Inhibition of PI3 kinase/Akt pathway is required for BMP2-induced EMT and invasion

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Abstract. Although dysregulation of bone morphogenetic protein (BMP) signaling has been linked to various types of cancers, the relationship between abnormal activation of these signaling pathways and tumorigenesis is not clear. The purpose of the current study was to clarify how BMP2 is involved in colon cancer aggressiveness. The data showed that SW480 and DLD-1 cells displayed different responses to short- and long-term exposure to BMP2. During the first 24 h of exposure to BMP2, these cells were growth-inhibited, whereas surviving cells became resistant to growth inhibition, showing epithelial-to-mesenchymal transformation (EMT) and enhanced motility and invasiveness. Interestingly, in highly metastatic mesenchymal colon carcinoma cells (CT26), blockade of BMP2 signaling by BMP2 siRNA prevented EMT, motility and invasiveness; rather, blockade of BMP2 signaling caused a mesenchymal-to-epithelial transition (MET). The levels of phosphorylated Akt were very different between the two cell types; the BMP2-sensitive SW480 and DLD-1 cells had much higher levels of expression than the BMP2-resistant SW480 and DLD-1 and CT26 cells. CT26 cells, following exposure to BMP2 and activation of Akt, escaped the EMT-induced cellular motility and invasiveness. Moreover, LY294002 treatment of BMP2-sensitive SW480 cells blocked cell growth and enhanced motility and invasiveness. Together, these results suggest that suppression of the PI3 kinase/Akt pathway is correlated with the development of BMP2 resistance and invasion in BMP2-induced EMT in colon cancer.

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Introduction

The bone morphogenetic proteins (BMPs) were originally identified as osteo-inductive cytokines that promote bone and cartilage formation when injected into mice (1,2). BMPs that are members of the TGF-ß family have been described for >20 different BMP isoforms in mammals and *Drosophila* (3,4). BMPs mediate their biological effects by binding to heteromeric type I/II receptor complexes that contain serine/threonine kinase domains. Upon BMP binding, the receptor complex mediates intracellular signaling via phosphorylating Smad 1/5/8. Then, these phosphorylated Smads associate with Smad4 and translocate to the nucleus to activate the transcription of downstream targets (5-7). More recently, multiple non-Smad pathways have been implicated in mediating BMPs signaling. Studies in colon cancer cell lines have shown that BMP down-regulates PTEN via RAS/ERK in a SMAD4-null environment that contributes to cell growth, and constitutes a SMAD4-independent, but BMP-responsive signaling pathway (8). BMPs has also been shown to induce proliferation and colony formation of fetal pancreatic cells through phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways (9).

BMP2 plays complicated roles, including the promotion of cell survival, regulation of cell proliferation, functional differentiation, cell motility, apoptosis and self-renewal of embryonic stem cells. In tumorigenesis, BMP2 signaling plays dual roles, functioning as cell context-dependent tumor suppressors or oncogenes. In mature colonic epithelial cells, BMP2 acts as a tumor suppressor by inhibiting proliferation and inducing apoptosis (10). Therefore, perturbations in BMP2 signaling may lead to increased tumorigenesis. The ability of BMP2 to enhance tumorigenicity *in vivo* is mediated by stimulating angiogenesis, inhibiting immune surveillance, or promoting the degradation of ECM (11). Studies have previously shown that BMP2 stimulates the development of a neovasculature in developing tumors and promotes local invasion and metastasis in melanoma (12) and lung cancer cell lines (11,13,14). The ectopic expression of BMP2 in lung cancer cells significantly increases lung metastasis in nude mice (14). Furthermore, a higher level of BMP2 expression is correlated with poor survival in stage I non-small cell lung carcinomas (15).

The mechanisms by which the BMP2 regulates cancer cells are poorly understood. Integration of the BMP2 pathway with other signaling cascades may modulate BMP2 responses. In the N1511 chondrocytic cell line, it has been reported that BMP2 rapidly induces the phosphorylation of Akt and NF-κB activation and prevents an apoptotic effect of these cells (8,16). In colon cancer cells, BMP-SMAD signaling and growth suppression is facilitated by p21(WAF1), but modulated by oncogenic K-RAS to reduce the growth suppression directed by this pathway (8).

