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TGF-beta and BMP in breast cancer cell invasion

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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:methocel 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:methocel 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'-GGTTCTCCAGCTTCAGGTAAT-3'; integrin α_v forward 5'-CAGGCTTGCAACCCATTCTT-3' and reverse 5'-GAATGTGAGCCTGTCGACTAATGTT-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.

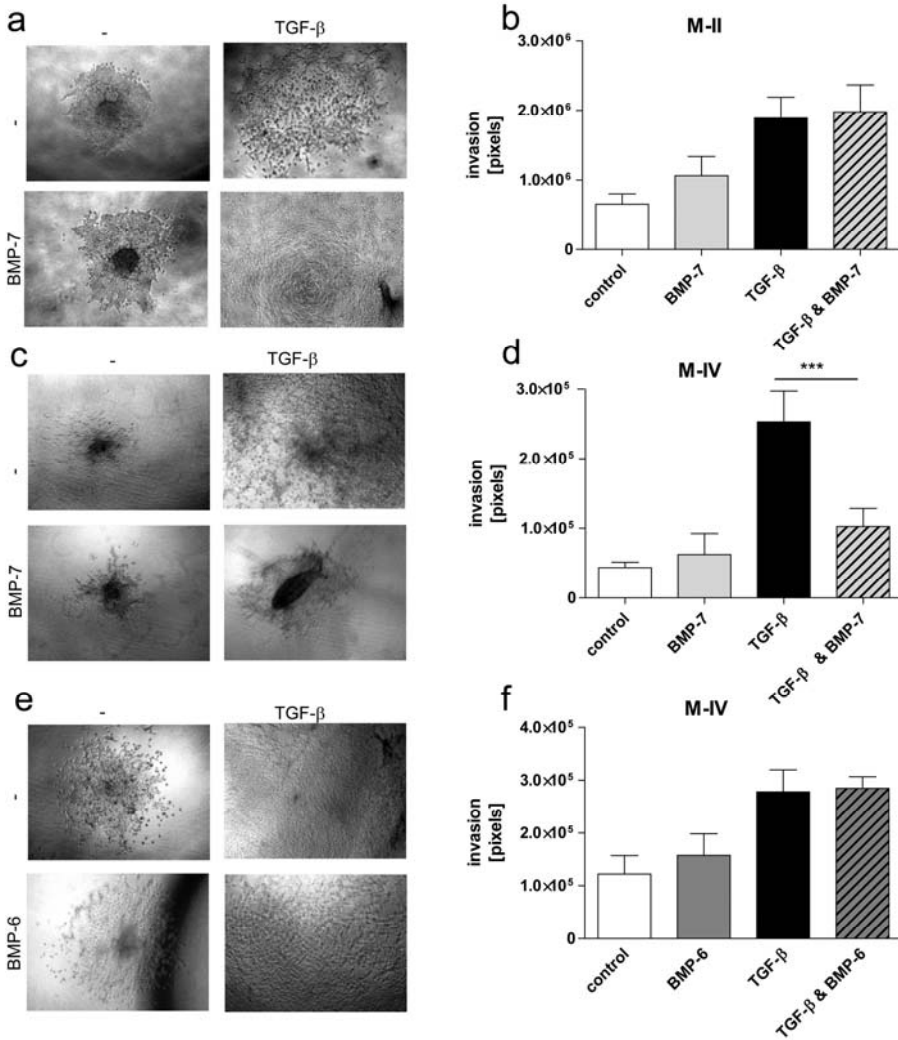


Fig. 1 BMP-7, but not BMP-6, inhibits TGF- β -induced invasion in M-IV, but not in M-II. (a-d) Spheroids were allowed to invade collagen in the presence of BMP-7 (100 ng/ml), TGF- β (5 ng/ml) or TGF- β & BMP-7. (a) Pictures of representative spheroids of M-II. (b) Quantification of invasion of M-II experiments 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

