase	-	up
MMP2; matrix metalloproteinase	-	up
IL-11; cytokine	up	-
CXCL-1; chemokine ligand	-	up
CTGF; growth factor	up	-
ANGPTL4; angiopoietin-like 4	-	up
VCAM-1; cell adhesion molecule	down	up
SPARC; cell adhesion molecule	-	up

solely induce lung metastasis suggesting that a whole program of events has be initiated for metastatic recurrence [260, 264].

Comparison of the MDA-MB-231 bone metastatic gene signature with primary human breast tumors of patients which presented with distant recurrent skeletal metastasis revealed that CTGF, IL-11, and CXCR4 were indeed up-regulated in the primary mammary tumor tissues [263]. In line with these observations, a similar study in GI101A human breast cancer cells and the lung metastatic clone, GILM2, identified *CXCL-1*, *CTGF*, *ID1*, and *ID2* as being highly expressed in lung metastatic cells versus parental cells along with *IL-8* and *E2F2* [266].
In these reports, tumor metastasis were initiated by systemically inoculating breast cancer cells either intracardially or via the tail vein. Such metastasis models obviate the critical steps of invasion and intravasation and could fail to identify genes involved in early stages of metastasis [99].

A recent report identified a 31 gene profile which could predict whether primary mammary cancers would metastasis to bone with a 50 % probability [267, 268]. Transcriptomic profiling of microdissected RNA from 107 primary tumors with known relapse to the bone, identified the trefoil protein encoding genes *TFF1* and *TFF3* to have the highest differential expression profile in osteotropic recurrent tumors [269]. Also, the FGFR-MAPK signaling cascade was up-regulated in tumors with bone preferences [269]. The gene fingerprint identified correlated to findings in osteotropic MDA-MB-231 versus lung signature MDA-231 cells [263].
Furthermore, bone sialoprotein and osteopontin, two secreted glycoproteins regulated by TGF-β, play important roles in bone turnover and were found to be highly overexpressed in malignant breast tissue and correlate with tumor grade [270]. In patients, serum and mRNA levels of bone sialoprotein and osteopontin were identified to be prognostic indicators for osteotropic metastasis [270].

Moreover, an elegant study by McAllister and Weinberg et al. [271], illustrated how an actively growing tumor inoculated at one site in a host animal can induce tumori-

genesis in a collaterally xenografted dormant tumor [271]. The pro-tumorigenic stimuli was regulated by endocrine activation and migration of host bone marrow cells to the dormant tumor site by the initiating tumor. This was in part induced by systemic secretion of osteopontin by the pro-active tumor [271]. Distant activation of tumors is not a novel observation. In 1985 Dolberg et al. [272] found that a tumor xenografted in wings of chickens could migrate collaterally to the opposite wing where a wound was inflicted and establish growth at this secondary site [272].

Together, these findings stratify the critical role of the host microenvironment and the systemic activation of bone marrow derived cells in tumorigenesis. The fact that metastatic gene signatures can be identified in primary breast cancers and distant metastases suggests that the metastatic phenotype is acquired early during tumorigenesis in the parental cancer population [261]. Many genes have been identified whose expression correlate with metastasis and specific tropism of cancer cells and some have been shown to play a causal role in this process [263, 260, 264, 273, 262]. Delineation of specific signatures for each step of the metastatic cascade will lead to a better understanding of the pathobiology of metastases and the molecular mechanisms predictive of distant metastatic recurrence in women diagnosed with breast cancer.

1.5 Disrupting TGF-β signaling in breast cancer metastasis

Metastatic breast cancer remains an incurable disease [113, 251]. Currently available therapies reduce patient morbidity but cannot induce regression of established bone metastasis [119, 110]. New drug candidates are therefore in desperate need in the clinic along with biomarkers for early detection of breast cancer patients with predilection for bone metastasis. The dichotomous role of TGF-β in malignancy represents a great therapeutic challenge. Targeted strategies must be directed towards the pro-metastatic functions of the signaling pathway and protect the normal homeostatic role of TGF-β, its potent anti-inflammatory actions and the anti-mitogenic effects of TGF-β on primary tumors [274, 275]. In this section, we will review the novel insights and current status of anti-TGF-β therapeutics and present the clinical complications encountered in the fight against cancer.

Several modalities have been attempted to target the TGF-β signaling pathway. These include applications of neutralizing antibodies to TGF-β ligands, small-molecular weight antagonists inhibiting receptor kinase activity, administration of soluble receptors or antisense oligonucleotides to TGF-β (reviewed in [274]). Figure 1.9 and table 1.6 provides overviews of the inhibitory approaches attempted to manipulate TGF-β signaling.

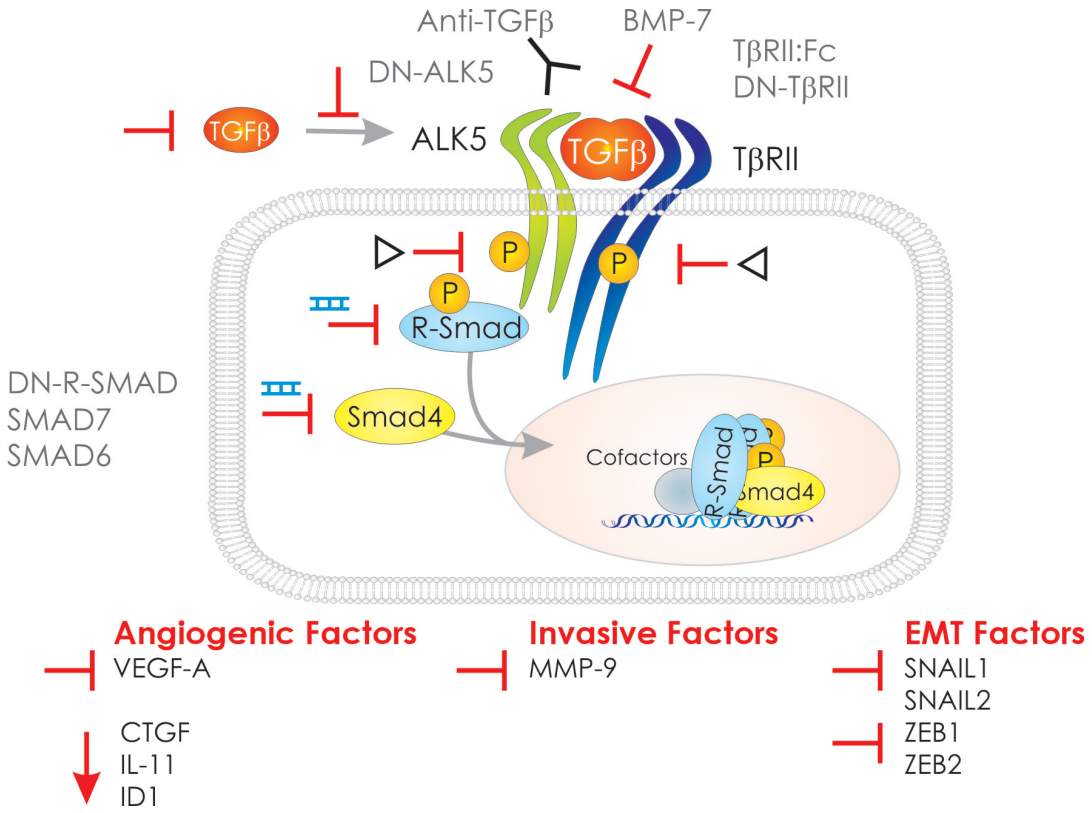


Figure 1.9: Targeting the TGF-β superfamily in breast cancer bone metastasis
Several strategies for targeting the TGF-β pathway have been attempted in cancer models including administration of anti-TGF-β antibodies, small-molecule inhibitors of ALK5 and TβRII, soluble Fc:TβRII, overexpression of dominant negative (DN) receptors or kinase inactivated ALK5, ectopic expression of mutated R-Smads and I-Smads, by inhibiting activation of latent-TGF-β or deletion or overexpression of downstream target genes of TGF-β or BMP signaling and finally by overexpression of BMP-7. Symbol: $\triangle$, synthetic inhibitor; $\nearrow$, antibody; $=$, shRNA.

1.5.1 Targeting ligands

Functional antagonism of TGF-β and BMP signaling can be conferred by anti-ligand antibodies, administration of soluble receptors or natural antagonists, or by silencing ligand expression by RNAi. A neutralizing TGF-β antibody (1D11, Genzyme), recognizing all three isoforms, was used to treat orthotopic metastasis of 4T1 cells in nude mice [276, 270]. A clear reduction in the amount of lung metastatic lesion was observed in mice treated with 1D11 [270]. A downstream mediator of TGF-β signaling, bone sialoprotein was shown to directly mediate the pro-metastatic functions of TGF-β in both MDA-MB-231 and 4T1 cells. This factor was identified by comparing metastasis from treated versus untreated animals by gene array analysis [270, 277]. Artega et al. similarly administered the pan-TGF-β-neutralizing mouse antibody 2G7 to mice with subcutaneous MDA-MB-231 tumors [278]. They similarly found that the antibody could

suppress primary growth and lung metastasis. This was correlated with an increase in the activity of natural killer cells in athymic nude mice [278]. Though antibody administration regimens are approaching the clinic for treating renal diseases these agents have not been tested in a cancer setting [279, 280, 281, 282]. The pharmacokinetics and selectivity of an antibody regimen is generally preferred in the clinic compared to the use of small-molecule kinase inhibitors even though, synthetic inhibitors have better tissue delivery properties (reviewed in [283, 274]).

Targeted knockdown of TGF-β1 by RNAi was pursued in PyVmT and the MDA-MB-435 melanoma cell lines [284, 255]. Loss of TGF-β1 had minor or some effects on primary orthotopically implanted tumor growth, but significantly reduced the number of mice with lung metastasis [284, 255]. The reverse experiment was performed by Muraoka-Cook et al. who engineered a doxycycline-inducible TGF-β1 transgenic mouse model [284]. They observed that enhanced expression of TGF-β1 in late stages of mammary tumorigenesis markedly accelerated metastasis formation [284].

Additionally, the drug tranilast, which is a clinically used anti-inflammatory agent that suppress protein synthesis of all TGF-β isoforms was found to inhibit TGF-β-induced Smad2 phosphorylation [285, 286]. It was shown to inhibit growth of gliomas *in vivo* supposedly through inhibition of invasion and migration of malignant cells [285]. In addition, oral administration of tranilast reduced primary tumor growth of xenografted 4T1 mammary carcinoma cells and potently blocked metastasis to lungs from the orthotopic site [286].

Another agent which acts through a similar mechanism is the naturally occurring proteoglycan, decorin. This protein neutralizes the biological function of TGF-β1 by binding the ligand and sequestering it extracellularly [287]. This results in reduced TGF-β gene transcription and protein synthesis [288]. When decorin was delivered to tumor bearing rats by gene-therapy it significantly prolonged survival of treated animals [288]. Furthermore, systemic administration of decorin to mice with orthotopic mammary tumors reduced primary tumor progression and prevented pulmonary metastasis [289]. Finally, agents which target the catalytic activation of latent TGF-β isoforms are being explored and include small molecule and antibody therapy to αvβ6 integrin [290].

1.5.2 Targeting TGF-β receptors

Over the last decade several groups have studied the function of the TGF-β receptors, ALK5 and TβRII, and examined the changed tumorigenic and metastatic characteristics of breast cancers when expression of these is altered. A few mechanistic studies will be described here and additional information on small molecule antagonists of ALK5 will be described in a later section.

An elegant study by Safina et al. [178] compared ectopic expression of the inactive K232R-ALK5 (KD-ALK5) mutant versus the continuous active T204D-ALK5 (caALK5) mutant in MDA-MB-231 cells. When injected in the tail-vein of SCID mice, the caALK5 cells gave rise to four fold more lung metastasis compared to control mice. Tumors expressing caALK5 grew significantly faster orthotopically than tumors expressing control

and KD-ALK5 cells and the lung metastatic potential of the caALK5 cells was enhanced in athymic mice [178]. There was no difference in primary tumor growth rate of the KD-ALK5 expressing cells versus controls however, KD-ALK5 tumor-bearing mice developed significantly less metastases. Angiogenesis was enhanced in caALK5 expressing tumors compared to control and KD-ALK5 tumors [178]. Depletion of MMP-9 by siRNA in the caALK5 MDA-MB-231 cells inhibited their angiogenenic, tumorigenic and metastatic potential to levels in control injected animals [178]. In a similar approach, caALK5 was found delay Neu-induced mammary tumorigenesis and promote metastasis to lungs [291, 292]. These findings were correlated to enhanced extravasation potential in caALK5 tumor cells [292].

In the tumor same model, expression of TβRII lacking the intracellular kinase domain, resulted in accelerated tumor growth. However, the number of lung metastases was reduced due to the impaired ability of these cells to extravasate the lung parenchyme [292]. Yin et al. overexpressed a dominant negative TβRII in MDA-MB-231 and induced metastasis by intracardiac inoculation [249]. Mice bearing DN-TβRII expressing cells exhibited prolonged survival, decreased tumor burden and reduced bone osteolysis. This was directly ascribed to diminished TGF-β-induced PTHrP secretion in DN-TβRII expressing tumors [249]. In correlation with previous findings, expression of DN-TβRII in the tumor cells had no effect on xenografted tumor growth [249]. The inhibitory effects on breast cancer bone metastasis could be circumvented by expression of the T204D caALK5 receptor in the DN-TβRII in the tumor cells [249].

The development of TGF-β receptor antagonists was initially focused on large-molecule inhibitors such as antibody and antisense therapies [274]. Now, several small-molecule competitive antagonists of the ALK5 kinase have been developed (see table 1.6 for details). These inhibitors can block the ATP-binding site in the ALK5 kinase domain and efficiently prevent downstream signaling [37, 293, 274, 294]. This results in abrogation of TGF-β-mediated responses such as target gene regulation, EMT, migration, and invasion [37, 192, 194]. Several groups have systemically administered ALK5 kinase inhibitors *in vivo*, in small animal models, and reported selective inhibition of TGF-β without adverse side-effect (see 1.6). A few lead compounds have been used in the treatment of metastatic breast cancer and these include Ki26894 [194], an inhibitor from Eli Lily [295, 296], and SD-208 [187].

Ki26894 was given orally to immunodeficient mice (at 0.08 mg/kg) in a preventive setting. It efficiently decreased metastasis formation of MDA-MB-231, reduced osteolytic bone resorption, and increased survival [194]. Another ALK5 inhibitor from Eli Lily was systemically administered (at 25 and 100 mg/kg/day intraperitoneal) to mice injected intracardiac or in the mammary fat pad with MDA-MB-435 cells. This compound had no effect on primary tumor growth but significantly inhibited the incidence and size of pulmonary and skeletal metastasis [295]. The suggested mechanism for reduced metastasis is blockage of TGF-β-induced αvβ3 integrin expression [295]. Furthermore, SD-208 was recently applied in a glioma mouse model and found to enhance survival [297]. Interestingly, these effects were conferred through inhibition of the immunosuppressive effect of TGF-β alone without affecting tumor angiogenesis, proliferation or apoptosis [297]. In

addition, syngeneic 129S1 mice inoculated with 4T1 or R3T mammary tumors received the SD-208 (at 20 or 60 mg/kg/day) in a curative treatment regimen [187]. SD-208 caused a growth delay of both tumor types and dose-dependently reduced the number of metastasis. However, SD-208 had no effect on tumor growth or metastatic potential of R3T mammary tumors in athymic mice [187].

Together these lead compounds hold great promises for the future use of TGF-β inhibitors in clinical applications. Though, one thing to keep in mind is that these inhibitors also target the structurally related type I receptors ALK4 and ALK7 which are used by activin and nodal, respectively [37]. The impact of abrogation of these signaling pathways in tumor biology is currently not known (reviewed in [274, 88]).

Finally, targeting TGF-β signalling intracellularly was attempted through inhibiting endogenous Smad molecules either by gene transfer of the inhibitory Smad7 or by treatment with SARA peptide aptamers [54, 157, 298], both of which can inhibit TGF-β mediated EMT [298].

Table 1.6: Therapeutic strategies to target TGF-β. Abbreviations: NSCLC, Non-small cell lung carcinoma; Vac., vaccine.

Drug	**Target**	**Inhibitory effect**	**Clinical Phase**	**Company and Ref**
GW788388	ALK4,5,7 TβRII	EMT,fibrosis	Pre-clinical	GSK [37, 293]
Ki26894	ALK4,5,7	Metastasis	Pre-clinical	Kirin [194]
SM305	ALK4,5,7	Fibrosis	Pre-clinical	Biogen [299]
SD-208	ALK4,5,7	Metastasis	Pre-clinical	Tocris [187, 297]
Ki26894	ALK4,5,7	Metastasis	Pre-clinical	Kirin [194]
A-83-01	ALK4,5,7	EMT	Pre-clinical	Kyoto Uni. [192]
SX-007	ALK4,5,7	Glioma	Pre-clinical	Scios [274]
LY580276	ALK4,5,7	Oncology	Pre-clinical	Eli Lilly [274]
LY2109761	ALK4,5,7	Oncology	Pre-clinical	Eli Lilly [300]
Tranilast	TGF-β	Metastasis	Clinic	Nuon Ther. [285, 286]
Lerdelimumab	anti-TGF-β2	Fibrosis	Phase III	Cam. A. Tech. [274]
Metelimumab	anti-TGF-β1	Fibrosis	Phase I/II	Cam. A. Tech. [274]
AP-12009	TGF-β2 antisense	Oncology	Phase III	Antis. Phar. [274, 301]
AP-11004	TGF-β2 antisense	Oncology	Pre-clinical	Antis. Phar. [274, 301]
Lucanix	Anti-TGF-β2 vac.	NSCLC	Phase III	NovaRx [302]
Glionix	Anti-TGF-β2 vac.	Glioma	Phase I/II	NovaRx [274]
rhBMP-7	TGF-β	EMT,Metastasis	Pre-clinical	[134, 303]

1.5.3 Alteration of BMP and accessory receptors

Targeted disruption of the BMP signaling pathway has been studied in breast cancer bone metastasis. This was achieved through ectopic expression of a dominant negative BMP receptor, DN-ALK3, in MDA-MB-231 cells. When used in a metastasis model in nude mice DN-ALK3 was shown to reduce breast cancer-induced bone osteolysis compared to

controls [133]. Surprisingly, overexpression of DN-ALK3 blocked IL-11 expression and secretion in comparison DN-TβRII had no effect on the expression of this cytokine [133].

Loss of function of β-glycan impaired motility, invasiveness and induced apoptosis of MDA-MB-231 cells [304]. When orthotopically implanted, β-glycan knockdown cells grew slower and showed no sign of distant metastasis [304]. Thus, suggesting a tumorigenic role of β-glycan in MDA-MB-231 breast cancer cells. In contrast, overexpression of β-glycan could sequester active TGF-β and when MDA-MB-231 cells were subcutaneously inoculated in athymic mice a reduced tumor incidence and growth rate was observed [305]. Overexpression of endoglin in MDA-MB-231 enhanced the invasive properties of the cells *in vitro* through enhanced proteolysis and migration in a 3D co-culture model [306]. Moreover, elevated plasma levels of soluble endoglin in breast carcinoma patients were correlated with shorter overall survival [307] and high metastatic risk [308].

1.5.4 Disrupting the common Smad4 and R-Smads

Overexpression of mutated R-Smads or ALK5 mutated at the R-Smad binding site was shown to disrupt TGF-β signaling in MCF10A breast cancer cells [309, 144]. These cells displayed enhanced primary tumor growth whereas invasion and metastasis to lungs were reduced after tail vein injection [309, 144]. Overexpression of activated Smad2/3 was shown to increase cell motility in a squamous skin tumorigenesis models [196]. In contrast, homozygous deletion of Smad2 triggered complete EMT in skin tumors [147]. Recently, Daly et al. found that TGF-β could activate Smad1 and Smad5 signaling in MDA-MB-231 cells and that this activation mediate the complex formation of mixed BMP and TGF-β R-Smads [35]. Smad1/5 activation by TGF-β did not induce BMP target genes. However, when silencing Smad1 and Smad5 in metastatic EpHRasXT cells the TGF-β-induced tumorigenicity was lost *in vitro* [35].
Depletion of Smad4 in MDA-MB-231 cells increased metastasis-free survival in nude mice and reduced the growth rate of bone-metastatic lesions after intracardiac inoculation [259, 195]. Smad4 depletion correlated with loss of TGF-β-induced IL-11 secretion [195]. Neither intra-osseous nor s.c. injection of the shRNA Smad4 MDA-MB-231 in nude mice affected tumor progression suggesting that Smad4 is crucial for extravasation and initial growth of the micrometastases formation [195]. Together, these results strongly imply that Smad4 signaling is needed for breast cancer progression.

1.5.5 Targeting I-Smads, co-repressors and ubiquitin ligases

Ectopic expression of Smad7 in mouse mammary and human melanoma cells reduced their capacity to form metastasis in lungs and bone, respectively [54, 157]. Osteolysis was significantly reduced and survival prolonged in mice with Smad7 over-expressing metastases compared to controls [157]. These effects are thought to be a result of reduced MMP-2 and MMP-9 activity and reduced expression of PTHrP, IL-11, CXCR4, and Osteopontin in response to TGF-β [157]. Strikingly, ectopic expression of Smad6 had no effect on the metastatic potential of the murine mammary carcinoma cells [54].

Targeting the co-repressors of Smads, Ski and SnoN, in MDA-MB-231 cells enhanced TGF-β signaling and rescued TGF-β-induced growth arrest [61, 310]. The EMT inducers *HMGA2* and *SNAIL2* were up-regulated in response to knockdown of Ski [310] and *Twist* in cells depleted of SnoN [61]. Whereas loss of SnoN in MDA-MB-231 cells suppressed subcutaneous tumor growth in nude mice [61] knockdown of Ski displayed no inhibitory effect on primary tumor growth [310]. Targeted deletion of either SnoN or Ski enhanced metastasis formation after intracardiac inoculation in nude mice [61, 310]. To recapitulate, SnoN may thus play a dual role tumorigenesis which can be inversely correlated with the role of TGF-β signaling in breast cancer metastasis [259, 195, 249, 61]. Ski, on the other hand, has no or little effect on primary tumorigenesis but display anti-metastatic effects in breast cancer [310].

Lastly, knockdown of either Smurf1 or Smurf2 expression by siRNA in MDA-MB-231 reduced their invasive and migratory potential *in vitro* [311]. Ectopic expression of these proteins increased the number of lung lesions of metastatic MCF10A breast cancer cells after tail vein injection in nude mice [311]. An E3 ligase-defective mutant of Smurf2, significantly abrogated the pro-metastatic effect Smurf2 [311]. Surprisingly, neither knockdown nor overexpression of Smurfs induced a concomitant change in TGF-β signaling at Smad2, Smad3, or ALK5 level [311].

1.5.6 Downstream mediators of TGF-β and BMP

Twist is a master regulator of EMT and its expression is enhanced upon TGF-β stimulation in breast epithelial cells [201, 117, 99]. In the 4T1 *in vivo* model system Twist was identified as a key player in invasion and intravasation. Loss of Twist had no effect on primary tumor growth but drastically reduced the lung metastatic potential of orthotopically implanted tumor cells [99]. Blood colony formation was inhibited and the EMT phenotype and *in vitro* migratory potential of the cells abrogated in Twist knockdown cells [99]. High expression of Twist in human breast cancers clustered with invasive lobular carcinoma which directly correlated with loss of E-cadherin expression [99].

Another key player in EMT is Snail1. Silenced expression of this protein significantly reduced the metastatic potential of MDA-MB-231 cells [312].

Onder et al. recently studied the function of E-cadherin, either through protein depletion by shRNA or expression of a truncated form of E-cadherin lacking the ecto-domain (DN-Ecad) [313]. Both methods disrupted cell-cell adhesion of primary HMECs though cells expressing shRNA additionally displayed a fibroblast like morphology [313]. The metastatic potential of the shRNA E-cadherin, tumors, when inoculated orthotopically, was enhanced compared to controls and DN-E-cad [313]. When additionally silencing β-catenin in these cells their ability to form lung metastasis was significantly impaired. In response to E-cadherin knockdown N-cadherin, vimentin, fibronectin, *TWIST*, and *ZEB-1* were highly up-regulated. Thus, loss of E-cadherin results in the induction of its own repressors *TWIST*, and *ZEB-1* [313].

Expression of the homeobox gene *Goosecoid* (*GSC*) is highly elevated in DCIS and

IDC compared to matched healthy breast tissue [314]. TGF-β can induce the expression of *GSC* in HMECs. Ectopic expression of GSC in HMECs or MDA-MB-231 resulted in a morphological transformation to a mesenchymal-like phenotype with increased migratory potential. Tail vein inoculation of GSC-MDA-MB-231 cells highly increased the number of lung metastasis in immunodeficient mice [314].

1.5.7 Interplay between TGF-β and BMPs

We have learned that TGF-β signaling is active in the bone microenvironment in established skeletal metastasis and that TGF-β plays a key role in the self-amplifying process of bone metastasis [119, 315, 113, 224]. BMP-7 can reverse TGF-β-induced responses such as EMT and restore an epithelial morphology possibly through induction of MET, as introduced in section 1.3 (reviewed in [89, 104]). In primary breast cancer, loss of BMP-7 mRNA expression is correlated with formation of clinically overt bone metastases in patients [134]. Also, in line with these clinical observations, BMP-7 expression is inversely related to tumorigenicity and invasive behavior of human breast cancer cell lines [134]. Systemic administration of human recombinant BMP-7 to mice bearing MDA-MB-231 bone metastasis significantly inhibited tumor growth and reduced the cancer-induced osteolytic response *in vivo* [134]. To support of these findings, BMP-7 was overexpressed in MDA-BO2 and skeletal metastases were induced by either tail vein or direct intra-osseous injection. BMP-7 overexpression significantly reduced the number and size of metastatic bone lesions of MDA-BO2 cells [134]. Similar results were obtained in models of metastatic prostate cancer [303]. Taken together, these findings strongly suggest that BMP-7 can inhibit micro-metastasis formation and progression of osteolytic breast cancer. Thus, BMP-7 could be considered a clinically relevant TGF-β antagonist and be used in the treatment of osteolytic bone metastasis.

1.5.8 BMP antagonists

Extracellular BMP antagonists include Noggin, Chordin, twisted gastrulation (TSG), and DAN family members such as DAN and Gremlin1 (reviewed in [48]). Negative regulators of BMPs have been found to be involved in the establishment and progression of osteolytic bone metastasis. Gremlin1 is undetectable in normal and benign breast tissues but highly expressed in tumor-associated stromal cells. Furthermore, 55% of breast cancer tissues, in a large selection of patient samples, were positive for *GREMLIN1* [244]. In line with these observations, the BMP2 and BMP4 antagonists *TSG1* and *CHORDIN* were similarly expressed at higher levels in tumor tissues compared to normal tissue sections [244]. Noggin inhibits BMP-2, BMP-4 and BMP-7, albeit with much lower affinity for BMP-7 [252, 48]. This BMP antagonist was shown to be highly expressed in osteolytic MDA-MB-231 versus the osteo-inductive T-47D or ZR-75-1 breast cancer cells lines [252]. In addition, when Noggin was overexpressed in osteotropic cancer cells progression of cancer-induced bone metastatic lesions was inhibited [252]. Thus, negative regulators of BMPs might confer important restrictions on BMP signaling in the primary

tumor but probably more importantly to the bone stroma and cells herein.

1.5.9 Targeting the breast cancer stroma

Loss of myoepithelial cells and basement membrane leads to progression of breast cancer from DCIS to invasive carcinoma (reviewed in [107]). These observations suggest that breast tumorigenesis is determined not only by somatic or heritable properties of tumor epithelial cells but also by complex interaction with cell types that compose the breast cancer microenvironment. TGF-β is one of the signaling pathways that regulate myoepithelial cell differentiation and play a crucial role in protecting the basement and myoepithelial architecture [316]. This was directly evident when TβRII or Smad4 was depleted which resulted in loss of myoepithelial cells and accelerated tumor progression [316]. Also, resident fibroblasts within the interstitial compartment of a mammary carcinoma transform from a quiescent to an activated state. Such mesenchymal cells support malignant progression through enhanced invasive behavior, increased proliferation, and induction of angiogenesis (reviewed in [317, 107]). Snail1, is a crucial regulator of the aggressive gene expression program. Its expression is not only induced in cancer cells but also in tumor infiltrated mesenchymal cells [317]. Strikingly, *Snai1*-deficient fibroblast showed impaired tissue-invasive and angiogenic functions *in vivo* due to defects in MT1-MMP activity [317]. Thus, Snail1 activity is induced both in neoplastic epithelial cells and in the interstitial fibroblasts and exerts key regulatory mechanisms in malignant progression [317].

Collectively, these observations reinforce the findings that various types of human cancers and cancer-associated stromal cells induce expression of normally latent, transcription factors to acquire characteristics needed to execute invasion and metastasis. Interference with the microenvironment could therefore also be targeted for treatment of DCIS or metastatic disease.

Established minimal residual disease is incurable and although current palliative therapies reduce morbidity, tragically they do not eliminate established bone metastasis [113, 219, 119]. Clinically, at the time of diagnosis, tumor cells might already be present at secondary sites. Therefore, inhibiting later stages of metastasis is a more appropriate for therapy. Furthermore, targeting an inefficient biological process, such as progression of micro- to macrometastatic lesion, should in theory be easier since fewer cells would have to be inhibited [318]. Modulating the paracrine signaling between tumor cells with the local host microenvironment present novel regimens for such therapies. Moreover, careful stratification of patient groups according to the secondary site of metastasis is crucial. Also, identification of markers of metastatic likely-hood and understanding of the mechanisms and factors that influence organ-specific metastatic growth of breast cancer are key steps towards developing such treatment regimes. Thus, tailored drugs which target both the seed and the congenial soil hold great promises for future drug discoveries in breast cancer.

1.6 Pathobiology of TGF-β in fibrosis

In many pathological conditions the production of TGF-β is enhanced such as in diseases of chronic fibrosis. This section will focus on the pro-fibrotic functions of TGF-β in chronic organ fibrosis and the rational for directing therapies towards this pathway.

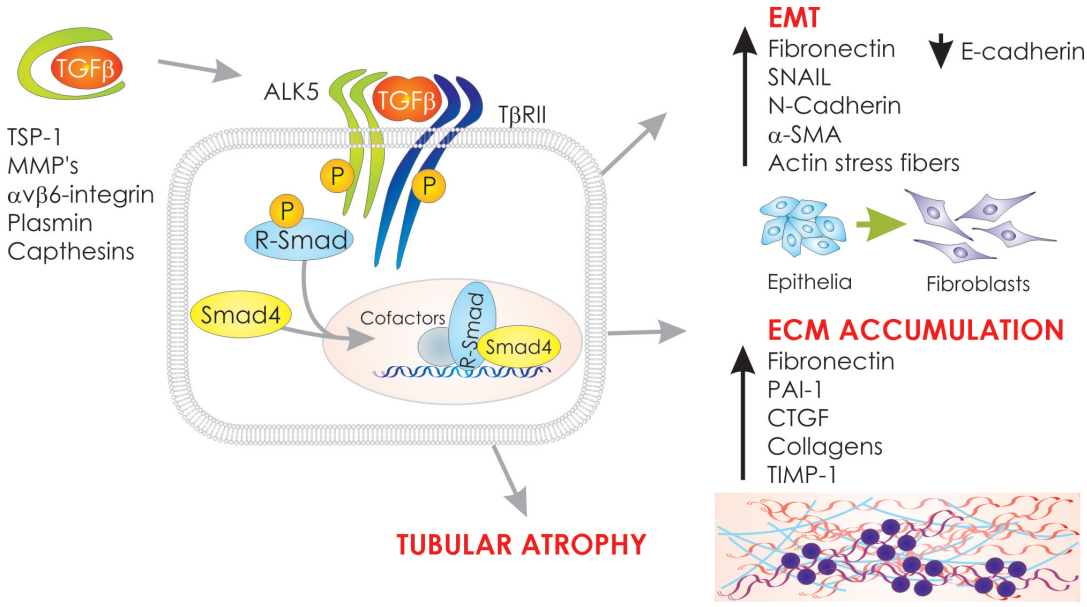


Figure 1.10: Schematic illustration of TGF-β signaling and its role in fibrogenesis. The TGF-β signaling pathway contributes to the pathogenesis of fibrosis. Latent TGF-β is proteolytically activated by matrix enzymes and the dimeric chemokine signal via heteromeric TβRII and ALK5 receptor complexes and the intracellular mediators the Smads. TGF-β induce the transcription or repression of a vast number of target genes. In fibrosis, enhanced TGF-β signaling results in excess accumulation of ECM, inhibition of MMP activity, induction of EMT, and epithelial cell apoptosis (reviewed in [86, 294, 274]). Thus, TGF-β promotes renal fibrogenesis by increasing the synthesis of ECM components and inhibiting matrix degradation. Abbreviations: α-SMA, alpha smooth muscle actin; CTGF, connective tissue growth factor; ECM, extracellular matrix; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; TIMP-1, tissue inhibitor of metalloproteinase-1; TSP-1, thrombospondin-1.

Fibrosis, is a disease that results from abnormal development of excess fibrous connective tissue which typically induced by chronic inflammation [154, 86, 319]. In patients with diabetic kidney disease, up-regulation of TGF-β1 mRNA and protein is found in the glomerulus and tubulointerstitium [320, 321]. Patients suffering from diabetic nephropathy present with increased urine and serum levels of TGF-β1 and this isoform is thought to be predominantly responsible for fibrosis [322]. It is primarily produced by circulating monocytes and tissue macrophages and secreted in an inactive complex with latency associated peptide (LAP). Several agents can activate the latent TGF-β by proteolytic cleavage of the LAP and include thrombospondin-1 (TSP-1), MMP's, αvβ6 integrin, plasmin, and cathepsins (see figure 1.10). TGF-β is a pro-fibrotic factor operating at several stages of fibrotic disease pathogenesis. One mechanism is through sustained

deposition of extracellular matrix components which is triggered by TGF-β-induced expression of laminin, fibronectin, proteoglycans, type I collagen, type III collagen and type IV collagen in tubular epithelial cells and glomerular mesangial cells (reviewed in [154, 86, 323, 294]). *CTGF* expression is also enhanced upon TGF-β stimulation [324] and this leads to further ECM accumulation through up-regulated expression of fibronectin and type III and IV collagen. Furthermore, TGF-β-induced expression of tissue inhibitors of metalloproteinases (TIMPs) and PAI-1 result in suppression of MMP's. The net effect being less matrix degradation [294, 154, 86]. Thus, TGF-β act on several levels of fibrogenesis by enhancing synthesis of ECM components and inhibiting matrix degradation thereby promoting pathological fibrosis (see figure 1.10). Moreover, an additional pathomechanism of TGF-β is the induction of EMT in tubular epithelial cells which transform into activated myofibroblasts [325, 326]. These myofibroblasts may be responsible for additional deposition of interstitial matrix as they produce vast quantities of ECM which accumulates in the kidneys.

Distinct functional roles for Smad2 and Smad3 have been identified in the TGF-β1 induced fibrosis [326, 327]. Smad2 induce an anti-mitogenic response distinct from Smad3 [327] and is specifically required for maintenance of an epithelial phenotype [327]. This is hypothesized to occur independently of TGF-β. In contrast, Smad3 is required for TGF-β1-induced EMT [327] and necessary for CTGF, SNAIL1, PAI-1, and collagen I expression and E-cadherin repression [326, 327]. Smad2 is required for the induction of secreted MMP-2 [326] and both R-Smads are needed for the up-regulation of α-SMA in response to TGF-β1 [326].

An additional cellular pathomechanism, whereby the TGF-β signaling pathway contributes to fibrosis is by inducing apoptosis in tubular epithelial cells. This results in tubular atrophy and is an indicator of poor prognosis in patients presenting with chronic renal failure (reviewed in [154]).

The pro-fibrotic functions of TGF-β can be antagonized by exogenous addition of BMP-7 [205, 106, 328]. This results in amelioration of glomerular and interstitial fibrosis in experimental models. Moreover, in interstitial and mesangial renal cells, the physiological renal functions were restored by the antagonizing actions of BMP-7 on TGF-β-induced responses. In detail, TGF-β-mediated up-regulation of PAI-1 was reduced which lead to restoration of MMP2 activity resulting in regular ECM degradation [328]. Additionally, systemic administration of BMP-7 reversed the EMT response induced by TGF-β through MET resulting in restoration of the normal renal epithelial morphology in animal models of chronic renal fibrosis [106, 325, 105].

1.6.1 Targeted therapy to TGF-β in fibrosis

Fibrosis is one of the largest groups of disorders for which no effective therapy is available. As for anti-TGF-β cancer drug discovery, recognizing the pleiotropic function of TGF-β is critical when designing anti-fibrotic drugs targeting TGF-β [283, 294, 274, 329]. Identification of key down-stream mediators of the pro-fibrotic actions of TGF-β could represent potential targets for anti-fibrotic therapy. Current drugs under investigation

as anti-fibrotic agents and their status in the pipeline towards the clinic are reviewed here and some are shown in table 1.6.

Several modalities have been pursued in the search for anti-TGF-β therapies targeting various levels of the signaling pathway. Inhibiting the function and expression of TGF-β ligands have been attempted with anti-TGF-β antibodies, antisense oligonucleotides, and with TGF-β binding proteins. In a model of diabetic nephropathy, long-term administration of neutralizing anti-TGF-β antibodies, recognizing all isoforms, prevented glomerulosclerosis and attenuated expression of matrix proteins [279]. Furthermore, adenoviral-delivery of antisense TGF-β prevented liver fibrosis in a similar model (reviewed in [274]). Also, the natural inhibitor of TGF-β, decorin, was found to attenuate the overproduction of ECM components in a experimental mouse model of glomerulonephritis [330].

On the level of TGF-β-receptors, administration of the a soluble extracellular domain of TβRII [331] or local administration of adenovirus-expressing dominant-negative TβRII both displayed anti-fibrotic effects in a model of liver fibrosis [332]. Similar results were found with administration of synthetic peptides of the TβRIII β-glycan [333]. In addition, small molecule antagonists to ALK5 can block the actions of TGF-β *in vitro* such as TGF-β-induced EMT, up-regulation of critical pro-fibrotic target genes, and TGF-β-induced apoptosis [37, 294, 274]. When such ALK5 antagonists were administered to animals with renal disease, several compounds significantly ameliorated the TGF-β-induced fibrotic responses [37, 293, 334, 335, 299]. No notable systemic side-effects were observed with any of these therapeutic strategies [37, 279, 332].

Another class of anti-fibrotic drugs are aimed at inhibiting bioactivators of latent TGF-β. Such candidate targets include thrombospondin (TSP)-1, αvβ6 integrin, plasmin, cathepsins and MMPs [336, 47]. Antisense oligonucleotides to TSP-1 were shown to inhibit activation of TGF-β and downstream Smad signaling resulting in diminished accumulation of ECM and reduced glomerulonephritis in rats with progressive renal disease [336].

Moreover, CTGF, the downstream mediator and target gene of TGF-β was recently identified to activate TGF-β signaling. Hence, functioning in a feed-forward proteolytic autocrine loop [177]. Targeting CTGF by antisense oligonucleotides in experimental mouse models of diabetic kidney disease resulted in inhibition of TGF-β activation, decreased ECM accumulation and reduced albuminuria [337]. A clinical trial is underway where patients with diabetic nephropathy will be treated with anti-CTGF antibodies (reviewed in [329, 283]).

Clinical disadvantages of TGF-β inhibition is the abrogation of immunosuppressive and anti-tumor functions of this growth factor which could lead to hyper-inflammatory and pro-tumorigenic states (reviewed in [283, 274]). Local and targeted drug administration are therefore recognized as the most promising methods for inhibiting fibrosis. Though it has yet to be shown that fibrosis progression can be halted by inhibiting TGF-β signaling in patients, more and more evidence in animal models suggest that manipulation of the TGF-β signaling pathway is a promising approach to cure fibrotic diseases.

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1.7 Outline of the thesis

Since the discovery and cloning of TGF-β tremendous scientific effort has led to a sophisticated understanding of the multifunctional actions of this pleiotropic growth factor. TGF-β regulates a myriad of processes in normal tissues and in disease pathogenesis. In cancer, TGF-β often suppress early tumorigenesis and later enhance tumor progression. The pathogenic role of TGF-β signaling is an attractive target for therapeutical intervention. However, in order to specifically direct therapy to this branch of TGF-β signaling a deeper understanding of its cellular actions in specific contextual settings is crucial. Several studies are presented in this thesis, which aim at unraveling the molecular mechanisms of TGF-β in the pathogenesis of breast cancer, bone metastasis and in renal fibrosis.