In the present study, the potential function and mechanism by which BMP2 acts as a promoter of human tumor development was investigated. Specifically, we analyzed the response of colon cancer cells to both acute and sustained exposure to BMP2. Sustained exposure of colon cancer cells to BMP2 resulted in growth inhibition that gave rise to actively proliferating cells which had undergone epithelial-to-mesenchymal transformation (EMT) and invasion. However, blockade of BMP2 in CT26 cells, which are refractory to the BMP2 growth-inhibitory response, induced mesenchymal-to-epithelial transition (MET) and abolished invasiveness. The differences in colon cancer cell responses to BMP2 treatment results from down-regulation of the PI3K/Akt pathway.

Materials and methods

Cell cultures. The human colon carcinoma cell lines, SW480, DLD-1, or CT26, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained according to the ATCC's instructions.

Reagents and antibodies. Recombinant human BMP2 was purchased from R&D Systems (Minneapolis, MN). LY294002 was purchased from Calbiochem (San Diego, CA). Myc-Akt^{Myr}, Myc-Akt^{K17M} and Myc were purchased from Upstate Biotechnology (Lake Placid, NY).

Cell proliferation assay. Cells were seeded at a concentration of $4x10^3$ cells per well in 96-well culture plates and then incubated for 24 h at 37˚C in an atmosphere of 5% CO_2. The medium was then changed to RPMI-1640 that contained 0.5% FBS in the presence or absence of 100 ng/ml recombinant human BMP2. After incubating for 2 or 7 days, the number of viable cells was determined in triplicate wells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Roche Molecular Biochemical, Indianapolis, IN) according to the manufacturer's instructions.

RT-PCR analysis. Total RNA extraction was performed using the TRIzol reagent (Life Technologies, Rockville, MD), according to the manufacturer's instructions. Amplification of transcripts was performed using 1 μg/μl of total RNA and the reverse transcriptase-polymerase chain reaction (RT-PCR) using Molony murine leukemia virus reverse transcriptase (MMLV; Gibco/BRL, Gaithersburg, MD) and oligo-$d(T)_{15}$ primer (Roche). PCR amplification was performed using the following primers: BMP2, forward: 5'-TAA GTT CTA TCC CCA CGG AGG-3', reverse: 5'-GGT ACA GCA TCG AGA CCA ACG-3'.

Wound-healing assay. Cells were seeded at $1x10^5$ cells/well in 12-well plates and then pre-incubated for 24 h in serum-free RPMI-1640 (Invitrogen, Carlsbad, CA) before creating a wound across the cell monolayer with a plastic tip. Cells were then grown in culture medium with 0.5% FBS that was treated with various drugs in the presence or absence of 100 ng/ml BMP2. Cell migration into the wound surface was then monitored by microscopy after 24 h and reported as the estimated ratio of the remaining wounded area relative to the initial wound area. Quantification of the closure of the monolayer was performed using the NIH Image program and the results are expressed as the percentage of wound closure. This assay was repeated three times independently.

Matrigel invasion assay. For the Matrigel invasion assay, $1x10^5$ cells/well were seeded in the upper chamber, which was coated with Matrigel (Calbiochem) and serum-free medium containing 100 ng/ml BMP2 or control vehicle was added to the lower chamber. After 24 h of incubation, non-migrating cells were removed from the upper chamber with a cotton swab and cells present on the lower surface of the insert were stained with Diff-Quik Stain (Biochemical Sciences, Inc., Swedesboro, NJ). The invading cells were then counted by microscopy. All experiments were repeated three times.

Small interfering RNA transfection. Small interfering RNA (siRNA) duplex specific to BMP2 were synthesized at Invitrogen. The siRNA sequences used were as follows: BMP2 siRNA, 5'-ACA CCA GGU UAG UGA AUC ATT-3', 5'-UGA UUC ACU AAC CUG GUG UTT-3'. As a non-specific control siRNA, scrambled siRNA duplex was used. Transfection was done using Lipofectamine 2000 reagent (Invitrogen), following the manufacturer's instruction.

Western blotting. Whole-cell extraction was conducted using RIPA buffer [50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1% nadeoxycholate (pH 7.4)] supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml peptasin A, 10 μg/ml aprotinin and 5 μg/ml leupeptin). Protein concentrations were then measured using Bio-Rad protein assay kits (Bio-Rad, Hercules, CA). Next, the protein lysates were resolved by SDS-PAGE and then transferred onto nitrocellulose membranes (Hybond™ -P; Amersham Biosciences, Piscataway, NJ), blocked with PBS containing 0.2% Tween-20 and 5% non-fat dry milk and incubated with primary antibody. Primary antibodies were as follows: anti-Akt (Cell Signaling Technology, Beverly, MO), anti-phospho-Akt (Ser 473, Cell Signaling Technology), anti-E-cadherin (BD Bioscience, San Diego, CA), anti-ß-actin (Sigma, St. Louis, MO), anti-N-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Slug (Santa Cruz Biotechnology).

