Research Article

Bone morphogenetic protein – 4 and – 5 in pancreatic cancer—Novel bidirectional players

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ABSTRACT

Bone morphogenetic proteins (BMPs) are multifunctional signaling molecules that have gained increasing interest in cancer research. To obtain a systematic view on BMP signaling in pancreatic cancer we first determined the mRNA expression levels of seven BMP ligands (BMP2–BMP8) and six BMP specific receptors in pancreatic cancer cell lines and normal pancreatic tissue. BMP receptor expression was seen in all cancer and normal samples. Low expression levels of BMP5 and BMP8 were detected in cancer cells compared to the normal samples, whereas BMP4 expression was elevated in 25% of the cases. The impact of BMP4 and BMP5 signaling on cell phenotype was then evaluated in five pancreatic cancer cell lines. Both ligands suppressed the growth of three cell lines (up to 79% decrease in BMP4-treated PANC-1 cells), mainly due to cell cycle changes. BMP4 and BMP5 concurrently increased cell migration and invasion (maximally a 10.8-fold increase in invaded BMP4-treated PANC-1 cells). The phenotypic changes were typically associated with the activation of the canonical SMAD pathway, although such activation was not observed in the PANC-1 cells. Taken together, BMP4 and BMP5 simultaneously inhibit the growth and promote migration and invasion of the same pancreatic cells and thus exhibit a biphasic role with both detrimental and beneficial functions in pancreatic cancer progression.

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Introduction

Bone morphogenetic proteins (BMPs) are signaling molecules originally identified based on their ability to form bone [1,2]. They constitute a subfamily of secreted signaling ligands belonging to the transforming growth factor-β (TGF-β) superfamily [3]. In humans over 20 BMPs have been identified and based on their sequence similarity they can be divided into distinct subgroups [3]. BMPs signal through two types of transmembrane serine-threonine kinase receptors, known as type I and type II receptors [4,5]. Upon ligand binding, activated BMP receptor complex phosphorylates receptor-activated SMAD proteins (R-SMADs), SMAD1, -5, and -8 [6–8] which then form a heteromeric complex with SMAD4 (Co-SMAD), translocate to the nucleus and regulate the transcription of target genes [9]. This signaling pathway is referred to as the canonical SMAD pathway. Conversely, the BMPs can activate other pathways such as mitogen-activated protein kinases (MAPK), ERK1/2 and/or p38 [10–12].

At present it is widely acknowledged that the functions of BMP ligands go beyond their role in osteogenesis. BMPs have been shown to regulate tissue development and diverse biological processes such as cell proliferation, differentiation, and apoptosis...
Because of these versatile and essential functions, BMPs have now become of great interest in cancer research. There are undoubtedly differences in the expression of BMP family members between normal tissue and cancer cells in many cancer types [15–17]. Another strong evidence for the role of BMP signaling in cancer came when it was found that mutations in the BMP receptor BMPR1A cause a rare inherited colorectal cancer predisposition syndrome known as familial juvenile polyposis [18,19]. Despite of these findings the actual biological role of BMPs in tumorigenesis is still mostly uncovered. Functional data is limited and the results from different studies are partly controversial. Antitumorigenic functions have been described for example in prostate and colon cancer [20–23], whereas other studies in prostate cancer and those in breast cancer report protumorigenic or metastasis promoting effects [24–26].

Recent observations suggest that alterations in BMP signaling may play a role also in pancreatic cancer progression. It has been shown that the mRNA levels of BMPR1A, BMPR2 and BMP2 are up-regulated in pancreatic cancer [27], however the molecular and cellular consequences of aberrant BMP signaling have been investigated in very small extent [27–30]. Thus far comprehensive BMP ligand and receptor gene expression analysis has not been performed, neither do we have sufficient functional data to create a profound understanding of this complicated signaling pathway in pancreatic cancer development. Therefore, our interest was first to determine the gene expression profiles of BMP ligands (BMP2–BMP8) and all BMP specific receptors (ACVR1, BMPR1A, BMPR1B, ACVR2A, ACVR2B, and BMPR2) in normal pancreatic tissues and in a large panel of pancreatic cancer cell lines. Secondly, we wanted to further study the potential role of BMP signaling in pancreatic cancer pathogenesis by examining the capacity of BMP4 and BMP5 to modulate growth, migration and invasion of pancreatic cancer cell lines.