- In chapter 2 we characterize the role of two downstream mediators of TGF-β signaling, namely Smad2 and Smad3, in breast cancer metastasis. We identify crucial differences in their target gene regulation patterns and their individual role in breast cancer metastatic bone disease.

- Chapter 3 describes the function of HMGA2 a TGF-β-inducible, Smad-dependent, gene. We identify how TGF-β employs HMGA2 to elicit epithelial to mesenchymal transition of breast cancer cells.

- BMP-7 has been shown to inhibit progression of metastasis possibly through antagonizing TGF-β-induced EMT. In chapter 4, we characterize the functional type I receptor for BMP-7 in breast cancer cells and perform a head-to head comparison of breast cancer cells over-expressing either BMP-7 ligand or a continuously activated BMP-7 receptor. By this mean we analyze whether BMP-7 acts directly on the breast cancer cells and/or on the cells in the bone stromal environment.

- In chapter 5 we characterize the *in vitro* and *in vivo* functions of a novel TGF-β receptor kinase inhibitor, GW788388. We focus on the inhibitory effects of this compound in TGF-β-induced EMT and growth arrest. Furthermore we administer the compound to mice presenting with severe renal fibrosis caused by diabetes.

- Chapter 6 recapitulates the findings described in this thesis and we discuss how our research contributes to the wider context of the TGF-β field.

Chapter 2

Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis

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Abstract

Transforming growth factor (TGF)-β can suppress and promote breast cancer progression. How TGF-β elicits these dichotomous functions and which roles the principle intracellular effector proteins Smad2 and Smad3 have therein, is unclear. Here we investigated the specific functions of Smad2 and Smad3 in TGF-β-induced responses in breast cancer cells in vitro and in a mouse model for breast cancer metastasis. We stably knocked down Smad2 or Smad3 expression in MDA-MB-231 breast cancer cells. The TGF-β-induced Smad3-mediated transcriptional response was mitigated and enhanced by Smad3 and Smad2 knockdown, respectively. This response was also seen for TGF-β-induced vascular endothelial growth factor (VEGF) expression. TGF-β induction of key target genes involved in bone metastasis, were found to be dependent on Smad3 but not Smad2. Strikingly, whereas knockdown of Smad3 in MDA-MB-231 resulted in prolonged latency and delayed growth of bone metastasis, Smad2 knockdown resulted in a more aggressive phenotype compared to control MDA-MB-231 cells. Consistent with differential effects of Smad knockdown on TGF-β-induced VEGF expression, these opposing effects of Smad2 versus Smad3 could be directly correlated with divergence in regulation of tumor angiogenesis in vivo. Thus, Smad2 and Smad3 differentially affect breast cancer bone metastasis formation in vivo.

Abbreviations

ALK, activin receptor-like kinase; BLI, bioluminescent imaging; BMP, bone morphogenetic protein; CTGF, connective tissue growth factor; EMT, epithelial to mesenchymal transition; GAPDH, glyceraldehyde 3'phosphate dehydrogenase; GFP, green fluorescent protein; IL-11, interleukin 11; miR RNAi, micro RNA interference; MMP, matrix metalloproteinase; N-T control, non-targeting control; PAI-1, plasminogen activator inhibitor 1; PTHrP, parathyroid hormone-related protein; SDS-PAGE, sodium dodecyl sulphate-polyacrylaminde gel electrophoresis; P-Smad, phosphorylated Smad; R-Smad, receptor regulated Smad; Smad, small phenotype and mothers against decapentaplegic related protein; TGF-β, transforming growth factor β; TβRII, TGF-β type II receptor; VEGF, vascular endothelial growth factor.

Introduction

Metastatic breast cancer is one of the leading causes of death from cancer in women. Transforming growth factor (TGF)-β is frequently overexpressed in human breast tumors and the tumor-associated stroma and its expression correlates with poor prognosis and metastasis [1, 2, 3, 4]. TGF-β is the prototypic member of a large family of evolutionarily conserved pleiotropic cytokines, including three TGF-β isoforms, activins, and bone morphogenetic proteins (BMPs) [5, 6, 7]. TGF-β family members have critical and specific roles during embryogenesis and later in maintaining tissue homeostasis. Perturbations in their signaling pathways have been linked to a diverse set of developmental disorders and diseases, including cancer, fibrosis and auto-immune diseases [8]. These factors signal through specific sets of type I and type II serine/threonine kinase receptors. TGF-β bind to the TGF-β type II receptor (TβRII) which in turn trans-activates the TGF-β type I receptor, also termed activin receptor-like kinase (ALK)5. Activated ALK5 recruits and phosphorylates the receptor-regulated Smads (R-Smads) Smad2 and Smad3. These can then form heteromeric complexes with Smad4, translocate to the nucleus, and control the activation or repression of target genes [9]. Smad2 and Smad3 are also used by activin as downstream signaling mediators [10], whereas BMPs use the R-Smads Smad1, 5, and 8 [11]. Smad2 and Smad3 are highly conserved proteins with 83.9% amino acid sequence identity (Fig. 1A) The major structural difference between Smad2 and Smad3 is in the mad homology 1 domain where Smad2 has two short peptide inserts, amino acids 21-30 and 79-108 [12]. The latter insert imposes steric constraints that prevent Smad2 from binding to DNA [12]. Smad3 on the other hand readily binds DNA in complex with Smad4.

TGF-β has a dual role in tumorigenesis [5]. It inhibits growth of early carcinomas whereas in advanced stages of carcinogenesis TGF-β promotes tumor growth. TGF-β can further stimulate tumor progression and metastasis by inducing epithelial to mesenchymal transition (EMT) and invasion of epithelial cancer cells, [6, 13] and by suppressing anti tumor immune responses [6, 8]. Furthermore, several studies have shown that TGF-β can promote tumor angiogenesis and thereby create a favorable microenvironment for rapid tumor growth and dissemination [13, 14]. In breast cancer bone metastasis target genes of TGF-β are essential for cell homing, establishment of micrometastatic lesions, and in the self-amplifying process of tumor-induced bone resorption [15, 16, 17].

The TGF-β signaling pathway has been extensively studied in cancer patients and in animal models of xenografted tumors and metastasis. In human cancers diffuse phosphorylated Smad2 (P-Smad2) staining has been observed indicative of active TGF-β signaling [18, 19, 20]. Disrupting TGF-β signaling in human breast cancer cells induced tumorigenesis but inhibited invasion and metastasis to lungs after tail vein injection. This was studied by ectopic overexpression of mutated R-Smads or receptors mutated at the R-Smad binding site [21, 22, 23, 24]. Also, when using ALK5 inhibitors *in vivo* primary tumor growth was inhibited and the number of metastases was reduced [25, 26]. In a model of mouse breast cancer metastasis of 4T1 cells, administration of anti-TGF-β antibody to mice reduced the number of metastasis by 50-60% [27]. In the 4T1 and

the MDA-MB-231 tumor models, systemic administration of a soluble TβRII protein or dominant negative TβRII overexpression, respectively, displayed anti-metastatic effects [16, 28].

Several studies have provided evidence that Smad2 and Smad3 have different transcriptional functions and profiling studies have revealed distinct target genes for Smad2 and Smad3 [29, 30]. Also, whereas mice deficient in Smad2 are embryonic lethal, Smad3 deficient mice are viable [31, 32, 33]. These observations clearly suggest that Smad2 and Smad3 have distinct functions *in vivo*. In a skin cancer model in mice, homozygous deletion of Smad2 in keratinocytes triggered an EMT phenotype in tumors. This was observed by down-regulation of E-cadherin expression and induction of Vimentin, α-smooth muscle actin, and the E-Cadherin repressor Snail [34]. In contrast, overexpression of activated Smad2/3 was shown to increase cell motility in a squamous skin tumorigenesis models [21]. Nevertheless, the specific functions for Smad2 and Smad3 in breast cancer are not known.

We and others previously reported that silencing Smad4 in breast cancer cells delayed the formation of bone metastasis *in vivo* [19, 35]. To particularly study the contribution of TGF-β signaling in metastasis and explore the exact role of either Smad2 or Smad3, we stably silenced these molecules in an osteotropic clone of the human breast cancer cell line MDA-MB-231. A mouse model of bone metastasis was used to study the differential role of the R-Smads in metastatic progression [35, 36]. Tumor growth and metastasis were quantified *in vivo* by bioluminescent imaging (BLI). Our results show that, depending on the type of R-Smad silenced the metastatic potential of the human breast cancer cells is differentially and significantly affected.

Results

Specific silencing of Smad2 or Smad3 using miR RNAi

The TGF-β signaling cascade plays crucial roles in breast cancer metastasis. We previously found that silencing Smad4 in MDA-MB-231 cells inhibits bone metastasis formation *in vivo* [35]. In order to examine direct effects of TGF-β signalling and in particular of the TGF-β R-Smads, we designed and cloned miR RNAi constructs specifically targeting either Smad2 or Smad3. These R-Smads are highly homologous with the exception of the MH1 domain (Fig. 1A) where Smad2 has two additional short peptide inserts compared to Smad3 [12]. The targeting miR RNAi sequences were therefore designed to the additional peptide stretches in the MH1 domain of Smad2 and to the flanking sequences of this region in Smad3. We first examined the efficiency of knockdown of the miR RNAi constructs in a pcDNA 3.1 vector by co-transfection with FLAG-tagged Smad2 or Smad3 in COS cells. A non-targeting (N-T) miR RNAi was used as control. As seen by immunoblot analysis (Fig. 1B lane 4 and 8) both Smad2 and Smad3 were specifically and efficiently silenced by the corresponding miR RNAi. The miR RNAi pcDNA constructs were tested on a specific TGF-β-inducible luciferase reporter in COS and MDA-MB-231 cells, the TGF-β Smad3/Smad4 responsive CAGA-luciferase reporter. The miR RNAi

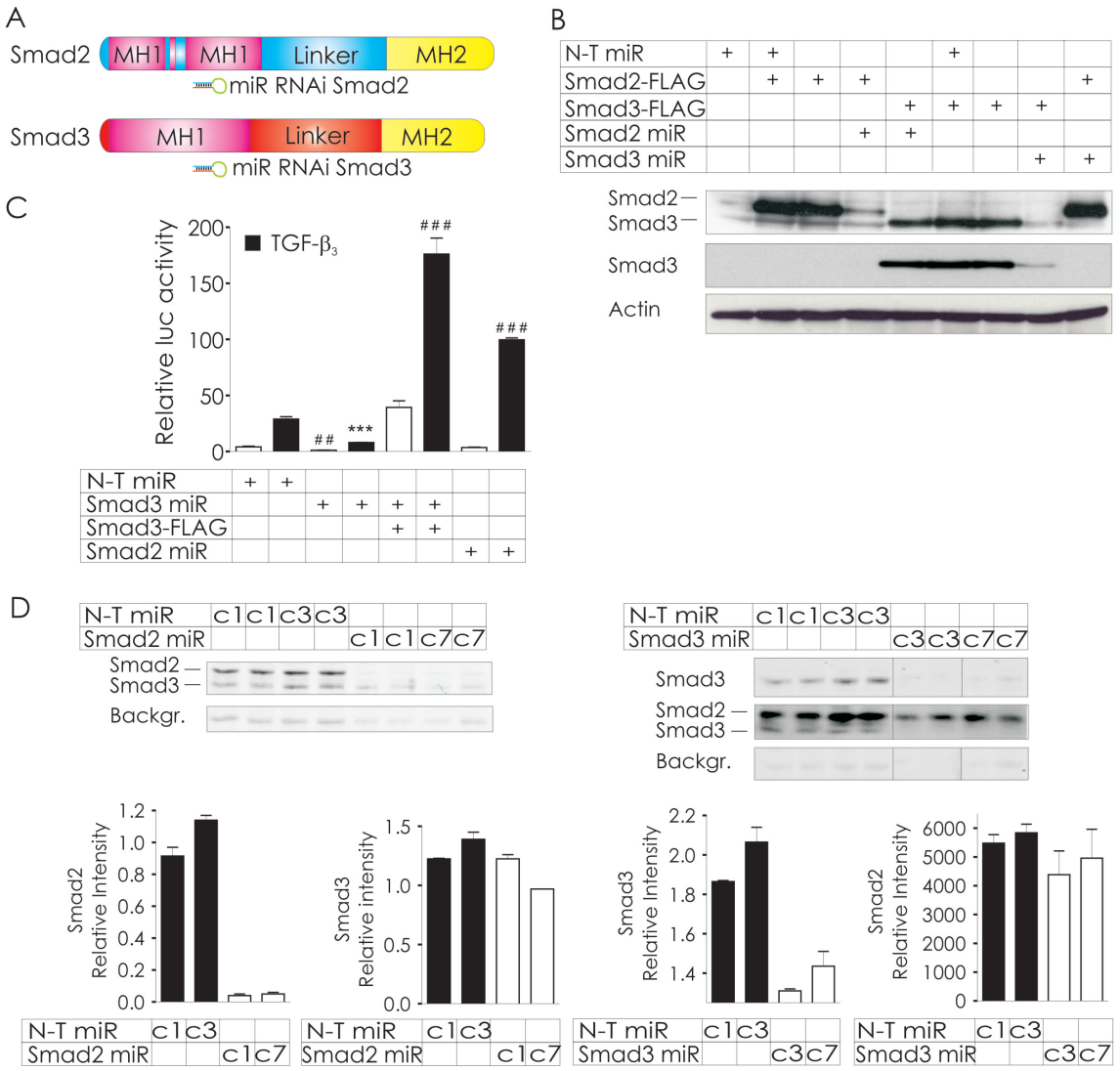


Figure 2.1: Specific silencing of Smad2 or Smad3 using miR RNAi vectors. (A) Schematic illustration of the Smad2 and Smad3 proteins. (B) Immunoblot analysis of Smad2 and Smad3 in COS cells. FLAG-tagged Smad2 or Smad3 were co-transfected with miR RNAi DNA constructs. Protein lysates were separated by SDS-PAGE and analyzed by western blot analysis. Antibodies recognizing Smad3 and Smad2/3 were used. Actin served as a loading control. (C) CAGA-renilla luc reporter transiently co-transfected in COS cells with miR RNAi and Smad3-FLAG constructs in the absence (white bars) or presence (black bars) of TGF-β. Non-targeting (N-T) vs Smad3 miR RNAi $^{\#\#}$P (P=0.0088), TGF-β-induced control vs TGF-β-induced Smad2 miR RNAi ***P (P=0.0006), TGF-β-induced control vs TGF-β-induced Smad3 miR RNAi with Smad3-FLAG $^{\#\#\#}$P (P=0.0005). (D) Quantitative immunoblot analysis of lentiviral infected MDA-MB-231 clones stably expressing miR RNAi's for Smad2, Smad3 or a N-T control. The relative band intensities of Smad2 or Smad3 are presented below. Values were corrected for equal loading with a non- specific background band. Error bars indicate mean±S.D.

targeting Smad3 significantly reduced the TGF-β-induced CAGA-luc activity (Fig. 1C).

The basal level of activity was similarly reduced suggesting that knockdown of Smad3 inhibits autocrine TGF-β signaling. This effect could be rescued by overexpression of Smad3-FLAG. Smad2 miR RNAi significantly potentiated the TGF-β-induced activity (Fig. 1C), suggesting that more Smad3 is accessible at the promoter when Smad2 is eliminated. Neither of the miR RNAi constructs had an effect on the BMP-responsive reporter indicating that the miR RNAi's selectively target Smad2 or Smad3 and not BMP R-Smads or Smad4 (data not shown). The miR RNAi constructs were sub-cloned into lentiviral vectors and MDA-MB-231-luc cells were infected. Several single cell clones stably expressing miR RNAi's were selected and characterized. As shown in Fig. 1D, the stable clones showed efficient knockdown for Smad2 or Smad3. The protein levels of Smad2 in the Smad2 miR RNAi clones were 90-95% reduced. The best knockdown clones with miR RNAi for Smad3 showed a 70-80% reduction of Smad3 protein levels. No cross-targeting was observed for either construct.
Thus, Smad2 and Smad3 are potently and specifically silenced by lentiviral delivered miR RNAi. Binding of TGF-β to its receptor leads to trans-phosphorylation of R-Smads. In order to determine if the levels of phosphorylated Smad2 were elevated in clones silenced for Smad3 and vice versa, we stimulated the stable cell lines with TGF-β (Fig. S1). We further confirmed that a very sufficient knockdown is obtained with the miR RNAi's since no P-Smad2 or P-Smad3 were observed in Smad2 and Smad3 silenced clones, respectively. Furthermore, we found that the level of phosphorylation of one R-Smad was unchanged when the other was silenced. This suggests that the TGF-β-induced activation of R-Smads in MDA-MB-231 cells is non-competitive.

Proliferation of miR RNAi stable clones

Next, we examined if silencing Smad2 or Smad3 would affect *in vitro* cellular proliferation of MDA-MB-231 cells. Relative cell growth profiles for Smad2 miR RNAi, Smad3 miR RNAi, and N-T control miR RNAi stable cell lines were monitored for four consecutive days (Fig. 2A). The Smad3 and the N-T control miR RNAi clones displayed very similar growth curves. The *in vitro* proliferation of the Smad2 miR RNAi clone was significantly lower compared to the other two cell lines (Fig. 2A).

Smad2 and Smad3 are crucial for TGF-β-induced migration

Altered migratory and tumorigenic potential of cancer cells can be simulated by *in vitro* model systems such as transwell migration. TGF-β induced transwell migration of MDA-MB-231 cells and this was blocked by the ALK5 inhibitor, SB431542 (Fig. 2B). We tested if silencing either Smad2 or Smad3 would affect the TGF-β-induced migratory phenotype of these cells. When Smad2 or Smad3 were knocked down TGF-β failed to stimulate migration. Thus, both Smad2 and Smad3 are critical for TGF-β induced migration of MDA-MB-231 cells *in vitro*. To characterize the tumorigenic potential of the cells we performed a colony formation assay in soft agar. We observed no difference in the number of colonies formed or in the size of the aggregates (data not shown).

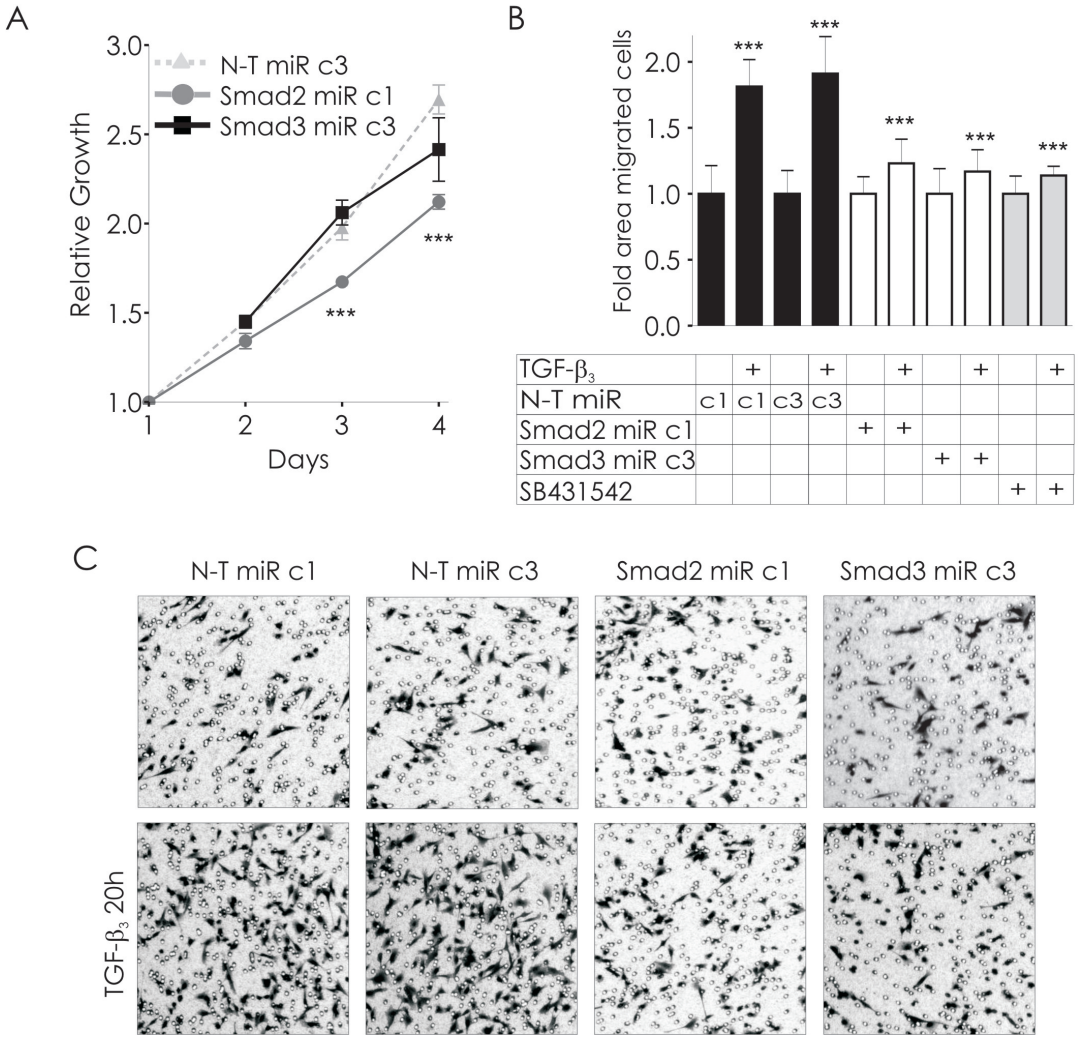


Figure 2.2: Effect of miR-mediated knockdown of Smad2 or Smad3 on MDA-MB-231 cell viability and TGF-β induced migration. (A) Cell proliferation assay of MDA-MB-231 stable clones. The relative growth of Smad2 miR RNAi c1 (circle), Smad3 miR RNAi c3 (square), and N-T miR RNAi c3 (triangle) cells was followed for four consecutive days by measuring mitochondrial activity (MTS assay). Bars represent the mean of four measurements ±S.D. N-T control and Smad3 miR RNAi versus Smad2 miR RNAi at day 3 and 4 ***P (B) Quantification of transwell migration of MDA-MB-231 cells with or without TGF-β for 20 h. N-T miR RNAi cell lines c1 and c3 (black bars). MiR RNAi silenced Smad2 c1 and Smad3 c3 (white bars). TGF-β induced migration was inhibited in cells treated with the ALK5 inhibitor, SB431542 (grey bars). Values are given as mean ±S.D. ***P of TGF-β-induced Smad2 miR RNAi (P=0.0006), Smad3 miR RNAi (P=0.0002), and SB431542 (P≤0.0001) versus TGF-β-induced N-T control c3. (C) Representative images of MDA-MB-231 stable clones on transwell filters, fixed and stained with crystal violet after migration.

Silencing Smad3 affects TGF-β target genes

Important TGF-β responsive genes involved in the vicious cycle include interleukin (IL)-11, parathyroid hormone-related protein (PTHrP), and connective-tissue growth factor

(CTGF) [15, 16, 35, 37]. We hypothesized that silencing either Smad2 or Smad3 would have an effect on the regulation of TGF-β target genes and perhaps give an indication of the metastatic potential of the cells. In order to look at early target genes of TGF-β we extracted RNA at 6 and 24 hours post stimulation and performed quantitative real time PCR analysis. An increment of IL-11 mRNA levels was seen after 6 hours stimulation with TGF-β in N-T control and Smad2 miR RNAi cells (Fig. 3A). When Smad3 was silenced this induction was significantly inhibited in multiple clones. Also, TGF-β-induced PAI-1 expression levels were significantly reduced in Smad3 knockdown cells compared to the control and Smad2 knockdown cells (Fig. 3B). We also examined the TGF-β-induced up-regulation of CTGF (Fig. 3C). CTGF mRNA levels were significantly increased after TGF-β stimulation in the N-T control cells. In Smad2 and Smad3 miR RNAi cells both basal and TGF-β-induced CTGF mRNA levels were reduced. However, the fold induction with TGF-β stimulation was comparable to the N-T control. PTHrP mRNA levels were 5 fold induced by TGF-β in all three cell lines (Fig. 3D). To control for off-target effects of the miR RNAi's we used Smad2 and Smad3 shRNAi lentiviral constructs. This resulted in 70-80% knockdown of endogenous protein levels (Fig. S2A). The gene expression analysis was repeated and the effect on TGF-β-induced target genes were confirmed in MDA-BO2 cells stably expressing shRNAi for Smad2 and Smad3 (Fig. S2B-F). Thus, independent approaches for Smad2 or Smad3 knockdown gave nearly identical results indicating that effects of the miR RNAi's are on-target.

Smad2 and Smad3 differentially affect bone metastasis

Our observations *in vitro* suggest that both R-Smads are necessary for TGF-β-induced migration, whereas Smad3 appears to be more important in the regulation of TGF-β target genes. We evaluated the specific effect of either Smad2 or Smad3 in an experimental mouse model of bone metastasis where osteotropic MDA-MB-231-luc cells were inoculated into the left heart ventricle [35, 36]. This model recapitulates late stages of metastatic progression, namely, survival in the circulation, extravasation, and establishment of metastases at secondary sites. Mice were injected with breast cancer cells stably silenced for either Smad2, Smad3, or a N-T control. Establishment and growth of bone metastatic cells was followed in time with BLI (Fig. 4A). The metastatic growth was plotted as the average total body flux of each experimental group in time (Fig. 4B). Smad2 miR cells showed a significantly more aggressive phenotype compared to both the N-T control and Smad3 miR cell lines ($P \leq 0.001$ versus Smad3 miR RNAi). In contrast, Smad3 silenced cells displayed a prolonged lag time of tumor growth in the bones (Fig. 4B). This can be more readily observed from the insert of Fig. 4B which demonstrates the tumor growth from day 21 to 35. At this stage the Smad2 silenced metastases are growing at an exponential rate, whereas the Smad3 miR RNAi cells are still in a "lag phase" ($P=0.046$ Smad2 versus Smad3 miR RNAi). N-T control miR RNAi cells are also starting to grow exponentially at this phase. However, at the end of the experiment there was no significant difference in the total BLI emission from Smad3 miR versus N-T control miR injected mice (Fig. 4B). No significant differences were detected in the

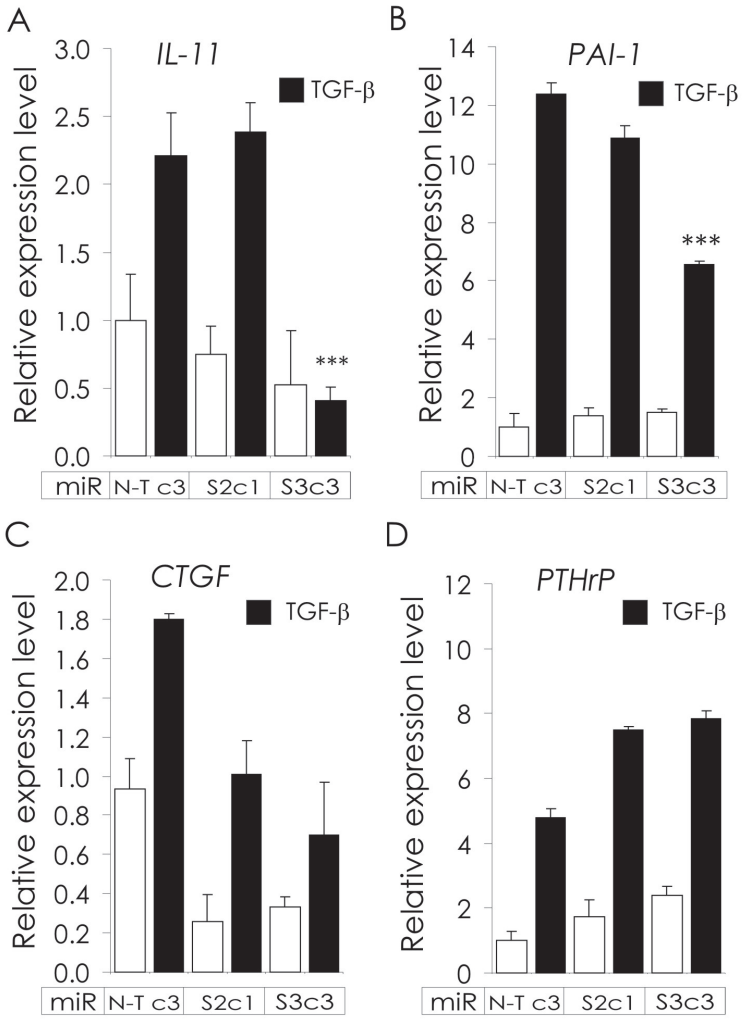


Figure 2.3: Knockdown of Smad2 or Smad3 differentially affects TGF-β-induced target gene expression in MDA-MB-231 cells. Quantitative RT-PCR analysis of TGF-β-induced target genes. Relative expression level of IL-11 (A), PAI-1 (B), CTGF (C), and PTHrP (D) correlated to GAPDH in stable MDA-MB-231 miR RNAi knockdown clones. Cells were stimulated with (black bars) or without (white bars) TGF-β for 6 hours. ***P of stimulated N-T control miR RNAi versus Smad3 miR RNAi cells of IL-11 and PAI-1 mRNA expression levels. Error bars indicate mean±S.D.

total number of metastases per animal in each of the three groups (Fig. 4C). However, it appears that there is a tendency for more metastases at an earlier stage in the Smad2 miR RNAi group compared to the N-T and Smad3 miR RNAi groups. Fluorescence imaging was used to visualize the spatial volume of the tumors *in vivo* and examine if the miR RNAi were still actively being expressed. This can be done, since the lentivirally inserted miR RNAi co-cistronically express GFP. As seen in Fig. 4D, showing a Smad2 miR RNAi injected mouse, the bone metastases were highly GFP positive. Radiographies of the same animal reveal the existence of osteolytic bone lesions at the same sites.

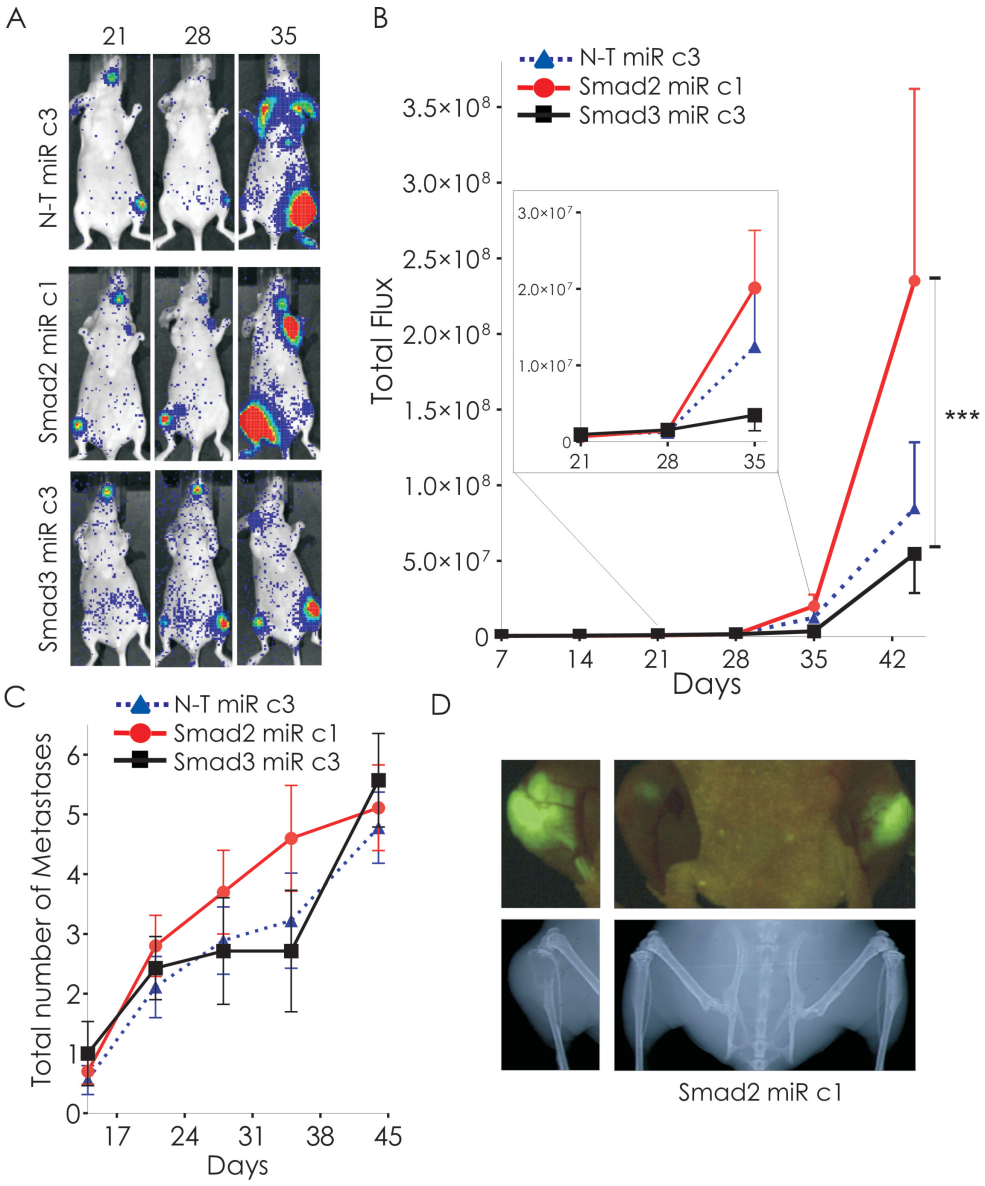


Figure 2.4: Knockdown of Smad2 and Smad3 differentially affect the metastatic profile of MDA-MB-231 cells. (A) *In vivo* BLI at day 21, 28, and 35 of three representative mice injected with MDA-MB-231 luc cells stably expressing N-T control, Smad2, or Smad3 miR RNAi. Ventral images are shown. (B) Average total flux of BLI from each experimental group followed in time. N-T miR RNAi (Triangle, dotted line), Smad2 miR RNAi (circle) Smad3 miR RNAi (square). Asterisks indicate statistically significant difference in total flux at day 45 between Smad2 and Smad3 miR RNAi metastases ***P. Insert show an enlargement of the lower graph from day 21-35. At day 35 Smad2 miR tumor-bearing animals have significantly higher tumor burden compared to Smad3 miR mice *P (P=0.0462). A trend for reduced total flux in Smad3 miR animals compared to N-T control animals was observed (P=0.162). (C) Average amount of metastases per animal in each experimental group. (D) Fluorescent *in vivo* imaging of Smad2 miR RNAi mice and below the corresponding radiographies.

By re-establishment of cell lines from bone marrow aspirates of metastases we found that the stable cell lines were continuously silenced for the respective R-Smad even after *in vivo* passaging (Fig. S3).
Bone metastases are detected as areas of low mineral density where the bone has been extensively resorbed by tumor-induced osteoclasts (Fig. 5). All experimental groups displayed strong osteolytic metastases. In general, the bone metastases were located in the distal femur, proximal tibia, vertebra, mandibula, and os coxae (Fig. 5A arrow heads). When comparing radiographies of mice from different experimental groups, with similar BLI emission, we found no apparent differences in bone destruction (Fig. 5A). This observation was further substantiated by histomorphologic analysis (Fig. 5A and B). Masson-Goldner staining revealed extensive bone loss and nearly complete replacement of the bone marrow with breast cancer cells.

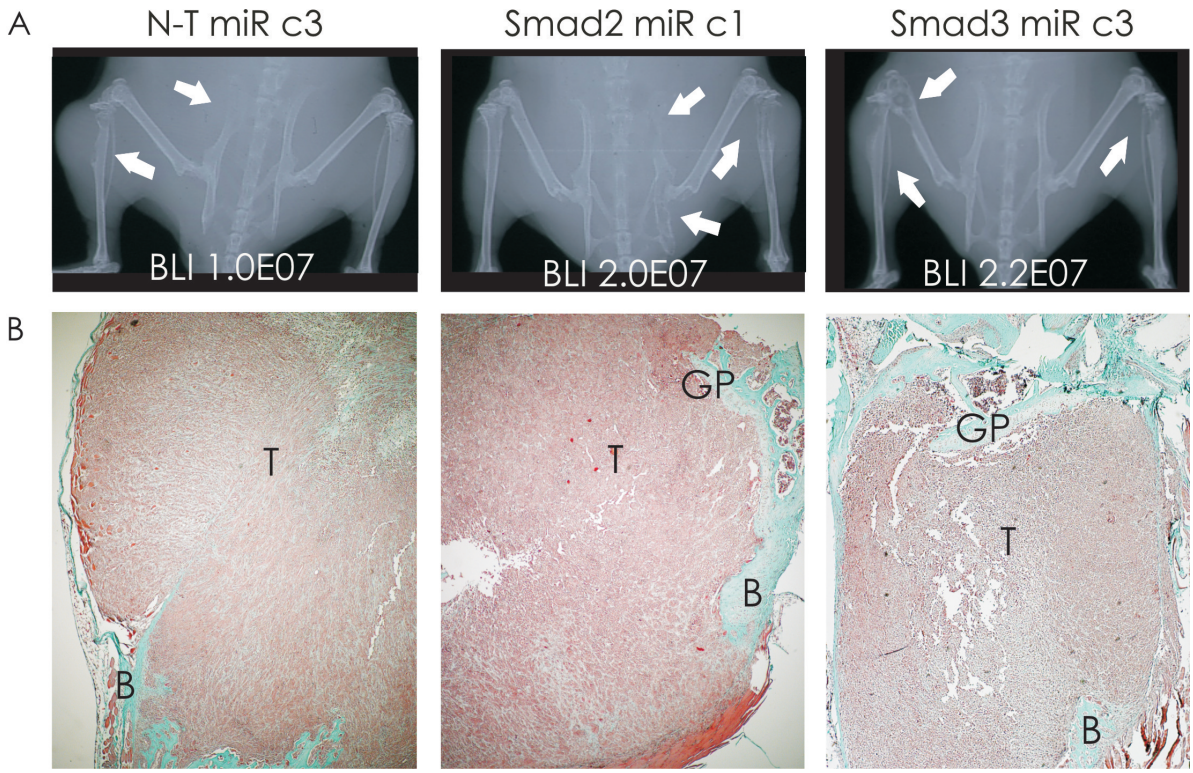


Figure 2.5: Figure 5 Radiographic and histological analysis of bone metastases in mice intracardially injected with MDA-MB-231 cells with or without Smad2 or Smad3 specific knockdown (A) Radiographies of three representative mice with N-T control, Smad2, and Smad3 miR RNAi MDA-MB-231 bone metastases. Arrowheads indicate sites of osteolytic lesions. The BLI values of the tibial regions is given in the lower part of the X-ray. Dorsal side of mice are shown. (B) Histological analysis of tibial proximal metaphyses corresponding to the radiography above. Massons-Goldner trichrome staining of sections to visualize mineralized bone. 4 x magnification. Abbreviations: B, bone; GP, growth plate; T, tumor.

Smad2 and Smad3 differentially regulate angiogenesis

We observed significant differences in metastatic growth of the Smad2 and Smad3 miR RNAi cell lines *in vivo*. This was seen already at early stages of the metastatic process where the initial growth of Smad3 miR RNAi tumors at the metastatic site was inhibited. At this phase angiogenesis is critically important for metastatic growth [15, 38]. We therefore hypothesized that Smad2 and Smad3 could have differential effects on angiogenesis. VEGF is a key regulator of angiogenesis and directly associated with worse prognosis in patients with invasive breast cancer metastasis [39]. Inhibition of VEGF signaling results in inhibition of breast cancer metastasis [40]. VEGF is a direct TGF-β target gene and Smad3, but not Smad2, was previously shown to mediate TGF-β-induced VEGF production [41, 42].

We therefore analyzed the expression of *VEGF-A* in the stable miR RNAi clones after 6 and 24 hour TGF-β stimulation (Fig. 6A). TGF-β significantly induced *VEGF* expression at both time points. Interestingly, when Smad2 was silenced the basal *VEGF* levels were dramatically increased. In cells lacking Smad3 the TGF-β-induced *VEGF* expression was lost. We next examined the amount of VEGF secreted in conditioned medium from the miR RNAi clones after TGF-β stimulation (Fig. 6B). In the N-T control miR RNAi cells, TGF-β induced VEGF secretion by 3 fold. In Smad2 miR RNAi cells, 3 fold more VEGF was secreted compared to the N-T control and Smad3 miR RNAi under un-stimulated conditions. This secretion could be further enhanced by TGF-β stimulation. In Smad3 miR RNAi cells TGF-β was unable to induce VEGF production. Thus, the VEGF secretion studies confirmed our transcription profiling results.

