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TGF- β and BMP in breast cancer cell invasion

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Chapter 1

General introduction

1. TGF- β superfamily

The Transforming Growth Factor (TGF)- β superfamily comprises a large collection of structurally related cytokines which regulate a multitude of biological processes. These include TGF- β s, Bone Morphogenetic Proteins (BMPs), Growth Differentiation Factors (GDFs), Activins, Inhibins, Nodal and Anti-Müllerian Hormone (AMH) [1]. Its prototypical member, TGF- β , was discovered as a factor capable of transforming the cellular morphology and promoting growth of normal rat kidney cells in soft agar [2]. Three isoforms of TGF- β exist in humans, namely TGF- β 1, TGF- β 2, TGF- β 3, and they share 60-80 % amino acid homology in their mature forms [3]. Knockout mice of either of these isoforms showed severe defects and died *in utero* or shortly after birth [4-9] (Table 1). These phenotypes emphasize the importance of TGF- β during development. In adult life, dysregulation of TGF- β signaling has been reported in vascular disorders, fibrosis and cancer.

BMPs were initially discovered for their capacity to induce bone formation [10-12]. Subsequent studies showed that they have an important role in patterning during embryonic development [13]. Deletion of specific BMP isoforms in mice can lead to defects in the vascular system, cardiac system, nervous system or the skeleton [14-22] (Table 1), thus highlighting the diversity in function of the different BMPs. However, some BMP knockout mice have only a mild phenotype [23,24] (table 1), suggesting redundancy between BMPs. Indeed, BMP5/7 double knockout mice die during early development, whereas the single mutants are not embryonic lethal [25].

1.1 Smad signaling

TGF- β family members usually act as homodimers, although some may act heterodimers, such as the BMP-2/BMP-7 dimer [1]. These dimers mediate their effects through binding to type I and type II transmembrane receptors, which have a strong Ser/Thr kinase activity and a weaker Tyr-kinases activity [26]. Ligand binding can be regulated by co-receptors, the type III receptors. Upon ligand-induced complex formation, the type II receptor phosphorylates the type I receptor. The type I receptor phosphorylates receptor regulated

Gene	Phenotype	Ref.
<i>Tgfb1</i>	Defective yolk sac vasculogenesis and hematopoiesis. Embryonic lethal (E9.5-11.5)	[4] [6,9]
<i>Tgfb2</i>	Cardiac, lung, craniofacial, limb and urogenital defects. Perinatal lethal	[8]
<i>Tgfb3</i>	Cleft palate, delayed lung maturation. Postnatal lethal	[5,7]
<i>Bmp2</i>	Malformation of the amnion. Cardiac defects. Embryonic lethal (E7.5-10.5)	[22]
<i>Bmp3</i>	Increased bone density. Viable	[15]
<i>Bmp4</i>	Defective gastrulation and mesoderm formation. Embryonic lethal (E7.5-9.5)	[21]
<i>Bmp5</i>	Mild defects in skeletal patterning. Viable and fertile.	[23]
<i>Bmp6</i>	Mild defects in skeletogenesis. Viable and fertile	[24]
<i>Bmp7</i>	Defective kidney and eye development. Defects in skeletal patterning. Perinatal lethal	[16,18]
<i>Bmp10</i>	Cardiac defects. Embryonic lethal (E9.5-10.5)	[14]
<i>Bmp12</i>	Defective neural cell differentiation. Hydrocephalus after birth.	[17]
<i>Bmp13</i>	Defects in joint, ligament and cartilage formation. Viable	[19]
<i>Bmp14</i>	Defects in skeletal limb formation. Viable	[20]

Table 1 Knockout studies of TGF- β s and BMPs in mouse models. *Tgfb1* phenotype depends on genetic background

Smads (R-Smads) at their C-terminal Ser-residues [1,27-32].

Smads consist of two Mad homology (MH) domains, with a linker region in between. In R-Smads, these two MH domains (MH1 and MH2) bind to each other, thus inhibiting each other. Phosphorylation of the R-Smad by the type I receptor releases the MH1 and MH2 domain of the R-Smads from each other. This allows the MH2 domain of the R-Smad to associate with the common-Smad (Co-Smad), Smad4. In addition, the nuclear localization signal of the R-Smad is unmasked, enabling the complex to translocate into the nucleus. In the nucleus the Smad complex can affect transcription together with other transcription factors [1,26] (Figure 1).

TGF- β binds initially to the TGF- β type II receptor (TGF- β RII) on the cell surface. Because of its low affinity, TGF- β 2 also requires binding to the TGF- β RIII β -glycan before binding to the type II receptor [32]. Binding of TGF- β to the type II receptors induces a conformational change in the type II receptor resulting in increased affinity for the type I receptor,

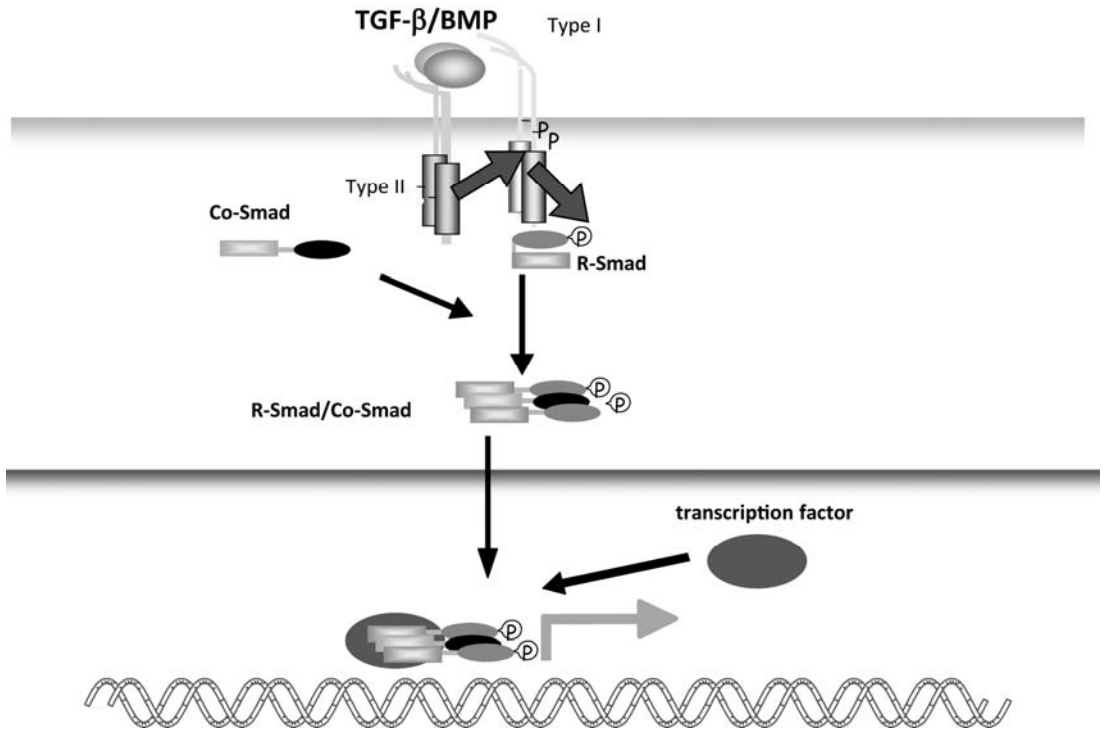


Figure 1 Basic signaling scheme. Upon binding of TGF- β /BMP to the receptors, the type II receptor phosphorylates the type I receptor, which then phosphorylates the R-Smad. This induces a conformational change in these Smads, enabling them to interact with the Co-Smad and these complexes translocate into the nucleus, where they affect transcription, in conjunction with other transcription factors.

ALK5 [33]. ALK5 phosphorylates the R-Smads 2 and 3 (Table 2).

Upon translocation into the nucleus, Smad3 can bind directly to the DNA at GTCT/AGAC sequences through its MH1 domain. Smad2 is not able to bind directly to DNA, because it has additional amino acids in its MH1 domain [1]. A plethora of transcription factors has been shown to interact with Smad2/3, such as CREB binding protein (CBP)/p300, SP1 [39] and c-Jun [40]. Some transcription factors only interact only with Smad3, such as FOXO family members [41] and ATF3 [42].

Formation of the BMP-receptor complexes occurs through simultaneous recruitment of the type I and type II receptor [33]. BMPs use Activin receptor-like kinase (ALK) 1,2, 3 or 6 as type I receptor and BMP type II receptor (BMPRII) and Activin type II receptor (ActRII) A or B as type II receptor. Not all BMPs can bind to all these receptors. For example, BMP-9 can bind to ALK1 and ALK2 [34,35], whereas BMP-7 can bind to ALK2, 3 and 6, but not to ALK1 [36]. An exception is BMP-3, as it signals via ALK4 and ActRII to Smad2, to

<i>Ligand</i>	<i>Type I receptor</i>	<i>Type II receptor</i>	<i>Co-receptor</i>	<i>R-Smad</i>
TGF- β	ALK5 ALK1 ALK2,3	TGF β RII TGF β RII TGF β RII	betaglycan (necessary for TGF- β 2) endoglin	Smad2, 3 Smad1, 5, 8 Smad1, 5
BMP-2	ALK3, 6	BMPRII	RGMa,b, c	Smad1, 5, 8
BMP-3	ALK4	ActRIIB		Smad2
BMP-4	ALK3, 6	BMPRII	RGMa, b, c	Smad1, 5, 8
BMP-6	ALK2, (3) 6	BMPRII, ActRIIA, B		Smad1, 5, 8
BMP-7	ALK2, (3), 6	BMPRII, ActRIIA, B		Smad1, 5, 8
BMP-9	ALK1, 2	BMPRII, ActRIIA	endoglin	Smad1, 5, 8
BMP-10	ALK1			Smad1, 5, 8
BMP-11		ActRIIB		Smad1, 5, 8
BMP-13	ALK6, (3)	BMPRII		Smad1, 5, 8
BMP-14	ALK6,(3)	BMPRII, ActRIIA, B		Smad1, 5, 8
BMP-15	ALK6	BMPRII		Smad1, 5, 8

Table 2 Receptor binding of the ligands. Receptors in brackets indicate that only weak binding to this receptor has been observed

counteract bone differentiation [37,38]. Ligand-receptor binding of BMP-2 and 4 is enhanced by GPI-anchored proteins of the repulsive guidance molecule (RGM) family, such as RGMb/DRAGON and RGMc/hemojuvelin [30]. Activation of ALK1, 2, 3 or 6 by type II receptors results in phosphorylation of the R-Smads 1,5 and 8 (Table 2). Upon translocation to the nucleus, these BMP-R-Smads can bind directly to the DNA at sites containing BMP Response Elements and GC-rich sequences [26]. In addition, BMP-R-Smad complexes also interact with transcription factors, such as Runx2 and Schnurri2 [30].

In certain cell types, TGF- β also induces activation of R-Smads 1 and 5 through the type I receptor ALK1. ALK5 was required for this activation, whereas ALK1 inhibited ALK5-

Smad2/3 signaling [43]. The co-receptor endoglin (CD105) potentiates TGF- β -ALK1 signaling over ALK5 signaling [44]. Recent reports also suggested that TGF- β could signal through the type-I receptor ALK2 and 3 to activate Smad1 and 5 in epithelial cells (Table 2). This process also requires ALK5 and resulted in the formation of so-called "mixed" R-Smad complexes, consisting of BMP-R-Smads and TGF- β -R-Smads [45].

1.2 Non-Smad signaling

Besides Smad activation, TGF- β and BMPs may induce non-Smad signaling, such as MAPK and Rho GTPase signaling. For example, activation of p42/44 MAP kinases by TGF- β occurred through direct phosphorylation of ShcA by ALK5. Phosphorylated ShcA recruits Grb2 and Sos. This complex activates RAS, which activates the Raf-MEK-ERK cascade [46]. Interestingly, recruitment of Shc and Grb2 to the TGF- β type II receptor was enhanced by phosphorylation of Thr284 by Src at the type II receptor, but this resulted p38 activation [47].

TGF- β and BMPs also induce activation of TGF- β activated kinase-1 (TAK-1). TAK-1 is able to activate p38 and JNK pathways in response to TGF- β and BMPs. Activation of TAK-1 by TGF- β occurred by the ubiquitin ligase Tumor Necrosis Factor α Associated Factor (TRAF)-6 in the TGF- β receptor complex, possibly through poly-ubiquitination of TAK-1 [48]. TAK-1 is also activated in response to BMPs, although it is not able to bind BMP receptors by itself. This requires interaction with the X-linked inhibitor of apoptosis (XIAP), which acts as a scaffold [49].

Conflicting data exist about the role of RhoA in TGF- β signaling. Initial studies showed that RhoA is activated by TGF- β [50,51]. However, in the tight junctions of polarized epithelial cells, TGF- β RII phosphorylated Par6, leading to recruitment of the E3 ligase Smurf1 and subsequent ubiquitination and degradation of RhoA [52,52]. Perhaps the localization in the cell determines if the response is either inhibition or stimulation of RhoA activity.

1.3 Regulation of TGF- β and BMP signaling

Because of their important role in development and tissue homeostasis, activity of TGF- β and BMPs are tightly regulated. This regulation occurs at multiple stages of signaling, ranging from regulated secretion to degradation in the nucleus. A large number of these regulatory proteins are induced by TGF- β and BMPs themselves, thus functioning in feedback loops.

1.3.1 Extracellular control of TGF- β /BMP signaling

TGF- β and BMPs are synthesized as large precursor proteins with a long N-terminal propeptide and a shorter C-terminal mature peptide [53]. Disulfide bridges link the two peptides at conserved cysteine knots, with the exception of BMP-15 [26]. Cleavage of the prodomain occurs in the Golgi apparatus by furin proteases. The propeptide remains non-covalently attached to the mature peptide. The TGF- β propeptide is referred to as latency associated peptide (LAP) as it prevents binding of the protein to its receptor. In most cells, the LAP is covalently bound to latent TGF- β binding proteins (LTBPs). This protein links the complex to the extracellular matrix (ECM), especially to the ECM component fibrillin [53]. Release of the complex from the ECM occurs through proteolytic cleavage by elastase or the protease BMP-1 [1,26,53,54]. TGF- β is subsequently released from the LAP by degradation of LAP by matrix metalloproteinases (MMPs) or scavenging of LAP by other proteins such as thrombospondin or integrins $\alpha_v\beta_6$, $\alpha_v\beta_5$, $\alpha_v\beta_8$, [53,55,56] (figure 2A).

The prodomain of all BMPs, except BMP-11, does not interfere with their activity, but may regulate mature protein stability and processing [26]. Regulation of BMPs may already occur at the level of secretion. For example, the BMP antagonist Gremlin binds intracellularly to BMP-4, thereby preventing secretion [57]. Also Sclerostin inhibits BMP-7 secretion by binding to both the prodomain and mature domain of BMP-7 [58] (Figure 2E).

Bio-availability of BMPs is generally regulated extracellularly by natural antagonists, such as Noggin, twisted gastrulation (Tsg), the Chordin protein family and the DAN protein family [59]. These proteins bind to BMPs, thus preventing binding to the receptor (Figure 2B). One antagonist usually targets a subset of BMPs. For example, Noggin binds with high affinity to BMP-2, 4 and 7, but not to other BMPs [60]. Some of the BMP-antagonist complexes are able to form ternary complexes with other inhibitory proteins. For example, the binding of Chordin to BMP4 is enhanced by Tsg [59].

TGF- β and BMPs can also be bound by the decoy receptor BMP and Activin membrane-bound inhibitor (BAMBI). BAMBI resembles the type I receptors, but lacks an active

kinase domain and consequently sequesters ligands from the active receptors [61] (Figure 2C). Another mechanism of modulating ligand activity extracellularly is shedding of the extracellular domain of the receptor. This results in receptors that are unable to bind ligands, whereas the soluble extracellular domain may prevent the ligands from binding to functional receptors by scavenging them. For example, the TGF- β co-receptors endoglin and betaglycan can be cleaved by matrix metalloproteinase (MMP) 14 [62,63]. In addition, cleavage of the extracellular domain of ALK5 by TNF α converting enzyme (TACE) resulted in decreased Smad3 activation [64] (figure 2D).

1.3.2 Regulation at the receptor level

Signaling can be enhanced by accessory proteins which facilitate the recruitment of R-Smads to the receptors. Recruitment of R-Smads 2 and 3 to ALK5 occurs through Smad anchor for receptor activation (SARA) [26,32]. The transmembrane protein Transmembrane prostate androgen-induced RNA (TMEPAI) competed with SARA for interacting with R-Smads, thus sequestering Smads from the activating receptor complex [76]. For BMP signaling, the R-Smads 1, 5 and 8 can be recruited to the type I receptors by endofin [77] or CD44 [78], also resulting in enhanced Smad signaling.

A common mechanism of regulating receptor signaling is internalization and degradation of the receptors. Also the TGF- β receptors can be internalized and degraded, which occurs through caveolin-Smad7 positive vesicles. However, internalization through clathrin coated pits promotes Smad signaling, which are enriched in SARA [79]. Similarly, the

BMPRII can be targeted for degradation through caveolae, whereas endocytosis of BMP receptors via clathrin coated pits promotes the transcriptional activity of BMP-Smads [80]. Recently Smad2 and 3 were shown to be dephosphorylated by MTMR4 in endosomes [81]. If these phosphatase regulate signaling in clathrin coated vesicles or aid the termination of signaling in caveolin positive vesicles remains to be determined.

Another posttranslational modification which modulates protein function is covalent linkage of the small ubiquitin-like protein SUMO. For example, activated ALK5 can be sumoylated by the ligase Ubc9, which results in enhanced recruitment of Smad3 [82]. If other SUMO-ligases function similarly for the other type-I receptors remains to be established.

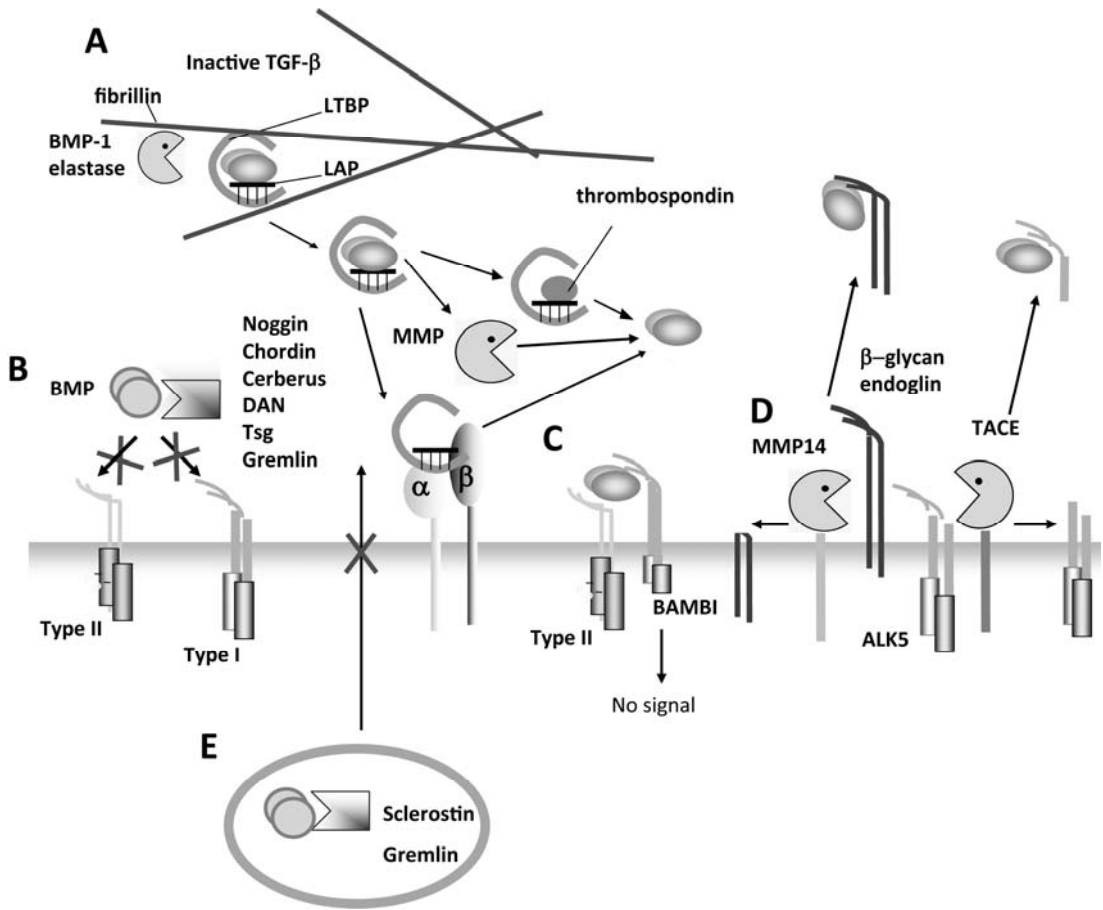


Figure 2 Extracellular regulation of TGF- β superfamily signaling. A. Inactive TGF- β is bound to latency associated peptide (LAP) which is covalently linked to latent TGF- β binding protein (LTBP). This complex is targeted to the extracellular matrix component fibrillin, from which it can be released by BMP-1 and elastase. Further processing of the complex is achieved through MMPs or by scavenging the LAP-LTBP to integrins or thrombospondin. B. Natural antagonists are secreted by cells and bind to BMPs thus preventing receptor ligation. C. The decoy receptor BAMBI binds ligands and forms a receptor complex, but can not transduce signals. D. ALK5 or the co-receptors endoglin and β -glycan can be cleaved by TACE or MMP14, respectively. Shedding of the extracellular domain scavenges ligands from receptors. E. Sclerostin and Gremlin prevent the secretion of BMPs

ALK5 is also known to interact with regulatory subunits of protein phosphatase 2 (PP2) [83,83]. More recently, two different regulatory subunits of PP2, B α and B δ , were shown to have different effects on TGF- β signalling, with B α promoting and B δ inhibiting signalling [84]. However, it remains to be established if PP2 dephosphorylates the receptor. ALK3 can be dephosphorylated by the phosphatase Dullard, which also promotes internalization and degradation of the BMPRII [85].

1.3.3 I-Smads

Inhibitory (I)-Smads, Smad6 and Smad7, are well-known negative regulators of TGF- β superfamily signaling. I-Smads have a similar MH2 domains as the R-Smads, enabling them to interact with the activated type I receptors and thus competing with R-Smads for binding [1]. Smad7 binds both to TGF- β and BMP receptors, whereas Smad6 has a preference for BMP-receptors [26]. In addition, I-Smads are involved in the degradation of TGF- β RI. They interact with the E3 ligases Smurf 1 and 2, resulting in the degradation of the I-Smads and the bound type I receptor [65,66]. Smad7 is also able to recruit the E3 ligases WWP1/Tiul and Nedd4-2/Nedd4L which also resulted in degradation of the type I receptor [67-69]. Furthermore, Smad7 may recruit PP1c, a regulatory subunit of PP1, thus promoting dephosphorylation of ALK5 [70]. A similar mechanism involving Smad7 and PP1c has been implicated in the regulation of ALK1 [71].

Although the I-Smads predominantly act on the type I receptor, they also inhibit R-Smad and Smad4 function. Smad6 competed with Smad4 for binding to Smad1, thus inhibiting propagation of the BMP signal [72]. Smad7 acted as an adaptor protein for ubiquitination of Smad2 and Smad4 by Nedd4-2 and WWP1, thus promoting its degradation [68,69,73].

Furthermore, Smad6 might be a transcriptional repressor of BMP target genes [74]. Smad7 competed with Smad2/Smad4 complexes in the nucleus for binding sites in the promoters of targets genes [75] (Figure 3).

1.3.4 Smad linker phosphorylation

Besides phosphorylation at their C-terminal Ser residues by the type I receptor, R-Smads may also be phosphorylated at different sites. These phosphorylations modulate the stability and nuclear localization of the Smads. For example, Smad2 phosphorylation by calmodulin kinase II at Ser240, prevents its nuclear localization [86]. Protein kinase C phosphorylated Smad3 at Ser37 and Ser70, which interferes with Smad3 DNA binding [87]. Smad1 can be phosphorylated in the linker region between the two MH domains by MAP kinases in response to mitogens [88]. This primes Smad1 for linker phosphorylation by glycogen synthase kinase (GSK) 3 β and promotes its interaction with Smurf1, resulting in

ubiquitination and degradation [89]. GSK3 β also phosphorylates Smad3 at Thr66, which promotes its ubiquitination and subsequent degradation [90]. Thus, the phosphorylation of Smads in the linker region integrates signals from different other signaling pathways, such as the Wnt and mitogen pathways, into TGF- β and BMP signaling pathways.

However, linker phosphorylation of Smad1 or Smad2/3 is also induced by BMPs or TGF- β themselves. Casein kinase (CK) 1 γ phosphorylated Smad3 in a ligand dependent manner and promotes its ubiquitination and degradation [91]. This might be mediated by the ubiquitin ligase ROC1, which was the first ubiquitin ligase identified for Smad3 [92]. The kinases cyclin dependent kinase (CDK) 8 and 9 phosphorylates both BMP and TGF- β R-Smads in the linker region [93]. This creates a docking site for Smurf1 in Smad1, whereas the ubiquitin ligase Nedd4-2 binds to linker phosphorylated Smad2 and 3, resulting in their degradation [93,94]. This process could be enhanced by further linker phosphorylation by GSK3- β [95]. However, the CDK8/9 phosphorylation sites also served as docking site for positive regulators, such as the transcriptional co-activator Yes Associated Protein (YAP) for Smad1 [93].

This mechanism might also explain why MAPK-mediated linker phosphorylation of Smad2/3 seems to have opposing effects. Initial reports suggested that Smad2/3 linker phosphorylation prevents nuclear localization [96,97]. Others, however, reported that Smad2 is stabilized by MAPK phosphorylation, thus promoting transcriptional activity [98]. The amount of YAP versus Smurf1 and Nedd4/2 present in the cell may determine the outcome of linker phosphorylation.

1.3.5 Ski and SnoN

The co-repressors Ski and SnoN regulate TGF- β and BMP signaling in the nucleus. They bind simultaneously to Smad4 and the R-Smad, resulting in dissociation or inactivation of the complex [99,100]. Both Ski and SnoN are able to bind to Smad2/3/4, whereas Ski is also able to bind Smad1 and 5 [101]. Thus, SnoN acts as an inhibitor for TGF- β signaling, whereas Ski also inhibits both TGF- β and BMP signaling.

Another level of regulation of TGF- β signaling is the regulation of these negative

regulators. The ubiquitin ligase Arkadia binds to Smad7 and reduces its levels [102]. More recently, it was discovered that Arkadia also targets Ski and SnoN for degradation [103-105]. This occurs upon ligand stimulation and required phosphorylated Smad2 or Smad3 [103,104]. This mechanism provides a positive feedback loop in TGF- β signaling.

1.3.6 Smad nucleocytoplasmic shuttling

Although the described signaling model implies that Smads only are present in the nucleus upon ligand stimulation, Smad2, 3 and 4 are continuously shuttling between cytoplasm and nucleus. Stimulation with TGF- β increased the nuclear pool of Smads [106] by reducing the rate by which Smads were exported from the nucleus [107]. Mathematical modeling of the kinetics implied that Smad complexes are imported faster than exported and Smads are dephosphorylated in the nucleus [108]. Indeed, Smad1,2 and 3 were dephosphorylated by the nuclear phosphatase protein phosphatase, Mg^{2+}/Mn^{2+} dependent (PPM)1A [109,110]. The faster import of Smad2/3/4 complexes may be mediated by the transcriptional regulator Tafazzin (TAZ), which enhanced Smad nuclear import [111].

Furthermore, posttranslational modifications could alter Smad localization. For example, mono-ubiquitination at Lys519 of Smad4 by ectodermin (Ecto) inhibits Smad2/3 complex formation and promotes Smad4 nuclear export [112], a process which was reversed by the deubiquitinating enzyme FAM/ USP9x [113]. However, Smad4 is also mono-ubiquitinated at Lys507, resulting in enhanced formation of Smad3/4 complexes [114].

Smads are also modified by sumoylation. Smad4 can be sumoylated by protein inhibitor of activated STAT (PIAS)y and ubiquitin conjugating enzyme (Ubc) 9, which results in increased stability and transcriptional activity [115,116]. However, sumoylation of Smad3 by PIASy resulted in decreased transcriptional activity [117], by promoting its nuclear export [118]. On the other hand, the SUMO ligase PIAS3 recruited the co-activator CBP/p300, hereby promoting signaling [119]. Thus, the effect of sumoylation on TGF- β signaling depends on which Smad and ligase are involved.

Recently, poly-ADP-ribose polymerase (PARP) 1 was identified as a mediator of Smad-ADP ribosylation. This modification occurred in Smad2/3/4 complexes at the promoters,

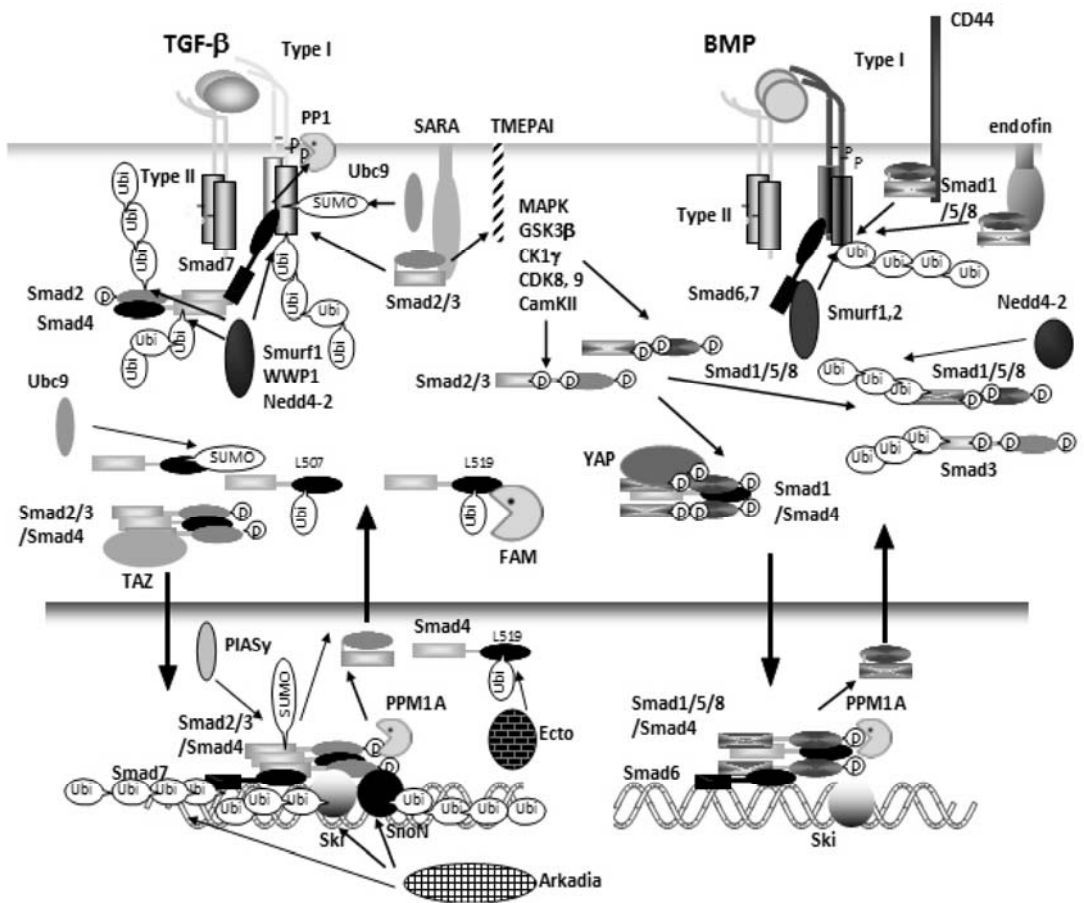


Figure 3 Intracellular regulation of TGF- β and BMP signaling. SARA enhances signaling by presenting Smad2-3 to the type I receptor. TMEPAI competes with SARA for Smad2/3 and inhibits TGF- β signaling. BMP-Smads (1/5/8) can be presented by endofin or CD44. Smad6 competes with Smad1/5/8 for the type I receptor. Smad7 competes with all R-Smads for the type I receptor and recruits ubiquitin ligases, such as Smurf1, 2, WWP and Nedd4-2 to target the receptors, Smad2 and Smad4 for degradation. Smad7 also recruits the phosphatase PP2 to the type I receptor. Sumoylation of the TGF- β type I receptor (ALK5) and Smad4 by Ubc9 enhances signalling. Phosphorylation of Smads at the linker sites may result in ubiquitination and degradation, but may also result in enhanced signalling, for example through association with the transcription factor YAP. Nuclear entrance of Smads is promoted by TAZ. In the nucleus, R-Smads can be dephosphorylated by PPM1A, thus terminating signalling. Also Ski, SnoN, Smad6 and Smad7 may inhibit Smad-dependent transcription, which on their turn are targeted for degradation by the ubiquitin ligase Arkadia. Sumoylation of Smad3 by PIASy promotes nuclear export. Mono-ubiquitination of Smad4 at L519 by Ecto also promotes nuclear export, which can be reversed by the de-ubiquitinating enzyme FAM. Mono-ubiquitination of Smad4 at another residue, L507 promotes nuclear entrance.

resulting in dissociation of the complex and decreased transcriptional responses [120].

Whether a similar mechanism operates in BMP signaling remains to be determined.

1.4 Interplay between TGF- β and BMP signaling

The TGF- β and BMP signaling pathways share several common mediators, such as Smad4, which may result in antagonism between these two pathways. The Smad4 pool may thus be a limiting factor if BMP and TGF- β signaling pathways are activated simultaneously, thus resulting in reduced activation in one of the pathways. Indeed, competition for Smad4 has been observed during *Xenopus* development [121]. Furthermore, TGF- β and BMPs have opposing effects on the transcription of genes, such as Id2 and Id3 [122]. However, studies on digit chondrogenesis in chicken embryos demonstrated positive effects of the TGF- β pathway on the BMP pathway by repression of Smad6 and BAMBI [123]. The effect of TGF- β on BMP signaling depended on timing and duration of the co-stimulation [124], thus emphasizing the importance of the context of the signals.

The interplay between TGF- β and BMP-7 in fibrosis has been extensively studied. TGF- β promotes fibrosis, which can be reversed by BMP-7 [125-128]. This action of BMP-7 occurs through inhibition of TGF- β -induced transdifferentiation of epithelial cells to mesenchymal cells [128]. A similar phenomenon was reported in prostate cancer [129]. Further studies have demonstrated that BMP-7 antagonizes Smad3 signaling by inducing SnoN expression [130].

2. Breast cancer

Breast cancer is one of the leading causes of death in women. Most morbidity and mortality does not arise from the primary tumor, but from distant metastasis, especially bone metastasis which causes severe pain and increased risk of pathologic fractures [131]. Gene expression profiling primarily distinguishes between estrogen receptor (ER) positive and estrogen negative breast tumors, with the latter having worse prognosis [132-134]. ER negative breast cancer cells can be further classified into Erbb2/Her2/Neu positive, basal cell like and normal breast-like subtypes, with the basal cell like breast cancer having the worst prognosis and the normal breast-like the best prognosis [132,133,135,136]. ER negative breast cancer can be further classified into luminal

subtype A, B and C, with luminal C having the worst and luminal A having the best prognosis [132,133,135,136]. Furthermore, the clinical outcome can be predicted based on a 70 gene signature in the primary tumor [134]. This led to the hypothesis that metastatic traits are already acquired during early tumorigenesis [137].

2.1 3-dimensional culture models

Most of the data on cancer cell biology are based on studies of cell monolayers grown on flat and hard plastic. However, this does not recapitulate the 3 dimensional (3D) organization and multicellular complexity of tissues and tumors *in vivo* [138-140]. Several 3D models have been developed over the past decades. Cells can be either completely embedded within in a matrix or placed on top of a matrix or a polymeric scaffold [138,139]. This can be achieved using single cell suspensions or multicellular spheroids. Spheroids are clustered masses of cells, which are formed under non-adhesive conditions [139].

These models have been proven to recapitulate tissues in 3D. For example, non-transformed breast epithelial cells organized themselves into acinar structures when grown in a 3D basement membrane matrix (Matrigel), thus resembling the architecture of a normal breast duct [141]. Transformed cells failed to organize themselves in such a manner [142]. The composition of the matrix provides important cues for this organization, since normal breast epithelial cells could not organize themselves in a collagen type I matrix [143]. Increased expression of this collagen has been associated with metastasis [144] and dense matrixes containing high levels of collagen I promoted tumorigenesis, invasion and metastasis [145]. Increased matrix density resulted in increased signaling through the focal adhesion kinase (FAK) and ERK, thus promoting cell growth [146]. Further crosslinking of collagen by lysyl oxidase also promoted tumor progression through enhanced signaling by adhesion molecules [147].