Materials and methods

Cell culture and reagents

Total of 16 established pancreatic cancer cell lines were used in this study. Thirteen of them (AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HPAC, HPAF-II, HS 700T, HS 766T, MIA PaCa-2, PANC-1, SU.86.86, and SW 9000) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and three (DanG, Hup-T3, and Hup-T4) from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultured under recommended conditions. Commercially available normal pancreatic total RNA samples were obtained from Ambion (Austin, TX, USA), two different samples from BioChain Institute (Hayward, CA, USA) and one from Clontech (Mountain View, CA, USA). BMP4 and BMP5 were obtained from R&D Systems (Minneapolis, MN).

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

RNA was isolated from cells using the RNAeasy Mini Kit (Qiagen, Valencia, CA). RNA was reverse transcribed to cDNA by using SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) with random hexamers (50 ng/μl) as described [31]. The qRT-PCR was performed using the LightCycler equipment (Roche, Mannheim, Germany) as described [32]. Transcript levels were determined using the LightCycler FastStart DNA Master Hybridization probe kit (Roche) and gene specific primers and hybridization probes (TIB MOLBIOL, Berlin, Germany; Supplementary Table 1). The expression levels were normalized against the reference gene TBP (TATA box binding protein), which was selected from a panel of ten different housekeeping genes (G6PD, PPIA, GAPD, TBP, B2M, GUSB, PBDG, HPRT, ACTB, and PGK1) since it had the most consistent expression levels across the sample set examined in this study.

Immunohistochemistry in primary tumors

BMP4 protein expression was studied in seven pancreatic ductal adenocarcinoma samples using immunohistochemistry. Immunostaining was performed using automated Ventana Benchmark staining system according to the manufacturer's instructions (Ventana Medical Systems, Tuscon, AZ). BMP4 protein was detected with mouse anti-BMP4 monoclonal antibody (1:10 dilution, Chemicon Millipore, Temecula, CA) and ultraView Universal DAB Detection Kit (Ventana Medical Systems) and the slides were counterstained with hematoxylin–eosin. The entire tissue core was evaluated and BMP4 staining pattern classified as negative, low or strong staining.

Cell proliferation assay

Cells were plated (day 0) overnight in 24-well plates and subsequently incubated in 10% serum medium containing either BMP4 or BMP5 ligand (250 ng/ml, unless otherwise indicated) or an equivalent volume of vehicle (4 mM HCl, 0.1% BSA). Due to very high proliferation rate of Mia PaCa-2 cells, which led to overgrowth during the experimental period, they were cultured in 1% FBS and these culture conditions were also used in all the subsequent functional experiments. Fresh medium with BMP ligand or vehicle was added every third day. Cell number was counted 3 (day 4) and 6 (day 7) days after the first addition of the ligand or vehicle was added every third day. Cell number was counted 3 (day 4) and 6 (day 7) days after the first addition of the ligand or vehicle using Z1 Coulter Particle Counter (Beckman Coulter, Fullerton, CA). All experiments were performed in 3–6 replicates and were repeated at least twice.

Cell cycle and apoptosis analysis

Cell cycle and apoptosis analyses were performed after 48 h after the first addition of BMP4, BMP5 or vehicle in a 6-well plate. Both floating and adherent cells were collected and each assay was performed in triplicate and repeated at least twice. Cell cycle analysis was performed by propidium iodide (PI) staining and the apoptosis assay was done using the Annexin V-FITC apoptosis detection kit (Calbiochem, La Jolla, CA) as described [33]. Cell cycle distribution was analyzed with the ModFit LT Version 3.1 program (Verity software house, USA) and apoptosis with EXP032 ADC Version 1.2 analysis software (Beckman Coulter) according to the manufacturer's instructions.

Migration and invasion assay

Migration and invasion was studied using BD Bioscience cell culture inserts (8.0 μm pore size) and BD Biocoat Matrigel Invasion chambers (8.0 μm pore size, BD Biosciences, Bedford, MA) as
described [24]. Briefly, cells were treated with BMP4, BMP5 or vehicle for 72 h after which 0.1–2.0 × 10^5 cells, depending on the cell line, were transferred in serum free medium to the upper migration or invasion chamber. In the lower chamber standard cell culture medium with 10% FBS was added, and cells were allowed to migrate or invade for 22 h at 37 °C. PANC-1 cells were allowed to migrate only for 3 h and MIA PaCa-2 cells for 6 h to ascertain that the effects on cell migration and invasion were not caused by differences in cell proliferation. The fixed and stained migration and invasion membranes were scanned with Aperio ScanScope® XT (software version 9; Aperio Technologies, USA) and analyzed with ImageJ software [34] as described [24].

