TGF-beta and BMP in breast cancer cell invasion

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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 (TβRII) and type I (TβRI) receptors. Upon TGF-β-induced heteromeric complex formation, TβRII phosphorylates TβRI. The activated TβRI 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-b, and components of the TGF-b pathway are frequently inactivated in a subset of cancer specimens [12]. However, breast and other cancers often only carry defects in downstream cytostatic TGF-b targets, show normal TGF-b 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), 0.5μg/ml hydrocortisone (Sigma), 10 μg/ml insulin (Sigma), 100U/ml penicillin and 50μg/ml streptomycin (Gibco). All cell lines were grown in a humidified incubator at 37°C and 5% CO_2.

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 10^4 cells/ml. 100μl 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 10^3 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-β3 (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 puromycine; 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 $CaCl_2$) 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

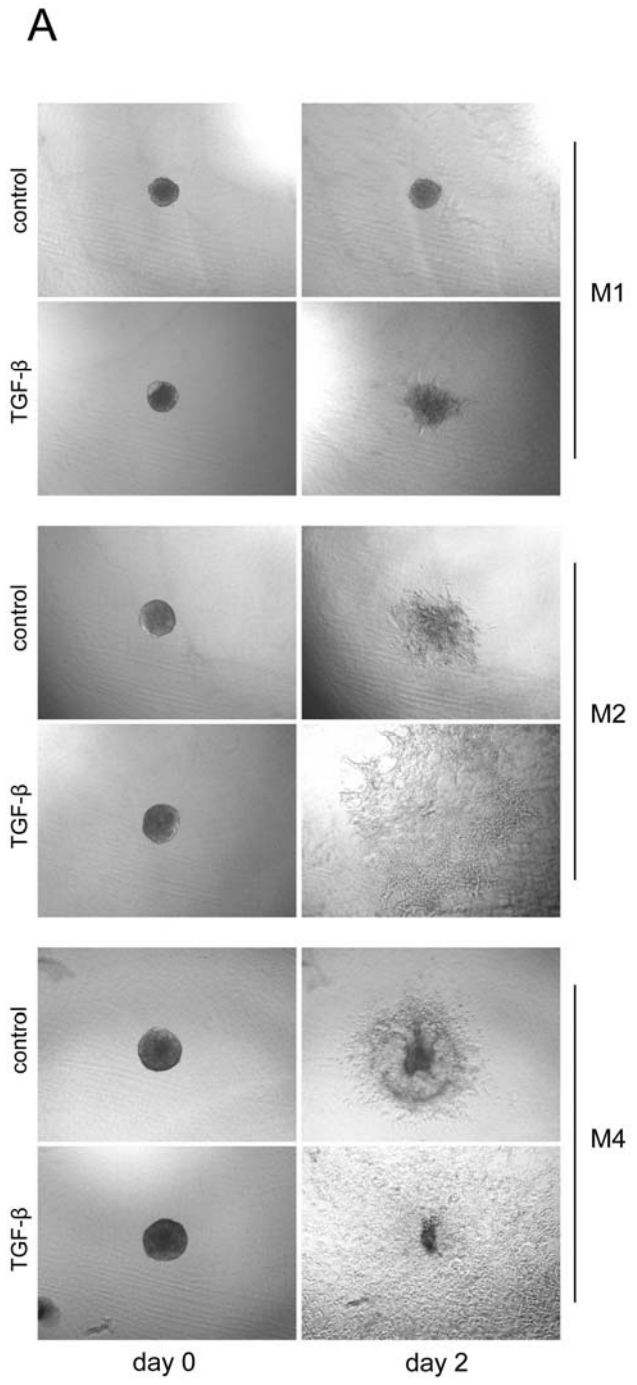
Assays for cell proliferation, transcriptional reporter activity, Western blot analysis, RNA isolation, cDNA synthesis and quantitative PCR are found in supplementary methods

