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Parallel inhibition of cell growth and induction of cell migration and invasion in breast cancer cells by bone morphogenetic protein 4

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Abstract

Bone morphogenetic proteins (BMP) are extracellular signaling molecules that belong to the transforming growth factor β (TGF β) superfamily. BMPs have diverse roles during development where they regulate proliferation, differentiation, and apoptosis in many different cell types by modulating the transcription of specific target genes. BMPs have also been implicated in both promotion and inhibition of cancer progression. We have recently shown that BMP4 is commonly expressed in breast cancer but its functional significance has not been previously explored. Our data demonstrate that in all nine breast cancer cell lines studied, BMP4 treatment leads to a dramatic growth suppression as a result of the induction of G1 arrest of the cell cycle. At the same time, BMP4 stimulates cell migration and invasion in a subset of these breast cancer cell lines. The BMP4 induced phenotypic changes were mediated through the activation of the canonical SMAD signaling pathway whereas no activation of MAP-kinases ERK1/2 or p38 was detected. Our results thus implicate that BMP4 is an important regulator of key phenotypic characteristics of cancer cells, cell growth, cell migration, and invasion, and that, similar to TGF β , it possesses both tumor suppressive and oncogenic properties in breast cancer.

Introduction

Bone morphogenetic proteins (BMPs) are extracellular signaling molecules that belong to the transforming growth factor β (TGF- β) superfamily. BMPs signal through two types of transmembrane serine-threonine kinase receptors, type I and type II. Ligand binds to type II receptor which then activates type I receptor by phosphorylation. Activated type I receptor subsequently phosphorylates receptor regulated SMAD proteins (R-SMADs, SMAD1, -5, and -8). In cytosol, phosphorylated R-SMADs are released from type I receptor and interact with the common SMAD, SMAD4. This complex then translocates into the nucleus where it regulates gene transcription. In addition to the above described canonical BMP signaling pathway, BMPs are also capable of activating several mitogen-activated protein kinases (MAPKs), including ERKs, JNKs, and p38 kinases [1-2]. However, different ligands have different signaling properties and, for example, BMP4 is known to activate p38 and ERK but not JNK pathways [2-5].

BMPs were originally identified as bone and cartilage inducing factors, but they are also known to have critical roles in vertebrate development for example in neural patterning, kidney development, and spermatogenesis [6]. Of specific BMPs, BMP4 has been found to be essential for development since *Bmp4* null mice die early during embryogenesis [7]. More recent analyses have pinpointed an important role for BMP4 in limb, inner ear, kidney, and heart development [8-11]. Interestingly, BMP4 is also involved in mammary gland formation [12-13].

In addition to their actions during vertebrate development, BMPs have also been implicated in many human tumors but their exact role and contribution to cancer

pathogenesis is still under debate. The expression patterns of different BMPs have been studied in multiple tumor types either at mRNA or protein level. To mention a few, BMP6 is overexpressed in prostate cancer [14], BMP7 in colorectal cancer [15], and *BMP2* in pancreatic cancer [16]. In contrast, *BMP2* is downregulated in breast cancer [17]. The functional consequences of such aberrant BMP expression in cancer have been elucidated mainly using cell line model systems. These studies have revealed that BMPs are able to inhibit the growth of breast [18-22], prostate [23-24], and colon cancer cells [25]. These growth inhibitory effects were shown to be mediated by induction of G1 cell cycle arrest [18-20, 23-25]. In lung cancer BMP2 induced cell growth both *in vitro* and *in vivo* [26] whereas in pancreatic cancer both growth inhibitory and stimulatory effects have been observed after BMP2 treatment [16]. These contradictory growth responses to BMPs are often dependent on the cell line or culture conditions used as shown in breast and prostate cancer [22, 27-28]. In breast cancer there are only few studies that have examined the role of BMPs using *in vivo* mouse models and both inhibitory and stimulatory effects of BMPs have been reported. BMP7 has been shown to inhibit primary tumor and bone metastasis growth [29] whereas BMP2 has tumor growth and bone metastasis promoting effect [30-32]. Taken together, BMPs can exert diverse phenotypic effects in cancer cells.

Rather little is known about the possible effects of BMP4 on cancer cells. It has been shown to inhibit the proliferation of myeloma [33], lung cancer [34], and undifferentiated human cancer cells [35]. On the other hand, BMP4 increased the growth of prolactinoma cells [36] and induced migration and/or invasion of colon [37], ovarian [38], and pancreatic cancer cells [39]. BMP4 has also been implicated in the induction of

epithelial-mesenchymal transition (EMT) in pancreatic cancer [39-40] and ovarian cancer [38]. In breast cancer, we have previously shown that BMP4 is commonly expressed both in breast cancer cell lines and primary tumors [41]. Shon and coworkers [42] recently reported reduced migration and invasion of MDA-231 breast cancer cells after BMP4 treatment but there are no other reports on the possible functional relevance of BMP4 in breast cancer. In this study, we show using a large breast cancer cell line panel that BMP4 induces a G1 cell cycle arrest leading to a dramatic growth inhibition and that it, at the same time, promotes breast cancer cell migration and invasion.