To further verify if our observations *in vitro* would explain the differences in tumor burden *in vivo*, we visualized the micro-vascular network in the bone metastastatic sections by CD31 (PECAM-1) immunolocalization [36, 38]. Images of the preparations were evaluated by blinded quantification of the micro-vascular density (MVD) and computer analysis of the % capillary area (Figure 6C, 6D, and 6E). Significantly more vascular structures were observed in bone metastases originating from Smad2 miR RNAi cells compared to N-T and Smad3 miR RNAi cells. Furthermore, the expression of the pro-angiogenic factors hypoxia-inducible factor-1α and placenta growth factor (PlGF) was enhanced in RNA isolated from Smad2 miR RNAi metastases compared to N-T and Smad3 miR RNAi (Fig. S4). Together these results demonstrate that Smad2 and Smad 3 differentially regulate tumor angiogenesis thus, providing an explanation for the observed differences in tumor growth at bone metastatic sites.

Discussion

The concept that the TGF-β signaling pathway plays an important role in tumorigenesis and metastases of breast cancer is well established [5] in clinical and *in vivo* studies [43]. Recently, we and others demonstrated a pro-metastatic role of Smad4 in breast cancer bone metastasis of MDA-MB-231 cells [19, 35]. Due to the nature of Smad4 as a central

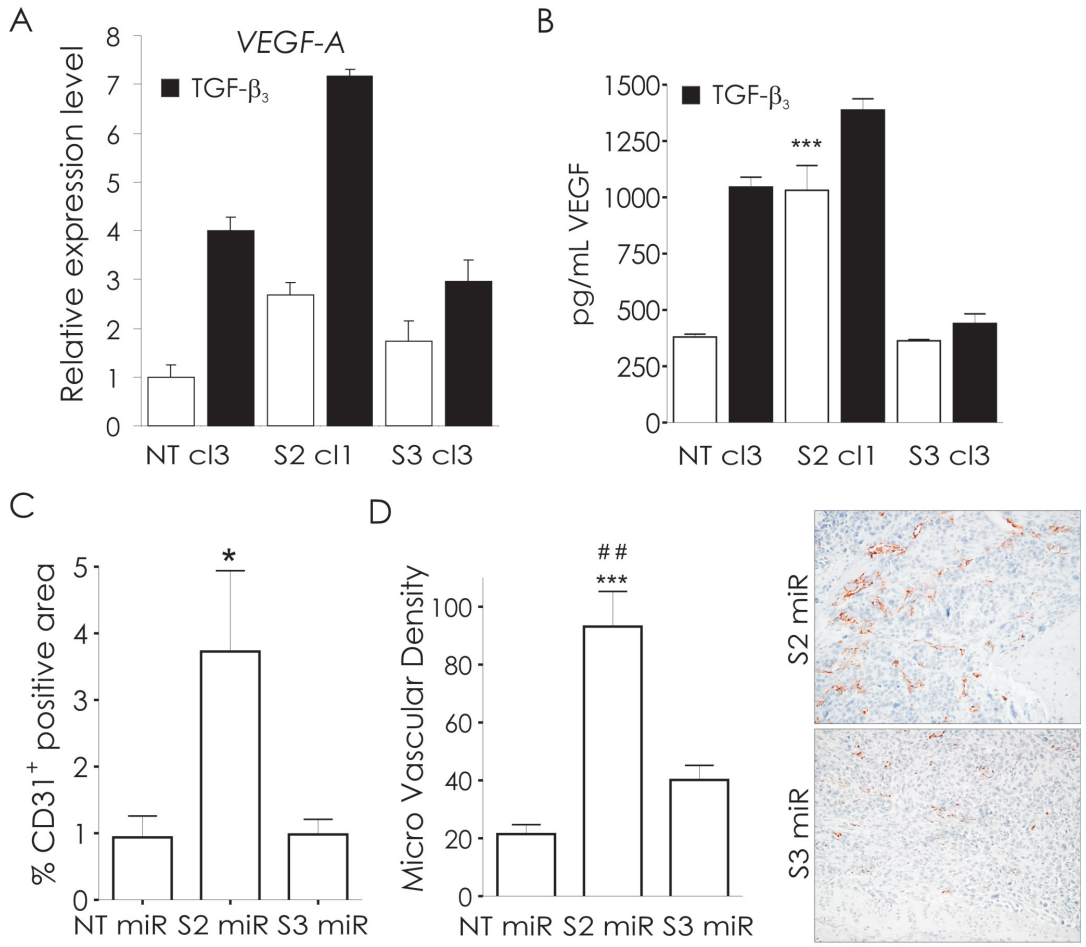


Figure 2.6: Smad2 and Smad3 differentially affect tumor induced angiogenesis (A) Real-time Q PCR analysis of the relative *VEGF-A* mRNA expression in N-T control, Smad2, and Smad3 miR RNAi MDA-MB-231 clones. *GAPDH* was used as a housekeeping gene. Cells were stimulated with TGF-β (black bars) or without (white bars) for 6 hours. (B) VEGF protein secretion in the three stable miR RNAi cell lines measured by ELISA. Data is presented at amount of VEGF secreted per ml conditioned medium. ***P of Smad2 miR RNAi versus Smad3 and N-T control miR RNAi cells. Black bars indicate samples stimulated with TGF-β for 15 hours. Error bars indicate mean±S.D. (C) Quantification of CD31 staining of bone tumor sections as the % stained area. Asterisks indicate statistically significant more CD31 staining in Smad2 miR RNAi metastases *P versus Smad3 (P=0.033) and N-T control miR RNAi (P=0.046) metastases. (D) Micro vascular density (MVD) in CD31 stained sections from N-T control, Smad2, and Smad3 miR RNAi metastases. A significant higher MVD is observed in Smad2 miR RNAi metastases ***P versus N-T control and $^{\#\#\#}$P (P=0.0086) versus Smad3 miR RNAi metastases. Error bars indicate mean±S.E.M. (D, right) Representative images of CD31 staining in bone sections from mice injected with Smad2 and Smad3 miR RNAi MDA-MB-231 cells.

regulator of both TGF-β and BMP signaling [35, 36] we decided to further study the role of the TGF-β Smad-dependent pathway in breast cancer metastasis by specific

knockdown of either Smad2 or Smad3.

Smad2 or Smad3 expression was eliminated by miR RNAi in the highly aggressive breast cancer cell line MDA-MB-231 and the effect of knockdown was evaluated in several *in vitro* assays. Furthermore, the metastatic potential of the cells was characterized in a mouse model of bone metastasis [38]. Our *in vivo* model recapitulates late events in the metastatic cascade including survival in the circulation, homing to bone, extravasation in the bone, and establishment of micrometastatic disease [44]. We show, for the first time, that Smad2 and Smad3 have distinct roles in osteotropic breast cancer. Strikingly, Smad2-silenced MDA-MB-231 cells were considerably more aggressive *in vivo* than cells silenced for Smad3 or a non-targeting control, despite the fact that they displayed slightly slower proliferation *in vitro*. In contrast, Smad3 knockdown cells showed a prolonged lag phase of tumor growth in the bone microenvironment. These observations are in line with recent findings by Hoot et al [34] who reported that homozygous deletion of Smad2 potentiated EMT and tumor aggressiveness in a skin cancer model. In these cells a direct up-regulation of critical EMT target genes such as SNAIL and Vimentin was observed along with a reduced E-Cadherin expression in the Smad2$^{-/-}$ skin tumors compared to wild type tumors. We were unable to study the role of EMT in our model which recapitulates later stages of tumorigenesis [35]. Also, Smad2 heterozygous mice displayed accelerated tumor formation and progression compared to wild type control mice [45] and reduced P-Smad2 staining was correlated with a shorter overall survival in patients with stage II breast cancer [20]. Taken together, these observations support our findings regarding the pro-metastatic effects of Smad2 knockdown in breast cancer and suggest that Smad2 has a tumor suppressor role. Bone metastases originating from Smad3 knockdown cells took considerably longer to develop into overt bone lesions. Nevertheless, at the end of the experiment growth of the Smad3 miR RNAi tumors was similar to the N-T control miR RNAi tumors perhaps due to the fact that the knockdown efficiency of Smad3 is not absolute (70-80%). Alternatively, the tumor might induce alternative responses to compensate for the loss of Smad3 as suggested from our findings on HIF-1α and PlGF expression in Smad3 miR RNAi metastases ([46, 47]). The increased lag phase of bone metastatic growth of Smad3-silenced cancer cells may be explained by altered expression of TGF-β target genes that were no longer responsive to TGF-β when Smad3 was silenced, whereas Smad2 knock down had limited effect on these target genes.

In particular, critical genes involved in the vicious cycle of osteolytic bone metastases, including IL-11, CTFG, and PAI-1, were affected. IL-11 is an important osteolytic factor secreted by the cancer cells to stimulate osteoclastic bone resorption. CTGF and PAI-1 have been reported to stimulate survival in the bone microenvironment through the induction of angiogenesis [15, 48]. Comparable observations were reported for MDA-MB-231 cells knocked down for Smad4 [19, 35].

Blocking the function of endogenous Smad3 in the MCF10A-derived breast cancer cells strongly suppressed formation of metastatic foci in lungs of mice [22, 23], thus supporting our Smad3 findings. In the same study overexpression of a defective binding mutant of Smad3 enhanced malignancy of primary tumors [23]. This is in line with previous findings

where modulation of TGF-β receptors had no effect or even promoted primary tumor growth and at later stages of tumorigenesis significantly reduced invasion and metastatic progression [24, 25, 26, 27]. In our hands, TGF-β-induced migration of MDA-MB-231 cells was dependent on both Smad2 and Smad3. Overexpression of a C-terminal truncated mutant of Smad3 was previously shown to have the same effect on TGF-β-induced migration of MCF10A cells [22]. Taken together these results suggest that Smad3 is critical for stimulation of tumor growth and metastasis.

Angiogenesis is critically important for metastatic growth when the tumor reaches a size that outgrow the normal blood supply [38, 44]. We reckoned that Smad3 miR RNAi metastases grow slower in this phase, which can be explained by a diminished ability to stimulate angiogenesis in the bone metastases. Indeed, significant differences in *VEGF-A* mRNA expression and VEGF protein secretion were observed. In the Smad3 miR RNAi cell line the TGF-β-induced up-regulation of *VEGF* was lost, whereas in the Smad2 miR RNAi cell line both *VEGF* expression and secretion was significantly enhanced. Enhanced angiogenesis was also observed in tumor metastatic sections in Smad2 miR RNAi inoculated animals in agreement with the notion that VEGF directly correlates with the degree of malignancy [39]. In line with our findings, Smad2 was found to mediate secretion of factors with anti-angiogenic properties, whereas Smad3 induced the secretion of pro-angiogenic factors by other epithelial cells [49]. Furthermore, conditioned medium from Smad2 knockout fibroblasts induced proliferation of endothelial cells, whereas medium from Smad3 knockout cells had no effect [49]. These findings support our observations namely that loss of Smad2 potentiate angiogenesis and loss of Smad3 inhibit tumor angiogenesis in breast cancer cells.

We show, for the first time, that Smad2 and Smad3 play distinct roles in breast cancer bone metastasis of MDA-MB-231 cells. Loss of Smad2 significantly increase the metastatic potential whereas loss of Smad3 shows a delayed growth of micro-metastases. These differences can be explained by the distinct roles of Smad2 and Smad3 in tumor-induced angiogenesis. In conclusion, our observations provide evidence that the Smad3 pathway mediates pro-metastatic activities in invasive cells and suggest that Smad2 has tumor suppressor activities. Current therapeutic strategies are aiming at antagonizing the TGF-β receptors thereby completely blocking signaling of both Smad2 and Smad3. Despite the validity of this approach our findings indicate that selectively targeting of Smad3 may lead to more effective therapeutic responses in the treatment of bone metastasis.

Materials and Methods

Cell culture and reagents

MDA-MB-231-luc, MDA-BO2-luc, COS, HEK293T cells were maintained as previously described [35, 36]. Cells were stimulated with TGF-β3 and BMP6 at 5 and 100 ng/ml, respectively. SB431542 (Tocris bioscience, Bristol, UK) was used at 10 μM as previously reported [50].

Constructs and cloning

We used BLOCK-iT Pol II micro RNA interference (miR RNAi) technology (Invitrogen, Breda, The Netherlands) for transient and stable knockdown of Smad2 and Smad3 (see supplementary methods for details).

Animals and surgical procedures

4-5 week old female BAlb-c nu/nu mice (Charles River, Maastricht, The Netherlands) were anaesthetized with isofluorane 0.8 l/min and $1x10^5$ freshly harvested MDA-MB-231-luc miR RNAi cells in 100 μl PBS were inoculated into the left heart ventricle (n=10 per group) [35, 36]. Injections were done with 27G syringes. All animal experiments were approved and carried out according to the guidelines provided by the local animal welfare committee.

Statistical analysis

All results are expressed as the mean±S.D. or mean±S.E.M. as indicated. Two-way ANOVA followed by Bonferronis multiple comparison test and two-tailed Students t-test were used where applicable. $P \leq 0.05$ was considered to be statistically significant.

Additional procedures

Descriptions of additional experimental procedures used are given in Supplementary Methods.

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Supplementary Material

Supplementary Figures

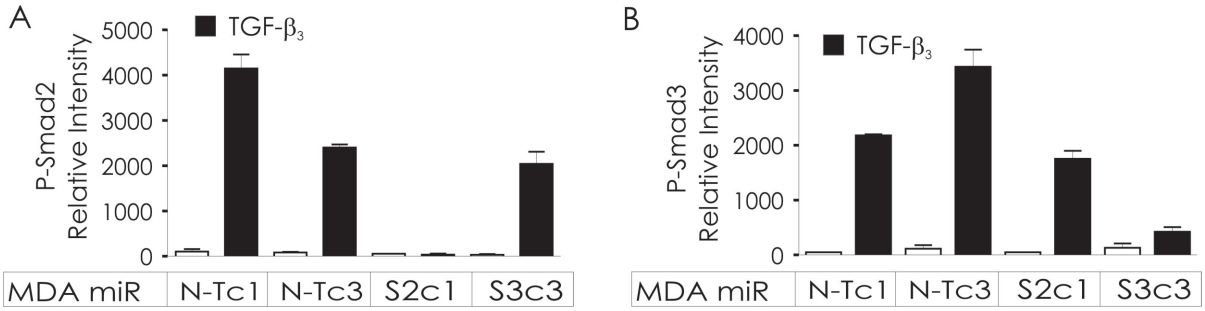


Figure 2.7:

Supplementary figure 1.

Relative densitometric analysis of western blot for P-Smad2 (A) and P-Smad3 (B). Cells were stimulated for 1 hour with TGF-β (black bars) where indicated. Protein levels were normalized with a non-specific background band.

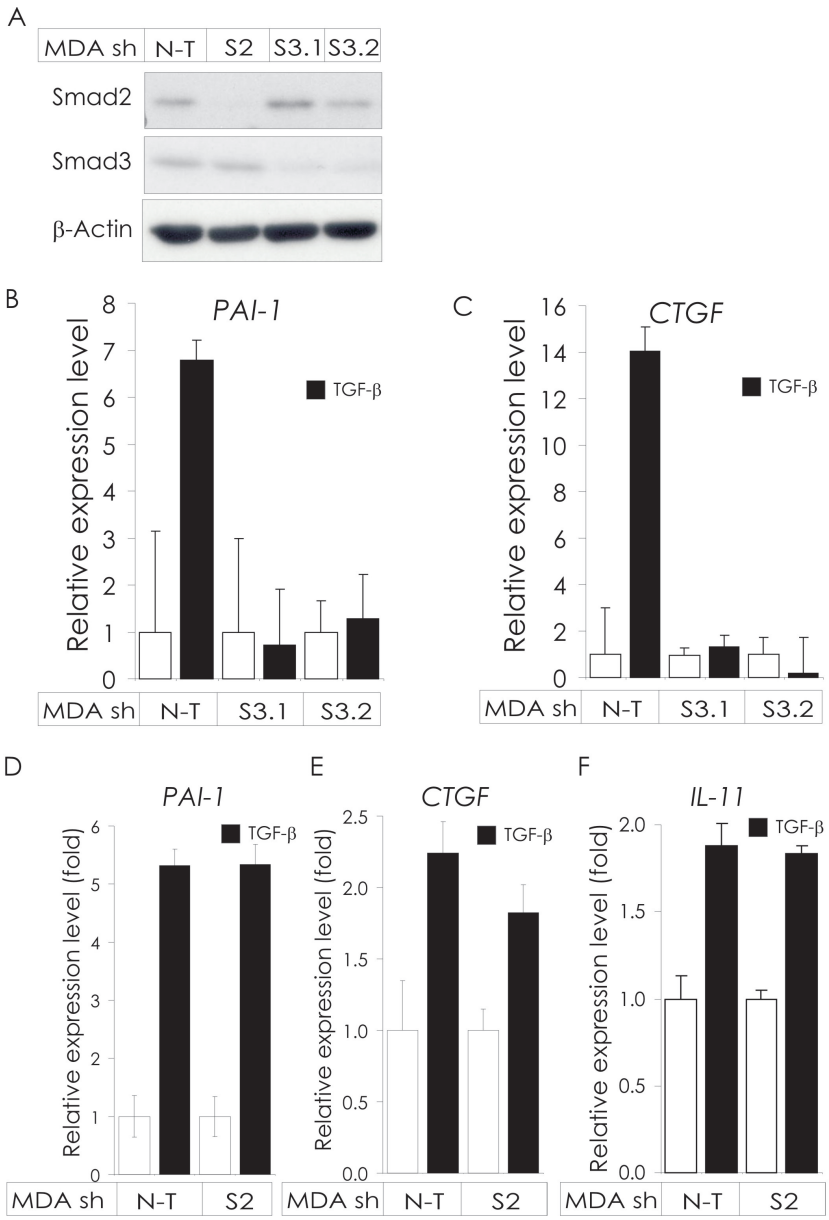


Figure 2.8:

Supplementary figure 2. (A) Immunoblot analysis of Smad2 and Smad3 protein levels in MDA-B02 cells stably knocked down for Smad2 or Smad3. Control cells were infected with a non-targeting (N-T) control shRNAi vector. MDA-B02 cells were lentivirally infected with specific shRNAi expressing vectors and selected for uptake of the shRNA vector by culturing the cells with puromycin. Real-time analysis of PAI-1 (B) and CTGF (C) in Smad3 shRNAi MDA-B02 cells and PAI-1 (D), CTGF (E), and IL-11 (F) in Smad2 shRNAi MDA-BO2 cells after 6 hours TGF-β stimulation (black bars).

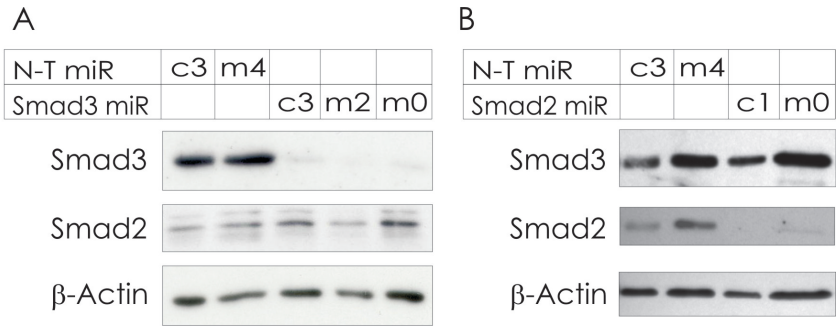


Figure 2.9:
Supplementary figure 3. Immunoblot analysis of MDA-MB-231 cells stably expressing miR RNAi for N-T control and the cell line m4 originating from a N-T control miR RNAi metastasis. Smad3 miR RNAi cells and the re-established cell lines m0 and m2 both originating from Smad3 miR RNAi metastases.

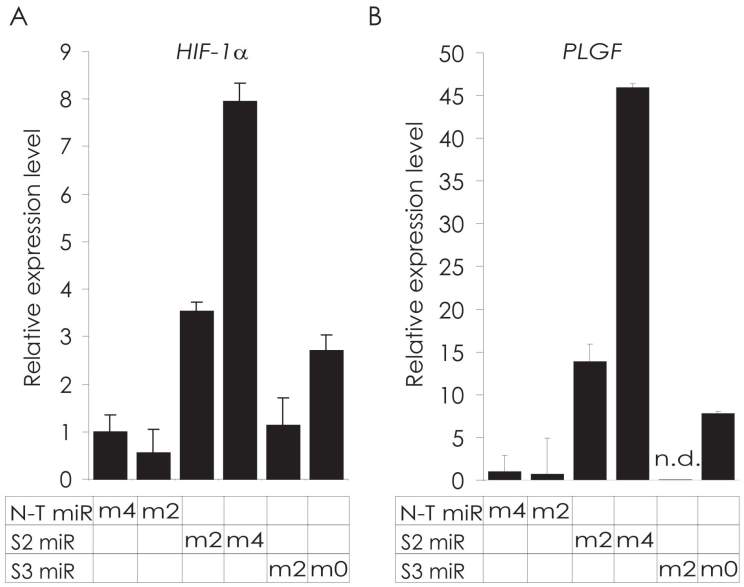


Figure 2.10:
Supplementary figure 4. Real-time PCR analysis of hypoxia inducible factor 1 α (HIF-1α) and placenta growth factor (PlGF) in bone metastasis from mice inoculated with N-T control, Smad2 miR RNAi or Smad3 miR RNAi breast cancer cells.

Supplementary Methods

Constructs and cloning

We used the BLOCK-iT Pol II micro RNA interference (miR RNAi) technology (Invitrogen) for transient and stable silencing of Smad2 and Smad3. The targeting sequence for Smad2 was 5'-ACC AAG CAC TTG CTC TGA AAT-3' and for Smad3, 5'-AGA ACG TCA ACA CCA AGT GCA-3'. The non-targeting (N-T) sequence was 5'-ACG TCT CCA CGC AGT ACA TTT-3'. Oligonucleotides were cloned into pcDNA6.2 GW-EmGFPmiR expression vector which co-cistronically express the miR RNAi and emerald green GFP (EmGFP) and transferred to the pLenti6/V5-DEST vector (Invitrogen). Cells were lentivirally infected and single cell clones selected with 5 ng/ml blasticidin (Invitrogen). We used shRNAi targeting Smad2 or Smad3 from the MISSION lentiviral library (Sigma). After lentiviral infection transfectants were selected with 1 μg/ml puromycin. ShRNAi for Smad2 was TRCN-0000010477 and for Smad3 TRCN-0000020011 and TRCN-0000020012. As controls a non-targeting sequence and a GFP targeting sequence were used. The N-terminally tagged Flag-Smad2 and Flag-Smad3 expression plasmids were previously described [51].

Cell transfection and cell viability assay

Cells were seeded in 24-well plates and the following day transiently co-transfected with miR RNAi constructs and CAGA$_{12}$-*Renilla* or the BRE-*Renilla* luciferase reporter constructs in which the firefly was replaced with *Renilla* luciferase ([52, 53] and unpublished data). The ratio of reporter construct versus miR RNAi was 1:6. We used Lipofectamine (Invitrogen) or FugeneHD (Roche) according to the manufacturers protocol. Two days after transfection, cells were stimulated for 15 hours with the respective ligands and the relative *Renilla*/firefly luciferase activity was measured. CMV-firefly luciferase was used to control transfection efficiency. Each transfection was done in triplicate and representative experiments are shown. Cell viability was performed as previously described [50].

Western blot analysis

Proteins were separated on SDS-PAGE and subjected to Western blotting using standard techniques [50]. Antibodies recognizing phosphorylated Smad2 (P-Smad2) and phosphorylated Smad 1/5 (P-Smad1/5) are described in [54]. P-Smad1/3 was a kind gift from Dr. E. Leof (Rochester, Minnesota, USA). Smad2/3 (BD transduction laboratories, Belgium), Smad3 (Zymed, CA, USA or AbCam, MA, USA), and -Actin (AC-15, Sigma, Netherlands). Secondary antibodies were either HRP or near infrared (NIR) labeled and detection performed with chemiluminescence or fluorescence scanning on the LI-COR Odyssey.

Quantitative real-time PCR

Total RNA and cDNA synthesis were performed as previously described [50]. Samples were run in triplicates for each primer set. Gene expression levels were assessed as the threshold cycle (Ct) values of the target gene and reference gene normalized to *GAPDH* ($\Delta\Delta$Ct method). Relative expression levels are presented as mean$\pm$S.D. The following human primers were used; *CTGF*, forward 5'-TTG CGA AGC TGA CCT GGA AGA GAA-3' and reverse 5'-AGC TCG

GTA TGT CTT CAT GCT GGT-3'; *PAI-1* forward 5'-TCT TTG GTG AAG GGT CTG CT-3' and reverse 5'-CTG GGT TTC TCC TCC TGT TG-3'; HIF-1α forward 5'-GCA AGC CCT GAA AGC-3' G and reverse 5-GGC TGT CCG ACT TTG A-3'; PlGF 5'-ACG TGG AGC TGA CGT TCT CT-'3 and reverse 5'-CAG CAG GAG TCA CTG AAG AG-'3; *IL-11* 5'-ACT GCT GCT GCT GAA GAC TC-3' and reverse 5'-CCA CCC CTG CTC CTG AAA TA-3'; *PTHrP* forward 5'-ACC TCG GAG GTG TCC CCT AAC-3' and reverse 5'-TCA GAC CCA AAT CGG ACG-3'; *VEGF-A* forward 5'-AGC CTT GCC TTG CTG CTC TA-3' and reverse 5'-GTG CTG GCC TTG GTG AGG-3'.

Transwell migration

Transwell migration was performed in 24 well plates with filter inserts of a pore size of 0.8 μm (Corning Costar, CA, USA). 30.000 pre-starved cells were seeded in the upper chamber in 200 μl medium. 300 μl medium with or without stimuli was added to the lower chamber. Experiments were done in triplicates. Cells were fixed after 20 hours with 4% paraformaldehyde and stained with 0.1% crystal violet. Pictures were acquired with phase-contrast microscopy at 10 x magnification. Three fixed positions were imaged of each membrane. The area covered by cells was quantified by binary image analysis using the NIH/ImageJ software.

VEGF secretion

VEGF secretion in conditioned media from stable miR RNAi MDA-MB-231 cells was analyzed by quantitative sandwich ELISA. Media were harvested 15 hours with or without stimulation with TGF-β and the analysis was performed as previously described [55].

In vivo imaging and Radiography

Metastatic tumor growth was followed weekly by live BLI with the IVIS 100 (Caliper Life Sciences) as previously described [35, 36]. The BLI signal intensity was quantified as the sum of photons within a region of interest given as the total flux (photons per second). Fluorescence-based imaging of EmGFP was measured with the CRi Maestro FLEX system [56] (Cambridge Research Instrumentation). The skin was carefully removed from the tibial regions prior to imaging. GFP emission was measured at 550 nm with an automatically estimated scan time of 360 ms. Radiographs were taken after *in vivo* fluorescent measurements as previously described [35, 36].

Establishment of *in vitro* cell lines from metastases

Whole bone metastases were surgically excised for histochemical analysis and *in vitro* tumor cell growth. Cell lines were established by removal of the tibial diaphysis and aspirating the metaphysis with cell culture medium. Bone marrow aspirates were plated in tissue culture asks and selection with neomycin was initiated the following day. This selects only for MDA-MB-231 cells expressing the CMV-luciferase construct enabling us to determine if cells have retained Smad2 or Smad3 knockdown caused by the expression of the miR RNAi.

RNA isolation from bone metastases

Whole bone metastases were surgically excised for RNA extraction. Samples were pulverized with a hammer under sterile conditions and processed as previously described [35].

Histochemistry and immunohistochemistry

Tissue samples were embedded in paraffin and 5 μm sections were stained with the Masson-Goldners method to visualize mineralized bone [35, 36]. The micro-vasculature in metastases was visualized by CD31 staining using standard techniques [37] and images were acquired with phase-contrast microscopy. The area covered by CD31 positive staining was quantified with image analysis and quantification of the micro vascular density was done by counting vessels in a given area.

Chapter 3

Transforming growth factor-β employs HMGA2 to elicit epithelial mesenchymal transition

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Abstract

Epithelial-mesenchymal transition (EMT) occurs during embryogenesis, carcinoma invasiveness, and metastasis and can be elicited by transforming growth factor-β(TGF-β) signaling via intracellular Smad transducers. The molecular mechanisms that control the onset of EMT remain largely unexplored. Transcriptomic analysis revealed that the high mobility group A2 (HMGA2) gene is induced by the Smad pathway during EMT. Endogenous HMGA2 mediates EMT by TGF-β, whereas ectopic HMGA2 causes irreversible EMT characterized by severe E-cadherin suppression. HMGA2 provides transcriptional input for the expression control of four known regulators of EMT, the zinc-finger proteins Snail and Slug, the basic helix-loop-helix protein Twist, and inhibitor of differentiation 2. We delineate a pathway that links TGF-β signaling to the control of epithelial differentiation via HMGA2 and a cohort of major regulators of tumor invasiveness and metastasis. This network of signaling/transcription factors that work sequentially to establish EMT suggests that combinatorial detection of these proteins could serve as a new tool for EMT analysis in cancer patients.

Abbreviations

ChIP, chromatin immunoprecipitation; EMT, epithelial mesenchymal transition; Gapdh, glycer-aldehyde-3'-phosphate dehydrogenase; HMG, high mobility group; Id, inhibitor of differentiation; ZO-1, zonula occludens 1.

Introduction

Epithelial mesenchymal transition (EMT) converts polarized epithelial cells to motile mesenchymal cells [1]. EMT operates during embryonic cell layer movements and tumor cell invasiveness [2]. During EMT, the epithelial proteins E-cadherin and zonula occludens 1 (ZO-1) are downregulated, and the mesenchymal proteins vimentin, α-smooth muscle actin, and fibronectin are up-regulated.

Receptor tyrosine kinase, Wnt, Notch, and TGF-β pathways trigger EMT [2]. TGF-β binds to receptor serine/threonine kinases, which activate intracellular Smad and other signaling pathways that regulate gene expression [3]. TGF-β inhibits epithelial cell growth, acting as a tumor suppressor, but it also promotes carcinoma progression and metastasis [4]. The tumor-promoting effects of TGF-β are based on its ability to induce (a) EMT, matrix invasiveness, and blood vessel intravasation by carcinoma cells [2]; (b) cytostatic effects on surveilling immune cells [5]; and (c) proangiogenic effects [6]. TGF-β elicits EMT and in vivo metastasis via Smads and complementary non-Smad effectors, such as Rho GTPases and p38 MAPK [7]. TGF-β represses inhibitor of differentiation (Id) 2 and 3 expression and induces expression of the Notch ligand Jagged-1, which are critical events during EMT [8, 9, 10].

Here, we describe the role of high mobility group (HMG) A2, also known as HMGI-C, as an effector of TGF-β that causes EMT. HMGA2 and -1 constitute a family of nuclear factors that bind AT-rich DNA sequences [11, 12]. HMGA factors contribute to transcriptional regulation by organizing nucleoprotein complexes such as enhanceosomes [13]. HMGA2 is expressed during embryogenesis and becomes silent in adult tissues [12]. However, HMGA2 is abundantly expressed by transformed cells or tumors of mesenchymal and epithelial origin (for reviews see [14, 12]). In contrast, depletion of HMGA2 by antisense cDNA in thyroid cells eliminates their transformation by myeloproliferative and Kirsten murine sarcoma viruses [15]. Here, we show that HMGA2 regulates the transcription factors Snail, Slug, Twist, and Id2, thus linking TGF-β signaling to regulators of tumor invasiveness and metastasis.

Results and discussion

TGF-β/Smad signaling induces *Hmga2* transcription

Transcriptomic analysis of TGF-βinduced EMT in mammary epithelial NMuMG cells identified *Hmga2* as a prominent TGF-β target [16]. *Hmga2* mRNA increased after 2 and 8 h and returned to basal levels after 36 h of TGF-β stimulation (Fig. 1 A). In contrast, *Hmga1* mRNA was not regulated and dropped significantly upon cell confluence at 36 h (Fig. 1 A). HMGA2 protein increased after 8 h and peaked at 12 h of TGF-β stimulation (Fig. 1 B). A TGF-β type I receptor kinase inhibitor (LY580276; [17]) did not affect basal HMGA2 levels, demonstrating the absence of autocrine TGF-β (Fig. 1 B). *Hmga2* mRNA induction by TGF-β was not impaired by the protein synthesis inhibitor cycloheximide while it was blocked by the RNA polymerase inhibitor II actinomycin

D (Fig. S1 A). A constitutively active form of the TGF-β type I receptor increased *Hmga2* expression more efficiently than TGF-β itself, whereas a kinase-dead mutant of this receptor inhibited it (Fig. S1 B). When the constitutively active type I receptor was expressed at higher levels, it often failed to induce *Hmga2* at higher levels than TGF-β [16]. This reflects mechanisms of pathway desensitization, as TGF-β signaling is controlled in a timed fashion by activation and inactivation of receptor and Smads. The

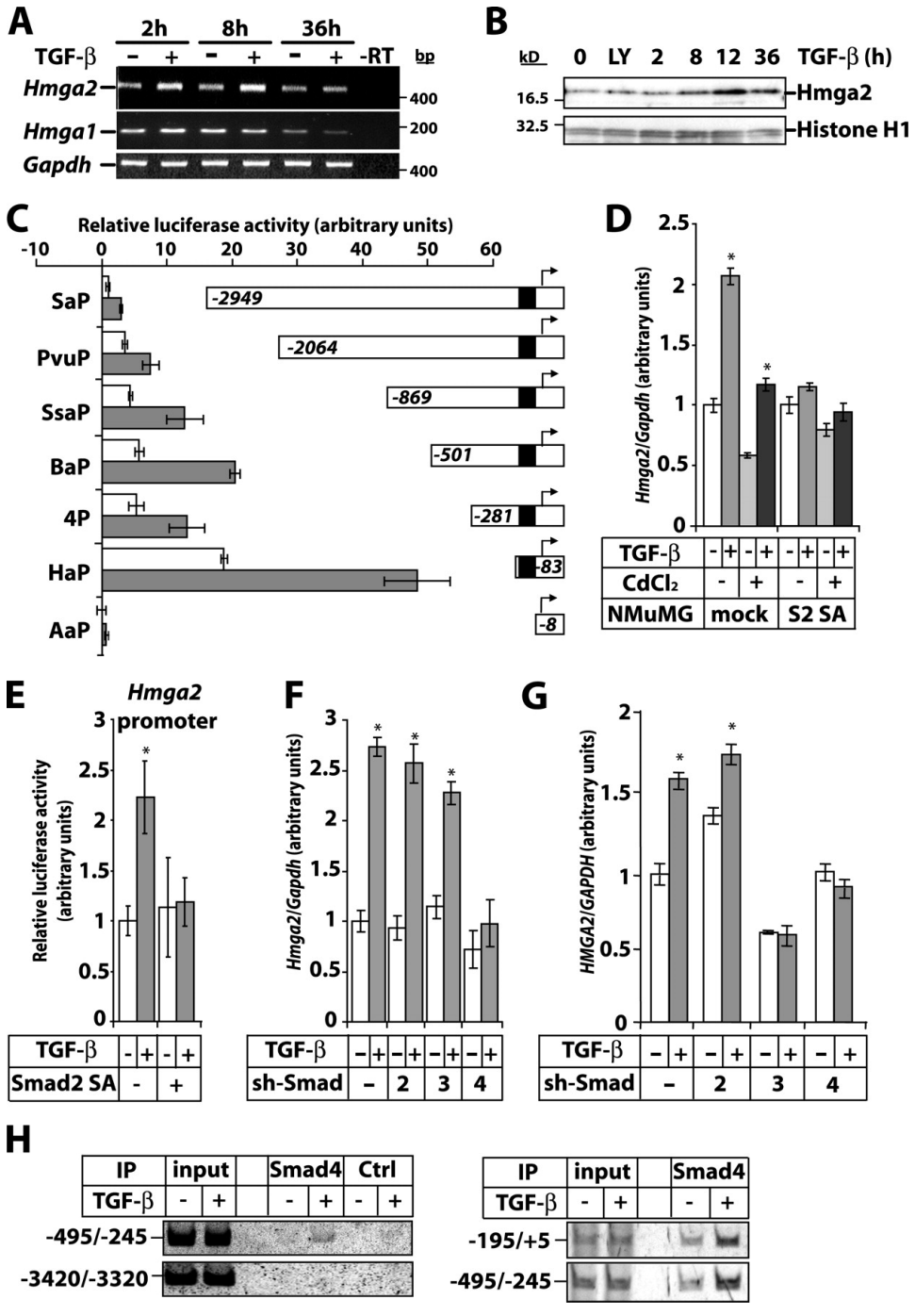


Figure 3.1: TGF-β/Smad signaling induces Hmga2 transcription. (A) RT-PCR analysis of *Hmga2* and *Hmga1* expression in NMuMG cells stimulated with 5 ng/ml TGF-β for the indicated times. (B) Immunoblot analysis of endogenous Hmga2 in NMuMG cells treated with vehicle (0), TGF-β type I receptor inhibitor LY580276 (2.5 μM; LY) for 4 h, or stimulated with TGF-β for the indicated periods of time. Histone H1 serves as a loading control. Molecular size markers are in bp (A) and kDa (B).(C) *Hmga2* promoter assays of the indicated deletion constructs in HepG2 cells stimulated (grey bars) or not (white bars) with 5 ng/ml TGF-β for 24 h. The black box in the *Hmga2* promoter corresponds to a TCC repeat-rich sequence. (D) Quantitative RT-PCR analysis of *Hmga2* expression in NMuMG clones expressing dominant-negative Smad2 (S2 SA) or empty vector (mock) induced or not with 10 μM CdCl2 for 24 h, before stimulation with 5 ng/ml TGF-β for 4 h. (E) Promoter assays of the Hmga2 BaP construct in HepG2 cells transfected with Smad2 SA and stimulated (grey bars) or not (white bars) with 5 ng/ml TGF-β for 24 h. (F, G) Quantitative RT-PCR analysis of Hmga2 expression in NMuMG (F) and MDA-MB-231 (G) clones expressing short hairpin vectors (sh-Smad) directed against Smad2, Smad3 and Smad4, or the empty vector and treated with 5 ng/ml TGF-β for 6 h. Stars indicate statistically significant gene expression or promoter activity differences between TGF-β-stimulated and non-stimulated conditions ($p \leq 0.05$). (H) ChIP assays in NMuMG cells treated or not with 5 ng/ml TGF-β for 2 h using a Smad4 antibody or a preimmune serum (Ctrl) and amplification of Hmga2 promoter fragments.

results suggest that *Hmga2* is a direct TGF-β target.

Mouse *Hmga2* promoter analysis showed that basal promoter activity varied according to the deletion construct used, and TGF-β stimulation led to a 2.53-fold induction (Fig. 1 C). Basal promoter activity and induction by TGF-β were lost when the proximal region containing TCC repeats was deleted. Sequence inspection of 4 kbp upstream from the transcription initiation site showed few noncanonical Smad binding elements between -700 and -100 bp (unpublished data). We now examine the role of these elements on *Hmga2* transcriptional induction by TGF-β.

Hmga2 mRNA induction and promoter activation by TGF-β was blocked in cells expressing dominant-negative Smad2 (Smad2 SA; Fig. 1, D and E), Smad2 SA cannot be phosphorylated by the TGF-β type I receptor and blocks TGF-βinduced EMT [16]. Knockdown of Smad2 by 80% or Smad3 by 65% after RNAi had no effect on *Hmga2* induction by TGF-β or on the EMT response (Fig. 1 F and Fig. S1, C and D). However, knockdown of the common partner of Smad2 and 3, Smad4, by 95% effectively blocked *Hmga2* induction by TGF-β and the EMT response (Fig. 1 F and Fig. S1, C and D; [18]). The lack of effect by knockdown of Smad2 or 3 may indicate that the protein depletion achieved was insufficient. Alternatively, both Smad2 and 3 may be involved in EMT, as we previously proposed [16], and for effective block of EMT, both Smad2 and 3 need to be depleted. Experiments are under way to test this possibility. In another cell line, metastatic breast cancer MDA-MB-231 cells, TGF-β weakly induced *HMGA2* expression, and knockdown of Smad3 or 4 blocked this response, whereas knockdown of Smad2 did not (Fig. 1 G and Fig. S1, E and F). Based on these data, it appears that single Smad3 or 4 knockdown is sufficient in blocking TGF-βinduced *HMGA2* expression (Fig. 1 G and Fig. S1 F). A more robust knockdown of Smad2 is needed to reach final

conclusions about the role of this Smad isoform in *HMGA2* regulation and EMT.