Results

Establishment of a spheroid cell culture model to study TGF-b-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 $CAGA_{12}$-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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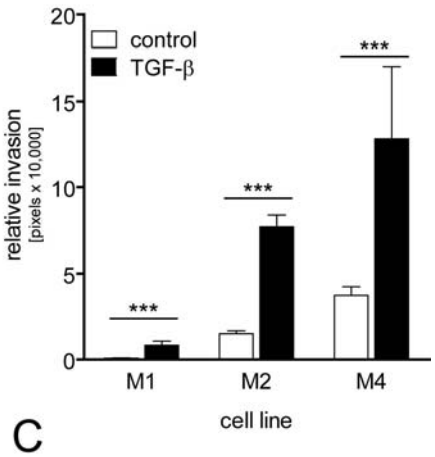
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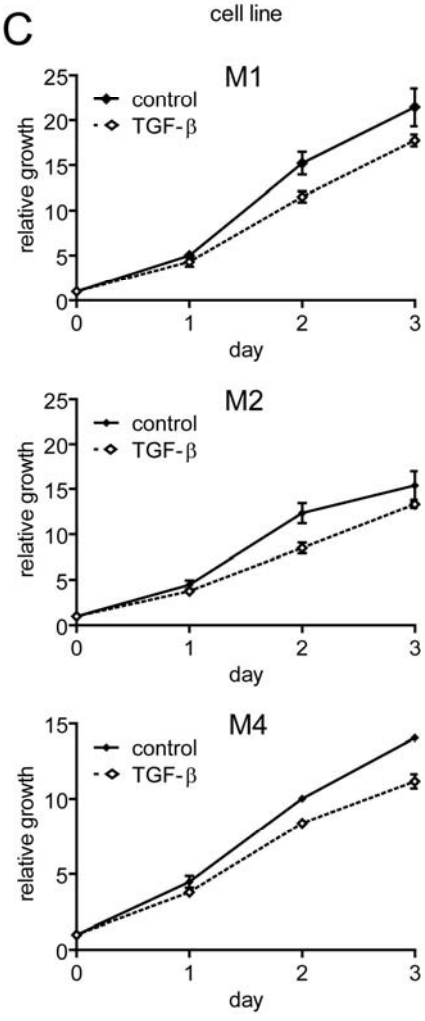
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Figure 1 TGF-β-induced invasion of spheroids of normal MCF10A1 (M1), RAS- transformed MCF10AneoT (M2) and its metastatic derivative MCF10CA1a (M4). M1, M2 and M4 spheroids embedded into collagen were treated with 5ng/ml of TGF-β for 48 hr as indicated (**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 ± 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 ± SEM. of three independent experiments performed in triplicate. Significance: ***P<0.001.