Furthermore, multicellular spheroids showed the same resistance to cytotoxic drugs as their parental cell line *in vivo*, whereas cells in monolayers failed to do so [148]. In subsequent studies, cells in 3D cultures were more resistant to cytotoxic drugs compared to cells in monolayers [149-153]. A possible mechanism might be that cells are more

resistant to apoptosis, because adhesion to the matrix provides survival signals [149]. Furthermore, expression of drug transporters was higher in 3D cultures [154]. On the other hand, breast cancer cells showed increased sensitivity to the Her2 antibody Trastuzumab in spheroids, because Her2 homodimerization is preferred over Her2/Her3 heterodimerization compared to monolayers [155]. Furthermore, breast cancer cells in 3D were more sensitive to MEK inhibitors than in monolayers [153]. These studies demonstrated the usefulness of 3D models in cancer biology.

2.2 TGF- β in breast cancer

TGF- β has a dual role in carcinogenesis. In the early stages it has a growth inhibitory and pro-apoptotic effect, whereas at the later stages of cancer, TGF- β promotes invasion and metastasis [27,29]. This was elegantly demonstrated using the MCF10A system of cell lines [156]. The MCF10A cell lines originate from MCF10A1 (M-I), a spontaneously immortalized breast cell line [157]. This cell line was transformed with oncogenic RAS, resulting in MCF10AT (M-II), which forms premalignant lesions in mice [158]. From the carcinomas, which arose after xenografting in nude mice, MCF10CA1h (M-III) and MCF10CA1a were isolated (M-IV). M-III was isolated from a low grade carcinoma, while M-IV was isolated from a high grade carcinoma, which metastasized to the lung [159]. Loss of TGF- β signaling caused oncogenic transformation in the premalignant M-II and a more aggressive phenotype in low-grade M-III. In M-IV however, inhibition of TGF- β signaling resulted in decreased metastasis [156]. In line with its tumor suppressor function, loss of TGF- β RII expression in benign hyperplastic lesions correlated with progression to invasive breast carcinoma [160]. Furthermore, inhibition of TGF- β RII expression correlated with tumor grade [161]. In line with its stimulatory role in cancer progression, TGF- β is frequently overexpressed in breast cancer and its expression correlates with poor prognosis and metastasis [162-165]. Moreover, studies in mouse models demonstrated that inhibition of TGF- β -signaling in breast cancer cells reduces metastasis [166-170]. Also gene signatures representing presence or absence of TGF- β stimulation have been used to identify correlations between TGF- β signaling and clinical outcome of breast cancer. In ER negative breast tumors, the TGF- β response gene signature correlated with lung metastasis [171].

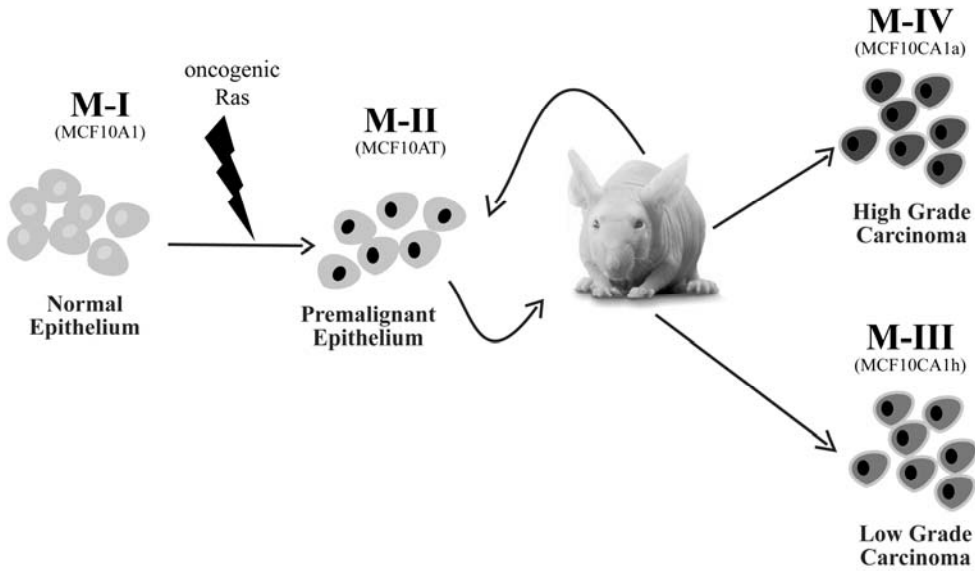


Figure 4 The MCF10A system of cell lines. MCF10A1 (M-I) is a spontaneously immortalized normal epithelial breast cell line. This cell line was transformed with oncogenic RAS to give rise to MCF10AT (M-II). M-II was xenografted several times in nude mice, which ultimately gave rise to tumors. From these tumors, MCF10CA1h (M-III) was isolated, a low grade carcinoma, as well as MCF10CA1a (M-IV), a high grade tumor which metastasized to the lung.

However, in ER positive breast cancer a TGF- β signaling deficient gene signature correlated with poor prognosis, especially in luminal A breast carcinomas [172]. (reviewed in [28]). These findings suggest that the role of TGF- β is context-dependent.

2.3 BMPs in breast cancer

The role of BMPs in breast cancer is not so well characterised. Blockade of BMP signaling by a dominant negative BMPRII stimulates cell growth [173]. Expression analysis has shown that BMP-4, 6 and BMP-7 mRNA are expressed in breast cancer, while BMP-2 mRNA expression is decreased compared to normal tissue [174-176]. BMP-7 expression is reported to be associated with bone metastasis [177]. However, BMP-7 expression has also been inversely correlated with bone metastasis and BMP-7 reduced metastasis in a mouse model [178]. A study on the effects of BMP-7 in different breast cancer cell lines demonstrated differential effects on proliferation and migration, thus indicating that the

effect of BMP-7 might be dependent on the cellular context [179]. In prostate cancer the response to BMP-7 depended on androgen dependency of the prostate cancer cells [180]. Similarly, estrogen dependency might affect the outcome of BMP signaling in breast cancer. This is supported by the notion that expression of ALK6 correlates with high tumor grade and poor prognosis of ER positive breast cancer only [181]. Interestingly, Smad4 has been proposed to be a co-repressor for ER α [182].

BMP-2 has been shown to inhibit breast cancer cell proliferation through induction of p21 [183-185]. Another study demonstrated BMP-2 promoted the expression of the tumor suppressor PTEN [186]. On the other hand, BMP2 induced tumor angiogenesis, invasion and hormone independent growth [187,188]. The effects of BMP-2 on breast cancer cells might be biphasic, since different genes are expressed in response to prolonged exposure, compared to short exposure to BMP-2 [189].

The effect of BMPs on breast cancer cell growth may depend on other growth factors present. For example, BMP-4 enhanced Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF) or Hepatocyte Growth Factor (HGF) induced cell growth at suboptimal concentrations, whereas BMP-4 alone had no effect [190]. In addition, BMP-6 and BMP-7 inhibited estrogen receptor-induced proliferation [191].

Other BMPs implicated in breast cancer include BMP-10 and BMP-15, whose expression correlated with good prognosis [192,193]. BMP-10 negatively affected cell growth and migration in MDA-MB231 cells [193]. How BMP-15 inhibits tumor progression remains to be established.

2.4 Breast cancer metastasis

The process of metastasis can be divided in different steps. First, a cancer cell must shed many of its epithelial characteristics, invade into the surrounding tissue to enter the circulation, subsequently survive in the circulation, then extravasate and colonize a metastatic niche [194].

2.4.1 *Losing epithelial characteristics: epithelial to mesenchymal transition (EMT)*

Epithelial to mesenchymal transition (EMT) is a process in which epithelial cells acquire a more motile, mesenchymal phenotype. This process occurs normally during embryonic development. However, it also occurs in pathologic conditions, such as fibrosis and cancer [195]. Cancer cells with a more mesenchymal phenotype were observed at the invasive edge of the tumor [196]. This led to the hypothesis that the process of EMT is involved in the invasion and escape of the cancer cells from the tumors. The observation that cancer cells which have undergone EMT resembled the cancer stem cells, a few cells which can establish a whole tumor [197], suggests the importance of EMT in the pathology of cancer.

In normal breast tissue, epithelial cells form a tight layer of cells. The cells are polarized and are connected to each other through adherens junctions and desmosomes. Epithelial are also tightly associated with the underlying basement membrane. During EMT these epithelial characteristics are lost, marked by the loss of the adherens junctions proteins E-cadherin and Zona Occludens (ZO)-1. At the same time mesenchymal markers are induced such as α -smooth muscle actin (α -SMA), vimentin and N-cadherin [195,198-200].

The downregulation E-cadherin is the most studied event in EMT. Several transcription factors are able to bind to so-called E-boxes in the E-cadherin promoter, such as Snail [201,202], Slug [203], ZEB1/ δ EF1 [204] and ZEB2/SIP1 [205]. Snail and Slug are zinc finger proteins of the Snail family that recognize E2 box type elements C/A (CAGGTG). Zinc finger E-box binding homeobox (ZEB)1 and ZEB2 are zinc finger proteins that bind to bipartite E-boxes (CACCT and CACCTG) [198]. Furthermore, the basic Helix Loop Helix (bHLH) factor Twist is also able to induce EMT, although it does not directly regulate E-cadherin [200]. The expression of these transcriptional regulators has been associated with poor prognosis and metastasis (reviewed in [198,199]).

These transcription factors also downregulate components of the adherens junctions [206-208] and desmosomes [209,210]. Recent studies have identified target genes that are involved in maintaining the polarity of epithelial cells, such as Crumbs3 and Lethal giant larva (Lgl) [211-213]. Furthermore, these transcription factors regulate microRNAs, small

single non-coding RNAs which bind to complementary sequence in mRNAs and prevent their translation. They induced either a miRNA which targets epithelial genes [214] or repressed a miRNA which targets positive regulators of EMT, such as the transcription factor themselves [215-217].

TGF- β is a potent inducer of EMT, by inducing these transcription factors. This is mediated by the TGF- β -ALK5-Smad pathway [166,218,219]. In addition signaling through the polarity protein Par6 in adherens junctions, also plays a role in TGF- β -induced EMT [52]. Furthermore, activation of MAPK signaling in specialized domains of the cell membrane, lipid rafts, has also been implicated in TGF- β -induced EMT [220].

HMGA2 is a Smad dependent target gene and acts as a master regulator of EMT. It is involved in the upregulation of Snail, Slug and Twist by TGF- β [221]. TGF- β -induced upregulation of ZEB1 and ZEB2 is regulated by Ets transcription factors in a Smad dependent manner [222]. TGF- β not only induces expression of Slug, Snail, ZEB1, ZEB2 and TWIST, but TGF- β -activated Smads may also cooperate with these transcription factors. For example, Snail interacted with the Smad3/4 complex to repress the transcription of E-cadherin [223]. Also the induction of Snail by HMGA2 involved interaction between Smads and HMGA2 [224].

TGF- β also cooperates with other pathways to induce EMT [195]. For, example TGF- β induced components of the Notch signaling pathway, which are essential for TGF- β -induced EMT [225]. In addition, RAS enhanced TGF- β -induced EMT [226]. Further analysis revealed that the downstream Raf-Erk pathway is involved in this process, whereas the PI3K pathway merely protects from TGF- β induced growth arrest [227]. Cooperation of TGF- β and the Raf-ERK pathway resulted in synergistic activation of Snail [228].

During development, BMPs are involved in EMT during neural crest cell induction [195,200]. The role of BMPs in oncogenic EMT has been less well studied. BMP-2 induced an EMT phenotype in colorectal cancer cells in the absence of PI3K signaling [229]. BMP-4 induced EMT in ovarian cancer cells, possibly by activation of RhoA [230]. BMP-2, 4 and to a lesser extent BMP-7 also induced EMT in pancreatic cancer cells, which was enhanced by downregulation of betaglycan [231]. Furthermore, the constitutive active form of ALK3

also induced EMT [230,231].

On the other hand, BMP-7 antagonized TGF- β -induced EMT in kidney cells, normal mammary epithelial cells and prostate cancer cells [123,122]. Rather, BMP-7 induces the reverse process, mesenchymal to epithelial transition (MET) [232]. In the developing kidney, BMP-7 induced MET by inducing p38-ATF-2 and integrin linked kinase (ILK) activity [233,234]. This effect of BMP-7 was biphasic, since low doses stimulated p38 activity, whereas high doses of BMP-7 inhibited p38 activity [233].

BMP-6 has been shown to induce E-cadherin expression by repressing ZEB1 in breast cancer cells [235]. This repression might be the consequence of repression of AP-1 and expression of miR192 [236]. The repression of both ZEB1 and AP1 reduced levels of mir21 [237], which has been implicated in TGF- β -induced EMT [238]. Taken together, BMP-2 and 4 seem to promote EMT, whereas BMP-6 and 7 seem to inhibit EMT.

2.4.2 Invasion

Having lost their epithelial phenotype, cancer cells are able to invade the surrounding tissue, allowing them to reach the vasculature. Several factors are known to induce invasion of breast cancer cells [187,229,241,242], whereas BMP-7 and BMP-10 inhibited this process [Chapter 4, [193]. Cancer cells can either invade as a single cell or collectively as a cohesive mass [243]. Intravital imaging studies revealed that both modes of motility occur in experimental breast tumors, while TGF- β stimulated the single cell motility [244]. These single cells moved along collagen fibers towards blood vessels [245,246]. The process of invasion requires interaction with the extracellular matrix (ECM). Adhesion to the ECM provides the traction necessary for movement, which is mediated by integrins [247]. Integrins are heterodimeric cell surface receptors that recognize specific sequences in the ECM, such as the RGD sequence. At least 24 integrins heterodimers exist by the combination of one of the 18 α -subunits with one of the 8 β -subunits [248-250]. Upon adhering to the ECM, integrins cluster in the plane of the membrane, which induces downstream signaling. Most integrins activate FAK and Src family kinases [247-249]. A subset of integrins, namely $\alpha_5\beta_1$, $\alpha_1\beta_1$, $\alpha_v\beta_3$ and $\alpha_4\beta_6$, recruits the adaptor protein SHC in a Src-dependent manner, which can activate RAS through the GRB2-SOS adaptor proteins [249]. These

signals, in concert with signals from growth factors, regulate migration [247-249] in an invasion-promoting manner.

In breast cancer, expression of integrin $\alpha_6\beta_4$ correlated with increased tumor grade and decreased survival [248], although this integrin mediates firm adhesion to the basement membrane in hemidesmosomes in normal breast tissue [251]. However, phosphorylation of integrin β_4 by EGF-receptor-activated Src kinase Fyn or protein kinase C results in destruction of hemidesmosomes and more invasive behavior of cancer cells [252,253]. This process might be enhanced by TGF- β , which induced clustering of this integrin with the EGF receptor Her2 by activating Src kinase [254]. Furthermore, expression of integrin $\alpha_6\beta_4$ in the β_4 negative breast cancer cell line MD-MB465 promoted its invasive behavior [255]. Integrin $\alpha_6\beta_4$ interacts also with the receptor for HGF, Met, and is required for invasive growth in response to this growth factor [256].

Expression of integrin $\alpha_v\beta_3$ correlated with bone metastasis in breast cancer [257,258]. Overexpression of this integrin enhanced migration [259] and bone metastasis of cancer cells [260,261]. Invasion is further promoted by association and cooperation of integrin $\alpha_v\beta_3$ with growth factor receptors, such as the EGFR or TGF- β RII, which enhanced growth factor signaling [247,250,262]. Integrin $\alpha_v\beta_3$ is a TGF- β target gene [263] and promoted TGF- β signaling through phosphorylation of TGF β -RII by Src [47,264], thus functioning in a feed forward loop. Integrin $\alpha_v\beta_3$ is also able to activate latent TGF- β *in vitro*, but it remains unresolved if this also occurs *in vivo* [56]. Furthermore, integrin $\alpha_v\beta_3$ enhanced invasion through association with MMP2 and MMP9, thus promoting local ECM degradation at the cell surface [265,266].

MMP2 and MMP9 belong to the protein family of matrix metalloproteinases, which cleave the components of the ECM [267,268]. MMPs are secreted as inactive zymogens, which are activated upon cleavage. Activity of the MMPs is controlled by endogenous inhibitors, such as thrombospondin [267]. Both MMP1 and MMP2 expression has been correlated with breast cancer metastasis, whereas MMP9 seems to have a dual role in cancer [268].

MMPs do not only promote tumor progression through degradation of the ECM, but also by processing signaling and adhesion molecules [267,268]. For example, cell-surface-bound MMP9 activates TGF- β , thus promoting invasion through TGF- β signaling [269]. Since TGF- β is able to induce the expression of MMP9 [270-272], TGF- β promotes activation of other latent TGF- β present. Furthermore, TGF- β induced sialoprotein (BSP), which is able to accelerate activation of MMP2, MMP3 and MMP9 [273,274]. BSP is also able to associate with integrin $\alpha_v\beta_3$ and MMP2 and this complex enhanced tumor invasion [275]. Thus, TGF- β may promote invasion through induction of several key players of invasion.

2.4.3 Intravasation

Having reached a vessel, a cancer cell can enter the blood or lymphatic system. Using intravital imaging, it has been observed that cancer cells enter the blood through amoeboid movement through the vessel wall [245]. This process is stimulated by perivascular macrophages by secretion of EGF and Colony Stimulation Factor (CSF) 1 [276]. Proteolytic activity, provided both by MMPs and urokinase plasminogen activator (uPA) is important for the intravasation process [277].

4.4 Survival in the circulation

Once in the blood stream, cancer cells often aggregate with platelets and leukocytes, which protects them from shear forces. This process is mediated through integrins and selectins [249]. For example, integrin $\alpha_v\beta_3$ enhances binding of breast cancer cells to platelets [278]. Furthermore, platelets protect tumor cells from natural killer cells [279]. Protection from lysis by the complement system may be achieved by expression of BSP [274].

2.2.4.5 Extravasation

Extravasation of cancer cells appears in a similar fashion as leukocyte extravasation, although different molecules are involved. First cells adhere loosely to the endothelium in a process called rolling, then the cells firmly adhere to the endothelium and transmigrate [280]. Leukocytes may aid extravasation by acting as a bridge between tumor cells and endothelium, thus promoting adhesion [281]. Tumor cells may also adhere directly to

endothelial cells through integrin $\alpha_v\beta_3$ [282]. Tissues with fenestrated capillaries, such as the bone marrow and the liver, are more readily traversed by tumor cells than tissues with capillaries fully lined with endothelial cells, such as the lung [283].

Expression of genes involved in vascular remodeling, namely epiregulin, cyclo-oxygenase 2, MMP1 and MMP2 enabled extravasation of breast tumor cells into the lung [284]. Induction of angiopoietin-like 4 by TGF- β in the primary tumor enhanced vessel permeability in the lung, thus promoting extravasation in the lung [171]. Similar to intravasation, macrophages enhanced breast cancer extravasation in lung metastasis [285].

Extravasation into the brain is hampered by the blood-brain barrier [283]. Therefore, breast cancer cells that are able to metastasize to the brain not only express the same vascular remodeling genes as cells which metastasize to the lung, but also ST6GALNAC5. This sialyltransferase catalyzes the addition of sialic acid to cell surface molecules, which are important for adhesion to brain endothelial cells and their passage through the blood brain barrier [286].

2.4.6 Colonization

Most breast cancer that infiltrate into distant organs die due to restrictions in the host microenvironment [283]. However, other organs provide an environment in which the disseminated breast cancer cells may survive. Already in 1889, Paget discovered that breast cancer cells are osteotropic, leading to the "seed and soil hypothesis"; the cancer cell ("the seed") can only grow in a suitable environment ("the soil") [287]. In the bone environment, breast cancer cells usually induce destructive osteolytic lesions. Less common are osteoblastic lesions, in which bone is synthesized [131]. Noggin seems to play an important role in determining which type of lesions occurs [288]. Other "soils" include the lungs and brain. The focus of this section will be on bone metastasis.

Once extravasated, breast cancer cells do not immediately colonize the bone [289]. Instead, the single cells or cells micrometastatic clusters reside in the bone marrow, either being dormant or proliferating at a slow rate [283]. Survival of breast cancer cells may be mediated by Src, which can be induced by several growth factors present in the bone marrow [290].

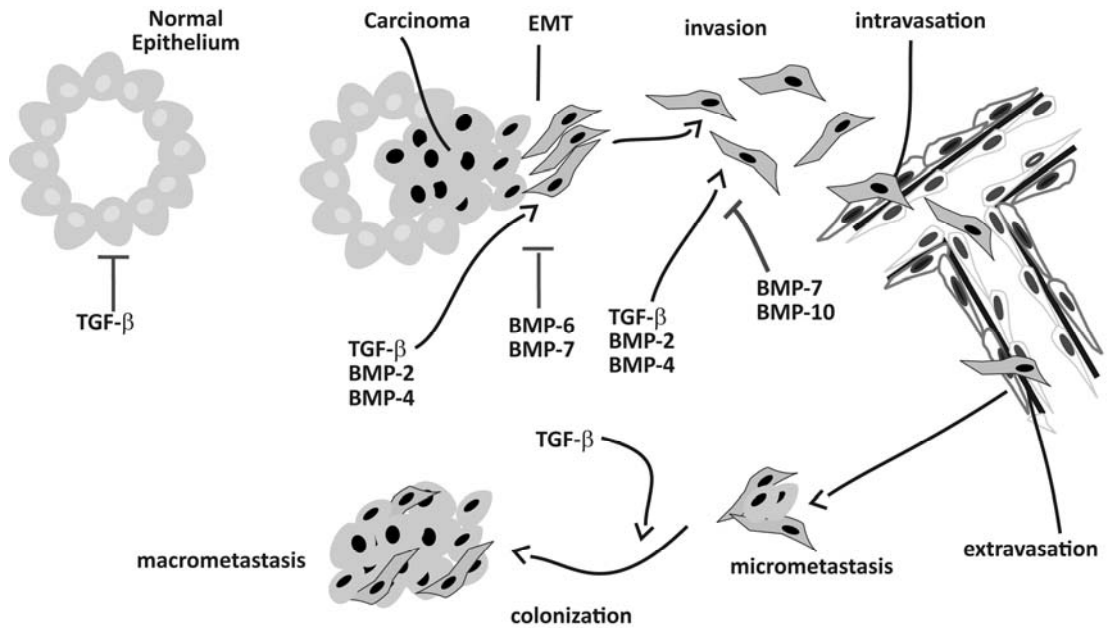


Figure 5 Role of TGF- β and BMPs in the metastatic cascade. In normal epithelium TGF- β inhibits cell growth. Upon malignant transformation, TGF- β , as well as BMP-2 and BMP-4 promote epithelial to mesenchymal transformation (EMT) resulting in more motile mesenchymal cells. BMP-6 and BMP-7 counteract this process. Invasion of these cells is promoted by TGF- β , BMP-2 and BMP-4, whereas BMP-7 and BMP-10 inhibit it. Cancer cells then intravasate, survive in the circulation and extravasate, where they establish micrometastasis. TGF- β promotes further colonization, which allows the cancer cells to grow into a micrometastasis.

The presence of bone marrow micrometastasis correlated with metastasis [291], indicating that the cells in the micrometastasis eventually acquire the ability to colonize or manipulate the environment to enable colonization. TGF- β is one of the growth factors highly present in the bone environment and active TGF- β signaling has been detected in breast cancer metastasis [292,293]. However, abrogation of TGF- β signaling in macrometastasis had no effect, indicating the importance of TGF- β signaling in early stages [292]. In early stages of tumor growth, angiogenesis is crucial for a metastasis to grow. TGF- β induced expression of VEGF, a critical mediator of angiogenesis, in a Smad3 dependent manner. Indeed, breast cancer cells with Smad3 knockdown grew slower due to reduced angiogenesis [169]. The parathyroid hormone-related protein (PTHrP) is involved in bone metastasis formation by activating the bone-resorbing cells, osteoclasts [131]. Ectopic

expression of PTHrP reverted the reduction in osteolytic lesions observed in metastasis of cells expressing a dominant negative TGF- β RII, thus indicating an important role for this protein in TGF- β -induced bone metastasis formation [170]. Furthermore, TGF- β is able to induce a set of genes which have been identified as drivers of breast cancer metastasis, namely IL-11, CTGF, CXCR4 and MMP1 [294]. These genes were found to be expressed in breast cancer cells which preferentially metastasize to the bone and overexpression of these genes cooperatively promoted bone metastasis [295]. TGF- β also induced expression of cyclo-oxygenase 2 (COX2), which enhanced breast cancer bone metastasis [296].

All of the above mentioned studies used MDA-MB231 cells, which form osteolytic lesions. In osteoblastic breast cancer lesions, endothelin-1 is an important mediator of bone formation, which can also be induced by TGF- β [294,297]. Since BMPs are also potent inducers of bone formation, one would expect a role for them in osteoblastic metastasis. Indeed, in prostate cancer BMP-6 promotes osteoblastic metastasis [298]. However, expression of BMP-2 in MDA-MB231 cells still gives rise to osteolytic lesions [299]. Thus, different BMPs might have different roles in bone metastasis.

3. Outline of the thesis

A large number of studies has been performed to understand the biology of TGF- β and BMPs. These have highlighted the dual role of TGF- β in cancer cells; in early stages it hampers proliferation and promotes apoptosis, whereas in later stages it promotes invasion and metastasis. However, TGF- β also acts on the environment of the tumor, the tumor stroma. This will be discussed in Chapter 2.

To investigate TGF- β -induced invasion of breast cancer cells experimentally, we established a 3D spheroid invasion model, which is introduced in Chapter 3. Using this model, we characterized the role of Smads and MMPs in breast cancer cell invasion, which is described in Chapter 4.

BMP-7 has been shown to inhibit bone metastasis of breast cancer, possibly by antagonizing TGF- β -induced EMT. In Chapter 5, we exploited our spheroid system to study the crosstalk between TGF- β and BMP-7 in invasion. We also characterized the genes which play a crucial role in this crosstalk.

EMT has been proposed to be a crucial step before invasion. In Chapter 6, we analyzed the role of the key mediators of EMT in invasion of breast cancer cells.

Compared to TGF- β -signaling, BMP-signaling has been less well studied. Therefore, we sought to identify kinases involved in BMP-signaling. The results are described in Chapter 7.

Finally, the conclusions of the results described in this thesis are summarized and discussed in Chapter 8.

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Chapter 2

Role of TGF- β in the tumor stroma

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Abstract

Recent findings have demonstrated that the tumor stroma actively contributes to tumorigenesis. The communication of malignant cells and tumor stromal components is orchestrated in part by a network of growth factors. One of these growth factors is transforming growth factor- β (TGF- β), a secreted multifunctional protein that acts in a highly cellular contextual manner. TGF- β can either stimulate or inhibit the tumor-promoting effects of the different components of the tumor stroma. In this review, we discuss our current understanding on how TGF- β influences different stromal compartments.

Introduction

For many years, cancer research focused on the genetic and phenotypic changes in malignant cells in order to understand progression of tumor growth and metastasis. However, it is evident today that the tumor stroma is more than an innocent bystander. The involvement of stroma may already occur as early as during transformation of the epithelial cells. This was demonstrated in animal studies in which either irradiation or treatment with carcinogenic chemicals of stromal cells, but not of epithelial cells, is sufficient to give rise to tumors from normal epithelial cells [1,2]. Moreover, genetic studies in breast tumors revealed that mutations also occur in the stromal compartment [3-5].

The stroma in the vicinity of the tumor consists of various cell types such as fibroblasts, immune cells, cells lining the blood and the lymphatic vessels, which are embedded in extracellular matrix (ECM) and produce soluble factors. The relative size and composition of the tumor stroma differs between tumors, and is distinct from normal tissue stroma. Malignant cells produce growth factors which can induce a 'reactive' stroma that will favor tumor cell proliferation, migration and invasion and angiogenesis [6]. This 'reactive stroma' has also been described as desmoplasia [7]. Tumor stroma resembles the stroma usually found in injured tissues. However, wound 'reactive' stroma is resolved after the tissue is repaired, whereas tumor stroma is not. Therefore, tumors have been described as 'wounds that do not heal' [8]. TGF- β is an important regulator of wound healing [9].

TGF- β is well-known for its dual role in malignant cancer epithelial cells: in early stages it blocks tumor growth, whereas in later stages it stimulates invasion and metastasis of tumor progression (reviewed in [10]). In this review, we will focus on the effects of TGF- β on the different components of the tumor stroma.

Cancer Associated Fibroblasts

Fibroblasts are the predominant cells in tumor stroma of almost all primary carcinomas [11], suggesting that fibroblasts may support or induce tumor progression. Moreover, progression to invasive carcinoma is accompanied by the differentiation of fibroblast to myofibroblasts or so-called cancer-associated fibroblasts (CAF) [6,12]. CAFs are an important source of growth factors, such as insulin growth factor (IGF), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), hepatocyte growth factor (HGF) and TGF- β [13-16], as well as matrix metalloproteases (MMPs) [17] and ECM components [18].

The origin of CAFs still remains controversial. CAFs can be derived *in situ* by transdifferentiation of cells of the connective tissue surrounding the carcinoma. Immunohistochemical analysis of primary tumors revealed that fibroblasts are the main source of myofibroblasts in breast cancer [19]. Interestingly, it has been shown that TGF- β can transdifferentiate fibroblasts into myofibroblasts *in vitro* [20,21]. However, TGF- β may not be the essential factor in CAF differentiation, since TGF- β receptor type II (T β RII) knockout fibroblasts are still able to promote tumorigenesis (see below).

There is also evidence that malignant cells attract mesenchymal stem cells (MSC) to differentiate into CAFs. Studies exploring the potential of MSC to deliver anticancer agents showed that these cells are incorporated in tumors [22-26]. Co-transplantation of MSCs with breast tumor cells enhances metastasis to the lung [27]. Moreover, transplantation of eGFP+ hematopoietic stem cells demonstrated that stem cells can give rise to fibroblasts as well as perivascular cells [28]. TGF- β can induce fibroblast migration [29], but whether TGF- β is involved in MSC migration and their transformation into CAFs remains to be determined.

Other studies have suggested that epithelial cells can transdifferentiate into myofibroblasts via TGF- β dependent epithelial mesenchymal transition (EMT) [30,31]. EMT occurs normally during embryonic development, but has also been observed in the progression of some tumors. Thus, malignant epithelial cells could transdifferentiate into fibroblasts themselves. The observation that stromal and breast cancer cells share genetic alterations supports the idea that tumor cells undergo EMT and differentiate to CAFs [5]. Moreover, a fibroblast cell line established from breast tumor stroma carried the same X-chromosome inactivation pattern as the malignant epithelial cells and expressed certain epithelial markers, suggesting that these fibroblasts are of epithelial origin [32]. A process similar to EMT is the so-called endothelial to mesenchymal transition. Recent evidence suggests that endothelial cells can give rise to CAFs through this process, which can be induced by TGF- β *in vitro* [33].

TGF- β signaling

TGF- β family members are multifunctional cytokines with pivotal roles in several cellular functions including cell growth, differentiation, extracellular matrix production, motility and regulation of the immune system. Three TGF- β isoforms (1, 2 and 3) have been identified [34]. TGF- β is initially secreted as a latent complex that is activated by components of the extracellular matrix. Activated TGF- β can bind to its cell surface receptors. There are three types of transmembrane TGF- β receptors, namely type I, II and III (T β RI, T β RII and T β RIII). T β RI and T β RII are serine/threonine kinase receptors, whereas T β RIII are accessory receptors with short intracellular domain that lacks catalytic activity. Mature TGF- β 1 and 3 bind directly to T β RII, whereas TGF- β 2 binds to T β RII only in the presence of betaglycan, a T β RIII. Once TGF- β binds to T β RII, the T β RI receptor is recruited into the complex and is phosphorylated by T β RII on serine and threonine residues in a glycine-serine rich juxtamembrane intracellular domain [35]. T β RI, in turn, phosphorylates specific members of the Smad family, i.e. the receptor-regulated (R)-Smads Smad2 and Smad3 at two carboxy terminal serine residues. Receptor phosphorylated Smad2 and 3

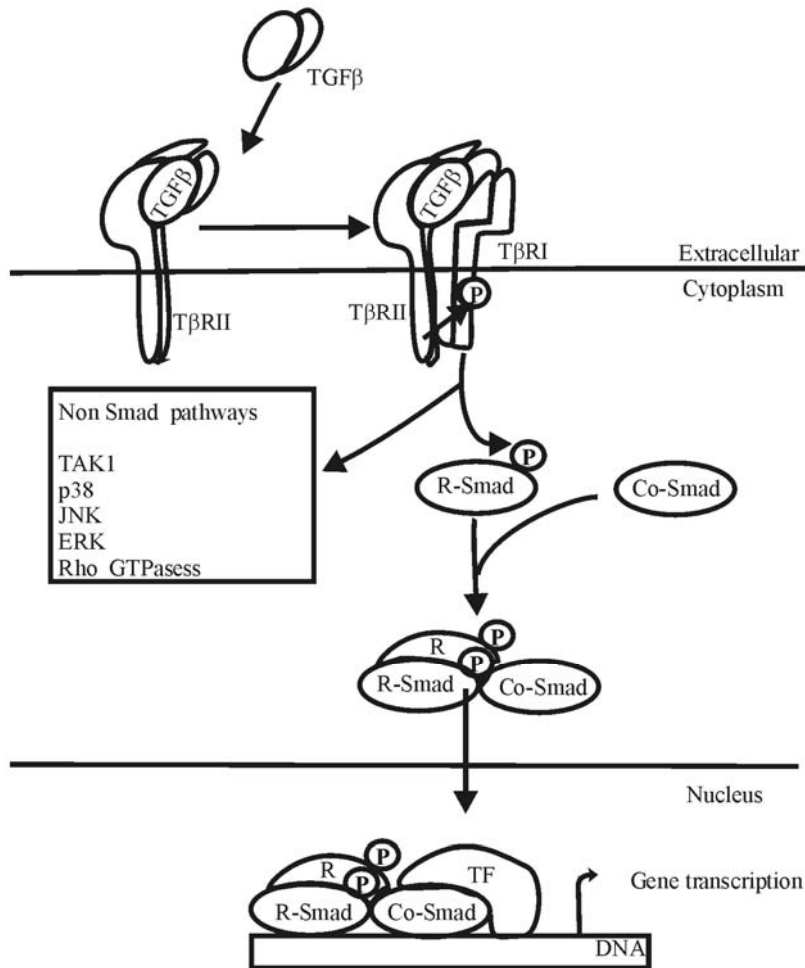


Figure 1. Overview of the TGF-β signaling. TGF-β homodimer binds to TβRII receptor resulting in recruitment and phosphorylation of TβRI receptor. Subsequently, TβRI phosphorylates receptor regulated Smads (R-Smads), which then can associate with the common Smad (Co-Smad) in a heterotrimeric complex. This complex translocates to the nucleus, where it affects gene transcription in cooperation with other transcription factors (TF). In addition to the Smad-dependent pathways, non-Smad pathways can be activated by TGF-β.

form heterotrimeric complexes with the common mediator Smad (Co-Smad) Smad4.

These complexes then translocate to the nucleus, where they regulate transcription of TGF-β target genes in collaboration with other transcription factors (TF) (Figure 1) [34].

In most cells types activin receptor-like kinase 5 (ALK5) is the predominant TβRI receptor. In certain cell types TGF-β can signal via another TβRI. In endothelial cells besides

ALK5, ALK1 can also be activated in response to TGF- β . ALK5 signaling results in the activation of Smad2 and Smad3 and ALK1 activation results induces Smad1, Smad5 and Smad8 phosphorylation [36,37] (reviewed in [38]).

In addition to the previously described TGF- β Smad pathway, TGF- β receptor activation results in activation of several other non-Smad signaling pathways. Non-Smad signaling pathways can involve TGF- β -activated kinase-1 (TAK-1), extracellular regulated kinase (ERK), Jun kinase (JNK), p38 and Rho GTPases, which can cross-talk with the Smad pathways [34,39].

Role of TGF- β in tumor stroma interactions

Studies on the role of TGF- β signaling in stromal-epithelial interactions in mammary gland development suggested that stromal fibroblasts can regulate epithelial cell morphogenesis and development. Specific overexpression of a dominant negative TGF- β type II receptor in mouse mammary stromal fibroblasts resulted in an increase in lateral branching of the ductal epithelium that was correlated with increased HGF mRNA expression [40]. In tumors, TGF- β is also important for the communication between epithelial malignant cells and stromal cells. For instance, inhibition of TGF- β signaling by a soluble extracellular domain of T β RII, a latency-associated peptide (LAP) or TGF- β 1 neutralizing antibody resulted in inhibition of tumor growth and angiogenesis in a reactive stroma xenograft model [41]. Moreover, transplantation of TGF- β 1 overexpressing fibroblasts with normal human epithelial cells gave rise to breast tumors [42].