Finally, immunoprecipitation of chromatin bound Smad4 from NMuMG cells confirmed a TGF-βinducible association of Smad4 in the proximal (-195/+5) and upstream (-495/+245) but not in the distal (-3420/-3320) promoter region of the *Hmga2* gene (Fig. 1 H). The -500 to +5 *Hmga2* promoter region where Smad4 binds overlapped with putative Smad binding elements. The results establish that Smad signaling is involved in *Hmga2* induction by TGF-β, with Smad4 clearly being implicated. However, we cannot conclude whether Smad4 cooperates with Smad2, Smad3, or both during Hmga2 regulation.

Ectopic HMGA2 weakly inhibits epithelial cell proliferation

To address the functional role of HMGA2, we established stable NMuMG clones inducibly expressing human HMGA2. Ectopic HMGA2 was expressed in the absence of an inducer, and induction increased its expression further (Fig. 2 A). Ectopic HMGA2 localized in the nucleus as expected, and its localization was not affected by TGF-β (Fig. 2 B). In the absence of TGF-β, HMGA2 clones grew slower than mock cells (Fig. 2 C). TGF-β inhibited growth of mock and HMGA2-expressing cells (Fig. 2 D). Thus, TGF-β induces growth arrest despite ectopic HMGA2 expression.

HMGA2 mediates EMT in response to TGF-β

Mock NMuMG clones treated with an inducer displayed characteristic polarized epithelial morphology (Fig. 3 A). TGF-β caused EMT, as mock cells acquired elongated, fibroblast-like morphology. HMGA2 clones were constitutively elongated and lost cell-cell contacts, suggesting induction of EMT, which was enhanced further by TGF-β (Fig. 3 A). EMT in HMGA2 clones was confirmed by visualizing actin cytoskeleton rearrangements and the loss of ZO-1 and E-cadherin from cell junctions (Fig. 3 B) and by measuring the loss of expression of *E-cadherin* and *Mucin-1* mRNA (Fig. 3 C). Moreover, mRNAs of the mesenchymal markers *PAI-1*, *Timp-3*, and *Fibronectin-1* were constitutively expressed, and *Vimentin* was increased to a lesser extent. Immunoblot analysis confirmed E-cadherin protein downregulation and enhanced expression of mesenchymal N-cadherin in HMGA2 clones (Fig. 3 D). These experiments demonstrate that ectopic HMGA2 causes EMT.

The fact that ectopic HMGA2 mimicked the TGF-β response (Fig. 3, C and D) raises the question of whether HMGA2 activates autocrine TGF-β, leading to EMT. In mock NMuMG cells, the LY580276 inhibitor blocked TGF-βmediated EMT, as cells kept a cortical actin distribution and did not downregulate E-cadherin (Fig. S2). However, in HMGA2 clones, LY580276 had no effect on the elongated morphology, actin stress fiber network, or lack of E-cadherin. Similar results were obtained with a TGF-βneutralizing antibody added to the medium of HMGA2 clones for several days (unpublished data). These experiments demonstrate that the profound effects HMGA2 shows on EMT cannot be accounted for by the induction of autocrine TGF-β that signals in a constitutive

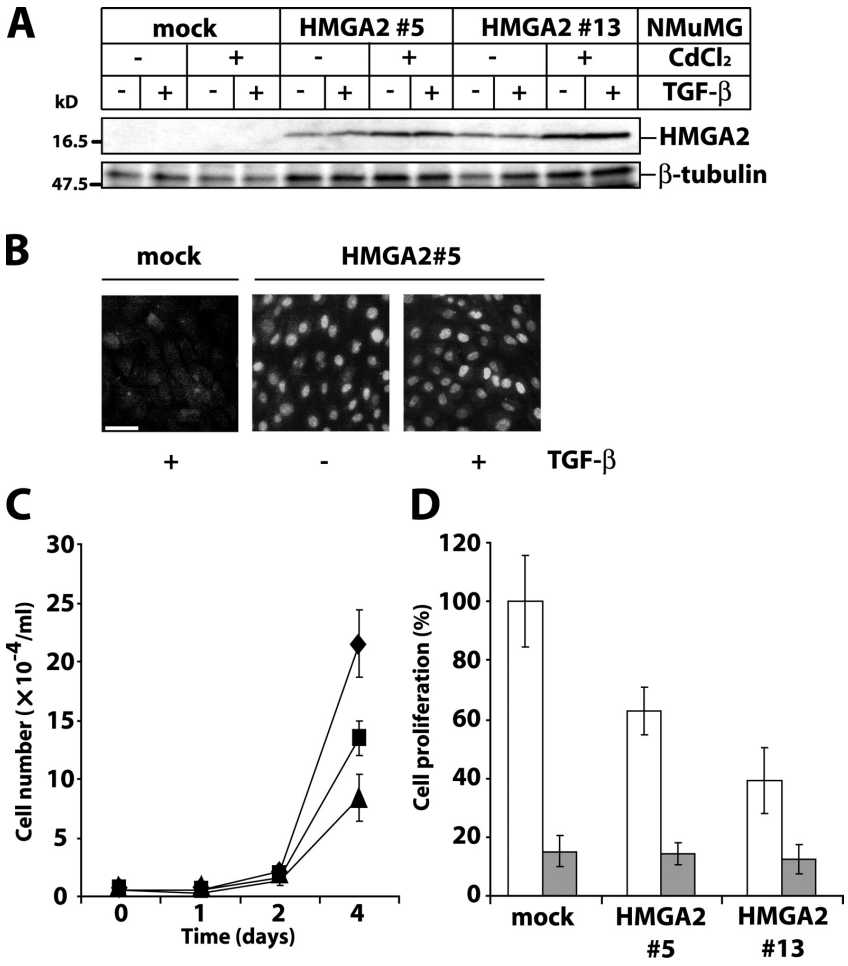


Figure 3.2: HMGA2 inhibits cell proliferation. (A) Analysis of ectopic *HMGA2* expression in NMuMG clones (mock, 5, 13) transfected with empty or HMGA2 vector. Cells were stimulated with 5 ng/ml TGF-β for 36 h, 24 h after induction with 10 μM CdCl2. Immunoblots were incubated with anti-HA antibody. β-tubulin is loading control. Molecular size markers are in kDa. (B) Immunostaining with anti-HA antibody of mock NMuMG and HMGA2-clone 5 stimulated with 5 ng/ml TGF-β for 36 h (Bar = 10 μm). (C) Cell proliferation assays with mock (diamonds) and HMGA2-clones (5, squares; 13, triangles). (D) Cell proliferation assays with mock, HMGA2 clones 5 and 13 stimulated (grey bars) or not (white bars) with TGF-β for 4 days.

manner.

Transfection of NMuMG cells with *Hmga2* siRNA resulted in an 70% decrease in basal and TGF-βinduced *Hmga2* mRNA expression (Fig. 4 A). An even stronger reduction was seen of the endogenous HMGA2 protein level (Fig. S3). Weak nuclear HMGA2 was seen in control cells; TGF-β dramatically increased the nuclear HMGA2 levels, as the cells became elongated and fibroblast like (Fig. S3). Upon HMGA2 depletion, its nuclear staining was barely detectable. Immunoblot analysis of endogenous HMGA2 protein upon chromatin extraction (Fig. 1 B) was not efficient enough in these transfected cells to quantitatively monitor the degree of protein depletion upon RNAi (unpublished data).

We reproducibly observed that the mammary epithelial cells enlarged their diameter by

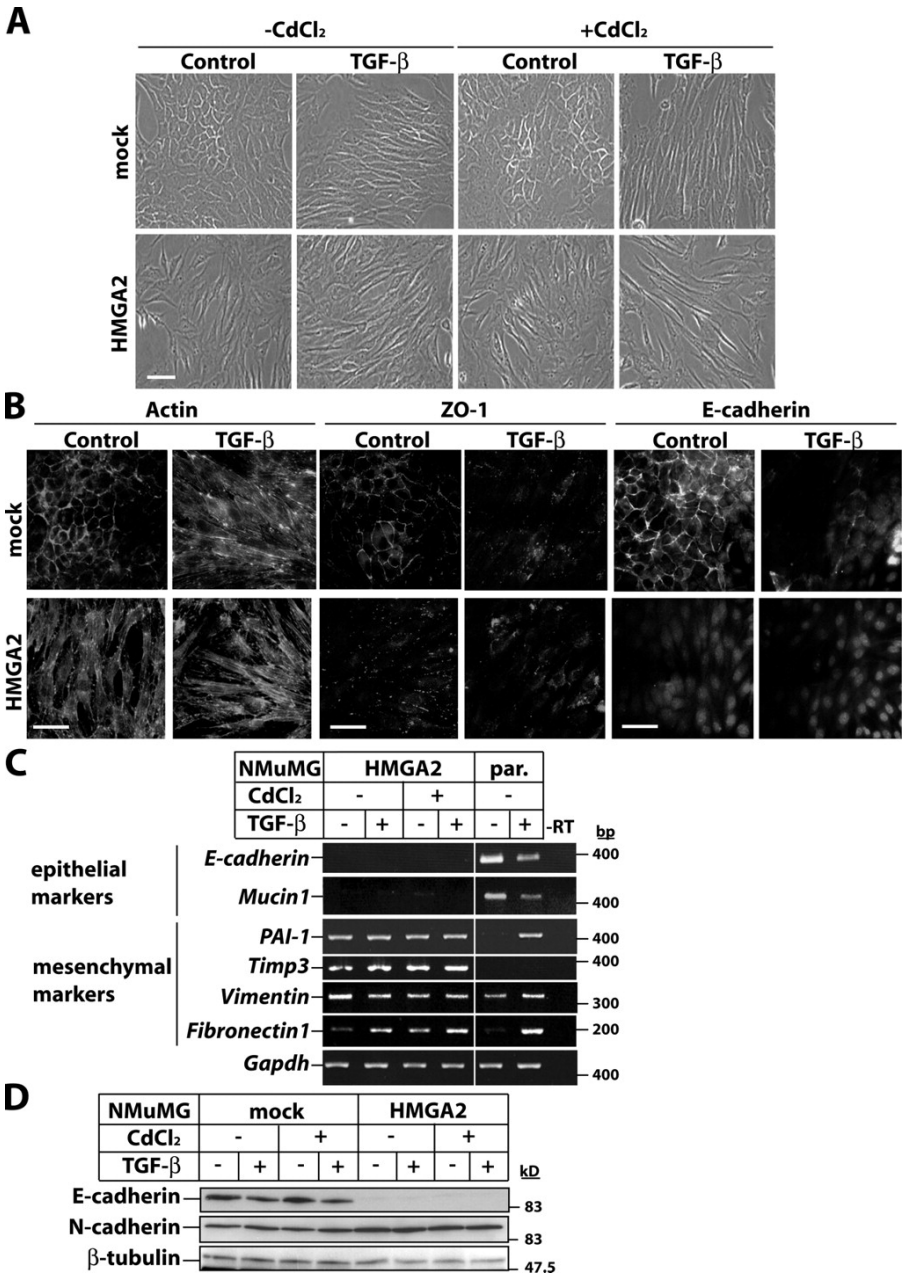


Figure 3.3: HMGA2 induces EMT. (A) Phase-contrast microscopy of mock and HMGA2-clones. HMGA2 induction with 10 μM CdCl2 was followed by vehicle (control) or 5 ng/ml TGF-β stimulation for 36 h. (B) Visualization of actin cytoskeleton and the epithelial markers ZO-1 and E-cadherin by immunostaining of mock and HMGA2-clones treated with vehicle (control) or 5 ng/ml TGF-β for 36 h (Bars = 10 μm). (C) RT-PCR analysis of EMT markers. HMGA2-clones were treated as in (A). Parental NMuMG (par.) were treated with 5 ng/ml TGF-β for 36 h. (D) Immunoblot analysis of E- and N-cadherin in cells treated as in (A). Molecular size markers are in bp (C) and kDa (D).

roughly 1.82.2-fold when HMGA2 was depleted (Fig. S3). This effect was specific for the *Hmga2* siRNA, as we did not observe size changes by control (siLuc) or a panel of siRNAs that target unrelated genes in this cell line (Fig. S3 and unpublished data).

TGF-β induced EMT in NMuMG cells transfected with control siRNA. In contrast, cells transfected with *Hmga2* siRNA do not undergo EMT. Indeed, these cells maintained polarized morphology, ZO-1, and E-cadherin at their junctions and decreased the TGF-βinducible levels of N-cadherin and Fibronectin-1 (Fig. 4, BE; and Fig. S3). Although *Hmga2* knockdown restored to a large extent epithelial tight and adherence junctions, we observed only a weak block of total ZO-1 and E-cadherin protein down-regulation by TGF-β (unpublished data). We conclude that endogenous HMGA2 is required for TGF-βinduced EMT.

HMGA2 regulates expression of key regulators of EMT

The zinc-finger transcription factors Snail, Slug, δEF-1/ZEB-1, and SIP-1/ZEB-2 or the basic helix-loop-helix factor Twist repress *E-cadherin* expression during embryonic EMT [19] and promote tumor cell metastasis or cancer recurrence after therapy [20, 21]. The extreme down-regulation of *E-cadherin* expression in HMGA2 clones (Fig. 3, C and D) prompted us to analyze some of these transcriptional repressors. In parental NMuMG cells, *Snail*, *Slug*, and to a lesser extent *Twist* mRNA were induced by TGF-β (Fig. 5, A and C). Repressor expression was dramatically up-regulated in HMGA2 clones even in the absence of TGF-β. Similar to regulation of endogenous *Snail* and *Twist* mRNA, TGF-β induced *Snail* and *Twist* promoter activity (Fig. 5 B). Notably, cotransfection of HMGA2 enhanced *Snail* and *Twist* promoter activity to significantly higher levels than TGF-β stimulation alone. As specificity control, the Smad3/Smad4 dependent promoter reporter $CAGA_{12}$-Luc was not regulated by HMGA2 (unpublished data). Upon RNAi-mediated knockdown of *Hmga2*, endogenous *Snail* mRNA induction by TGF-β was reduced by 50% (Fig. 5 D). This explains why total E-cadherin and ZO-1 protein levels were still repressible by TGF-β after *Hmga2* knockdown (unpublished data). We conclude that partial depletion of endogenous *Hmga2* by RNAi is sufficient to restore epithelial differentiation in NMuMG cells and establishment of cellcell junctions; however, it is not sufficient to block strongly enough *Snail* induction by TGF-β.

TGF-β down-regulates Id2 to induce EMT [8, 9]. *Id2* mRNA and protein expression were down-regulated in HMGA2 clones compared with mock cells, and TGF-β further repressed Id2 levels (Fig. 5, E and F). The results demonstrate that HMGA2 regulates *Snail*, *Slug*, *Twist*, and *Id2*, all key players of EMT. TGF-β induces *Snail* expression either via Smad3 or via MAPK signaling [22, 23]. Our results add HMGA2 as a novel regulator of *Snail* expression downstream of Smads. Whether HMGA2 cooperates with Smad3 or MAPK signals to induce *Snail* is being explored. *Twist* is another gene target of HMGA2 that is weakly induced by TGF-β. We conclude that TGF-β, via HMGA2, primarily affects the Snail pathway and, to a lesser extent, the Twist pathway.

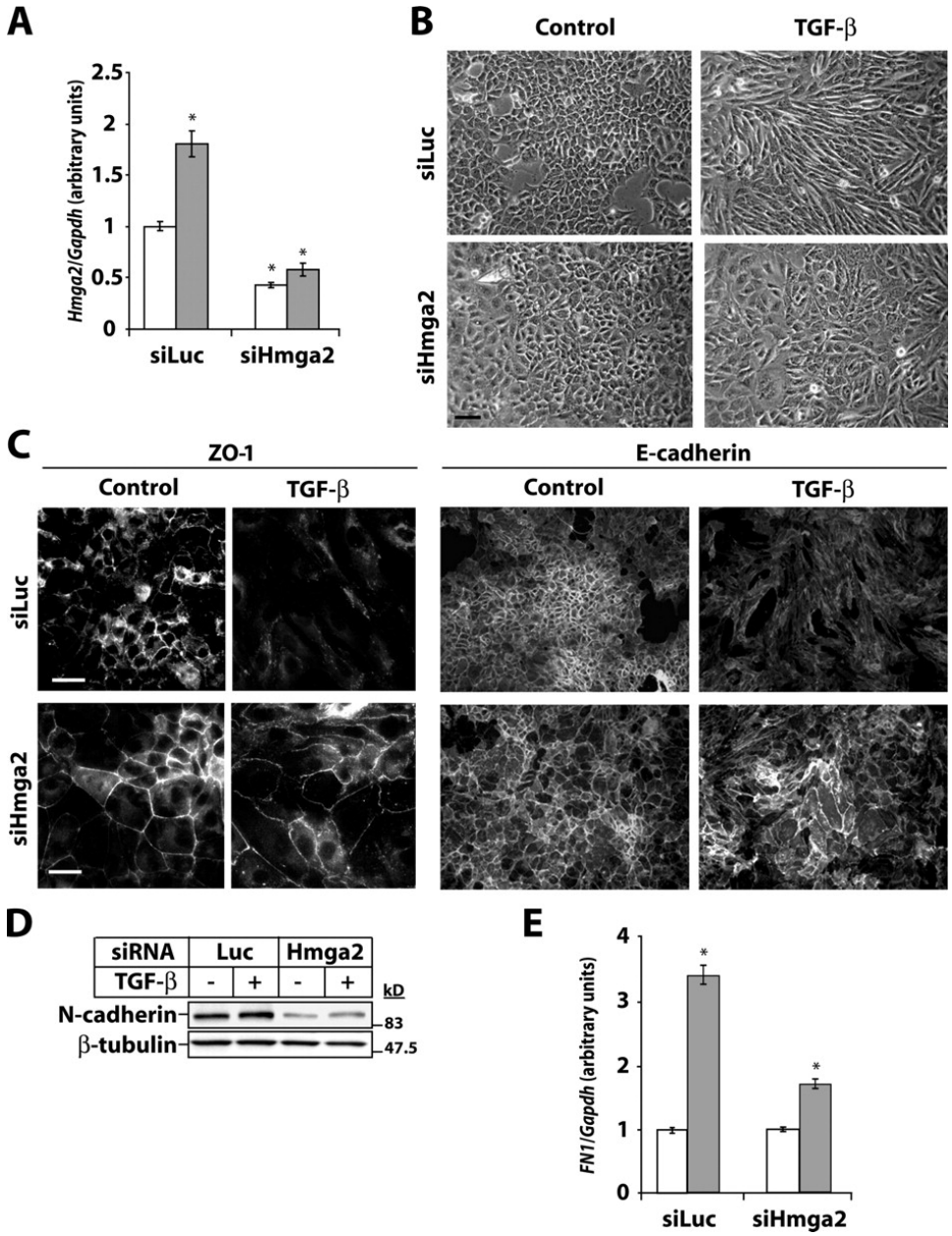


Figure 3.4: HMGA2 mediates EMT by TGF-β. (A) Quantitative RT-PCR analysis of *Hmga2* expression in NMuMG cells transfected with control (siLuc) or specific siRNA against *Hmga2* (siHmga2) and treated with vehicle (white bars) or 5 ng/ml TGF-β (grey bars) for 12 h. (B) Phase-contrast images, (C) indirect immunofluorescence of ZO-1 and E-cadherin and (D) immunoblot analysis of N-cadherin in NMuMG cells transfected as in (A) and treated with vehicle (control) or 5 ng/ml TGF-β for 36 h (Bars = 10 μm). β-tubulin is loading control. Molecular size markers are in kDa. (E) Quantitative RT-PCR analysis of Fibronectin-1 (FN1) expression in cells treated as in (A). Stars indicate statistically significant gene expression differences compared to the ground condition ($p \leq 0.05$).

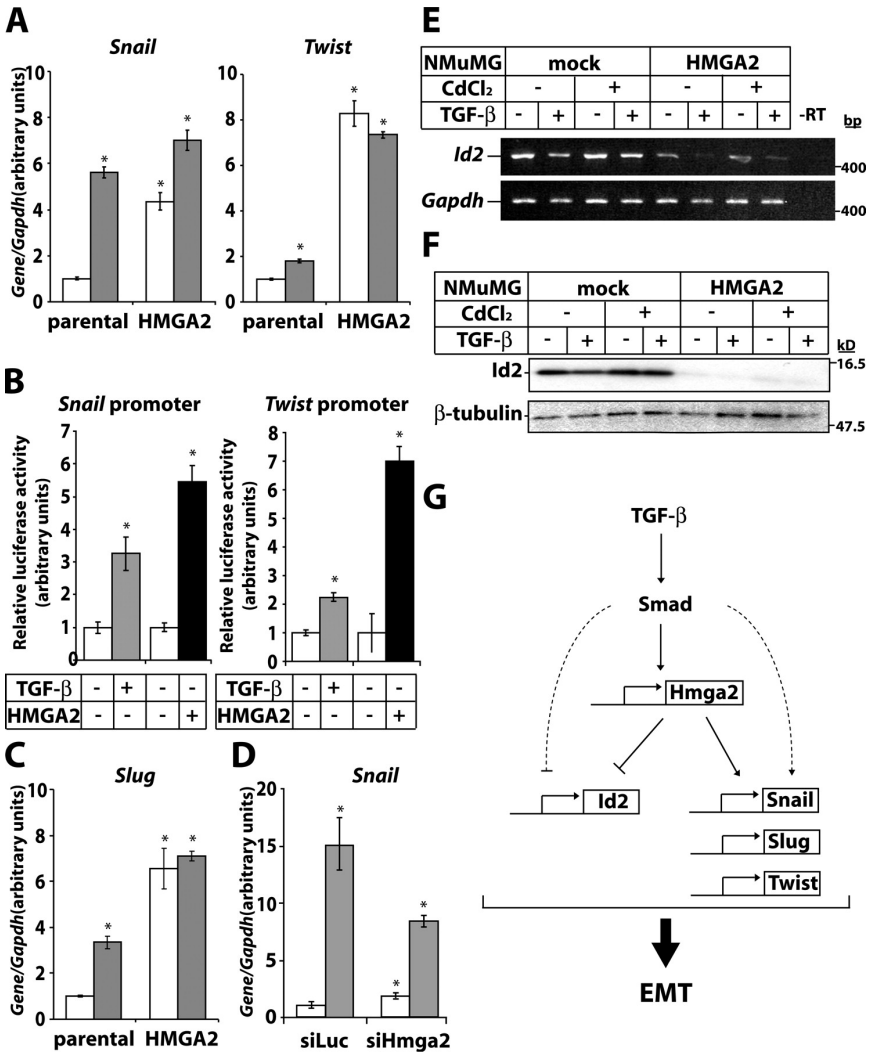


Figure 3.5: HMGA2 regulates expression of key regulators of EMT. (A) Quantitative RT-PCR analysis of Snail and Twist expression in parental or HMGA2-clones stimulated (grey bars) or not (white bars) with 5 ng/ml TGF-β for 36 h. (B) Luciferase reporter assays of Snail and Twist promoter constructs in HepG2 cells transfected with mock (-) or HA-hHMGA2 (+) vector and treated with 5 ng/ml TGF-β for 24 h. (C) Quantitative RT-PCR analysis of Slug expression under conditions as in (A). (D) Quantitative RT-PCR analysis of Snail expression in NMuMG cells transfected with control (siLuc) or Hmga2 (siHmga2) siRNA and treated with vehicle (white bars) or 5 ng/ml TGF-β (grey bars) for 12 h. Stars indicate statistically significant gene expression or promoter activity differences compared to the ground condition ($p \leq 0.05$). Expression pattern of Id2 using RT-PCR (E) or protein (F) analysis in mock and HMGA2-clones induced with 10 μM CdCl2 24 h before stimulation with 5 ng/ml TGF-β for 36 h. Molecular size markers are in bp (E) and kDa (F). (G) Diagram of the role of HMGA2 in TGF-β-induced EMT.

Concluding remarks

We describe a new target of TGF-β signaling, the nuclear factor HMGA2, and a new transcriptional circuitry that mediates EMT by TGF-β (Fig. 5 G). HMGA2 links TGF-

β signaling to major factors of tumor invasiveness and metastasis. This work suggests that HMGA2 acts not only as an architectural chromatin factor as previously thought but also as a gene-specific regulator that responds to signals from extracellular factors.

HMGA2 is overexpressed in a variety of tumors primarily of mesenchymal origin (for review see [12]). However, the mechanism of HMGA2 action is not yet known. This study demonstrates for the first time that HMGA2 is involved in EMT. Our results are consistent with the specific presence of HMGA2 at the invasive front of squamous carcinomas [24], a place where EMT occurs during cancer progression. On the other hand, overexpression in MCF7 mammary carcinoma cells of HMGA1b, another member of the HMGA family, but not that of the related HMGA1a, led to invasive tumor growth in nude mice [11]. Histochemical and transcriptomic analysis of tumor samples from such mice indicated that HMGA1b induced expression of genes with links to EMT.

Our study demonstrates that HMGA2 is necessary and sufficient for TGF-βinduced EMT. Considering the variety of tumors where HMGA2 expression has been detected, it will be interesting to determine in how many of them TGF-β is the upstream inducer of tumor HMGA2 expression and to what extent the signaling network outlined here explains the tumor promoting properties of TGF-β.

Materials and methods

Cells, adenovirus, and reagents

Mouse mammary epithelial NMuMG, human hepatocarcinoma HepG2, human MDA-MB-231-Eco cells, and stable NMuMG clones expressing Smad2 SA (mutant with alanines in place of two C-terminal serines that become phosphorylated by receptors) have been described [9, 16, 18]. Adenoviruses expressing control GFP (Ad-GFP) were a gift from B. Vogelstein (Johns Hopkins Medical Institutions, Baltimore, MD); adenoviruses expressing C-terminally HA-tagged constitutively active TGF-β type I receptor (activin receptor-like kinase 5) ALK-5(TD) and HA-tagged kinase-inactive ALK5(KR) receptor were a gift from K. Miyazono (University of Tokyo, Tokyo, Japan) and have been described [16].

The mouse *Hmga2* siRNA (available from GenBank/EMBL/DDBJ under accession no. NM-010441), which was a single RNA oligonucleotide (D-043585-03), and control siRNA against the luciferase reporter vector pGL2 (accession no. X65324) were obtained from Dharmacon Research, Inc. Human HMGA2 cDNA, cloned by PCR from total normal human mRNA in pcDNA3-HA C-terminally of the HA tag using EcoRIXhoI as restriction sites, resulted in pcDNA3-HA-hHMGA2. The N-terminally HA-tagged hHMGA2 HindIIIXhoI fragment was subcloned in the inducible vector pMEP4 to produce pMEP4-HA-hHMGA2.

The LY580276 inhibitor for the TGF-β type I receptor kinase was a gift from J.M. Yingling (Eli Lilly and Company, Indianapolis, IN; [17]). Recombinant mature TGF-β1 was obtained from PeproTech and TGF-β3 from K.K. Iwata (OSI Pharmaceuticals, Farmingdale, NY). TGF-β1 was used in most experiments and TGF-β3 in experiments with stable NMuMG and MDA-MB-231 clones for Smad shRNAs (Fig. 1 and Fig. S1). TGF-β1 and TGF-β3 have indistinguishable effects on EMT or Hmga2 gene regulation.

Cell counting

Cell monolayers were washed with PBS, trypsinized, and resuspended in PBS, and cell numbers were calculated using a Z1 cell counter (Beckman Coulter).Numbers are plotted as means from triplicate determinations with standard errors.

Cell transfections

pMEP4 and pMEP4-HA-hHMGA2 were transfected into NMuMG using Lipofectamine 2000 (Invitrogen). 2 d after transfection, cells were cultured in 400 μ/ml hygromycin-B (Calbiochem), and individual antibiotic-resistant clones were derived. For HMGA2 induction, cells growing in hygromycin-B were treated with 10 μM CdCl2 (Sigma-Aldrich) for 24 h, and cells were incubated with vehicle or TGF-β for another 24-48 h.

NMuMG cells were transiently transfected with *Hmga2* siRNAs using Dharmafect 4 (Dharmacon Research, Inc.). After 2 h of stimulation with TGF-β, cells were retransfected with siRNAs to a final concentration of 50 nM for 10 h (RNA assay) or 36 h (protein/immunofluorescence assay).

NMuMG and MDA-MB-231-Eco clones with Smad knockdown were established after infection with retroviral supernatants derived from cells transfected with pRetroSuper-expressing shRNA against Smad2, 3, or 4 and were provided by M. van Dinther and P. ten Dijke (Leiden University Medical Center, Leiden, Netherlands; [18]). The shRNA oligonucleotide sequences were 5-GGA GTG CGC TTG TAT TAC A-3 (mouse/ human Smad2), 5-CGT CAA CAC CAA GTG CAT C-3 (mouse/human Smad3), and 5-CCA GCT ACT TAC CAT CAT A-3 (mouse/human Smad4).

RT-PCR

Total DNA-free cellular RNA was extracted with the RNeasy kit (Qiagen). Reverse transcription (RT)-PCRs were performed as described [9] and analyzed using specific primers (Table I and II). Primers for mouse glyceraldehyde-3'-phosphate dehydrogenase (Gapdh) were used for reference. Lack of DNA contamination was verified by omitting reverse transcriptase(-RT). Quantitative real-time PCR reactions were as described [16]. Gene expression levels were determined with the comparative Ct method using Gapdh as reference. The ground condition was set to 1 and expression data are presented as bar-graphs of average values plus standard deviations.

Perchlorate extraction of chromatin

NMuMG cells were lysed in 140 mM NaCl, 1.5 mM MgCl2, 10 mM Tris-HCl pH 8.6, 0.5% NP-40, supplemented with protease inhibitors. After centrifugation (3 min, 6000 x g, 4 °), nuclear pellets were resuspended, vortexed 30 s and rotated at room temperature for 1 h in 5% perchloric acid. Perchloric acid supernatants (5 min, 6000 x g, 25 °) were precipitated by 8 volumes cold ethanol, centrifuged (15 min, 10000 x g 4 °) and pellets were resuspended in lysis buffer and analyzed by immunoblotting.

Immunoblotting and immunofluorescence microscopy

Total protein extracts subjected to SDS-PAGE were analyzed by immunoblotting, as described [9]. Mouse monoclonal anti-β-tubulin (T8535), anti-β-actin (AC-15) were from Sigma-Aldrich; mouse monoclonal anti-HA (12CA5) from Roche Applied Science; mouse monoclonal anti-E-cadherin (C20820) from BD Transduction Laboratories; rat monoclonal anti-ZO-1 (MAB1520) from Chemicon Int.; mouse monoclonal anti-histone H1 (AE-4), anti-Smad2/Smad3 (H-2) and anti-Smad4 (B-8) from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-Hmga2 was raised against a synthetic peptide (CSPSKAAQKKAETIGE where S maps at residue 59 of mouse Hmga2), and recognizes mouse but not human HMGA2 (unpublished results). Secondary anti-mouse-IgG and anti-rabbit-IgG coupled to horseradish peroxidase were from Amersham Biosciences. The enhanced chemilluminescence detection system was from Santa Cruz Biotechnology, Inc. For immunofluorescence, cells were treated as indicated in the figure legends, fixed and stained with tetramethyl-rhodamine isothiocyanate (TRITC)-labeled phalloidin (Sigma-Aldrich) or with rat anti-ZO-1, mouse anti-E-cadherin and rabbit anti-HMGA2 antibodies as primary antibodies and TRITC-conjugated goat anti-rat-IgG and FITC-conjugated anti-mouse- or anti-rabbit-IgG antibodies as secondary antibodies from Jackson ImmunoResearch Laboratories, Inc. as described [9]. Photomicrographs were obtained by a Zeiss Axioplan 2 microscope with a Hammamatsu C4742-95 digital camera, using the Zeiss Plan-neofluar 40 x/0.75 objective lens and photographing at ambient temperature in the absence of immersion oil. Primary images were acquired with the camera's QED software. Image memory content was reduced and brightness-contrast was adjusted using Adobe Photoshop 6.0.

Promoter-reporter assays

HepG2 cells were transiently transfected with calcium phosphate and a panel of mouse Hmga2 promoter luciferase constructs, as described [25]. The Snail and Twist promoter luciferase constructs were provided by A. Cano, Universidad Autónoma de Madrid, Madrid, Spain and L. R. Howe, Weill Medical College of Cornell University, New York, NY, USA respectively. All promoter constructs were co-transfected with the normalization reporter plasmid pCMV- -Gal and the expression vector pcDNA3 (mock vector) or pcDNA3-HA-hHMGA2 for Snail and Twist promoter analysis or pcDNA3-HA-Smad2 SA for Hmga2 promoter analysis. The enhanced luciferase assay kit from BD PharMingen, Inc. was used. Normalized promoter activity data are plotted in bar graphs representing average values from triplicate determinations with standard deviations. Each independent experiment was repeated at least twice.

Chromatin immunoprecipitation (ChIP) assays

The equivalent of 10^7 cells was used per ChIP reaction. Crosslinking was performed using 1% formaldehyde followed by neutralization with 0.125 M glycine. Cells were lysed in 1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1 plus protease inhibitors on ice. DNA was sheared by sonication to 200-1000 bp length. Sonicated cell pellets were diluted 10 times in a buffer containing 0.01% SDS and were precleared with protein A-Sepharose in the presence of bovine serum albumin (BSA) and salmon sperm DNA prior to incubation with 5 μg rabbit anti-Smad4 antibody (H-552; Santa Cruz Biotechnology Inc.) or preimmune rabbit antiserum as a negative control. Protein-DNA complexes were precipitated with protein A-Sepharose in the presence of BSA and salmon sperm DNA. Immunoprecipitated complexes were washed once with 150

mM NaCl, 0.2% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, once with an identical buffer containing 500 mM NaCl, once with 0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1, and twice with 10 mM Tris-EDTA. Immunoprecipitated complexes were eluted with 1% SDS, 0.1 M NaHCO3. After reversal of crosslinks by heating in 0.2 M NaCl, proteinase K treatment, purification by classical phenol-chloroform extraction and ethanol precipitation, DNA pellets were resuspended in 20 μl water. Input material prior to immunoprecipitation corresponds to one third of the immunoprecipitated material. Input DNA pellets were resuspended in 50 μl of water. PCR was performed using 2 μl of immunoprecipitated or 0.5 μl of input material using Taq DNA polymerase (Invitrogen Corp.). Amplification was carried out for 30 cycles for immunoprecipitated DNA and 25 cycles for input DNA. Primer sets used to amplify different regions of the *Hmga2* promoter are described in Table II.

Statistical analysis

Statistical analysis of real time PCR quantification and promoter assays was performed by 2-tailed paired Student's t-test. Significance was considered at P value ≤ 0.05.

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Supplemental material

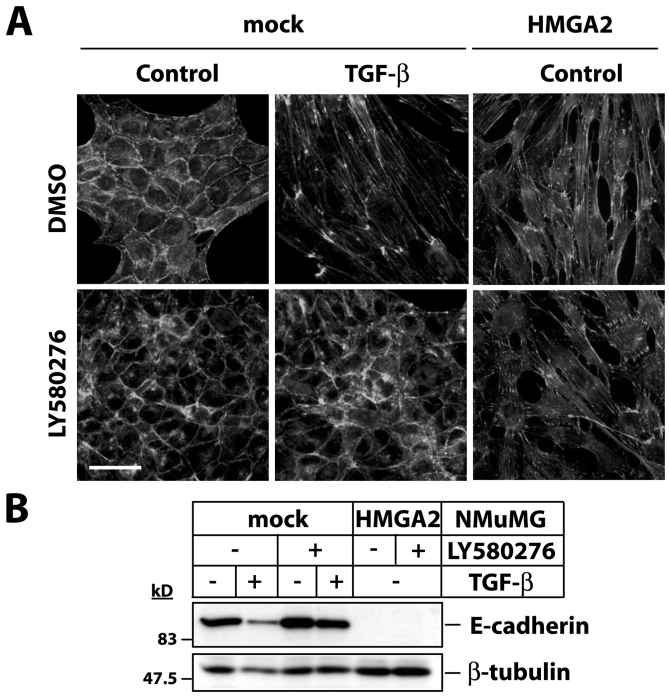


Figure 3.6: Figure S1. Hmga2 expression is induced by the TGF-β/Smad pathway. (A) Quantitative RT-PCR analysis of *Hmga2* expression in NMuMG cells stimulated (grey bars) or not (white bars) with 5 ng/ml TGF-β in presence or not (NT) of 5 g/ml cycloheximide (CHX) or 1 g/ml actinomycin D (ActD) for 1 h. (B) Quantitative RT-PCR analysis of *Hmga2* expression in NMuMG cells infected with control adenovirus GFP (Ad-GFP) or adenoviruses expressing constitutively active (TD) or dominant-negative (KR) forms of ALK5 and stimulated or not with TGF-β (5 ng/ml) for 4 h. Stars indicate statistically significant differences between TGF-β/receptor-stimulated and non-stimulated condition. (C) Phase contrast images of NMuMG cell lines expressing short hairpin RNAi vectors (sh-Smad) directed against Smad2, Smad3 or Smad4, or the empty vector (pRS) treated or not (control) with 5 ng/ml TGF-β for 48 h. A bar indicates 10 μm. (D) Immunoblot analysis of Smad2, Smad3 and Smad4 levels in cell lysates from cells used in (C). In the Smad3 knockdown cells only Smad2 and Smad3 were assayed (middle shorter immunoblot). (E) Immunoblot analysis of Smad2, Smad3 and Smad4 levels in cell lysates from MDA-MB-231 cells used in the HMGA2 expression analysis of panel (F) and Fig. 1G. (F) Semi-quantitative RT-PCR assay of HMGA2 expression in MDA-MB-231 stable clones with specific Smad knockdown, in the absence or presence of stimulation with 5 ng/ml TGF-β.

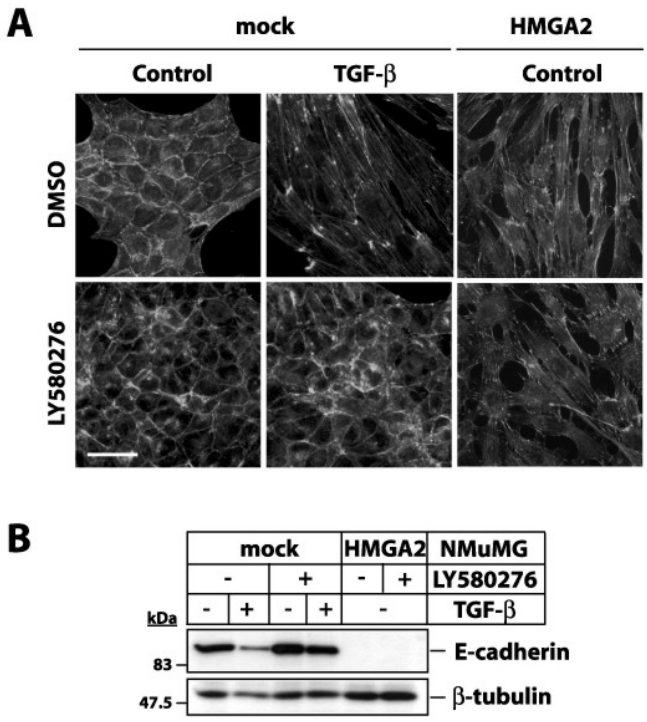


Figure 3.7: Figure S2. HMGA2 does not initiate an autocrine TGF-β loop. (A) Phalloidin staining of actin cytoskeleton in mock or HMGA2-expressing cells in the presence (LY580276) or absence (DMSO) of the specific TGF-β type I receptor inhibitor LY580276 (2.5 μM) and treated (TGF-β) or not (control) with 5 ng/ml TGF-β for 36 h (Bar = 10 μm). (B) Immunoblot analysis of E-cadherin using protein extracts from cells treated as in panel (A).