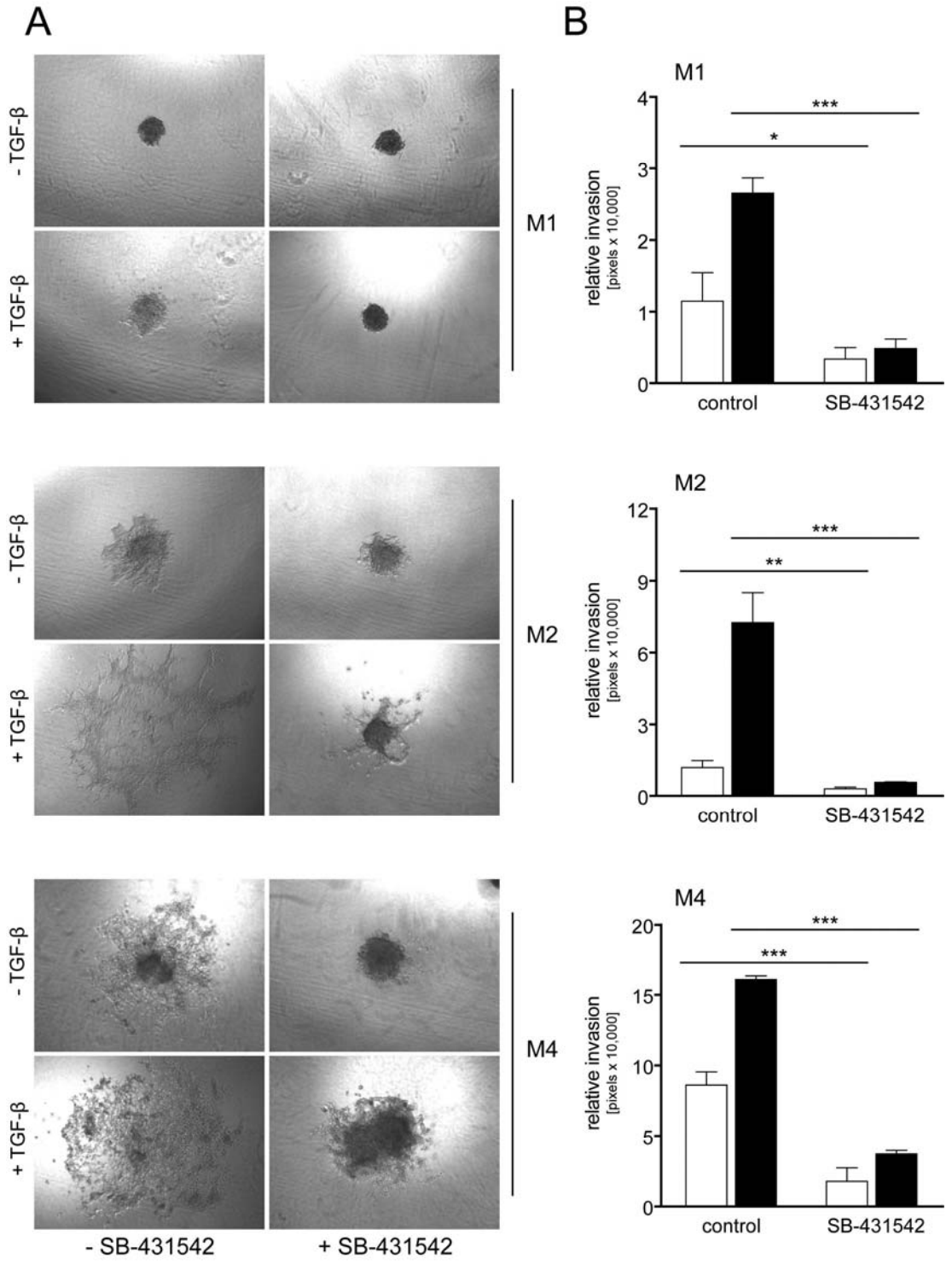
receptor binding and transcriptional activity did not correlate with the levels of spheroid invasiveness of the three cell lines.

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 TβRI kinase. For this purpose we used SB-431542, an ATP analogue and selective inhibitor of the kinase activity of TβRI, activin type IB receptor (ActRIB) and activin receptor-like kinase (ALK)7 [34]. As expected, SB-431542 potently inhibited TGF-b-induced invasion of M1, M2 and M4 cells (Fig 2a, b), indicating that TGF -β-induced invasion is TβRI-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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- TGF-β
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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 10μM 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 ± s.d. (M1: n=3, M2 and M4: n=4). Significance: ***P<0.001, **P<0.01, *P<0.05.

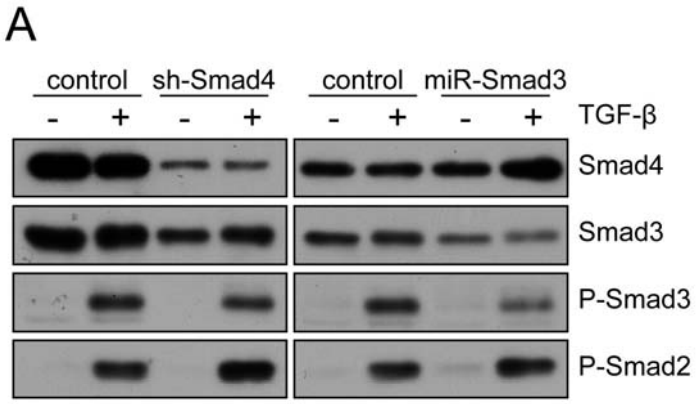
ActRIB and ALK7. However, Activin-A failed to induce invasion (data not shown) and only marginally induced $CAGA_{12}$-luciferase transcriptional activity, possibly caused by low expression of activin receptors in these cells (Suppl. Fig. 1a).

