BMP2 Induces PANC-1 cell invasion by MMP-2 overexpression through ROS and ERK

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1. ABSTRACT

The emerging roles of bone morphogenetic proteins (BMPs) in the initiation and progression of multiple cancers have drawn great attention in cancer research. We hypothesized that BMP2 promotes cancer metastasis by modulating MMP-2 secretion and activity through intracellular ROS regulation and ERK activation in human pancreatic cancer. Our data showed that stimulation of PANC-1 cells with BMP2 induced MMP-2 secretion and activation, associated with decreased E-cadherin expression, resulting in epithelial-to-mesenchymal transformation (EMT) and cell invasion. Blockade of ROS by the ROS scavenger, 2-MPG, abolished cell invasion, inhibited the EMT process and decreased MMP-2 expression, suggesting ROS accumulation caused an increase in MMP-2 expression in BMP2-stimulated PANC-1 cell invasion. Furthermore, treatment of PANC-1 cells with 2-MPG or ERK inhibitor PD98059 reduced the phosphorylation of ERK, resulting in attenuation of BMP2-induced cell invasion and MMP-2 activation. Taken together, these results suggest that BMP2 induces the cell invasion of PANC-1 cells by enhancing MMP-2 secretion and acting through ROS accumulation and ERK activation.

2. INTRODUCTION

Pancreatic cancer is a devastating disease which is the fourth leading cause of cancer death worldwide (1). Pancreatic cancer is notorious due to its late presentation, early and aggressive local invasion, metastatic potential and poor outcome (2-4). Epithelial-derived cancer cells within a primary tumor partially become metastatic, undergoing the epithelial to mesenchymal transition (EMT), which enables cells to penetrate the basement membrane and access the bloodstream (5). EMT is characterized by loss of epithelial cell markers such as E-cadherin and is associated with an increase in matrix metalloproteinases (MMPs) that remodel the extracellular matrix (6).

Bone morphogenetic proteins (BMPs) are signaling molecules, which belong to the transforming growth factor-β (TGF-β) superfamily, and were first identified based on their ability to form bone (7,8). More interestingly, BMPs have been found not only to act in osteogenesis, but also to play an important role in regulating tissue development and diverse biological process such as cell proliferation, differentiation and apoptosis (9,10). Because of these versatile and essential

functions, BMPs have drawn much attention in cancer research. Virtanen *et al* (11) demonstrated that BMP4 and BMP5 simultaneously functioned as anti-proliferative 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. According to recent work, multiple BMP family numbers including BMP2, BMP4 and BMP7 induce EMT in the human pancreatic cancer cell line PANC-1 through BMP2-p38/ERK-Msx2, as demonstrated by morphological alterations and loss of E-cadherin expression (12).

MMPs are a family of zinc-containing proteolytic enzymes that break down extracellular matrix proteins (12). Thus MMPs are believed to be associated with cancer invasion through degradation of the basement membrane, which can lead to tumor or cancer cells crossing tissue barriers. Studies report that increased expression of MMPs is correlated with poorer prognosis, shorter survival time and/or the presence of local invasion of cancer (13-15). In the MMP family, MMP-2 is type-IV collagenase secreted by cells as a pro-enzyme and then activated in the extracellular matrices to execute its proteolytic activity (16,17). MMP-2 shows substrate specificity toward type IV collagen, the major component of basement membrane, and its expressions are strongly correlated with tumor metastasis (18,19). Furthermore, high expression and activation levels of MMP-2 have been found in human pancreatic cancer tissues (15). However, the signaling pathways that regulate MMP expression in pancreatic cancer cells are still under investigation. Previously, the BMP-signaling pathway was demonstrated to be intact and functional in human pancreatic cancer cells where several BMP signaling components and transcriptional targets were increased in cancer specimens (20). Recent studies (21) also demonstrated that TGF-β1, which is in the same superfamily as BMP2, increases invasiveness of human pancreatic cancer cells through the Rac1/ROS/NF-κB/IL-6/MMP-2 signaling pathway.

NADPH oxidase-medicated production of reactive oxygen species (ROS) is known to promote cell proliferation and considered as an anti-apoptotic factor in pancreatic cancer cells (22,23). Studies have demonstrated that high levels of ROS correlate with an increase in MMP-2 levels (24). ROS activity and ERK activation is tightly linked with MMP-2 activity (25,26). Therefore, in the present study, we evaluated the hypothesis that ROS and ERK signaling pathways play predominant roles in BMP2-induced pancreatic cancer cell invasion and EMT through MMP-2 regulation.