Immunofluorescence staining. Cells were seeded on dishes containing glass coverslips, 1 cm in diameter and then grown in culture medium that was treated or untreated with various drugs in the presence or absence of 100 ng/ml BMP2 for various times. Cells were fixed in 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 10 min and blocked with 3% bovine serum albumin for 30 min at room

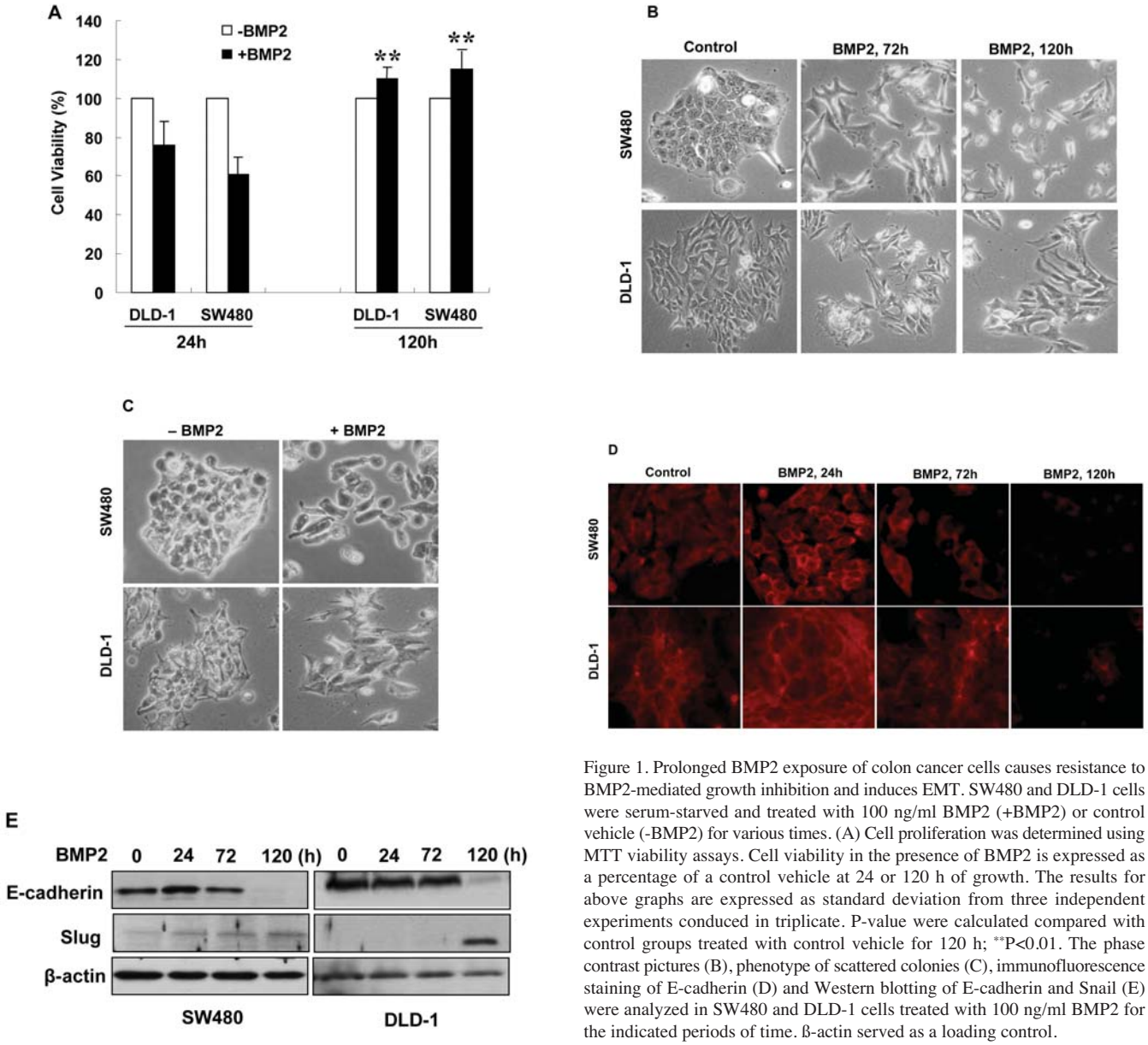

Figure 1. Prolonged BMP2 exposure of colon cancer cells causes resistance to BMP2-mediated growth inhibition and induces EMT. SW480 and DLD-1 cells were serum-starved and treated with 100 ng/ml BMP2 (+BMP2) or control vehicle (-BMP2) for various times. (A) Cell proliferation was determined using MTT viability assays. Cell viability in the presence of BMP2 is expressed as a percentage of a control vehicle at 24 or 120 h of growth. The results for above graphs are expressed as standard deviation from three independent experiments conduced in triplicate. P-value were calculated compared with control groups treated with control vehicle for 120 h; $^{**}P<0.01$. The phase contrast pictures (B), phenotype of scattered colonies (C), immunofluorescence staining of E-cadherin (D) and Western blotting of E-cadherin and Snail (E) were analyzed in SW480 and DLD-1 cells treated with 100 ng/ml BMP2 for the indicated periods of time. ß-actin served as a loading control.

temperature. After blocking, cells were incubated overnight at 4˚C with primary antibodies, washed with PBS and incubated for 30 min at room temperature with secondary Alexa fluor-594-conjugated secondary antibody (Molecular Probes, Eugene, OR) or FITC-conjugated secondary antibody (Sigma). Samples were co-stained with 2 μM 4',6-diamidino-2-phenylindole (DAPI) fluorescence (Molecular Probes) at 37˚C. Following three washings with PBS, the slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and visualized with fluorescence microscopy.

Statistical analysis. Statistical comparisons were performed by two-tailed Student's t-test. Data are given as mean ± SEM. Significance was established when $P<0.05$.

Results

Prolonged BMP2 exposure of colon cancer cells causes resistance to BMP2-mediated growth inhibition and induces EMT and increased cellular motility. BMP2 pathway is considered to be a tumor suppressor pathway that negatively regulates cell growth (10). Acquired resistance toward growth inhibition is one of the hallmarks of tumor development. Here, to understand how cancer cells regulate BMP2-induced growth inhibition in tumorigenesis, we developed cell lines with acquired BMP2 resistance from BMP2-sensitive parental SW480 and DLD-1 cells by continuously exposing them to BMP2 for 120 h. As shown in Fig. 1A, initial treatment (24 h) of these cells with BMP2 showed sensitivity to BMP2-induced growth inhibition, whereas sustained exposure to BMP2 (up to 120 h), resulted in normal cell proliferation and expression of a BMP2-resistant phenotype. We then assessed the change in cell morphology in response to BMP2. During the first 24 h of BMP2 stimulation, SW480 cells acquired slight fibroblastoid phenotype, but in DLD-1 cells, no major change was apparent in cell morphology compared with control vehicle (Fig. 1B). After 120 h, BMP2-treated SW480 and DLD-1 cells exhibited a dramatic change in cell morphology, from a more epithelial-like to a spindle-shaped cell morpho-