Materials and methods

Cell lines and primary breast tumors

Twenty-one breast cancer cell lines (BT-474, CAMA-1, DU4475, HCC38, HCC1419, HCC1954, MCF-7, MDA-134, MDA-231, MDA-361, MDA-415, MDA-436, MDA-453, SK-BR-3, T-47D, UACC3133, UACC732, UACC812, UACC893, ZR-75-1, and ZR-75-30) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and MPE600 cells were originally provided by Dr. Helene Smith. Normal human mammary epithelial cells (HMEC) were obtained from Clonetics (Walkersville, MD, USA). Cells were cultured as recommended, except in multi-well plate experiments where MDA-361 cells were cultured in DMEM containing 2mM glutamine, 1% penicillin/streptomycin, and 10% FBS.

Thirty-nine freshly frozen primary breast tumor specimens were acquired from the Department of Pathology, Tampere University Hospital. The clinicopathological

characteristics of the tumor samples are presented in Table 1. The use of these samples in this study was approved by the Ethics Committee of the Pirkanmaa Hospital District and the National Board of Medicolegal Affairs.

Real time quantitative RT-PCR (qRT-PCR) and semiquantitative RT-PCR

The *BMP4* mRNA levels were measured by qRT-PCR using the LightCycler equipment (Roche, Mannheim, Germany). Total RNA was extracted either using the TRIzol-reagent (Invitrogen, Carlsbad, CA) or the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed with SuperScriptTM First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) as described [43]. The housekeeping gene *TBP* (TATA box binding protein, reference) and *BMP4* transcript levels were determined using the LightCycler FastStart DNA Master Hybridization probe kit (Roche, Mannheim, Germany) and gene specific primers (*BMP4* primers: sense: 5'-GGGACTTCGAGGCGACA-3'; antisense: 5'-TTCTCCAGATGTTCTTCGTGGT-3') and hybridization probes (TIB MOLBIOL, Berlin, Germany) as described [44]. The expression levels of *BMP4* were normalized to those of *TBP*. Finally, E-cadherin (*CDH1*) and vimentin (*VIM*) mRNA levels were determined using semiquantitative RT-PCR as described [45].

BMP4 treatment

Recombinant human BMP4 protein was purchased from R&D Systems (Minneapolis, MN). For functional assays 2-10 x 10⁴ cells (24-well plates) or 10-50 x 10⁴ cells (6-well plates) were seeded. After 24h the culture medium was replaced with medium containing

BMP4 (100ng/ml, unless otherwise indicated) or an equivalent volume of vehicle (4mM HCl, 0.1% BSA). BMP4 or vehicle containing fresh medium was added to the cells every second or third day. Functional assays were performed at the indicated time points.

BMP4 silencing

For BMP4 silencing experiments, 3×10^4 (24-well plates) or 20×10^4 (6-well plates) T-47D cells were seeded. After 24h, cells were transfected with BMP4 On-Target plus Smartpool siRNAs (Thermo Scientific Dharmacon, Lafayette, CO, USA) or control siRNAs targeting the firefly *luciferase* (*PPYLUC*) gene using Interferin reagent (Polyplus-Transfection, San Marcos, CA) as described [46].

Cell proliferation

Cell proliferation assays were performed using 24-well plates. Cells were counted using Z2 Coulter Counter (Beckman Coulter, Fullerton, CA) at the indicated time points after the first addition of BMP4 or vehicle or after the siRNA transfection. All experiments were done in six replicates and were repeated at least twice.

Cell cycle and apoptosis assays

For cell cycle and apoptosis analyses, cells were cultured either using 24- or 6-well plates and were analyzed 2 days after the first addition of BMP4 or vehicle. Cell cycle analyses were done using propidium iodide (PI) staining as previously described [47]. For the apoptosis analyses, Annexin V FITC apoptosis detection kit (Calbiochem, La Jolla, CA) was used as described [47]. The cell cycle distribution was analyzed with ModFit LT

Version 3.1 (Verity software house, USA) and apoptosis with EXPO32 ADC Version 1.2 analysis software (Beckman Coulter). All experiments were done in three to six replicates and repeated at least twice.

Migration and invasion assays

Cell migration and invasion was studied using BD Falcon™ cell culture inserts and BD BioCoat™ Matrigel invasion chambers (both 8.0µm pore size, BD Biosciences, Bedford, MA) as described [22]. Cells were collected after 3 days of BMP4 (100ng/ml) or vehicle treatment or siRNA transfection and $1.75\text{-}20 \times 10^4$ cells, depending on the cell line, were transferred to the upper chambers and were allowed to migrate/invade for 22 hours. In the case of MDA-231 cells, BMP4 or vehicle were alternatively added directly to the upper chamber of migration insert. The migration and invasion membranes were scanned with Aperio ScanScope® XT (software version 9; Aperio Technologies, USA) using a lossless image format as primary output. The total area of migrated or invaded cells (area percent) or cell count on each membrane was determined with ImageJ software [48]. The assays were performed with three to six replicates and repeated twice.