3. MATERIALS AND METHODS

3.1. Antibodies and reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from GIBCO/Invitrogen (Carisbad, CA). Trans-well chambers (8 μm pore-size) and Matrigel were purchased from BD Biosciences (San Jose, CA). Recombinant human BMP2 was purchased from R&D Systems (Minneapolis, MN). 2-MPG and PD98059 were provided by Calbiochem (San Diego, CA). Antibodies specific for MMP-2, and E-cadherin were purchased from Abcam (Abcam, USA). ERK, phosphor-ERK and β-actin antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA).

3.2. Cell culture

Human pancreatic carcinoma, epithelial-like cell line PANC-1 was obtained from ATCC, (Manassas, VA) and maintained in DMEM with with 10% fetal bovine serum at 37C in a 5% CO_2 humidified atmosphere.

3.3. Western blotting

After the addition of lysis buffer, cell lysates were centrifuged at 16,000 rpm, 4°C for 15 min followed by adding 5× sample loading buffer. Cleared lysates were then boiled for 5 min at 100C. The samples were electrophoresed on 12.5% polyacrylamide gels at 200 V and transferred to nitrocellulose membranes (Millipore). The membranes were then blocked with 5% non-fat dry milk, and incubated with the primary antibody as follows: anti-MMP-2, anti-E-cadherin, anti-ERK, anti-p-ERK, and anti-β-actin. Western films were scanned and the signal intensity for each band was determined using Quantity One software (Bio-Rad).

3.4. MMP-2 activity assay

Gelatin zymography was performed for quantification of active and pro-MMP-2 in the supernatants of PANC-1 cells as demonstrated in previous studies (27). After 72 hours treatment, cells were cultured in serum-free media in the absence or presence of BMP2 for 24 h. The medium (30 μL) was then removed and mixed with an equivalent amount of Laemmli sample buffer (without β-mercaptoethanol). Samples were separated using 10% SDS–PAGE electrophoresis containing 0.1% gelatin at 4C. After electrophoresis, the gel was incubated in 2.5% Triton X-100 for 1 h followed by gentle rinsing and incubation in activation buffer for 16 h at 37C. Gels were stained with Coomassie Blue for 45 min and destained with methanol–acetic acid in water for 15 min. The activity of the lytic bands was determined by Quantity One. Pro-MMP-2 levels and active-MMP-2 levels were expressed as a fold-increase over the controls.

3.5. Invasion assay

Twenty-four-well Matrigel-coated trans-well chambers (BD Biosciences) were used for cell invasion assays. PANC-1 cells were seeded in the upper chamber (1×10^5 cells/well) in serum-free media, with conditioned medium in the lower compartment of the chambers. After 48 h incubation at 37C, non-migrating cells on the upper surface were completely removed with a cotton swab, while migrating cells on the underside were fixed and stained with crystal violet. The invading cells were then counted under a NIKON inverted microscope (200×).