TGF-β-induced invasion is Smad3/4 dependent

As TGF-β signaling potently 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 $CAGA_{12}$-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-b-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 knockdown 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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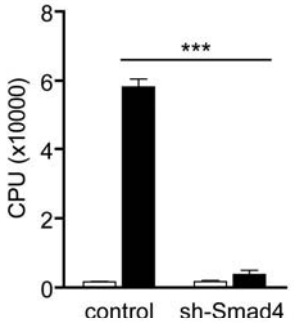
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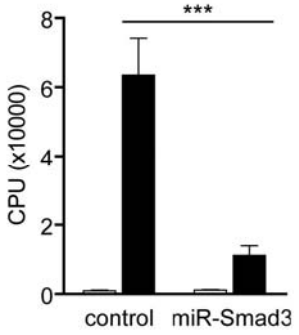
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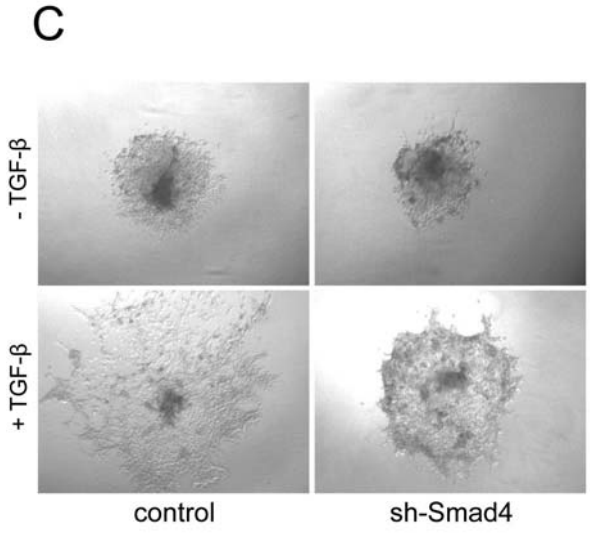
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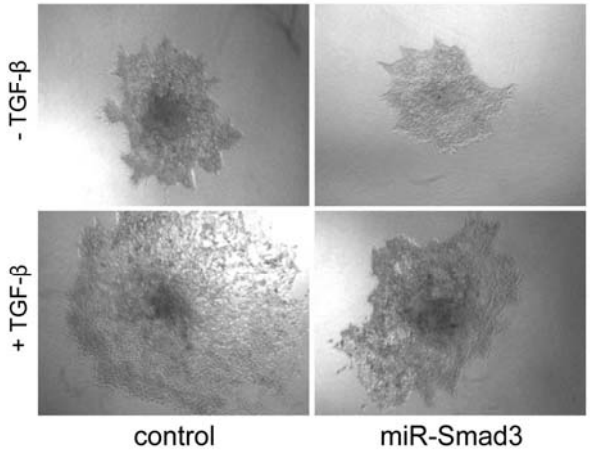
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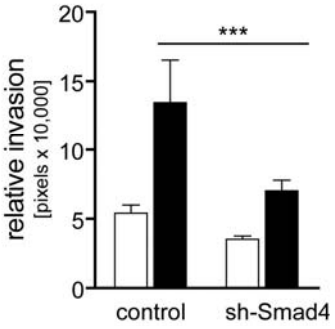
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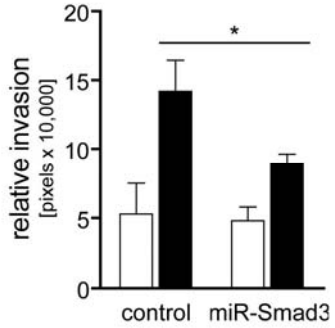
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Figure 3 TGF-β-induced spheroid invasion is Smad3/4 dependent. MCF10CA1a (M4) cells were transduced with lentiviruses expressing shRNA against Smad4 or miRNA against Smad3 and the appropriate control viruses. **a** Cells were stimulated with 5ng/ml 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 $CAGA_{12}$-luc transcriptional reporter and subsequently stimulated with 5ng/ml of TGF-β for 16h. Relative luciferase activity (CPU) is expressed as mean ± 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 ± 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