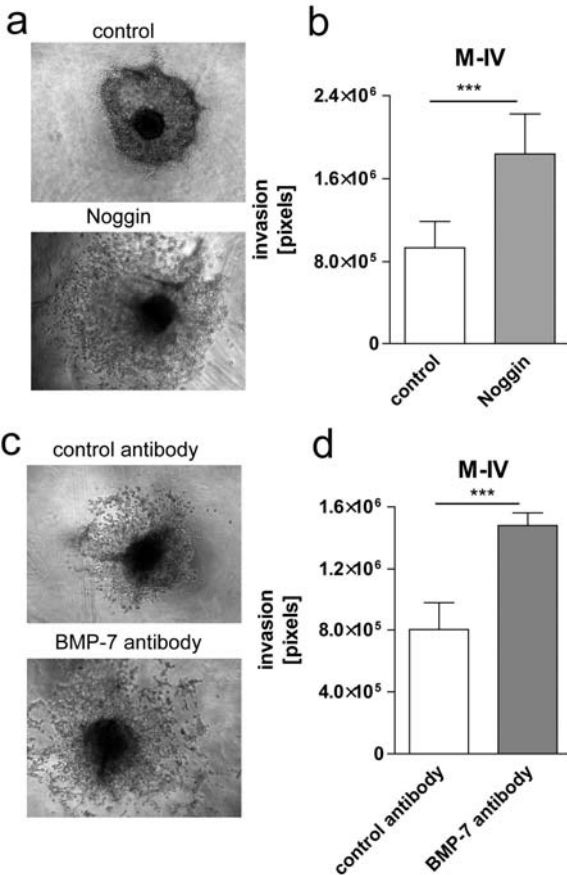


Fig. 2 Noggin enhances basal invasion through inhibition of BMP-7. Spheroids of M-IV were allowed to invade collagen in the absence or presence of Noggin (200 ng/ml). (a) Pictures of representative spheroids. (b) Quantification of spheroids of experiments shown in (a). (c) Spheroids of M-IV were allowed 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.

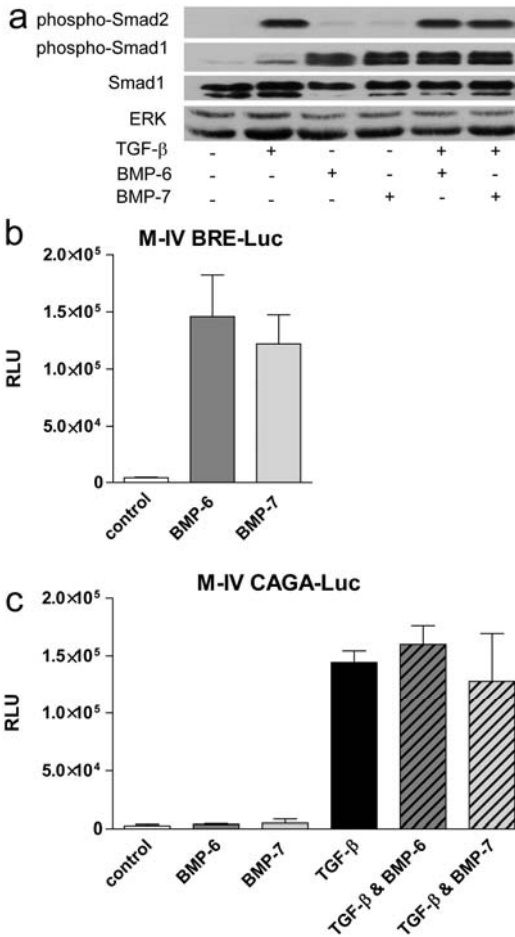


Fig. 3 BMP-6 and BMP-7 induce BMP-Smad signalling, but do not affect TGF- β Smad signaling. (a) Cells were treated with the indicated ligands (BMP-6 and BMP-7 at 100 ng/ml, TGF- β at 5 ng/ml) for 15 min, lysed and subjected to SDS-PAGE and Western blotting. Blots were incubated with phospho-Smad2, phospho-Smad1 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