3.6. ROS production

Free ROS production was measured by incubating PANC-1 cells with 10 μM CM-H2DCFDA using a ROS Assay Kit (Invitrogen, Eugene, Oregon). ROS levels were expressed as arbitrary units (AU) of DCF
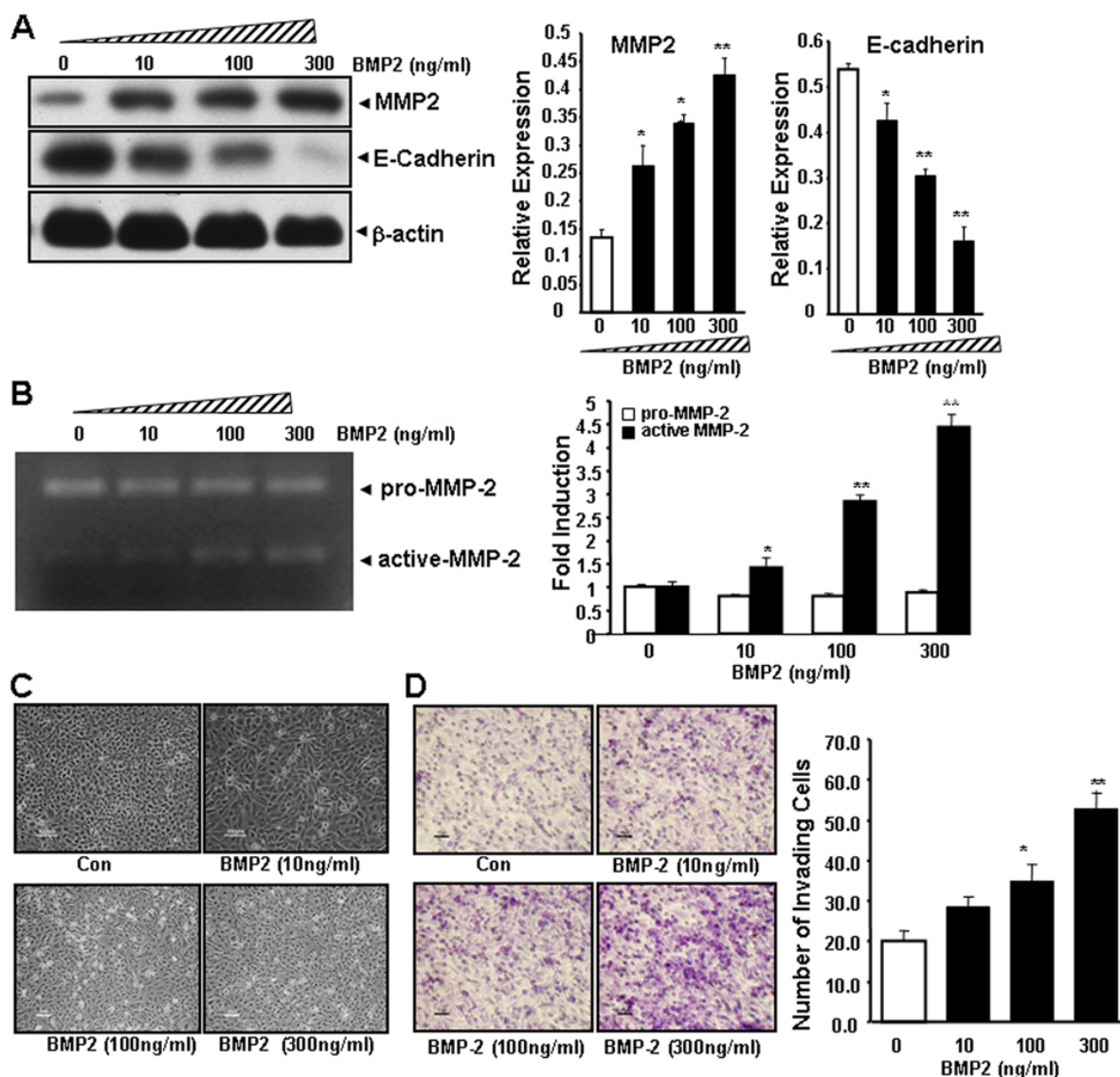
Figure 1. BMP2 was functional in PANC-1 cells. PANC-1 cells were treated with BMP2 at three concentrations: 10 ng/mL, 100 ng/mL and 300 ng/mL. (A) Western blot images and quantification showed BMP2 increased MMP-2 protein expression while decreased E-cadherin expression in a dose-dependent manner; (B) MMP-2 activity was dose-dependently increased by BMP2, while the level of pro-MMP-2 remained unchanged; (C) BMP2 also resulted in cell morphology changes, which were consistent with E-cadherin decrease; The invasion study showed that BMP2 increased cell migration in a dose-dependent manner (D).

fluorescence, which was measured in a stirred cuvette at 37C in a Hitachi H-7000 (Hitachi Limited, Tokyo, Japan) spectrofluorometer with excitation at 488 nm and emission at 530 nm. ROS production was expressed as fold increase compared with ROS levels in control conditions.

3.7. Statistical analysis

All experiments were done in triplicate, and data correspond to at least three independent experiments. Results are shown as mean ± SEM. The data were evaluated using the two-tailed Student's t test. Significance was established at values of p < 0.05.