**Western blotting analysis**

To examine the effects of BMP stimulation on pancreatic cancer cell on molecular level, cells were collected after 6 hour incubation with BMP4, BMP5 or vehicle. Protein extraction, SDS-PAGE gel electrophoresis and blotting were done as previously described [35]. The following primary antibodies were used: rabbit Phospho-SMAD 1/5/8, rabbit phospho-p44/42 MAP kinase, rabbit phospho-p38 MAP Kinase, rabbit p38 MAP Kinase, rabbit p44/42 MAP Kinase, and rabbit SMAD5 (1:1000 dilution, Cell Signaling Technology, Inc., Beverly, MA). The latter three antibodies were used as loading controls. Blots were incubated with primary antibodies overnight at +4 °C and proteins visualized using BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit) (Roche Diagnostics GmbH) as described [24]. Western films were scanned and the signal intensity for each band was determined using ImageJ software [34].

**Statistical analysis**

The Mann–Whitney test was used to statistically compare the medians of the expression levels in the cancer cell line and normal sample group, as well as the BMP-treated and control (vehicle-treated) groups. A P value <0.05 was considered statistically significant.

**Results**

**Expression of BMP ligands and receptors in normal pancreas and pancreatic cancer cells**

We determined the relative expression values of BMP type I and type II receptors ACVR1, BMPR1A, BMPR1B, ACVR2A, ACVR2B, and BMP2 in 16 pancreatic cancer cell lines and in 4 normal pancreatic samples using qRT-PCR. As seen in Fig. 1A, detectable expression of all six BMP specific receptors was seen in all pancreatic cancer cell lines as well as in normal pancreas samples. The range in receptor expression levels was wide both in the cancer cell line group and in the normal sample group, and did not permit classification of the samples into distinct groups. Interestingly, the median expression level of all the studied receptors, except for BMPR2, was lower in cancerous cell lines compared to normal cells. This difference was statistically significant for ACVR1 (P = 0.034) and reached close to significance also for ACVR2A (P = 0.065) and BMPR1B (P = 0.081). Notably the expression levels of BMPR1B were somewhat lower than those of the other receptors. The individual relative expression level for each receptor is shown in Supplementary Table 2.

We next characterized the mRNA expression of the seven BMP ligands (BMP2–BMP8) in the same set of cancer cell lines and normal pancreatic samples (Fig. 1B, Supplementary Table 2). In general, the expression level of BMP2 was considerably higher compared to the other studied ligands, both in the cancer cell line group and the normal sample group. A slightly higher BMP2 median expression level was revealed in the normal sample group compared to the cancer cell line group, but the difference was not statistically significant. BMP3, BMP6 and BMP7 were expressed at very low levels both in cancer cell lines and in normal pancreatic samples, and their expression did not notably differ between these two groups. Of note, each of these ligands were highly expressed in few cell lines, for example BMP3 in Hup-T4 cells, BMP6 in Hs 700T and AsPC-1 cells, and BMP7 in HPAF-II and Hs 700T cells (Supplementary Table 2). However, for the majority of the cancer cell lines the expression of these ligands was similar to that detected in the normal sample group.

BMP4, BMP5 and BMP8 were distinct from the other studied ligands due to their different expression patterns between the cancer cell line and the normal sample groups (Fig. 1B). Interestingly, BMP4 was expressed at very low level or below detection in all normal samples whereas a subset (4/16) of the cancer cell lines had highly elevated expression. HPAF-II was one of the cancer cell lines with very high expression of BMP4 (Fig. 1C). Analysis of BMP4 protein expression levels in primary pancreatic tumors demonstrated a similar pattern with five out of seven cases having negative or low expression and the remaining two cases showing strong expression. On the contrary, there was a dramatic and statistically significant (P = 0.013) decrease in the relative expression level of BMP5 in cancer cell lines compared to the normal pancreas samples. In fact, PANC-1 was the only cell line in which BMP5 expression was detected (Fig. 1C). BMP8 has two very similar transcript variants, BMP8A and BMP8B, which were studied here together as BMP8. Similar to BMP5, the median expression level of BMP8 in the cancer cell lines was significantly lower (P = 0.021) than that of the normal pancreas samples.