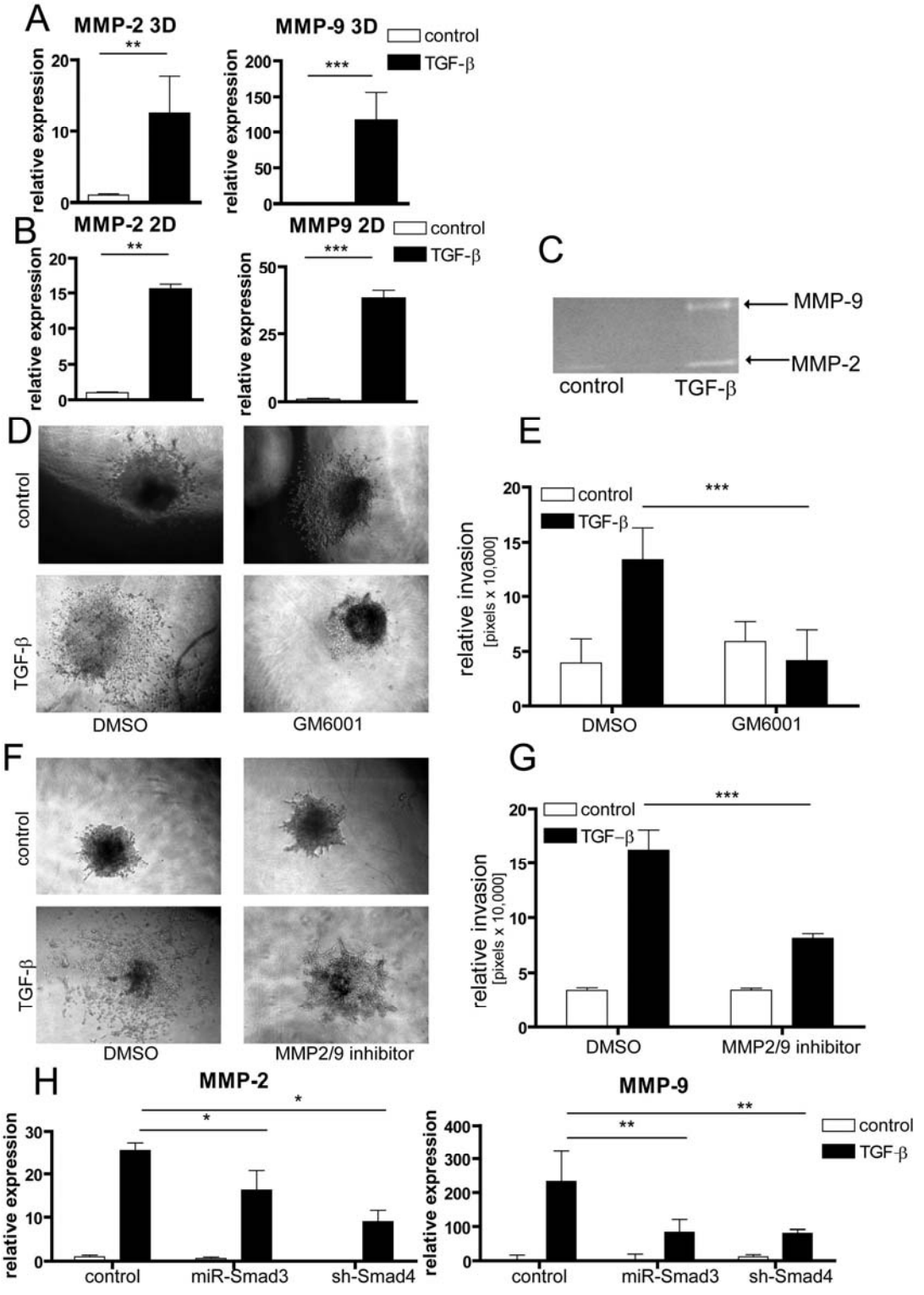
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Figure 4 TGF-β-induced spheroid invasion is dependent on matrix metalloproteinase activity. **a,b** RNA was isolated from spheroids (**a**) or monolayer (**b**) of M4 cells stimulated for 24 hr with or without TGF-β (5ng/ml). Q-PCR for MMP2 and MMP9 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 (1μM) (**d,e)** or selective MMP 2/9 inhibitor (1μM) (**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 ± 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-b-induced invasion of M4 spheroids was dependent on Smad3/Smad4 (Fig. 3), we next examined the role of Smad3 and 4 on TGF-b-induced MMP2 and 9 expression. Both knockdown of Smad3 and Smad4 resulted in strongly reduced TGF-b-induction of MMP2 and 9 in M4 cells (Fig. 4h). Taken together, TGF-b-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 -b 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 coworkers 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 TβRI 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 TβRI-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 TβRI 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.