4. RESULTS

4.1. BMP2 increases MMP-2 secretion and activity in PANC-1 cells

MMP-2 is secreted by cells as a pro-enzyme and then activated in the extracellular milieu to execute its proteolytic activity. Here we investigated whether BMP2 alters MMP-2 secretion or activity in the PANC-1 cells. Stimulation of PANC-1 cells with BMP2 significantly increased MMP-2 protein expression levels in a dose-dependent manner. Even at a low concentration of 10 ng/mL, BMP2 was able to increase MMP-2 activity by 1.8
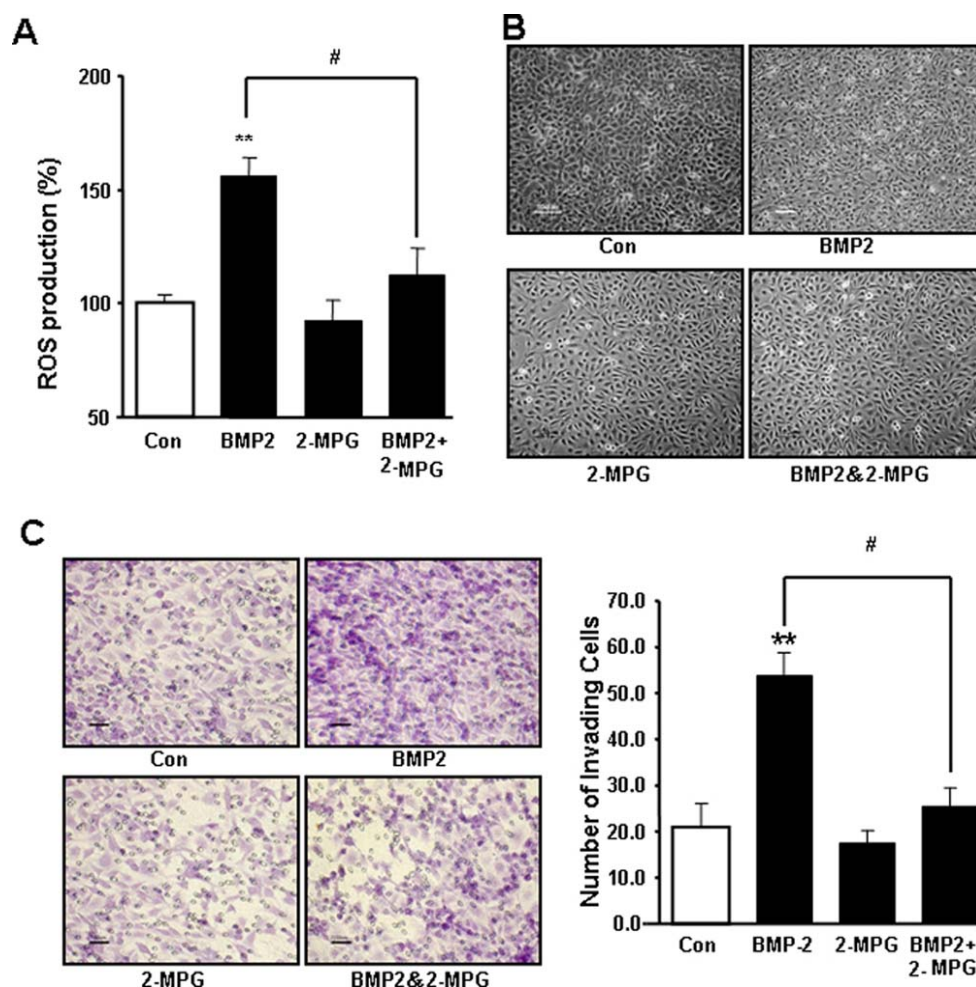
Figure 2. ROS scavenger, 2-MPG, partially reversed BMP2–induced cell morphology changes and cell invasion. PANC-1 cells were stimulated with one or both of 300 ng/mL BMP2 and 30 μg/L 2-MPG, or vehicle as control. The fluorescence reading showed ROS production was dramatically increased by BMP2 treatment, which was mainly blocked by 2-MPG (A). 2-MPG treatment partially inhibited BMP2-induced cell morphology changes (B) and cell invasion (C).

fold . At high concentrations, a 2.4- and 3-fold increase in MMP-2 activity was observed when cells were treated with 100 and 300 ng/mL of BMP2, respectively. EMT is an important indicator of pancreatic cancer progression (6) and loss of E-cadherin expression is a common marker of EMT. Here we have shown that the expression of E-cadherin decreased in response to the increase in BMP2 concentration (Figure 1A). BMP2 was able to alter MMP-2 activity in a dosage-dependent manner in PANC-1 cells. The increase in BMP2 concentration significantly increased total MMP-2 protein expression levels, as well as the active form of MMP-2. Meanwhile, the level of pro-MMP-2 was not altered by BMP2 induction (Figure 1B).