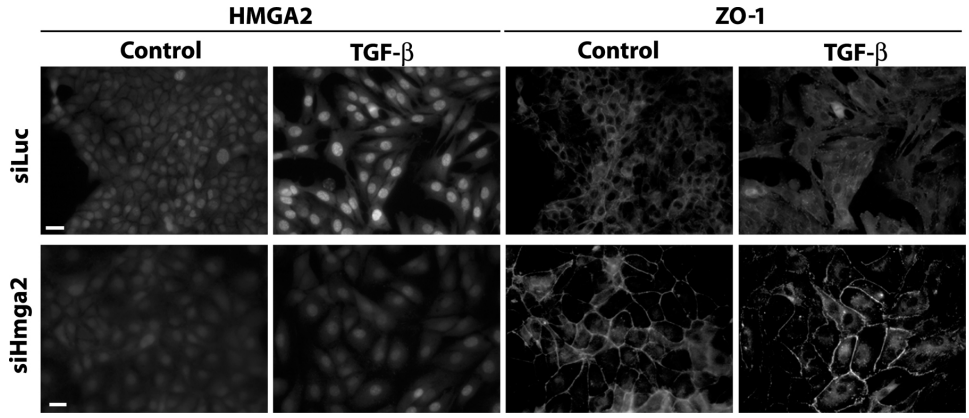


Figure 3.8: Figure S3. HMGA2 mediates EMT by TGF-β Localization of endogenous HMGA2 and ZO-1 by indirect immunofluorescence in NMuMG cells transfected with control (siLuc) or specific siRNA against Hmga2 (siHmga2) and treated with vehicle (control) or 5 ng/ml TGF-β for 36 h (Bars = 10 μm).

Chapter 4

Constitutive Activation of Activin Receptor-like Kinase 2 in Human Breast Cancer Cells inhibits Metastatic Progression and Osteolytic Bone Lesions

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Abstract

Transforming growth factor-β (TGF-β) is a crucial player in metastasis of breast cancer cells to bone. We previously reported that bone morphogenetic protein-7 (BMP-7) can counteract the effects of TGF-β in vitro and inhibit the progression and formation of osteotropic MDA-BO2 human breast cancer bone metastasis in vivo. Whether BMP-7 acts directly on the breast cancer cells and/or on the cancer-associated bone stroma has remained elusive. Here, we demonstrated that activin-like kinase receptor 2 (ALK2) is the functional BMP-7 type I receptor in MDA-BO2 cells. Subsequently, to restrict BMP-7-induced actions to MDA-BO2 cells we engineered these cells to ectopically express a constitutively active variant of ALK2, denoted caALK2. We demonstrate that caALK2 continuously activates the BMP signaling pathway as measured by Smad phosphorylation, BMP transcriptional reporter activity and target gene responses, and antagonized basal and TGF-β-induced transcriptional reporter activation. In vivo tumor-induced osteolysis and metastatic progression was mitigated in mice inoculated with MDA-BO2 cells over-expressing caALK2 compared to a control cell line. These observations suggest that cell autonomous activation of caALK2 or BMP7 over-expression in breast tumor cells inhibit osteolysis and metastatic progression possibly by interfering with the TGF-β-mediated self-amplifying cycle of bone destruction.

Abbreviations ActR-II, Activin receptor type II; ALK, Activin receptor-like kinase; caALK, constitutively active ALK; BMP, bone morphogenetic protein; BMPR-II, BMP receptor type II; BRE, BMP-responsive element; caALK2, constitutively active ALK2; EMT, epithelial mesenchymal transition; GFP, green fluorescent protein; HLH, helix-loop-helix; ID, inhibitor of differentiation/DNA binding; μ-CT, micro-computed tomography; MET, mesenchymal epithelial transition; P-Smad, phosphorylated Smad; I-Smad, inhibitory Smad; Smad, small phenotype and mothers against DPP related protein; TGF-β, transforming growth factor β.

Introduction

Women diagnosed with advanced breast cancer are at high risk of developing metastatic bone disease [1]. Tumor-induced bone remodeling result in severe patient morbidity [2, 1]. At present, no curative therapies are available for the treatment of bone metastasis and a better understanding of the underlying mechanisms of metastatic disease and the interplay between the cancer cells and the bone microenvironment is critically important in the search for new anti-metastatic therapies.

Bone morphogenetic protein (BMP)-7 is a dimeric cytokine and a member of the transforming growth factor (TGF)-β family that have pleiotropic functions in development, tissue homeostasis and in cancer progression [3]. BMPs signal via assembly of heterotetrameric BMP-receptor complexes comprised of type I and type II serine/threonine kinase receptors [4, 5, 6]. The type I receptors for BMPs include activin-like kinase (ALK)1, ALK2, ALK3, and ALK6 whereas the type II receptors are BMPRII, ActRII, and ActRIIB. ALK2 has been described as predominant type I receptor for BMP-7 [5, 6, 7, 8, 9]. Type I receptors are phosphorylated and activated by type II receptors and determine the signaling specificity within the heterotetrameric complex [10]. Upon ALK2 activation receptor-regulated (R-)Smad1, Smad5, and Smad8 can be phosphorylated and form heteromeric complexes with the common mediator Smad4. These transcriptional complexes modulate target gene expression in concert with various co-factors [5, 11, 12]. Inhibitory Smads (I-Smads), Smad6 and Smad7 antagonize activation of R-Smads [13, 14, 15].

BMP-7 plays key regulatory roles during early embryogenesis [16, 17, 18, 19]. In a pathological setting, loss of BMP-7 mRNA expression in primary tumor is associated with increased bone metastasis and invasiveness of breast cancers cell lines [20]. Moreover, BMP-7 was shown to counteract TGF-β-induced tumorigenic responses in breast cancer cells in particular through inhibition of TGF-β-mediated epithelial mesenchymal transition (EMT) and induction of mesenchymal epithelial transition (MET) [17, 20, 21]. The dichotomous roles of TGF-β and BMP-7 on EMT and MET can be explained, at least in part, by the differential regulation of inhibitors of differentiation/DNA binding (ID) proteins [22].

Breast cancer has high predilection for the skeleton and once micro-metastatic lesions are established they induce osteolytic bone destruction through a vicious cycle of paracrine signaling between the tumor cells and the bone microenvironment [1, 2, 23]. In brief, cancer cells stimulate osteoclastic progenitor maturation and bone resorption resulting in release of matrix-bound growth factors such as TGF-β, insulin-like growth factor and others. These cytokines can then act back on the tumor cells and stimulate further growth, osteoclastogenesis and bone destruction [1, 2, 21, 23]. The MDA-BO2 breast cancer cell line is an osteotropic sub-clone of the MDA-MB-231 cell line which gives rise to highly aggressive and osteolytic bone metastasis in immunodeficient mice [24]. Over-expression of BMP-7 in MDA-BO2 cells or systemic administration of human recombinant BMP-7 to MDA-BO2 tumor-bearing animals inhibited the metastatic potential and the progression of these breast cancer cells *in vivo* [20]. Whether the BMP-7

exerts its effects directly on the cancer cells directly and/or on the cancer-associated stroma is not known. In this study, we engineered MDA-BO2 cells to over-express a constitutively active form of, the BMP-7 receptor, ALK2 to restrict BMP-7-induced responses solely to breast cancer cells. This cell line was characterized *in vitro* and compared to BMP-7 over-expressing cells in a bone tumor model *in vivo*. The outcome of our studies demonstrates that inhibition of *de novo* formation of bone metastases and tumor cell-induced osteolysis is mediated primarily through direct effects of BMP-7 on the cancer cells.

Results

ALK2 is the functional BMP-7 type I receptor in MDA-BO2 cells

We first examined the expression profile of BMP type I and type II receptors in MDA-BO2 cells by quantitative real-time PCR (Fig. 1A). *ALK2* was expressed in these cells however the expression levels of *ALK3* was four fold higher. Some *ALK6* was expressed, whereas *ALK1*, a type I receptor for BMPs in endothelial cells, was not detected as expected. The BMPRII was expressed at high levels whereas ActRII and ActRIIB were detected at lower ratios (Fig. 1A).

The physiological binding property of BMP-7 to cell surface BMP type I receptors of MDA-BO2 cells was analyzed by affinity labeling with radiolabeled BMP-7 to a monolayer of cells. Ligand-receptor complexes were cross-linked and lysates immunoprecipitated with anti-sera for all BMP type I receptors. BMP-7 specifically bound to ALK2 (Fig. 1B). No detectable binding to ALK3 or ALK6 was observed for BMP-7 (Fig. 1B).

Ligand receptor binding triggers trans-phosphorylation of BMP-specific R-Smads; i.e. Smad1, Smad5, and Smad8 and subsequently heteromeric complex formation with Smad4 [13]. To ascertain that BMP-7 signals via ALK2, we transiently co-transfected cells with shRNA plasmids targeting ALK2 and the BMP responsive reporter construct (BRE-luc) which contains repeated promoter sequences of the BMP target gene ID1 [25]. When endogenous levels of ALK2 were depleted in MDA-BO2 cells both the basal and the BMP-7-induced reporter activity was significantly inhibited (Fig. 1C). This was observed with two independent shRNAs for ALK2 and strongly suggests that BMP-7 indeed signals via ALK2 in MDA-BO2 cells.

Mimicking a state of continuous BMP-7 signaling can be achieved through expression of a constitutively active form of the ALK2 receptor (caALK2). This genetically modified receptor contains an activating mutation, Q207D, in the GS domain [26]. MDA-BO2 cells with isogenic stable expression of caALK2 were generated by Gateway Flp-In recombinase. Cells stably expressing GFP were used as controls along with MDA-BO2 cells over-expressing BMP-7 [20]. The stable caALK2 cells expressed 8 fold more *ALK2* compared to physiological levels in control GFP (Fig. 1D) and parental MDA-BO2 cells (data not shown).

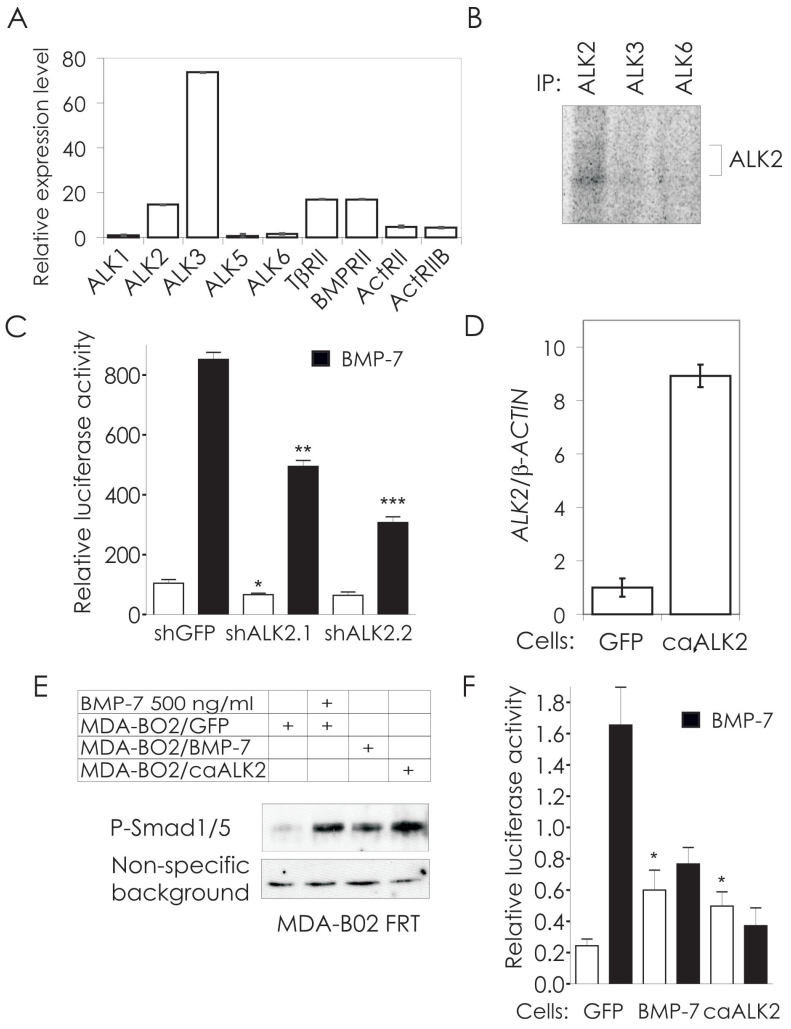


Figure 4.1: Ectopic of constitutive activate BMP-7 type I receptor, ALK2, in MDA-BO2 cells. (A) Quantitative real-time PCR analysis of the BMP specific type I receptors *ALK1*, *ALK2*, *ALK3*, and *ALK6* and the type II receptors *ActRII*, *ActRIIB*, and *BMPRII* in MDA-BO2 cells. Relative gene expression levels were corrected to *β-ACTIN* using the $\triangle\triangle$Ct method. Values are presented as relative mean ± S.D. (B) MDA-MB-231 cells were affinity-labeled with [^{125}I]-BMP-7, cross-linked and immunoprecipitated with anti-sera to ALK2, ALK3 and ALK6. Proteins were resolved by SDS-PAGE and radioactivity detected on a Phosphoimager. (C) MDA-BO2 cells were transiently co-transfected with the BRE-luc reporter and shRNA constructs targeting ALK2. A shRNA plasmid targeting GFP was used as control. Cells were stimulated with (black bars) or without (white bars) BMP-7 for 16 hours and the relative luciferase activity determined. The BMP-7-induced BRE-luc activity was significantly inhibited when shALK2.1 ($P \leq 0.01$) or shALK2.2 ($P \leq 0.001$) were co-transfected compared to shGFP control transfected cells. Also, basal reporter activity was inhibited by shALK2.1 compared to shGFP control transfected cells ($P \leq 0.05$). (D) Quantitative real-time PCR analysis of the relative ALK2 gene expression in GFP control or caALK2 overexpressing MDA-BO2 cells. Data is presented as mean ± S.D corrected for *β-ACTIN* expression levels. (E) Immunoblot analysis of P-Smad1/5 in stable MDA-BO2 cell lines. GFP control cells stimulated with BMP-7 for 45 minutes were used as a positive control. A non-specific protein band was used to control for equal loading. (F) Cells with ectopic expression of control GFP, BMP-7 or caALK2 were transiently transfected with the BRE-luc reporter and stimulated with (black bars) or without (white bars) BMP-7 for 16 hours. Basal levels of BRE-reporter activity in BMP-7 and the caALK2 cells were induced compared to control GFP cells ($P \leq 0.05$).

We next analyzed whether ectopic expression of caALK2 resulted in continuous activation of the BMP signaling cascade. Constitutive active ALK2 cells showed high endogenous levels of P-Smad1/5 compared to the control GFP cell line as observed by western blot analysis (Fig. 1E lane 4). The amount of phosphorylated Smad1/5 (P-Smad1/5) was equivalent to the P-Smad1/5 intensity in GFP control cells stimulated with 500 ng/ml BMP-7 (Fig. 1E lane 2) and slightly higher than P-Smad1/5 levels in BMP-7 over-expressing cells (Fig. 1E lane 3). When stable cell lines were transfected with the BRE-luc reporter, significant higher basal luciferase activity was observed in caALK2 and BMP-7 cells compared to control GFP cells (Fig. 1F). Exogenous addition of BMP-7 did not further increase the luciferase activity in either caALK2 or BMP-7 over-expressing cells suggesting that a plateau in BMP signaling is reached by constitutive receptor activation and BMP-7 expression (Fig. 1F). The amount of BMP-7 produced by BMP-7 over-expressing cells was estimated by harvesting conditioned medium from the cells and using this medium in a BRE-luc reported assay. This was estimated to approximately 200 ng/ml (Fig. S1). Thus, stable isogenic expression of caALK2 continuously activates the BMP signaling cascade to similar extent as ectopic expression of BMP-7 in MDA-BO2 cells.

Ectopic expression of caALK2 inhibits TGF-β signaling

Exogenous addition of BMP-7 can inhibit TGF-β mediated signaling and counteract the induction of downstream responses such as EMT [20, 17]. We hypothesized that ectopic expression of caALK2 would result in a similar inhibition of the TGF-β pathway. To explore this, we transiently transfected the three stable MDA-BO2 lines with, CAGA-luc, a Smad3/4-dependent TGF-β responsive reporter [27].

Basal and TGF-β-induced luciferase activities were significantly reduced in cells expressing caALK2 (Fig. 2A and B) and similar results were observed in BMP-7 over-expressing cells (Fig. 2A). Together, these results suggest that the interplay between BMP-7 and TGF-β signaling pathways is mutually competitive and may depend on formation of mixed Smad complexes [7] or bioavailability of intracellular signaling molecules such as Smad4 or transcriptional co-activators [28].

Ectopic expression of caALK2 mimics BMP-7-induced gene responses

Ectopic expression of BMP-7 or caALK2 induces similar profiles of Smad1/5 activation and BRE-luc reporter activation. The expression levels of the BMP-inducible target genes *ID2*, *SMAD6*, and *SMAD7* were greatly induced after 24 hours BMP-7 stimulation in control GFP cells (Fig. 3). Elevated mRNA levels of *ID2* were also observed in cells over-expressing BMP-7 and caALK2. The expression was comparable to control cells stimulated with BMP-7 (Fig. 3A). Similar enhanced basal levels of *SMAD6* and *SMAD7* mRNA were observed in these lines (Fig. 3B and C). *SMAD6* was expressed at two fold higher levels in caALK2 cells compared to BMP-7 cells (Fig. 3B). Exogenous

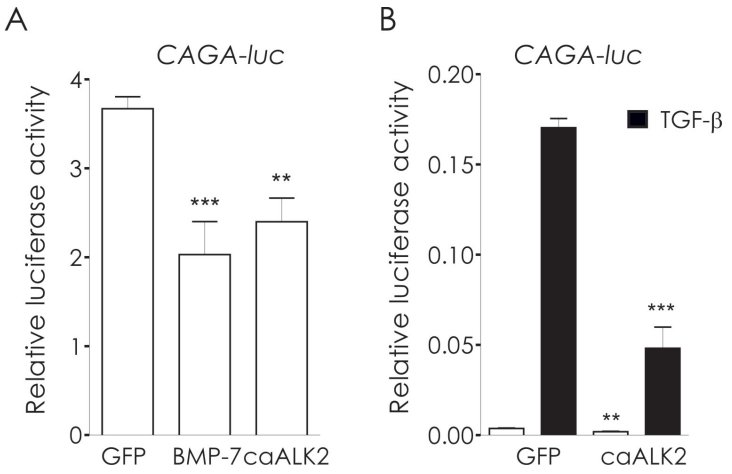


Figure 4.2: Ectopic expression of caALK2 inhibits TGF-β signaling. (A) The stable MDA-BO2 cell lines were transiently transfected with the TGF-β-inducible CAGA-luc reporter and the relative luciferase activity measured. Autocrine TGF-β activity was inhibited in cells with ectopic expression of caALK2 (P$\leq$0.01) or BMP-7 (P$\leq$0.001) compared to GFP control cells. (B) GFP control and caALK2 cells were transient transfected with the CAGA-luc reporter and cells were stimulated with (black bars) or without (white bars) TGF-β for 16 hours. The TGF-β-induced reporter activity was significantly inhibited in caALK2 cells (P$\leq$0.001) versus control GFP cells.

addition of BMP-7 to the BMP-7 MDA-BO2 cells further enhanced these gene responses by 2 fold whereas the response in caALK2 cells was saturated (Fig. 3B and C). Thus, activation of BMP-7 signaling pathways in MDA-BO2 via either over-expression of ligand or continuous activation of the receptor results in comparable target gene responses.

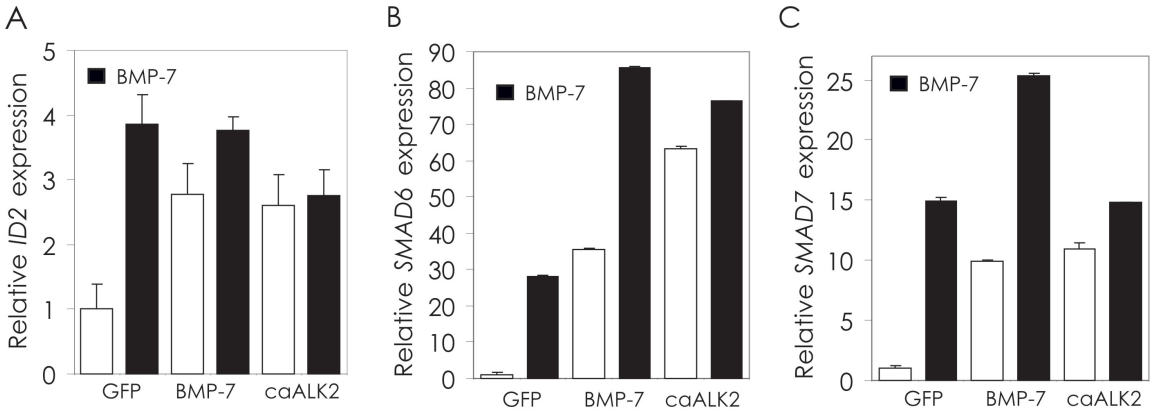


Figure 4.3: Target gene responses in cells over-expressing GFP, caALK2 or BMP-7. Quantitative real-time PCR analysis of (A) *ID2*, (B) *SMAD6*, and (C) *SMAD7* in cells stimulated with (black bars) or without (white bars) BMP-7 for 24 hours. Data is presented as the relative gene expression compared to *GAPDH* as a mean of three measurements $\pm$ S.D.

We next examined if expression of either BMP-7 or caALK2 affects the viability of MDA-BO2 cells *in vitro*. Viability profiles for the control GFP, BMP-7, and caALK2

cells were monitored for four consecutive days and similar growth kinetics were observed for the three cell lines (Fig. S2). Furthermore, no differences in growth or sizes of colonies were found in anchorage-independent growth assays with the three cell lines (data not shown).

Constitutive activation of ALK2 inhibits osteolysis and metastatic progression *in vivo*

Bone metastases from breast cancers are typically osteolytic as a result of the establishment of a vicious cycle of tumor-induced osteoclastic bone resorption and tumor progression. The net results being osteolysis and enhanced release of growth factors stored in the bone matrix, which can then act back on the cancer cells [1, 23]. This ultimately results in dramatic alterations in the micro-architecture of the bone and extensive loss of mineral content. We previously found that BMP-7 suppress the incidence and progression of metastatic bone disease [20, 29]. In order to determine if the inhibitory actions of BMP-7 is a result of direct effects of BMP7 on the breast cancer cells and/or mediated indirectly via the surrounding bone-marrow stroma, we compared the growth in bone of cells over-expressing BMP-7 versus cells over-expressing caALK2 in MDA-BO2 cells *in vivo*. Alternatively, MDA-BO2 cells were inoculated into the systemic circulation of immunodeficient mice via the left heart ventricle giving rise to osteolytic bone metastases [20, 29, 30]. Metastatic progression was followed in time by bioluminescent imaging (BLI) and osteolytic lesions were detected by radiography and/or micro-computed tomography (μ-CT) scanning as areas of low mineral density at the end of the experiment [30, 31].

For the intra-osseous tumor growth model, series of longitudinal and transverse μ-CT and radiographs were compared to assess the extent of tumor-mediated osteolysis in the three experimental groups (Fig. 4). Animals injected with caALK2 or BMP-7 over-expressing tumor cells displayed significantly less bone destruction compared to mice inoculated with control MDA-BO2 GFP cells. ($P=0.04$ and $P=0.017$ for caALK2 and BMP-7 ectopic expressing cells, respectively; Fig. 4A and B). The bone density was measured by transverse image analysis of the proximal tibiae at one fixed position and given as the % area covered by mineralized bone. The μ-CT volume measurements were significantly reduced in MDA-BO2 GFP xenografted animals compared to non-tumor bearing tibiae ($P\leq0.001$), as expected (Fig. 4C). The radiographic observations were further supported by morphometric analysis by Masson-Goldner histological staining of bone sections (Fig. 4D). This revealed extensive bone loss and complete replacement of the bone marrow with breast cancer cells in bone sections from mice inoculated with MDA-BO2 GFP cells. Osteolytic bone destruction was again significantly less in mice inoculated with both BMP-7 and caALK2 expressing cells (Fig. 4C and D).

We next used the experimental *in vivo* model of bone metastasis, which recapitulates later stages of metastatic progression namely, survival in the blood stream, extravasation, bone marrow colonization and establishment of bone metastases [32, 31, 20]. Animals were injected with stably expressing GFP or caALK2 MDA-BO2 cells and metastatic

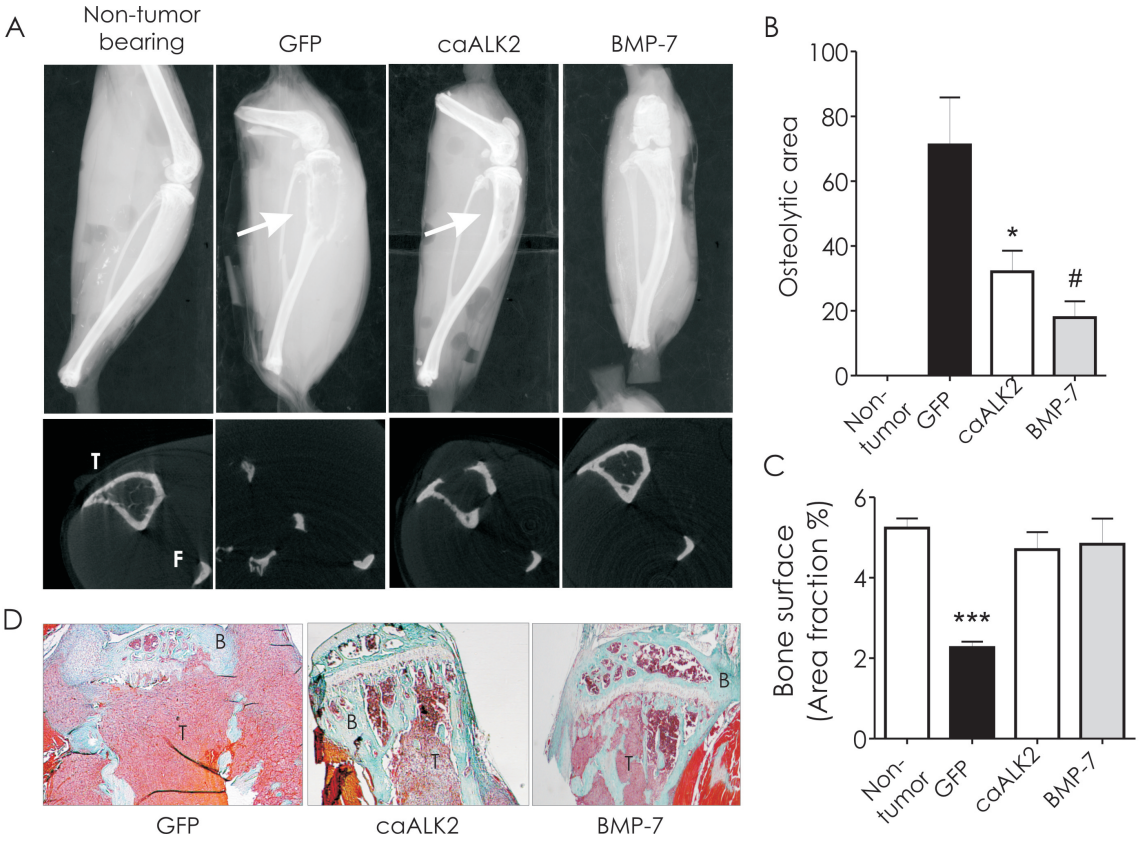


Figure 4.4: Active BMP signaling in MDA-BO2 cells inhibits osteolytic bone resorption. (A) Representative μ-CT images of the longitudinal plane and the corresponding transversal sections acquired below the growth plate of non-injected tibiae or mice injected with GFP control, caALK2 and BMP-7 MDA-BO2 cells. Micro-CT scans were acquired at the end of the experiment 45 days after tumor cell inoculation. Mice injected with GFP control cells display extensive tumor-induced osteolysis and complete loss of normal bone architecture (arrows indicate osteolytic lesions). Animals injected with caALK2 or BMP-7 over-expressing cells display significantly less osteoclastic bone destruction. This is quantified in the figure bone surface area. Abbreviations: F, fibula; T, tibia. (B) Quantification of the tumor burden and (C) percentage bone volume area on μ-CT images (n=6) measured by binary image analysis. The tumor burden and osteolytic bone destruction were significantly higher in GFP control MDA-BO2 versus BMP-7 and caALK2 MDA-BO2 inoculated mice $^{***}P \leq 0.001$ and $^{*}P \leq 0.05$ in caALK2 and BMP-7 MDA-BO2 $^{\sharp}P \leq 0.05$ versus GFP MDA-BO2 inoculated mice. Data is presented as mean $\pm$ S.D. (D) Histomorphometric analysis of proximal tibiae. Masson's-Goldner trichrome staining was used to visualize mineralized bone. Massive loss of cortical and trabecular bone is observed in control GFP injected animals. Images were captured at 4 x magnification. Abbreviations: B, bone; T, tumor.

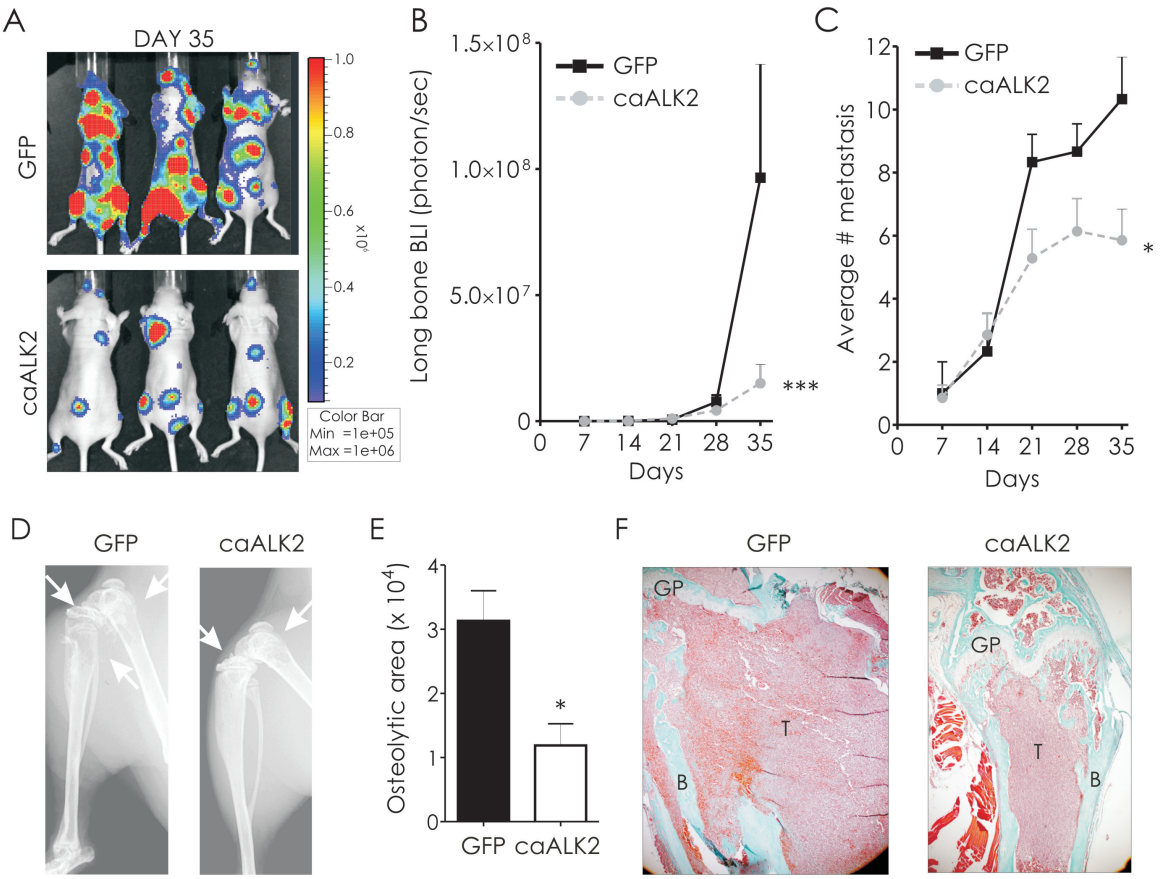


Figure 4.5: Ectopic expression of caALK2 in MDA-BO2 cells inhibits breast cancer metastasis. (A) *In vivo* BLI at day 35 of three representative mice injected with MDA-BO2 luc cells stably expressing GFP control or caALK2. Dorsal images are shown. (B) Average total flux of BLI from long bones of each experimental group followed in time. MDA-BO2 GFP (black square, black line) and caALK2 (grey circle, grey dotted line). Asterisks indicate statistically significant difference in total flux at day 35 between GFP MDA-BO2 and caALK2 MDA-BO2 long bone metastases ***P≤0.001. (C) Average number of metastases per animal in each experimental group. Significant less metastases are observed in the animals inoculated with caALK2 versus GFP control cells at day 35 *P≤0.05. (D) Representative radiographies of GFP (left) and caALK2 (right) inoculated mice at day 35. (E) Quantification of the average area of osteolytic lesion measured on radiographies on hind legs of mice with bone metastases. Results are expressed at mean relative area ± S.E.M. *P≤0.05 compared to GFP MDA-BO2 inoculated mice. (F) Histology of tibiae with metastases. Near complete loss bone architecture is observed in control GFP MDA-BO2 injected animals. Images were acquired at 4 x magnification. Abbreviations: B, bone; GP, growth plate; T, tumor.

progression was assessed by BLI (Fig. 5A and B). Tumor burden in long bones (Fig. 5B) was significantly decreased in mice inoculated with caALK2 MDA-BO2 cells compared to GFP control MDA-BO2 tumor-bearing mice ($P \leq 0.001$). Also, the average number of metastasis at the end of the experiment was significantly reduced in caALK2 MDA-BO2 inoculated mice compared to GFP MDA-BO2 metastases-bearing animals ($P \leq 0.05$) (Fig. 5C). In line with these findings, mice inoculated with the caALK2 over-expressing cells displayed less osteolytic lesions (Fig. 5D and E) compared to mice with GFP control MDA-BO2 bone metastases ($P \leq 0.05$) as evaluated by radiography. Histomorphometric analysis further confirmed these findings (Fig. 5F). Mice inoculated with GFP control cancer cells displayed a near complete loss of normal bone architecture and tumor cells had largely replaced the bone marrow.

To verify that tumor cells maintained the expression of BMP-7 or caALK2 *in vivo* tumor cells derived from bone tumors or metastases were analyzed by quantitative real-time PCR (Fig. S3). BMP-7 and caALK2 over-expression was retained to similar levels as in the parental cell lines. In addition, functionality of caALK2 in bone metastases was confirmed by immunohistological staining of P-Smad1. Enhanced nuclear P-Smad1 staining was observed in caALK2 expressing sections compared to control GFP metastases (Fig. 6).

Taken together, our observations suggest that the inhibitory action of BMP-7 on tumor growth and metastatic progression is mediated via direct effects on breast cancer cells.

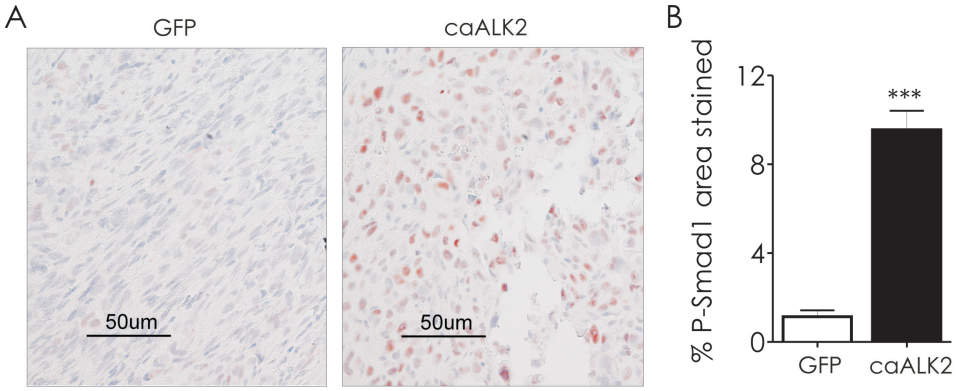


Figure 4.6: Immunohistological staining of P-Smad1 in breast cancer metastasis (A) P-Smad1 immunostaining of GFP and caALK2 overexpressing tumor metastasis. (B) Image quantification of P-Smad1 staining in metastatic bone sections. Significant more P-Smad1 staining is observed in caALK2 versus GFP control bone metastases ($P \leq 0.001$). Error bars indicate mean ± S.D.

Discussion

Skeletal metastases are observed in the majority of breast cancer patients diagnosed with advanced disease [33, 34]. Evidence is mounting that the formation and progression of skeletal metastasis is critically dependent on tumor-induced osteoclastic bone

destruction, which requires a complex interplay of cancer cells and the bone stromal microenvironment. [1, 2, 23, 31]. TGF-β is a major inducer of this vicious cycle of skeletal metastasis and understanding the multifaceted mechanisms and the role of TGF-β and BMP signaling herein is critically important for the development of anti-metastatic therapies.

We previously showed that over-expression of BMP-7 in osteotropic breast cancer cells or systemic administration of human recombinant BMP-7 to tumor-bearing animals inhibit *de novo* formation and progression of osteolytic metastasis [20, 29]. However, whether BMP-7 confers these anti-metastatic effects by acting directly on the cancer cells and/or via the bone microenvironment has remained elusive.

Here, we demonstrate that BMP-7 specifically binds and signals via the type I receptor ALK2 in MDA-MB-231/BO2 cells. We engineered MDA-BO2 cells to stably express a constitutively active form of ALK2 [26]. This enabled us to study ligand-independent BMP-7 signaling confined to the cancer cells and compare to BMP-7 over-expressing MDA-BO2 cells which in addition secrete ligands into the surrounding microenvironment. Ectopic expression of caALK2 in MDA-BO2 cells exhibited sustained phosphorylation of Smad1/5/8 and elevated BMP-mediated transcriptional activity, thus mimicking a state of continuous BMP-7 signaling in the cancer cells. Furthermore, BMP-responsive target genes such as ID2, Smad6, and Smad7 were highly elevated in caALK2 MDA-BO2 cells. Autocrine and TGF-β-induced transcriptional reporter activity was mitigated by over-expression of caALK2 or BMP-7, as was previously observed when MDA-BO2 cells were co-stimulated with BMP-7 and TGF-β [20, 29].

We next compared the metastatic ability and intra-bone tumor growth of these cell lines *in vivo*. Forced expression of caALK2 or BMP-7 in MDA-BO2 cells resulted in significantly decreased tumor-induced bone resorption. In addition to intra-bone tumor growth, over-expression of caALK2 strongly inhibited the development of bone metastases in nude mice after intra-cardiac inoculation. Also, the number of metastasis was reduced and osteolytic lesion area decreased. These findings are in line with our previously reported observations with BMP-7 over-expressing MDA-BO2 cells or recombinant BMP-7 treatment of mice with breast cancer metastasis [20].

Together, these results suggest that *de novo* bone metastasis formation and subsequent osteolytic bone destruction is regulated by tumor cell autonomous signaling. The antagonistic actions of over-expressing either caALK2 or BMP-7 on TGF-β signaling seem to play an important anti-metastatic role. Inhibition of bone metastasis formation and osteolytic progression was previously reported when TGF-β signaling was blocked through ectopic expression of a dominant negative TGF-β type II receptor [23] or by systemic treatment with kinase inhibitors to ALK5 [35, 36, 37].

Though the exact downstream mediators which are responsible for the dichotomous roles of BMP-7 and TGF-β in metastasis remain unknown, it is thought to be, in part, dependent on the differential regulation of ID proteins [22]. TGF-β is known to repress ID expression, whereas BMPs induce and stabilize expression of ID genes [22, 38]. We found that both ID1 promoter activity and ID2 expression were enhanced in caALK2 cells. Conversely, ID1 and ID3 have also been identified as prime mediators of lung

metastases in MDA-MB-231 cells [39] and targeted silencing of ID1 in 4T1 breast cancer cells inhibited lung metastatic spread of 4T1 cells [40]. Thus, mechanisms of metastasis might be distinct depending on the route of administration and the specific cell subtype applied. To elucidate the underlying mechanisms of inhibition of metastatic bone disease by which active BMP signaling inhibits osteolysis we examined a set of bone-metastatic genes reported to mediate different steps of the metastatic cascade [23, 41, 42]. However, we found no difference in the expression levels of IL-11, CTGF or PTHrP in caALK2 and BMP-7 over-expressing cells compared to GFP control MDA-BO2 cells.

We further show that Smad6 and Smad7 were significantly up-regulated in caALK2 and BMP-7 over-expressing cells indicative of enhanced proteasomal degradation of type I receptors and R-Smads [43, 44, 45]. Sustained up-regulation of these genes could have dramatic effects on TGF-β and BMP signaling in the tumor cells. Over-expression of Smad7 in melanoma and breast cancer cells was recently shown to inhibit the formation of metastasis [14, 15]. We speculate that since the BMP signaling pathway is continuously activated, by either caALK2 or BMP-7, the potential inhibitory actions of Smad7 may be more pronounced on TGF-β signaling compared to the effects on BMP-7 signaling pathway. In order to elucidate this, knockdown studies of Smad7 in cells ectopically expressing caALK2 or BMP-7 would have to be performed.