Effects of TGF- β on CAFs

One of the first studies on the role of TGF- β in CAFs was done in a tissue specific T β RII knockout mouse model using the fibroblast specific protein-1 (FSP1, S100A4) promoter to drive expression of Cre recombinase *in vivo* [43]. The endogenous FSP1 promoter is active in a subset of the stromal fibroblasts that reside in tissues including prostate, forestomach, and skin. FSP1-Cre inducible T β RII knock out mice developed squamous cell carcinoma of the forestomach and prostatic intraepithelial neoplasia, accompanied by an

increased abundance of fibroblasts. Disruption of TGF- β signaling appears to result in tissue-specific fibroblast activation, with increased proliferation and upregulation of growth factor expression such as HGF, with parallel increased levels of phosphorylated c-Met, the HGF receptor [43].

In order to investigate the role of TGF- β signaling in the reactive stroma in breast cancer Cheng and colleagues used the FSP1 Cre lox system to conditionally delete the T β RII in mouse mammary fibroblasts [44]. Co-implantation of mouse mammary carcinoma cells with T β RII-FSP knockout fibroblasts under the renal capsule resulted in increased tumor growth and invasion compared to tumors derived from implantation of the mammary carcinoma cells with T β RII-FSP wild type cells. Moreover, the T β RII-FSP knockout tumors displayed increased tumor cell survival, proliferation, and tumor angiogenesis. This phenotype was correlated with increased expression of macrophage-stimulating protein (MSP), HGF and TGF- α [44]. The effects of T β RII downregulation on tumor progression could be blocked with a c-Met pharmacologic inhibitor (EXEL-7592) and with siRNA targeting c-Met [45].

The above mentioned studies clearly indicate a suppressive role for TGF- β in the tumor-promoting activities of CAFs. However, there are studies suggesting that TGF- β promotes the tumor-supportive effects of CAFs. Verona et al examined the role of TGF- β in a xenograft co-transplantation model of fibroblasts and prostate tumor cells [46]. In their study, tumor latency is prolonged when fibroblasts expressing dominant negative T β RII are cotransplanted, suggesting that intact TGF- β signaling is needed for tumor progression. Micro-array analysis revealed that TGF- β 3 induced expression of genes involved in ECM remodeling as well as growth factors and factors typical for myofibroblasts [46]. In a similar prostate tumor mouse model, it was found that co-transplantation of T β RII knockout or dominant negative Smad3 CAFs resulted in decreased tumor growth and microvessel density. This was accompanied by a decrease in stromal fibroblast growth factor 2 (FGF-2) staining. Re-expression of FGF-2 in the CAFs rescued the effect of impaired TGF- β signaling [47]. The discrepancy between these results and the results discussed before can be explained by the different models used in

these studies, i.e. syngeneic and spontaneous tumor models versus human xenograft models. Moreover, the level of downregulation of TGF- β pathway in the different models might be different. For example, overexpression of dominant negative T β RII or Smad3 might not result in complete abrogation of TGF- β signaling or the non-Smad signaling pathways might be perturbed. Further investigation is needed to elucidate the mechanisms behind this dual effect of TGF- β .

TGF- β and the local road: the ECM

One of the main characteristics of reactive stroma is remodeling and changes in the composition of ECM. Cancer cells and CAFs secrete ECM proteins, such as collagen, fibronectin, as well as ECM-degrading enzymes, such as MMPs. ECM remodeling results in changes in the microenvironment that facilitate local proliferation, invasion and metastasis of cancer cells. Collagen fibers serve as tracts along which cancer cells can migrate [48]. In addition, the ECM network accommodates transport of nutrients and waste products [49].

Several studies have shown an important role of TGF- β in ECM synthesis. TGF- β induces expression of collagens, fibronectin, tenascin and basement membrane components such as laminin. Moreover, TGF- β regulates also expression of MMPs, and induces expression of protease inhibitors such as tissue inhibitors of metalloproteases (TIMPs) and plasminogen activator inhibitor-1 (PAI-1) [50-52]. Changes in the ECM are sensed by integrins. This allows the cells to organize the cytoskeleton and activate intracellular signaling pathways. Cancer cells tend to express integrins that activate signaling pathways that promote proliferation, survival and migration [53]. TGF- β has been shown to induce expression of the latter integrins, thus contributing to invasion [54,55].

TGF- β and tumor angiogenesis

Tumor growth over a certain size requires formation of new blood vessels, to provide nutrients and oxygen and remove waste and toxins, a process referred to as tumor

angiogenesis. Several *in vitro* and *in vivo* studies have revealed the importance of TGF- β signaling in physiological and tumor angiogenesis, since TGF- β regulates both endothelial cells and the surrounding vascular smooth muscle cells and pericytes in a context-dependent manner (reviewed in [56]). Several studies have provided evidence for the role of TGF- β on tumor angiogenesis. High TGF- β mRNA expression in breast tumors or high levels of circulating TGF- β in patients with carcinoma correlate with increased angiogenesis and poor patient prognosis [57]. Prostate carcinoma cells overexpressing TGF- β showed enhanced tumor angiogenesis in tumor xenografts, while local administration of neutralizing antibodies to TGF- β 1 strongly reduced tumor angiogenesis. Moreover, intraperitoneal injection of TGF- β 1 antibodies reduced angiogenesis and tumorigenicity of a renal carcinoma cell line *in vivo* [58].

In endothelial cells, TGF- β can signal through both ALK5 and ALK1 pathways, which have opposing effects on angiogenesis. Signaling through ALK1 stimulates endothelial cell proliferation and migration, whereas signaling through ALK5 inhibits these processes [36]. The balance between these two pathways is skewed towards an ALK1 response by endoglin, another T RIII [59]. TGF- β plays also important role in the differentiation of MSC into vascular smooth muscle cells and pericytes [56].

Besides the direct effects of TGF- β on the vasculature, TGF- β can indirectly induce tumor angiogenesis by inducing production of angiogenic factors such as VEGF, FGF, connective tissue growth factor (CTGF) and platelet derived growth factor (PDGF) from different cell types in the tumor microenvironment [60,61]. In addition, TGF- β may regulate angiogenesis through induction of pro-angiogenic proteases such as urokinase Plasminogen Activator (uPA) [62] and MMPs [50,63,64]. Degradation of ECM components by MMPs may release certain angiogenic growth factors and specific cleavage of angiogenic growth factors may activate latent growth factors [65].

TGF- β and the immune system

Another important component of reactive stroma is the immune system. Infiltrations of cells of both the innate and the adaptive immune system have been found to affect both tumor initiation and progression [66]. TGF- β plays an important role in regulation of immune responses [67]. Numerous studies have demonstrated the importance of tumor associated macrophages (TAM) in tumor progression [68,69]. TAMs are an important source of pro-angiogenic factors [70-72] and have been implicated in tumor invasion [73]. TGF- β stimulates chemotaxis of monocytes, the precursors of macrophages, in head-and-neck squamous-cell carcinomas [74]. The phenotype of TAMs is similar to that of a subset of macrophages, which are poor stimulators of anti-tumor immunity. Differentiation of monocytes towards this phenotype is possibly regulated by TGF- β [75].

Other innate immune cells that have been found in tumors are neutrophils and mast cells. Depletion of mast cells ablated tumor progression in a mouse model of skin tumors. Mast cells may contribute to progression by secretion of pro-angiogenic proteases [76]. Neutrophils can promote tumor destruction, but also tumor growth by release of VEGF [77] and MMP9 [78]. As has been observed during wound healing, TGF- β might recruit mast cells and neutrophils in tumors [67].

Tumor infiltrating T cells have a T-helper cell 2 (Th2) phenotype which skews the immune response towards activation of B cells [66]. Although the antibodies produced by B cells can have anti-tumorigenic activity, there is also data indicating that B cells play a role in the recruitment of innate immune cells to tumors [79]. Kim and colleagues showed that T cell specific deletion of Smad4 in mice, results in aberrant expansion of the gastrointestinal stromal compartment, increased infiltration of antibody-producing B cells and T cell maturation towards the Th2 phenotype [80]. Interestingly, T cell specific deletion of Smad4 resulted in spontaneous development of gastrointestinal carcinomas and oral squamous cell carcinomas. These tumors are similar to tumors observed in Familial Juvenile Polyposis (FJP), in which germline mutations in Smad4 are frequently found [80]. Additionally, disruption of TGF- β signaling in T cells by transgenic expression of a dominant negative T β RII, has been shown to increase azoxymethane-induced colon carcinogenesis [81]. The above

studies demonstrate that deregulation of TGF- β signaling in the immune cells can initiate and promote carcinomas *in vivo*, independently of epithelial cell defects.

Concluding remarks

Recent research has aroused the view that cancer cells do not solely direct cancer progression. Instead, the tumor stroma actively regulates tumor growth, invasion and metastasis. One of the critical factors of tumor progression is TGF- β . TGF- β plays an important role in regulating the balance between cancer cells and tumor microenvironment (summarized in Figure 2).

Several therapeutic strategies targeting TGF- β signaling have been developed for treatment of cancer patients (reviewed in [9]). However, TGF- β can have tumor-suppressive and tumor-promoting effects on cancer cells and stimulate as well as inhibit the tumor-supporting role of the stromal cells. Thus, it is important to specifically tackle the pro-tumorigenic action of TGF- β , while keeping the tumor-suppressive actions of TGF- β intact. This could be achieved by specifically targeting certain cells in the stroma.

A recent study using low doses of a T β RI kinase inhibitor shows promising results. Low doses only affected pericytes in the vasculature, thereby enhancing the delivery of nanodrugs [82]. Moreover, endoglin, a T β RIII, is strongly upregulated in the tumor-associated angiogenic vasculature compared to nonmalignant tissue. Systemic administration of endoglin antibodies conjugated with immunotoxins and immunoradioisotopes efficiently suppressed tumor growth in murine models of breast and colon carcinoma [83]. Thus inhibition of the endoglin pathway may be another target for therapeutic strategies.

TAM is another stromal cell type in which inhibition of TGF- β signaling may result in inhibition of tumor growth. Further studies for the elucidation of the role of TGF- β in tumor microenvironment will give more insights in the complex tumor-stroma interactions and may reveal new potential targets for therapeutic strategies.

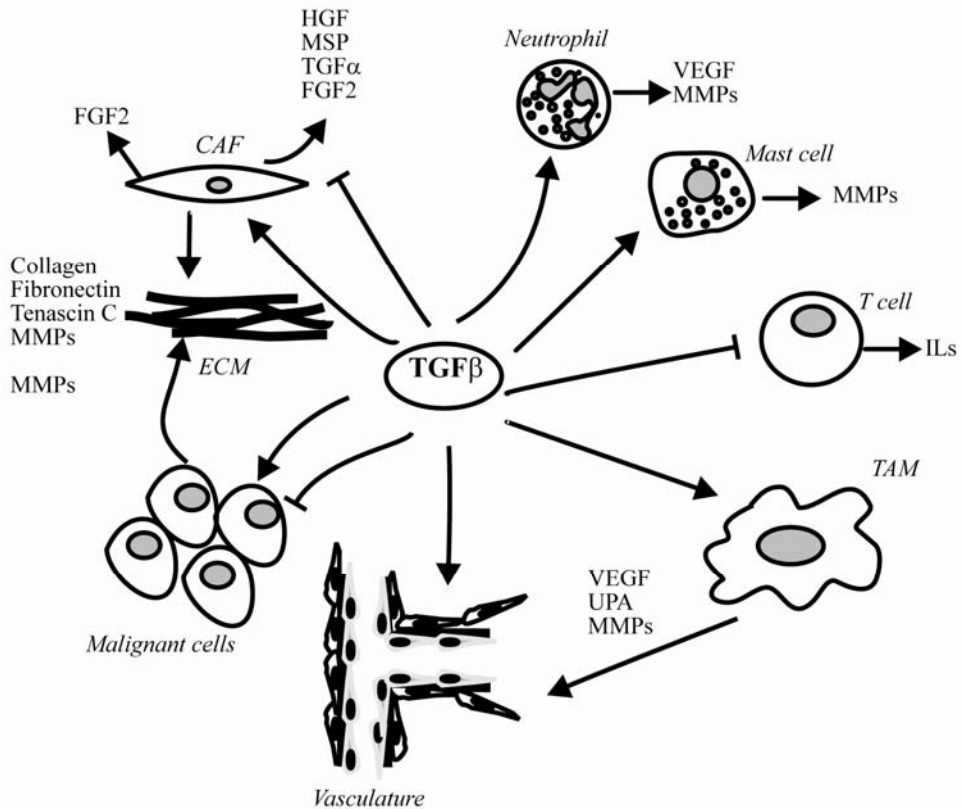


Figure 2. Effect of TGF- β on the different tumor compartments. TGF- β plays an integral role in cancer by regulating tumour initiation, progression and metastasis. TGF- β can act both as a tumor suppressor or a tumor promoter depending on the cellular context. The role of TGF- β in cancer is not limited to autonomous signaling of the malignant epithelial cell, but also on the tumor stroma. TGF- β contributes to generation of CAFs, ECM remodeling and expression of growth factors, from various cells in the tumor microenvironment, that can affect tumor growth and tumor angiogenesis. TGF- β can also recruit and regulate immune cell function in the tumor stroma.

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Chapter 3

Spheroid assay to measure TGF- β -induced invasion

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Abstract

An assay to quantitatively measure Transforming Growth Factor (TGF)- β -induced invasion in 3-dimensional collagen gels is described. This assay takes advantage of the MCF10A series of cell lines, which represent different stages of breast cancer development. This method can be adopted to be used with other cell lines and might be used to investigate other potential activators or inhibitors of invasion.

Introduction

TGF- β has opposing roles in breast cancer progression by acting as a tumor suppressor in the initial phase, but stimulating invasion and metastasis at later stage [1,2]. Moreover, TGF- β is frequently overexpressed in breast cancer and its expression correlates with poor prognosis and metastasis [3,4]. The mechanisms by which TGF- β induces invasion are not well understood.

TGF- β elicits its cellular responses via TGF- β type II (T β RII) and type I (T β RI) receptors. Upon TGF- β -induced heteromeric complex formation, T β RII phosphorylates the T β RI. The activated T β RI initiates its intracellular canonical signaling pathway by phosphorylating receptor Smads (R-Smads), i.e. Smad2 and Smad3. These activated R-Smads form heteromeric complexes with Smad4, which accumulate in the nucleus and regulate the transcription of target genes [5]. In addition to the previously described Smad pathway, receptor activation results in activation of several other non-Smad signaling pathways, for example Mitogen Activated Protein Kinase (MAPK) pathways [6].

To study the role of TGF- β in different stages of breast cancer, we made use of the MCF10A cell system. This system consists of spontaneously immortalized MCF10A1 (M1) breast epithelial cells [7], the H-RAS transformed M1-derivative MCF10AneoT (M2), which produces premalignant lesions in mice [8], and the M2-derivative MCF10CA1a (M4), which was established from M2 xenografts and forms high grade carcinomas with the ability to metastasize to the lung [9]. This MCF10A series offers the possibility to study the responses of cells with different grades of malignancy that are not biased by a

different genetic background.

For the analysis of TGF- β -induced invasion, we generated homotypic MCF10A spheroid cell cultures embedded in a 3D collagen matrix *in vitro* (Figure 1). Such models closely resemble human tumors *in vivo* by establishing a gradient of oxygen and nutrients, resulting in active and invasive cells on the outside and quiescent or even necrotic cells in the inside of the spheroid [10]. Spheroid based assays have also been shown to better recapitulate drug resistance than monolayer cultures [11]. This MCF10 3D model system allowed us to investigate the impact of TGF- β signaling on the invasive properties of breast cells in different stages of malignancy.

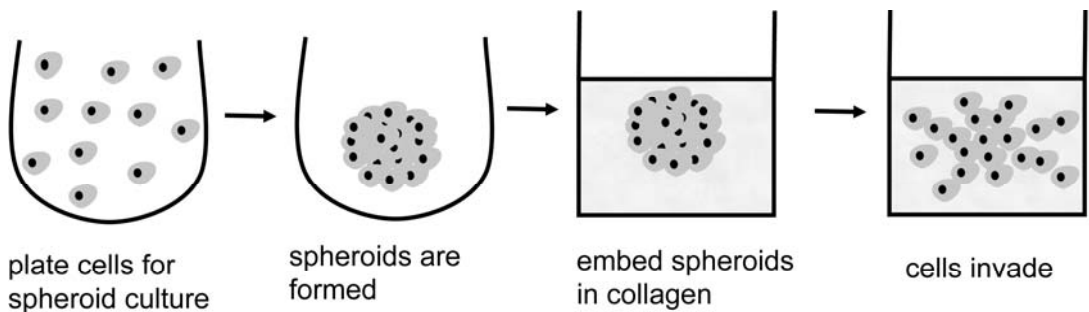


Figure 1 Flow chart of the 3D spheroid assay. First, cells are plated under non-adherent conditions in u-shaped suspensions plates. Cells aggregate into multicellular spheroids and can be embedded into a collagen matrix. Into this collagen matrix, they are able to invade.

Protocol

1. Preparation of the methocel solution (500 mL)

1. Autoclave 6 g of methylcellulose in a 500 ml bottle containing a magnetic stirrer.
2. Dissolve the methylcellulose in 250 ml of preheated DMEM/F12 without serum (60°C) for 20 min.
3. Add 250 ml of DMEM/F12 (RT) containing 10% horse serum. Mix overnight (4°C).
4. Clear the solution by centrifugation (5000g, 2h, 4°C).

5. Take the clear highly viscous solution to a new tube (about 90-95% of the stock solution).
6. Store at 4°C until use.

2. Spheroid culture

1. Prepare a 20% methocel solution (10 ml methocel and 40 ml growth medium).
2. Wash cells twice with PBS, add 0.1% trypsin and incubate cells at 37 °C
3. Resuspend cells in growth medium and count the cells.
4. Prepare a suspension of 10 000 cells per ml in 20% methocel.
5. Add the cell suspension to the 96 well suspension plates (100 µl per well).
6. Next day, check the spheroid formation in the suspension plates. Spheroids will be ready for use after 1-3 days.

3. Preparation of collagen

1. Prepare on ice a solution of 8 ml collagen, 1 ml 10x PBS, 1 ml 0.1 N NaOH and 3 drops 0.1 N HCl. Check if pH is 7.4 by spotting some of the solution on pH indicator strips. Adjust pH if out of range.
2. Add 50 µl of neutralized collagen solution in the wells of a 96 well plate
3. Incubate at 37°C until the collagen is solidified (90 min).
4. Prepare methocel-collagen-ligand solutions on ice: for each ligand pipette 1 part of collagen solution. Add 20 ng per ml of collagen TGF-β. After dilution with methocel and diffusion over the different layers, the final concentration of TGF-β will be 5 ng/ml Mix by vortexing. Add 1 part of methocel-solution. Mix by vortexing.

4. Spheroid embedding

1. Place a 200 µl tip which has approximately 5 mm of the tip cut off, on a 200 µl pipette. Suck up one spheroid with the pipette and carefully dispense the medium into an

empty plate. Watch if the spheroid stays inside the tip. The spheroid is visible as a white dot. Without releasing the plunger, suck up 100 μ l collagen-methocel mixture and dispense the spheroid in the collagen mixture into a collagen-coated 96 well. Do this for 12 spheroids for each condition.

2. Let collagen solidify at least 30 minutes at 37°C

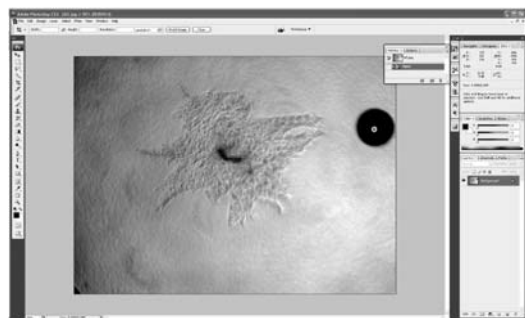
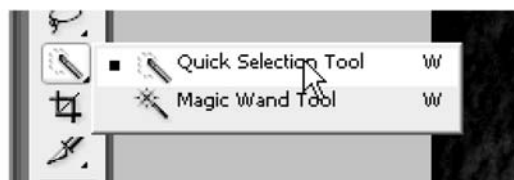
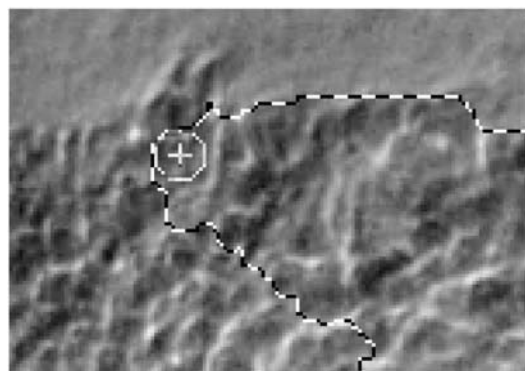
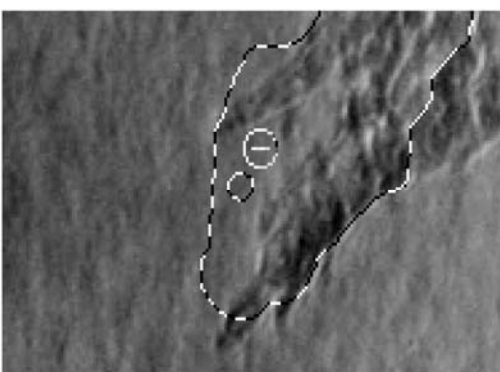
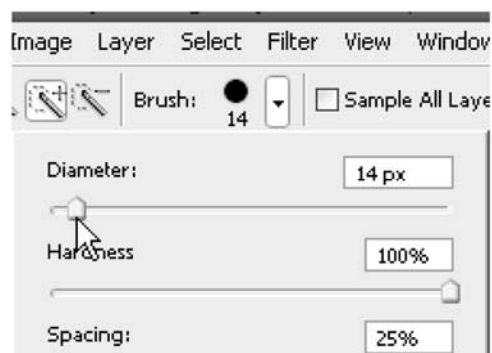
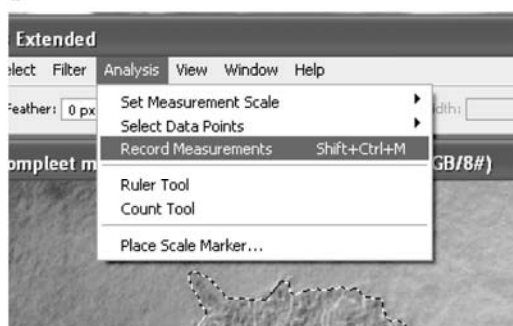
3. Remove air bubbles with a 200 μ l tip.

4. Add 50 μ l of medium with 1.6 % serum and inhibitors dissolved in DMSO. For SB-431542 add 4 μ l of stock solution (10 mM) to 1 ml of medium. After diffusion, the final concentration of the inhibitor will be 10 μ M. TGF- β and inhibitors will stay the course of the experiment. Take pictures of the spheroids under the microscope using 40X magnification.

5. After 48h of stimulation with TGF- β and/or inhibitors, take pictures of the spheroids under the microscope using 40X magnification.

Measuring the area of a spheroid: open the picture from the spheroid in Adobe Photoshop Extended (figure 2A) and select the Quick Selection tool (figure 2B). The mouse pointer changes into a circle with a 'plus' (+) sign (figure 2C). While dragging the cursor over the spheroid, Photoshop will recognize the borders of the spheroid. If an area outside the spheroid is selected, this region can be excluded from the selection by pressing the Alt key or by using the negative selection tool which is indicated by a (-) sign in the toolbar. The cursor changes into a circle with a 'minus' (-) sign and by dragging the cursor over the wrongly selected area, this area is removed from the selection (figure 2D). If necessary, multiple regions can be selected by pressing the Shift button. To work more accurately, the size of the mouse pointer can be adjusted by changing the brush diameter in the toolbar (figure 2E). When the whole spheroid is selected, the area of the selection is calculated via ANALYSIS-MEASURE in the menu bar or by pressing Ctrl + Shift + M (figure 2F). The measurement recording window will appear showing the file

Figure 2 Quantification of the area of the spheroids using Adobe Photoshop Extended. (A) The picture of the spheroid is opened in Adobe Photoshop Extended (B) Selection of the Quick Selection tool (C) Dragging the cursor over the spheroid to select the area of the spheroid (D) Removal of a wrongly included area using the negative Quick Selection tool (E) Adjustment of the brush size of the Quick Selection tool to more accurately select the area (F) Selection of the measurement command to record (G) Example of a measurement record.

A**B****C****D****E****F****G**

A screenshot of a software interface showing the 'Measurement Log' table. The table contains the following data:

Label	Date and Time	Document	Source	Scale	Scale Units	Scale Factor	Count	Area	Perimeter	Circularity
0001 Measurement 3	4/20/2011 7:23:51 PM	b5_gsd	Selection	1 pixels = 1.0000 pixels	pixels	1.000000	1	542927.00...	5719.405387	0.208563

name and the area of the selection in pixels, as well as other data (figure 2G). If multiple selections are measured, the first line shows the sum of all the areas. The measurements of the individual selections are presented in the lines below. The data of all the measurements can be exported into a text file and opened in Excel.

Representative results

An example of the spheroid assay with MCF10A1 (M1) normal breast epithelial cells, H-RAS transformed MCF10AneoT (M2) cells and M2-derived MCF10CA1a (M4) is given in Figure 3. M1 cells showed only weak invasion without stimulation, but invaded significantly better upon stimulation by TGF- β . In contrast, the RAS-transformed M1-derivative M2 invaded already efficiently without addition of stimuli, and this was further increased 4-5 fold upon TGF- β treatment. M4 cells showed the strongest invasion, both with and without TGF- β addition.

Next, we examined whether we could interfere with TGF- β -induced invasion in this spheroid model using chemical inhibitors. For this purpose we used SB-431542, an ATP analogue and selective inhibitor of the kinase activity of T β RI, as well as activin type IB receptor (ActRIB) and activin receptor-like kinase (ALK)7 [12]. As expected, SB-431542 potentially inhibited TGF- β -induced invasion of M1, M2 and M4 cells (Figure 4), indicating that TGF- β -induced invasion is T β RI-kinase dependent. In addition, the basal invasion of the spheroids was also strongly inhibited, suggesting that basal invasion is also dependent on TGF- β .

Discussion

We established a spheroid model in which MCF10A1 cells and its malignant derivatives invade into a collagen matrix in a TGF- β -dependent manner. When comparing the invasive properties of the different MCF10A1 cell lines we found that basal invasion as well as TGF- β -induced invasion increased from the relatively benign M1 cells, to higher levels in the RAS-transformed M2 derivative, to even higher levels in the metastatic M4

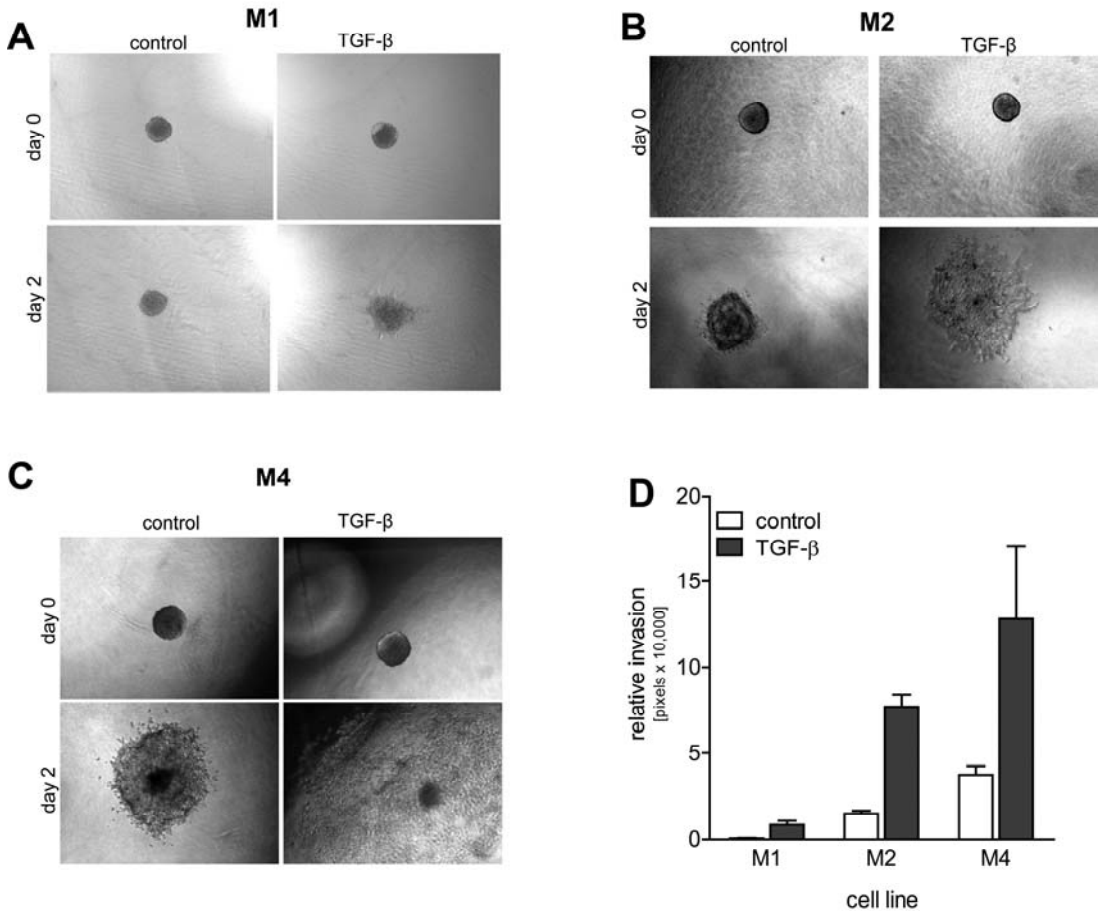


Figure 3 TGF- β -induced invasion of spheroids of spontaneously immortalised MCF10A1 (M1) (A), RAS-transformed MCF10AneoT (M2) (B) and its metastatic derivative MCF10CA1a (M4) (C). M1, M2 and M4 spheroids embedded into collagen were treated with 5ng/ml of TGF- β for 48h as indicated. **A-C** Representative pictures taken at the day of embedding and two days later. **D** Relative invasion was quantified as area difference on day 2 minus day 0. The results are expressed as mean $\pm$ s.d. Adapted from [13].

variant. Thus, the invasive properties correlated with the relative state of aggressiveness of these three cell lines [7,8,14].

The embedding of the spheroid (step 4.1) is a critical step in the assay. The detection of the spheroid in the pipette tip might be difficult when performing the assay for the first few times. One can avoid this by collecting the spheroids in a tube, spinning them down, removing supernatant and resuspend th spheroids in collagen-methocel mixture. This requires more spheroids as input as around 50% of the spheroids is lost during this process. Furthermore, multiple spheroids end up in one well and might lie too

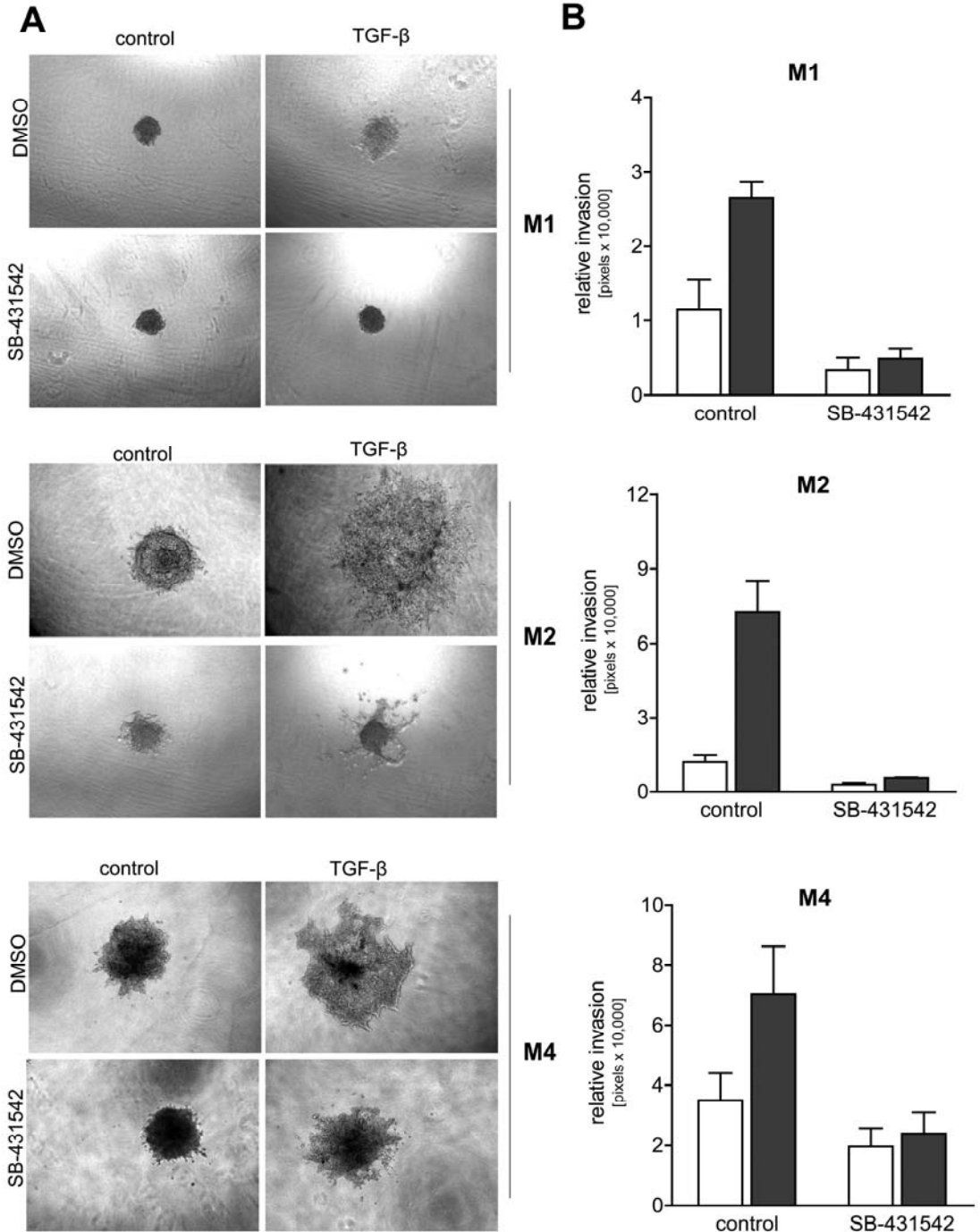


Figure 4 Basal and TGF- β -induced spheroid invasion is inhibited by SB-431542. M1, M2 and M4 spheroids were embedded into collagen and treated with 5ng/ml TGF- β and/or 10 μ M SB-431542 as indicated. Representative pictures taken after 2 days (A). Relative invasion was quantified as area difference on day 2 minus day 0 (B). The results are expressed as mean $\pm$ s.d. Adapted from [13].

close to each other to analyse. Therefore the resuspension step is a critical step using this method.

A limitation of this assay is that we analyze a 3 dimensional assay in a 2 dimensional plane. As can be seen in Figures 3 and 4, most cells usually invade in the same plane. However, spheroids that are located very near the edge of the well invade generally more in depth and are for that reason excluded from the analysis. Of course it would be possible to perform the analysis in 3D, in order to get a more accurate result. But this requires sophisticated hardware and software and the benefit of calculating volume instead of using the area may not outweigh the trouble. Especially since we found that the variation in starting size of the 3-dimensional spheroids as measured in 2D, is generally only 5-10%. Another disadvantage is that the imaging is a time consuming step, since the spheroids are located at different heights, which hampers automated imaging. Also the analysis step is time consuming and requires pictures with good contrast from spheroid to matrix. In case the contrast is too low, one can easily adapt the brightness and contrast using the CTRL + B and CTRL + L commands in Photoshop. As an alternative, one could draw the area around the spheroid manually using ImageJ. Another alternative is to use a vital fluorescent dye.

The protocol can be adapted to investigate invasion of other cell lines, provided that the cells are able to form spheroids. If spheroids do not form in suspension plates, one could also try poloy-HEMA coated plates. Furthermore, the optimal concentration of TGF- β to induce invasion of the cell line might be different. In all our cell lines, TGF- β induces invasion at 5 ng/ml, although M4 performs better when using 2 ng/ml.

In addition, this assay can be adapted to measure invasive responses to other growth factors, such as epithelial growth factor (EGF) or hepatocyte growth factor (HGF).

In our assays the basal and TGF- β -induced invasion was strongly inhibited by treatment with SB-431542, an inhibitor of type I receptor for TGF- β . This is a proof of principle that chemical inhibitors can be used in this assay. Other chemical inhibitors, such as MMP inhibitors, have also been used successfully at concentrations recommended by the manufacturer for monolayer cell culture. It is advisable to add chemical inhibitors dissolved in

DMSO not directly into the collagen, since the DMSO precipitates in the icecold collagen-methocel mixture. Therefore, we add these compounds in the medium on top of the collagen.