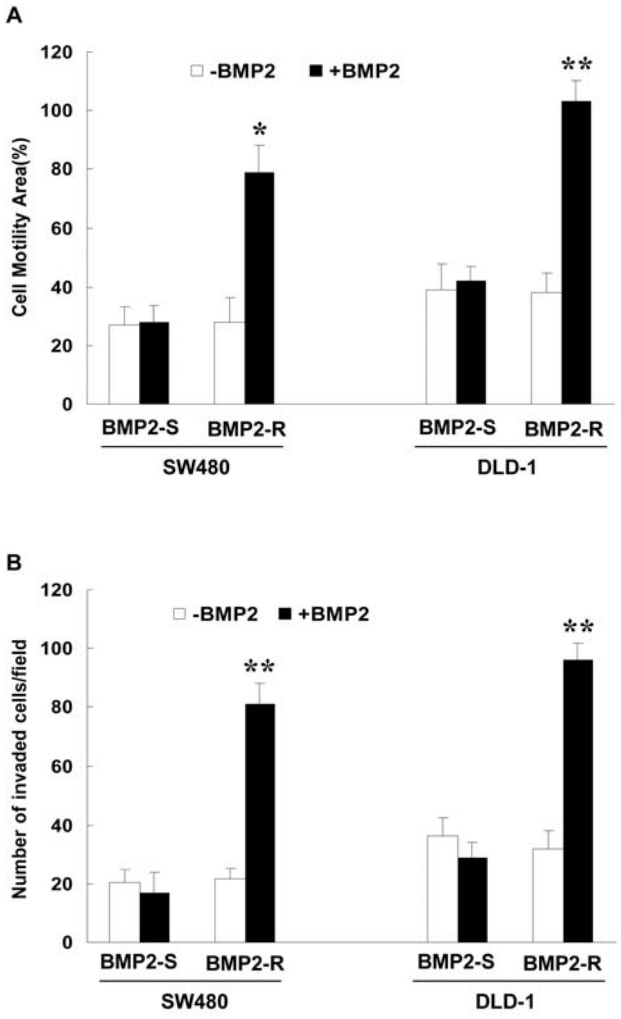

Figure 2. Prolonged BMP2 exposure causes increased cellular motility and invasiveness of colon cancer cells. (A) SW480 and DLD-1 cell monolayers with responsive (BMP2-S) or resistance (BMP2-R) to BMP2-induced growth inhibition were wounded with a pipette tip and then treated with 100 ng/ml BMP2 (+BMP2) or control vehicle (-BMP2). Cell migration to the wound area was then monitored by microscopy for 24 h post-wound. The percentage of total area covered by cells was assessed using the NIH Image program. (B) The cells ($1x10^5$ cells/well) were seeded in the upper chamber, which was coated with Matrigel and serum-free medium containing 100 ng/ml BMP2 (+BMP2) or control vehicle (-BMP2) was added to the lower chamber. After 24 h of incubation, the cells that invaded the lower surface of the insert were stained with Diff-Quik Stain and counted by microscopy. Bars represent the standard deviation of three independent experiments conducted in triplicate. P-value were calculated compared with control groups treated with control vehicle, $^{*}P<0.05$, $^{**}P<0.01$.

logy, which is typical for mesenchymal cells. Those cells also showed a scattered phenotype with a loss of the cell-cell contact that is observed in mesenchymal cells (Fig. 1C).

A morphologic change of the cells from a cuboid, epithelial-like shape to a spindle, fibroblastic-like appearance is often accompanied by a decrease or loss of epithelial markers and a gain of mesenchymal markers. To determine if delayed exposure to BMP2 resulted in a loss/decrease of the epithelial phenotype of colon cancer cells and an increase in mesenchymal markers, we performed immunofluorescence staining for an EMT marker. Concomitant with the change in phenotype, in response to BMP2, strong cell-cell boundary staining pattern for the epithelial marker protein E-cadherin persisted for up to 72 h in SW480 and DLD-1 cells (Fig. 1D). In contrast, in the cells that were exposed to BMP2 for up to 120 h, there was little or no detectable expression of E-cadherin. We further verified the switch of marker expression using Western blotting. As expected, E-cadherin expression was abundantly expressed during the first 72 h of BMP2 treatment and the treated cells showed strong suppression of E-cadherin 120 h after addition (Fig. 1E). In contrast, low expression of Slug, which is known to repress expression of the E-cadherin gene, was detected in control cells, but strong expression of these proteins was detected in the treated cells. Taken together, these findings strongly suggest that the sustained exposure to exogenous BMP2 induce both the resistance to BMP2-mediated growth inhibition and morphologic change of colon cancer cells through the EMT process.

Sustained exposure of SW480 and DLD-1 colon cancer cells to BMP2 caused resistance to growth inhibition in normal epithelial cells and EMT. We therefore investigated the role of BMP2 signaling as it relates to the motility and invasive phenotypes of those cells. A wound closure assay was used to examine the effects of BMP2 signaling on the motility of DLD-1 cells. As shown in Fig. 2A, cells (BMP2-R) with resistance to BMP2-induced growth inhibition exerted a significant increase in cell motility in response to BMP2 stimulation, whereas the addition of BMP2 resulted in no significant difference from control vehicle in cells (BMP2-S) responsive to BMP2-induced growth inhibition. In the Matrigel invasion assay, as shown in Fig. 2B, the ability of those cells to invade Matrigel was determined by the 24 h response to BMP2. BMP2-R cells had a 4-fold increase in cell invasion toward the BMP2-containing well compared with the control vehicle-containing compartment. Conversely, no significant changes existed in BMP2-S cells. These two assays indicated that sustained BMP2 exposure blocked BMP2-induced growth inhibition and induced cellular motility and invasion.