**BMP4 and BMP5 inhibit the proliferation of human pancreatic cancer cell lines**

As indicated above our results show that BMP4, BMP5 and BMP8 mRNAs are expressed differently in pancreatic cancer cell lines compared to normal pancreatic samples. In order to determine the possible functional significance of this aberrant expression, we evaluated the effects of BMP4 and BMP5 on the proliferation of pancreatic cancer cell lines. BMP8 was not included in this study because we were unable to separate the two variants’ (BMP8A and BMP8B) expressions profiles from each other.

The proliferation response to BMP4 and BMP5 treatment was investigated in a panel of five pancreatic cancer cell lines which had either low or no endogenous BMP4 and BMP5 expression (AsPC-1, SU.86.86 and MIA PaCa-2) or high endogenous BMP4 (HPAF-II) or BMP5 (PANC-1) expression (Fig. 1C). BMP5 has not been used earlier in pancreatic cancer studies, thus we tested different ligand concentrations (50 ng/ml, 100 ng/ml and 250 ng/ml) to find the most effective dose and counted the cells after 3 and 6 days of first addition of BMP5 or vehicle. The MIA PaCa-2 cells were selected for
Fig. 1 – BMP ligand and BMP specific receptor mRNA expression. A, mRNA expression of six BMP specific receptors and B, seven BMP ligands were determined in sixteen pancreatic cancer cell lines (■) and four normal pancreatic samples (Δ) by qRT-PCR. Median value of relative expression is indicated by a horizontal line. C, BMP4 and BMP5 expression levels in five selected cell lines (AsPC-1, HPAF-II, MIA PaCa-2, PANC-1, and SU.86.86).
this analysis since they do not show any endogenous expression. The most pronounced response was obtained with 250 ng/ml dose (Fig. 2A). For BMP4, doses of 100 ng/ml and 250 ng/ml were tested (Fig. 2A), and 250 ng/ml was selected for subsequent analyses.

BMP4 treatment led to growth inhibition in three out of five studied pancreatic cancer cell lines as compared to vehicle-treated cells. Most distinct statistically significant decrease in cell number was seen in PANC-1 cells (on average 54% on day 4 and 79% on day 7) and in MIA PaCa-2 cells (37% on day 4 and 30% on day 7) (Fig. 2B). The anti-proliferative response was slightly smaller, but statistically significant (P<0.01) in SU.86.86 cells (21% on day 4 and 22% on day 7, data not shown). HPAF-II and AsPC-1 cells did not respond to BMP4 treatment with apparent growth stimulation or inhibition. In a similar fashion, BMP5 addition to pancreatic cancer cells resulted in a significant growth suppressing effect in PANC-1 (on average a 22% decrease in cell number on day 4 and 36% on day 7), HPAF-II (16% on day 4 and 36% on day 7), and MIA PaCa-2 (26% on day 4) compared to vehicle-treated cells (Fig. 2B). SU.86.86 and AsPC-1 cells did not show any consistent growth response to BMP5 treatment (data not shown).

Decreased cell growth is mainly mediated by cell cycle alterations

To more carefully investigate the growth regulating action of BMP4 and BMP5 in these cancer cell lines, we analyzed their effects on cell cycle distribution after 48 h of stimulation. In the PANC-1 cell line, an increased fraction of cells in G1-phase of the cell cycle was detected after BMP4 (71% vs. 55%, P=0.002) and BMP5 treatment (61% vs. 55%, P=0.002) compared to vehicle-treated cells (Fig. 3A, Table 1). Similarly, the amount of MIA PaCa-2 cells in the G1-phase was increased after BMP4 treatment (75% vs. 67%, P=0.002) and BMP5 treatment (72% vs. 67%, P=0.002) (Table 1). These changes were accompanied by a concomitant decrease of cells in S-phase. No changes in cell cycle profile were observed in HPAF-II cells after BMP5 treatment (Table 1). BMP4-treated SU.86.86 cells had a small but significant difference (50% vs. 47%) in the fraction of cells in G1-phase compared to control-treated cells, correlating well with their less dramatic decrease in cell growth (Table 1).