Upon mastering this techniques, one can test the effect of chemical inhibitors, knock-down or overexpression of genes on invasion. Furthermore, by scaling up the assay to a 24 well format, one can isolate RNA using a phenol-chloroform-based method (e.g. Trizol® or Tripure) followed by a silica-column based clean-up. This RNA can be used for quantitative real-time PCR. Our spheroid invasion assay resembles the *in vivo* process more closely than 2D invasion models, because cells in spheroids are in different metabolic states and interact in more natural fashion with their surroundings [10]. We used type collagen I, since high levels of this collagen promoted breast cancer formation and invasion [15]. In addition, collagen I was associated with a greater incidence of metastasis [16]. Several 3D models have been developed over the past decades. Cells can be either completely embedded within in a matrix or placed on top of a matrix or a polymeric scaffold [17,18]. In a 3D model developed by Bissell and co-workers, cells were grown in a reconstituted basement membrane (rBM) matrix. This model provides a rapid assay to distinguish between normal and malignant mammary epithelium, but focuses on cell morphology [19,20]. Morphogenesis and organization of the distinct MCF10A cell lines inversely correlated with malignancy [21,22]. In other 3D models multicellular spheroids showed the same resistance to cytotoxic drugs as their parental cell line *in vivo*, whereas cells in monolayers failed to do so [11]. Also 3D cultures of MCF10A cell lines have been used to assess sensitivity to kinase inhibitors [23]. Our spheroid model complements these assays by specifically focusing on invasion.

Acknowledgements

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Chapter 4

The TGF- β /Smad pathway induces breast cancer cell invasion through the up-regulation of matrix metalloproteinase 2 and 9 in a spheroid invasion model system.

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Abstract

Transforming growth factor- β (TGF- β) has opposing roles in breast cancer progression by acting as a tumor suppressor in the initial phase, but stimulating invasion and metastasis at later stages. In contrast to the mechanisms by which TGF- β induces growth arrest, the pathways that mediate tumor invasion are not well understood. Here, we describe a TGF- β -dependent invasion assay system consisting of spheroids of MCF10A1 normal breast epithelial cells (M1) and RAS-transformed (pre-)malignant derivatives (M2 and M4) embedded in collagen gels. Both basal and TGF- β -induced invasion of these cell lines was found to correlate with their tumorigenic potential; M4 showing the most aggressive behavior and M1 showing the least. Basal invasion was strongly inhibited by the TGF- β receptor kinase inhibitor SB-431542, indicating the involvement of autocrine TGF- β or TGF- β -like activity. TGF- β -induced invasion in premalignant M2 and highly malignant M4 cells was also inhibited upon specific knockdown of Smad3 or Smad4. Interestingly, both a broad spectrum matrix metalloproteinase (MMP) inhibitor and a selective MMP2 and MMP9 inhibitor mitigated TGF- β -induced invasion of M4 cells, while leaving basal invasion intact. In line with this, TGF- β was found to strongly induce MMP2 and MMP9 expression in a Smad3- and Smad4-dependent manner. This collagen-embedded spheroid system therefore offers a valuable screening model for TGF- β /Smad- and MMP2- and MMP9-dependent breast cancer invasion.

Keywords invasion · matrix metalloproteinase · spheroids · MCF10A · Smad · TGF- β

Introduction

Breast cancer is one of the leading causes of death in women. Morbidity and mortality are predominantly caused by metastasis, but not by the primary tumor. A critical event in the metastatic cascade is tumor cell invasion. However, the mechanisms by which primary tumor cells acquire an invasive phenotype are not well known. One of the inducers of invasion is transforming growth factor- β (TGF- β). TGF- β is thought to play a dual role in cancer progression [1,2]. It can act as a tumor suppressor in the early stages, but promote cancer

progression in later stages of the disease. Moreover, TGF- β is frequently overexpressed in breast cancer and its expression correlates with poor prognosis and metastasis [3–6].

TGF- β elicits its cellular responses via TGF- β type II (T β RII) and type I (T β RI) receptors. Upon TGF- β -induced heteromeric complex formation, T β RII phosphorylates T β RI. The activated T β RI initiates its intracellular canonical signaling pathway by phosphorylating receptor Smads (R-Smads), i.e. Smad2 and Smad3. These activated R-Smads form heteromeric complexes with Smad4, which accumulate in the nucleus and regulate the transcription of target genes [7,8]. Tumor suppressor activities of TGF- β /Smad signaling include amongst others repression of c-myc [9] and induction of cyclin-dependent kinase inhibitors [10,11].

During cancer progression accumulation of genetic mutations as well as epigenetic changes can render cells insensitive to the cytostatic effects of TGF- β , and components of the TGF- β pathway are frequently inactivated in a subset of cancer specimens [12]. However, breast and other cancers often only carry defects in downstream cytostatic TGF- β targets, show normal TGF- β signaling from receptors to Smads, and retain or gain other TGF- β responsive properties [13–16]. One of the TGF- β -induced processes contributing to tumor migration and invasion is epithelial-to-mesenchymal transition (EMT). Both Smad and non-Smad signaling pathways have been implicated in TGF- β -induced EMT of breast cancer cells [17–19]. In addition, matrix metalloproteinases (MMPs) appear to play a critical role in TGF- β -induced migration and invasion [20–22].

To study the role of TGF- β in different stages of breast cancer, we made use of the MCF10A cell system. This system consists of immortalized MCF10A1 (M1) normal breast epithelial cells (derived from human fibrocystic mammary tissue [23]), the H-RAS transformed M1-derivative MCF10AneoT (M2), which produces premalignant lesions in mice, and the M2-derivative MCF10CA1a (M4), which was established from M2 xenografts and forms high grade carcinomas with the ability to metastasize to the lung [24,25]. Since RAS and TGF- β have been reported to collaborate in promoting malignant progression of breast cancer [26,27], this MCF10A series offers the possibility to study the responses of cells with different grades of malignancy that are not biased by a different genetic background.

For the analysis of TGF- β -induced invasion we generated homotypic MCF10A spheroid cell cultures embedded in a 3D collagen matrix *in vitro*. Such models closely resemble human tumors *in vivo* by establishing a gradient of oxygen and nutrients, resulting in active and invasive cells on the outside and quiescent or even necrotic cells in the inside of the spheroid [28,29]. Spheroid based assays have also been shown to better predict drug resistance than monolayer cultures [30,31]. This MCF10 3D model system allowed us to investigate the impact of TGF- β signaling on the invasive properties of breast cells in different stages of malignancy. Our results show that both Smads and MMP2 and in particular MMP9 are critical for TGF- β -induced invasion of the malignant breast cancer cells.

Materials and methods

Cell culture

MCF10A series of cell lines, i.e. MCF10A (M1), transformed MCF10AneoT (M2) and its derivative MCF10CA1a (M4) were obtained from Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, USA). All MCF10A cell lines were maintained in DMEM/F12 (Gibco) supplemented with 5% horse serum (Gibco), 20ng/ml epidermal growth factor (EGF) (Upstate), 100ng/ml cholera toxin (Calbiochem), 0.5 μ g/ml hydrocortisone (Sigma), 10 μ g/ml insulin (Sigma), 100U/ml penicillin and 50 μ g/ml streptomycin (Gibco). All cell lines were grown in a humidified incubator at 37°C and 5% CO₂.

3D spheroid invasion assays

Semi-confluent MFC10A cells were trypsinized, counted and re-suspended in medium containing 2.4mg/ml methylcellulose (Sigma) at the concentration of 10⁴ cells/ml. 100 μ l of suspension was added into each well of U-bottom 96-well-plate allowing the formation of one spheroid per well. All spheroids consisted of 10³ cells. Two days after plating spheroids were harvested and embedded into collagen. Flat-bottom 96-well-plate was coated with neutralized bovine collagen-I (PureCol, Advanced BioMatrix) according to

manufacturer's protocol. Single spheroids were embedded in a 1:1 mix of neutralized collagen and medium supplemented with 12mg/ml of methylcellulose. TGF- β 3 (generous gift of Dr. K. Iwata, OSI Pharmaceuticals, Inc, New York, USA) and/or SB-431542 (Sigma) were directly added to the embedding solution. GM6001 and MMP2/9-inhibitor II (Calbiochem) were added in medium on top of the collagen. Invasion was monitored during the next two days and quantified by measuring the area occupied by cells using ImageJ software. Pictures were taken at day 0 and day 2 after embedding.

Lentiviral transduction

Lentiviral constructs expressing short hairpin RNAs targeting Smad4 (sh-Smad4) or a non-targeting control were obtained from the Sigma Mission shRNA library. The targeting sequence for Smad4 was 5'-GTACTTCATACCATGCCGATT-3'. The non-targeting sequence was 5'-AGAACGTCAACACCAAGTGCA-3'. Smad3 knockdown was performed using miR-Smad3 lentivirus using BLOCK-iT Pol II micro RNA interference technology (Invitrogen) as described previously [32]. Cells were infected at multiplicity of infection (MOI) 2, selected with appropriate antibiotics (sh-Smad4 and the non-targeting control cells with puromycin; miR-Smad3 and miR-control cells with blasticidine), and used for experiments one week later.

Gelatin zymography

Conditioned medium was harvested from cells after 48 hr of stimulation and resuspended in 4x Laemli buffer (250mM Tris-HCl pH6.8, 8% SDS, 40% glycerol, 0.004% bromophenol blue). Samples were ran on a 10% acrylamide gel containing 0.8mg/ml gelatin, washed with 2.5% Triton and incubated at 37°C with Brij solution (50mM Tris-HCl pH7.4, 0.05% Brij-35, 10mM CaCl₂) overnight. Gels were stained with Coomassie Brilliant Blue (25% methanol, 15% acetic acid, 0.1% Coomassie Brilliant Blue R).

Statistical analysis

For statistical comparison of two groups, a two-tailed Student's *t*-test was used where applicable. Statistical analysis of three or more groups was done by one-way ANOVA followed by Bonferroni's multiple comparison test. *P*<0.05 was considered as statistically

significant.

Other assays

Assays for cell proliferation, transcriptional reporter activity, Western blot analysis, RNA isolation, cDNA synthesis and quantitative PCR are found in supplementary methods

Results

Establishment of a spheroid cell culture model to study TGF- β -induced invasion of breast cancer cells

To analyze the role of TGF- β -signaling in 3D cultures, spheroids of MCF10A1 (M1) normal breast epithelial cells, H-RAS transformed MCF10AneoT (M2) cells, or M2-derived MCF10CA1a (M4) cells were embedded into collagen matrix and stimulated with TGF- β (Fig. 1a). M1 cells showed only weak invasion without stimulation, but invaded significantly better upon TGF- β stimulation. In contrast, the RAS-transformed M1-derivative M2 invaded already efficiently without any stimulus, which was further increased 4-5 fold upon TGF- β addition. M4 cells showed the strongest invasion, both with and without TGF- β addition (Fig. 1a, b), in line with their metastatic capacity. Other TGF- β family ligands, such as Activin-A, BMP-6, BMP-7 or BMP-9 failed to induce invasion in any of the cell lines (data not shown). Thus, the invasiveness in our spheroid model *in vitro* correlates with the grade of aggressiveness of the M1, M2 and M4 cells *in vivo* [24,25].

Next, we examined whether the differences in invasion between M1, M2 and M4 cells can be explained by differences in TGF- β responsiveness. All three cell lines were found to express type I, II and III receptors as shown by TGF- β affinity crosslinking assays (Suppl. Fig. 1b), which is in line with a previous study [33]. To investigate if all cells activate TGF- β /Smad transcriptional activity to the same extent, we made use of the CAGA₁₂-luciferase reporter, which contains 12 repeats of the Smad binding element CAGA. All cells showed TGF- β /Smad transcriptional activity in response to TGF- β . However, induction in M2 was markedly lower than in M1 and M4 (Suppl. Fig. 1a). Furthermore, all cells showed Smad2 phosphorylation upon TGF- β stimulation (data not shown). Thus, the differences in

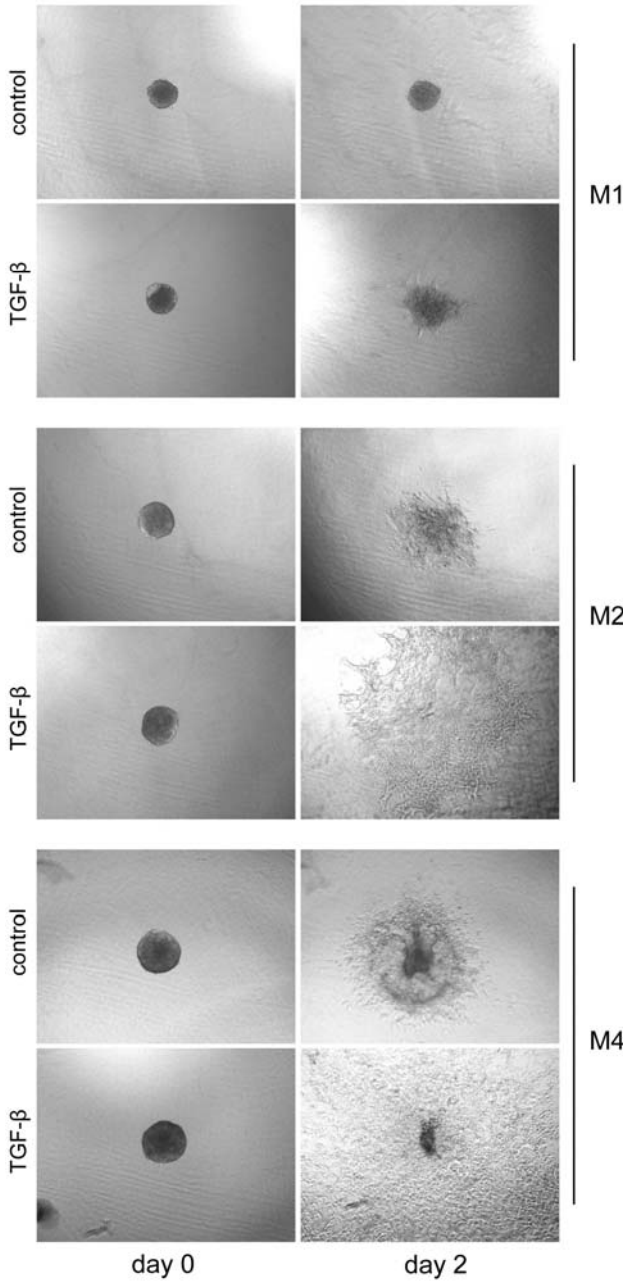
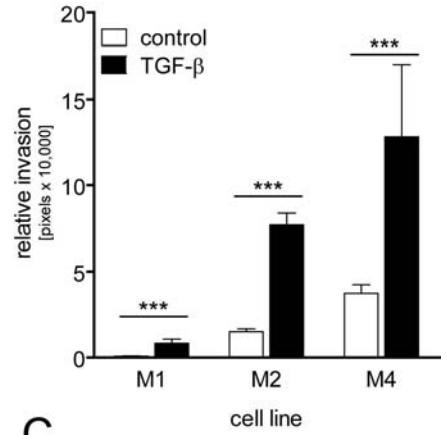
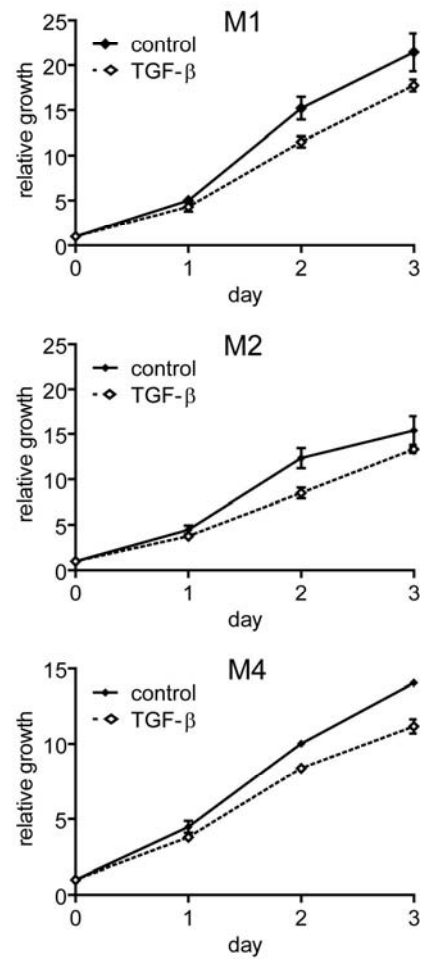
A**B****C**

Figure 1 TGF- β -induced invasion of spheroids of normal MCF10A1 (M1), RAS- transformed MCF10AneoT (M2) and its metastatic derivative MCF10CA1a (M4). M1, M2 and M4 spheroids embedded into collagen were treated with 5ng/ml of TGF- β for 48 hr as indicated (**a,b**). **a** Representative pictures taken at the day of embedding and two days later. **b** Relative invasion was quantified as area difference on day 2 minus day 0. The results are expressed as mean $\pm$ s.d. (M1: n=7, M2 and M4: n=4). **c** Proliferation of M1, M2 and M4 cells in the presence and absence of TGF- β . Cell viability was measured 0, 24, 48 and 72 hr after 5ng/ml TGF- β treatment using the MTS assay. Relative growth was determined by calculating the absorbance at 490 nm for each time point relative to 0 hr measurement. The results are expressed as mean $\pm$ SEM. of three independent experiments performed in triplicate. Significance: ***P<0.001.

receptor binding and transcriptional activity did not correlate with the levels of spheroid invasiveness of the three cell lines.

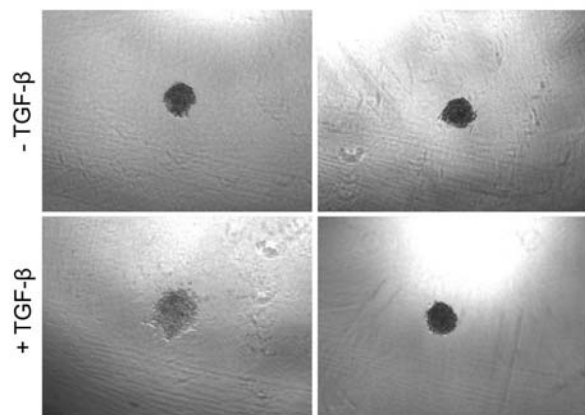
TGF- β is a potent inhibitor of proliferation of epithelial cells and this inhibitory effect is often lost during cancer progression. To test whether the differences in basal and TGF- β -induced invasion between M1, M2 and M4 cells is associated with differences in growth rate, we performed proliferation assays. Interestingly, the growth of all three cell lines was slightly inhibited by TGF- β , but no major differences were detected between the cell lines (Fig. 1c).

Taken together, our results demonstrate that the described spheroid invasion assay closely reproduces the differences in the aggressive properties of the three cell lines [24,25,33], whereas the observed differences are not due to differences in proliferative capacity or responsiveness to TGF- β .

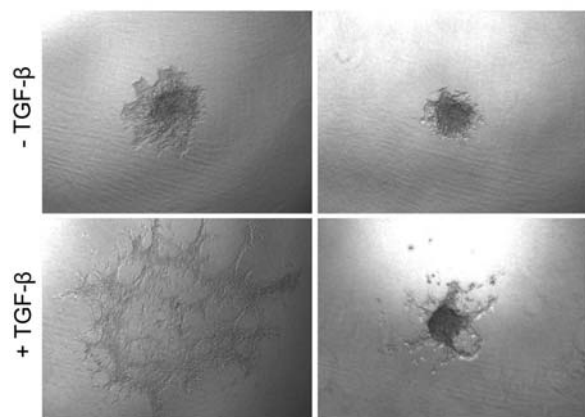
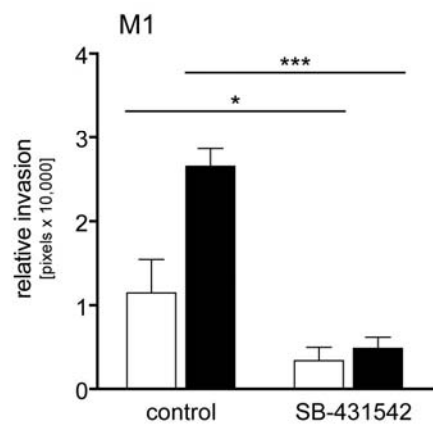
SB-431542 inhibits basal and TGF- β -induced invasion

Next, we examined whether the basal and TGF- β -induced invasion in this spheroid model is dependent on the T β RI kinase. For this purpose we used SB-431542, an ATP analogue and selective inhibitor of the kinase activity of T β RI, activin type IB receptor (ActRIB) and activin receptor-like kinase (ALK)7 [34]. As expected, SB-431542 potently inhibited TGF- β -induced invasion of M1, M2 and M4 cells (Fig 2a, b), indicating that TGF- β -induced invasion is T β RI-kinase dependent. Surprisingly, the basal invasion of the spheroids was also strongly inhibited, suggesting autocrine TGF- β signaling, since we found these cells to express all TGF- β isoforms. The basal invasion of M1, M2 and/or M4 cells might also be mediated by ligands other than TGF- β , e.g. ligands that signal via

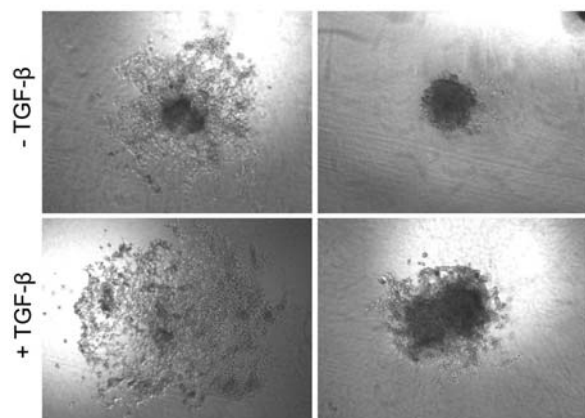
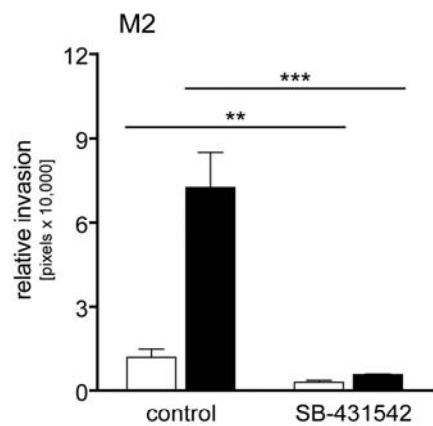
A



M1



M2



M4

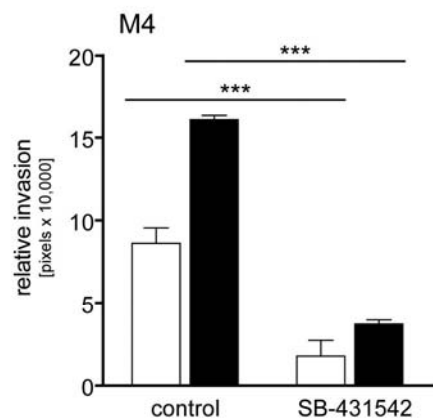


Figure 2 Basal and TGF- β -induced spheroid invasion is inhibited by SB-431542. M1, M2 and M4 spheroids were embedded into collagen and treated with 5ng/ml TGF- β and/or 10 μ M SB-431542 as indicated. **a** Representative pictures taken after 2 days. **b** Relative invasion was quantified as area difference on day 2 minus day 0. The results are expressed as mean $\pm$ s.d. (M1: n=3, M2 and M4: n=4). Significance: ***P<0.001, **P<0.01, *P<0.05.

ActRIB and ALK7. However, Activin-A failed to induce invasion (data not shown) and only marginally induced CAGA₁₂-luciferase transcriptional activity, possibly caused by low expression of activin receptors in these cells (Suppl. Fig. 1a).

TGF- β -induced invasion is Smad3/4 dependent

As TGF- β signaling potentially induced invasion of the aggressive and metastatic M4 cells, we used these cells to further investigate the role of the TGF- β pathway in metastasis-associated invasion. Recent studies by our group and others have demonstrated that Smad3 and Smad4 are key players in inducing metastasis of MDA-MB231 cells [17,32,35]. To investigate the role of Smad3 and Smad4 in M4, we knocked-down Smad3 and Smad4 through lentiviral vectors. The Smad4 shRNA construct used strongly reduced Smad4 protein, but did not affect the TGF- β -induced phosphorylation of Smad2 and Smad3 (Fig. 3a). The Smad4 knockdown vector also strongly reduced TGF- β /Smad-induced transcription as measured by a CAGA₁₂-luciferase reporter (Fig. 3b). Moreover, M4 spheroids embedded in collagen showed a severe defect in TGF- β -induced invasion upon Smad4 knockdown (Fig. 3c and d), indicating that TGF- β -induced 3D invasion is Smad4-dependent.

Although the knockdown of Smad3 was less efficient than the knockdown of Smad4, it significantly reduced the levels of phospho-Smad3, while leaving TGF- β -induced phosphorylation of Smad2 unaffected (Fig. 3a). Moreover, this level of Smad3-knockdown, like knockdown of Smad4, severely inhibited TGF- β /Smad-induced transcription (Fig. 3b). Knockdown of Smad3 also reduced the levels of TGF- β -induced invasion of M4 cells in collagen (Fig. 3c and d).

To further investigate the role of Smads in TGF- β -induced invasion we performed knock-down experiments in the premalignant cell line M2 (Suppl. Fig. 2a). Also in these cells Smad3 or Smad4 knockdown caused inhibition of TGF- β -induced invasion (Suppl. Fig. 2b and c). Taken together, these results emphasize the importance of the Smad3/4 pathway

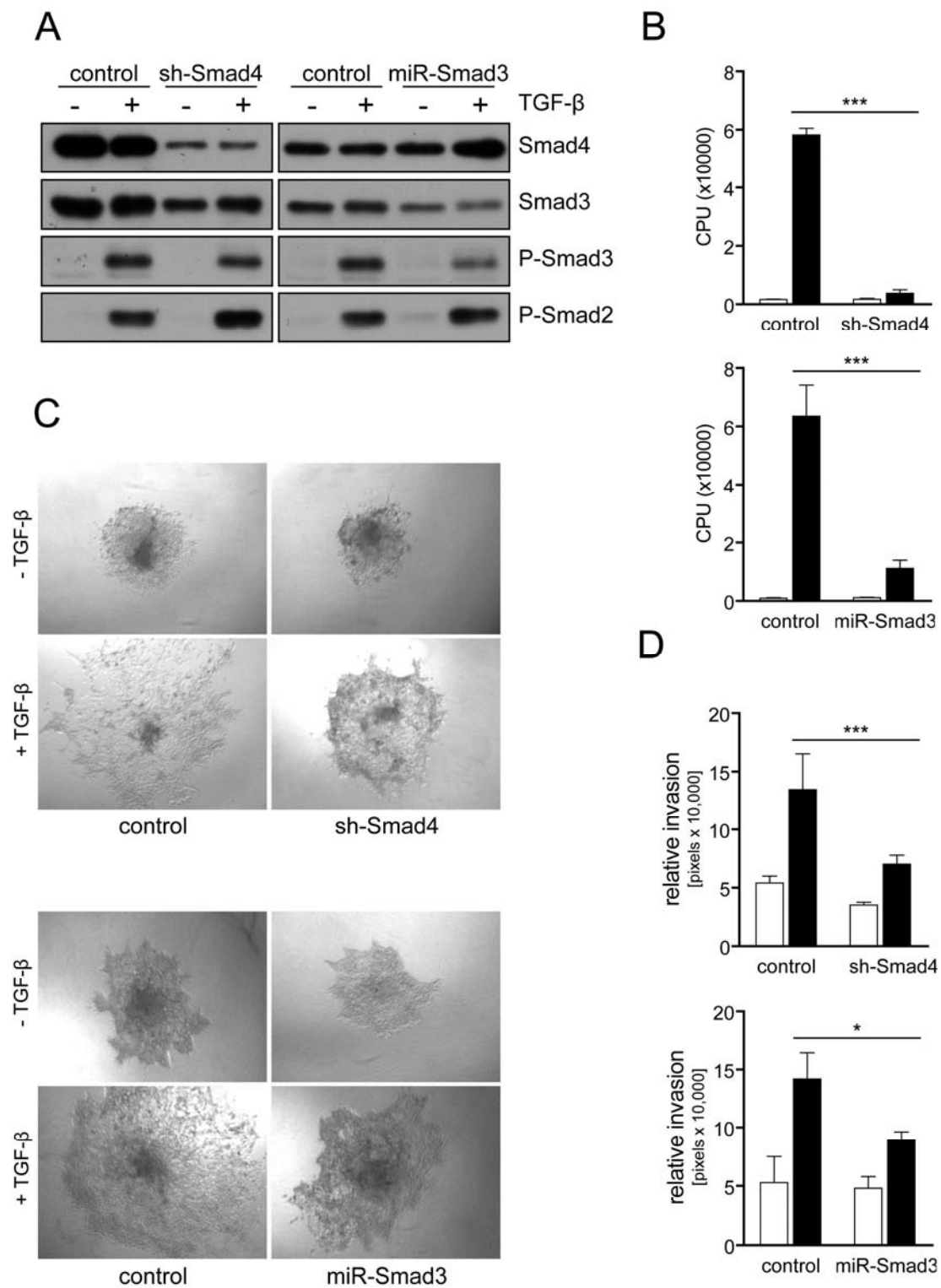


Figure 3 TGF- β -induced spheroid invasion is Smad3/4 dependent. MCF10CA1a (M4) cells were transduced with lentiviruses expressing shRNA against Smad4 or miRNA against Smad3 and the appropriate control viruses. **a** Cells were stimulated with 5ng/ml of TGF- β for 16h. Protein lysates were separated by SDS-PAGE and subjected to western blot analysis using Smad3-, Smad4-, phospho-Smad2- and phospho-Smad3-specific antibodies. **b** Cells were transfected with CAGA₁₂-luc transcriptional reporter and subsequently stimulated with 5ng/ml of TGF- β for 16h. Relative luciferase activity (CPU) is expressed as mean $\pm$ s.d. of triplicate cultures. **c,d** Spheroids were embedded into collagen and treated with 5ng/ml of TGF- β for 2 days. **c** Representative pictures taken after 2 days. **d** Relative invasion was quantified as area difference on day 2 minus day 0. Results are expressed as mean $\pm$ s.d. (sh-Smad4: n=4, miR-Smad3: n=3). Significance: ***P<0.001, *P<0.05.

in TGF- β -induced invasion.

Critical role of MMP2 and 9 in TGF- β -induced invasion

Next, we asked ourselves which downstream target genes are necessary for TGF- β -induced invasion in this model system. MMPs play important roles in tumor cell migration and invasion, both through degradation of components of the extracellular matrix, and by activation of growth factors and cleavage of adhesion molecules [36]. Analysis of TGF- β -induced mRNA expression identified MMP1, MMP2 and MMP9 among the strongest TGF- β -responsive genes in M4 spheroids (Suppl. Fig. 3a and Fig. 4a, b, respectively). Since the spheroid assay is performed in collagen I matrix, MMP1, a known collagen I protease, was expected to be a critical mediator of TGF- β -induced invasion. However, knockdown experiments showed that MMP1 is not necessary for TGF- β -induced invasion in this system (Suppl. Fig. 3b-d).

We next examined the involvement of the gelatinases MMP2 and MMP9, as these proteins have been correlated with poor prognosis and lung metastasis in breast cancer [37,38]. TGF- β was found to strongly induce MMP2 and MMP9 mRNA levels both in spheroids (3D) and in monolayer (2D): MMP2 expression was induced approximately 10-fold upon TGF- β stimulation in both 2D and 3D (Fig. 4a and 4b); MMP9 approximately 100-fold and 40-fold in spheroids and monolayer respectively (Fig. 4a and b). To confirm that this induction was seen also at the protein level, we analyzed conditioned medium by gelatin zymography. As shown in Fig. 4c, TGF- β induces pro-MMP2 and both pro- and active MMP9 in M4 cells.

To further investigate the role of MMPs in 3D invasion, spheroids of M4 were analyzed in

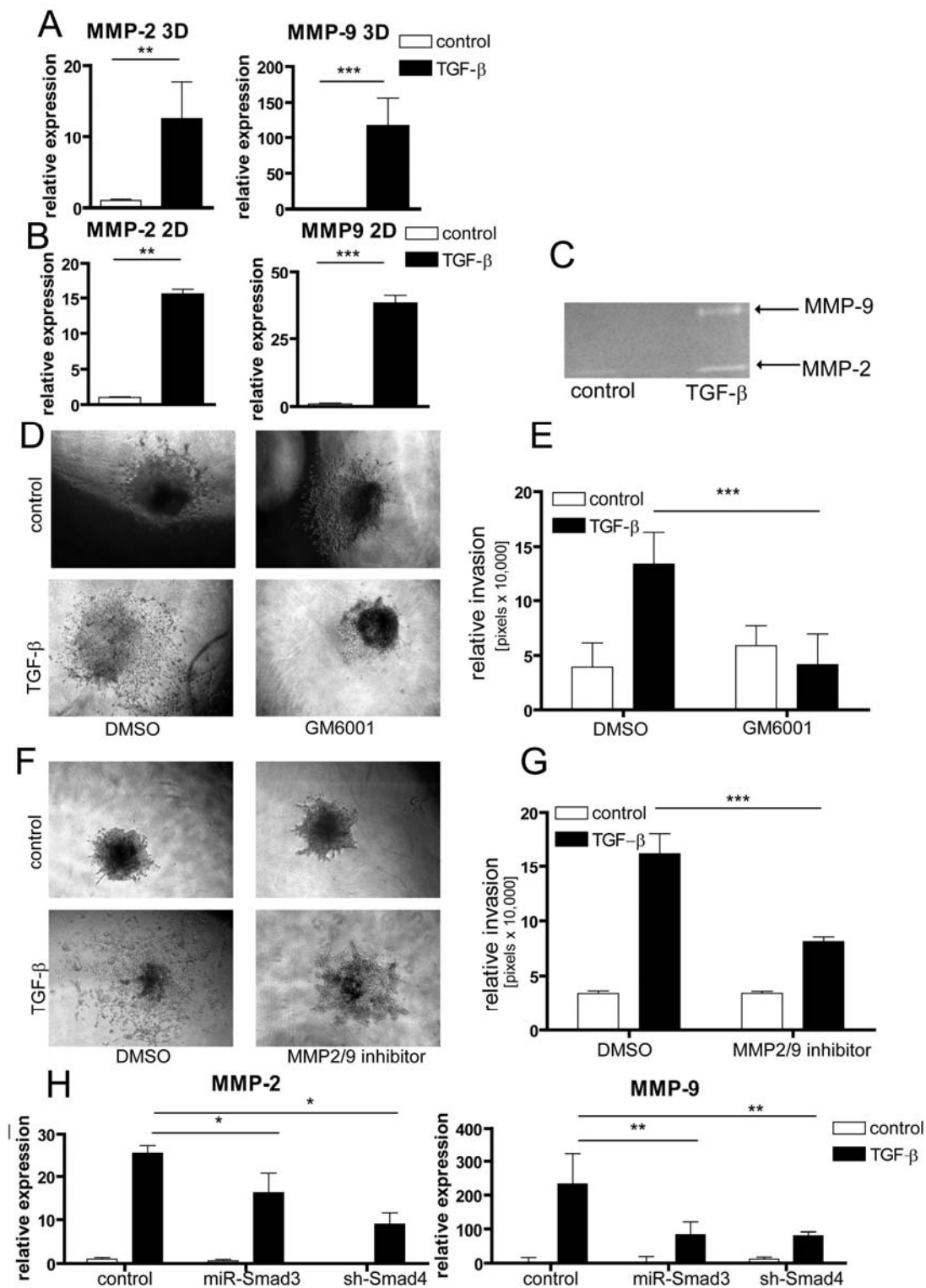


Figure 4 TGF- β -induced spheroid invasion is dependent on matrix metalloproteinase activity. **a,b** RNA was isolated from spheroids (**a**) or monolayer (**b**) of M4 cells stimulated for 24 hr with or without TGF- β (5ng/ml). Q-PCR for MMP2 and MMP9 was performed using ARP as internal control. **c** Conditioned medium was harvested from M4 cells stimulated with or without TGF- β (5 ng/ml) for 48 hr and subjected to gelatin zymography **d-g** Inhibition of matrix metalloproteinases (MMPs) hampers TGF- β -induced invasion in MCF10CA1a (M4). M4 spheroids were embedded into collagen and incubated with 5ng/ml TGF- β , and/or general MMP inhibitor GM6001 (1 μ M) (**d,e**) or selective MMP 2/9 inhibitor (1 μ M) (**f,g**) as indicated. **d,f** Representative pictures taken after 2 days. **e,g** Invasion was quantified as area difference on day 2 minus day 0. Results are expressed as mean $\pm$ s.d (**e**: DMSO: n=7; GM6001: n=8; **g**: DMSO: n=9,12; inhibitor: n=23,24). **h** RNA was isolated from M4 cells transduced with Smad knock-down or non-targeting control viruses and stimulated for 24 hr with 5ng/ml TGF- β . Q-PCR for MMP2 and MMP9 was performed using ARP as internal control. One representative experiment out of three independent experiments is shown. Significance * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the presence or absence of the general, broad-spectrum, MMP-inhibitor GM6001. Interestingly, GM6001 strongly inhibited TGF- β -induced invasion (Fig. 4d, e), but had no significant effect on the basal invasion. We subsequently analyzed the effect of a selective MMP2/9 inhibitor [39]. Importantly, treatment of M4 spheroids with selective MMP2/9 inhibitor like the broad-spectrum inhibitor reduced TGF- β -induced invasion without an effect on basal invasion (Fig 4f, g).