Blockade of BMP2 signaling induces MET and suppresses invasion in late-stage, metastatic carcinoma CT26 cells. Next, we determined whether BMP2-activated signaling is required to maintain the metastatic tumor cell phenotype. CT26 cells, derived from a chemically-induced colon carcinoma, are highly tumorigenic in syngeneic or nude mice and also induce blood-borne lung metastases from the primary tumor site with a high incidence (17). In culture, CT26 cells were not only resistant to BMP2-induced growth inhibition, but also displayed a spindle-like, mesenchymal phenotype and a loss of E-cadherin in membranes of cells in contact (data not shown). Moreover, CT26 cells treated with BMP2 for 24 h showed a significant increase in cell motility and invasiveness compared with cells treated with control vehicle (Fig. 3A). Here, we investigated whether inhibition of signal transduction by BMP2 would inhibit EMT and interfere with cell motility and invasiveness. Transient transfection of BMP2 siRNA into CT26 cells efficiently down-regulated BMP2 expression (Fig. 3B). Expression of BMP2 siRNA in CT26 cells caused reversion of the mesenchymal, spindle cell phenotype of CT26 cells towards a classic, cobble-stone morphology compared with non-specific control siRNA (Fig. 3C). CT26 cells expressing non-specific control siRNA are characterized by a complete