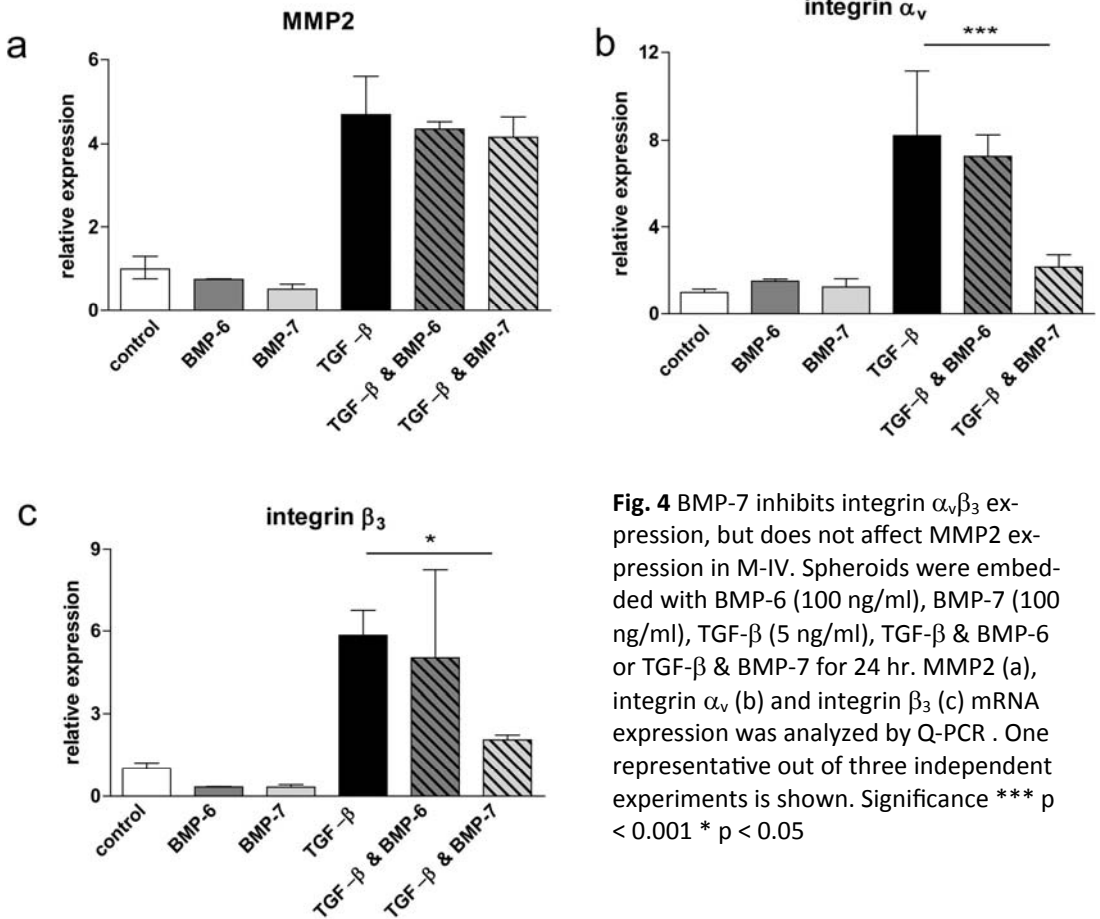


Fig. 4 BMP-7 inhibits integrin $\alpha_v\beta_3$ expression, but does not affect MMP2 expression in M-IV. Spheroids were embedded with BMP-6 (100 ng/ml), BMP-7 (100 ng/ml), TGF- β (5 ng/ml), TGF- β & BMP-6 or TGF- β & BMP-7 for 24 hr. MMP2 (a), integrin α_v (b) and integrin β_3 (c) mRNA expression was 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.