To examine the possible effects of BMP4 and BMP5 on apoptosis we analyzed the cell viability by annexin V-FITC/PI staining after 48 h of BMP treatment. BMP4, which had the most dramatic effect on PANC-1 cell growth, clearly increased the number of apoptotic PANC-1 cells (Fig. 3B) whereas BMP5 had no effect. In the other four cell lines, BMP4 or BMP5 ligand treatments did not induce any consistent changes on programmed cell death (data not shown).

Fig. 2 – BMP4 and BMP5 decrease pancreatic cancer cell growth. A, MIA PaCa-2 cells were treated with different doses of BMP4 (100 and 250 ng/ml) or BMP5 (50, 100 and 250 ng/ml) or vehicle in medium containing 1% FBS. Cell count was determined 3 days (day 4) and 6 days (day 7) after the first addition of the ligand. Day 0 denotes the number of cells seeded. The mean cell count of 3–6 replicates with error bars (SD) is shown. B, PANC-1, MIA PaCa-2 and HPAF-II cells were treated with 250 ng/ml BMP4, 250 ng/ml BMP5 or vehicle. BMP-treated and vehicle-treated cells were counted at day 4 and day 7. The fraction (%) of BMP-treated cells as compared to vehicle-treated cells is shown. Mean value of 6 replicates with SD from a representative experiment is shown. The BMP-treated group was statistically compared to a vehicle-treated group, *P<0.05, **P<0.005.
BMP4 and BMP5 promote migration and invasion of pancreatic cancer cells

To assess whether BMP4 and BMP5 treatments induce transcriptional changes that influence the ability of the cells to migrate and invade, AsPC-1, HPAF-II, Mia PaCa-2, PANC-1, and SU.86.86 cells were first treated with BMP4, BMP5 or vehicle for 72 h and then subjected to migration and invasion assays. BMP4 treatment clearly increased the migration of PANC-1, MIA PaCa-2, and HPAF-II cells (6.0-, 2.4-, and 2.9-fold as compared to vehicle-treated cells, Figs. 4A–B). Similarly, the migration of BMP5-treated PANC-1 and HPAF-II cells increased significantly (1.8- and 3.0-fold, respectively, Figs. 4A–B). For MIA PaCa-2 cells, there was a clear tendency for increased migration after BMP5 treatment but this change was not statistically significant. Furthermore, the enhanced migration was reflected in increased invasion through Matrigel in all these cells after BMP4 or BMP5 treatment with the exception of BMP5-treated PANC-1 cells. The most dramatic increase (10.8-fold) was detected in the amount of invaded PANC-1 cells after BMP4 treatment (Fig. 4A).

In contrast, BMP4- or BMP5-treated AsPC-1 and SU.86.86 cells did not exhibit significant changes in their migration or invasion capability as compared with vehicle-treated cells (data not shown). In conclusion, BMP4 and BMP5 treatment exhibited a simultaneous anti-proliferative response and induction of cell migration and invasion phenotype in a subset of pancreatic cancer cell lines.

BMP treatment can activate SMAD pathway in pancreatic cancer

Finally, we wanted to evaluate whether the phenotypic changes observed after BMP4 and BMP5 stimulation were caused by activation of the canonical SMAD pathway or through the MAP kinases p38 or ERK1/2. Phosphorylation levels of SMAD1/5/8, p38 and ERK1/2 were determined after a 6 h treatment with BMP4,
BMP5 or vehicle. Clear induction of SMAD1/5/8 phosphorylation was observed in AsPC-1 and MIA PaCa-2 cells after BMP4 and BMP5 treatment (Fig. 5). BMP5 treatment also increased SMAD1/5/8 phosphorylation in HPAF-II cells whereas BMP4 did not (Fig. 5). In contrast, BMP4 treatment increased the phosphorylation level of SMAD1/5/8 in SU.86.86 cells while BMP5 had no influence (Fig. 5). No changes were detected in the activation of the canonical SMAD pathway after either BMP4 or BMP5 treatment in PANC-1 cells (Fig. 5). Interestingly, with the exception of AsPC-1, some baseline SMAD1/5/8 phosphorylation was detected in all of the studied cell lines. ERK1/2 MAP kinase was not affected by BMP4 or BMP5 treatment in any of the cell lines (data not shown). However, it has to be noted that all cells exhibited very high basal levels of activated ERK1/2 which made it difficult to assess the possible additional phosphorylation induction of this protein. No differences in the phosphorylation levels of p38 were detected with the exception of AsPC-1 showing a small increase in the p38 phosphorylation levels after BMP4 treatment.