As the TGF- β -induced invasion of M4 spheroids was dependent on Smad3/Smad4 (Fig. 3), we next examined the role of Smad3 and 4 on TGF- β -induced MMP2 and 9 expression. Both knockdown of Smad3 and Smad4 resulted in strongly reduced TGF- β -induction of MMP2 and 9 in M4 cells (Fig. 4h). Taken together, TGF- β -induced MMP2 and 9 expression is dependent on both Smad3 and Smad4, and is critical for TGF- β -induced invasion of M4 cells in this spheroid model system.

Discussion

To increase our understanding of breast cancer cell invasion and the role of TGF- β therein, we established a spheroid model in which MCF10A1 cells and its malignant derivatives invade into a collagen matrix in a TGF- β -dependent manner. When comparing the invasive properties of the different MCF10A1 cell lines we found that basal invasion as well as TGF- β -induced invasion increased from the relatively benign M1 cells, to higher levels in

the RAS-transformed M2 derivative, to even higher levels in the metastatic M4 variant, and thus nicely correlated with the relative state of aggressiveness of these three cell lines [24,25,33]. In contrast, there was no correlation between invasion and cell proliferation, and the growth inhibitory effect of TGF- β on the cell lines was only weak. The fact that the relatively benign M1 cells showed only a minor growth-inhibitory effect upon TGF- β treatment might be due to chromosomal rearrangements during immortalization, leading to loss of the CDKN2A locus and amplification of MYC [40,41].

Our spheroid invasion assay resembles the *in vivo* process more closely than 2D models, because cells in collagen I-embedded spheroids are amongst others in different metabolic states and interact in more natural fashion with their surroundings [29]. Moreover, the composition of extracellular matrix in breast cancer is often altered, resulting in fibrotic stiff foci with a high collagen I content. It has been demonstrated that increased collagen I content promotes breast cancer formation and invasion [42] and is associated with greater incidence of metastasis [43]. In a previously developed 3D model by Bissell and co-workers cells are grown in a reconstituted basement membrane (rBM) matrix. This model provides a rapid assay to distinguish between normal and malignant mammary epithelium but focuses mainly on cell morphology [44]. In this assay morphogenesis and organization of the distinct MCF10A cell lines inversely correlates with malignancy [45,46]. In addition, 3D cultures of MCF10A cell lines have been used to assess sensitivity to kinase inhibitors [47]. Our spheroid model complements these assays by specifically focusing on invasion. Moreover, as it is easy to conduct and very quantifiable it can also be used for screening.

In our assays the basal 3D invasion was strongly inhibited by treatment with SB-431542, an inhibitor of type I receptors for TGF- β , Activin and Nodal. This suggests that cells produce TGF- β -like activity and stimulate invasion in an autocrine manner. This is in agreement with a recent report in which it is shown that the ECM can induce TGF- β signaling in the absence of TGF- β [48]. We cannot exclude that Activins or Nodal also contribute to basal invasion. In fact, Activin and Nodal have also been implicated in breast cancer progression [49–51], However, Activin-A only very weakly induced transcriptional activity and failed to induce invasion in our spheroid model.

Our results reveal a critical role for Smad3 and Smad4 in TGF- β -induced invasion of M4 cells. This is consistent with our previous findings that had demonstrated a key role for Smad3 and Smad4 in TGF- β -induced EMT and breast cancer metastasis [17,32,52].

Abrogation of the Smad pathway in M4 cells by overexpression of a Smad-binding defective mutant of T β RI or a dominant negative Smad3 mutant was also reported to suppress metastasis [53,54]. However, our results do not exclude a role for non-Smad intracellular signaling pathways downstream of TGF- β receptors I. Various studies have shown that RAS and TGF- β collaborate in promoting malignant progression of breast cancer [26,27]. Both TGF- β and RAS can activate the MAP kinases ERK, JNK and p38 and their downstream transcription factors, such as AP-1 family members. Interestingly, in the MCF10A cell lines Smads, MAPKs as well as AP-1 proteins have been reported to contribute to TGF- β -induced migration and MMP induction [26,53]. However, it remains to be established whether and which MAPK and AP-1 members collaborate with Smad proteins to mediate basal and/or TGF- β -induced invasion of the MCF10A cell lines in our spheroid model system.

Our studies reveal a key role for MMP2 and MMP9 in TGF- β -induced invasion of the highly aggressive M4 spheroids. MMP2 and 9 were initially known for their ability to degrade native type IV collagen [55], in line with the idea that the main function of MMPs in invasion was degradation of the extracellular matrix, in particular the basal lamina [20]. Various matrix components that can be degraded by MMP2 and MMP9 are in fact produced by the epithelial cells themselves. However, besides degradation of extracellular matrix MMPs can also activate growth factors, including TGF- β , and cleave adhesion molecules, such as E-cadherin [36]. It is likely that these functions of MMP2 and 9 also contribute to TGF- β -induced invasion.

MMP2 and 9 appear attractive targets for therapeutic intervention of breast cancer invasion. However, MMPs are multifunctional proteins and may also have anti-tumor actions. The latter appears in particular an issue for targeting of MMP9 [22]. Tumors of K14-HPV16 mice in a null MMP9 background were found to be more aggressive, indicating that MMP9 inhibits certain aspects of tumor progression [56]. On the other hand,

knockdown of MMP9 reduces metastasis of T β RI-expressing cells [57]. This does not appear to be the case for MMP2, as overexpression in MDA-MB-231 cells augments orthotopic tumor growth and metastasis in nude mice [58]. In human cancer cells, MMP2 is one of the predictors for lung metastasis [38]. The development of specific MMP inhibitors remains a challenge, as the enzymatic pocket of these proteins does not differ from one another.

One striking finding is that the basal invasion of M4 spheroids is not affected by the MMP inhibitors. This is in contrast to the results obtained with the T β RI inhibitor, which blocks both basal and TGF- β -induced invasion. One could speculate that basal invasion depends on other factors induced by TGF- β , whereas MMPs are necessary for overt invasion.

In conclusion, we have established a 3D model for TGF- β -induced invasion. Using this model, we demonstrate that Smad3 and Smad4 are crucial for TGF- β -induced invasion by inducing MMP2 and MMP9. This supports the idea that targeting these molecules might aid in the prevention of metastasis in patients. The 3D assay also offers a valuable screening model system to further study TGF- β /Smad- and MMP2- and MMP9-dependent breast cancer invasion.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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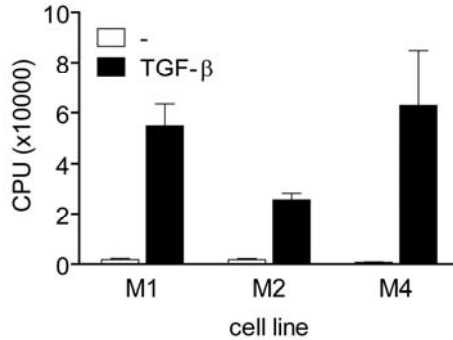
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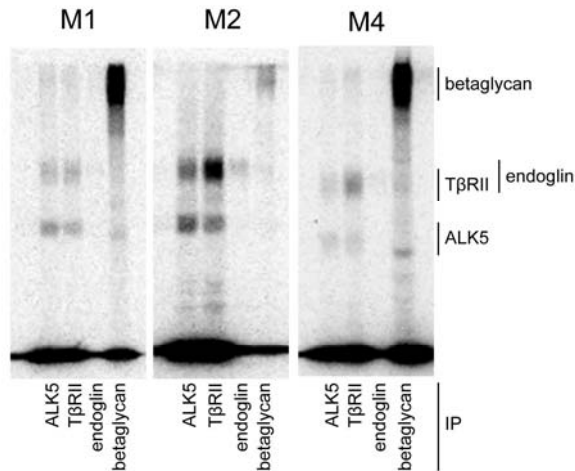
Supplementary materials

Supplementary figures

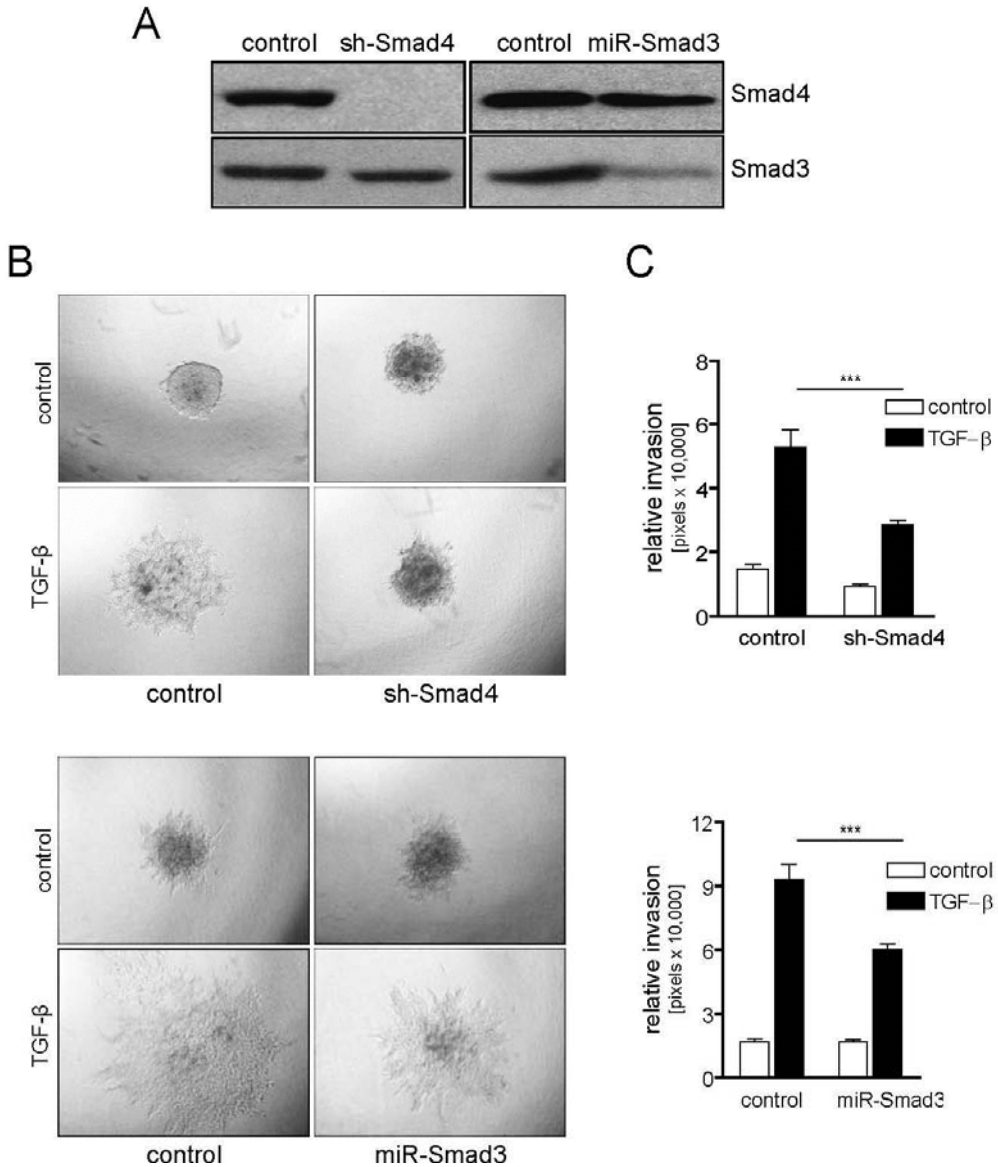
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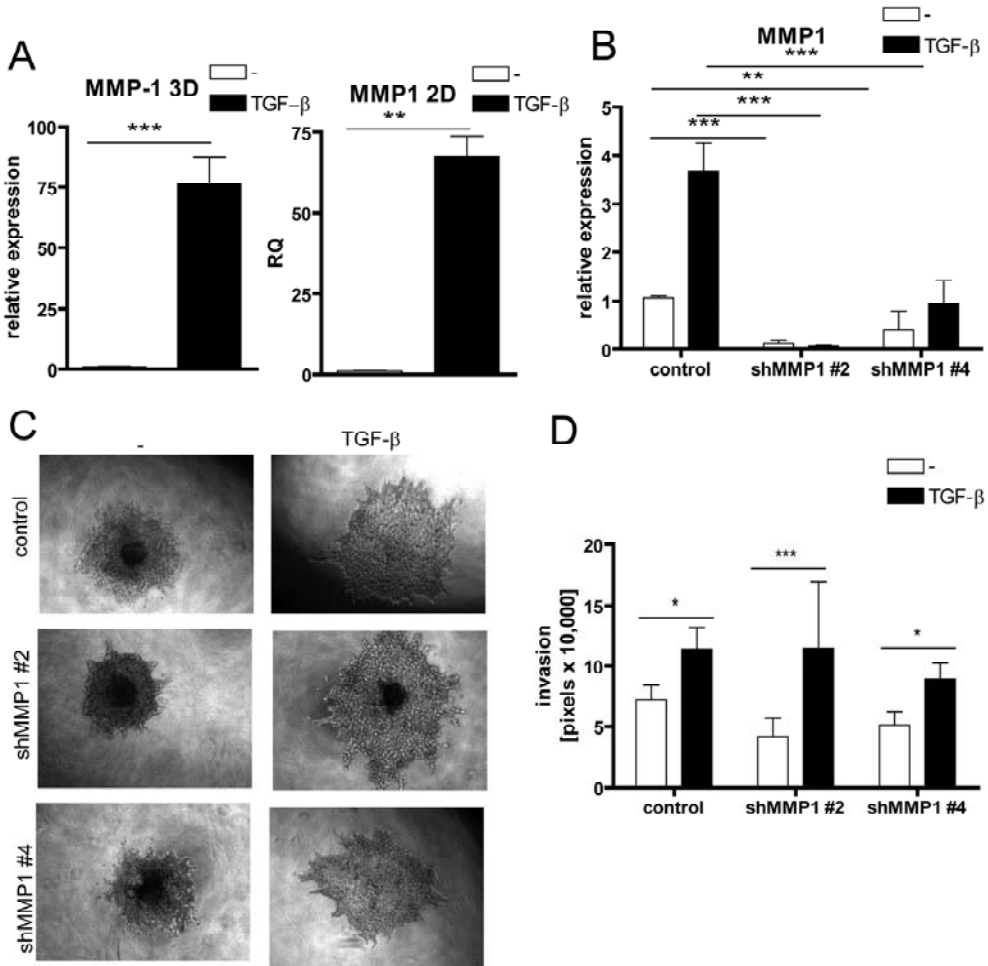
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Supplementary figure 1 A. M1, M2 and M4 cells were transfected with CAGA₁₂-luc transcriptional reporter and subsequently stimulated with 5ng/ml of TGF-β or 20ng/ml of Activin-A for 16h. Relative luciferase activity (CPU) is expressed as mean ± s.d. of triplicate cultures and corrected for differences in transfection efficiency. All three cell lines respond to TGF-β. **B.** TGF-β binds with high affinity to type I, II and III receptors on all three cell lines. Cells were affinity-labeled with [¹²⁵I]TGF-β, subsequently TGF-β-receptor complexes were immunoprecipitated with specific antisera (indicated underneath the scans) and subjected to SDS-PAGE and scanned. TGF-β-receptor cross-linked complexes are indicated on the right side: betaglycan (also termed TGF-β type III receptor), endoglin, TβRII and TβRI. M1 and M4 show intense TGF-β binding to betaglycan, and binding of TβRII is particularly strong in M2. The TGF-β-endoglin complex is only weakly visible. At the bottom (front) of the gel the TGF-β bound to the receptors but not crosslinked is visible.



Supplementary figure 2 TGF- β -induced invasion in M2 is Smad3/4 dependent. Cells were transduced with lentiviruses expressing shRNA against Smad4 or miRNA against Smad3 and the appropriate control viruses. **A**, Protein lysates were separated by SDS-PAGE and subjected to western blot analysis using Smad3- or Smad4-specific antibodies. **B**, **C** Spheroids were embedded into collagen and treated without ligand (n=5) or 5ng/ml of TGF- β (n=5) for 2 days. **B** Representative pictures taken after 2 days. **c** Relative invasion was quantified as area difference on day 2 minus day 0. One representative experiment out of three independent experiments is shown. Significance: ***P<0.001.



Supplementary figure 3 **A** RNA was isolated from spheroids (left graph) or monolayer (right graph) of M4 cells stimulated for 24 hr with or without TGF- β (5ng/ml). Q-PCR for MMP2 and MMP9 was performed using ARP as internal control. **B** M4 cells were transduced with control lentivirus or lentivirus encoding shRNA against MMP1. Cells in a monolayer were stimulated with or without TGF- β (5ng/ml) and RNA was harvested for Q-PCR analysis to determine knockdown. **C, D** Spheroids of M4 cells transduced with control lentivirus were incubated without ligand (n=5) or TGF- β (5ng/ml; n=7). Spheroids of M4 cells transduced with shMMP1 #2 lentivirus were incubated without ligand (n=6) or TGF- β (5ng/ml; n=4). Spheroids of M4 cells transduced with shMMP1 #4 lentivirus were incubated without ligand (n=5) or TGF- β (5ng/ml; n=8). **(C)** Representative pictures taken after 2 days. **(D)** Invasion was quantified as area difference on day 2 minus day 0. Results are expressed as mean $\pm$ s.d.

Supplementary materials and methods

[¹²⁵I]TGF- β receptor binding assay

Iodination of TGF- β 3 was performed according to the chloramine T method followed by affinity-labeling of the cells as described before [1,2]. In brief, cells were incubated on ice for 4 hours with the radioactive ligand, subsequently washed and crosslinked using 54mM disuccinimidyl suberate (DSS) and 3mM bis(sulfosuccinimidyl)suberate (BS3, Pierce) for 15 minutes. Cells were washed, scraped and lysed. Immunoprecipitation was performed using specific receptor antisera that have been previously described [3,4]. Samples were subjected to SDS-PAGE. Gels were dried and scanned with the STORM imaging system (Amersham).

Cell proliferation

Cells were seeded at a density of 5×10^2 cells/well in 96-well plates. The next day, medium was refreshed and TGF- β was added. Cell number was determined at days 0, 1, 2 and 3 by adding MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, Promega), followed by measuring the absorbance at 490 nm.

Transcriptional reporter assay

Cells were seeded in 24-well plates and transiently transfected with CAGA₁₂-luciferase reporter construct [5] using Lipofectamine2000 (Invitrogen) according to manufacturer's protocol or polyethylenimine (Sigma-Aldrich). An expression plasmid encoding β -galactosidase was co-transfected to correct for differences in transfection efficiency. Cells were stimulated overnight with TGF- β 3. Luciferase and β -galactosidase activity were determined as previously described [5]. Each transfection was carried out in triplicate and the mean $\pm$ s.d. is shown.

Western blot analysis

2×10^5 cells were seeded in 6-well-plates. The following day cells were stimulated with 5ng/ml of TGF- β 3 for 16h. Cells were lysed in RIPA-buffer and 20 μ g of protein was subjected to SDS-PAGE and western blotting. Anti-phospho-Smad3 antibody was kindly provided by Dr. Ed Leof (Mayo Clinic, Minnesota, USA); anti-phospho-Smad2, anti-Smad4 and

anti-Smad3 antibodies were obtained from Cell Signaling Technology, Sigma and Zymed Laboratories, respectively.

RNA isolation, cDNA synthesis and quantitative real-PCR

RNA isolation from monolayer was performed using RNA extraction kit (Macherey-Nagel) according to manufacturer's instructions. For RNA isolation from 3D cultures, spheroids embedded in collagen were homogenized in TriPure (Roche), cleared by centrifugation and extracted with phenol-chloroform. The aqueous phase was diluted 1:1 with 70% ethanol, applied to RNA binding mini-columns (Macherey-Nagel) and further processed following manufacturer's instructions. cDNA synthesis and quantitative real time PCR were performed as previously described [6], using a StepOne Plus (Applied Biosystems) instrument and Sybr Green (Applied Biosystems) as detection reagent. All samples were analyzed in triplicate for each primer set. Gene expression levels were determined with the comparative ΔC_t method using *ARP* as reference and the non-stimulated condition was set to 1. Relative expression levels are presented as mean $\pm$ s.d.. The following primers were used: MMP2, forward 5'-AGATGCCTGGAATGCCAT-3' and reverse 5'-GGTTCTCCAGCTTCAGGTAAT-3'; MMP9, forward 5'-TACTGTGCCTTTGAGTCCG-3' and reverse 5'-TTGTGGCGATAAGGAAG-3', ARP forward 5'- CACCATTGAAATCCTGAGTGATGT -3' and reverse 5'- TGACCAGCCGAAAGGAGAAG -3'.

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Chapter 5

BMP-7 inhibits TGF- β -induced invasion of breast cancer cells through inhibition of integrin β_3 expression

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Abstract

The transforming growth factor (TGF)- β superfamily comprises cytokines such as TGF- and Bone Morphogenetic Proteins (BMPs), which have a critical role in a multitude of biological processes. In breast cancer, high levels of TGF- β are associated with poor outcome, whereas inhibition of TGF- β -signaling reduces metastasis. In contrast, BMP-7 inhibits bone metastasis of breast cancer cells. In this study, we investigated the effect of BMP-7 on TGF- β -induced invasion. BMP-7 inhibited TGF- β -induced invasion of the metastatic breast cancer cell line MCF10CA1a, but not of its premalignant precursor MCF10AT in a spheroid invasion model. The inhibitory effect appears to be specific for BMP-7, as its closest homolog, BMP-6, did not alter the invasion of MCF10CA1a spheroids. To elucidate the mechanism by which BMP-7 inhibits TGF- β -induced invasion, we analyzed invasion-related genes. BMP-7 inhibited TGF- β -induced expression of integrin $\alpha_v\beta_3$ in the spheroids. Moreover, targeting of integrins by a chemical inhibitor or knockdown of integrin β_3 negatively affected TGF- β -induced invasion. On the other hand, overexpression of integrin β_3 counteracted the inhibitory effect of BMP7 on TGF- β -induced invasion. Thus, BMP-7 may exert anti-invasive actions by inhibiting TGF- β -induced expression of integrin β_3 .

Key words: breast cancer, invasion, TGF- β , BMP-7, integrin β_3

Abbreviations used throughout the text:

ALK	activin receptor-like kinase
BMP	Bone Morphogenetic Protein
EMT	epithelial to mesenchymal transition
M-II	MCF10AT
M-IV	MCF10CA1a
MMP	matrix metalloproteinase
Smad	Sma and Mad related gene product
TGF- β	Transforming growth factor- β

Introduction

Breast cancer is one of the major causes of death in women. However, most mortality and morbidity does not arise from the primary tumor, but from distant metastasis. High morbidity is especially caused by bone metastasis. In order to metastasize a cancer cell must shed many of its epithelial characteristics, invade the surrounding tissue to enter the circulation, subsequently survive in the circulation, extravasate and proliferate in the metastatic niche [1]. Invasion is therefore a key step in the metastatic cascade. Several classes of proteins have been shown to play a role in this process, such as the integrin family of adhesion receptors. Integrins, which consist of an α and β subunit, are important for adhesion of cells to the extracellular matrix [2]. Cancer cells express specific integrin combinations, whose signaling favors migration and invasion [3]. Integrin $\alpha_v\beta_3$ is strongly expressed by breast cancer cells, especially by cells residing in bone metastasis [4]. Its expression promotes bone metastasis of breast cancer cells [5,6], whereas inhibition of $\alpha_v\beta_3$ diminishes their osteotropism [7].

Transforming Growth Factor (TGF)- β has a dual role in carcinogenesis. In the early stages it has a growth inhibitory and pro-apoptotic effect, whereas at the later stages of cancer, TGF- β promotes invasion and metastasis [8,9]. In line with its stimulatory role in cancer progression, TGF- β is frequently overexpressed in breast cancer and its expression correlates with poor prognosis and metastasis [10-12]. Moreover, studies in mouse models have demonstrated that inhibition of TGF- β -signaling in breast cancer cells reduces metastasis [13-16].

TGF- β and Bone Morphogenetic Proteins (BMPs) signal via comparable mechanisms. Upon binding of the ligand, the type II receptor phosphorylates the type I receptor. The type I receptor, in turn, phosphorylates the receptor-regulated (R)- Smad and Mad related gene product (Smad). Phosphorylated R-Smads form heteromeric complexes with the common mediator Smad (Co-Smad) Smad4. These complexes then translocate to the nucleus, where they regulate transcription of target genes in collaboration with other transcription factors [17]. In most cell types, TGF- β signals through the TGF- β receptor type II (T β RII)

and the TGF- β type I receptor, also termed activin receptor-like kinase 5 (ALK5). ALK5 mediates the phosphorylation of Smad2 and Smad3 [18]. BMPs signal through their specific BMP receptor type II (BMPRII) and BMP type I receptors (BMPRI) which induce the activation of Smad1, Smad5 and Smad8 [19]. In addition to the previously described Smad pathways, receptor activation results in activation of several other non-Smad signaling pathways, for example Mitogen Activated Protein Kinase (MAPK) pathways [20].

TGF- β is thought to be pro-invasive by inducing epithelial-to-mesenchymal transition (EMT). During this process, carcinoma cells acquire a more motile, mesenchymal phenotype [21]. In normal breast and kidney epithelial cells BMP-7 can inhibit TGF- β -induced EMT [22]; BMP-7 has also been shown to inhibit EMT and bone metastasis in breast and prostate tumors [23,24]. However, the effect of BMP-7 on tumor invasion is unknown.

Previously, we have set up a spheroid invasion model to study TGF- β -induced invasion [25,26]. In this assay, spheroids made from MCF10A series of cell lines invade in response to TGF- β . The MCF10A cell lines originate from MCF10A1, a spontaneously immortalized breast cell line [27]. This cell line was transformed with oncogenic RAS, resulting in MCF10AT (hereafter referred to as M-II), which forms premalignant lesions in mice that upon grafting progressed into carcinoma [28]. From these carcinomas MCF10CA1a was isolated (hereafter referred to as M-IV), which metastasizes to the lung [29]. Using these cell lines in the invasion assay, we have shown the importance of Smad signaling for TGF- β -induced invasion [25]. In the current paper, we have analyzed the role of BMP-7. We show that BMP-7 can inhibit TGF- β -induced invasion through inhibition of TGF- β -induced integrin β_3 expression.

Materials and methods

Cell culture

MCF10AT (M-II) and MCF10CA1a (M-IV) were obtained from Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, USA) and maintained as described [25].

Invasion spheroid assays

The invasion spheroid assay was performed essentially as described in [25]. Briefly, 10^3 cells were allowed to form spheroids and spheroids were embedded in a 1:1 collagen:metacel mixture onto a collagen coated plate. Recombinant human TGF- β 3 (a generous gift of Dr. K. Iwata, OSI Pharmaceuticals, Inc, New York, USA), BMP-6, BMP-7 (both a kind gift of K. Sampath, Creative Biomolecules, Inc, Hoptinton, USA), isotype control antibody (Santa Cruz) or human BMP-7 neutralizing antibody (R&D Systems) were added directly into the collagen:metacel mixture at 4 °C. After 30 minutes incubation at 37 °C medium was added on top of the collagen, either containing no solvent, DMSO or the integrin inhibitor GLPG0187 (Galapagos NV, Mechelen, Belgium). Invasion was monitored during the next two days and quantified by measuring the area using ImageJ or Adobe Photoshop.

Western blot analysis

2×10^5 cells were seeded onto a six well plate and one day later starved for 16 hr in medium containing 2.5% horse serum (Gibco), 10ng/ml epidermal growth factor (EGF) (Upstate), 50ng/ml cholera toxin (Calbiochem), 0.25 μ g/ml hydrocortisone (Sigma), 5 μ g/ml insulin (Sigma), 100U/ml penicillin and 50 μ g/ml streptomycin (Gibco). After starvation, cells were stimulated, washed and lysed in RIPA buffer or sample buffer. Lysates were subjected to SDS-PAGE & Western blotting. Phosphorylation of Smad1/5/8 was detected by anti phospho-Smad1 antibody as described before [30]. Phospho-Smad2 antibody was purchased from Cell Signaling. Total Smad1 antibody was from Zymed.

Transcriptional reporter assay

1×10^5 cells were seeded onto a 24-well plate and one day later transfected for 4 hr with the reporters CAGA₁₂-Luc [31] or BRE-Luc [32] using either Lipofectamine 2000 or LTX (Invitrogen). An expression plasmid for β -galactosidase was co-transfected and used to correct for transfection efficiency. One day after transfection, cells were serum starved overnight in medium containing 2.5% horse serum (Gibco), 10ng/ml epidermal growth factor (EGF) (Upstate), 50ng/ml cholera toxin (Calbiochem), 0.25 μ g/ml hydrocortisone

(Sigma), 5 µg/ml insulin (Sigma), 100U/ml penicillin and 50µg/ml streptomycin (Gibco) and subsequently stimulated for 8 hr. Luciferase and β-galactosidase activity were determined as previously described [31]. Each transfection was carried out in triplicate and representative experiments are shown.

RNA isolation, cDNA synthesis and quantitative PCR

RNA isolation, cDNA synthesis and quantitative PCR (Q-PCR) were performed as previously described [25]. The following primers were used: acidic ribosomal phosphoprotein (ARP) forward 5'- CACCATTGAAATCCTGAGTGATGT -3' and reverse 5'- TGACCAGCCGAAAGGA-GAAG -3' ; matrix metalloproteinase 2 (MMP2) forward 5'-AGATGCCTGGAATGCCAT-3' and reverse 5'-GGTCTCCAGCTTCAGGTAAT-3'; integrin α_v forward 5'-CAGGCTTGCAACCCATTCTT-3' and reverse 5'-GAATGTGAGCCTGTGCGACTAATGTT-3'; integrin β₃ forward 5'-ACCTGCTTGCCCATGTTTG-3' and reverse 5'-GCGGGTCACCTGGTCAGTTA-3'. All samples were analyzed in duplicate for each primer set. Gene expression levels were determined with the comparative ΔCt method using *ARP* as reference and the non-stimulated condition was set to 1. Relative expression levels are presented as mean ± S.D.

Lentiviral transduction

Constructs TRCN0000003234, TRCN0000003235, TRCN0000003236 and TRCN0000003237 from the Mission library (Sigma) were used to target integrin β₃ with construct SHC001 as a negative control. Cells were transduced in the presence of 4 µg/ml polybrene (Sigma) overnight. After recovery cells were selected and maintained in growth medium containing 0.5 µg/ml puromycin. For overexpression of integrin β₃, M-IV cells were transduced with a lentiviral integrin β₃ expression construct [33], kindly provided by Dr. Deborah Novack, Washington University, St. Louis, USA.

Statistical analysis

All results are expressed as the mean ± S.D. Statistical differences were examined by one-way ANOVA followed by Bonferroni's multiple comparison test. *P*<0.05 was considered as statistically significant.

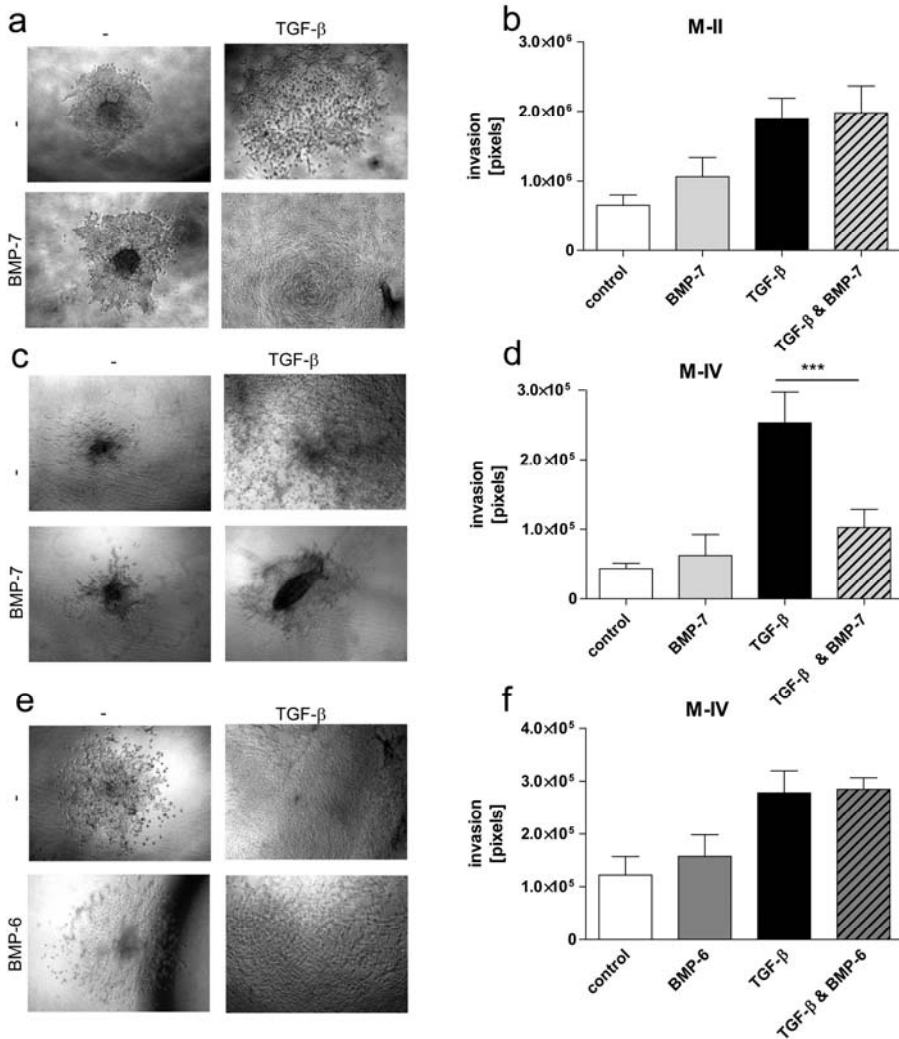


Fig. 1 BMP-7, but not BMP-6, inhibits TGF- β -induced invasion in M-IV, but not in M-II. (a-d) Spheroids were allowed to invade collagen in the presence of BMP-7 (100 ng/ml), TGF- β (5 ng/ml) or TGF- β & BMP-7. (a) Pictures of representative spheroids of M-II. (b) Quantification of invasion of M-II experiments shown in (a). (c) Pictures of representative spheroids of M-IV. (d) Quantification of invasion of M-IV experiments shown in (c). (e) Spheroids of M-IV were allowed to invade collagen in the presence of BMP-6 (100 ng/ml), TGF- β (5 ng/ml) or TGF- β & BMP-6. Pictures of representative spheroids are shown. (f) Quantification of invasion of experiments shown in (e). The results are expressed as mean $\pm$ S.D. of at least three spheroids per condition. One representative out of three independent experiments is shown. Significance *** $p < 0.001$

Results

BMP-7 inhibits TGF- β -induced invasion in the metastatic cell line M-IV, but not in the premalignant M-II cell line

BMP-7 inhibits TGF- β -induced EMT of both normal breast cells [22], as well as of invasive, osteotropic breast and prostate cancer cells, which is associated with inhibition of bone metastasis [23,24]. To investigate whether BMP-7 also has an inhibitory effect on TGF- β -induced invasion, we analysed its effect on M-IV in a spheroid invasion assay. The response of this metastatic cell line was compared to a precursor cell line, the RAS-transformed cell line M-II and not M-I, since RAS has been reported to enhance TGF- β -induced responses [34]. In the absence of TGF- β , BMP-7 had no inhibitory effect on the invasion of M-II and M-IV cells, while TGF- β induced invasion of both cell types (Figure 1 a-d). However, BMP-7 strongly inhibited the TGF- β -induced invasion of the metastatic M-IV cells, but not of the premalignant M-II cells (Figure 1a-d). This suggests that BMP-7 specifically inhibits TGF- β pro-invasive mechanisms exploited by metastatic cells.

BMP-7 does not affect proliferation in M-IV cells

To rule out that the observed effects of BMP-7 on TGF- β -induced invasion are due to effects on cell growth, we performed proliferation assays. As reported previously [25], TGF- β has a minor growth-inhibitory effect on M-IV. BMP-7 on its own did not affect cell proliferation (Supplementary Figure 1). BMP-7 also did not affect TGF- β -induced growth inhibition. Thus, the inhibitory effect of BMP-7 on TGF- β -induced invasion is not due to effects on the growth of these cells.