Western analyses

To collect total protein, cells were washed twice with PBS and then lysed into RIPA-buffer (1% PBS, 1% non-idet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and PhosStop phosphatase inhibitor cocktail (Roche). The protein content of cell lysates was determined using Bradford reagent (Sigma-Aldrich Chemie

GmbH, Steinheim, Germany). Gel-electrophoresis and blotting were done as described previously [22]. The following primary antibodies were used: rabbit polyclonal SMAD-5, rabbit polyclonal p44/42 MAP kinase (identifies ERK1/2 MAP kinases), rabbit polyclonal p38 MAP kinase, rabbit polyclonal Phospho-SMAD1/5/8, rabbit polyclonal Phospho-p44/42 MAP kinase (identifies phospho-ERK1/2 MAP kinases), rabbit polyclonal Phospho-p38 MAP kinase (all 1:1000 dilution, Cell Signaling Technology, Inc., Beverly, MA), and mouse monoclonal Smad4 (1:200 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Anti-mouse/rabbit IgG-POD antibody was used as secondary antibody (1:5000 dilution, Roche Diagnostics GmbH). Primary and secondary antibody incubations and protein detection were done as described earlier [22].

Statistical analyses

The Mann-Whitney test was used to calculate statistical differences between *BMP4* expression level and standard clinicopathological parameter of the primary breast tumors as well as *BMP4* and vehicle treated cells in cell proliferation, cell cycle, apoptosis, and cell migration assays.

Results

Breast cancer cell lines and primary breast tumors express BMP4 with varying levels

Our group has previously reported frequent *BMP4* expression in breast cancer using standard semiquantitative RT-PCR [41]. Here we used quantitative real-time RT-PCR to

obtain more accurate information on *BMP4* mRNA expression levels in a panel of twenty-two breast cancer cell lines as well as in normal human mammary epithelial cells (HMEC). *BMP4* expression was detected in 20 of the 22 cell lines examined and in general the expression levels varied widely from one cell line to another (Fig. 1a). There was no endogenous *BMP4* expression in the HCC1419 and UACC3133 cells and very low levels were detected in MDA-436. The highest *BMP4* mRNA levels were seen in the HCC38, UACC732, DU4475, MDA-453, T-47D, and MDA-231 cell lines. Especially in the case of HCC38, the *BMP4* expression level was extremely high, about hundred times higher compared to HMEC (Fig. 1a).

To validate the cell line data, *BMP4* mRNA levels were also measured in 39 primary breast tumors. Eleven out of the 39 tumor samples had no detectable *BMP4* expression. Twenty-five samples (64%) expressed *BMP4* at a level similar to that seen in the majority of the breast cancer cell lines (Fig. 1b). Although none of the primaries displayed such highly elevated *BMP4* expression as was observed in few of the cell lines, there was a group of three tumors with relatively high expression, exceeding that detected in the normal mammary gland sample (Fig. 1b). Overall, similar wide spectrum of *BMP4* expression was thus revealed both in breast cancer cell lines and primary tumors. Comparisons between *BMP4* expression levels and standard clinicopathological tumor characteristics (size, grade, nodal status, ER, PR, and ERBB2) revealed an inverse correlation to tumor grade. Grade III tumors had significantly ($P = 0.04$) lower *BMP4* expression level than grade II tumors. It is especially interesting that 10 of the 11 primary tumors with no *BMP4* expression were of grade III.

BMP4 decreases breast cancer cell growth

To study the possible effects of BMP4 on breast cancer cell growth, we selected nine cell lines (HCC38, HCC1419, HCC1954, MCF-7, MDA-231, MDA-361, SK-BR-3, T-47D, and ZR-75-30) which represent the entire endogenous *BMP4* expression range from no to extremely high level of expression. First, to determine the optimal dose of BMP4 to be used in these assays, HCC1419 and SK-BR-3 cells were treated with four different concentrations (10, 50, 100, and 250 ng/ml) of recombinant BMP4 or equivalent volume of vehicle. All doses of BMP4 decreased cell proliferation (Fig. 2a). The growth reduction was observed already at day 2 for HCC1419 cells, but only at day 4 for SK-BR-3 cells. The effect was concentration dependent, although there was no clear difference between the two highest doses (100 and 250 ng/ml). Thus, the lowest dose with the maximum growth inhibitory effect (100 ng/ml) was chosen for all subsequent experiments.

The remaining seven cell lines (HCC38, HCC1954, MCF-7, MDA-231, MDA-361, T-47D, and ZR-75-30) were treated with 100 ng/ml of BMP4 or vehicle and their growth was monitored for several days. In line with the data from HCC1419 and SK-BR-3 cells, a statistically significant growth inhibition was observed for all cell lines, regardless of their level of endogenous *BMP4* expression (Fig. 2b, Supplemental Table 1). The most dramatic growth reduction (62% at day 3 and 79% at day 6 as compared to vehicle treated cells) was seen in T-47D cells with rather high endogenous *BMP4* expression. This growth inhibitory effect was already observed at 12h (5% growth reduction) and 24h (20%) after BMP4 treatment (data not shown). Intriguingly in the HCC38 cell line with the highest endogenous *BMP4* expression, the magnitude of the

growth change was smaller and statistically significant only at day 6 (24% reduction as compared to vehicle treated cells).