In a recent report, continuous activation of ALK3, a receptor for BMP-2 and BMP-4 [46], was shown to mediate invasion and metastasis of MDA-MB-231 breast cancer cells. This pro-metastatic function of caALK3 could be blocked by over-expression of a mutated dominant negative ALK3 receptor [47]. We specifically show that BMP-7 signal via ALK2 in MDA-BO2 cells and that over-expression of caALK2 gives rise to a less metastatic phenotype *in vivo*. Thus, active BMP-7 signaling in tumor cells distinctively induce anti-tumorigenic properties in these human breast cancer cells. Together, these observations highlight the importance of discriminating between different BMPs when characterizing their functions.

In summary, we demonstrate that continuous activation of BMP-7 signaling via ectopic expression of caALK2 or BMP-7 ligand can inhibit tumor-induced osteolysis and progression of breast cancer bone metastases. Our results suggest that therapeutic targeting of TGF-β signaling and enhancement of BMP-7 signaling can prevent progression of skeletal metastases. We are currently analyzing potential downstream target genes of TGF-β and BMP-7 signaling that are affected by active BMP-7 signaling. Identifying such genes could potentially allow us to target the pro-metastatic function of TGF-β or augment the anti-metastatic activities of BMP-7 signaling.

Materials and Methods

Cell culture and reagents

The human breast cancer cell lines MDA-MB-231 luc and MDA-MB-231/BO2 FRT luc cells (referred to as MDA-BO2) were cultured as previously described [20, 48]. Human recombinant BMP-7 (RD Systems) was used at 500 ng/ml unless otherwise indicated. Human recombinant

TGF-β3 was used at 5 ng/ml and obtained from K. Iwata (OSI Pharmaceuticals, Inc., Melville NY, USA). Antibodies recognizing BMP type I and type II receptors are described in [8] and antibody to phosphorylated Smad 1/5 (P-Smad1/5) is described in [11]. Lentiviral shRNA constructs targeting ALK2 were TRCN 0000 000 442 and TRCN 0000 000 444 (Sigma-Aldrich). The non-targeting control lentiviral shRNA plasmid was SCH002 (Sigma-Aldrich).

Cloning and generation of caALK2 over-expressing cell line

The caALK2 plasmid was kindly provided by Dr. K. Miyazono [26]. Constitutive active ALK2 was enzymatically cleaved from pENTR backbone and inserted into the pEF5/FRT/V5 vector by Gateway technology (Invitrogen). MDA-BO2/luc Flp-In cells were transiently cotransfected with the pOG44 Flp recombinase expression plasmid and the pEF5/FRT/V5/caALK2 construct and selected with antibiotics to generate stable isogenic caALK2 expressing cells [20].

[^{125}I]-BMP-7 binding assay

Iodination of BMP-7 was performed according to the chloramine T method and cells were affinity-labeled with the radioactive ligand (Amersham) as previously described [49, 5, 8]. In brief, a monolayer of cells were incubated with [^{125}I]-BMP-7 for 3 hours on ice and cross-linked with 54 mM disuccinimidyl suberate (DSS) and 3 mM bis (sulfosuccinimidyl) suberate (BS3, Pierce). Cell lysates were incubated with specific anti-sera for type I and type II receptors for 3 hours and immune complexes were precipitated with proteinA Sepharose (Amersham). Samples were boiled in SDS sample buffer and separated by SDS-PAGE. Gels were dried and scanned with the STORM imaging system (Amersham).

Quantitative real-time PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen) and first strand cDNA synthesis and real-time PCR performed as previously described [50]. Samples were run in triplicates for each primer set and the relative gene expression levels were assessed as the threshold cycle (Ct) values of the target and reference gene normalized for *GAPDH* or *ACTIN*. Values are presented as mean ± S.D. The following primers were used; ID2, forward: 5'-TCA GCC TGC ATC ACC AGA GA-3' and reverse: 5'-CTG CAA GGA CAG GAT GCT GAT -3'. SMAD6 forward: 5'-TCT CCT CGC GAC GAG TAC AAG-3' and reverse: 5'-GGA GCA GTG ATG AGG GAG TTG-3'. SMAD7 forward: 5'-AGA GGC TGT GTT GCT GTG AAT C-3' reverse: 5'- GCA GAG TCG GCT AAG GTG ATG-3'.

Cell transfection and cell viability assay

Cells were transiently transfected with BRE renilla luciferase [25] or the CAGA renilla-luc [27] reporter constructs using FugeneHD (Roche) according to the manufacturer's protocol. The following day, cells were stimulated 15 hours (or after three days for shRNA experiments) with the respective ligand. Cells were washed, lysed and the relative renilla/firefly luciferase activity measured [50, 20]. Transfection was done in triplicate and representative experiments are shown. For shRNA co-transfection assays, a ratio of 1:6 was used of BRE-luc versus shRNA. Cell viability was measured performed as previously described [32, 50].

Western blot analysis

Cells were stimulated with BMP-7 for 45 minutes, washed with PBS and lysed in SDS-sample buffer. Proteins were separated on SDS-PAGE and subjected to western blotting as previously described [50].

In vivo intra-bone tumor growth and bone metastasis models

Six week old female BALBc nude mice (Charles River) were anesthetized with isofluorane and inoculated with 100.000 freshly harvested MDA-BO2-luc cells suspended in 10 μl sterile PBS (n=5 per group). The surgical procedure was as follows; an incision was made just below the knee and two holes were drilled in the longitudinal plane of the proximal diaphysis using a 27G needle. Tumor cells were then directly inoculated into the bone marrow cavity and the wound closed with sutures [20]. For the bone metastasis model $1x10^5$ freshly harvested cells in 100 μl PBS were inoculated into the left heart ventricle (n=10 per group) [20]. Injections were done with 27G syringes. Tumor growth progression was followed weekly by bioluminescent imaging on the IVIS-100 (Caliper Life Sciences). All animal experiments were approved and carried out according to the guidelines provided by the local animal welfare committee.

Radiography and μ-CT imaging

Radiographies were acquired as described in [20] and μ-CT scanning was performed with Skyscan 1076 micro-CT (Skyscan) on fixed tibial samples. Acquisition parameters were as follows; 750 ms exposure, 0.9 degree rotation at a source voltage of 40kV. 3D scans were reconstructed using the NRecon and Dataviewer software from Skyscan. Series of tibial longitudinal and transversal plane sections were analyzed. Tibial cross-sections were quantified by binary image analysis and presented as the percentage cone comprising area in a given image.

Immuno- and histochemical analysis

Bone tumor sections were imbedded in paraffin and sectioned at 5-6 μm. Histological Masson's-Goldner staining was performed as previously described [20, 30]. The relative tumor burden and area of osteolytic bone resorption were quantified by image analysis with ImageJ software.

Statistical analysis

All results are expressed as the mean $\pm$ S.E.M unless otherwise stated. One-way ANOVA followed by Bonferronis multiple comparison test and two-tailed Students t-test was used where applicable. $P \leq 0.05$ was considered to be statistically significant.

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Supplemental material

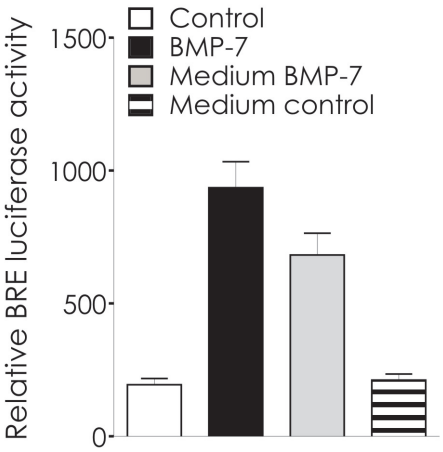


Figure 4.7: Figure S1. BMP secretion in conditioned medium from BMP-7 over-expressing cells. (A) Culture medium was collected from BMP-7 over-expressing cells two days after stimulation and added to MDA-BO2 cells transfected with BRE-luc reporter. A positive control was treated with 200 ng/ml BMP-7 (black bar). Data is presented at mean ± S.D of triplicate samples.

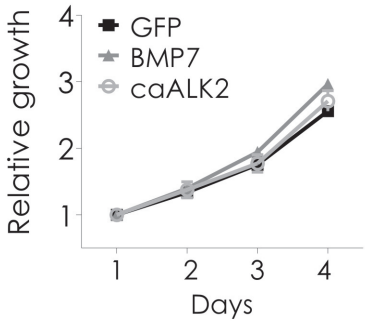


Figure 4.8: Figure S2. Tumor cell proliferation *in vitro*. Cell viability of stable MDA-BO2 cell lines measured for four consecutive days. The relative growth rates of GFP control (black square), BMP-7 (grey triangle), and caALK2 over-expressing cells (grey open circle) is presented. Error bars indicate mean ± S.D. of four measurements, a representative experiment is shown.

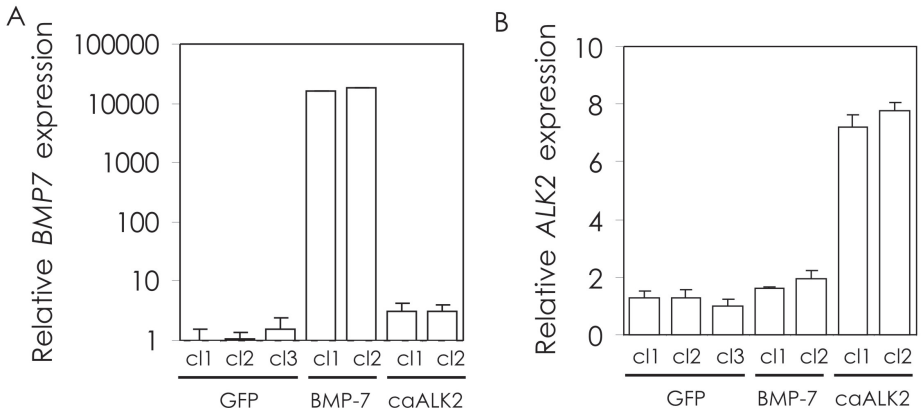


Figure 4.9: Figure S3. Continuous over-expression of BMP-7 or caALK2 *in vivo*. The relative expression level of *BMP-7* (A) or *ALK2* (B) compared to *GAPDH* determined by quantitative real-time PCR in MDA-BO2 cell clones (cl) established from bone tumors and metastases. Error bars indicate mean ± S.D.

Chapter 5

Oral administration of GW788388, a kinase inhibitor of the TGF-β type I and type II receptors, reduces renal fibrosis in db/db mice.

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Abstract

Progressive kidney fibrosis precedes end-stage renal failure in up to a third of patients with diabetes mellitus. Elevated intra-renal transforming growth factor-β (TGF-β) is thought to underlie disease progression by promoting deposition of extracellular matrix and epithelialmesenchymal transition. GW788388 is a new TGF-β type I receptor inhibitor with a much improved pharmacokinetic profile compared with SB431542. We studied its effect in vitro and found that it inhibited both the TGF-β type I and type II receptor kinase activities, but not that of the related bone morphogenic protein type II receptor. Further, it blocked TGF-β-induced Smad activation and target gene expression, while decreasing epithelial- mesenchymal transitions and fibrogenesis. Using db/db mice, which develop diabetic nephropathy, we found that GW788388 given orally for 5 weeks significantly reduced renal fibrosis and decreased the mRNA levels of key mediators of extracellular matrix deposition in kidneys. Our study shows that GW788388 is a potent and selective inhibitor of TGF-β signalling in vitro and renal fibrosis in vivo.

Abbreviations

ActRII, activin type II Receptor; ALK, activin receptor-like kinase; caALK, constitutively active ALK; β-gal, β-galactosidase; BMP, bone morphogenic protein; BMPRII, BMP type II receptor; BRE, BMP responsive element; COL, collagen; CTGF, connective tissue growth factor; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GW788388, 4-(4-(3-(Pyridin-2-yl)-1H-pyrazol-4-yl) pyridin-2-yl)-N-(tetrahydro-2Hpyran-4-yl) benzamide; MMP, matrix metalloproteinase; NMuMG, namru murine mammary gland; PAI-1, plasminogen activator inhibitor 1; PAS, picric acid stain; PSmad, phosphorylated Smad; RCC4, renal cell carcinoma; RT-PCR, reverse transcriptase polymerase chain reaction; SB431542, 4-(5-benzo(1,3) dioxol- 5-yl-4-pyridin-2-yl-1H -imidazol-2-yl)-benzamide; Smad, small phenotype and mothers against DPP related protein; α-SMA, α-smooth muscle actin; TGF-β, transforming growth factor-β; TβRII, TGF-β type II receptor; TIMP, Tissue inhibitor of metalloproteinase; VHL, von Hippel Lindau.

Introduction

Diabetic nephropathy leads to end-stage renal failure in 20 to 30% of patients with type 1 or type 2 diabetes mellitus[1].1 The multifunctional cytokine, transforming growth factor-β (TGF-β), is elevated in patients with diabetic nephropathy and is likely a prime mediator in the progression of renal disease [2, 3].

Specimens from patients with diabetic nephropathy show elevated TGF-β mRNA and protein levels in glomeruli and the tubulointerstitium [4]. Also, urinary and serum levels of TGF-β are significantly increased in diabetes patients [5]. In experimental animal models of type 1 and type 2 diabetes, similar patterns of increased TGF-β expression and secretion have been observed [6]. Nuclear accumulation of downstream TGF-β effector proteins was observed in diabetic kidneys. Furthermore, elevated levels of the TGF-β type II receptor (TβRII) have been reported in diabetic mice compared with non-diabetic controls [7].

One of the mechanisms by which TGF-β induces fibrogenesis is through stimulation of extracellular matrix (ECM) proteins and inhibition of matrix degradation. Expression of key matrix components is enhanced upon TGF-β treatment, both in glomerular mesangial cells and renal tubular epithelial cells [8, 9, 10]. These factors include fibronectin (FN), type I collagen (COL-1), type III collagen (COL-III), type IV collagen (COL-IV) and laminin [11]. TGF-β further stimulates ECM accumulation through enhancing expression of connective tissue growth factor, which in turn induces FN and COL-III expression [12]. Also, activated TGF-β suppress the activity of matrix metalloproteinases [13] through increased expression of tissue inhibitor of metalloproteinases and plasminogen activator inhibitor 1 (PAI-1) [14]. Thus, TGF-β promotes renal fibrogenesis by increasing the synthesis of ECM components and inhibiting matrix degradation.

An additional cellular pathomechanism whereby TGF-β promotes fibrosis is through the mediation of epithelial to mesenchymal transition (EMT), a process whereby polarised epithelial cells are transformed into highly migratory fibroblastoid cells. Epithelial cells lose polarity, epithelial markers, and cellcell contact. The cells undergo cytoskeletal remodelling and gain mesenchymal markers essential for cell-ECM association. The net result being enhanced cell motility and invasiveness [15, 16]. In renal fibrosis, the pathological significance of tubular EMT has become increasingly recognised. Epithelia can contribute to the ECM overproduction by creating new fibroblasts through the induction of EMT [17].

TGF-β and the superfamily members, activins and bone morphogenic proteins (BMPs), signal through related type I and type II transmembrane serine/threonine kinase receptors. The kinases act in sequence, with the ligand-specific type I receptor acting as a substrate for the type II receptor. In most cell types, TGF-β signals via the TGF-β type I receptor also termed activin receptor-like kinase (ALK)5 [18]. In endothelial cells, however, TGF-β signals via ALK1 and ALK5 [19]. In contrast, BMP signals through ALK2, ALK3, or ALK6 and activin, and nodal through ALK4 and ALK7 [20, 21]. For TGF-β/ALK5 and activin, the signal is transduced into the cytoplasm through phosphorylation of the receptor-regulated Smads (R-Smads), Small phenotype and mothers

against DPPrelated protein (Smad)2, and Smad3. For TGF-β/ALK1 and BMP, the signal is via phosphorylation of the R-Smads, Smad1, 5, and 8 [22]. Phosphorylated and activated R-Smads dissociate from the receptor complex and associate with Smad4 in a heteromeric manner. The activated complexes shuttle to and accumulate in the nucleus. Here they regulate expression of a large array of genes in a cell-type-specific and ligand dose-dependent manner [23].

To directly address the therapeutic potential of TGF-β inhibitors in renal disease, small-molecule competitive antagonists of the ALK5 kinase activity have been developed. These inhibitors interact with the ATP-binding site, thereby preventing phosphorylation of Smad proteins [24, 25]. The commonly used ALK5 inhibitor, 4-(5-benzo(1,3)dioxol-5-yl-4-pyridin-2-yl-1H-imidazol-2-yl)-benzamide (SB431542), is an ATP competitive kinase inhibitor [26]. Another mechanism for abrogating TGF-β signalling has been through long-term treatment with monoclonal anti-TGF-β antibody. In diabetic rodents, this effectively prevented glomerulosclerosis and renal insufficiency [7, 27, 28, 29]. Also, antisense TGF-β oligonucleotides were found to reduce kidney weight and expression of matrix components in diabetic mice [30]. Recently, a soluble fusion protein of the TβRII was reported to reduce albuminuria in a chemically induced model of diabetic nephropathy in rats [31].

A limited number of studies have been reported on the use of small-molecule inhibitors of TGF-β signalling *in vivo* [32, 33]. SB525334 was shown to significantly reduce pro-collagen 1a(I), in rat kidneys, in an induced model of nephritis [34]. Also the inhibitor IN-1130 reduced obstructive nephropathy in rats [35]. These data provide a strong foundation for using type I receptor kinase inhibitors in clinical testing.

Recently, 4-(4-[3-(Pyridin-2-yl)-1H-pyrazol-4-yl]pyridin- 2-yl)-N-(tetrahydro-2Hpyran-4-yl) benzamide (GW788388) was developed, as an alternative to the ALK5 inhibitor, SB431542, with better *in vivo* exposure (Figure 1). GW788388 is orally active and has a good pharmacokinetic profile, with an elimination half-life of 1.3 h and a systemic plasma clearance of 20 ml/min/kg in rats. It was previously shown to reduce the fibrotic response in a chemical induced model of fibrosis in rats and improve liver histology [32].

In this study, we further characterised the potency and selectivity of this novel inhibitor, GW788388. We show that this compound effectively blocks both the ALK5 and to some extent the TβRII. In renal epithelial and cancer cell lines, we assess the inhibitory effects on TGF-β-mediated biological responses such as EMT and fibrogenesis. We examine the effect of blocking TGF-β signalling on renal fibrosis and kidney function in the db/db mouse model of spontaneous diabetic nephropathy, resembling the pathogenic phenotype observed in patients with type 2 diabetes mellitus. We show that GW788388 effectively inhibits TGF-β signalling *in vitro* and reduces renal fibrosis *in vivo*.

Results

GW788388 is a selective inhibitor of ALK5 and TβRII

Structures of GW788388 and SB431542, two ATP competitive inhibitors of the kinase domain of ALK5 are shown in Figure 1a. In a biochemical binding assay, using the intracellular kinase domain of ALK5, GW788388 was found to have an IC50 for GST-ALK5 of 0.018 ± 0.08 TGF-μM [32]. To test the specificity of GW788388, we performed an *in vitro* kinase assay on full length receptors. Human embryonic kidney 293T cells were transiently transfected with expression plasmids encoding constitutively active ALK (caALK) 5, TβRII, BMP type II receptor (BMPRII) or activin type II receptor (ActRII). Receptors were immunoprecipitated and challenged with [γ^{32}P]-labelled ATP and 10 μM of compounds. GW788388 potently inhibited autophosphorylation of ALK5, TβRII and to some extent the ActRII (Figure 1b). The compound had no effect on the BMP type II receptor kinase activity.

To address if GW788388 was cytotoxic, we treated cells with a dilution range of the compound and measured cell viability after 72 hours. GW788388 showed no toxicity in Namry murine mammary gland (NMuMG) (Figure 1c), MDA-MB-231, renal cell carcinoma (RCC)4, or U2OS cells (data not shown) when treated with dilutions from 4 nM to 15 μM. Similar results were obtained with the SB431542 inhibitor (Figure 1c).

GW788388 inhibits TGF-β-induced Smad2 phosphorylation and Smad2/3 nuclear translocation

Since GW788388 could block the kinase activity of ALK5 and TβRII, we next studied the inhibitory effect on TGF-β, activin, and BMP-induced R-Smad phosphorylation and nuclear translocation. GW788388 inhibited TGF-β-induced Smad2 phosphorylation in a dose-dependent manner in NMuMG (Figure 2a; Figure S1a), MDA-231-MB (Figure 2b), and renal cell carcinoma (RCC4)/von Hippel Lindau (VHL) (data not shown). TGF-β-mediated Smad1/5 phosphorylation, which requires ALK5 and TβRII, was also inhibited by GW788388 (Figure 2a and b). In T47D cells, GW788388 and SB431542 inhibited the activin-induced phosphorylation of Smad2 (Figure 2c).

Upon phosphorylation, R-Smads form complexes with Smad4 and accumulate in the nucleus. TGF-β-induced Smad2/3 nuclear translocation was dose-dependently inhibited when NMuMG cells were treated with GW788388 (Figure 2d). We tested if GW788388 inhibited the BMP signalling cascade by analyzing the effect of the compound upon BMP-induced phosphorylation of Smad1/5. As shown in Figure 2e, GW788388 had no inhibitory effect on Smad1/5 phosphorylation by BMP. SB431542 was shown to have some inhibitory effect on the mitogen-activated protein kinase p38α at 10 μM.25 We therefore tested whether GW788388 could inhibit sorbitol-activation of stress-induced kinases such as the mitogen-activated protein kinases p38 and ERK 1/2. GW788388 had no inhibitory effect on these mitogenactivated protein kinases (data not shown). Thus, GW788388 selectively inhibits TGF-β and activin Smad signalling and not the

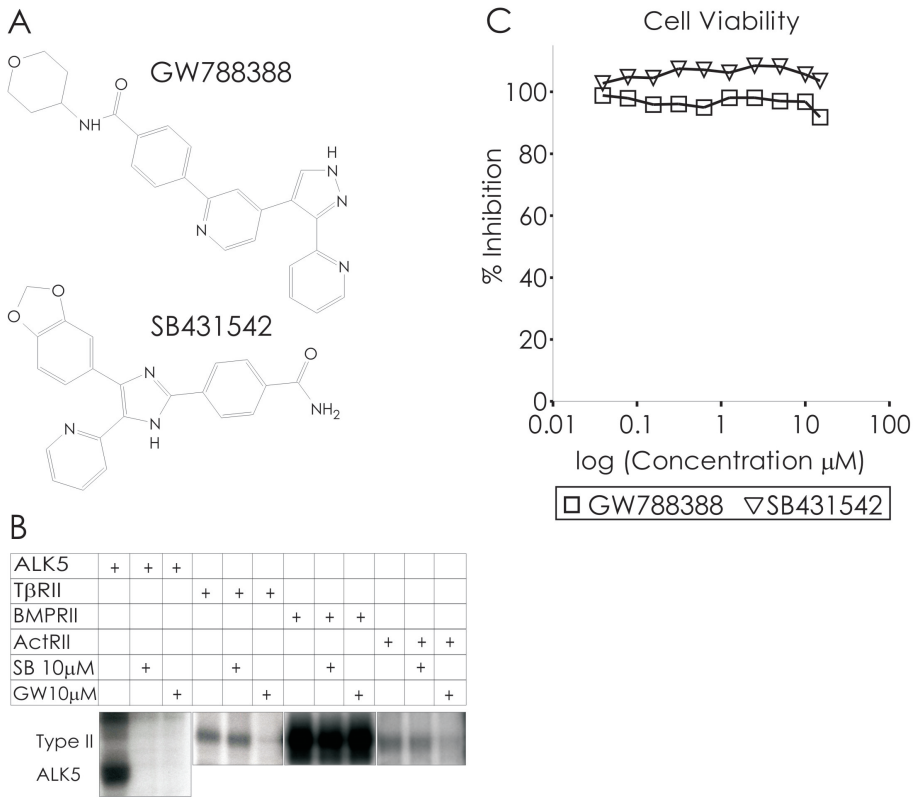


Figure 5.1: GW788388 inhibits both ALK5 and TβRII. (A) Chemical structures of GW788388, 4-(4-(3-(Pyridin-2-yl)-1H-pyrazol-4-yl) pyridin-2-yl)-N-(tetra-hydro-2Hpyran- 4-yl) benzamide and SB431542, 4-(5-benzo(1,3) dioxol- 5-yl-4-pyridin-2-yl-1H -imidazol-2-yl)-benzamide. (B) Effect of GW788388 and SB431542 on autophosphorylation of caALK5, TβRII, ActRII and BMPRII kinase activity. HEK293T cells were transfected with plasmids encoding full length actively signalling receptors. These were immunoprecipitated and the *in vitro* kinase assays were performed with gamma ^{32}P-labelled ATP in the presence of 10 μM GW788388 (GW) or 10 μM SB431542 (SB). (C) Cell viability assay. NMuMG cells were treated with dilutions of GW788388 (squares) and SB431542 (triangles) for 72 hours. Viability was measured with MTS assay. Data is presented as % inhibition compared to vehicle control. Bars represent mean $\pm$s.e.m.

closely related BMP-signalling cascade.

GW788388 selectively inhibits ALK4, ALK5 and ALK7

To further confirm the selectivity of GW788388, U2OS cells were transiently transfected with expression plasmids encoding the constitutively active full-length receptors caALK2, caALK3, caALK4, caALK5, caALK6, and caALK7 [36]. These mutationally active receptors signal independently of ligand and their type II receptors. They were cotransfected with the corresponding luciferase reporter constructs. The TGF-β-inducible reporter CAGA $_{12}$-Luc contains Smad-responsive elements from the PAI-1 promoter, which specifically bind Smad3/Smad4 and drive the luciferase reporter gene

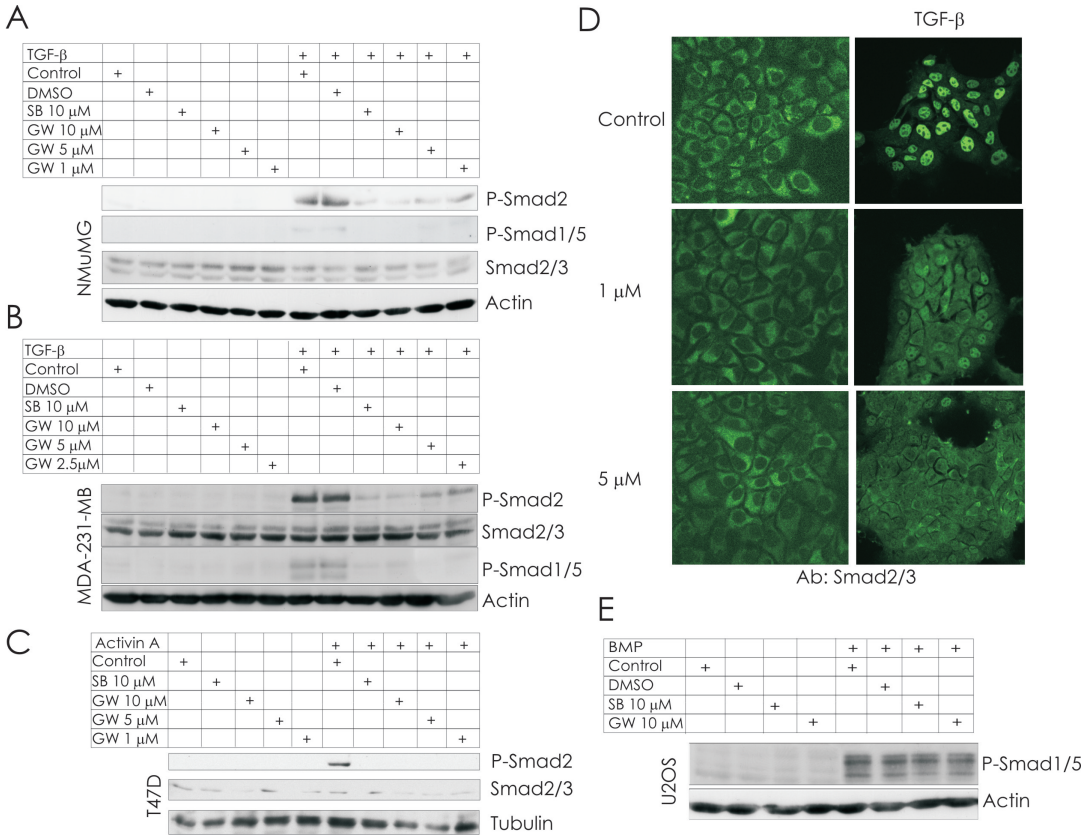


Figure 5.2: GW788388 inhibits TGF-β-induced Smad activation dose-dependently. NMuMG (A) and MDA-MD-231 cells (B) were treated with GW788388 (GW) or SB431542 (SB) in the presence or absence of TGF-β for 1 hour. Protein expression of PSmad2, PSmad1/5 and Smad2/3 was analysed by western blot analysis. β-Actin served as a loading control. (C) Immunofluorescent staining of Smad 2/3 in NMuMG cells treated with vehicle or GW788388 $\pm$ TGF-β for 1 hour. Images were captured with confocal microscopy. (D) Western blot analysis of U2OS cells treated with GW788388$\pm$BMP6 for 1 hour. Control denotes non treated cells and DMSO was used as vehicle.

[37]. The BMP-inducible luciferase reporter, BMP-responsive element-Luc, contains a BMP-responsive elements from the inhibitor of DNA-binding 1 promoter [38].

GW788388 inhibited the TGF-β response, very efficiently, by blocking signalling through caALK5, caALK4, and caALK7 in a dose-dependent manner (Figure 3a). The SB431542 inhibitor was used for comparison and similar results were obtained with all caALKs (data not shown). In agreement with the phosphorylation data, GW788388 had no inhibitory effect on the constitutively active BMP receptors (Figure 3b). In addition, TGF-β and activin (Figure S1b) but not BMPinduced reporter activity was blocked by GW788388 (data not shown). Thus, GW788388 is a selective inhibitor of the TGF-β type I receptors ALK5, ALK4, and ALK7.

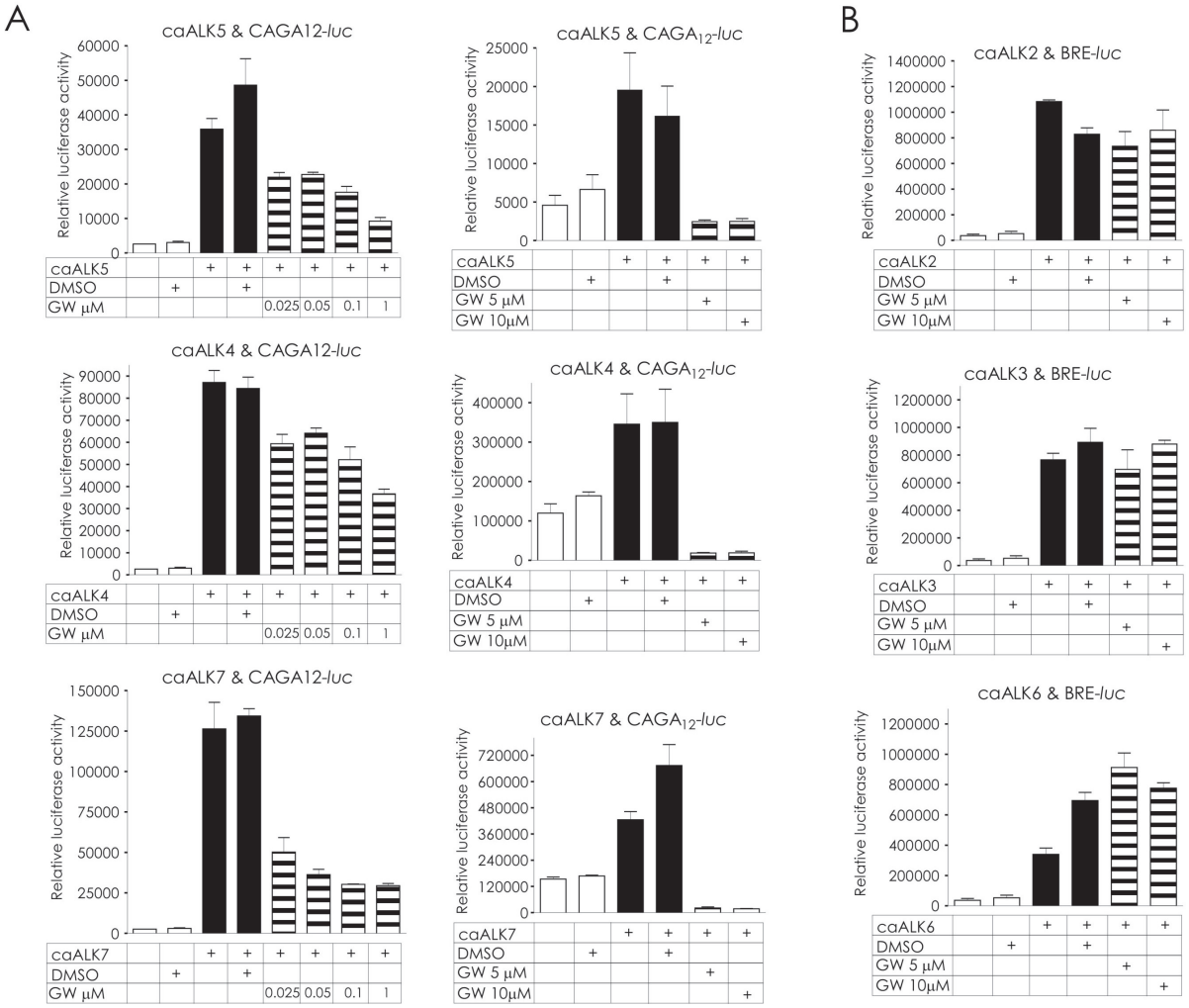


Figure 5.3: GW788388 inhibits ALK5, ALK4 and ALK7 in a dose-dependent manner and has no effect on ALK2, ALK3 and ALK6. (a) U2OS cells were transfected with caALK4, caALK5 or caALK7 together with the TGF-β specific luciferase reporter construct CAGA12-luc. Cells were treated with doses of GW788388 (GW) or vehicle. (b) U2OS cells were transfected with caALK2, caALK3 or caALK6 together with the BMP responsive BRE-luciferase reporter. Measurements are presented as luciferase activity normalised to β-gal activity. Error bars indicate mean $\pm$s.e.m. of three measurements, one representative experiment is shown.

GW788388 inhibits TGF-β-induced EMT and growth inhibition

In epithelial cells, the TGF-β-mediated growth inhibitory response and EMT are two cellular processes that have been extensively explored. The NMuMG cells are a widely used *in vitro* model system for studying these TGF-β-mediated responses [39]. To test if GW788388 could inhibit the TGF-β-induced growth inhibitory response, we measured cell proliferation with serial dilutions of GW788388. As shown in Figure 4a, increasing concentrations of GW788388 inhibits TGF-β-induced growth inhibition. These re-

sults were further supported by light microscopy images, demonstrating detachment of NMuMG cells from the tissue culture surface in response to TGF-β treatment, suggestive of programmed cell death (Figure 4b). Thus, GW788388 blocked TGF-β mediated growth arrest.

With phase-contrast microscopy, we observed the transition of the cells from an epithelial to a fibroblastoid phenotype upon stimulation with TGF-β for 48 h (Figure 4b). In cells stained by immunofluorescence with epithelial and mesenchymal markers, we observed actin stress fiber formation (Figure 4c), loss of E-cadherin expression at cell-cell junctions, and gain of N-cadherin expression (data not shown) in response to TGF-β treatment. This EMT response, mediated by TGF-β, was completely inhibited with GW788388 (Figure 4b and c).

We confirmed these visual observations by analyzing protein and mRNA expression by western blot analysis and semi-quantitative reverse transcriptase-polymerase chain reaction. In NMuMG and RCC4/VHL cells, we analyzed the changes in the expression of epithelial and mesenchymal markers after 48 h of TGF-β stimulation (Figure 5). E-cadherin protein expression was reduced upon TGF-β treatment, whereas the expression of the mesenchymal markers N-cadherin and α-smooth muscle actin was increased (Figure 5a and b). GW788388 attenuated these TGF-β-induced EMT responses. The inhibitory effect of GW788388 was also observed on mRNA levels of critical TGF-β target genes involved in EMT, such as E-cadherin, FN, and the transcriptional repressor of E-cadherin, SNAIL (Figure 5c). In conclusion, GW788388 blocks the TGF-β-induced growth inhibitory and EMT response.

GW788388 inhibits the TGF-β-induced fibrotic responses *in vitro*

Since GW788388 inhibited important TGF-β-induced target gene responses required for EMT, we sought to examine whether the drug also could inhibit TGF-β responses involved in ECM remodelling. We investigated this by quantitative RT-PCR and western blot analysis. We found that GW788388 could prevent the TGF-β-induced up-regulation of CTGF, PAI-1, and COL-I mRNA expression in the renal epithelial cells RCC4/VHL (Figure 6a) and FN in NMuMG cells (Figure 5c). On protein levels, we confirmed that GW788388 blocks the TGF-β-induced expression of COL-I and FN (Figure 6b). Thus, GW788388 inhibits the TGF-β-mediated expression of important players in fibrogenesis both on mRNA and protein levels.

GW788388 potently attenuates renal fibrosis *in vivo*

We have demonstrated that GW788388 is a potent inhibitor of TGF-β signaling in several *in vitro* models. We next sought to examine the effects of GW788388 *in vivo*. First, we compared the i.v. pharmacokinetic profiles of GW788388 compared to SB431542, in Sprague-Dawley rats. Clearance was 34 $\pm$ 12.2 ml/min/kg for GW788388 versus 37.5 $\pm$ 13.5 ml/min/kg for SB431542. The half-life of GW788388 was 4.1 $\pm$ 1.8 hours versus 28.5 $\pm$ 16.1 minutes for SB431542 (data not shown). Hence, GW788388 is far more

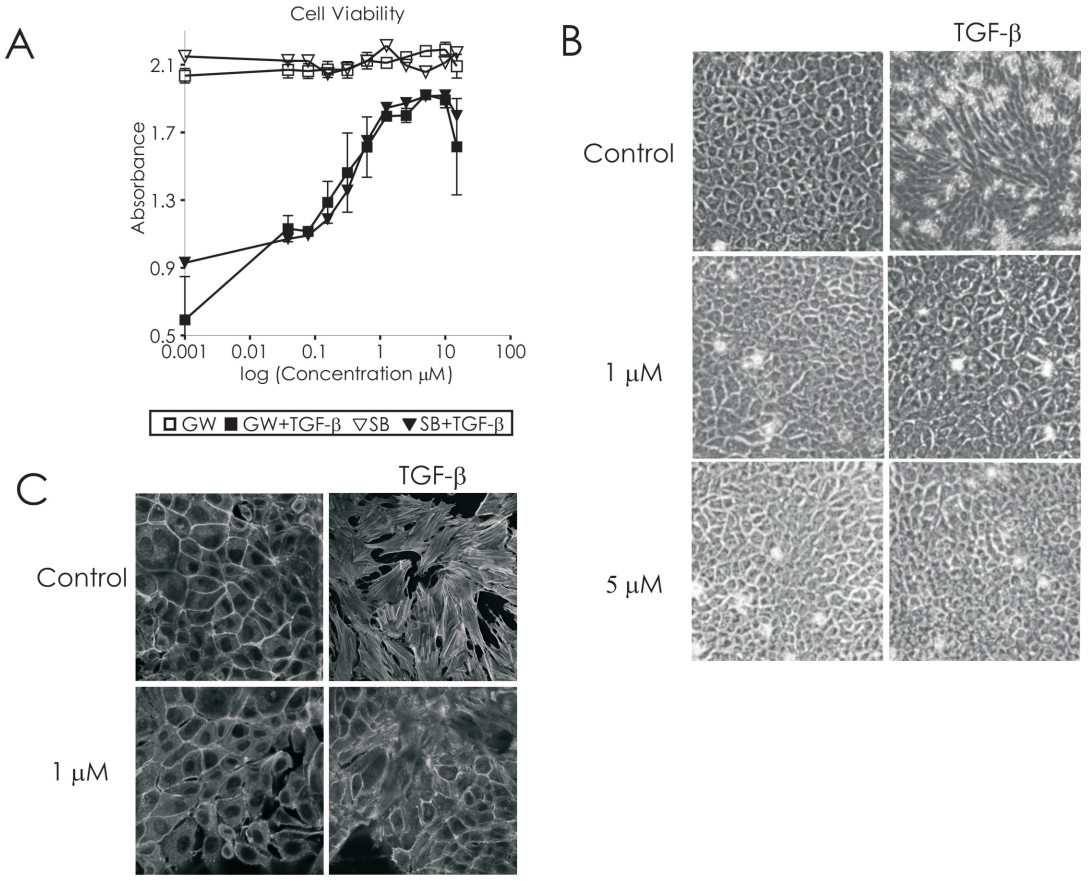


Figure 5.4: GW788388 inhibits TGF-β-mediated EMT and apoptosis. NMuMG cells were treated with GW788388, the vehicle control DMSO and TGF-β where indicated, for 48 hours. (A) NMuMG cell proliferation measured after 72 hours drug stimulation with dilution series of GW788388 (GW) (squares) and SB431542 (SB) (triangles) in the presence (closed symbols) or absence (open symbols) of TGF-β. Metabolically active cells were measured with a cell proliferation/viability assay. Bars represent means of three independent measurements ±s.e.m. (B) Phase contrast images of TGF-β-induced EMT. (C) Immunofluorescent staining of actin stress fibre formation. Images were captured with confocal microscopy.

suitable for *in vivo* applications than SB431542.