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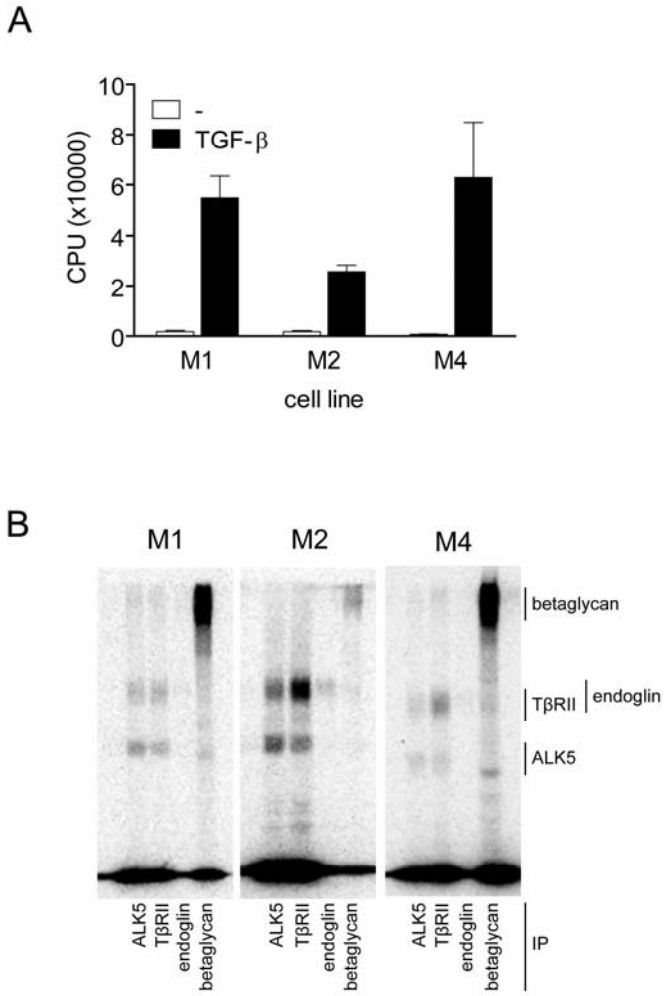