**Discussion**

In recent years, BMPs and BMP signaling have drawn much attention in cancer research. There is a growing body of studies...

![Fig. 4](image-url) – BMP4 and BMP5 increase the migration and invasion of pancreatic cancer cell lines. Cells were treated with BMP4 (250 ng/ml), BMP5 (250 ng/ml) or vehicle for 72 h and then subjected to migration and invasion assay. The total area of migrated or invaded cells (in pixels) was determined from four images per membrane. A. The mean total area of migrated (upper chart) and invaded (lower chart) cells from six replicate membranes is shown with SD. The BMP-treated group was statistically compared to the vehicle-treated group. *P<0.05, **P<0.005. B. Representative images of BMP-treated and vehicle-treated migrated PANC-1, MIA PaCa-2 and HPAF-II cells (20× objective) are shown.

![Fig. 5](image-url) – BMP4 and BMP5 activate the SMAD pathway in pancreatic cancer cells. Activation of SMAD1/5/8 was determined in five pancreatic cell lines after six hour treatment with BMP4 (250 ng/ml), BMP5 (250 ng/ml) or vehicle using Western blot. The band intensities were quantitated by ImageJ software and the phosphorylated SMAD1/5/8 values were normalized against the unphosphorylated SMAD5 expression levels. Fold change (FC) values under each lane indicate the normalized P-SMAD1/5/8 intensities in BMP4 or BMP5-treated samples as compared to corresponding vehicle control.
showing the association between BMP pathway and different cancers, but the functional data is still very limited, especially in pancreatic cancer. In this study we took a unique approach by first performing a comprehensive expression survey of all six BMP specific receptors as well as seven BMP ligands (BMP2–BMP8) in a large panel of sixteen pancreatic cancer cell lines and four normal pancreatic samples to identify appropriate model systems for subsequent evaluation of the functional role of BMPs. This is the first time BMP receptors and ligands have been studied to this extent in pancreatic cancer.

Our results show that all BMP specific receptors are expressed in pancreatic cancer cell lines thus demonstrating that this part of the BMP signaling cascade is indeed functional. The median expression level of all BMP receptors, except for BMPR2, was lower in cancerous pancreatic cell lines compared to normal cells. However, only for ACVR1 this difference was statistically significant. This common trend seems, however, interesting and might suggest down-regulation of BMP receptors in pancreatic cancer. In fact, loss of BMP receptor expression has been previously shown to correlate with high tumor grade in prostate cancer [36]. The expression levels of BMP receptors BMPR1A, BMPR1B and BMPR2 have earlier been studied by Northern blot analysis [27] and RT-PCR [28] in pancreatic cancer. There is a good concordance in the receptor expression patterns when one compares the same pancreatic cancer cell lines studied here and in these two previous reports. However, both studies [27,28] reported elevated BMPR1A and BMPR2 mRNA levels in primary pancreatic tumors. One must keep in mind that primary tumors contain multiple cell types whereas cancer cell lines are homogeneous in their cellular origin. More importantly, cancer cell lines are typically derived from more advanced disease and thus it is conceivable that the receptor expression levels change during tumor progression.