We also observed a dramatic morphological change of the PANC-1 cells as BMP2 concentration increased, in the context of EMT. Cells treated with BMP2 resulted in a spindle-like phenotype, as compared with untreated cells (Figure 1C). We measured the effect of BMP2 treatment on the invasiveness of the PANC-1 cells, as cell invasion is closely linked with EMT. The invasive capacity increased as

the BMP2 concentration increased. After treatment with 100 and 300 ng/mL BMP2, the number of invading cells increased significantly compared to untreated cells. These results suggest BMP2 is capable of inducing EMT and increasing cell invasion in PANC-1 cells (Figure 1D).

4.2. BMP2-stimulated ROS activity induces EMT and increases cell invasion in PANC-1 cells

Since certain doses of ROS have been known to increase pancreatic cancer cell proliferation and decrease apoptosis (28), we first examined the level of ROS production in BMP2-stimulated PANC-1 cells. ROS levels for control cells dramatically increased (9-fold) following BMP2 treatment. We then analyzed the effect of N-(2-mercaptopropionyl) glycine (2-MPG), a scavenger of ROS, in BMP2-treated PANC-1 cells. As expected, 30 μg/L 2-MPG was able to reduce the 300 ng/mL BMP2-stimulated ROS production by 67%. Treatment of PANC-1 with 2-MPG served as a negative control and we did not observe any increase of the basal ROS level in the absence of BMP2 stimulation (Figure 2A). Accordingly, cell
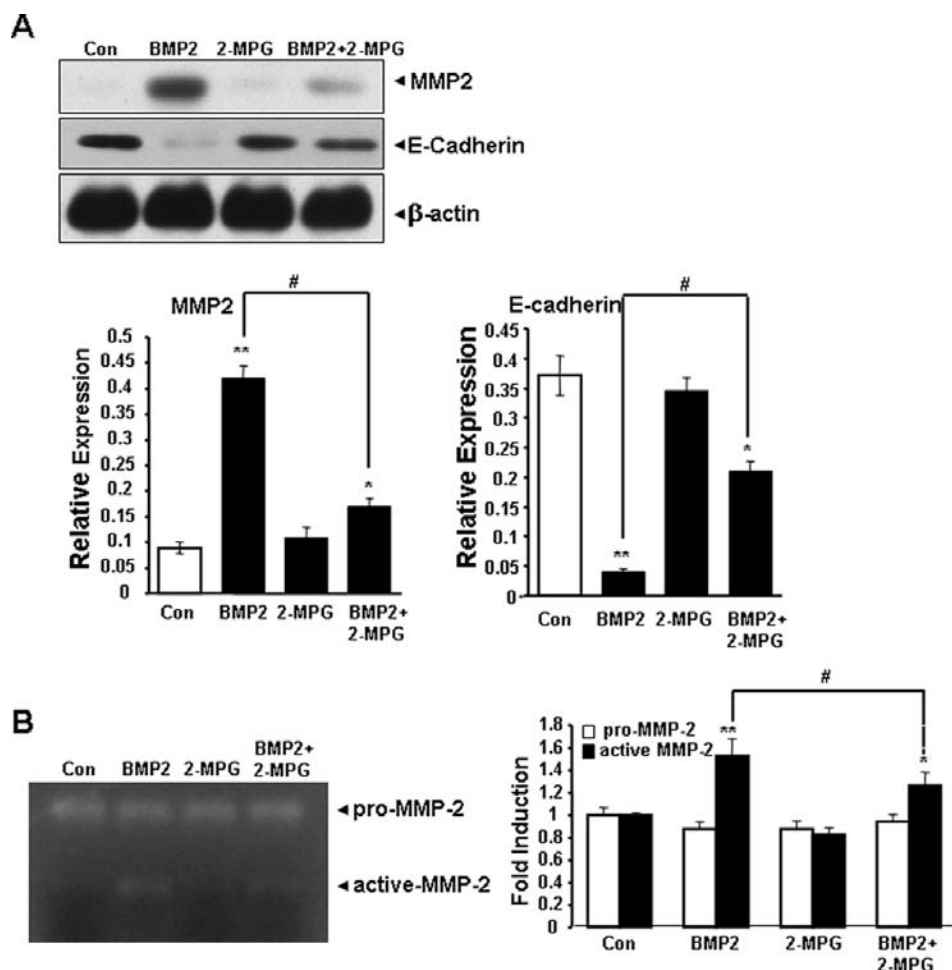
Figure 3. ROS accumulation increased MMP-2 secretion and activity in BMP2-stimulated PANC-1 cells. PANC-1 cells were stimulated with one or both of 300 ng/mL BMP2 and 30 μg/L 2-MPG, or vehicle as control. 2-MPG treatment showed little effect on MMP-2 or E-cadherin expression levels of by itself, but could significantly inhibit BMP2-induced increase in MMP-2 and decrease in E-cadherin (A). 2-MPG also induced less MMP-2 activity when combined with BMP2, compared with BMP2 treatment only, while pro-MMP-2 level did not change (B).