The db/db mouse model of spontaneous diabetic nephropathy was chosen for further *in vivo* characterisation of GW788388. Six month old mice were used, with advance stage renal disease, significant glomerular changes and elevated albuminuria [40]. Mice were treated for 5 weeks with oral administration of 2 mg/kg/day of GW788388. No adverse side-effects were observed with the treatment.

Figure 7a shows diabetic mouse kidneys stained with Masson's Trichrome stain. Collagen deposits are observed in blue. Robust collagen deposits were seen in glomeruli and minimal to mild glomeropathy was evident in most diabetic animals (left panel). Treatment with GW788388 at 2 mg/kg/day resulted in a reduced collagen staining (Figure

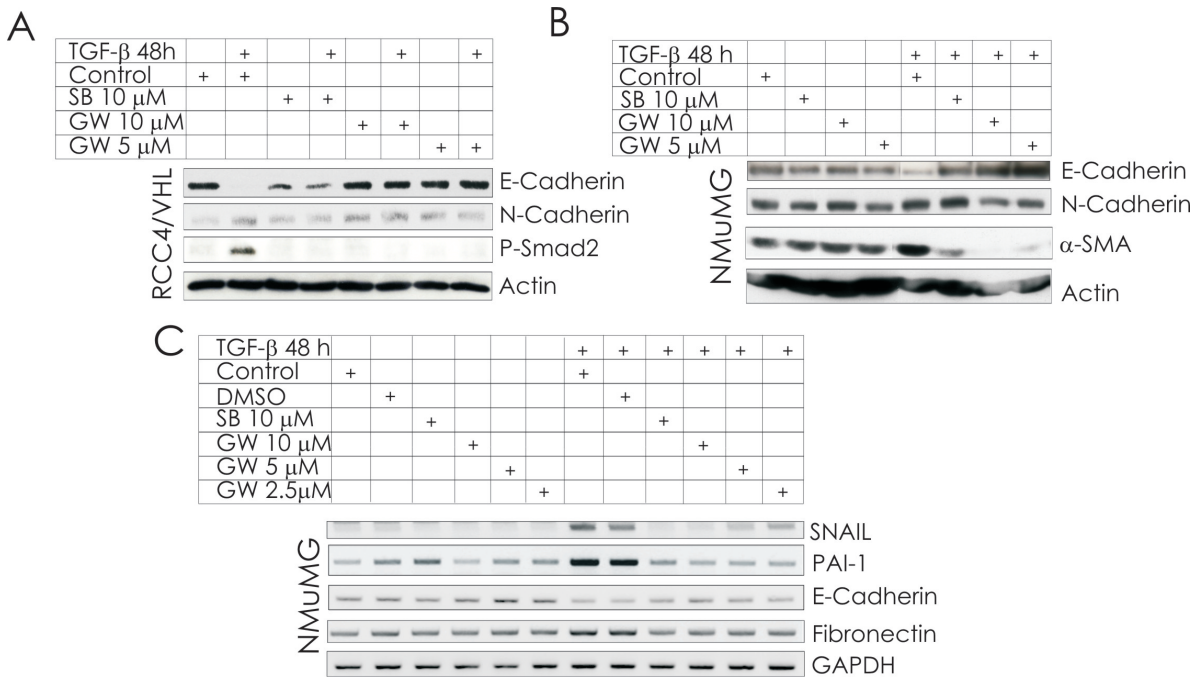


Figure 5.5: TGF-β-induced EMT is inhibited by GW788388. Western blot analysis of epithelial and mesenchymal protein markers in RCC4/VHL (A) and NMuMG (B) cells after 48 hours drug and TGF-β stimulation. Control is DMSO treated cells. β-Actin was used as a loading control. (C) RT-PCR semiquantitative analysis of SNAIL, PAI-1, E-Cadherin and FN in NMuMG cells after GW788388 (GW) or SB431542 (SB) treatment and TGF-β stimulation for 48 hours. GAPDH was included as loading control. Control depicts non treated cells and DMSO vehicle treated cells.

7a right panel). Glomerulopathy was assessed independently on PAS stained sections, scored blinded. Diabetic mice had significant glomerulopathy marked by mesangial matrix expansion, mesangial hypertrophy, proliferation and glomerular basement membrane thickening. This was significantly reduced when treated with GW788388 (Figure 7b). Urinary albumin excretion was additionally measured and corrected for creatinine concentrations. In diabetic mice urinary albumin levels were significantly elevated (Figure 7c). GW788388 appeared to decrease urinary albumin concentrations, although not statistically significant, suggesting that the underlying glomerular dysfunction persisted in the treated animals. To confirm that the observed changes, in glomerulopathy and the trend for reduced albuminuria, correlated with inhibition of TGF-β target genes *in vivo* RNA was extracted from kidneys and the levels of matrix mRNAs examined. FN, COL-I, PAI-1 and COL-III expression levels were significantly increased in diabetic mice as compared to their lean littermates (Figure 7d). Treatment with 2 mg/kg/day of GW788388 significantly reduced the mRNA levels of PAI-1, COL-I and COL-III to nearly the same levels as seen in the non-diabetic lean littermates. Taken together, these results indicate that GW788388 attenuates TGF-β signalling in vivo and effectively reduces hallmarks of fibrogenesis in mice suffering from late stage diabetic nephropathy.

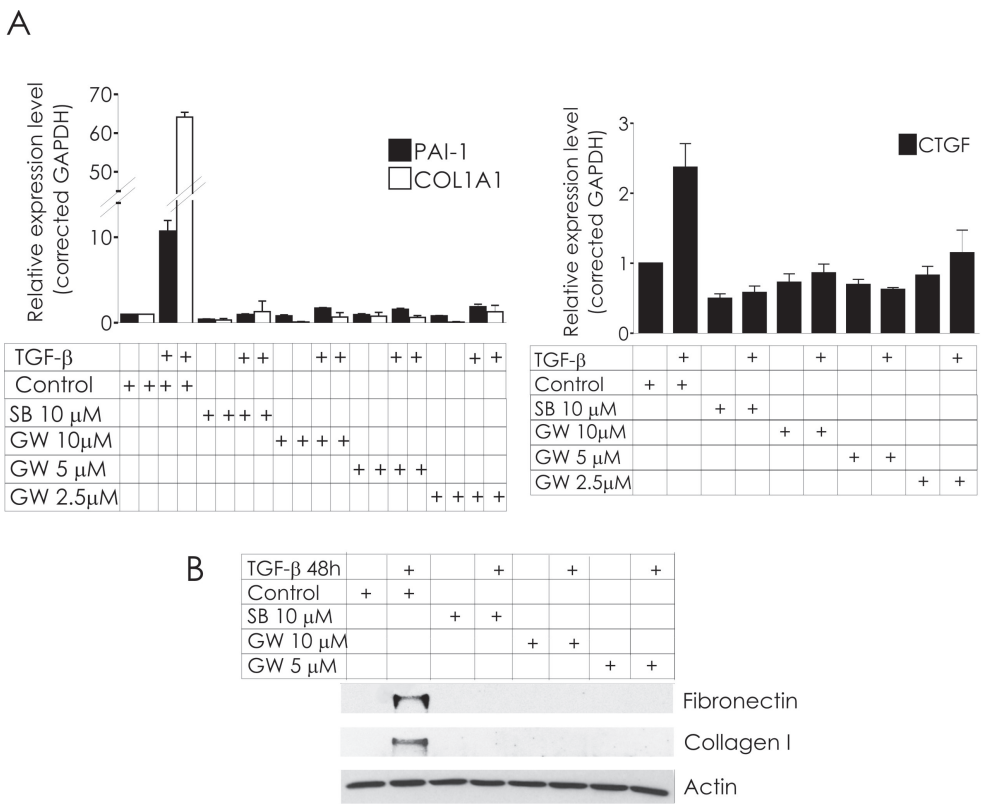


Figure 5.6: GW788388 inhibits the TGF-β-induced fibrotic response *in vitro*. (A) The effect of GW788388 (GW) on TGF-β-induced mRNA expression of the ECM genes PAI-1, COL-1αI and CTGF were analysed by real time Q-PCR. RNA was extracted from RCC4/VHL renal epithelial cells stimulated with drug $\pm$ TGF-β for 48 hours. GAPDH was used as a reference housekeeping gene. Results are presented as means $\pm$ SD of three measurements, the experiment was repeated twice. (B) GW788388 inhibits TGF-β-induced FN and COL-I on protein level, β-actin was used as a loading control. Controls were treated with DMSO.

Discussion

TGF-β is suggested to be a key factor in the generation of tissue fibrosis [8, 28, 29, 41]. In the diabetic kidney, TGF-β plays an important role in early and late manifestations of nephropathy such as renal hypertrophy and mesangial matrix expansion [7]. These pathomechanisms result in destruction of functional renal tissue and eventually loss of renal function. Blocking TGF-β signalling is therefore considered a promising therapeutic approach in the treatment of renal disease. We studied a new TGF-β inhibitor, GW788388 (results are summarised in Figure 8).

We show that GW788388 effectively inhibits TGF-β-mediated responses *in vitro* by blocking the kinase activity of both the type I and the type II receptors. Importantly, we show that oral administration of GW788388 to diabetic mice significantly reduces glomerulopathy in kidneys and attenuates expression of key components involved in fibrosis. These results encourage further studies to therapeutically target the TGF-β

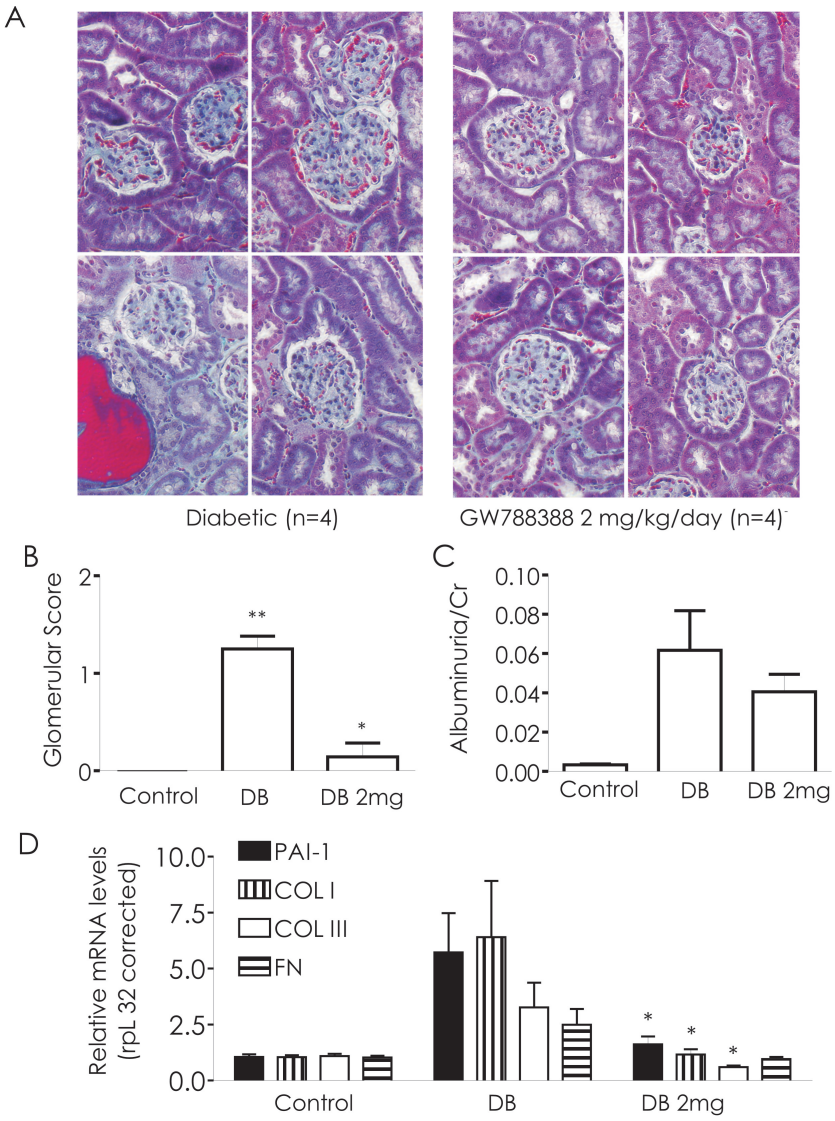


Figure 5.7: GW788388 attenuates renal fibrosis in db/db mice. GW788388 was orally administered to db/db mice for 5 weeks at 2 mg/kg/day. (A) Masson's Trichrome stained kidney sections. Representative images are shown for db/db control (left panel) and db/db mice treated with 2 mg/kg/day GW788388 (right panel). Blue stain indicates heavy collagen presence indicative of glomerulosclerosis. (B) Glomerulopathy blinded scores of PAS stained kidney sections. 40 tufts were scored/animal and the mean score ± s.d were tabulated for each animal. $^{**}P{\leq}0.001 versus lean control (n = 10), ^{*}P{\leq}0.01$ versus vehicle treated db/db mice (DB) (n=12). 2 mg/kg/day dose of GW788388 (2mg DB) (n=7). (C) Urinary albumin levels corrected for creatinine excretion. Lean controls (n=10), db/db control (DB) (n=11) and treated with 2 mg/kg/day (2mgDB) (n=6). (D) GW788388 reduced the expression of TGF-β-induced extracellular matrix target genes *in vivo*. RNA extracted from kidneys and analysed by real time PCR of lean controls (n=11), db/db control mice (n=12) and mice treated with 2 mg/kg/day (n=7). Bars represent mean ± s.e.m.

pathway in order to treat renal diseases.

GW788388 was identified as an orally active ALK5 inhibitor with much improved *in vivo* properties compared to SB431542 [24, 32]. In order to study the specificity of the compound we performed an *in vitro* kinase assay with ^{32}P-ATP. We found that GW788388 potently inhibits both the ALK5 and the TβRII kinase receptor activity but not the BMPRII. This contrasts what is seen for the related inhibitor, SB431542 [19, 22]. As a consequence of inhibiting the TGF-β receptors, we found that TGF-β-induced Smad2 phosphorylation and nuclear accumulation were potently blocked by GW788388. The specificity of GW788388 was further tested on all seven activated ALKs with reporter assays. We show that the compound could inhibit ALK5 along with the structurally similar receptors i.e. ALK4 and ALK7. GW788388 did not inhibit ALK2, ALK3 and ALK6. Previous studies using other TGF-β type I receptor inhibitors have shown similar results [34, 42, 43] the main distinction being that GW788388 also reduce the TβRII kinase activity.

TGF-β induces a growth inhibitory and an EMT response in NMuMG cells [39, 44]. We show that GW788388 dose-dependently inhibits these TGF-β responses. The TGF-β-mediated up-regulation of target genes, involved in excess ECM deposition is well described. Treating renal epithelial cells with TGF-β mimics the fibrotic response seen in renal disease, where mRNA levels of PAI-1 and COL-I are increased by TGF-β treatment [27]. In our hands, GW788388 could prevent the TGF-β-mediated up-regulation of CTGF, PAI-1 and COL-I RNA levels and FN and COL-I on protein levels in the renal epithelial cell line RCC4/VHL. Recently, SNAIL was described to directly induce renal fibrosis and strong expression was found in fibrotic human kidney sections [45]. With GW788388, we could block SNAIL mRNA expression in epithelial cells. Taken together, these data indicate that GW788388 selectively and efficiently inhibits TGF-β-mediated responses *in vitro*.

Since GW788388 inhibits important components in the TGF-β-induced fibrotic response in cell models, we hypothesised that GW788388 could reduce markers of fibrosis in a mouse model of diabetic nephropathy. Our aim was to examine if TGF-β receptor inhibition could be effective in older mice with established renal disease, as would be observed in patients presenting with impaired renal function. We show that oral administration of GW788388 for 5 weeks in 6 month old db/db mice significantly attenuated glomerulopathy in mouse kidneys. This correlated with reduced mRNA expression of critical factors in ECM remodelling, namely PAI-1, collagen I, and collagen III by GW788388. These results are in agreement with the oral application of GW788388 to rats with chemical induced liver fibrosis [32] and with db/db mice treated with neutralizing anti-TGF-β antibodies [7].

Despite ALK5 inhibition having inhibitory effects on fibrogenesis and histological glomerulopathy, the effects on kidney function were not significant. Only a trend for a reduction in urinary excretion of albumin was observed. This suggests that longer treatments may be necessary to reverse the effects of fibrosis. Moreover, it is not clear if ALK5 inhibition would address the underlying glomerular pathology leading to albuminuria in the first instance. To address this hypothesis, treatment would need to

be started earlier before any albuminuria is observed. Thus, for ALK5 inhibition alone to be fully effective against a change in glomerular permeability, we hypothesise that earlier and longer treatment periods are needed in order to inhibit tissue remodelling within the kidney and allow restoration and/or preservation of glomerular morphology and function.

All together, these data provide a strong foundation for using TGF-β receptor kinase inhibitors in a clinical setting. Renal disease progresses slowly and halting this process with a TGF-β receptor inhibitor will presumably require chronic treatment. However, TGF-β is a pleiotropic cytokine which modulates a broad array of processes. The challenge in using TGF-β receptor inhibitors for anti-fibrotic treatment will be to balance the disease related fibrotic actions against the immune modulatory and tumour suppressor functions of TGF-β. Also, all ALK5 kinase inhibitors reported to date inhibits the kinase activity of the ALK4 and ALK7 [20, 24, 33, 43, 46] and GW788388 to some extend the ActRII. Long-term treatment may therefore affect activin and nodal dependent signalling.

In summary, we have demonstrated that GW788388 can inhibit TGF-β signalling *in vitro* and attenuate renal fibrosis *in vivo* (Figure 8). By blocking the action of the ALK5 and TβRII kinase receptors TGF-β-induced growth arrest, EMT and ECM deposition was inhibited *in vitro*. Through oral administration of GW788388 to db/db mice for 5 weeks, we were able to reduce glomerulopathy and prevent the TGF-β-mediated up-regulation of excess renal ECM deposition. Thus, we could reduce renal fibrosis in a mouse model for advanced diabetic nephropathy. Our results suggest that TGF-β receptor kinase inhibition should attenuate fibrogenesis and improve the fibrotic outcome for patients suffering from diabetic nephropathy. Whether prolonged or earlier treatment might restore or prevent declines in renal function and not just fibrosis remain to be determined.

Materials and Methods

Cell culture and reagents

The human breast carcinoma MDA-MB 231, the human osteosarcoma U2OS and the monkey kidney COS cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/ml penicillin and 50 μg/ml streptomycin. The human renal carcinoma cell line (RCC4) stably transfected with plasmid expressing the von Hippel Lindau (VHL) protein, was maintained in medium as above with neomycin. The murine breast epithelial cells, NMuMG were maintained in DMEM as above with 10 mg/ml insulin [44]. Cell lines were cultured at 37 $^\circ$ in 5% CO_2. SB431542 was from Tocris. Compounds were dissolved in DMSO. We used 5 ng/ml TGF-β3, 100 ng/ml BMP6 and 50 ng/ml activin a. Antibodies recognizing phosphorylated Smad2 (PSmad2) and phosphorylated Smad 1/5 (PSmad1/5) are described in [47] and TβRII antibody in [18]. Smad2/3, N-cadherin and E-cadherin antibodies were from BD transduction laboratories. Collagen type I antibody from Southern Biotechnology. FN antibody from Abcam. β-Actin (AC-15), α-smooth muscle actin (1A4) and FLAGM2 antibodies from Sigma. Hemagglutinin antibody was from Roche.

Cellular assays, immunodetection and RNA extraction

Immunofluorescence, western blotting, *in vitro* kinase assay, cell proliferation assays, transfection and transcriptional reporter gene assay were done as previously reported [19, 36, 37, 38, 39, 48]. RNA extraction RT-PCR and Q-PCR were described in [32, 44]. For detailed description of these methods see Supplementary Methods.

Histopathology

Kidneys were fixed in 10% formalin. Sections were stained with picric acid stain (PAS) and Masson's Trichrome at Research Pathology Services Incorporated (New Britain, PA). Stained sections were submitted to Pathology Associates, Incorporated for assessment of glomerular changes. Scoring system is outlined in Supplementary methods.

Animal experiments

Intravenous pharmacokinetics profiles were determined in Sprague-Dawley rats, using a crossover design on four separate study days (see Supplementary Methods). Male C57BLKS/J^{Lepr} db/db mice were used as a model for type 2 diabetes mellitus [40] (Jackson laboratory). Animals received GW788388 at 2 mg/kg/day mixed with powdered rodent chow, water *ad libitum*. After 5 weeks of drug treatment, a 24 hour urine collection was performed by individual housing in metabolic cages. Albumin concentrations corrected for creatinine were determined (Nephrat II enzyme-linked immunosorbent assay kit). Kidneys were snap-frozen for RNA analysis or fixed for histology. Plasma drug levels determined by HPLC/MS/MS. GW788388 was isolated from 50 μl of plasma (Sciex API 365). End plasma concentration was 10.4 $\pm$ 1.2 nM and urine concentration 0.9 $\pm$ 0.3 μM. All experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by US Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee.

Statistical Analysis

The experiment was completely randomised. One-way analysis of variance was performed with Bonferroni's multiple comparison test. $P \leq 0.05$ was considered to be statistically significant. Mean is presented either as $\pm$ s.e.m or $\pm$ s.d.

Disclosure

Authors from GlaxoSmithKline disclose a duality of interest.

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Supplementary information

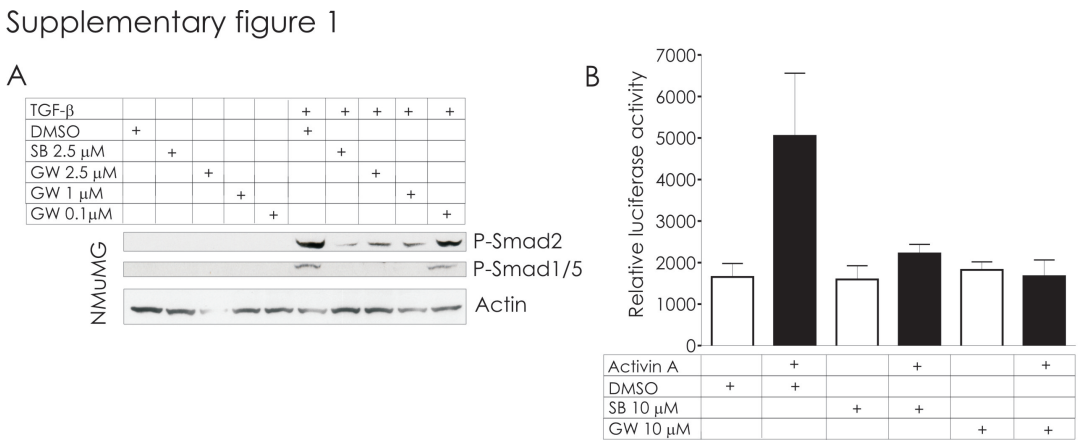


Figure 5.9: Supplementary Figure S1. TGF-β-induced Smad2 phosphorylation and activin-induced CAGA-luciferase activity are inhibited in a concentration-dependent manner. (A) Western blot analysis of Smad2 phosphorylation in NMuMG cells after 1 hour TGF-β stimulation. Smad2 phosphorylation is blocked with nanomolar concentrations of GW788388. (B) Activin a-induced CAGA-luciferase activity is inhibited by SB431542 and GW788388. Transiently transfected U2OS cells were stimulated with activin overnight and luciferase activity measured, corrected for β-gal activity. Error bars denote $\pm$ s.e.m of three measurements.

Supplementary methods

Transfection and reporter gene assay.

Transient transfection of cells were carried out using Lipofectamine (Invitrogen) [39]. Human constitutive active ALK (caALK) 1, 2, 3, 4, 5, 6, and -7 plasmids and the CAGA12 or the BRE luciferase reporter constructs have previously been described [36, 37, 38]. We measured on the Wallac 1420 VICTOR3.

RNA extraction, RT-PCR and Q-PCR.

Cells were treated with GW788388, SB431542 or DMSO and TGF-β the day after plating. Total RNA was isolated using the RNeasy kit (Qiagen). RT-PCR was performed for PAI-1, SNAIL, FN, E-cadherin and glyceraldehyde-3- phosphate (GAPDH) as previously described [44]. RNA was extracted from mouse kidneys and Q-PCR was performed using the ABI Prism 6700 Workstation [32]. RpL-32 or GAPDH were used as housekeeping genes.

In vitro kinase assay, cell viability assay and western blotting.

The kinase assay was performed as described in [7]. In brief, cells were transfected with caALK5, TβRII, BMP type II receptor (BMPRII) or the Activin type II receptor (ActRII). Immunoprecipitated receptors challenged with 14.8 kBq/ml [γ-32P]-ATP and 10 μM compounds.

Cell viability and proliferation assays were done according to the manufactures instructions (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, The Netherlands). Viability and proliferation were measured after 72 hours drug treatment in the presence or absence of TGF-β. Western blot analyses were performed using standard techniques [39].

Immunofluorescence.

Cells were seeded on coverslips and treated with GW788388 and TGF-β where indicated, for 1 or 48 h as previously described [39, 44]. Images were captured with confocal microscopy.

Histopathological scoring.

Stained sections were independently assessed for glomerular changes. Scoring was done on approximately 40 glomerular tufts (representing essentially all of the glomeruli present in a typical section) from each animal using the following categorical criteria for each glomerulus: 0 - well-defined glomerular tuft with essentially no significant accumulations of PAS positive mesangial matrix; 1 - slight, focal increases in PAS positive mesangial matrix or thin bands of increased PAS positive matrix running along the core of the glomerular tuft; 2 - Multiple small foci of increased mesangial matrix or thicker more dense bands of matrix; 3 - Increased matrix to the extent that some lobules of a glomerular tuft can be considered sclerotic. Mesangial cells may be increased in these areas; 4 - significant increases in mesangial matrix and frequently increased numbers of mesangial cells involving the entire glomerular tuft.

Pharmacokinetic profiling *in vivo*.

Intravenous pharmacokinetics profiles were determined in Sprague-Dawley rats, using a crossover design on four separate study days. Femoral vein catheters implanted for infusion of test compounds at least three days prior to the start of the study. On day one, animals received GW788388 (6 μmol/kg target dose) or SB431542 (2 μmol/kg target dose) by 30 minutes i.v. infusion (4 mL/kg). The dose (pH = 3.5), contained 3% DMSO prepared in 20% aqueous encapsin (Cerestar USA Inc., Hammond, IN). For SB431542 the dose solution was prepared in 10% PEG 400 and isotonic saline (pH = 3.5-4.0) and contained 1.0% DMSO.

Chapter 6

Summary and Discussion

Since the discovery of TGF-β nearly three decades ago [1, 2, 3, 4] tremendous scientific effort has led to a sophisticated understanding of the multifunctional actions of this pleiotropic cytokine. TGF-β regulates a myriad of processes in normal tissues and in cancer pathogenesis a characteristic is the frequent loss of TGF-β-induced growth arrest and abnormal secretion of this cytokine (reviewed in [5, 6, 7, 8, 9]). The pathogenic branch of TGF-β signaling is an attractive target for therapeutical intervention. However, in order to specifically direct therapy to this arm of TGF-β signaling a deeper understanding of its cellular actions and intercellular signal transduction pathways in specific contextual settings is crucial. Several studies are presented in this thesis, which are aimed at unraveling the mechanisms by which TGF-β is involved in the pathogenesis of breast cancer, breast cancer bone metastasis and in renal fibrosis.

The key findings presented in this thesis are:

1. The intracellular TGF-β effector proteins, Smad2 and Smad3, play differential roles in breast cancer bone metastasis. Smad2 mediates anti-angiogenic properties whereas Smad3 contributes with pro-angiogenic effects *in vitro* and in human breast cancer bone metastasis (chapter 2).

2. HMGA2 was identified as a Smad4-dependent TGF-β target gene in breast cancer cells. This factor is required for TGF-β-induced EMT of epithelial cells. Ectopic expression of HMGA2 results in a down-regulation of E-cadherin, which is mediated via enhanced expression of its transcriptional repressors Snail1, Snail2/Slug and Twist (chapter 3).

3. TGF-β-induces EMT, growth arrest and initiates specific gene responses in epithelial cells. The actions of TGF-β can be inhibited by administration of the ALK5 and TβRII antagonist GW788388 (chapter 5).

4. Expression of constitutively active ALK2, a BMP-7 type I receptor, in osteotropic breast cancer cells impairs tumor-induced osteolysis in an intra-bone tumor growth model (chapter 4).

5. Constitutive activation of the BMP-7 receptor, ALK2, in breast cancer cells led to the findings that the anti-metastatic actions of BMP-7, in breast cancer bone metastasis can occur via direct effects on the tumor cells. This mediates paracrine signaling in cells of the bone and tumor-associated microenvironment. We speculate that enhanced BMP signaling can counteract TGF-β in cancer cells hereby disturbing the vicious cycle of bone metastasis (chapter 4).

6. When the TGF-β inhibitor GW788388 was orally administered to diabetic *db/db* mice with advanced nephropathy, GW788388 significantly reduced renal fibrosis and decreased expression of key mediators of fibrosis in kidneys (chapter 5).

The significance of these observations and how we can relate them to the field of TGF-β research and potentially lead to clinical applications will be discussed in the following. A simplified schematic overview of our findings is given in figure 6.1.

6.1 Modulation of the TGF-β signaling pathway

TGF-β may exert dual roles in carcinogenesis. Acting at first as a tumor suppressor, in premalignant stages of tumorigenesis, via inhibition of proliferation and induction of apoptosis. At later stages of disease, TGF-β can shift to being a tumor promoter (reviewed in [5, 6, 7]). Frequently in cancer there is a loss of the TGF-β cytostatic responses and the switch to a tumor promoting role of TGF-β. Concomitantly, high dose secretion of active TGF-β is often observed in carcinomas. The net result being tumor progression and possibly metastasis (reviewed in [5, 8, 9]). The molecular determinants which mediate this switch remain poorly understood [10]. However, dissecting the individual steps of the metastatic cascade has led to pivotal findings and the mechanistic of breast cancer metastasis are gradually being unveiled.

Smad2, Smad3, HMGA2 downstream mediators of TGF-β signaling

We studied several aspects of the pro-tumorigenic functions of TGF-β in breast cancer cells and in breast cancer metastasis models *in vivo*. It was previously shown that knockdown of Smad4 inhibits the formation and progression of bone metastasis of MDA-MB-231 cells [11, 12]. However, Smad4 is a central regulator of both TGF-β and BMP signaling. In chapter 2 we describe a comparative analysis of the two direct downstream mediators of TGF-β signaling Smad2 and Smad3. The function of these signaling molecules were analyzed in the highly invasive and osteotropic breast cancer cells the MDA-MB-231. Smad2 or Smad3 expression was genetically knocked down by lentiviral transduction of microRNAi (miR RNAi).
When analyzing the effect of eliminating these TGF-β effector molecules we found that key metastatic TGF-β target genes [13] were critically dependent on Smad3 and not Smad2 (see figure 6.1). These genes include IL-11, PAI-1 and CTGF. That Smad3 is

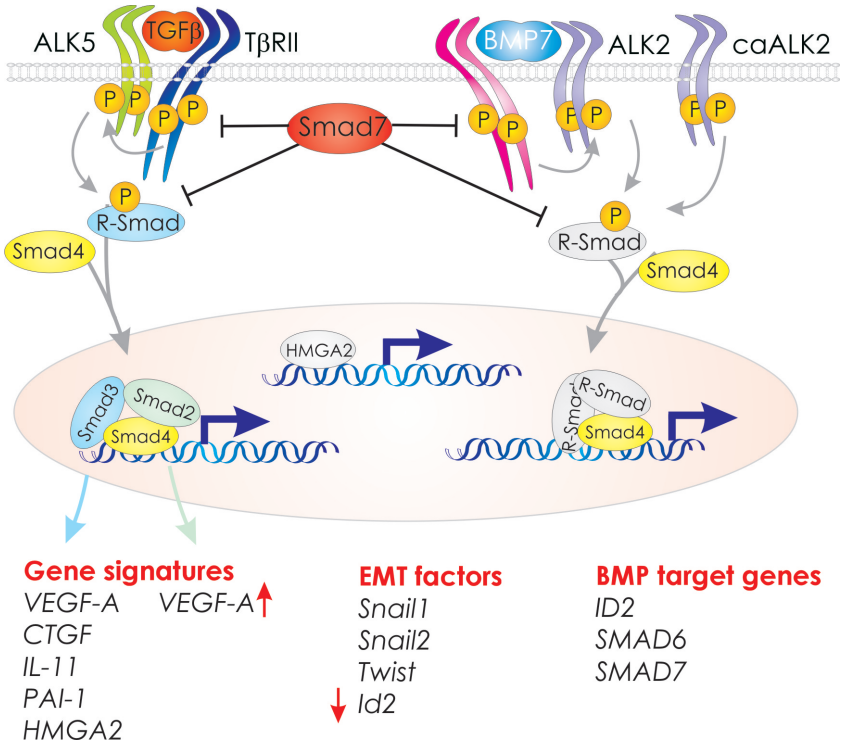


Figure 6.1: Modifying the TGF-β signaling pathway

We modified TGF-β signaling and downstream responses through several mechanisms in NMuMG and MDA-MB-231 breast cancer cells. By lentiviral transduced microRNAi we eliminated the expression of either Smad2 or Smad3. Smad2 and Smad3 were shown to play specific roles in MDA-MB-231 breast cancer cells. Smad3 was necessary for TGF-β inducible gene responses of IL-11, CTGF, PAI-1 and VEGF-A. In contrast, loss of Smad2 enhanced *VEGF* mRNA expression (*left side of figure*). We studied the function of HMGA2 a direct target gene of TGF-β and found HMGA2 to be dependent on Smad3 and Smad4 in MDA-MB-231 cells and on Smad2, Smad3, and Smad4 in NMuMG cells. Enhanced HMGA2 expression significantly induced the expression of the E-cadherin repressors *Snail1*, *Snail2* and *Twist*. Furthermore, expression of *Id2* was repressed when HMGA2 was overexpressed. Thus, ectopic expression of HMGA2 induce EMT of NMuMG cells independent of TGF-β and loss of HMGA2 inhibits TGF-β mediated EMT (*middle part of figure*). Continuous activation of the BMP-7 receptor caALK2 was shown to counteract TGF-β signaling in MDA-BO2 cells as observed in transcriptional reporter assays (*right side of figure*).

the major regulator of TGF-β target genes is in line with several other previous reports [10, 14, 15, 16, 17]. Also, specific knockout of Smad2 or Smad3 in hepatocytes identified Smad3 as the main transcriptional inducer or repressor downstream of TGF-β [10].

We further analyzed the effect of knockdown on the migratory properties of breast cancer cells. MDA-MB-231 cells readily migrate in response to TGF-β and this can be inhibited by administration of an ALK5 kinase inhibitor (chapter 2, [18]). We found that depletion of either Smad2 or Smad3 was sufficient to inhibit TGF-β-induced migration of MDA-MB-231 cells.

To specifically determine the actions of TGF-β in metastasis we used the Smad2 and Smad3 knockdown cells in a breast cancer metastasis model where cells are inoculated into the left heart ventricle and metastasize to bone [12]. Knockdown of Smad2 resulted in enhanced progression of metastasis compared to control and Smad3 knockdown tumor-bearing animals. In contrast, Smad3 knockdown cells displayed delayed growth kinetics in the early phases of the experiment. These findings were directly correlated with differential roles of Smad2 and Smad3 in tumor-induced angiogenesis (see figure 6.1 and 6.2). VEGF secretion was significantly enhanced in MDA-MB-231 cells depleted for Smad2. In Smad3 knockdown cells, VEGF-A expression and secretion were no longer induced by TGF-β.

To further examine this, we visualized and quantified the newly formed capillary networks in bone tumor metastasis by immunohistochemical CD31 staining. Strikingly, Smad2 knockdown metastasis showed a four fold increase in CD31 staining and the micro vascular density was significantly enhanced compared to Smad3 knockdown and control metastasis. Thus, Smad2 may inhibit tumor-induced angiogenesis by negatively regulating tumor-cell secretion of VEGF into the bone microenvironment. Smad3, on the contrary, induce angiogenesis by stimulating production of angiogenic factors (like VEGF) and chemotactic factors, which mediate the recruitment of inflammatory cells (see figure 6.2).

In agreement with our findings, Smad2 was found to mediate secretion of factors with anti-angiogenic properties, whereas Smad3 induced the secretion of pro-angiogenic factors in tubular epithelial cells [19].

A study by Ju et al. [10] clearly demonstrated differential roles of Smad2 or Smad3 in conditional knockout (KO) hepatocytes. Smad2KO cells displayed enhanced migratory potentials and spontaneously underwent mesenchymal transition suggesting that Smad2 is required to maintain a stable epithelial morphology [10]. In contrast, TGF-β was unable to induce EMT in Smad3KO cells. Moreover, Smad3 was found to be an essential mediator of apoptosis and TGF-β-induced G1 cell cycle arrest whereas in Smad2KO cells cyclinD1 and c-Myc were up-regulated [10]. Thus, Smad2 and Smad3 exert distinct roles in TGF-β-mediated cell cycle regulation in hepatocytes. In our breast cancer model we found that Smad2 knockdown cells proliferated slightly slower *in vitro* than control and Smad3 knockdown cells. However, we cannot exclude effects on apoptosis or survival since proliferation was analyzed in a cell viability assay. It would be interesting to examine the proliferative capacities of Smad2 miR RNAi cells versus control and Smad3 miR RNAi cells in our bone metastases sections with immunohistochemical markers for apoptosis and proliferation.

When analyzing TGF-β-induced responses in hepatocytes all genes examined were shown to be dependent on Smad3 [10]. Similarly, we found that most TGF-β-responsive genes were regulated by Smad3 not Smad2. Many of these genes were also dys-regulated in MDA-MB-231 Smad4 knockdown cells [11, 12]. However, even though many pro-metastatic genes lost their responsiveness to TGF-β in both Smad3 and Smad4 knock-down cells loss of Smad4 gave rise to a much less severe metastatic phenotype *in vivo*. Metastasis-free survival was significantly enhanced when Smad4 was depleted and the

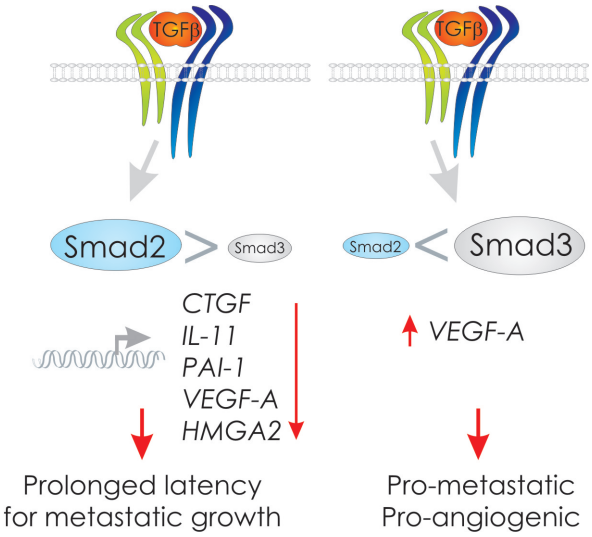


Figure 6.2: Smad2 and Smad3 play distinct roles in breast cancer bone metastasis. Model of the opposing roles of Smad2 and Smad3 i osteotropic MDA-MB-231 cells. Knock down of Smad3 (*left panel*) results in loss of TGF-β-inducible gene responses of critical metastatic inducers such as *IL-11*, *CTGF*, *PAI-1*, *VEGF-A* and *HMGA2*. When Smad3 knockdown cells were used in a bone metastatic model prolonged latency in metastatic growth was observed. In contrast, when Smad2 was eliminated (*right panel*) enhanced expression and secretion of VEGF was observed. This translated into enhanced metastatic potential of these cells *in vivo* and significantly enhanced tumor-induced angiogenesis. Together, these observations support a role for Smad2 as a tumor suppressor and Smad3 as a tumor promoter in breast cancer cells.

frequency of bone metastasis was reduced by 75% [11, 12]. In animal injected with Smad3 knockdown cells we observed a clear latency in tumor progression in the early phase of metastatic progression compared to control and Smad2 inoculated animals. Since Smad4 is a common transcription factor shared by both TGF-β and BMP these observations could suggest that abrogation of both signaling pathways result in a more efficient blockade of metastasis compared to only eliminating Smad3.