BMP4 induces a G1 cell cycle arrest in breast cancer cell lines

To reveal the mechanism for the decreased cell number after the BMP4 treatment, we searched for possible alterations in cell cycle and apoptosis in the breast cancer cells. There were no significant changes in the number of apoptotic cells between BMP4 and vehicle treated cells in any of the cell lines (data not shown). Conversely, BMP4 treatment resulted in a statistically significant increase in the G1 cell cycle fraction consistently in all of the studied cell lines at day 2 (Fig. 3). The extent of the G1-arrest was most distinct in the T-47D cells (G1 phase fraction 90% in BMP4 vs. 64% in vehicle treated cells) and MDA-361 (95% vs. 69%). HCC38, HCC1419, HCC1954, MCF-7, SK-BR-3, and ZR-75-30 cells showed less striking but nevertheless statistically significant effects (varying from 3.7% to 11.5% increase in the fraction of G1 cells as compared to vehicle treated cells, Supplemental Table 2). As expected, the increase in the G1 phase was reflected in simultaneous decrease in the fraction of cells in the S and G2 phases of the cell cycle (Supplemental Table 2).

BMP4 can either increase or decrease breast cancer cell migration and invasion

The possible effects of BMP4 on cell migration were studied in all nine cell lines. After three days of BMP4 treatment, the migration of HCC1954, MDA-231, and MDA-361 cells increased dramatically (2.5, 7.2, and 2.4-fold as compared to vehicle treated cells, respectively, Fig. 4a). In MDA-231 cells, we also tested short term treatment (22 hours)

with BMP4 by placing the ligand directly to the upper chamber of the migration insert and observed a 1.3-fold increase in cell migration. In contrast, migration of T-47D cells was decreased (70% reduction as compared to vehicle treated cells) after BMP4 treatment and no change was observed in HCC38, SK-BR-3, and ZR-75-30 cells (Fig. 4a). HCC1419 and MCF-7 cells did not have the ability to migrate in the assay either with or without BMP4 (data not shown). Subsequently, invasion analysis was performed for those four cell lines with an effect in the migration assay. BMP4 also increased invasion of HCC1954 and MDA-231 cells (3.9 and 2.9-fold, respectively, Fig. 4b), but had no consistent effect on the invasion capability of MDA-361 and T-47D cells (data not shown). To investigate whether the observed effects in cell migration and invasion were caused by induction of epithelial-mesenchymal transition (EMT), we measured E-cadherin and vimentin mRNA levels after 72 hour treatment with BMP4 or vehicle but no changes in the expression patterns of these epithelial and mesenchymal markers were detected (Supplemental Fig. 1).

The effects of BMP4 in breast cancer cells are mainly mediated through the canonical BMP signaling pathway

To evaluate which signaling routes are activated upon BMP4 treatment in the breast cancer cell lines, we determined the phosphorylation levels of SMAD1/5/8, p38, and ERK1/2 after 3h treatment with BMP4 or vehicle. All studied cell lines showed clear SMAD1/5/8 phosphorylation after BMP4 treatment whereas there were no differences in p38 or ERK1/2 phosphorylation levels between BMP4 and vehicle treated cells (Fig. 5). Finally, Western analysis also indicated that all breast cancer cell lines examined

expressed SMAD4 protein (Fig. 5) thus demonstrating the intactness of this part of the canonical BMP signaling pathway.

BMP4 silencing leads to opposite phenotypic effects than ligand treatment

To further characterize the role of BMP4 in the control of cell growth and migration, we used siRNA based silencing of *BMP4* in T-47D cells which possess high endogenous expression. An average of 72% downregulation of *BMP4* mRNA levels on day 3 was achieved in BMP4 siRNA treated cells as compared to control luciferase siRNA treated cells (Fig. 6a). A statistically significant increase in cell number (1.3- and 1.6-fold at day 3 and 5, respectively) as well as an 1.8-fold increase in cell migration were observed after BMP4 siRNA treatment (Fig. 6b-c). Thus *BMP4* silencing led to opposite phenotypic effects as compared to BMP4 ligand treatment.

Discussion

Bone morphogenetic proteins are important signaling molecules that have a key role in the regulation of cell proliferation, differentiation and apoptosis, especially during development. The BMP signaling pathway has also been implicated in cancer pathogenesis. For example, mutations in the components of the BMP pathway, such as BMP receptor type IA (*BMPRIA*) and *SMAD4*, have been implicated in the genetic predisposition to juvenile polyposis syndrome [49]. In sporadic cancers, differential expression of various BMP ligands has frequently been reported and functional studies

have suggested both cancer cell promoting and inhibitory effects for BMPs [16, 22, 27-28]. Our group has previously reported that *BMP4* is commonly expressed in breast cancer [41] but the functional consequences of BMP4 signaling on breast cancer cell phenotype have not been studied in detail.

Here we first confirmed using quantitative real-time RT-PCR that *BMP4* is in fact frequently expressed in both breast cancer cell lines and in primary breast tumors and that the expression levels vary widely from one sample to another. This data is in good concordance with our previous study where semi-quantitative RT-PCR was utilized [41]. Interestingly, we observed an inverse correlation between *BMP4* expression and tumor grade suggesting that reduced *BMP4* levels might be linked with loss of differentiation. The fact that the breast cancer cell lines and primary breast tumors show similar BMP4 expression patterns indicates that the cell lines indeed serve as good models in assessing the potential functional role of BMP4 in breast cancer.