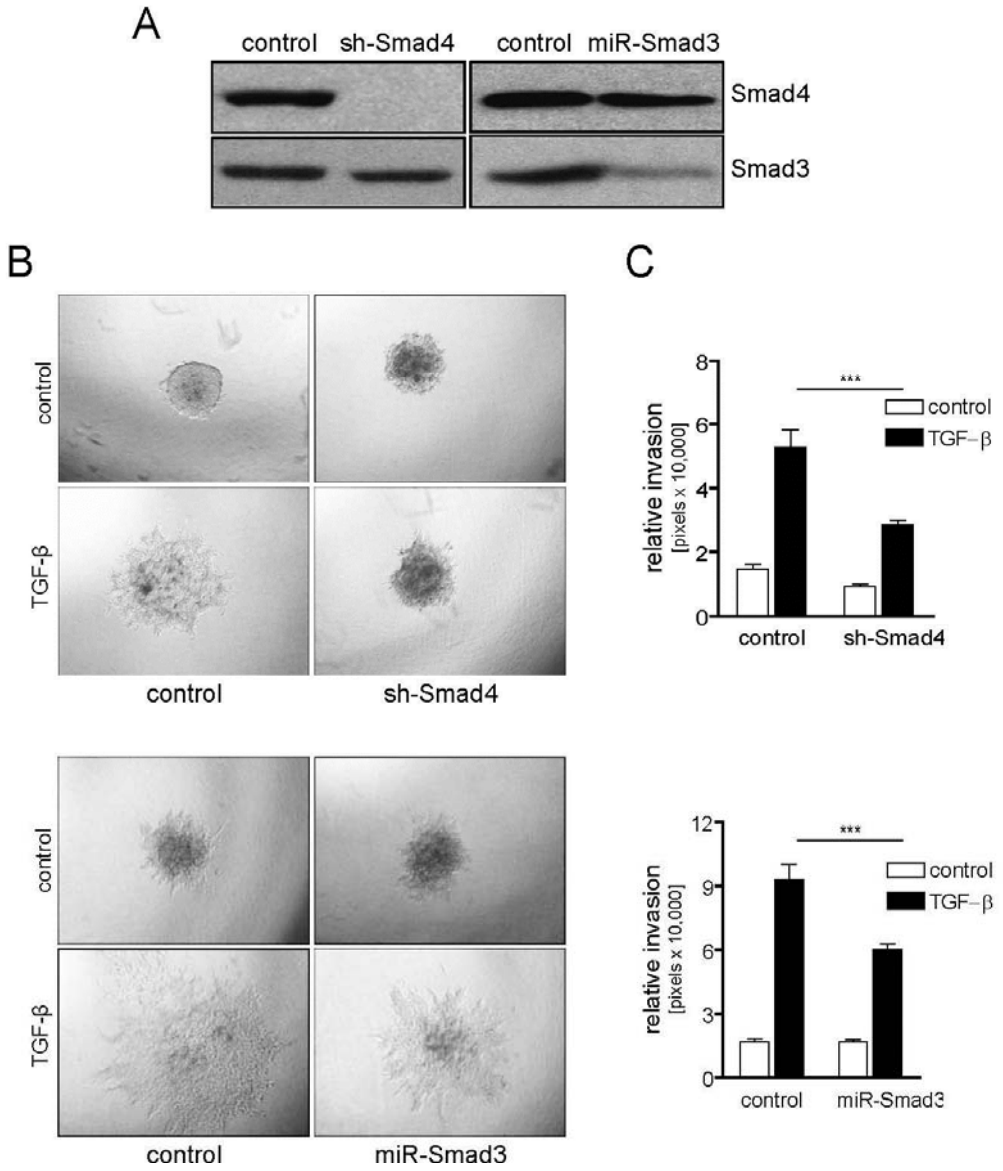
Supplementary materials