Here we also show that the relative expression levels of most BMP ligands did not differ remarkably between pancreatic cancer cell lines and normal samples. BMP3 and BMP6, the expression of which has not been previously studied in pancreatic cancer, as well as BMP7 were expressed at very low levels both in the great majority of cancer cell lines and in all normal pancreatic samples. BMP2 expression was in an earlier study shown to be highly increased in pancreatic cancer compared to normal pancreas [27]. In a like manner, we observed generally high level BMP2 expression but we did not detect a difference between normal and cancer samples. These results are likely to be due to different study materials, especially different normal samples, since data from four pancreatic cancer cell lines included in both studies are highly concordant. In addition, the assay methods (Northern analysis vs. qRT-PCR) used in these studies have quite different detection sensitivities which might influence the results. The expression levels of BMP5 and BMP8 have not been studied earlier in pancreatic cancer and only to a very small extent in other cancers. Here we demonstrated that BMP5 and BMP8 are present in cancer cell lines in significantly lower levels compared to normal samples. They thus appear to be silenced in pancreatic cancer and might have an antitumorigenic role. Earlier studies have shown BMP8 overexpression in lung cancer cell lines and in breast cancer [31,37]. BMP5 transcripts have been detected at very low levels in breast cancer cell lines and in primary tumors [31]. Down-regulation of BMP5 expression has also been seen in the adrenocortical carcinoma cells whereas overexpression was detected in squamous cell carcinoma [38,39]. Finally, the relative expression level of BMP4 was low both in normal pancreas samples and in the majority of the pancreatic cancer cell lines. However, 25% of the cell lines displayed highly elevated BMP4 levels. This up-regulation was also reported by Gordon et al. [28] in their analysis of publicly available microarray data. Furthermore, previous studies in breast cancer and malignant melanoma have reported high BMP4 expression [31,40], and in colorectal cancer the expression of BMP4 has been demonstrated to increase during tumor progression [41].

It is important to note that comparisons between pancreatic cancer cell lines and RNA isolated from normal pancreas are not straightforward. The cell lines originate from exocrine pancreas whereas normal samples contain both exocrine and endocrine tissue. However, as the exocrine part represents over 90% of the mass of the pancreas, normal tissue RNA is indeed a valid reference. To further address this issue, we compared our results with microarray based expression data derived from both normal pancreas and primary tumors (GeneSapiens, http://www.genesapiens.org/; Oncomine, https://www.oncomine.org/). As expected, this comparison revealed highly similar expression patterns in our four normal samples and the normal pancreas samples in the database. More importantly, the expression patterns in the cell lines were highly similar to those seen in primary pancreatic tumors. In particular, the expression levels of BMP5 were very low or missing in primary pancreatic tumors and BMP4 showed generally low expression with a small subset of primary tumors having elevated expression compared to normal pancreas. These patterns correspond extremely well to our cell line expression data. Hence this study provides useful information on BMP and BMP receptor expression and a resource of cell lines for further studies of BMP function in pancreatic cancer.

BMPs have been shown to influence cancer cell phenotype in many tumor types but in pancreatic cancer their biological role has not been adequately investigated. No clear association between BMP ligand or receptor expression and pancreatic cancer cell line characteristics, such as cell adhesion, migration, invasion, angiogenesis or tumorigenicity [42], was detected. Neither did the BMP status associate with the origin of the cell line (primary tumor vs metastasis) or the mutational patterns of KRAS, p53, p16 and SMAD4, genes most commonly altered in pancreatic cancer [42,43]. However, BMP4 expression might be related to a differentiation status of tumors since the majority of the cell lines derived from well-differentiated tumors [42] did express BMP4. To study the effects of BMPs in different cellular contexts, we evaluated the functional role of BMP4 and BMP5 in a panel of five pancreatic cancer cell lines with no, low or high endogenous expression. In general, both BMP4 and BMP5 reduced the growth of pancreatic cancer cells. A marked growth inhibition was observed in three out of five pancreatic cancer cell lines (MIA PaCa-2, SU.86.86 and PANC-1) after BMP4 treatment. This result is in agreement with earlier observations in other cancers [35,44,45]. BMP5 created similar but less dramatic growth reduction in three cell lines (HPAF-II, MIA PaCa-2 and PANC-1). This effect was independent of the endogenous expression level, since it was observed in both cell lines with high expression (PANC-1) and no expression (MIA PaCa-2 and HPAF-II). Reduced proliferation after BMP5 treatment has been previously reported in myeloma cells [46].

Consistent with the decreased proliferation, cell cycle analysis showed an increased fraction of cells in G1-phase and concomitant
decrease in S-phase after BMP4 and BMP5 stimulation in MIA PaCa-2 and PANC-1 cells. In BMP4-treated SU.86.86 cells, a smaller decrease in the S-phase fraction was seen, being consistent with a less dramatic reduction in cell number. In addition, BMP4 possessed pro-apoptotic activity in PANC-1 cells, thus further contributing to the observed reduction in cell growth. We were unable to show cytostasis or pro-apoptotic activity in HPAF-II cells after BMP5 treatment even though we did see a difference in their growth repeatedly. Thus we do not have conclusive evidence on the mechanisms of the reduced cell growth in these cells. However, it can be noted that the HPAF-II cells had a high number of apoptotic cells also in control-treated cells (data not shown) which might have masked the pro-apoptotic effect of the BMP5 treatment. Taken together, our data imply that the anti-proliferative effects of BMP4 and BMP5 in pancreatic cancer cells are generally due to cytostasis.