morphology and invasiveness following BMP2 and 2-MPG treatment were evaluated. EMT was observed following BMP2 treatment and was partially reversed by 2-MPG treatment (Figure 2B). In the invasion assay, BMP2 increased PANC-1 invasion while 2-MPG alone showed no impact on cell invasion. However, 2-MPG treatment significantly reduced invasiveness, which was induced by BMP2 stimulation (Figure 2C).

4.3. BMP2-stimulated ROS increases MMP-2 expression and activity in PANC-1 cells

Previous studies have shown that MMP expression is modulated by the intracellular ROS level (29). We investigated the effects of ROS inhibition on MMP-2 expression and activity in BMP2-stimulated PANC-1 cells. 2-MPG treatment significantly reduced BMP2-mediated MMP-2 expression by 60% (Figure 3A). Moreover, reduction of ROS activity by 2-MPG treatment also partially rescued the E-cadherin expression level, which was abolished in BMP2-stimulated PANC-1 cells.

We then proceeded to evaluate the effect of 2-MPG treatment on MMP-2 activity in BMP2-treated PANC-1 cells. The level of pro-MMP-2 remained unchanged following 2-MPG treatment, while the expression of active MMP-2 was significantly reduced after 2-MPG treatment (Figure 3B). These results suggest that BMP2 stimulation resulted in accumulation of ROS, which consequently mediated MMP-2 secretion and activation in PANC-1 cells.