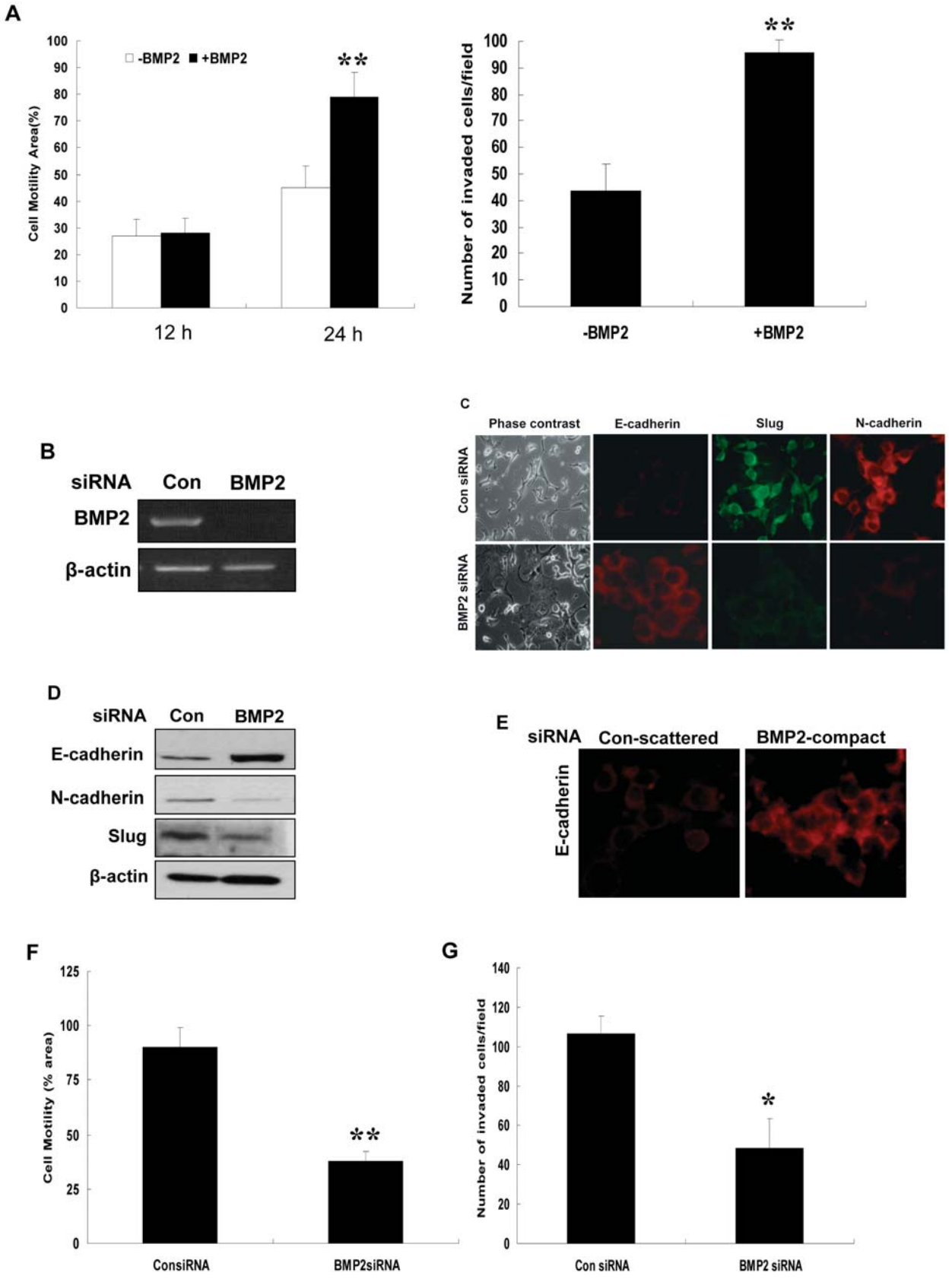

Figure 3. BMP2-activated signaling is required for both induction of EMT and maintenance of invasiveness during late-stage tumorigenesis. (A) CT26 cells were treated with 100 ng/ml BMP2 (+BMP2) or control vehicle (-BMP2) for 24 h and cell migration and invasiveness was determined using wound healing (left panel) and Matrigel invasion assay (right panel). The data are expressed as the means of three independent experiments ± SD. P-values were calculated compared with control groups treated with control vehicle, $^{**}P<0.01$. (B) CT26 cells were transfected with BMP2 siRNA or non-specific control siRNA and harvested 48 h later. RT-PCR for endogenous BMP2 transcripts was performed. ß-actin transcripts served as a loading control. (C) The left panel showed phase contrast pictures. The right panel showed immunofluorescence