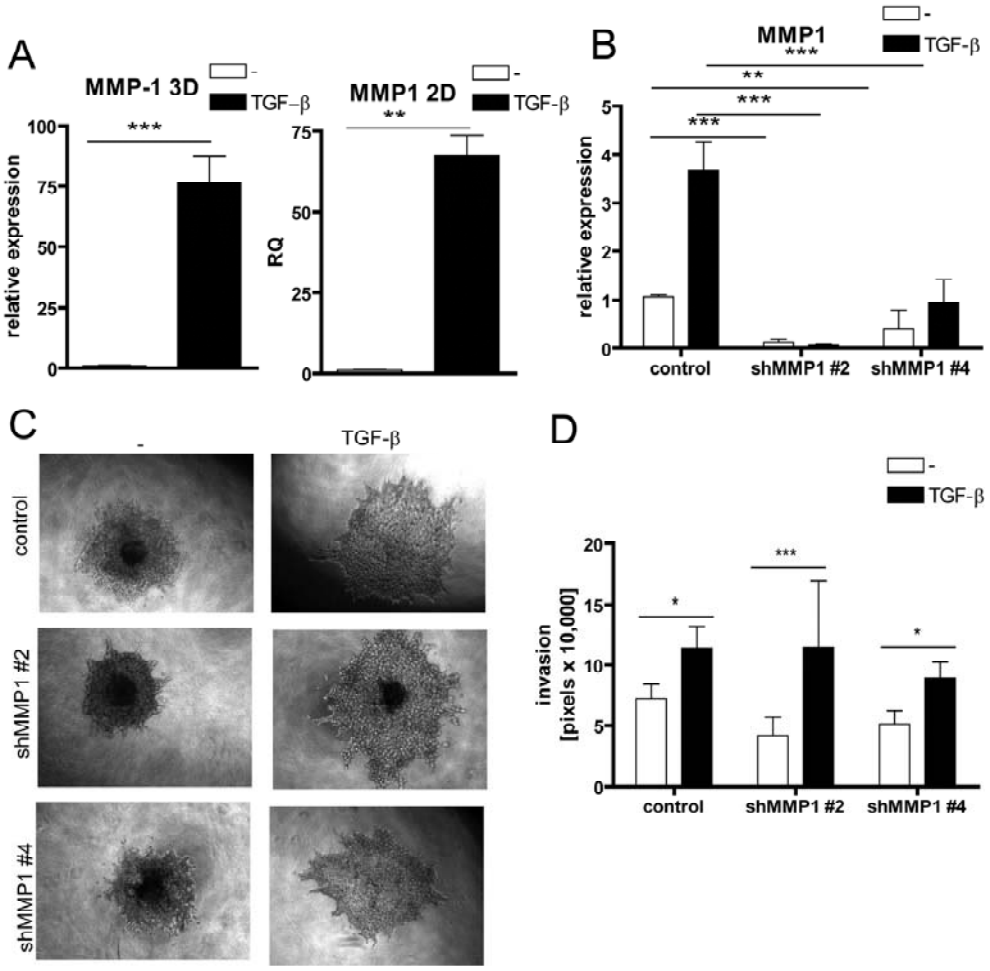
Supplementary figures



Supplementary figure 1 A. M1, M2 and M4 cells were transfected with $CAGA_{12}$-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 [^{125}I]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 Tβ RII 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 ± s.d.

Supplementary materials and methods

[^{125}I]TGF-β receptor binding assay

Iodination of TGF-β3 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 5x10^2 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 CAGA$_{12}$-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-β3. Luciferase and β-galactosidase activity were determined as previously described [5]. Each transfection was carried out in triplicate and the mean ± s.d. is shown.

Western blot analysis

2 x 10^5 cells were seeded in 6-well-plates. The following day cells were stimulated with 5ng/ml of TGF-β3 for 16h. Cells were lysed in RIPA-buffer and 20µg 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 ΔCt method using *ARP* as reference and the non-stimulated condition was set to 1. Relative expression levels are presented as mean ± 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'-TTGTCGGCGATAAGGAAG-3', ARP forward 5'- CACCATTGAAATCCTGAGTGATGT -3' and reverse 5'- TGACCAGCCGAAAGGAGAAG -3'.

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