4.4. ERK mediated MMP-2 activity and cell invasion in BMP2-stimulated PANC-1 cells

To evaluate the role of ERK in BMP2-induced MMP-2 activity, we first confirmed that ERK activity in PANC-1 cells could be activated by BMP2. The presence of BMP2 activated ERK activity whereas the addition of ROS scavenger 2-MPG alone could not phosphorylate ERK. As described in earlier experiments, intracellular ROS levels increased MMP-2 activity and pretreatment with ROS scavenger 2-MPG was capable of reducing MMP-2 activity. 2-MPG also reduced ERK
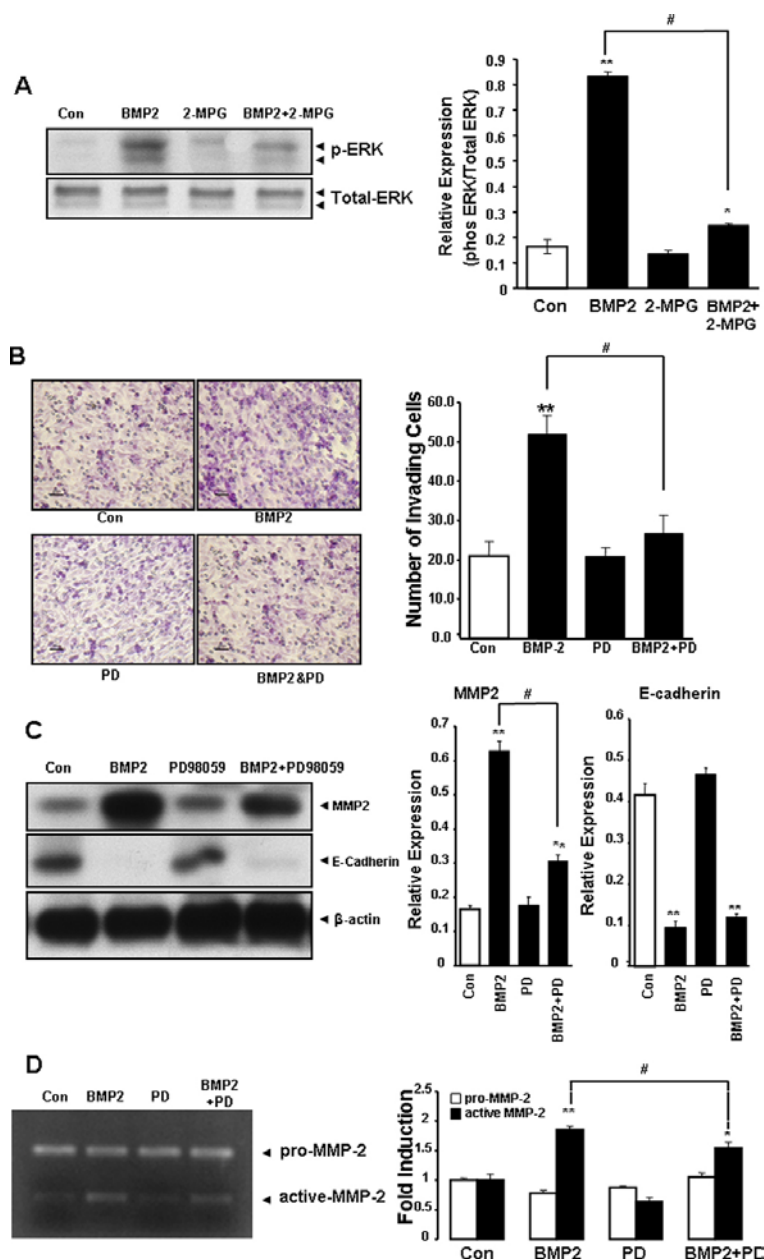
Figure 4. ROS regulated ERK activity; enhanced cell invasion and MMP-2 secretion/activity in BMP2-stimulated PANC-1 cells. After treatment with BMP2, PANC-1 cells showed an increase in ERK phosphorylation, which was partially blocked by 2-MPG; although 2-MPG itself did not show effect on p-ERK (A). ERK inhibitor, PD98059 (40 μmol/L), reduced BMP2-induced cell invasion as shown in the microscopy photographs (B). Additionally, it also reduced the BMP2-induced increase in MMP-2 expression, ; the ERK inhibitor had no affect on MMP-2 by itself (C). MMP-2 activity showed the same pattern with MMP-2 protein levels, while pro-MMP-2 levels did not change. This showed that PD98059 only reduced active MMP-2, which was increased by BMP2 (D). However, PD98059 showed little effect on E-cadherin either by itself or combined with BMP2 (C).

phosphorylation in the presence of BMP2. This suggests accumulation of ROS resulted in ERK activation, which subsequently increased MMP-2 expression (Figure 4A). Next we accessed the effects of ERK inhibition on the invasiveness of PANC-1 cells. Pretreatment with PD98059 (40 μmol/L), an ERK inhibitor dramatically reduced the invasiveness of BMP2-induced PANC-1 cells (Figure 4B). To confirm that ERK could mediate MMP-2 activity, we

then examined the effect of ERK inhibition on MMP-2 expression and activity. Incubation of PANC-1 cells with PD98059 significantly reduced the protein level of MMP-2 by approximately 50% in BMP2-stimulated cells. However, inhibition of ERK by PD98059 could not rescue the decrease of E-cadherin expression, induced by BMP2, indicating that ERK does not regulate E-cadherin expression directly (Figure 4C). We then analyzed the

effect of ERK inhibition on MMP-2 activity by PD98059 in BMP2-stimulated PANC-1 cells. We observed no change in the level of pro-MMP-2. However, ERK inhibitor significantly reduced the level of active MMP-2 (Figure 4D).

5. DISCUSSION

The BMP family belongs to the transforming growth factor-β superfamily and has been shown to be functional in cancer, contributing through autocrine and paracrine mechanisms to tumor development, invasion and metastasis (20,30,31). Studies involving lung, liver, prostate, melanoma, pancreatic, and ovarian cancer cells show that the BMP family induces changes in cell morphology, associated with increased cell motility and invasiveness (20,30,32).