The effects of BMP4 treatment on cell growth were evaluated in a panel of nine breast cancer cell lines representing different levels of endogenous *BMP4* expression. Addition of recombinant BMP4 to cell culture medium dramatically decreased cell growth in all cell lines studied. The magnitude of growth reduction varied from one cell line to another but there was no correlation between the endogenous *BMP4* mRNA level and the phenotypic response of cells to BMP4 treatment. This phenomenon is best illustrated by the fact that two cell lines with highest levels of *BMP4* expression, T-47D and HCC38, had the largest and smallest reduction in cell number after BMP4 treatment, respectively. Overall, our data demonstrates for the first time that BMP4 treatment triggers a consistent growth inhibitory effect in breast cancer cells. Moreover, *BMP4*

silencing resulted in an opposite phenotype, i.e. increase in cell growth, thus further corroborating the role of BMP4 in the regulation of cell growth in breast cancer. Similar to our data, growth inhibitory effects of BMP4 have been previously reported in myeloma [33], lung cancer [34], and undifferentiated human cancer cells [35]. In these studies, the BMP4 induced growth reduction was shown to be caused by accumulation of cells into the G1-phase of the cell cycle as well as simultaneous increase in the number of apoptotic cells [33, 35]. Our results illustrate that in breast cancer cells BMP4 treatment does not induce apoptosis but leads to a G1-arrest. In conclusion, BMP4 treatment may contribute to cell growth regulation in various cancer types through different mechanisms involved in the control of cell cycle and cell death.

In spite of its growth inhibitory effects, BMP4 treatment dramatically increased the migration of HCC1954, MDA-231, and MDA-361 cells. Moreover, in HCC1954 and MDA-231 cells BMP4 treatment also resulted in induction of cell invasion. Recently, Shon and coworkers [42] reported reduced migration and invasion of MDA-231 cells after BMP4 stimulation, but in line with our data, BMP4 has been commonly shown to promote migration and invasion of ovarian, colorectal, pancreatic, and hepatocellular carcinoma cells [38-39, 50-51]. Interestingly, one of the breast cancer cell lines studied here, T-47D, showed diminished cell migration after BMP4 treatment. This effect might be partly explained by the fact that BMP4 induces especially strong growth inhibition in these cells. However, we only observed a 20% reduction in cell growth within the 22 hour time period that the cells were allowed to migrate. Thus the growth inhibition alone does not explain the 70% reduction in the migration of T-47D cells. In accordance with these results, silencing of *BMP4* in T-47D cells led to an opposite phenotypic effect, i.e.

increased cell migration. Taken together, our data imply that although BMP4 systematically reduces the growth of breast cancer cells, it can at the same time either enhance or reduce cell migration and invasion. Thereby BMP4 possesses functions that are both detrimental and beneficial to breast cancer cells. That kind of a bidirectional role has been well established for TGF- β which is known to act as a tumor suppressor during the first steps of tumor pathogenesis, but later enhances metastasis formation [53]. Among BMP ligands, BMP7 has been shown to elicit diverse and opposite functional responses in both breast and prostate cancer cells [22, 28]. However, this is the first time that such a dualistic role has been proposed for BMP4.

Previous studies have linked BMPs with the induction of epithelial-mesenchymal transition (EMT), an event which is characterized by increased ability of the cells to migrate and invade [52]. We observed no changes in E-cadherin and vimentin (epithelial and mesenchymal markers, respectively) expression in the breast cancer cells after BMP4 treatment and thus the cell migration phenotype cannot be simply explained by induction of EMT.

It is intriguing to speculate that the different phenotypic responses in cell migration and invasion assays after BMP4 stimulation might be explained by differences in down stream signaling cascades. We have previously shown that the breast cancer cell lines studied here express all six BMP specific receptors [41] and thus have the ability to transmit BMP signals. Now the evaluation of the different BMP signaling pathways revealed that the canonical SMAD1/5/8 pathway is indeed activated in these breast cancer cell lines after BMP4 treatment. Most of the previous studies, for example in pancreatic, lung, and colorectal cancer, also show that BMP4 typically signals through

the SMAD pathway [21, 34, 39-40, 50, 54]. None of the studied cell lines showed activation of MAP kinases ERK1/2 or p38 in response to BMP4 even though such responses have been reported in human mammary epithelial cells [55] and in pancreatic cancer cells [40]. Nevertheless BMP signaling is known to be regulated in a complicated manner at multiple levels and thereby it is reasonable to expect some variability in the phenotypic responses in different cellular backgrounds.

In conclusion, we have shown for the first time that BMP4 has an important role in regulating breast cancer cell proliferation and migration. BMP4 possesses distinct tumor suppressive properties as it dramatically decreases the growth of breast cancer cells through a G1 arrest of the cell cycle. In addition to these growth inhibitory effects, BMP4 also elicits oncogenic characteristics by simultaneously enhancing the ability of breast cancer cells to migrate and invade. Taken together, BMP4 has a similar dualistic role in breast cancer pathogenesis as previously reported for the TGF- β .

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Table 1 Clinicopathological parameters of 39 primary breast tumors.