BMP-7, but not BMP-6, inhibits TGF- β -induced invasion in M-IV

To assess whether other BMPs are capable of inhibiting TGF- β -induced invasion, we studied the effect of BMP-6, which is the closest homolog of BMP-7 and shares 73% identity on the amino acid level. In contrast to BMP-7, BMP-6 did not affect the TGF- β -induced invasion of M-IV cells, and also did not inhibit basal invasion. (Figure 1e,f). These data

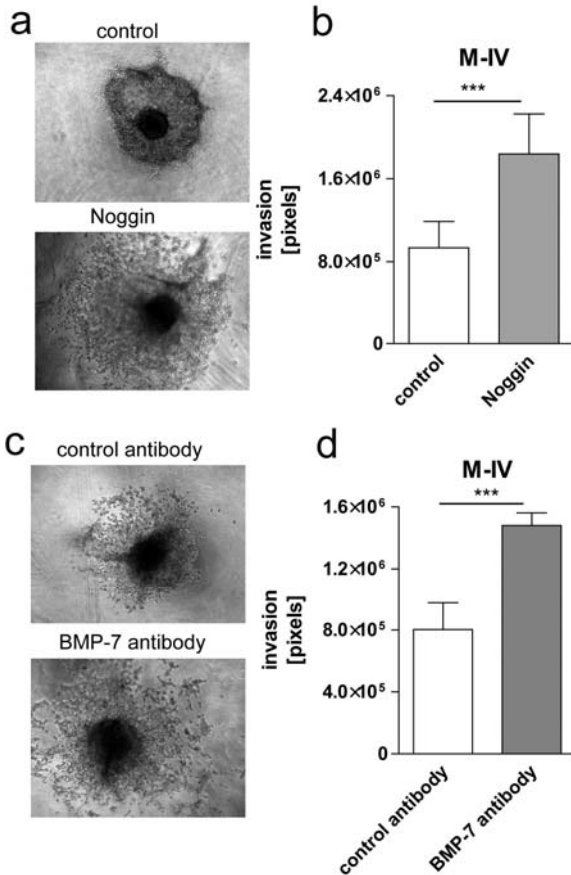


Fig. 2 Noggin enhances basal invasion through inhibition of BMP-7. Spheroids of M-IV were allowed to invade collagen in the absence or presence of Noggin (200 ng/ml). (a) Pictures of representative spheroids. (b) Quantification of spheroids of experiments shown in (a). (c) Spheroids of M-IV were allowed to invade into collagen in the presence of control antibody or BMP-7 blocking antibody (2 μ g/ml). Representative pictures are shown. (d). Quantification of spheroids of experiments shown in (c). The results are expressed as mean $\pm$ S.D. of at least four spheroids per condition. One representative out of three independent experiments is shown. Significance *** $p < 0.001$

indicate that the ability of BMP-7 to inhibit TGF- β -induced invasion is specific for BMP-7.

Noggin induces invasion in M-IV through blocking of BMP-7

Previous studies of our group have shown that the basal invasion of M-IV cells is dependent on TGF- β -signaling [25]. However we did not observe an inhibitory effect of BMP-7 addition on the basal invasion (Figure 1c,d). One explanation for this might be that BMP-7 is already present at saturating levels under these conditions. Indeed, we found that M-IV cells express BMP-7 mRNA and that the horse serum used in the assays contained BMP-7 protein (data not shown). Therefore, we tested the effect of a natural antagonist of BMP-2, 4 and 7, Noggin. As shown in Figure 2a and b, Noggin enhanced basal invasion 2.0-fold in M-IV. In the M-II cell line, in which TGF- β -induced invasion is not affected by BMP-7, addition of Noggin also had no effect on basal invasion (Supplementary Figure 2). Thus, inhibition of BMP-2, 4 and 7 induces invasion in M-IV cells, but not in M-II cells.

Since Noggin inhibits BMP-2, 4 and 7, we next determined if blockade of BMP-7 can also induce basal invasion of M-IV cells. For this, we added BMP-7 neutralizing antibodies to the spheroid invasion system. These antibodies enhanced basal invasion 1.8-fold compared to the control antibody (Figure 2c,d). This indicates that the induction of invasion by Noggin is mainly due to inhibition of BMP-7. Moreover, it suggests that TGF- β and BMP-7 also antagonize each other under basal conditions.

Both BMP-6 and BMP-7 induce Smad signaling

Next, we asked whether if the different effects of BMP-6 and BMP-7 on TGF- β -induced invasion in M-IV cells could be due to the possibility that BMP-7 is more potent in inducing BMP-signaling than BMP-6. To examine this, we analyzed activation of BMP-Smad signaling by two means. First, we analyzed whether BMP-6 and BMP-7 differ in their ability to induce phosphorylation of Smad1. However, Western blot analysis showed that Smad1 phosphorylation is induced to the same extent by both BMPs in M-IV cells, whereas the total levels of Smad1 were not affected (Figure 3a). Second, we investigated transcriptional activation of BMP-Smads, by using a luciferase-reporter driven by BMP Responsive Elements (BRE) [32]. Both BMP-6 and BMP-7 were found to activate the reporter ~30 fold and thus efficiently induced BMP-Smad transcriptional activity in M-IV cells (Figure 3b). Taken together, these results indicate that BMP-6 and BMP-7 do not differ in their ability to induce Smad signaling in this cell line.

BMP-7 does not affect TGF- β -induced Smad signaling

To investigate the molecular mechanism by which BMP-7 inhibits TGF- β -induced invasion, we analyzed the effects of BMP-7 and BMP-6 on TGF- β Smad signaling. First, we analyzed whether BMP-7 had an inhibitory effect on the phosphorylation of Smad2. However, both BMP-7 and BMP-6 did not affect TGF- β -induced Smad2 phosphorylation (Figure 3a). Second, we studied the effect of BMP-7 on TGF- β -induced Smad3 transcriptional activity. To this end, we used a luciferase reporter containing 12 repeats of the Smad3 binding sequence CAGA [31]. TGF- β induced the activity of this reporter ~55-fold, but both BMP-7 and BMP6 did not significantly affect the TGF- β -induced reporter activity (Figure 3c). Thus, BMP-7 has no effect on TGF- β -induced Smad signaling in M-IV cells.

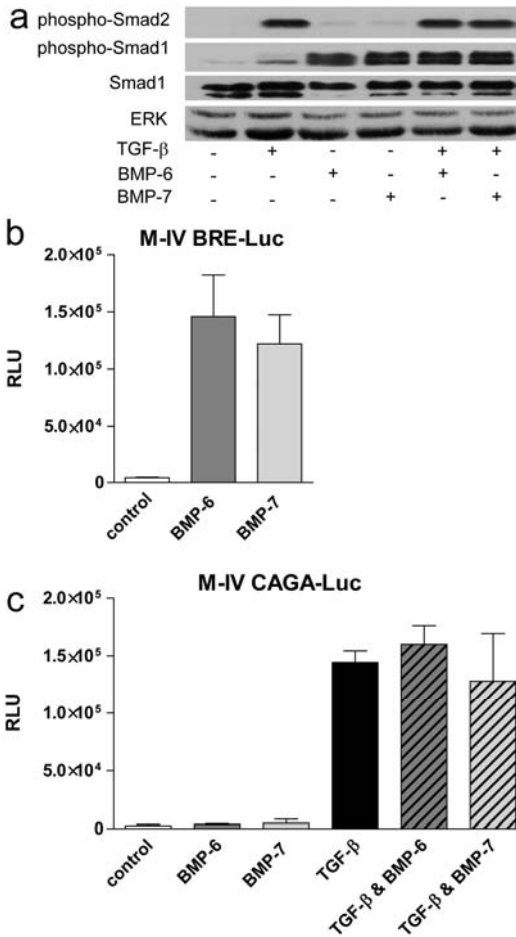


Fig. 3 BMP-6 and BMP-7 induce BMP-Smad signalling, but do not affect TGF- β Smad signalling. (a) Cells were treated with the indicated ligands (BMP-6 and BMP-7 at 100 ng/ml, TGF- β at 5 ng/ml) for 15 min, lysed and subjected to SDS-PAGE and Western blotting. Blots were incubated with phospho-Smad2, phospho-Smad1 and Smad1 antibodies. Total ERK was used as a loading control (b) Cells were transfected with the BRE-Luc reporter construct, starved and subsequently stimulated for 8 hr with BMP-6 or 7 (both at 100 ng/ml). Relative luciferase activity (RLU) is expressed as mean $\pm$ S.D. of triplicate cultures. (c) Cells were transfected with the CAGA₁₂-Luc reporter construct, starved and subsequently stimulated for 8 hr with the indicated ligands (BMP-6 and BMP-7 at 100 ng/ml, TGF- β at 5 ng/ml). Relative luciferase activity (RLU) is expressed as mean $\pm$ S.D. of triplicate cultures. One representative out of at least three independent experiments is shown

BMP-7 inhibits TGF- β -induced integrin $\alpha_v\beta_3$ expression

We next examined the effect of BMP-7 on the expression of TGF- β target genes involved in invasion. As we previously identified a critical role for matrix metalloproteinase 2 (MMP2) in TGF- β -induced invasion [25], we first analyzed if TGF- β -induced MMP2 expression is affected by BMP-7 in the spheroid model system. BMP-6 stimulation served as a negative control. However, as shown in Figure 4a, BMP-7 (and BMP-6) did not affect TGF- β -induced expression of MMP2 in M-IV spheroids in collagen. Since integrin $\alpha_v\beta_3$ has recently been shown to enhance TGF- β -induced EMT and inhibition of integrin $\alpha_v\beta_3$ reduces metastasis [7], we subsequently examined the expression of integrin α_v and β_3 . Importantly, TGF- β potently induced the expression of both subunits in the collagen-embedded spheroids, and BMP-7, but not BMP-6, inhibited the TGF- β -induced

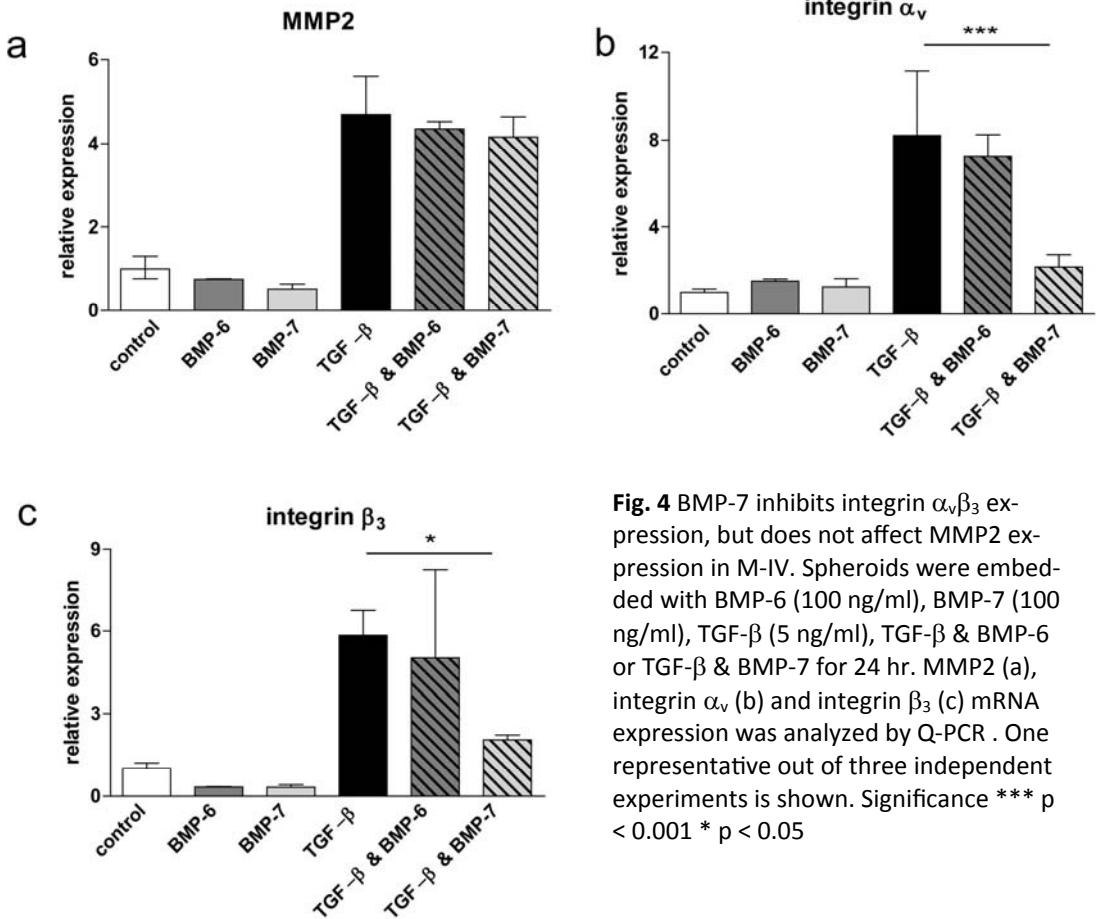


Fig. 4 BMP-7 inhibits integrin $\alpha_v\beta_3$ expression, but does not affect MMP2 expression in M-IV. Spheroids were embedded with BMP-6 (100 ng/ml), BMP-7 (100 ng/ml), TGF- β (5 ng/ml), TGF- β & BMP-6 or TGF- β & BMP-7 for 24 hr. MMP2 (a), integrin α_v (b) and integrin β_3 (c) mRNA expression was analyzed by Q-PCR. One representative out of three independent experiments is shown. Significance *** $p < 0.001$ * $p < 0.05$

expression of both α_v and β_3 (Figure 4b and c). Also, BMP-7 did not inhibit TGF- β -induced integrin α_v and β_3 expression in M-II (Supplementary Figure 3a,b). Thus, the inhibitory action of BMP-7 on TGF- β -induced collagen invasion of M-IV spheroids might be mediated through inhibition of TGF- β -induced integrin $\alpha_v\beta_3$ expression.

Integrin β_3 is necessary for TGF- β -induced invasion

To determine if integrin $\alpha_v\beta_3$ is important for TGF- β -induced invasion, we investigated if inhibition of α_v integrins blocks TGF- β -induced invasion in the spheroid model system. For this, we used a non-peptide integrin inhibitor, GLPG0187. This molecule exhibits high affinity for $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$ as well as $\alpha_5\beta_1$ in *in vitro* competitive binding assays (Supplementary Table 1). Treatment of M-IV spheroids with GLPG0187 inhibited TGF- β -induced invasion (Figure 5a,b). This indicates that integrin $\alpha_v\beta_3$ could be necessary for TGF- β -induced invasion of M-IV cells.

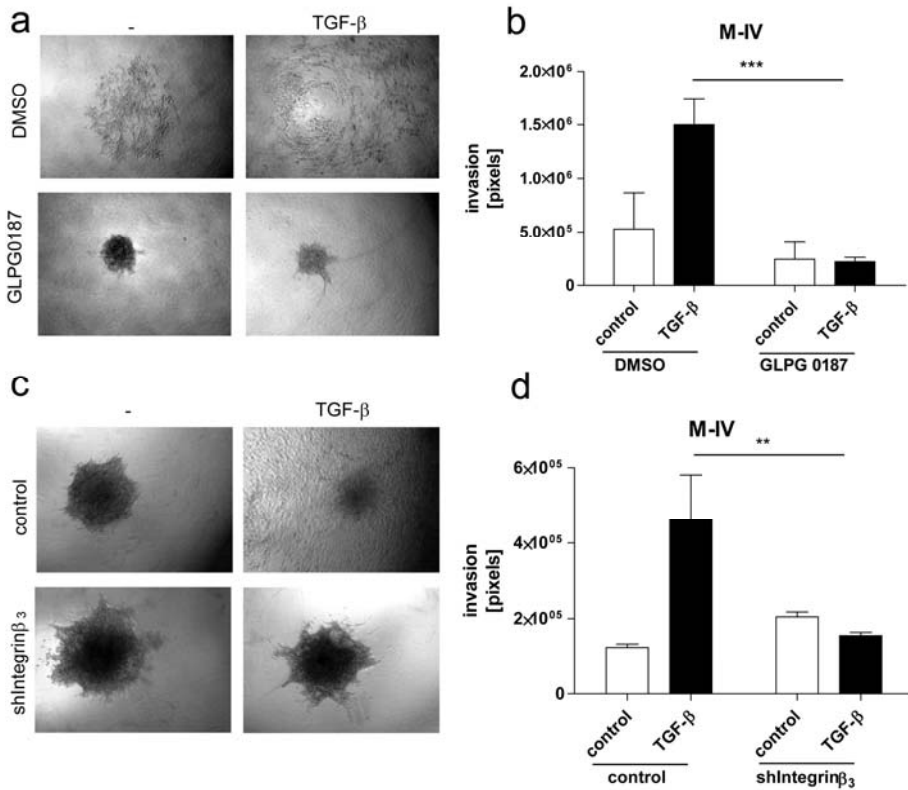


Fig. 5 Integrin $\alpha_v\beta_3$ is necessary for TGF- β -induced invasion. (a) Spheroids were allowed to invade into collagen in the presence of DMSO or GLPG0187 (80 nM) with or without TGF- β (5 ng/ml). Representative pictures of spheroids are shown. (b) Quantification of experiment shown in (a). (c) Cells were transduced with lentivirus encoding shRNA against integrin β_3 and control lentivirus.. Spheroids of these cells were allowed to invade in the absence or presence of TGF- β (5 ng/ml). Representative pictures of spheroids are shown. (d) Quantification of invasion shown in (c). At least four spheroids per condition. were quantified. One representative out of three independent experiments is shown. Significance ** $p < 0.01$

To further establish if integrin β_3 is necessary for TGF- β -induced invasion, we performed RNAi-mediated knockdown experiments. M-IV cells were transduced with viruses encoding 4 different shRNA constructs against integrin β_3 . These constructs inhibited TGF- β -induced integrin β_3 expression at the mRNA level (Supplementary Figure 4a). TGF- β -induced invasion was indeed inhibited by knockdown of integrin β_3 (Figure 5c,d), indicating that downregulation of integrin β_3 by BMP-7 contributes to BMP-7-mediated inhibition of invasion.

Overexpression of integrin β_3 rescues TGF- β -induced invasion from BMP-7 inhibition

To demonstrate that the inhibition of integrin β_3 expression by BMP-7 is critical for the inhibition of TGF- β -induced invasion, we used a lentiviral vector that overexpresses integrin β_3 [33]. As shown in Supplementary Figure 4b, M-IV cells transduced with this virus exhibit higher levels of integrin β_3 protein compared to wildtype (WT) cells. The overexpression of integrin β_3 did not affect TGF- β -induced invasion, but completely counteracted the BMP-7 inhibitory effect (Figure 6). Taken together, these data suggest that BMP-7 inhibits TGF- β -induced invasion by inhibiting TGF- β -induced integrin β_3 expression.

Discussion

In order to understand how BMP-7 exerts its anti-metastatic effects reported before [23,24], we investigated whether BMP-7 interferes with TGF- β -induced invasion of human breast cancer cells. We showed that BMP-7 inhibits TGF- β -induced invasion in the metastatic breast cancer cell line M-IV through inhibition of integrin β_3 expression. In addition, we showed that BMP-7 specifically inhibits the invasion of this metastatic cell line, and not the invasion of its premalignant precursor, M-II. Moreover, the inhibitory effect was specific for BMP-7, as its closest homolog, BMP-6 did not have any effect on TGF- β -induced invasion. To our knowledge, this is the first report that shows that BMP-7 inhibits TGF- β -induced invasion.

Since the antagonism between TGF- β and BMP-7 has been described before [22-24,35-37], the question arises whether the process that we describe occurs in other cell types as well. BMP-7 inhibits bone metastasis of the MDA-MB231 breast cancer cell line [23] and PC3 prostate cancer cell line [24]. However, in these cell lines BMP-7 inhibited TGF- β /Smad signaling [23,24], whereas under our experimental conditions we did not observe an inhibitory effect of BMP-7 on the TGF- β -Smad pathway. On the other hand, BMP-7 inhibited tumor progression of the uveal melanoma cell line OCM-1 without inhibiting the TGF- β -Smad pathway [37]. Thus, BMP-7 is able to inhibit the tumor-promoting effects of TGF- β through either Smad- and non-Smad- mechanisms, depending on the cellular context.

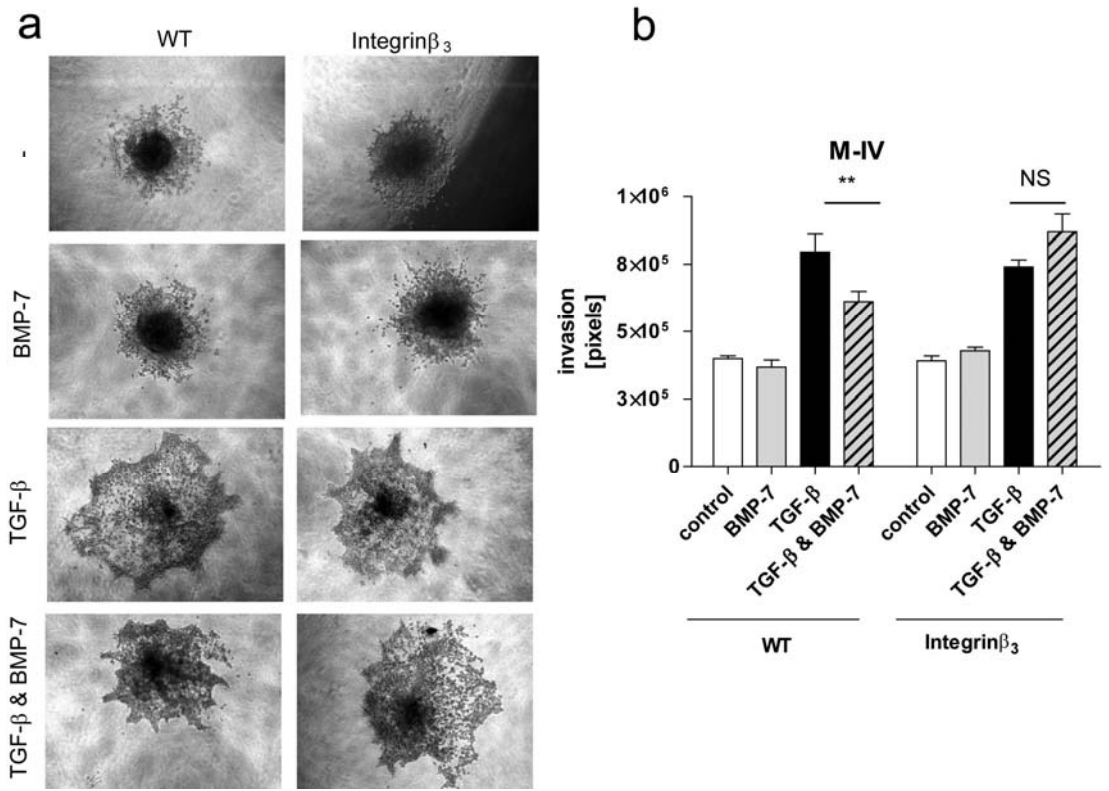


Fig. 6 Integrin β_3 overexpression rescues TGF- β -induced invasion from BMP-7 inhibition. Cells were transduced with lentivirus containing an integrin β_3 overexpression construct. Integrin β_3 overexpressing cells or wildtype (WT) cells were allowed to invade in BMP-7 (100 ng/ml), TGF- β (5 ng/ml) or TGF- β & BMP-7. (a) Representative pictures of spheroids are shown. (b) Quantification of spheroid experiment shown in (a). The results are expressed as mean $\pm$ S.D. of at least four spheroids per condition. One representative out of three independent experiments is shown. Significance ** $p < 0.01$ NS not significant.

Our data indicate that the inhibitory effect of BMP-7 on breast cancer invasion in part can be explained by inhibition of TGF- β -induced expression of integrin β_3 . Integrin β_3 is induced by TGF- β and enhances EMT in a feed-forward loop by inducing Src [38]. It is therefore tempting to speculate that the inhibition of TGF- β -induced integrin β_3 expression by -7 in EMT.

As mentioned before we did not detect a significant effect of BMP-7 on TGF- β Smad signaling, although we have shown that TGF- β -induced invasion is dependent on Smad3 and Smad4 [25]. Since TGF- β -induced integrin β_3 expression has been reported to be dependent on p38, but not on Smads [39], the inhibitory effect of BMP-7 on TGF- β -induced

invasion might be mediated via non-Smad pathways. However, we could not find a significant effect of BMP-7 on (TGF- β -induced) p38 phosphorylation that could explain the inhibition of BMP-7 on TGF- β -induced integrin β_3 expression (data not shown). It is therefore likely that the effect is mainly due to BMP-7 transcriptional targets, such as transcription factors or microRNAs, that interfere with TGF- β -induced integrin β_3 expression.

One striking finding is that BMP-7 is able to inhibit TGF- β -induced invasion, whereas BMP-6 is not, despite the fact that they share 73% amino acid homology and are able to bind to the same receptors [40-43]. Generally, BMP-6 is regarded as more potent than BMP-7 in inducing bone differentiation [40,44,45]. However, BMP-7 is inhibited by Noggin, whereas BMP-6 is resistant to inhibition by Noggin due to an additional lysine at residue 60 [45]. On the other hand, we found that BMP-6 and BMP-7 induce Smad1 phosphorylation and BMP-Smad transcriptional activity to the same extent in MIV cells. Possibly, BMP-6 or BMP-7 have different effects on non-Smad signaling pathways. However, we did not observe any significant differences between BMP-7 and BMP-6 on (TGF- β -induced) p38 and ERK phosphorylation in MIV cells (data not shown).

BMP-7 did not inhibit TGF- β -induced invasion of the premalignant precursor cell line M-II. Interestingly, overexpression of a dominant-negative TGF- β -receptor also had differential effects on these two cell lines *in vivo* [46]. It is likely that upon tumor progression the response to TGF- β is altered in such a manner that BMP-7 is able to inhibit certain TGF- β responses. On the genetic level M-IV has an extra copy of the long arm of chromosome 1, a type of alteration often observed in breast cancer [29]. A detailed survey of the genetic changes in the MCF10A series of cell lines revealed several genetic changes in M-IV compared to M-II, as well as differences in global gene expression [47]. It is likely that the ability of BMP-7 to inhibit TGF- β -induced invasion is highly dependent on these additional genetic changes.

In conclusion, we have shown that BMP-7 inhibits TGF- β -induced invasion through inhibition of TGF- β -mediated integrin β_3 expression. These data reinforce the rationale to either use BMP-7 or target integrin β_3 in breast cancer metastasis.

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Ethical standards

All experiments comply with the current laws of the country in which they were performed.

Conflict of interest

B.H. is employed at Galapagos SASU, which has a financial interest in GLPG0187.

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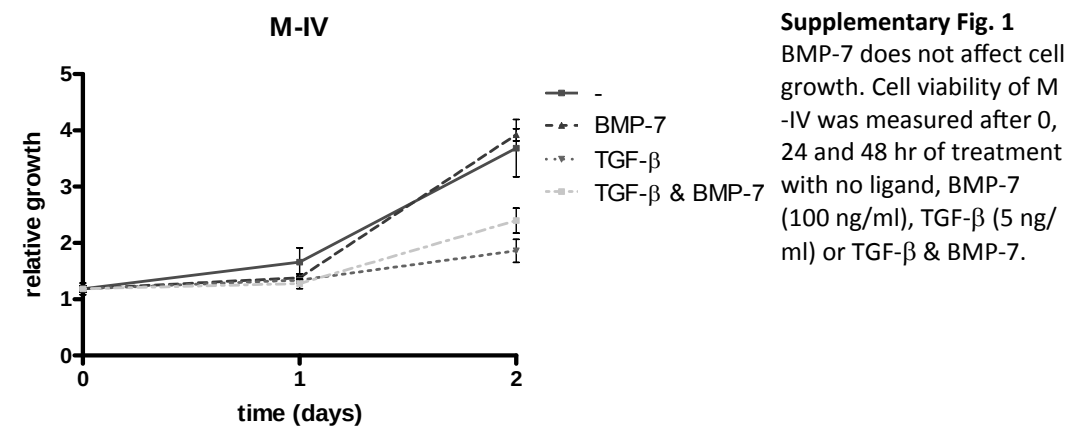
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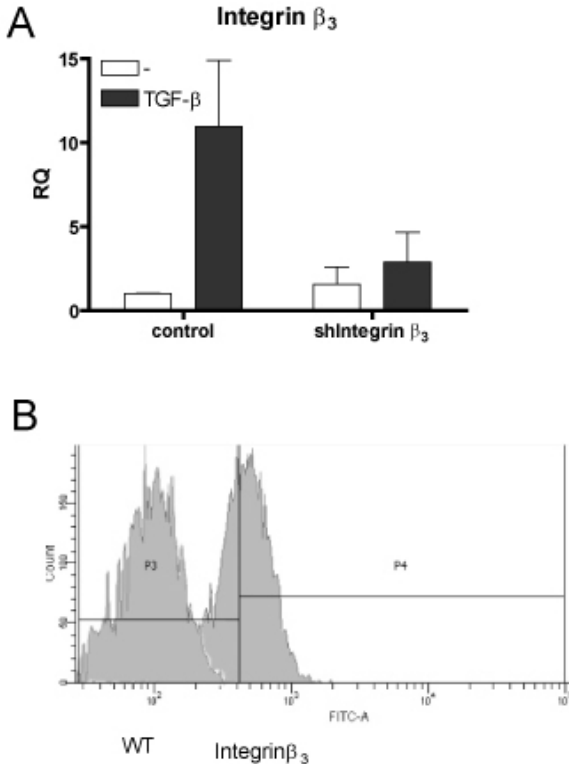
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Supplementary figures



	$\alpha_v\beta_1$	$\alpha_v\beta_3$	$\alpha_v\beta_5$	$\alpha_v\beta_6$	$\alpha_v\beta_8$	$\alpha_5\beta_1$	$\alpha_2\beta_1$	$\alpha_4\beta_7$	$\alpha_{IIb}\beta_3$
GLP G018 7	1.3 $\pm$ 0.1	3.7 $\pm$ 0.6	2.0 $\pm$ 0.6	1.4 $\pm$ 0.3	1.2 $\pm$ 0.3	7.7 $\pm$ 4.0	>10 ⁴	>10 ⁴	>10 ⁵

Supplementary Table 1
Specificity of GLPG0187 towards integrins. GLPG0187 was profiled in a solid phase assay for its ability to inhibit integrin-ligand interactions for integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_5\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_7$ and $\alpha_{IIb}\beta_3$. The table summarizes IC₅₀ values for GLPG0187 in nM. Data are expressed as mean $\pm$ S.E.M. from three independent experiments.



Supplementary Fig. 2 Confirmation of knockdown and overexpression of integrin β_3 . **(A)** M-IV cells were transduced with control lentivirus or a mixture of lentivirus encoding shRNA against integrin β_3 and selected with puromycin. Cells were treated with or without TGF- β (5 n/gml) for 24 hr before RNA was isolated and analyzed by Q-PCR for integrin β_3 expression. **(B)** M-IV cells were transduced with lentivirus encoding integrin β_3 and subjected to FACS analysis.

Supplementary material & methods

Cell proliferation

Cells were seeded at a density of 5×10^2 cells/well in 96-well plates. The next day, medium was refreshed and TGF- β was added. Cell number was determined at days 0, 1, 2 and 3 by adding MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, Promega), followed by measuring the absorbance at 490 nm.

Integrins and integrin ligands

Integrin $\alpha_2\beta_1$ was purified from detergent extracts of HT1080 fibrosarcoma cells as previously described (1). Recombinant integrin $\alpha_4\beta_7$ was purchased from R&D systems (Oxford, UK). Recombinant integrin $\alpha_5\beta_1$ was made as an Fc fusion protein as described before (2). Recombinant α_v integrins were made using a baculovirus expression system in insect cells.

Rat tail tendon collagen was obtained from Sigma-Aldrich (Poole, Dorset, UK) and biotinylated as described (1). Recombinant murine MAdCAM-1 Fc fusion protein was made in COS

cells. A recombinant fragment of human fibronectin containing type III repeats 6-10 was produced and biotinylated as described (3). Purified human vitronectin was obtained from Sigma-Aldrich. Recombinant human LAP (latency associated protein) was purchased from R&D systems, and biotinylated using sulfo-NHS-LC-biotin (Pierce) according to the manufacturer's protocols.

Solid phase assay

For solid phase assays the binding of ligands to integrins was measured in the presence of 1mM Mn^{2+} to activate the receptors. In brief, 96-well were coated with integrins (1-5 μ g/ml in Dulbecco's PBS) overnight at room temperature. Wells were then blocked for 1-3 h with 5% (w/v) BSA, 150 mM NaCl, 0.05% (w/v) NaN_3 , 25 mM Tris-Cl, pH 7.4. Wells were then washed three times with 200 μ l of 150 mM NaCl, 25 mM Tris-Cl, 1 mM $MnCl_2$, pH 7.4, containing 1 mg/ml BSA (buffer A). Ligands in buffer A were added to the wells in the absence or presence of inhibitors, and the plate was then incubated at room temperature for 2 h. The wells were washed three times with buffer A. For biotinylated ligands, bound ligand was quantitated by addition of 1:500 dilution of ExtrAvidin peroxidase conjugate (Sigma-Aldrich) in buffer A for 30 min at room temperature. For MADCAM-Fc, bound ligand was quantitated by addition of 1:1000 dilution of anti-human Fc peroxidase conjugate (Jackson) 30 min at room temperature. For vitronectin, bound ligand was quantitated by addition of 1:500 dilution of anti-human vitronectin antibody VIT-2 (Sigma-Aldrich) for 30 min at room temperature followed by 1:1000 dilution of anti-human IgM peroxidase conjugate (Jackson) for 30 min at room temperature. Wells were then washed four times with buffer A and the assay was developed using ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt), followed by measuring absorbance at 410 nm. Background binding of ligands to wells coated with BSA alone was subtracted from all measurements. IC_{50} = concentration of inhibitor for 50% of maximal inhibition of binding of ligand binding to the integrin used in the assay.

FACS analysis

Cells were detached by EDTA, filtered, washed and incubated with anti-integrin β_3 antibody (Abcam) or no antibody in 2% FBS/PBS for 1 hr at 4 C. Cells were washed and

incubated with anti-mouse-FITC (DAKO) for 30 minutes at 4°C and washed. Surface expression was measured on a FACSCanto (B&D).

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Chapter 6

Key regulators of EMT are essential for TGF- β -induced invasion by promoting single cell invasion

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In preparation

Abstract

TGF- β plays a dual role in cancer; in early stages it inhibits tumour growth, whereas in later stages it promotes invasion and metastasis. Recent data indicate that TGF- β promotes invasion of single cells, which move faster and are able to enter the bloodstream, in contrast to their collectively invading counterparts. TGF- β is thought to be pro-invasive by inducing epithelial-to-mesenchymal transition (EMT). Key players in EMT are transcriptional repressors, such as Slug, Snail and Twist. In this study, we investigated the role of these transcription factors in TGF- β -induced invasion. TGF- β induced the expression of the transcription factors Snail and Slug, but not of Twist in the premalignant H-Ras transformed breast cell line MCF10AT. In consequence, overexpression of Slug or Snail promoted invasion of breast cancer cells in an *in vitro* invasion assay. These cells invaded more as single, rounded amoeboid (cells compared to control cells. Knockdown of Slug inhibited TGF- β -induced invasion of these cells. Furthermore, in an embryonic zebrafish xenograft model, overexpression of Slug and Snail promoted invasion and metastasis. Also here, cells invaded predominantly as single cells. Thus, Slug and Snail appear to be critical mediators of TGF- β -induced invasion *in vitro* and *in vivo*.

Introduction

Transforming Growth Factor- β (TGF- β) is a pleiotropic cytokine involved a multitude of biological processes. Dysregulation of TGF- β signaling has been observed in several diseases, such as fibrosis and cancer [1]. In cancer, TGF- β plays a dual role; in early stages it inhibits tumour growth, whereas in later stages it promotes invasion and metastasis [2]. In line with its oncogenic role, TGF- β is frequently overexpressed in breast cancer [3-5]. Furthermore, inhibition of TGF- β signaling in breast cancer reduces metastasis in several mouse models of breast cancer [6-9].

TGF- β signals through a heteromeric receptor complex, composed of the TGF- β type I receptor Activin-receptor like kinase (ALK) 5 and the TGF- β type II receptor (TGF- β RII).

Within this complex, the TGF- β RII phosphorylates ALK5, which on its turn phosphorylates

Smad2 and Smad3. Phosphorylation of Smad2 and Smad3 induces a conformational change, which allows these Smad proteins to form heteromeric complexes with Smad4. This complex translocates to the nucleus, where it affects transcription of target genes [2,10-12]. In addition, receptor activation also results in non-Smad signaling, such as the mitogen activated protein kinases (MAPK) pathway [13].