The contribution of BMP5 to pancreatic cancer cell invasiveness has not been investigated before, and in case of BMP4 it has been studied only in two cell lines (PANC-1 and AsPc-1) [28]. Our results extend these findings by demonstrating that also BMP5 is able to increase the migration and invasiveness of pancreatic cancer cells. Importantly we now show that HPAF-II, MIA PaCa-2 and PANC-1 pancreatic cancer cell lines with a clear growth inhibitory effect after BMP treatment demonstrated a parallel increase in cell migration and invasion. In contrast, AsPc-1 and SU.86.86 cells that were less sensitive to BMP4 or BMP5 induced growth arrest did not display distinct changes in their migration/invadability. These data indicate that some cells are indeed more sensitive to BMP stimulation, and more significantly that BMP4 and BMP5 have functions that are both detrimental and beneficial to the pancreatic cancer progression.

There are several factors that might contribute to the cell line specific phenotypes observed in our study. Interestingly the endogenous BMP4 or BMP5 expression level did not seem to affect the results. Another interesting note is that BMP5 decreased the proliferation of HPAF-II cells whereas these cells were resistant to BMP4 proliferation controlling signals, and similarly SU.86.86 responded to BMP4 but not to BMP5 treatment. Thus there are distinct differences in the actions of BMPs and more importantly different cell lines show diverse responses to the same BMP ligand. On a molecular level, all cell lines but PANC-1 showed SMAD1/5/8 phosphorylation after BMP4 and/or BMP5 treatment but no MAP kinase activation, regardless of whether they showed proliferation or migration response or not. These findings argue that the BMP signal is typically transmitted through the canonical route. However, it is important to note that there was no direct association between the activation of the canonical BMP signaling pathway and a specific phenotypic response. This fact is exemplified by the AsPc-1 cell that showed marked phosphorylation of SMAD1/5/8 after both BMP4 and BMP5 treatment but no changes in cell growth or migration/invasion. All of the studied cell lines have wild type SMAD4, although in the case of AsPc-1 reports are divergent [42,43]. Thus this issue does not explain the differences in phenotypes. Taken together, it is conceivable that BMP4 and BMP5 signaling differs in pancreatic cancer, perhaps through BMP receptor usage or the use of completely separate signaling pathways and therefore it is important not to see them as functional equivalents. This feature of BMP signaling underlies its exceptionally pleiotropic and multifunctional nature.

One of the main findings of this study was the discovery of the dualistic functions of BMP4 and BMP5. They simultaneously acted as antiproliferative factors inhibiting cell growth mainly via induction of cell cycle arrest and as prometastatic factors through stimulation of cell migration and invasion within the same pancreatic cancer cells. Similar dualistic effects were recently reported after BMP4 treatment in breast cancer [35]. Furthermore, the well-known family member TGF-β has an established dualistic role in cancer development where it acts as a tumor suppressor during the first steps of tumorigenesis, but later stimulates metastasis formation [47]. It is in fact possible that cells have metastatic capability and at the same time are characterized by reduced proliferative activity. Anjomshoaa et al. recently reported that both metastasizing primaries and liver metastases have reduced proliferative activity relative to non-metastatic colorectal cancer cells [48]. These data agree well with our results where pancreatic cancer cells respond to BMP signals by growing slower but having increased ability to migrate and invade. Furthermore, it is important to note that not all pancreatic cell lines responded to BMP4 and BMP5 treatment. In similar fashion, BMP7 actions in prostate cancer were dependent on the cell phenotype [49]. These observations clearly suggest that BMPs have diverse roles in tumor progression even within the same tumor type. The effects of BMPs on cancer cells need to be further studied with special attention to cell type and environment where they are expressed, in order to achieve deeper understanding of BMP signaling, its consequences and the context in which it acts.

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Conflict of interest

The authors declare no conflict of interest.

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