Variable	n
Tumor types	
Ductal	33
Lobular	5
Mucinous	1
Grade ^a	
I	1
II	14
III	18
Tumor size	
<2 cm	21
≥2 cm	18
Lymph nodes	
Positive	13
Negative	22
n.a. ^b	4
ER	
Positive	32
Negative	7
PR	
Positive	22
Negative	17
ERBB2	
Positive	8
Negative	31

^aHistological grade was determined only from infiltrating ductal carcinomas.

^bNot available

Abbreviations: ER, estrogen receptor
PR, progesterone receptor

Supplemental Table 1 Reduced cell proliferation in breast cancer cells after BMP4 treatment. Breast cancer cells were treated with 100 ng/ml of BMP4 or equivalent volume of vehicle and cell numbers were determined at the indicated time points. The mean reduction in cell number (%) in BMP4 treated cells as compared to vehicle treated cells is shown. Data is from a single experiment with six replicates.

Cell line	day 2	day 3	day 4	day 6	day 7
HCC1419	14 [*]	-	36 ^{**}	-	63 [*]
SK-BR-3	ns	-	21 [*]	-	66 ^{**}
HCC38	-	ns	-	24 ^{**}	-
HCC1954	-	19 ^{**}	-	45 ^{**}	-
MCF-7	-	14 ^{**}	-	27 ^{**}	-
MDA-231	23 ^{**}	-	14 [*]	-	-
MDA-361	-	27 ^{**}	-	49 ^{**}	-
T-47D	-	62 ^{**}	-	79 ^{**}	-
ZR-75-30	-	28 ^{**}	-	54 ^{**}	-

^{*}p<0.05, ^{**}p<0.005

-, not done

ns, not significant

Supplemental Table 2 Cell cycle alterations after BMP4 treatment. Cell cycle fractions were determined 2 days after BMP4 (100ng/ml) or vehicle treatment using flow cytometry. The mean cell fraction (%) of six replicates is shown with standard error (SD) values.

Cell line	Treatment	Cell cycle phase, %					
		G1	+/- SD	S	+/- SD	G2	+/- SD)
HCC38	BMP4	54**	1.2	27**	1.3	19	0.9
	vehicle	50	0.2	31	1.2	19	0.9
HCC1419	BMP4	74**	0.8	14**	0.7	12	0.5
	vehicle	69	1.6	20	1.0	12	1.5
HCC1954	BMP4	58**	0.9	26**	1.1	16**	1.2
	vehicle	47	1.7	35	2.4	19	1.7
MCF-7	BMP4	64**	0.6	24**	1.3	12	1.0
	vehicle	61	1.1	28	1.6	11	0.5
MDA-361	BMP4	95**	0.3	3**	0.2	2**	0.1
	vehicle	69	0.6	23	0.9	8	0.5
SK-BR-3	BMP4	64**	0.7	25**	0.5	11	0.4
	vehicle	60	1.5	31	1.5	10	1.6
T-47D	BMP4	90**	1.2	3**	1.7	8**	1.5
	vehicle	64	0.6	17	0.6	19	0.9
ZR-75-30	BMP4	42*	2.2	19	10.0	38	8.5
	vehicle	38	3.3	28	9.5	34	6.4

* p<0.05, ** p<0.005

Figure Legends

Figure 1 Analysis of *BMP4* mRNA expression levels (a) in 22 breast cancer cell lines and normal mammary epithelial cells (HMEC), and (b) in 39 primary breast tumors and normal mammary gland using quantitative real time RT-PCR. Data from the breast cancer cell lines is also shown for reference in panel b. *BMP4* mRNA expression levels were normalized against the housekeeping gene TBP (TATA box binding protein).

Figure 2 BMP4 treatment leads to growth inhibition in breast cancer cells. (a) BMP4 influences cell growth in a concentration dependent manner. HCC1419 and SK-BR-3 cells were treated with different concentrations (10, 50, 100, and 250 ng/ml) of BMP4 or equal volume of vehicle. Cell numbers were counted at 2, 4, and 7 days after the first addition of ligand or vehicle containing medium. Day 0 denotes the number of cells seeded. Error bars indicate +/- SD of three replicates. (b) MDA-231, HCC38, HCC1954, MCF-7, MDA-361, T-47D, and ZR-75-30 cells were treated with 100 ng/ml of BMP4 or equivalent volume of vehicle. Cell numbers were counted at the indicated time points. Error bars indicate +/- SD of six replicates. * $P < 0.05$, ** $P < 0.005$.

Figure 3 BMP4 induces a G1 cell cycle arrest in breast cancer cells. The cell cycle distribution was determined at day 2 after BMP4 (100 ng/ml) or vehicle treatment by flow cytometry. The fraction of cells in G1 phase of the cell cycle (mean and SD of six replicates) is shown for each of the cell lines. * $P < 0.05$, ** $P < 0.005$.

Figure 4 BMP4 promotes cell migration and invasion *in vitro*. Cells were first treated with BMP4 (100 ng/ml) or vehicle for three days and then subjected to the migration or invasion assay. Fold changes in (a) migration and (b) invasion after BMP4 compared to vehicle treatment from six replicate membranes are shown with SD. * $P < 0.05$, ** $P < 0.005$. (c) Representative images of invaded HCC1954 cells after BMP4 or vehicle treatment are also shown.