EMT has been known to play a vital role in cellular trans-differentiation during tumor invasion and metastasis (33). During EMT cancer cells often show reduced expression of cell-cell adhesion protein such as E-cadherin. As a result, cells undergoing EMT gain mesenchymal cell-like functions including invasive potential and the ability to secrete extra cellular matrix (ECM) proteins and MMPs (34). Previous studies suggest that EMT is regulated by a number of growth factors such as TGF-β1, fibroblast growth factor, epithelial growth factor, and hepatocyte growth factor (35-37). Moreover, several BMPs, particularly BMP7 and BMP4, have been implicated in EMT in various organs such as the kidney and lung (38,39). Our data show that BMP2 induced EMT in the pancreatic cancer cell line, resulting in the reduction of E-cadherin and enhanced cell invasion of PANC-1 cells.

The crucial role of MMP-2 in cancer invasion and metastasis has been widely investigated both *in vitro* and *in vivo*, especially in pancreatic cancer cells, since increased expression and activation of MMP-2 has been found in human pancreatic cancer tissues (16,17). Our results provide solid evidence that BMP2 promotes the secretion and activation of MMP-2 in PANC-1 cell lines.

Recent studies have established that TGF-β1 is able to activate Rac1 followed by increased MMP-2 production and invasiveness in CAPAN-2 cells (21,40). EGF was also found to promote invasion by these tumor cells through Rac1/ROS dependent secretion and activation of MMP-2 (24), suggesting regulation of ROS is responsible for stimulation of MMP-2 expression. ROS is also suggested to play a substantial role in EMT, relevant to cancer progression (22.41). In our experiments, we demonstrate that BMP2 stimulation leads to an accumulation of intracellular ROS in PANC-1 cells, associated with enhanced cell invasion. Treatment of the BMP2-induced cells by ROS scavenger 2-MPG was able to reduce MMP-2 expression and increase E-cadherin, resulting in a reduction in the BMP2-induced cell invasion of the PANC-1 cells. These results suggest that ROS accumulation contributed to BMP2-induced EMT and MMP-2 activation. Furthermore, treatment of the PANC-1 cells with 2-MPG reduced ERK phosphorylation. Inhibition

of the ERK pathway also attenuated BMP2-induced MMP-2 secretion and activation. These results suggested that ERK activity is with a mediator in BMP2-stimulated MMP-2 secretion and activation of PANC-1 cells.

However, our data also show that inhibition of ERK activity by PD98059 decreased BMP2-induced cell invasion, MMP-2 secretion and activity, but could not rescue the reduction of E-cadherin. These results indicate that ERK activation alone does not contribute to EMT in BMP2–stimulated PANC-1 cells. EMT may be due to the more predominant role of ERK's upstream mediator Ras in E-cadherin regulation. Recent studies showed that the small GTPase Ras proteins are involved in cellular signal transduction leading to cell growth, differentiation and survival. Mutations of the ras genes will convert these genes into active oncogenes, which have been found in many types of cancer (42-44). It was previously shown that BMP2 stimulation and ROS signal led to activation of Ras (45,46). The presence of Ras is necessary and sufficient for ERK1 activation (47) and could induce downstream Snai1 transcription and consequently suppress E-cadherin expression (48). As Ras is involved in cross-talk between multiple signaling pathways, it also stimulates Wnt signaling and AKT/PKB pathway through the inhibition of glycogen-synthase kinase-3β (GSK3β), leading to a decreased E-cadherin level (49-51). GSK3β normally suppress Snai1 through the phosphorylation of Ser residues on Snai1 and inhibition of GSK3β led to increased Snai1 activity and hence a decrease in E-cadherin (52).

In summary, our study demonstrated for the first time, that BMP2 could increase cell invasion by enhancing MMP-2 expression and activity via ROS activation, resulting in decreased E-cadherin expression and EMT. As EMT and invasion are major contributors to cancer metastasis, which is the major cause of death in pancreatic cancer patients, these finding could provide potential novel therapeutic targets of signaling pathways involving EMT. Additionally, future studies will focus on the role of Ras/GSK3β/Snai1 activity in BMP-2/ROS/ERK/MMP-2 signaling pathway, EMT, and their roles in E-cadherin regulation.

6. ACKNOWLEDGEMENTS

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