TGF- β is thought to be pro-invasive by inducing epithelial-to-mesenchymal transition (EMT). During EMT, carcinoma cells acquire a more motile, mesenchymal phenotype. This process is marked by the loss of epithelial markers, such as E-cadherin, zona occludens (ZO)-1, EPCAM and keratin 18 (KRT18), and induction of mesenchymal markers, such as α -smooth muscle actin (α -SMA), vimentin and N-cadherin. Several transcription factors are able to downregulate E-cadherin expression by binding to E-boxes in the E-cadherin promoter, such as Snail and Slug. Snail and Slug are zinc finger proteins of the Snail family that recognize E2 box type elements C/A (CAGGTG). Furthermore, the bHLH factor Twist is also able to induce EMT, although it does not directly regulate E-cadherin [14]. Besides E-cadherin, Snail and Slug transcription factors downregulate components of the adherens junctions: desmosomes, polarity proteins and miRNAs [15]. TGF- β regulates EMT by inducing these transcription factors. Furthermore, Smad proteins also are able to interact with these transcription factors and jointly regulate target genes [16].

Although studies *in vitro* clearly have demonstrated that cancer cells are able to undergo EMT, the question if EMT also occurs in cancer *in vivo* has been subject of debate. The presence of more mesenchymal cells at the invasive edge suggested that EMT might occur in this area of the tumour [17]. Furthermore, cancer cells which have undergone TGF- β -induced EMT closely resemble cancer stem cells, a few cells of which can give rise to a whole new tumour [18]. Further studies revealed that Snail is partially responsible for the TGF- β -induced stem cell phenotype [19]. These studies strongly suggest the occurrence of EMT in cancer.

Whether EMT is necessary for invasion remains to be established. Cancer cells expressing E-cadherin have been observed to invade as a sheet of cells [20]. However, intravital imaging studies have shown that single breast cancer cells move faster than these sheets

and these individually moving cells had active TGF- β signaling [21]. Thus, EMT might be important in TGF- β -induced invasion. Therefore, we studied the role of key players of EMT, namely Snail, Slug and Twist, in TGF- β -induced invasion.

Materials & Methods

Cell culture

H-Ras transformed MCF10AT (M-II) were obtained from Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, USA) cultured and maintained as described before [22].

RNA isolation, cDNA synthesis and Q-PCR

2 x 10⁵ cells were seeded onto a six well plate and one day later starved for 16 hr DMEM/ F12 (Gibco/Invitrogen) containing 2.5% horse serum (Gibco/Invitrogen), 10ng/ml epidermal growth factor (EGF) (Upstate), 50ng/ml cholera toxin (Calbiochem), 0.25 μ g/ml hydrocortisone (Sigma), 5 μ g/ml insulin (Sigma), 100U/ml penicillin and 50 μ g/ml streptomycin (Gibco/Invitrogen). After starvation, cells were stimulated for 24 hr with no ligand or recombinant human TGF- β 3 (generous gift of Dr. K. Iwata, OSI Pharmaceuticals, Inc, New York, USA). RNA isolation was performed using a RNA extraction kit (Macherey-Nagel) according to manufacturer's instructions. cDNA synthesis and quantitative real time PCR were performed as previously described [23], on a StepOne Plus (Applied Biosystems) instrument and Sybr Green (Roche) as detection reagent. All samples were analyzed in triplicate for each primer set. Gene expression levels were determined with the comparative Δ Ct method using *ARP* or β -actin as reference and the non-stimulated condition was set to 1. Primers used are listed below.

Gene	Forward	Reverse
hARP	5'-CACCATTGAAATCCTGAGTGATGT -3'	5'-TGACCAGCCGAAAGGAGAAG -3'
h β -actin	5'- AATGTCGCGGAGGACTTTGATTGC -3'	5'-AGGATGGCAAGGGACTTCCTGTAA-3'
hSLUG	5'-ATGAGGAATCTGGCTGCTGT-3'	5'-CAGGAGAAAATGCCTTTGGA -3'
hSNAIL	5'-GCTGCAGGACTCTAATCCAGAGTT-3'	5'-GACAGAGTCCCAGATGAGCATTG-3'
hTWIST	5'-GACAGAGTCCAGATGAGCATTG-3'	5'-TTCTCTGGAAACAATGACATCTAGGT-3'
mSnail	5'- TCCAAACCCACTCGGATGTGAAGA-3'	5'- TTGGTGCTTGTGGAGCAAGGACAT-3'
mSlug	5'-CACATTCGAACCCACACATTGCCT-3'	5'- TGTGCCCTCAGGTTTGATCTGTCT-3'
hEPCAM	5'-CTTTATGATCCTGACTGCGATGAG-3'	5'-TCAGTGTCTTGTCTGTTCTTCTGA -3'
hKRT18	5'-TGGCGAGGACTTTAATCTTGGT-3'	5'- ACCACTTGGCATCCACTATCC-3'

Plasmids and transduction

For overexpression of Snail and Slug, mouse HA-Snail and mouse Slug were cloned into the lentiviral vector CMV-IRES-PURO. Lentiviral constructs expressing shRNA against human Slug and human Snail were from the Mission library (Sigma). The empty vector was used as a negative control. Transduction and selection of transduced cells was performed as described previously [24]. Knockdown and overexpression in M-II cells was verified by Q-PCR.

Spheroid invasion assay

The invasion spheroid assay was performed essentially as described [25]. Briefly, 10^3 cells were allowed to form spheroids in DMEM/F12 containing 5% horse serum and 20% methylcellulose in a 96 well round bottom plate and spheroids were embedded in a 1:1 collagen:methocel mixture onto a collagen coated plate. Recombinant human TGF- β 3 was added directly into the collagen/methocel mixture at 4 °C. After 30 minutes DMEM/F12 containing 1.6 % horse serum was added on top of the collagen. Invasion was monitored during the next two days and quantified by measuring the area using Adobe Photoshop.

Zebrafish embryonic xenograft model

Zebrafish and embryos were raised, staged and maintained according to standard procedures in compliance with the local animal welfare regulations. The transgenic line Tg (fli1:GFP) was crossed with the transparent casper mutant. Dechorionized 2d day old zebrafish embryos were anesthetized with 0.003% tricaine (Sigma) and positioned on a 10cm Petridish coated with 1% agarose. M-II cells overexpressing HA-Snail, Slug or the control vector were trypsinized into single cell suspensions, resuspended in PBS (Invitrogen), kept at room temperature before implantation and implanted within 3 hours. Cells were labeled with the fluorescent cell tracker CM-Dil (Invitrogen) according to the manufacturer's instructions. The cell suspension was loaded into borosilicate glass capillary needles (1mm O.D. x 0.78mm I.D.; Harvard Apparatus) and the injections were performed using a Pneumatic Pico pump and a manipulator (WPI). 50~400 cells, manually counted, were injected at approximately 60 μ m above the ventral end of the duct of Cuvier. Around 200 embryo's were implanted per cell line. After implantation, zebrafish

embryos (including non-implanted controls) were maintained at 34°C.

Invasion and subsequent metastasis was monitored after 1,3 and 5 days by imaging the zebrafish embryos with a confocal microscope. Invasion of perivascular tumour cells into the neighboring tail fin was quantified by counting the number of zebrafish embryo's with invading cells and is presented as the proportion of zebrafish embryo's with invasion over all zebrafish embryo's. Experimental micrometastasis in the tail fin of extravasated cells was quantified by counting the number of zebrafish embryo's in which experimental metastasis occurred and is presented as the proportion of metastasis positive embryo's over total number of zebrafish embryo's.

Statistical analysis

Representative results of single experiments are presented as the mean $\pm$ SD. Data shown from multiple independent experiments are shown as the mean $\pm$ SEM. Statistical differences were examined by one-way ANOVA followed by Bonferroni's multiple comparison test. $p < 0.05$ was considered as statistically significant.

Results

TGF- β induces mRNA expression of Slug and Snail in M-II

To identify which EMT regulators are of importance for TGF- β -induced invasion, we first tested which regulators are induced by TGF- β in this cell line. To this end, we stimulated MII cells with TGF- β (5 ng/ml) and after 24h we analyzed expression of Snail, Slug and Twist by quantitative real time PCR. TGF- β induced Slug mRNA 10 fold, whereas Snail mRNA was induced 5 fold (Figure 1A, B). No effect of TGF- β treatment on Twist mRNA could be detected (figure 1C). Thus, TGF- β induces expression of Snail and Slug, but not Twist in M-II cells.

Overexpression of Slug and Snail enhances invasion by promoting single cell motility

To study the function of Slug and Snail in invasion, we decided to mimic the effect of TGF- β on Slug and Snail expression by overexpression of Slug and Snail. This was achieved by

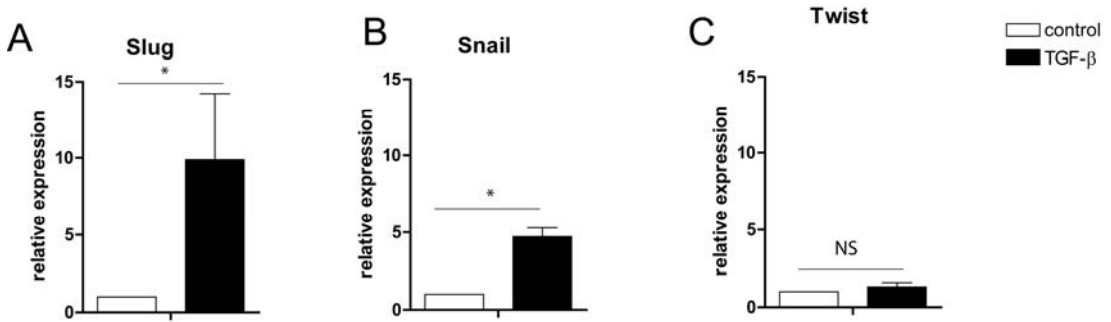


Figure 1 Induction of *Slug*, *Snail* and *Twist* by TGF- β in M-II. Spheroids of M-II were embedded with no ligand or TGF- β and RNA was isolated after 24 hr. RNA was analyzed by Q-PCR for *Snail* (A), *Slug* (B) or *Twist* (C). Data shown is the mean of two independent experiments $\pm$ SEM. Significance * $p < 0.05$ NS not significant

transduction of M-II cells with vectors containing either no insert (vector), mouse HA-Snail or mouse Slug. Overexpression was verified by quantitative real-time PCR for mouse *Snail* or *Slug* (Figure 2A,B). To test whether HA-Snail or Slug overexpression was functional, we performed real-time quantitative PCR for epithelial markers. As expected, EPCAM and keratin 18 (KRT18) were both downregulated by overexpression of HA-Snail or Slug (Figure 2C,D). Overexpression of HA-Snail or Slug did not affect directly TGF- β /Smad signaling, since the induction of the typical Smad target gene PAI-1 was equal in all cells (Figure 2E).

These cells were used in a spheroid *in vitro* invasion assay. Cells were allowed to form spheroids which were subsequently embedded in a collagen matrix in the presence or absence of TGF- β . Invasion into the collagen was measured after 24 hr. Overexpression of HA-Snail or Slug enhanced basal invasion of M-II cells compared to vector control cells, indicating that *Snail* and *Slug* mimic the effect of TGF- β on invasion (Figure 3A, B). In addition, invasion of HA-Snail or Slug overexpressing MII cells could be further enhanced by TGF- β , suggesting that other factors than *Snail* and *Slug* may also play a role in TGF- β -induced invasion.

When observing the invading spheroids more closely, we noticed that cells overexpressing -HA-Snail or *Slug* invaded in a different mode compared to control cells. Control cells remained invaded in a manner resembling collective cell migration, keeping cell-cell contacts intact and appearing rather epithelial (Figure 3C), whereas *Slug* or HA-Snail overexpressing cells invaded as single cells with a more rounded, amoeboid morphology (Figure

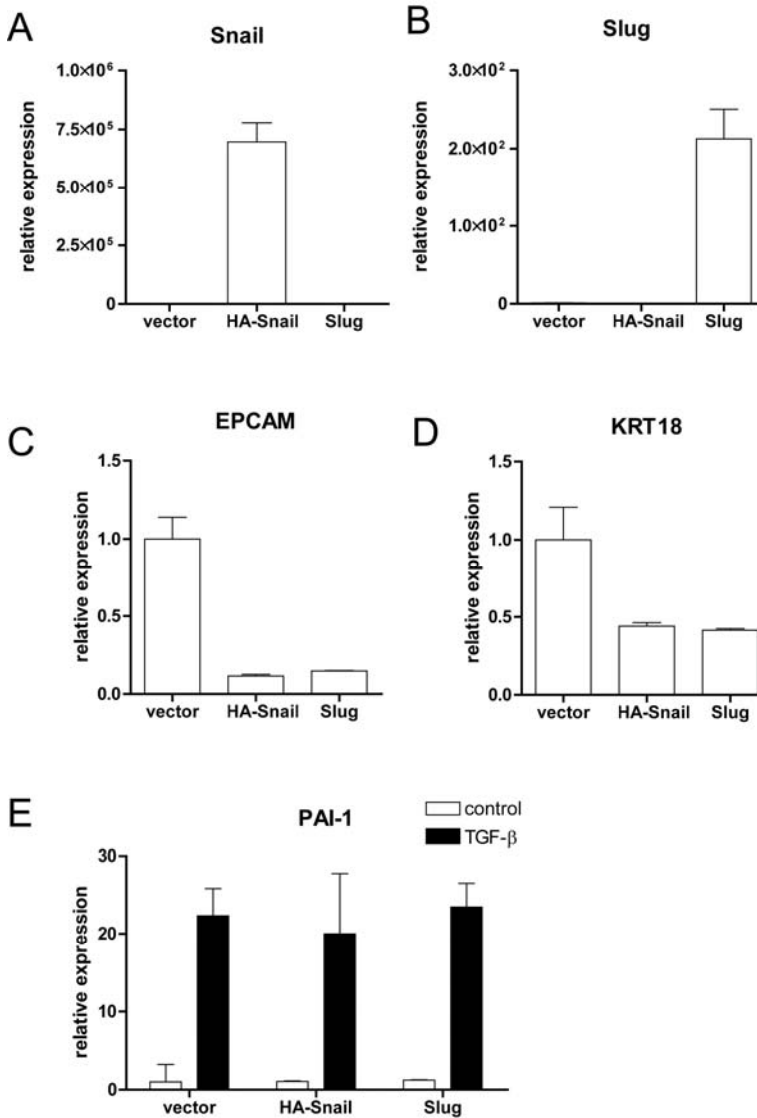


Figure 2 Functional overexpression of HA-Snail and Slug M-II cells were transfected with control plasmid, mouse Slug overexpression plasmid or mouse HA-Snail overexpression plasmid. After selection, cells were plated for RNA isolation. mRNA expression was analysed by Q-PCR for A mouse Snail B. mouse Slug C. human EPCAM D. human keratin 18 (KRT18) E. After selection cells were plated, serum starved and stimulated for 2 hr with no ligand or TGF- β (5 ng/ml). RNA was isolated and human PAI-1 expression was analysed by Q-PCR.

3C). This resembles the individually moving cells with active TGF- β signaling observed previously *in vivo*. These cells moved faster than their collectively invading counterparts [21]. Thus Slug and Snail may promote invasion by inducing single cell motility.

Slug is necessary for TGF- β -induced invasion

To confirm the importance of Slug for TGF- β -induced invasion, we performed shRNA-mediated knockdown experiments. MII cells were infected with a pool of lentiviral viruses expressing five different shRNA constructs for Snail or Slug respectively. Slug knockdown of MII cells was confirmed by quantitative real time PCR (figure 4A). Unfortunately,

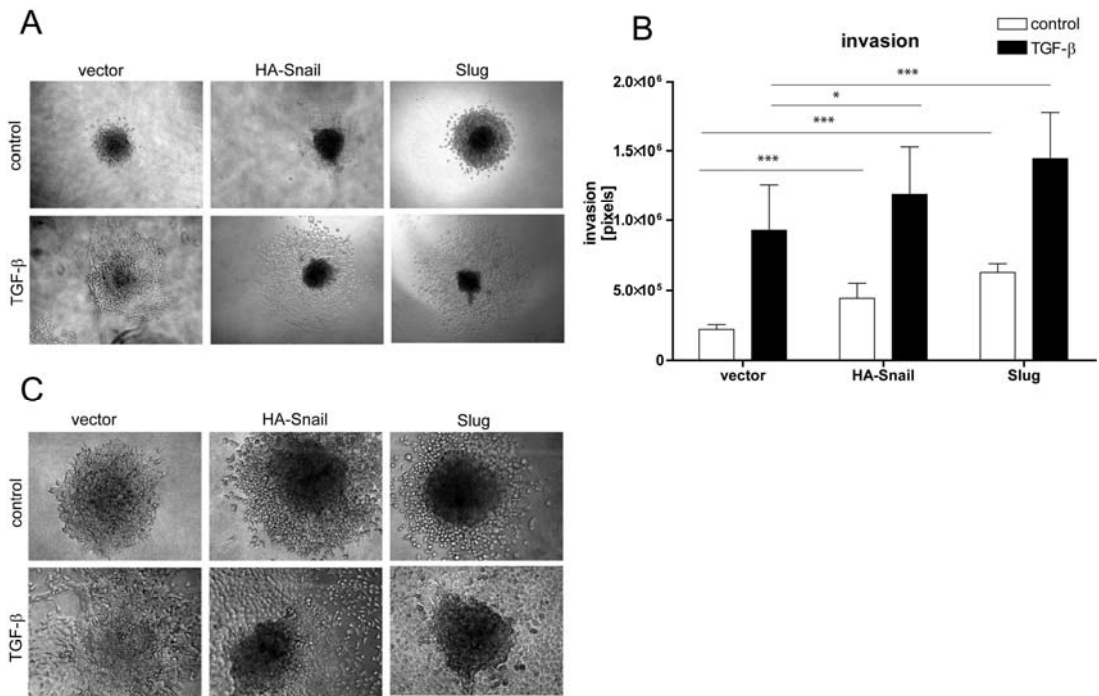


Figure 3 Overexpression of HA-Snail and Slug enhances invasion A. Cells overexpressing vector, mouse HA-Snail or mouse Slug were plated 8 hrs after transduction for spheroid culture and selection. These spheroids were embedded and allowed to invade into collagen. C. Quantification of invasion D. Higher magnification (100x) of spheroids after. Data shown is a representative of two independent experiments $\pm$ SD. Significance *** p < 0.001 * p < 0.05

we were not able to achieve sufficient knockdown of Snail (not shown).

To determine if knockdown of Slug affects TGF- β -induced invasion, we used the Slug knockdown cells in the spheroid invasion assay. Slug knockdown reduced basal invasion by 41%. (figure 4B,C). However, this did not reach statistical significance. Slug knockdown did significantly inhibited TGF- β -induced invasion by 66%. Thus, Slug is necessary for TGF- β -induced invasion.

Overexpression of Slug and Snail in M-II cells enhances their invasion and metastatic behaviour in zebrafish embryo's

Having demonstrated a role for Slug and Snail in *in vitro* invasion, we decided to validate the role of Snail and Slug in invasion *in vivo*. To this end, we made use of a zebrafish embryo xenograft model in which cells are injected in the ducts of Cuvier in the yolk sac of zebrafish embryo's, which then disperse through the bloodstream, invade the embryo

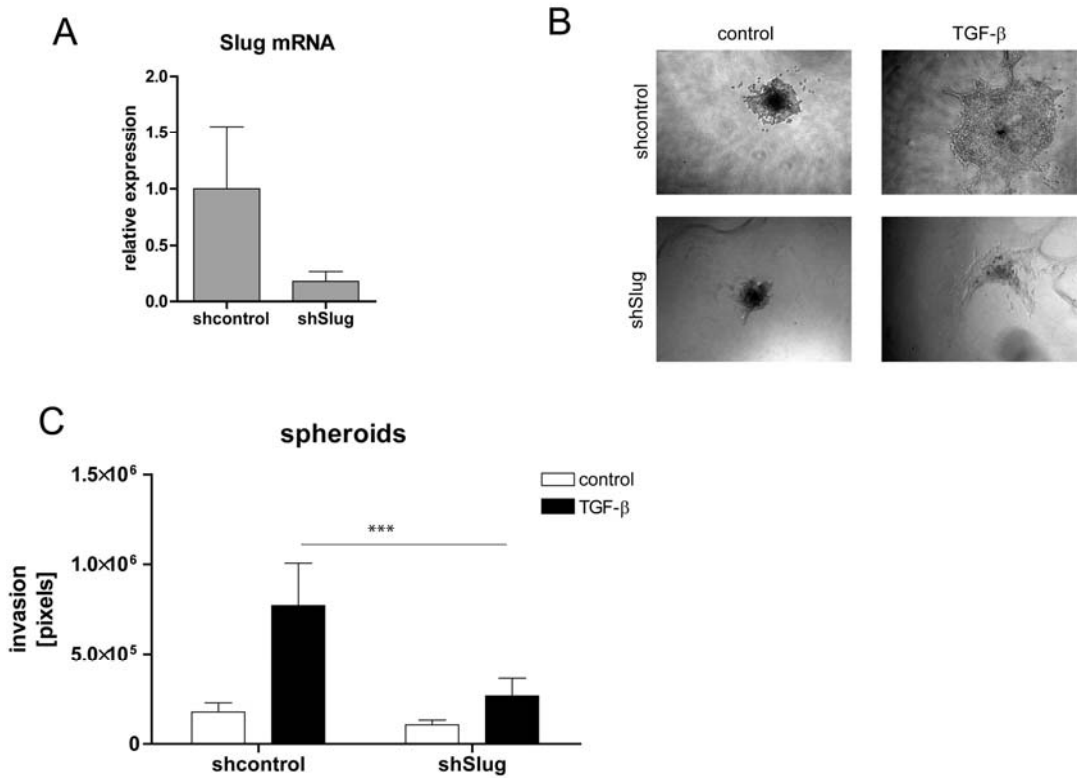


Figure 4 *Slug* is necessary for TGF- β -induced invasion in M-II. **A.** M-II was transduced with control shRNA or a combination of 5 shRNAs against *Slug*. After selection, knockdown efficiency was determined by Q-PCR. **B.** These cells were used for the spheroid invasion assay. **C.** Quantification of spheroids. Data shown is a representative of two independent experiments $\pm$ SD. Significance *** p < 0.001

an subsequently grow into metastasis [26]. When we injected M-II cells that overexpresses HA-Snail or *Slug*, we observed a dramatic increase in the amount of embryo's in which cells were invading (Figure 5A). This increase was evident from day 3 after injection, with only 13% of control embryo's showing invasion, whereas 31% and 39% of the embryos injected with HA-Snail and *Slug*, respectively, showed invasion. After 5 days, 38% of the control embryos showed invasion whereas 70% and 55% of the embryos injected with HA-Snail and *Slug* cells, respectively, showed invasion. Furthermore, the amount of embryo's displaying metastasis was also increased in cells overexpressing HA-Snail or *Slug*. While metastasis in embryo's injected with controls cells was rare after 3 days (0.29%) or 5 days (0.58%), embryo's injected with HA-Snail or *Slug* showed a more than tenfold increase in metastasis after 3 days (2.2% and 4.1%, respectively). This dramatic increase was

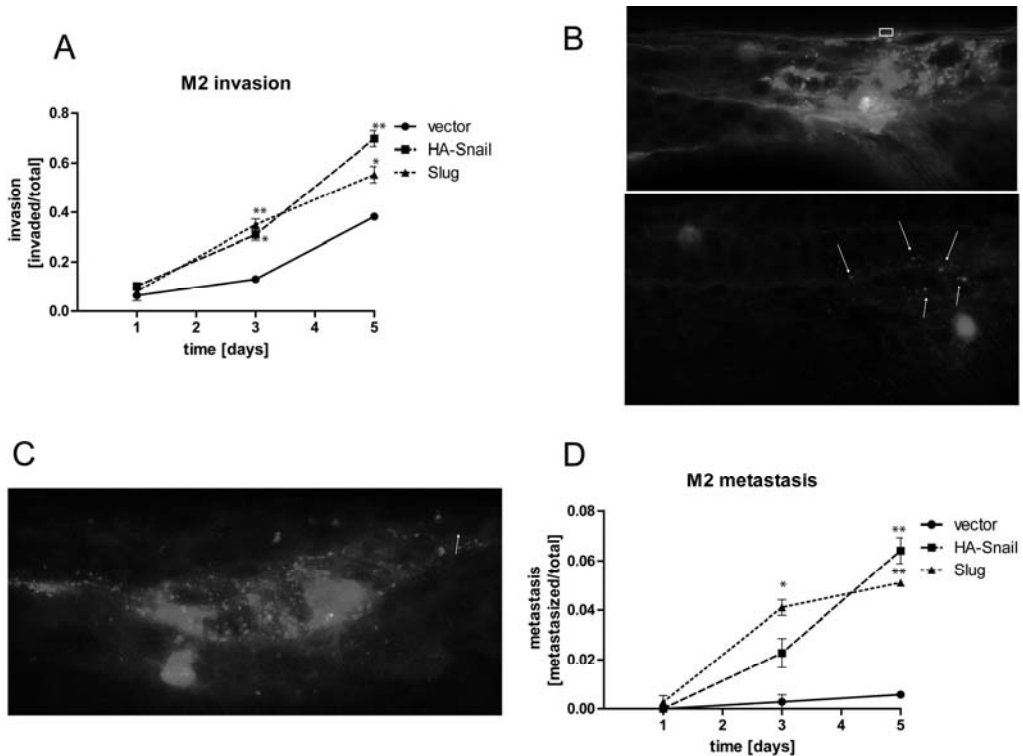


Figure 5 Overexpression of HA-Snail and Slug enhances invasion and metastasis *in vivo* M-II cells overexpressing HA-Snail, Slug or the control vector were dyed with CmDil and injected into the ducts of Cuvier of 2 days old Fli-GFPxCasper zebrafish embryo's. Cells were monitored after 1,3 and 5 days by imaging the zebrafish embryos with a confocal microscope. A. Invasion was quantified by counting the number of zebrafish embryo's where invading cells were observed and is expressed as the proportion of zebrafish embryo's with invasion over all zebrafish embryo's. B. Upper panel: Picture of a whole zebrafish embryo (green) with invading M2 cells overexpressing HA-Snail (red). Lower panel: larger magnification showing single invading cells (arrows) C. Picture of a whole zebrafish embryo (green) with invading M2 cells overexpressing Slug (red) D. Metastasis was quantified by counting the number of zebrafish embryo's in which metastasis occurred and is expressed as the proportion over total zebrafish embryo's. Data shown is the mean of two independent experiments $\pm$ SEM. Significance ** $p < 0.01$ * $p < 0.05$

maintained after 5 days (6,4% and 5.1% respectively). This suggests an important role for Slug and Snail in invasion and subsequent metastasis *in vivo*.

Discussion

In this study, we investigated if Snail and Slug, two key players in EMT, also have an important role during TGF- β -induced invasion. TGF- β induced the expression of Slug and

Snail mRNA in *H-Ras*-transformed MCF10AT (M-II) cells. Overexpression of Slug or Snail enhanced invasion in an *in vitro* invasion model. Most notably, these cells invaded as single cells. Using a zebrafish embryo xenograft model, we were able to observe the same effects *in vivo*. This study thus provides a link between TGF- β -induced EMT and single cell invasion and formation of experimental metastasis in zebrafish embryos.

Both Slug and Snail have been reported to induce MMP9 expression [27,28]. Since we have demonstrated a critical role of MMP9 in invasion [25], it is tempting to speculate that Slug and/or Snail are involved in TGF- β -induced MMP9 expression. However, MMP9 expression was not induced by Snail or Slug in our cells (data not shown).

Single cell invasion promoted by Slug and Snail resembles the amoeboid type of movement which occurs in the presence of MMP-inhibitors, without pericellular proteolysis [29,30]. Instead, cells generate enough actomyosin force to deform the extracellular matrix [31]. This type of movement is promoted by Rho-ROCK signaling [29]. Interestingly, RhoA is reported to be a Snail target gene [32], whereas RhoB is reported as a Slug target gene [33]. Furthermore, the RhoA-ROCK signaling axis has been implied in TGF- β -induced EMT and actin fiber rearrangements [34,35]. Thus, Snail and Slug might act via induction of Rho on TGF- β -induced invasion.

The single cell movement induced by Slug or Snail resembles cells invading *in vivo* which have active TGF- β -signaling. These cells invaded rapidly [21]. Since our data also implies Slug and Snail in single cell invasion *in vivo*, we speculate that Slug and Snail are mediators of TGF- β -induced single cell invasion, thus promoting metastasis.

TGF- β -induced EMT is also linked to the transition of cancers cells into cancer stem cells [18]. Snail has recently been implied in this transition [19]. Since a few of these cancer stem cells are able to establish a metastasis, it is likely that the Snail or Slug overexpressing cells also acquire some stem cell traits. It would be interesting to further investigate if these stem cell traits contribute to the invasive behaviour of these cells.

Taken together, we have shown a crucial role for the transcription factors Slug and Snail in TGF- β -induced single cell invasion of breast cancer cells. This further reinforces the role of these EMT mediators in metastasis.

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Chapter 7

Identification of kinases involved in BMP signalling

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In preparation

Abstract

Bone Morphogenetic Proteins (BMPs) play an important role in a variety of processes, such as bone formation and angiogenesis. In cancer, BMP-Smad signaling is often deregulated. In this study, we sought to identify protein kinases, which affect BMP signaling. Using an overexpression approach, we were able to identify known players in BMP signaling, such as ALK1, ActRIIA and ActRIIB. In addition, we identified two novel regulators of BMP signaling: ribosomal protein S6 kinase, 90kDa, polypeptide 4 (RPS6KA4) and Cyclin Dependent Kinase 8 (CDK8). RPS6KA4 enhanced BMP signaling, whereas CDK8 inhibited BMP signaling. Considering their involvement in cancer progression these protein kinases may play an important role in oncogenic BMP-Smad signalling.

Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF)- β superfamily of growth factors and are involved in a wide variety of processes, including bone formation, angiogenesis and cardiac development. A dozen of BMPs have been identified up to now, which all signal in a similar manner [1]. Ligand binding induces the formation of a heteromeric complex, consisting of a type I receptor and a type II receptor. BMPs are able to bind to the type I receptors activin receptor-like kinase (ALK) 1, 2, 3 and 6 and to the type II receptors BMP Receptor type II (BMPRII), Activin Receptor II (ActRII) A and B. Within this ligand-receptor complex, the type II receptor phosphorylates the type I receptor, which in turn phosphorylates Smad1, 5 and 8. This phosphorylation causes a conformational change in these Smads, which allows them to interact with Smad4 to form heteromeric complexes. This complex translocates into nucleus, where it affects transcription of target genes [1,2]. Besides, the induction of Smad signalling, BMPs may also induce non-Smad signalling pathways. For example, the BMP receptors activate TGF- β activated kinase-1 (TAK-1), which mediates the activation of p38 and JNK [1].

BMPs are necessary for tissue homeostasis and deregulation of BMP signalling leads to vascular disorders, such as primary pulmonary hypertension (PAH) and skeletal disorders, such as fibrodysplasia ossificans progressiva (FOP) [1]. In addition, disturbed BMP signalling has been implicated in cancer. Mutations in the *SMAD4* and *ALK3* genes result in juvenile polyposis, a hereditary form of colorectal cancer [3]. Furthermore, the BMP pathway is also inactivated by mutations in the BMP receptors in the majority of sporadic colorectal cancers during progression from adenoma to carcinoma [4,5]. In glioblastoma, BMP-4 treatment inhibited tumor growth [6]. However, in other types of cancers BMPs may either promote or inhibit tumorigenesis and metastasis. For example, loss of BMPRII has been correlated with poor prognosis in prostate cancer, whereas BMP-6 expression has been correlated with metastasis in prostate cancer [7]. The interplay with other growth factors or hormones may affect the outcome. For example, the effect of BMP-7 on prostate cancer cells relies on their hormone dependency. In androgen sensitive cell lines, BMP-7 inhibits proliferation, whereas it has no effect on androgen insensitive cell lines [8]. BMP-4 only stimulates cell growth in the presence of growth factors, such as Hepatocyte Growth Factor, Epidermal Growth Factor and [9]. Thus, the role of BMPs in cancer is highly context-dependent.

Interplay between the BMP-Smad signals and signals from other growth factors can be regulated by phosphorylation. For example, extracellular regulated kinase (ERK) phosphorylates Smad1 in the linker regions in response to growth factors, which results in exclusion of Smad1 from the nucleus [10]. Smad1 is also phosphorylated by Glycogen synthase kinase 3 (GSK3), which results in poly-ubiquitination and subsequent degradation of Smad1. Stimulation with Wnt decreases the levels of GSK3, with a concomitant increase in Smad1 protein levels and an increase in BMP signalling activity [11]. Thus, kinases play an important role in regulating BMP signalling.

In this study, we sought to identify novel kinases which modulate BMP-Smad signalling in cancer. To this end, we made use of a human kinome overexpression library [12]. Compared to a loss of function approach, use of this gain of function approach allows for identification of kinases that are not expressed by the cell type, act redundantly or

activate pathways only under pathological conditions. Using this library, we found known and novel regulators of BMP signalling. Further validation identified two novel kinases which regulate BMP-Smad signalling.

Materials and Methods

Cell culture

C2C12 mouse myoblast and human embryonic kidney (HEK) 293T transformed cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 50 µg/ml streptomycin (Gibco). C2C12 cells were not allowed to grow beyond 70-80% confluency. All cell lines were grown in a humidified incubator at 37 °C and 5 % CO₂.

Constructs

The cDNA kinase expression library [12] and BMP Responsive Element (BRE)-Luc reporter construct [13] have been described before. The *Renilla* luciferase construct pRL-SV40 was purchased from Promega. Constructs encoding human Ribosomal protein S6 kinase, 70kDa, polypeptide 1 (RPS6KB1), Mitogen Activated Kinase Kinase Kinase 10 MAP3K10, ribosomal protein S6 kinase, 90kDa, polypeptide 4 (RPS6KA4), ephrin type-A receptor 2 (EPHA2), ephrin type-B receptor 2 (EPHB2) and EPHB3 in pDNOR221 [12] were cloned into pcDNA DEST40 (Invitrogen) using the Gateway system (Invitrogen). Mouse Cyclin Dependent Kinase 8 (CDK8) shRNA constructs were from the TRC1 shRNA library (Sigma), namely constructs TRCN0000023107 and TRCN0000023104. Construct SHC002, expressing a non-targeting shRNA, was used as a negative control.

Kinase screen and data analysis

The kinase screen was performed in a 96-well plate format. Luciferase-reporter screening was performed in C2C12 cells, using 50 ng of protein kinase expression constructs and 50 ng of reporters (1:19 mix of control to pathway reporter) per well. The *Renilla* luciferase construct pRL-SV40 (Promega) was used as a control reporter; BRE-luc was used as a specific reporter for the BMP pathway. One day after transfection using FuGENE HD (Roche),

cells were serum starved over night and subsequently stimulated with BMP-6 (100 ng/ml; a kind gift of K. Sampath, Creative Biomolecules, Inc, Hoptinton, USA) in medium containing 0.5% serum. After 8 hr of stimulation, firefly and *Renilla* luciferase activities were determined using the dual-Luciferase kit (Promega), followed by subtraction of background luminescence counts from untransfected cells. Relative luciferase activities were calculated by dividing the pathway-specific firefly luciferase counts by the control *Renilla* luciferase counts separately for all replicates, and sample mean and standard deviation (SD) were calculated from these values ($n = 3$). Constructs that caused a severe drop in *Renilla* luciferase counts (below 15000 counts) were classified as toxic and were not analyzed further. Data was analyzed using GraphPad Prism.

Luciferase assays

C2C12 cells were seeded into a 24 wells plate and transfected the next day for 4 hr with 100 ng BRE-Luc and 100 ng of the kinase overexpression or knockdown construct using Lipofectamine (Invitrogen). An expression plasmid for β -galactosidase (200 ng) was co-transfected to correct for transfection efficiency. The next day, cells were serum starved overnight and stimulated with BMP-6 (100 ng/ml) for 8 hr. Luciferase and β -galactosidase activity were determined as previously described [14]. Each transfection was carried out in triplicate and representative experiments are shown.

Western blot

293T cells were seeded into a 6 wells plate and were transfected with 500 ng kinase overexpression construct using polyethylenimine (Sigma-Aldrich) and harvested two days after transfection in Laemmli buffer. C2C12 cells were transfected using Lipofectamine (Invitrogen), harvested 3 days after transfection in Laemmli buffer without bromophenol blue and β -mercaptoethanol. 10 μ g of protein was used for SDS-PAGE & Western blotting. V5-antibody was from Invitrogen and CDK8 antibody was from SantaCruz. Staining for β -actin (Sigma) was used as a loading control.