The molecular determinants that mediate the functional switch, which transform TGF-β from being a tumor suppressor to a tumor promoter remain poorly understood. We hypothesize that loss of Smad2 (genetic or epigenetic) during tumorigenesis may increase the metastatic potential, whereas loss of Smad3 could decrease the metastatic behavior of breast tumor cells (chapter 2, [10]). This is in line with findings in patients with stage II breast cancer where loss of P-Smad2 staining is correlated with shorter overall survival [20]. Thus, it is possible that the functional switch may be determined by changes of the relative balance of Smad2 (anti-metastatic signaling) and Smad3 (pro-metastatic signaling) in malignant cells (see figure 6.2) hereby affecting their distinct properties on the microenvironment [10]. It would therefore be of great interest to perform a more detailed genetic analysis of the exact roles of Smad2 and Smad3 both in response to TGF-β and in non-induced conditions and to analyze the impact of knock-

down or either Smad2 or Smad3 on the tumor-associated stroma in bone metastasis in further detail.

Thus, our findings suggest that Smad2 and Smad3 play distinct roles in mammary cancer metastasis. In hepatocytes, Smad2 knockout cells spontaneously acquired mesenchymal features with a pro-migratory phenotype [10]. Smad3, on the other hand, was necessary for TGF-β-induced EMT and migration [10]. Also, keratinocyte-specific knockout of Smad2 displayed clear mesenchymal characteristics [21]. Together, these studies suggest that Smad2 is necessary to maintain a stable epithelial phenotype and that the two TGF-β R-Smads have distinct functions in several cellular systems. The breast cancer model used in our study is not suited for studying aspects of EMT as MDA-MB-231 cells display partial hypermethylation of the E-cadherin promoter [22]. Moreover, we employ intracardiac inoculation of the cancer cells and hereby circumvent the critical steps of EMT.

EMT facilitates cell movement and the generation of new tissues in embryogenesis. In a cancer setting, gain of mesenchymal characteristics allow tumor cells to disseminate and intravasate and hereby escape from the primary tumor as extensively described in chapter 1 (reviewed in [5, 23, 6, 24]). The downstream mediators of TGF-β Smad2, Smad3 and Smad4 have all been extensively studied in normal epithelial cell models. Homozygous deletion of Smad2 triggered complete EMT in skin tumors [21]. In contrast, over-expression of activated Smad2/3 was shown to increase cell motility in a squamous skin tumorigenesis models [25]. In line with these findings, Valcourt et al. showed that ectopic expression of either Smad2 and Smad3, in combination with Smad4 could induce EMT in NMuMG cells [26]. In these cells, loss of Smad3 or Smad4 by RNAi completely abrogated TGF-β-induced EMT [17, 12]. Moreover, overexpression of the antagonist of TGF-β signaling, Smad7, in NMuMG cells blocked TGF-β-mediated EMT [26].

The discovery of HMGA2 as being a direct target gene of TGF-β and necessary for TGF-β-mediated EMT of mammary epithelial cells is described in chapter 3 (see figure 6.3). We found that knockdown of Smad4, in NMuMG cells blocked TGF-β-induced up-regulation of *Hmga2*. In MDA-MB-231 cells, knockdown of either Smad3 or Smad4 could block TGF-β-mediated *HMGA2* mRNA induction. When HMGA2 was over-expressed this alone could drive the EMT response through direct up-regulation of the E-cadherin repressors, i.e. Snail1, Snail2 and Twist. In addition, ID2, another key player in EMT, which expression is reduced in response to TGF-β [27], was down-regulated in HMGA2 over-expressing cells. Furthermore, NMuMG cells depleted for HMGA2 by RNAi no longer undergo EMT in response to TGF-β. Together these findings establish a strong role for HMGA2 as a mediator of EMT, which is directly induced by TGF-β [28].

In correlation with these observations, it was shown that HMGA2 is highly up-regulated during embryogenesis in cells which have not yet undergone overt differentiation [29, 30]. In normal tissues, the expression of HMGA2 is lost and various tumor types abundantly re-express HMGA2, which is correlated with enhanced malignancy or metastatic potential [29, 31]. Together, these findings suggests that HMGA2 indeed could be a key pro-tumorigenic mediator downstream of TGF-β.

We persistently tried to study the role of HMGA2 in the MDA-MB-231 breast can-

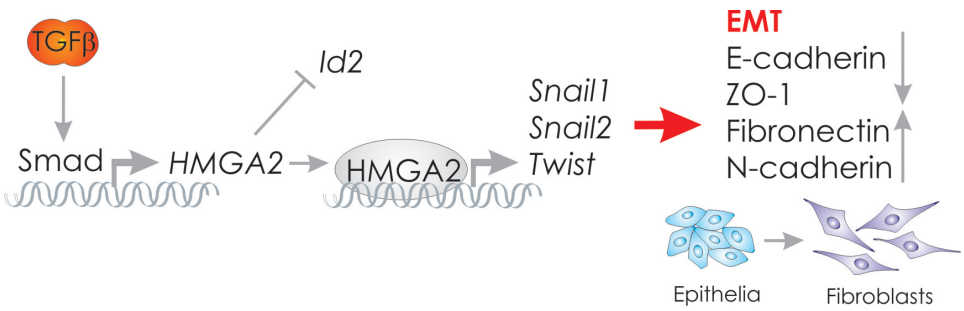


Figure 6.3: HMGA2 induce the expression of E-cadherin repressors resulting in EMT. TGF-β up-regulate HMGA2 via Smad-dependent mechanisms. Ectopic HMGA2 expression significantly induced the mRNA expression of *Snail1*, *Snail2* and *Twist* which functions as critical E-cadherin repressors. In addition, HMGA2 could repress the expression of *Id2*. Thus, ectopic expression of HMGA2 induce EMT of NMuMG cells independent of TGF-β as observed on morphological and EMT marker level.

cer bone metastasis model. This was done by knock down of HMGA2 expression by shRNAi and analyzing the effect in various tumorigenic assays. HMGA2 knockdown cells displayed reduced capacity to invade and migrate in matrigel coated transwell chambers (Thuault unpublished observations). No differences were observed in other *in vitro* models such as anchorage-independent growth or proliferation assays. When HMGA2 knockdown cells were used in our *in vivo* metastasis model we did not observe any differences in metastasis formation and progression between control cells and cells silenced for HMGA2. Despite technical difficulties in conducting these experiments we hypothesize that HMGA2 is important in a primary tumor setting where EMT is needed for migration and invasion to distant sites. This is in line with findings in squamous carcinomas where HMGA2 is observed at the invasive front in carcinoma tissue where tumor cells migrate into the connective tissue [31]. However, in later stages of the metastatic cascade HMGA2 may not be necessary for the establishment and progression of breast cancer bone metastasis.

We studied the knock down of HMGA2 in osteotropic MDA-MB-231 cells a highly metastatic cell line, which displays complete loss of E-cadherin [22]. This metastasis mode circumvents critical steps of EMT since the main pro-tumorigenic function of HMGA2 is the induction of the E-cadherin repressors this may explain the outcome of our preliminary *in vivo* study using cells in which there is no E-cadherin present to be repressed. HMGA2 may therefore be irrelevant in the later stages of tumorigenesis in MDA-MB-231 cells. Olmeda et al. showed that knock down of the E-cadherin repressor SNAIL in MDA-MB-231 could inhibit orthotopic growth *in vivo* [32]. This might have been a superior model for studying the effect of HMGA2 depletion or overexpression. Alternatively we could have used immortalized human mammary epithelial cells as described by Onder et al. who recently used this cell line to examine the function of E-cadherin in breast cancer [33]. Otherwise, the murine epithelial breast cancer line 4T1 which express E-cadherin and give rise bone-tropic metastasis could have been a better

suited model for these studies [34].

Counteracting TGF-β signaling by continuous activation of ALK2

Metastatic cancer affects millions of people worldwide yet the majority of patients with cancer metastasis remain incurable [6, 35]. Cancer research have largely been focussed on the cells from which a given cancer originates in a primary tumor setting. However, tumor growth depends on interactions between multiple inter-dependent cell types [36]. How the microenvironment and tumor-infiltrated stromal cells influence tumorigenesis and the role played by TGF-β herein are currently topics of intense investigation.

In patients with breast cancer, loss of BMP-7 mRNA expression in primary tumor is associated with the formation of bone metastasis [37]. In prostate cancer, the expression of BMP-7 was shown to be down-regulated compared to normal prostate tissue [38]. Moreover, in a panel of cancer cell lines the expression of BMP-7 negatively correlated with tumor cell aggressiveness [37, 38]. BMP-7 can counteract TGF-β signaling and reverse TGF-β-induced EMT. This is mediated through re-expression of the key EMT marker E-cadherin [39, 37, 40]. In MDA-MB-231 breast cancer cells, BMP-7 was shown to counteract TGF-β at the transcriptional level and reverse the TGF-β-induced up-regulation of vimentin [37]. When BMP-7 was ectopically expressed in MDA-BO2 cells or human recombinant BMP-7 systemic administered to MDA-BO2 tumor-bearing mice a significant inhibition in the number of osteolytic metastasis and progression of these lesions was observed [37].

In chapter 5, we describe the identification of the functional BMP-7 receptor, ALK2, in MDA-BO2 cells. Furthermore, we explore whether ectopic expression caALK2, a genetically constitutively activated receptor, can mediate similar responses as over-expression of BMP-7 ligand *in vitro* and *in vivo*. Ectopic expression of caALK2 resulted in continuous activation of downstream BMP signaling, thus mimicking a state of continuous BMP-7 signaling. When analyzing classical downstream target genes of BMPs, we found sustained up-regulation of *ID2*, *SMAD6*, and *SMAD7*. Moreover, TGF-β signaling was significantly abrogated at transcriptional level (see figure 6.1). Hence, these observations are directly in line with our findings in cells ectopically expressing the BMP-7 ligand [37].

Whether the inhibitory actions of BMP-7 on bone metastasis and intra-bone tumor growth are caused by direct cell autonomous actions on the cancer cells or through direct inhibitory signals on the tumor-associated stroma has remained elusive. With over-expression of caALK2 we mimic a situation where BMP signaling is active only in the tumor cells not in the entire microenvironment as is the case for BMP-7 over-expressing cells (see figure 6.4). The metastatic behavior of BMP-7 versus caALK2 over-expressing cells was studied in intra-bone tumor growth and metastasis models.

In the intra-bone tumor growth model the effects of either caALK2 or BMP-7 over-expression on tumor-induced bone remodeling was analyzed by quantifying changes in bone mass and bone architecture by μ-CT. We found that the formation of overt osteolytic lesions was significantly inhibited in mice injected with either caALK2 of BMP-7

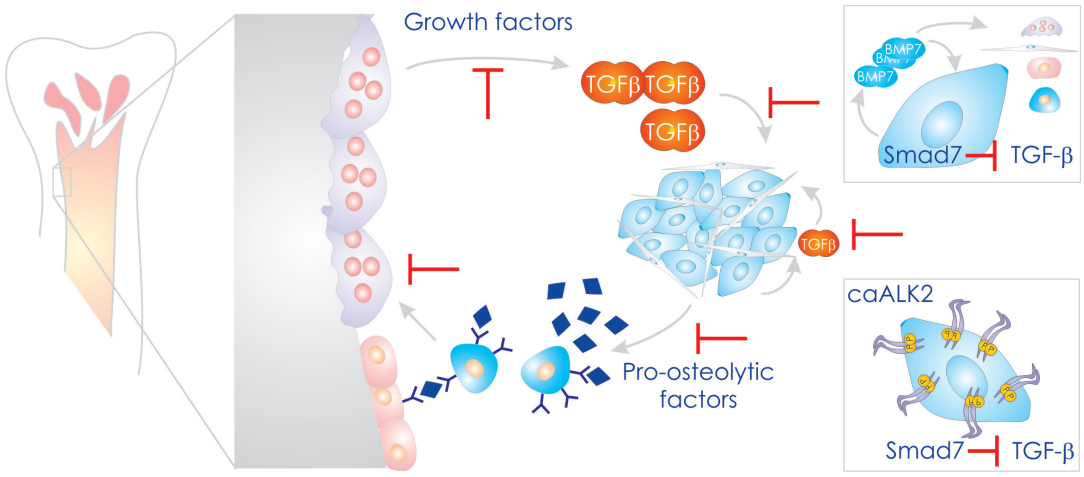


Figure 6.4: Over-expression of BMP-7 or caALK2 inhibits osteolytic breast cancer metastasis. A tumor cell-induced vicious cycle of osteolysis and tumor growth in the bone is dependent on active TGF-β signaling. Tumor cells secrete pro-osteolytic factors which stimulate osteoclastogenesis and osteoclastic activation hereby inducing bone resorption. Bone encapsulated growth factors such as TGF-β is released into the microenvironment and these act back on the cancer cells to stimulate further growth and osteolysis. We overexpressed BMP-7 (*top insert*) or caALK2 (*lower insert*) in osteotropic breast cancer cells. BMP-7 secreted from MDA-BO2 cells over-expressing the ligand act both on the tumor cells and cells in the bone microenvironment. Cells over-expressing caALK2 on the other hand solely activate BMP-7 signaling in the tumor cells. Both cell lines displayed significantly reduced tumor-induced osteolysis and metastatic progression. High up-regulation of Smad7, a well described inhibitor of TGF-β and BMP signaling was also observed in these cell lines. We hypothesize that this induction of Smad7 or alternatively ID proteins could play an important inhibitory role in breast cancer bone metastasis and intra-bone tumor growth.

expressing breast cancer cells compared to control inoculated cells.

We further characterized the metastatic potential of caALK2 MDA-BO2 cells in the bone metastasis model [41, 42]. Strikingly, ectopic expression of caALK2 inhibited the number and progression of osteolytic metastasis compared to control MDA-BO2 inoculated animals. Similar observations were seen with MDA-BO2 cells over-expressing BMP-7 [37]. Thus, progression and osteolytic bone destruction is largely dependent on tumor cell autonomous signaling.

One of the mechanisms whereby BMP and TGF-β signaling confer dichotomous actions is, in part, through differential regulation of ID proteins [27]. Thus, caALK2 or BMP-7 may antagonize TGF-β signaling and hereby mediate inhibitory actions on metastatic tumor growth through direct up-regulation of ID1 and ID2. Also, the negative regulator of TGF-β and BMP signaling, Smad7, was up-regulated in caALK2 and BMP-7 over-expressing cells. Smad7 targets type I receptors and R-Smads for degradation and inhibit complex formation with Smad4 [43, 44, 45, 46]. Ectopic expression of Smad7 in NMuMG cells can block TGF-β-mediated EMT [26]. Also, over-expression of Smad7 in melanoma cells significantly reduced in the progression of bone metasta-

sis [47]. Furthermore, adenoviral administration of Smad7 to mice bearing jygMC(A) murine mammary tumors or ectopic expression of Smad7 in these cells [48] significantly inhibited metastasis and increased metastasis-free survival [48].

Thus, Smad7 plays a crucial role in metastatic progression of various cancers. Stable overexpression of Smad7 inhibited the up-regulation of TGF-β-inducible genes such as *IL-11*, *CXCR4*, *PTHrP* and *Osteopontin* in melanoma cells [47]. We analyzed the expression levels of *IL-11*, *PTHrP* and *CTGF* in cell over-expressing caALK2 or BMP-7 MDA-BO2 compared to control cells and found no changes in expression patterns. In order to determine the contribution of either Smad7 or ID2 in our model it would be of great interest to genetically deplete these proteins from caALK2 and BMP-7 cells and analyze their changed metastatic behavior *in vivo*.

Continuous activation of ALK3, a receptor for BMP-2 and BMP-4 [49], was recently shown to mediate invasion and metastasis of MDA-MB-231 breast cancer cells. This pro-metastatic function of caALK3 could be blocked by over-expression of a dominant negative ALK3 [50]. We show that in MDA-BO2 cells BMP-7 signals via ALK2 and over-expression of continuous active ALK2 receptor gives rise to a less metastatic phenotype *in vivo*. Thus, active BMP-7 signaling in tumor cells distinctively induce anti-metastatic properties in these human breast cancer cells. Together, these observations highlight the importance of discriminating between different BMPs when characterizing their functions.

Taken together, these studies demonstrate that BMP-7 can block tumor progression in bone via directly inhibiting tumor cell-induced osteolysis possibly by antagonizing TGF-β signaling in the breast cancer cells (see figure 6.4). To determine the mechanism in detail a more thorough gene analysis would have to be performed to analyze the regulation of down-stream effectors in these cells.

6.2 Clinical applications and therapeutic opportunities

The dichotomous role of TGF-β in diseases represents a great therapeutic challenge. Targeted strategies must be directed towards the pathogenic functions of the signaling pathway and protect the normal homeostatic role of TGF-β such as its potent anti-inflammatory actions and the anti-mitogenic effects of TGF-β on primary tumors (reviewed in [7, 51]). Moreover, to properly stratify patient subpopulations, which may benefit from anti-TGF-β treatment regimens, new and robust diagnostic tools must be developed [52].

Abrogating TGF-β signaling by means of synthetic inhibitors have been attempted by several pharmaceutical companies and research groups, as introduced in chapter 1. In chapter 5 we describe the characterization of a novel TGF-β receptor antagonist GW788388 in epithelial cells of various origins [53, 54]. We show for the first time that GW788388 targets the ATP binding domain of both the TGF-β type I and the type II receptors. When analyzing downstream TGF-β signaling we found that GW788388

efficiently blocked TGF-β-mediated EMT. Furthermore, the compound inhibited TGF-β-induced growth arrest in NMuMG cells. Thus, down-steam mechanisms of TGF-β signaling can efficiently be inhibited by low doses of GW788388 [53]. These findings are summarized in figure 8 of chapter 5.

In a mouse model of diabetic nephropathy, the *db/db* model, we found that daily administration of GW788388 (2 mg/kg/day for 5 consecutive weeks) significantly attenuated glomerulopathy in mouse kidneys [53]. This was correlated with reduced mRNA expression of critical factors in ECM remodeling induced by TGF-β namely PAI-1, collagen I, collagen III and Fibronectin in GW788388 treated animals versus controls. All together, these data provide a strong foundation for using TGF-β receptor kinase inhibitors for treating advanced renal diseases. However, the use of such drugs for renoprotection may require chronic treatment and the impact of blocking of TGF-β signaling in perspective of its immunosuppressive and tumor suppressor functions could have adverse side effects [53, 55, 51].

In continuation of these studies, we initiated an curative drug administration regimen in mice induced with metastatic breast cancer. GW788388 was given daily (at 3 and 15 mg/kg) mixed with powdered rodent chow. However, due to technical difficulties and the publications of similar studies [18, 56, 57] during the course of these experiments this project was discontinued and our attention was focused to other research areas. Other groups elegantly showed that administration of small kinase inhibitors to ALK5 can inhibit metastasis formation in mice both in a preventive setting [18] and with curative treatment regimens [56, 57].

With genetic interference of the expression of downstream TGF-β signaling mediators, Smad2 or Smad3, we found that Smad2 can act as a negative regulator of breast cancer metastasis. In contrast, downstream TGF-β signaling was dependent on Smad3, which was shown to have a pro-metastatic function in metastatic breast cancer (chapter 2). Current therapeutic strategies aim at targeting TGF-β signaling through receptor inactivation [51]. This leads to a complete halt of downstream Smad2 and Smad3 signaling. Despite efficacious results using these approaches in animal models (reviewed in [51]) our findings (chapter 2) together with those of others [10, 21] suggests that selectively targeting Smad3 may lead to more effective anti-metastatic and anti-fibrotic therapy [10].

HMGA2 was identified as a critical Smad-dependent TGF-β target gene which directly induce EMT and is crucial for TGF-β-mediated EMT [28]. Our preliminary findings, in a breast cancer metastatic model, suggests that HMGA2 may be more important in a primary tumor setting compared to the progression of metastasis at a secondary site (unpublished observations). Thus, targeting HMGA2 in the primary tumor could lead to preservation of the epithelial morphology and hereby inhibit the acquisition of a migratory phenotype which facilitates metastatic progression. Since HMGA2 is lost in normal cells and highly re-expressed in various cancerous tissues it presents as an excellent target for anti-cancer therapy.

The let-7 family members of miRNAi's are characterized as tumor suppressors and clearly define an epithelial gene signature. Interestingly, the loss of miRNA let-7 and

enhanced expression of HMGA2 were recently suggested as superior prognostic markers with more clinical relevance than the classical markers E-Cadherin, vimentin and Snail1 in cancer [58].

We compared the intra-bone tumor growth of BMP-7 overexpressing breast cancer cells with cells ectopically expressing a continuous active ALK2 receptor (chapter 4). We found that BMP-7 can act as a negative regulator of TGF-β signaling likely by up-regulating the expression of Smad7 and by reversing TGF-β-induced EMT [37]. Thus, BMP-7 inhibits local tumor progression in the bone through tumor cell-autonomous actions. These findings suggests, that systemic or local administration of BMP-7 could be used to treat patients with breast or prostate cancer metastasis (reviewed in [37, 41, 59]).

Due to the pleiotropic nature of TGF-β the application of TGF-β receptor inhibitors or drugs which target this signaling pathway present unique challenges that must be considered in drug development programmes. Targeted drug delivery using liposome-based therapy can specifically deliver a drug to the site of interests. Systemic administration Bisphosphonate-coupled liposome encapsulated ALK5 inhibitors, to patients with bone metastasis, would allow specific delivery of biological relevant doses of compounds to sites of extensive bone remodeling and hereby prevent systemic toxicity and severe immune side-effects.

Also, novel studies examining combinatorial therapies targeting both HIF-1α and TGF-β signaling are currently being explored [60]. Ectopic expression of DN-TβRII and silenced expression of HIF-1α significantly enhanced survival of mice induced with cancer metastasis. Thus, targeting both TGF-β and HIF-1α showed additive inhibitory effects on tumor metastasis [60]. Whether combined therapy of human recombinant BMP-7 and an ALK5 inhibitor synergistically could provide a more potent anti-metastatic therapy would also be of great interest to examine in our osteotropic breast cancer models. In addition, Dr. Theresa Guise and colleagues are further exploring whether Halofuginone can block metastatic progression in mice models. This compound has been described to interfere with TGF-β signaling through down-regulation of TβRII expression and up-regulation of Smad7 [61]. In renal fibrosis, halofuginone was shown to prevent ECM deposition in *db/db* mice by inhibiting TGF-β signaling [62]. Together, these examples highlight the potential of targeting the TGF-β pathway in the treatment against cancer and fibrosis.

6.3 Perspectives

By understanding the stochastic nature of the metastatic cascade and the molecular mechanisms that facilitate metastasis we have begun to understand not only how metastasis proceeds but also why they occur in the first place [63, 64]. Understanding the role of the tumor-associated stroma and the cells herein on tumorigenesis is one field which holds great promise for future research and novel therapeutic strategies [36]. The pioneering work of Dr. Mina Bissell and many others have resulted in the design of complex 3D multi-cellular models *in vitro* which allow simulation of *in vivo* settings.

One model for tumorigenesis suggests that cancer develops through augmented cell-autonomy and rare cellular variants survive a darwinian selection process for enhanced metastatic abilities [65]. Another model proposes that metastatic traits are acquired through exposure of epithelial cancer cells to paracrine signals received from mesenchymal cell types in the tumor-associated stroma (reviewed in [66, 36, 67, 68, 69]). In support of this theory, Karnoub et al. recently showed that MSCs dramatically promote metastasis of breast cancer cells when these are co-injected orthotopically. When tumor cells were isolated and re-injected without MSCs they no longer possessed enhanced metastatic properties compared to controls [68]. Thus, supporting the hypothesis that acquisition of an invasive metastatic morphology is reversible and maintenance of this phenotype depends on continuous contact with stromal cells [68]. Furthermore, this suggests that the metastatic characteristics are expressed transiently in a minority of cells which respond to paracrine signals from the stromal compartment. Locating and identifying these key genes which mediate invasiveness should therefore be done by procedures rather than bulk analysis of the primary tumor [68] and focus should be aimed at analyzing the responsiveness of primary tumor cells to stromal signals and the effect of the cancer cells on the tumor stroma and cells herein [36, 69].

The observations described in this thesis are based on the current models available for studying metastatic bone disease [42, 41, 70]. Few human breast cancer cell lines give rise to bone metastasis and most of our studies are based on an osteotropic subclone of the MDA-MB-231 cell line. A cancer line originally isolated from a pleural effusion of a breast cancer patient suffering from widespread metastasis many years after removal of her primary tumors [65, 71]. That most research is based on this one model is a drawback and it is therefore of high relevance to identify new *in vivo* models for breast cancer bone metastasis. Furthermore, is it of great importance to correlate our finding in mouse models with observations in human material to produce clinical relevant data.

The discovery of miRNAs and their potential tumor suppressor and tumor promoting properties holds great promises for future drug design strategies [72] along with the use of miRNAs as prognostic biomarkers [73]. Also, the emerging field of tumor-initiating cancer stem cells (CSCs) and the pathways that regulate self-renewal and survival of breast CSCs may give rise to a promising novel cancer therapies. Moreover, the EMT program was recently shown to support the differentiation of mammary epithelial cells with self-renewal properties similar stem cell [74]. The impact of EMT on tumor-initiating CSCs and on miRNAs and how these observations can be translated into therapy will be interesting to follow in the near future.

On the prognostic side, the FDA recently approved an *in vitro* diagnostic test "the Mamma-print" which can predict the likelihood of distant metastatic recurrence in breast cancer based on a 70 gene signature [75, 76]. The application of expression profiling of primary human cancers is a promising approach to identify mechanisms responsible for tumorigenesis. Well-defined EMT and tumor models can complement the human cancer studies and the synergism between expression profiles could identify promising key target genes and pathways involved in late-stage tumorigenesis. This would support the identification of molecular markers and the development for anti-cancer therapies and

eventually stratify patient group which could benefit from such treatment regimens.

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Chapter 7

Miscellaneous

7.1 Samenvatting

Transforming growth factor-β (TGF-β) is een gesecreteerd eiwit dat zeer veel verschillende cellulaire functies kan reguleren. Genetische veranderingen en omgevingsfactoren kunnen zowel in als tussen cellen ontsporingen van TGF-β signaaloverdracht veroorzaken, welke in verband worden gebracht met een breed scala aan ziektes, waaronder kanker, fibrotische ziektebeelden, auto-immuunziekten, spierziekten, botziekten en vaatziekten. TGF-β maakt deel uit van een familie van 33 verschillende eiwit-liganden, waaronder eveneens de zogenaamde "bone morphogenic proteins" (BMPs) en activinen. TGF-β familieleden signaleren via specifieke type I en type II serine/threonine kinase receptoren, die gelegen zijn in de celmembraan. De type I receptoren worden ook wel activin-receptor-like kinases (ALKs) genoemd. Na ligand-binding en onderlinge complexvorming geven deze type I/type II receptorcomplexen op hun beurt, via fosforylering van specifieke targets, de door de liganden geïnitieerde signalen door aan meerdere intracellulaire second messengers, waarvan de Smad-transcriptiefactoren de belangrijkste zijn. De TGF-β eiwitten zelf activeren op deze manier Smad2 en Smad3, maar de BMP eiwitten activeren juist Smad1, Smad5 en Smad8. Na deze activatie kunnen deze Smad eiwitten de celkern binnendringen en daar specifieke, door TGF-β of BMP-reguleerbare genen activeren. Deze door TGF-β of BMP gecontroleerde genen zijn ondermeer betrokken bij remming van cel proliferatie, en bij celmigratie, celdifferentiatie en/of celdood.

De rol van TGF-β in kanker is complex. In de vroegste fasen van de tumor ontwikkeling (tumorigenese) remt TGF-β meestal de proliferatie van kanker (en normale) cellen en fungeert het dus als een tumorsuppressor. Maar in de latere fasen, wanneer de tumorcellen door (verdere) DNA veranderingen ongevoelig geworden zijn voor groeiremming door TGF-β en hoge hoeveelheden van TGF-β gaan maken, werkt TGF-β vaak als een tumorpromoter. Het verhoogd aanwezige TGF-β stimuleert dan de migratie en invasie van de tumorcellen, remt het immuun systeem en aktiveert de vorming van bloedvaten in en rondom de tumor. In geval van fibrose, een overmatige toename in de hoeveelheid bindweefsel die in veel verschillende organen kan plaatsvinden, is er ook een correlatie met verhoogde TGF-β expressie. In dit geval stimuleert TGF-β de vorming

van extracellulaire matrixeiwitten en differentiatie van fibroblasten in myofibroblasten.

De in dit proefschrift beschreven studies hadden als doel de moleculaire mechanismen te ontrafelen waarmee bepaalde leden van de TGF-β familie het gedrag van borstkankercellen beïnvloeden. In het bijzonder is gekeken hoe TGF-β en BMP eiwitten in het lichaam de kwaardaardige uitzaaiing van borstkankercellen naar bot kunnen reguleren. Ten tweede zijn de effecten van een specifieke remmer van TGF-β receptoren op fibrose in de nier bestudeerd. De belangrijkste resultaten die zijn verkregen, zijn hieronder samengevat.

1. De Smad2 en Smad3 transcriptiefactoren blijken door verschillende typen TGF-β-target genen te reguleren een tegengestelde rol te hebben bij TGF-β-gemedieerde uitzaaiing van borstkanker cellen naar bot. Smad3 stimuleert hierdoor de bloedvatvorming rondom de uitzaaiingen, terwijl Smad2 dit juist remt (hoofdstuk 2).

2. Het eiwit HMGA2 is geïdentificeerd als een Smad4-afhankelijk TGF-β-gereguleerd gen in borstkankercellen, en blijkt noodzakelijk te zijn voor de door TGF-β geinduceerde epitheliale naar mesenchymale transitie (EMT). Verhoogde expressie van HMGA2 veroorzaakt hierbij een verlaging van het epitheel-specifieke eiwit E-cadherine, wat belangrijk is voor cel-cel interactie. De E-cadherine verlaging wordt gemedieerd door de transcriptionele remmers Snail1, Slug en Twist (hoofdstuk 3).

3. De remmende werking van bepaalde BMP eiwitten op borstkankerprogressie is nader bestudeerd met behulp van een genetisch geactiveerde BMP receptor. Overexpressie van een actieve BMP type I receptor in de borstkankercellen bleek voldoende te zijn om zowel de uitzaaiing naar bot als ook de tumor-geassocieerde osteolyse te remmen (hoofdstuk 4).

4. De rol van TGF-β in nierfibrose van diabetische muizen met vergevorderde nefropathie is bestudeerd m.b.v. de TGF-β receptor kinase remmer GW788388. Deze remmer bleek zowel fibrose als de expressie van belangrijke mediatoren van fibrose in de nieren te remmen (hoofdstuk 5).

Deze resultaten laten zien dat het verloop van diverse ziekteprocessen in het lichaam beïnvloed kan worden door specifiek ingrijpen in de signaaltransductie routes die gecontroleerd worden door leden van de TGF-β familie. Verder onderzoek naar (meer) specifieke inhibitoren van de diverse componenten van deze routes kan daardoor leiden tot meer en betere therapeutische toepassingen.

7.2 Curriculum Vitae

Maj Petersen

Born 29^{th} November 1977 in Gentofte, Denmark.

Education

- PhD Marie Curie fellowship student, LUMC, Leiden, The Netherlands, 2005-2009
- Civil engineer in Biotechnology, Technical University of Denmark, 2000-2003
- Bachelor in Chemical engineering, Technical University of Denmark, 1996-2000
- Graduated as a European Baccalaureate from the European School of Brussels in 1996

Work experience

- LUMC, Leiden, The Netherlands. Post-doctoral fellow, 2009-
  Department of Urology in the group of Dr. Gabri van der Pluijm. The research is focused on the pathological and molecular mechanisms of TGF-β and BMP signaling pathways in breast cancer bone metastasis in order to identify novel treatment strategies for skeletal metastases.
- LUMC, Leiden, The Netherlands. PhD student, 2005-2009
  Department of Molecular cell biology in the group of Prof. Dr. Peter ten Dijke.
- Institute of Cancer Research, Sutton, UK. Research assistant 2004-2005
  Center for Cancer Therapeutics in the group of Angiogenesis and signal transduction under the supervision of Dr. Margaret Ashcroft. Worked on the identification of novel anti-cancer compounds targeting hypoxia inducible factor 1 in tumor angiogenesis.
- Imperial College of London, London, UK. Research assistant, 2003
  Employed in the group of Prof. Patrick Maxwell in the field of renal cell carcinoma and tumor angiogenesis.
- Leo Pharma, Ballerup, Denmark. Master student, 2002-2003
  Department of Biochemistry under the supervision of Dr. Lone S. Olsen. Identification and development of a functional screening assay for tumor angiogenesis inhibitors.
- NKT Research and Development, Glostrup, Denmark. Bachelor student, 1999
- L'Oréal, Research and Development laboratories, Paris, France. Bachelor student, 1999

Scientific conference proceedings

- Constitutive activation of Activin Receptor-like Kinase 2 in Human Breast Cancer Cells inhibits Metastatic progression and Osteolytic Bone Lesions, M. Petersen, J.T Buijs, E. Pardali, G. van der Horst, H. Cheung, P ten Dijke, and G. van der Pluijm. *The IX international meeting on Cancer Induced Bone Disease*, Virginia, USA. October 2009.

- Role of Smad2 and Smad3 in breast cancer metastasis to bone, M. Petersen, E. Pardali, G. van der Horst, H. Cheung, G. van der Pluijm, and P. ten Dijke. *The VII international meeting on Cancer Induced Bone Disease*, Edinburgh, Scotland. July 2008.

- Oral administration of GW788388, a kinase inhibitor of the TGF-β type I and type II receptors, reduces renal fibrosis in db/db mice, M. Petersen, M. Thorikay, M. Deckers, M. van Dinther, E.T. Grygielko, F. Gellibert, A-C. de Gouville, S. Huet, P. ten Dijke, N. J. Laping. *III Epithelial-Mesenchymal Transition meeting, EMBO workshop*, Krakow, Poland. September 2007.

Objectives

It is my objective to pursue an international career within the field of cancer research and take part in the small advantages that bring big changes to the lives of people touched by cancer.

Private life

In a relationship with Michael Lund Jensen and expecting there first child in the beginning of 2010. Enjoys outdoor sports such as alpine skiing and ski touring in the French Alps, horseback riding, sailing, mountain biking and hiking. Traveling is another great passion of mine.

7.3 List of Publications

- Oral administration of GW788388, an inhibitor of TGF-β type I and II receptor kinases, decreases renal fibrosis. **M Petersen**, M Thorikay, M Deckers, M van Dinther, E T Grygielko, F Gellibert, A-C de Gouville, S Huet, P ten Dijke, N J Laping. *Kidney International* **73**, 705 - 715 (December 2007).

- Transforming growth factor-β employs HMGA2 to elicit epithelialmesenchymal transition. S Thuault, U Valcourt, **M Petersen**, G Manfioletti, C-H Heldin, and A Moustakas. *J. Cell Biol.* **174**, 175 - 183 (July 2006).

- Smad2 and Smad3 have opposing roles in breast cancer bone metastasis by differentially affecting tumor angiogenesis. **M Petersen**, E Pardali, G van der Horst, H Cheung, G van der Pluijm, and P ten Dijke. *Oncogene* (December 2009).

- BMPs in osteotropic cancers. J T Buijs, **M Petersen**, G van der Horst and G van der Pluijm. *Current Pharm. Design* **16**, (2010).

- Constitutive Activation of Activin Receptor-like Kinase 2 in Human Breast Cancer Cells inhibits Metastatic Progression and Osteolytic Bone Lesions. **M Petersen**, J.T. Buijs, E Pardali, G van der Horst, H Cheung, P ten Dijke, and G van der Pluijm. (Manuscript submitted).

7.4 List of Abbreviations

Abbreviation	*Description*
ActRII	Activin type II receptor
ALK	Activin receptor-like kinase
AMH	Anti-müllerian hormone
ANGPTL	Angiopoietin-like
ANG	Angiopoietin
BAMBI	BMP and activin membrane-bound inhibitor
bHLH	basic helix-loop-helix
BLI	Bioluminescent imaging
BMP	Bone morphogenetic protein
BMPRII	BMP type II receptor
BRE	BMP-responsive element
caALK	Constitutively active ALK
Cdc	Cell division cycle
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
COL	Collagen
μ-CT	Micro-computed tomography
CSCs	Cancer stem cells
CTGF	Connective tissue growth factor
CXCL	CXC motif ligand
CXCR	CXC motif receptor
DCIS	Ductal carcinoma *in situ*
DN	Dominant negative
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FGFR	Fibroblast growth factor receptor
FN	Fibronectin
FOP	Fibrodysplasia ossificans progressiva
GADD	Growth arrest and DNA damage
GAPDH	Glyceraldehyde-3'-phosphate dehydrogenase
GDF	Growth/differentiation factor
GFP	Green fluorescent protein
GSC	Goosecoid
HMEC	Human primary mammary epithelial cell
HIF	Hypoxia inducible factor
HLH	Helix-loop-helix
HMG	High mobility group
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
hTERT	Human telomerase reverse transcriptase
HTT	Hemorrhagic telangiectasia
ID	Inhibitor of differentiation/DNA binding
IDC	Invasive ductal carcinoma
IL	Interleukin

Abbreviation	*Description*
INK	Inhibitor of CDK
JNK	Jun N-terminal kinase
LAP	Latency-associated peptide
LEF	Lymphoid enhancer binding factor
LOX	Loxyl oxidase
MAPK	Mitogen activated protein kinase
MDCK	Madin-Darby canine kidney
MET	Mesenchymal-epithelial transition
MH	Mad homology
miR	MicroRNA
miR RNAi	miR RNA interference
MKK	MAPK kinase kinase
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
MSI	Microsatellite instability
MT-MMP	Membrane-type MMP
NF-κB	Nuclear Factor Kappa Beta
NMuMG	Namru murine mammary gland
NSCLC	Non-small cell lung cancer
N-T control	Non-targeting control
OPG	Osteoprotegerin
PAI-1	Plasminogen activator inhibitor-1
PAH	Pulmonary arterial hypertension
PAS	Periodic acid-shiff
PI3K	Phosphatidylinositol 3-kinase
PlGF	Placenta growth factor
PTHrP	Parathyroid hormone related peptide
TAK	TGF-β activated kinase
TIF	Transcriptional intermediary factor
TIMP	Tissue inhibitor of metalloproteinase-1
TGF-β	Transforming growth factor-β
TβRII	TGF-β type II receptor
TSG	Twisted gastrulation
TSP	Thrombospondin
RANK	Receptor activator of nuclear factor-κB
RANKL	Receptor activator of nuclear factor-κB ligand
RCC	Renal cell carcinoma
SARA	Smad anchor for receptor activation
S.D.	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylaminde gel electrophoresis
S.E.M.	Standard error of mean
Ski	Sloan-Kettering virus
SHIP	Src homologue SH2 domain containing 5'inositol phosphatase
shRNA	Short hairpin RNA
SIP	Smad-interacting protein
siRNA	small interference RNA
α-SMA	α-smooth muscle actin
Smad	Small phenotype and mothers against decapentaplegic related protein

Abbreviation	*Description*
I-Smad	Inhibitory Smad
P-Smad	Phosphorylated Smad
R-Smad	Receptor-regulated Smad
Smurf	Smad ubiquitin regulatory factor
SnoN	Ski-related novel protein N
SNO	Spindle-shaped N-cadherin^{+} CD45^{-} osteoblast
SPARC	Secreted protein acidic and rich in cysteine
TFF	Trefoil protein
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	von Hippel Lindau
Wnt	Wingless int
ZEB	Zinc finger E-box binding homeobox
ZO-1	Zonula occludens-1

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