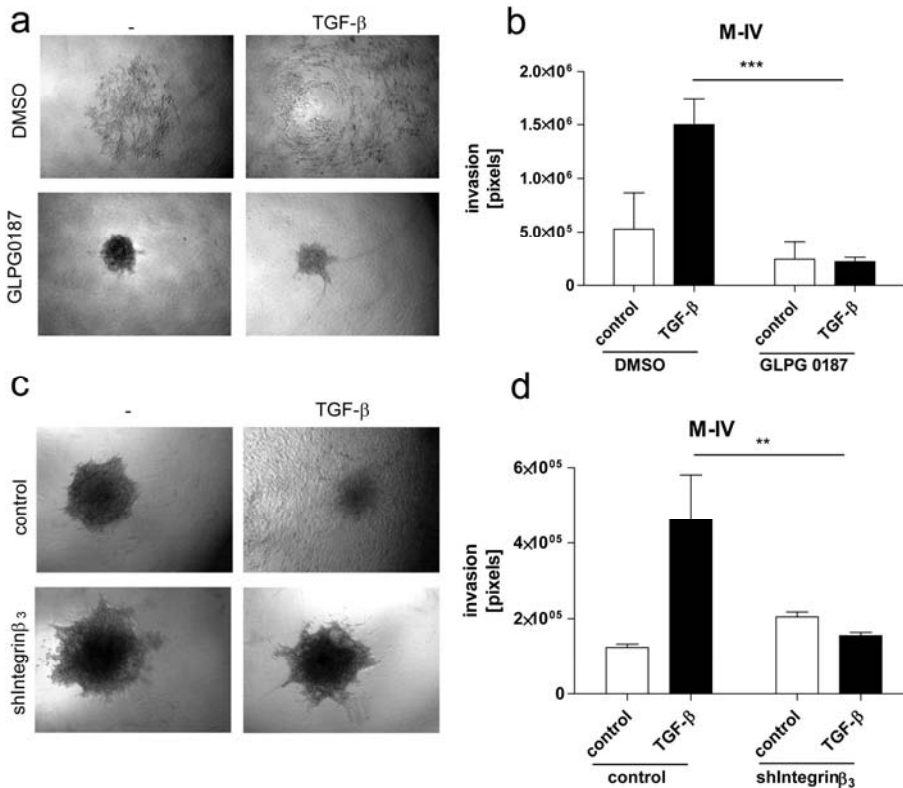


Fig. 5 Integrin $\alpha_v\beta_3$ is necessary for TGF- β -induced invasion. (a) Spheroids were allowed to invade into collagen in the presence of DMSO or GLPG0187 (80 nM) with or without TGF- β (5 ng/ml). Representative pictures of spheroids are shown. (b) Quantification of experiment shown in (a). (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.