Results

Kinase screening identifies putative positive regulators of BMP-Smad signalling

To identify new players in BMP-Smad signalling, we made use of a kinome cDNA expression library. We used activation of the transcriptional reporter BRE as a readout in the highly BMP responsive myoblast cell line C2C12. Without ligand stimulation, ectopic overexpression of protein kinases previously implicated in BMP-Smad signalling, namely ActRIIA, ActRIIB and ALK1, strongly induced promotor activity (figure 1A). Interestingly, ALK4 and ALK7, receptors for the TGF- β superfamily ligands Activin and Nodal, also enhanced basal BRE-reporter activity, 2.6 fold and 5.5 fold respectively. This suggests positive crosstalk between Activin/Nodal and BMP-Smad pathways. When cells were stimulated with the ligand BMP-6, overexpression of ActRIIA further enhanced reporter activity (figure 1B). The positive identification of known BMP-Smad pathway regulators proved that our high throughput system is suited for the search for new kinases involved in the control of BMP signaling

Further analysis of the results revealed a number of kinases which enhanced BMP-6 induced reporter activity more strongly than ActRIIA (Figure 1C, Figure 2A). Especially mitogen-activated protein kinase kinase kinase 10 (MAP3K10) had a strong effect on Smad1 transcriptional activity, whereas ribosomal protein S6 kinase 90kDa polypeptide 4 (RPS6KA4) enhanced reporter activity to a lesser extent. Interestingly, three members of ephrin receptor subfamily (EPH) of receptor protein tyrosine kinases, namely EPHA2, EPHA3 and EPHB2, enhanced reporter activity to the same extent as ActRIIA. Our results suggest that the kinases MAP3K10, RPS6KA4, EPHA2, EPHA3 and EPHB2 are potential positive regulators of BMP-Smad signalling.

Kinase screening identifies possible negative regulators of BMP-Smad signalling

A large number of kinases inhibited BMP-6 induced reporter activity. Among these were different splice variants of TGF- β activated kinase (TAK)-1 (Figure 1B), which has been shown previously to negatively effect BMP-Smad signalling by interfering with the DNA

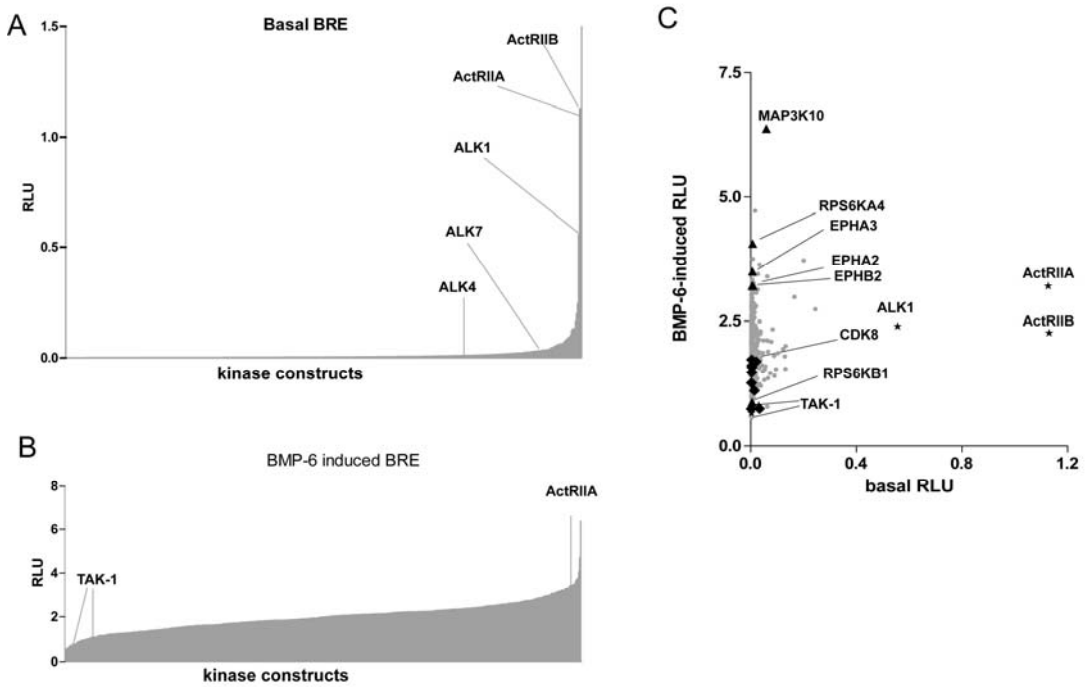


Figure 1 Kinase cDNA screen for novel BMP pathway regulators. (A and B) Effect of kinase expression on the BMP reporter BRE-Luc activity in C2C12 in the absence (A) or presence (B) of BMP-6 (100 ng/ml). The kinome overexpression constructs were co-transfected with the BRE-Luc reporter in C2C12 cells. Cells were starved and mock treated or BMP-6 (100 ng/ml) stimulated for 8 hours. The RLU of the kinases were plotted after sorting for RLU value. (C) Several kinases affect the BMP pathway. Basal RLU is plotted against BMP-6-induced BRE. Kinases for follow-up are indicated with triangles, kinases with a known function in the BMP pathway are indicated with stars and CDKs are indicated with diamonds. Relative luciferase activity (RLU) is expressed as mean of triplicate cultures.

binding domain of Smads [15]. One kinase negatively affecting BMP-6-induced reporter activity was ribosomal protein S6 kinase 70kDa polypeptide 1 (RPS6KB1) (Figure 1C, Figure 2B). Interestingly, cyclin dependent kinases, namely CDK2,4, 5, 8 and 9 all had an inhibitory effect on BMP-6-induced reporter activity (Figure 2B). These kinases are essential for cell cycle progression and they are frequently overexpressed in cancer [16]. Taken together, RPS6KB1, CDK2, 4, 5, 8 and 9 are possible negative regulators of BMP-Smad signalling.

RPS6KA4 is a positive regulator of BMP-Smad signalling

To further validate the effect of the different kinases on BMP-Smad signalling MAP3K10, RPS6KA4, EPHA2, EPHB2, EPHA3, and RPS6KB1 cDNAs were cloned into pcDNA DEST40 containing a C-terminal triple V5 tag. The expression levels of these constructs was analyzed in 293T cells by Western blotting following transfection of the different constructs (Figure 3A). In order to analyze their effects on BMP induced transcriptional activity using the BRE reporter construct. V5-Tagged MAP3K10, RPS6KA4, EPHA2, EPHB2, EPHA3, and RPS6KB1 were overexpressed in C2C12 cells together with the BRE reporter construct. As shown in Figure 3B, only the stimulatory effect of RPS6KA4 was reproducible, whereas ectopic expression of MAP3K10, RPS6KB1, EPHA2, EPHA3 and EPHB2 had no effect on BMP-6-induced BRE-Luc activity. CDK2 and CDK4 were the strongest inhibitors. These two CDKs were already known to phosphorylate Smad2 and 3, which are homologous to Smad1, 5 and 8, resulting in inhibition of their transcriptional activity [17]. Since CDK8 is also a colorectal oncoprotein [18] and BMP signalling is often inactivated in colorectal cancer [3], we decided to further characterise the effects of CDK8. We chose to analyze if knockdown of CDK8 had a stimulating effect on BMP-Smad signalling in C2C12 cells. Knockdown efficiency was determined by Western blot. Two constructs gave sufficient knockdown in C2C12 cells (Figure 3A). These constructs strongly enhanced BMP-6-induced BRE-luc activity, thus indicating that CDK8 is a negative regulator of BMP-Smad signalling.

Discussion

In this study, we performed a protein kinase overexpression screen to identify novel players in BMP-Smad signalling. The reliability of the screen was reinforced by the identification of kinases already known to be involved in BMP-Smad signalling. The receptors ALK1, ActRIIA and ActRIIB enhanced BMP reporter activity, whereas TAK-1 inhibited BMP signalling. The first ones are receptors for BMPs [1,2], whereas the latter has been reported to bind to the DNA binding domain of Smads to prevent transcriptional transactivation [15]. However, TAK-1 was shown to be essential for activation of BMP-Smad signalling during chondrogenesis [19,20]. The previous study showing negative effects of TAK-1 in BMP-

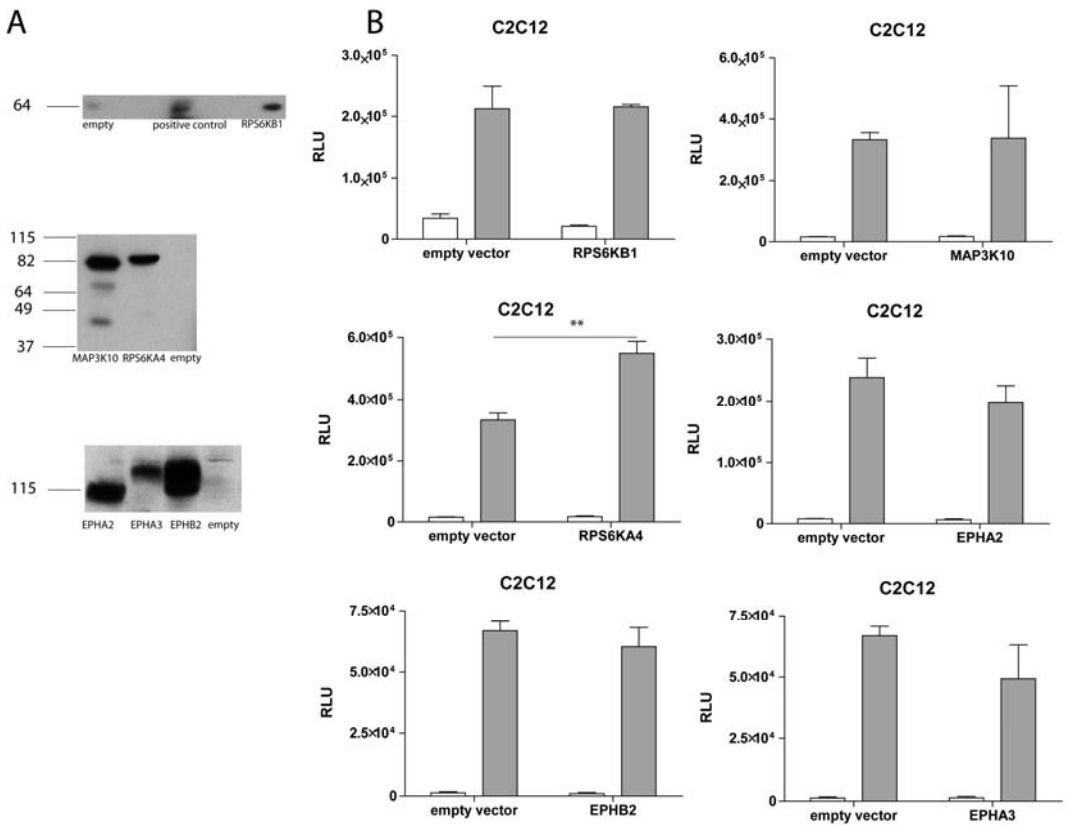


Figure 2 Validation of the functional role of the identified kinases from the cDNA screen on the BMP signalling. (A) Potential BMP pathway modifiers MAP3K10, RPS6KA4, EPHA2, EPHB2, EPHA3 and RPS6KB1 were cloned into pcDNA DEST40 vector to generate V5 tagged kinases. These constructs were transfected into 293T cells and expression was verified using a V5 specific antibody. (B) Kinase constructs were co-transfected with the BRE-Luc reporter into C2C12 cells. Cells were starved and mock treated or BMP-6 (100 ng/ml) stimulated for 8 hours. Relative luciferase activity (RLU) is expressed as mean $\pm$ S.D. of triplicate cultures. Significance ** $p < 0.01$

Smad signalling was performed in the murine mesenchymal stem cell line C3H10T $\frac{1}{2}$ during osteogenesis [15], whereas BMPs also induce osteoblastic differentiation in our C2C12 cells [21]. Thus, it is likely that the effect of TAK-1 is highly dependent on the cellular context.

Interestingly, the type I receptors for Activin and Nodal, ALK4 and ALK7, enhanced basal reporter activity. Such positive effect of the Activin signalling has been also described during chicken development where Activin signalling enhanced BMP signalling through membrane-bound inhibitor (BAMBI) [22].

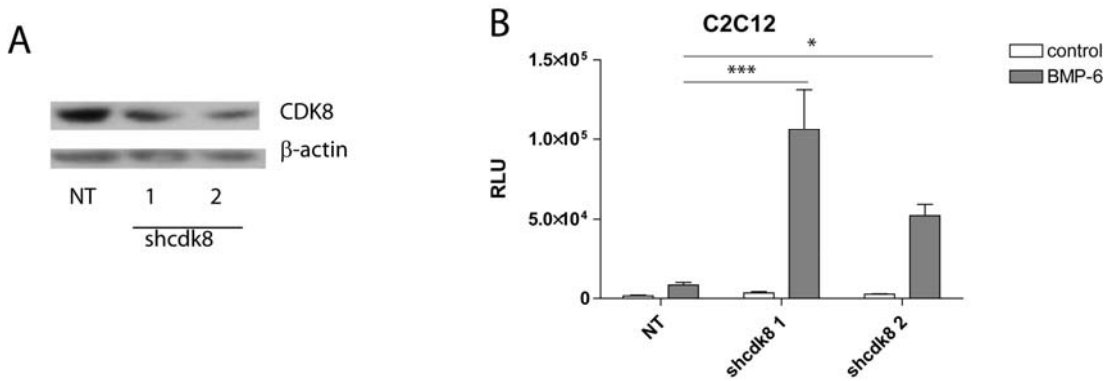


Figure 4 *CDK8 as a potential negative regulator of BMP signalling.* (A) C2C12 cells were transfected with the indicated constructs. Knockdown was verified by Western blot. (B) C2C12 cells were transfected with the indicated constructs and the BRE-Luc reporter construct, starved and stimulated with no ligand or BMP-6 (100 ng/ml). Relative luciferase activity (RLU) is expressed as mean $\pm$ S.D. of triplicate. Significance * $p < 0.05$ *** $p < 0.001$

A large number of kinases affected BMP signalling in the initial kinome overexpression screen. These included MAP3K10, RPS6KA4, EPHA2, EPHB2, EPHA3, RPS6KB1 and CDK8. However, the effect of most kinases could not be reproduced. This might be due to differences in assay set-up. Most notably, fold activation in the follow-up experiments was much lower than in the original screen. Another possibility is that the tag might influence the kinase activity, despite being rather small.

Validation identified RPS6KA4 as a positive regulator of BMP signalling. RPS6KA4, also known as Mitogen and Stress-activated Kinase 2 (MSK2) or Ribosomal S6 Kinase B (RSK-B), acts downstream of p38 and phosphorylates the transcription factor cAMP Responsive Element Protein (CREB) [23]. Phosphorylated CREB has been shown to form a transcriptional complex with Smad1/5/8 and CREB binding protein (CBP) resulting in enhanced BMP-Smad signalling [24]. Thus RPS6KA4 may potentiate BMP signalling by increasing the pool of phosphorylated CREB which can interact with Smad1 and CBP to activate transcription.

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Taken together, we have identified novel kinases regulating BMP-Smad signalling. If these kinases play a role in oncogenic BMP signalling is subject for further study.

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Chapter 8

Summary and discussion

Transforming Growth Factor- β (TGF- β) and Bone Morphogenetic Proteins (BMPs) are involved in a multitude of biological processes. Dysregulation of their signaling pathways is often observed in cancer. TGF- β has a dual role in cancer; in early stages it inhibits cell growth and promotes apoptosis, whereas in later stages it promotes invasion and metastasis. . Given its pro-tumorigenic role, many efforts have been made in developing TGF- β signaling inhibitors and some of them are currently in clinical trials (reviewed in [1]). However, given its growth inhibitory role, these inhibitors should be used with care. Therefore, these therapies might benefit from the use of additional inhibitors targeting downstream effectors of TGF- β that are involved in the pro-tumorigenic action of TGF- β .

To study invasion in more detail *in vitro*, a 3 dimensional model was developed, as described in **Chapter 3**. In this model, breast cancer cells, aggregated into spheroids, were allowed to invade into a collagen type I matrix. Collagen I is often found in breast cancer tissue [2] and is present to the so-called dense tissue in mammography, which is a poor prognosis factor for breast cancer [3]. An advantage of using spheroids in the invasion assay is that they have an organization similar to a tumour with an inner hypoxic core and more metabolically active cells at the outside [4]. In this way, cells organize themselves in structures which resemble more their parental tissue. In a model set up by the group of Bissell, mammary epithelial cells form acinar structures. Using that model one can easily distinguish normal mammary cells from malignant breast cancer cells, as malignant cells fail to organize themselves into acinar structures [5]. 3D models also better recapitulate resistance to chemotherapeutic drugs than cells in monolayers [6,7]. Our spheroid model complements these models by focusing on invasion. In our model, TGF- β potentially induces invasion. Using a series of cell lines representing different stages of cancer development, it was demonstrated that the invasiveness of the cell lines correlated with their malignancy. Thus, the spheroid invasion model allows to study TGF- β -induced breast cancer cell invasion.

Further characterization of the TGF- β -dependent invasive properties of these cells in **Chapter 4** revealed that invasion of the metastatic cell line was dependent on Smad3

and Smad4. This is in concordance with previous studies *in vivo* which demonstrated the importance of the Smad pathway by overexpression of a Smad binding defective mutant of ALK5 [8] or knockdown of Smad4 [9,10]. Also Smad3 has been shown to be involved in breast cancer metastasis, although this effect was mainly due to effects on angiogenesis [11].

Both Smad3 and 4 were necessary for induction of MMP2 and MMP9. This is in agreement with a previous study using the Smad-binding defective mutant of ALK5 [8]. Since MMPs especially MMP9, are involved in angiogenesis [12], Smad3 may promote both invasion and angiogenesis through the induction of MMPs. On the other hand, Smad2 has been shown to have anti-metastatic effects [11]. The role of Smad2 in our invasion model remains to be established.

Although Smads were shown to be crucial for TGF- β -induced invasion, we can not rule out that non-Smad pathways also contribute to invasion. Various studies have shown that RAS and TGF- β collaborate in promoting malignant progression of breast cancer [13,14,15]. TGF- β is able to activate RAS through phosphorylation of ShcA [16]. Downstream effectors of RAS include the Raf-MEK-ERK, PI3K-PKB and Ral pathways [17]. The PI3K-PKB pathway has been shown to primarily counteract TGF- β -induced growth arrest [18], although the PKB- β isoform was able to enhance invasion in a mouse model of breast cancer [19]. The Raf-MEK-ERK pathway appears to be the mediator of an invasive phenotype downstream of Ras [18,14]. The role of the small GTPase Ral in the interplay between Ras and TGF- β in invasion has not yet been investigated, although Ral has been implicated in migration of breast cancer cells and leukocytes [20,21]. In addition to the above-mentioned pathways, H-RAS is able to induce p38 activity [22]. In MCF10A cells, TGF- β and H-RAS cooperate to induce MMP2 expression through activation of p38 and ATF2 [23]. However, it remains to be established if this also occurs in the MCF10A metastatic derivative (M-IV) in our invasion system.

We established a crucial role for MMPs in our invasion model. MMP1, which is able to cleave type I collagen, was dispensable for TGF- β -induced invasion, whereas MMP2 and MMP9 proved to be necessary for invasion. Although these latter MMPs are not able to

cleave collagen I, their pro-invasive capacities may depend on cleavage of adhesion molecules, such as E-cadherin [24]. In addition, MMP2 and MMP9 are able to cleave latent growth factors. For example, MMP9 is able to cleave latent TGF- β , thus functioning in a feed-forward loop [12]. The individual roles of MMP2 and MMP9 in invasion remains to be established.

MMP2 and MMP9 appear attractive targets for therapeutic intervention of breast cancer invasion. Given the multifunctionality of MMPs, they may also have anti-tumorigenic effects. The latter appears in particular an issue for targeting of MMP9 [25]. Tumors in MMP9 null mice were found to be more aggressive, indicating that MMP9 inhibits certain aspects of tumor progression [26]. On the other hand, knockdown of MMP9 reduced metastasis of TGF- β RI-expressing cells [27]. MMP2 appears to be a better target, since overexpression in breast cancer cells augments orthotopic tumor growth and metastasis in mice [28]. In humans, MMP2 is one of the predictors for lung metastasis [29]. The development of specific MMP inhibitors remains a challenge, since the enzymatic pocket of these proteins are highly homologous.

The pro-invasive action of TGF- β is thought to be dependent on the ability of TGF- β to induce EMT, resulting in a more motile mesenchymal phenotype. Therefore, the role of key regulators in EMT in TGF- β -induced invasion, Snail and Slug, was studied in **Chapter 6**. Overexpression of the transcription factors Slug and Snail enhanced invasion, both in our *in vitro* spheroid invasion assay as well as in a *in vivo* zebrafish model, thus linking EMT to invasion. In addition, Slug was necessary for TGF- β -induced invasion *in vitro*. A role of Snail in TGF- β -induced invasion awaits further clarification.

Furthermore, we observed that either Slug or Snail overexpression enhanced single cell invasion. This type of movement resembles the amoeboid type of movement which occurs in the presence of MMP-inhibitors, without pericellular proteolysis [30,31]. Interestingly, this type of movement has been linked to TGF- β -signaling *in vivo* [32]. Amoeboid movement is promoted by Rho-ROCK signaling [30]. Rho GTPases are induced by Snail and Slug [33,34] and the Rho signaling axis is involved TGF- β -induced EMT and actin fiber rearrangements [35,36]. Therefore, it is tempting to speculate that the induction of Rho by

Slug or Snail contributes to their effects on migration. These data described in chapter 6 further emphasize the role of these EMT mediators in metastasis.

BMP-7 has been shown to negatively affect TGF- β -induced EMT [37] and BMP-7 inhibited bone metastasis of breast cancer cells [38]. Therefore, we investigated the effect of BMP-7 on TGF- β -induced invasion in **Chapter 5**. Indeed, BMP-7 inhibited TGF- β -induced invasion, whereas BMP-6, its closest homolog, was not able to do so. Although BMP-6 and BMP-7 share 73 % amino acid homology, differences between these two BMPs have been observed previously. For example, BMP-6 is more potent than BMP-7 in inducing bone formation [39]. However, in our cells BMP-6 and BMP-7 activated the BMP pathway to the same extent. If activation of non-Smad signaling pathways is differentially induced by these BMPs is still unresolved.

Interestingly, BMP-7 did not affect the growth inhibitory effect of TGF- β in these metastatic cells. Thus BMP-7 only inhibits pro-oncogenic actions of TGF- β . Furthermore, BMP-7 only inhibited TGF- β -induced invasion in a metastatic cell line, but not in its pre-malignant precursor cell line, further suggesting that BMP-7 only inhibits oncogenic TGF- β -signaling. Indeed, treatment with BMP-7 reduced bone metastasis in breast cancer [38], thus reinforcing the rationale that this cytokine might be useful in cancer therapy.

In chapter 5, it is described that BMP-7 inhibited the expression of integrin $\alpha_v\beta_3$, a critical factor for TGF- β -induced invasion. Overexpression of integrin β_3 could rescue TGF- β -induced invasion from inhibition by BMP-7, thereby demonstrating the importance of this inhibition. Integrin $\alpha_v\beta_3$ is able to interact with TGF- β RII and to enhance the activity of this receptor and resulting in increased TGF- β -induced EMT [40]. Furthermore, this integrin is able to interact with MMP2 and MMP9 to enhance invasion [41,42]. Interestingly, a complex between $\alpha_v\beta_3$, MMP2 and Bone Sialic Protein, another TGF- β target gene [43], promotes invasion. Given the importance of these MMPs in invasion, it is likely that this action of integrin $\alpha_v\beta_3$ also contributes to its pro-invasive effect.

Integrin $\alpha_v\beta_3$ appears to be an attractive target for therapeutic intervention in breast cancer invasion. The integrin complex is highly expressed in bone-metastasized breast cancer cells [44]. Overexpression of integrin $\alpha_v\beta_3$ promoted bone metastasis of breast cancer

cells [45], whereas inhibition of $\alpha_v\beta_3$ diminishes osteotropism of breast cancer cells [46]. Also, integrin β_3 identified cancer stem cells in mouse models of breast cancer [47]. Therefore, several drugs targeting integrin $\alpha_v\beta_3$ have been developed. The integrin $\alpha_v\beta_3/\alpha_v\beta_5$ inhibitor Cilengitide enhanced the tumoricidal effect of radioimmunotherapy in a mouse xenograft model [48], whereas Cilengitide alone decreased progression of bone metastasis [49]. Also CNTO 95, a humanized antibody directed against integrin $\alpha_v\beta_5$ and $\alpha_v\beta_3$ reduced the invasion and metastasis capacity of human breast cancer cells in pre-clinical breast cancer models [50].

Although we discovered which genes are involved in the inhibition of TGF- β by BMP-7, the signaling pathway involved is not clarified. A recent report demonstrated that BMP-7 could inhibit Smad3 translocation to the nucleus by inducing SnoN in kidney cells [51]. However, in M-IV cells BMP-7 did not affect Smad3 transcriptional activity (Chapter 5). Since induction of integrin β_3 by TGF- β has been reported to be dependent on p38 and Src in lung fibroblasts [52], BMP-7 could exert its effects through inhibition of non-Smad pathways. Further studies are awaited to shed more light into the mechanisms by which BMP7, but not BMP6, inhibit TGF- β induced invasion.

The BMP signaling pathway is far less well characterized than the TGF- β pathway. To identify novel kinases involved in the BMP signaling pathway, we performed an overexpression protein kinase screen. In **Chapter 7** we described the identification of both known and novel players in BMP signaling. Further validation revealed RPSKA4 as a novel positive regulator of BMP signaling. RPS6KA4 acts downstream of p38 to phosphorylate CREB [53]. Phosphorylated CREB is known to enhance BMP-Smad signaling [54]. Thus, RPS6KA4 may promote BMP signaling by increasing the amount of phosphorylated CREB. Further study is needed to characterize this mechanism and to show how this action affects the role of BMPs in cancer.

We also identified CDK8 as a negative regulator of BMP signaling. Recently, it was shown that CDK8 phosphorylated Smad1 at residues in the linker region. This creates docking sites for Smurf1, resulting in degradation of Smad1. However, also the transcriptional co-activator YAP is able to bind to these phosphorylated residues, resulting in enhanced

signaling [55]. Given the negative effect of CDK8 on BMP signaling, Smurf1 probably outcompetes the YAP present in the cells.

In this thesis, a novel model was described to study TGF- β -induced invasion of breast cancer cells *in vitro*. Key players in this process were identified, including MMP2, integrin $\alpha_v\beta_3$, Slug and ZEB, which hold therapeutic promise. The inhibitory effect of BMP-7 on TGF- β -induced invasion also provides a rationale for using this cytokine in breast cancer therapy. Further studies are needed to confirm these findings.

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Chapter 9

Nederlandse samenvatting voor niet- ingewijden

List of Publications

Curriculum vitae

Nederlandse samenvatting voor niet-ingewijden

De Transforming Growth Factor- β (TGF- β) familie omvat eiwitten zoals TGF- β , activines en Bone Morphogenetic Proteins (BMPs), die door cellen uitgescheiden worden en vervolgens in tal van biologische processen een belangrijke rol spelen. Het eiwit TGF- β werd ontdekt als een groeifactor die cellen kan aanzetten tot verandering in hun vorm (morfologie). BMPs werden geïdentificeerd als groeifactoren aanwezig in de botmatrix die botvorming kunnen induceren. Later werd ontdekt dat zowel TGF- β als BMPs ook belangrijk zijn voor de ontwikkeling van het embryo. In een volwassen organisme zijn TGF- β en BMPs van belang om het inwendige milieu van een cel in balans te houden. Verstoring van TGF- β en BMP signalering kan leiden tot ziektes, zoals bijvoorbeeld vaatafwijkingen, fibrose en kanker.

TGF- β en BMPs kunnen aan de buitenkant van cellen binden, via zogenaamde receptoren, en daarmee diverse processen in die cel op gang brengen. Binding van TGF- β aan deze receptoren leidt tot vorming van een complex tussen type I en type II receptoren, waarbij de type II receptoren de type I receptor fosforyleert. Deze receptor fosforyleert op zijn beurt andere signaleringseiwitten, waarvan de receptor Smad eiwitten (R-Smads) de belangrijkste zijn. Door deze fosforylering kunnen de R-Smads binden aan Smad4 waarna het R-Smad/Smad4-complex naar de kern van de cel verplaatst. In de kern van de cel bindt het R-Smad/Smad4 complex aan het DNA en beïnvloedt de expressie van een groot aantal genen.

TGF- β heeft een dubbel gezicht in kanker; in vroege stadia remt het de groei van cellen en induceert het de dood (apoptose) van kankercellen. Het werkt in eerste instantie dus als kanker-onderdrukkend eiwit, terwijl het in latere stadia juist invasie en het uitzaaien van kankercellen bevordert.

Om de oncogene rol van TGF- β beter te bestuderen, hebben wij een test opgezet waarbij TGF- β invasie induceert. Deze test wordt beschreven in **hoofdstuk 3**. Hierbij is gebruik gemaakt van gebruik van ronde bolletjes met borstkankercellen, zogenaamde

spheroiden. De organisatie in zo'n sferoid lijkt meer op de organisatie van cellen in een tumor dan in de bestaande modellen die gebruik maken van cellen die groeien in een enkele laag, een monolayer. Deze spheroids worden geïmplant in een collageen gel. Na 2 dagen is een duidelijk verschil te zien tussen spheroids die behandeld zijn met TGF- β en controle spheroids. Als in deze assay cellen worden gebruikt die verschillende stadia van het ontstaan van kanker vertegenwoordigen, namelijk normale cellen, cellen die goedaardige tumoren vormen en cellen die kwaadaardige tumoren vormen en cellen die kunnen uitzaaïen. De invasie van deze cellen correleert met hun kwaadaardigheid, dus de kwaadaardige cellen invaderen het meest.

Met dit model konden we belangrijke mediators van TGF- β -geïnduceerde invasie identificeren. Dit is beschreven in **hoofdstuk 4**. Remming van Smad4 of de R-Smad Smad3 leidde tot verminderde TGF- β -geïnduceerde invasie, wat een belangrijke rol voor deze signaleringseiwitten impliceert. Deze remming leidde ook tot verminderde transcriptie van induktie van de genen die coderen voor matrix metalloproteinases MMP2 and MMP9. Deze enzymen zijn belangrijk voor het afbreken van de extracellulaire matrix zodat cellen verder kunnen bewegen, maar ze activeren ook groeifactoren en breken eiwitten af die de hechting (adhesie) tussen cellen verzorgen, waardoor de cellen dus vrijelijker kunnen bewegen.

Remming van MMP2 en MMP9 verminderde ook de TGF- β -geïnduceerde invasie. Dus activatie van Smad3 en Smad4 door TGF- β leidt tot hogere expressie van MMP2 en MMP9, die een belangrijke rol spelen bij invasie van kankercellen.

Uiteraard kunnen ook andere factoren een rol spelen bij TGF- β geïnduceerde invasie. TGF- β induceert in epitheliale cellen de overgang van een epitheliaal naar een mesenchymaal uiterlijk (EMT). De meeste kankercellen hebben hun oorsprong in het epitheel; dit zijn cellen die stevig aan elkaar vastzitten en dus niet heel beweeglijk zijn. Tijdens EMT verandert de epitheliale kankercel in een meer beweeglijke, mesenchymale kankercel, waarbij de expressie van eiwitten zoals E-cadherine, die adhesie tussen cellen verzorgen, wordt onderdrukt. Een aantal regulatie eiwitten (transcriptiefactoren) kunnen de expressie van E-cadherine onderdrukken, waaronder Snail, Slug, ZEB1 and Twist. In de kanker cellen

induceerde TGF- β de expressie van Snail en Slug. Dit wordt beschreven in **hoofdstuk 6**. Overexpressie van Slug en Snail leidde tot meer invasie. Deze bevindingen suggereren dat inductie van EMT door TGF- β belangrijk is voor de invasie van borstkankercellen.

BMP-7 kan TGF- β -geïnduceerde EMT remmen. Ook remt BMP-7 het uitzaaien van borskankercellen naar de botten. Daarom werd in **hoofdstuk 5** het effect van BMP-7 op TGF- β -geïnduceerde invasie. BMP-7 remde de TGF- β -geïnduceerde invasie in een kwaadaardige tumor cellijn, maar niet in een goedaardige tumor cellijn. Maar BMP-7 liet de TGF- β -geïnduceerde groeiremming ongemoeid. Dit suggereert dat BMP-7 alleen oncogene invasie kan remmen.

Ook onderzochten we welke TGF- β -geïnduceerde genen door BMP-7 worden geremd. Hiertoe behoorden de integrine subunits α_v en β_3 , die samen integrine $\alpha_v\beta_3$ complex vormen. Integrines verzorgen de adhesie van cellen aan de extracellulaire matrix, waardoor cellen zich kunnen afzetten tegen de extracellulaire matrix om zich voort te bewegen. Verder induceert binding van een integrine aan de extracellulaire matrix een signaal in de cel die leidt tot een grotere beweeglijkheid. Remming van integrin $\alpha_v\beta_3$ leidde, net als BMP-7, tot remming van TGF- β -geïnduceerde invasie. Overexpressie van integrin β_3 zorgde er ervoor dat BMP-7 de TGF- β -geïnduceerde invasie niet meer kon remmen. Dus de remming van integrine $\alpha_v\beta_3$ door BMP-7 draagt bij aan het remmende effect van BMP-7 op TGF- β -geïnduceerde invasie. BMP signalering is vaak ontregeld in kanker. Hoewel BMP-7 meestal een anti-oncogene rol wordt toegedicht, hebben andere BMPs, zoals BMP-2 en BMP-4 een oncogene rol. Wat de rol van BMP signalering betreft in kanker is niet zoveel bekend als voor TGF- β signalering. In **hoofdstuk 7** wordt daarom een zoektocht (screen) naar nieuwe regulator eiwitten beschreven waarbij verschillende enzymen met fosforylerings activiteit (kinases) tot overexpressie werden gebracht in tumor cellen. Er werd vervolgens gekeken naar het effect op BMP signalering. In deze screen identificeerden we zowel bekende als nieuwe kinases die BMP signalering kunnen beïnvloeden. Zo heeft CDK8 een negatief effect op BMP-signalering, terwijl RPS6KA4 BMP de signalering stimuleert. Verder onderzoek zal uitwijzen hoe belangrijk deze kinases zijn voor oncogene BMP signalering.

Samenvattend, in dit proefschrift is onderzoek gedaan naar eiwitten welke een belangrijke

rol spelen bij TGF- β -geïnduceerde invasie van borstkankercellen, namelijk Smad3, Smad4, MMP2 en MMP9. Ook is onderzocht wat het effect van BMP-7 op deze invasie is en daarbij een belangrijke rol ontdekt voor integrine $\alpha_v\beta_3$. Daarnaast zijn er twee nieuwe kinases gevonden die mogelijk een rol hebben in BMP signalering. Tenslotte is gevonden dat Slug en Snail, twee belangrijke mediators van EMT, ook van belang zijn voor invasie. De kennis opgedaan in dit proefschrift zal op de lange termijn kunnen bijdragen aan het ontwikkelen van medicijnen gericht tegen de mediators van de oncogene rol van TGF- β .

List of publications

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Curriculum vitae

Hildegonda Petronella Henriëtte Naber werd op 29 januari 1983 geboren te 's-Gravenhage. Zij startte met gymnasium op St. Maartenscollege te Voorburg in 1995, welke zij afsloot met een diploma *cum laude* in 2001. Zij vervolgde haar opleiding aan de Universiteit van Amsterdam met de studierichting Bio-medische wetenschappen in 2001. Haar eerste wetenschappelijk stage liep zij in 2004-2005 op de afdeling Immunohematologie (later Blood Cell Research) van Sanquin in de groep van prof. dr. D. Roos en prof. dr. T. Kuijpers onder supervisie van N. Brouwer. Het onderzoek richtte zich op de oligomerisatie van Mannose Binding Lectin, een eiwit dat betrokken is bij de aangeboren immuniteit. Haar tweede stage liep zij in 2005-2006 op de afdeling pathologie van het Academisch Medisch Centrum in de groep van prof. dr. S. Pals and dr. M. Spaargaren onder supervisie van D.J.J. de Gorter. Hier bestudeerde zij de rol van de kleine GTPase Ral in Stromal Factor Derived-1 geïnduceerde migratie van B-cellen en multiple myeloma cellen. In augustus 2006 ontving zij haar Master diploma in de richting Immunologie *cum laude*.

In 2006 startte zij met haar promotie-onderzoek op de Afdeling Moleculaire Celbiologie van het LUMC onder supervisie van prof. dr. P. ten Dijke. Tot eind 2010 deed zij onderzoek naar de rol van Transforming Growth Factor- β (TGF- β) en Bone Morphogenetic Proteins (BMP) in invasie van borstkankercellen. De resultaten van dit onderzoek staan beschreven in dit proefschrift en in verschillende wetenschappelijke tijdschriften. Tevens heeft zij haar resultaten mondeling gepresenteerd op de KWF Tumor Cell Biology meeting in 2009 waarbij zij een prijs voor beste presentatie in ontvangst mocht nemen. In 2010 ontving zij een reisbeurs van het Leids Universitair Fonds om het Nature 2010 Miami Winter Symposium te bezoeken, waar zij een poster presenteerde.

Sinds 2011 is zij werkzaam bij Cell Signaling Technology Europe als Technical Support Specialist. Zij gebruikt hierbij haar wetenschappelijke kennis om klanten van dit bedrijf, veelal wetenschappers, te ondersteunen.