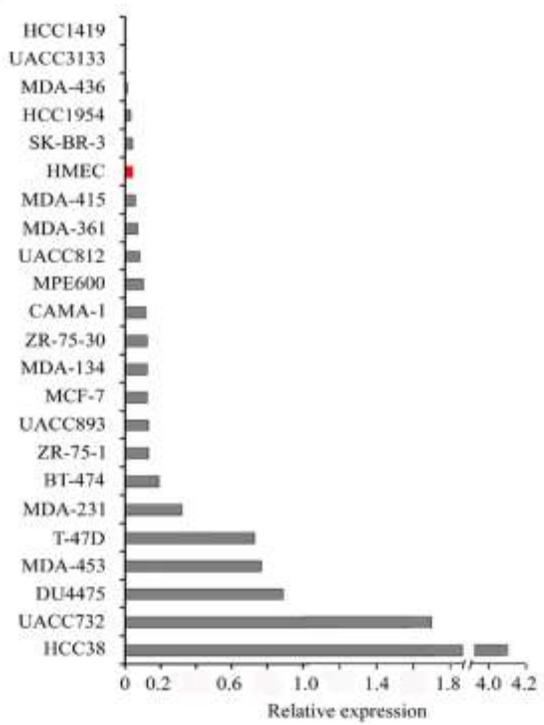
Figure 5 BMP4 treatment induces SMAD1/5/8 phosphorylation in breast cancer cells. Western blot analysis of phosphorylated SMAD1/5/8, ERK1/2, and p38 proteins after 3h treatment with BMP4 (100 ng/ml) or vehicle. Total SMAD5, ERK1/2, and p38 were used to measure the basal levels of these proteins. Basal SMAD4 expression is shown at the bottom of the figure.

Figure 6 *BMP4* silencing leads to opposite phenotypic effects than ligand treatment. T-47D cells were treated either with *BMP4* or control *luciferase* siRNAs and (a) relative *BMP4* mRNA expression levels were measured three days after transfection using qRT-PCR. (b) Cell numbers were counted at 3 and 5 days after the transfection. Day 0 indicates the number of cells seeded. (c) Cells were subjected to the migration assay three days after the transfection and the mean number of migrated cells was quantitated. Error bars indicate +/- SD of six replicates. * $P < 0.05$, ** $P < 0.005$.

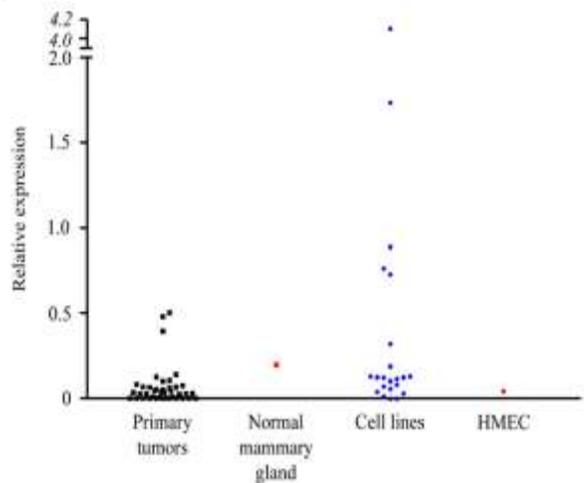
Supplemental Figure 1 BMP4 treatment does not induce epithelial-mesenchymal transition. Cells were treated with BMP4 (100 ng/ml) or vehicle for three days and then the E-cadherin and vimentin mRNA levels were measured using RT-PCR.

Figure 1.