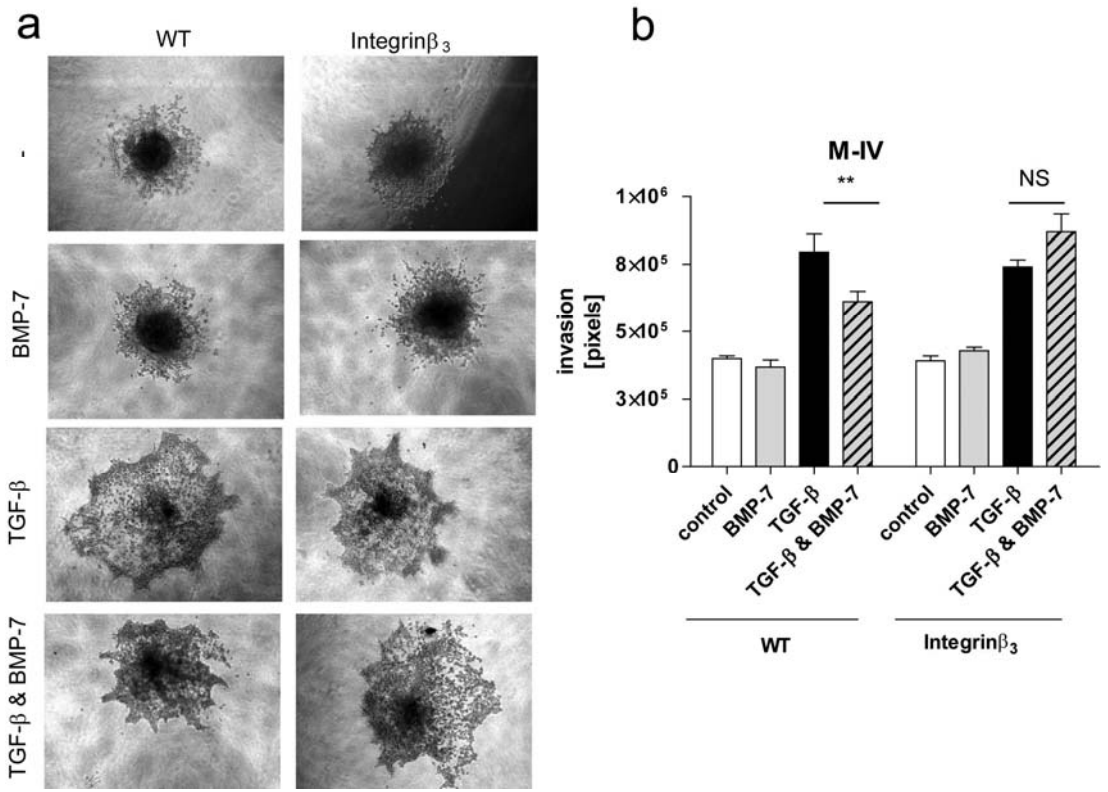


Fig. 6 Integrin β_3 overexpression rescues TGF- β -induced invasion from BMP-7 inhibition. Cells were transduced with lentivirus containing an integrin β_3 overexpression construct. Integrin β_3 overexpressing cells or wildtype (WT) cells were allowed to invade in BMP-7 (100 ng/ml), TGF- β (5 ng/ml) 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 BMP-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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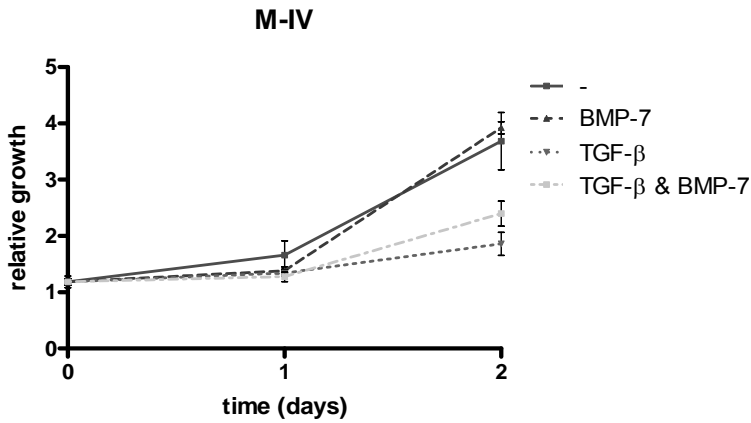
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Supplementary figures



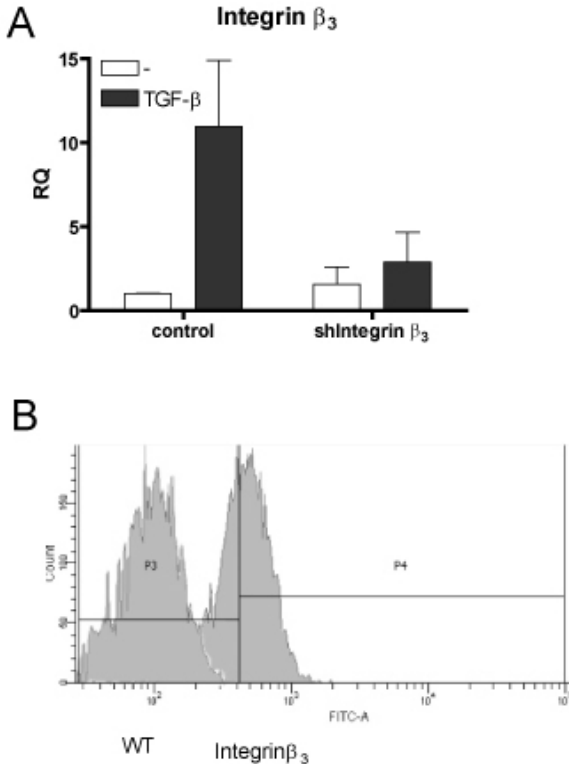
Supplementary Fig. 1

BMP-7 does not affect cell growth. Cell viability of M-IV was measured after 0, 24 and 48 hr of treatment with no ligand, BMP-7 (100 ng/ml), TGF- β (5 ng/ml) or TGF- β & BMP-7.

	$\alpha_v\beta_1$	$\alpha_v\beta_3$	$\alpha_v\beta_5$	$\alpha_v\beta_6$	$\alpha_v\beta_8$	$\alpha_5\beta_1$	$\alpha_2\beta_1$	$\alpha_4\beta_7$	$\alpha_{11b}\beta_3$
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 $\alpha_{11b}\beta_3$. 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 4C and washed. Surface expression was measured on a FACSCanto (B&D).

Supplementary references

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