a



b



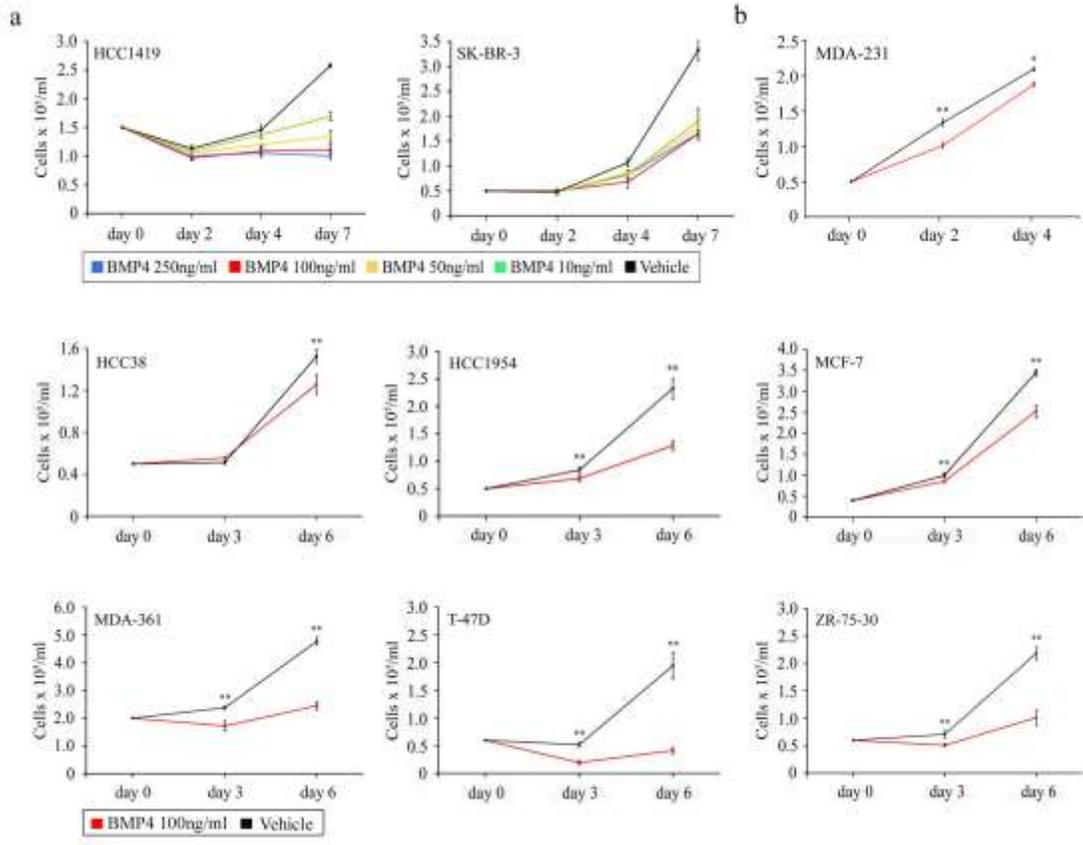


Figure 3.

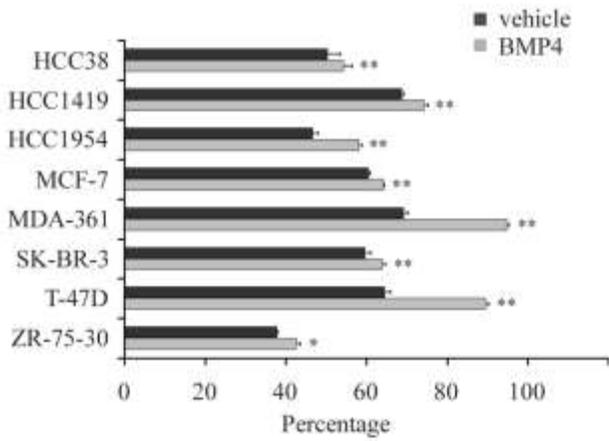


Figure 4.

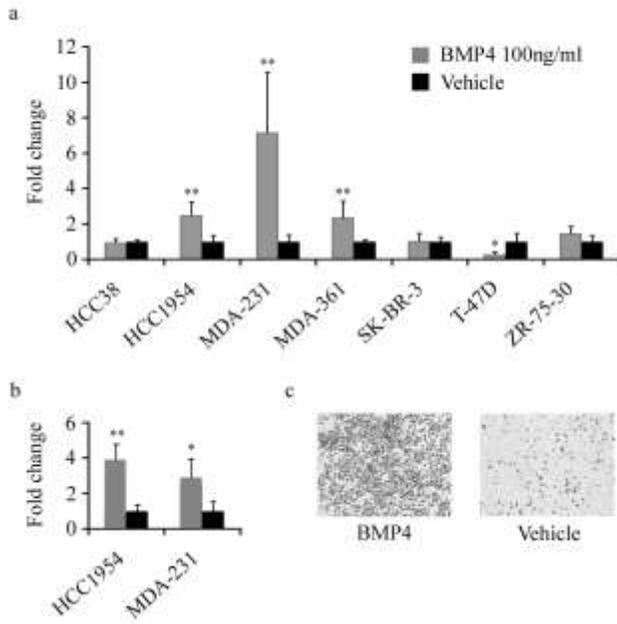


Figure 5.

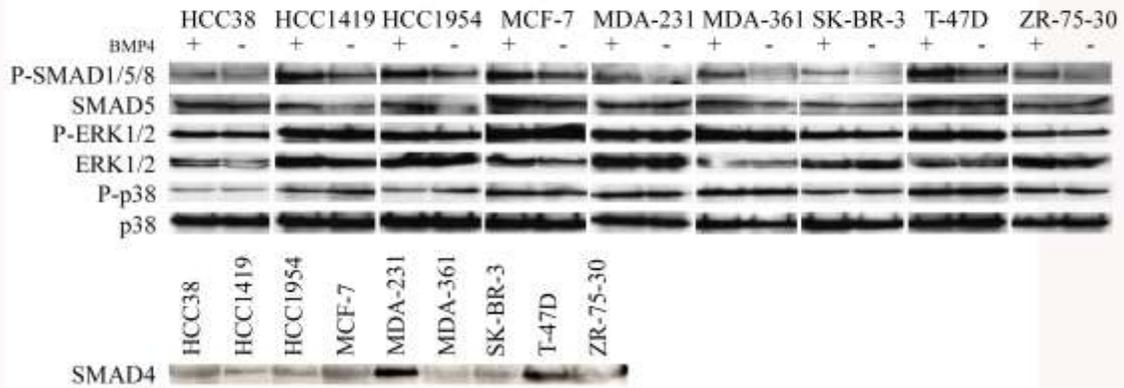


Figure 6.