staining of E-cadherin, Slug and N-cadherin. (D) Expression of E-cadherin, Slug and N-cadherin were determined by Western blotting. ß-actin served as a loading control. (E) E-cadherin in the scattered colonies was visualized by staining with anti- E-cadherin antibody. Cell migration (F) and invasiveness (G) was determined using wound healing and Matrigel invasion assay. All the experiments were are expressed as the means of three independent experiments ± SD. P-values were calculated compared with control groups treated with control siRNA, $^{*}P<0.05$, $^{**}P<0.01$.

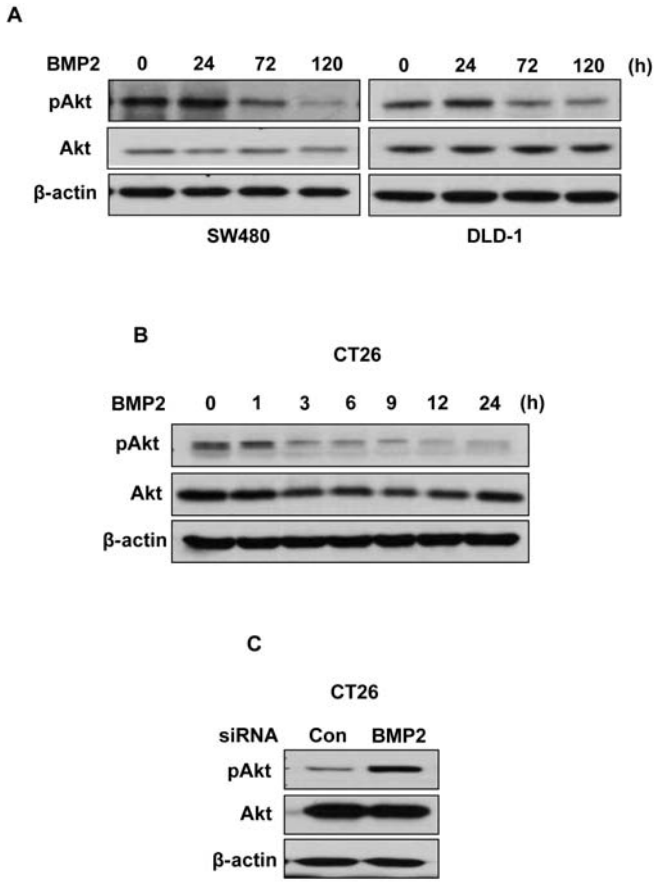

Figure 4. PI3K/Akt pathways were involved in the BMP2-mediated cellular response in colon cancer. SW480, DLD-1 (A) and CT26 (B) cells were serum-starved and treated with 100 ng/ml BMP2 for the times indicated. The total protein was harvested and resolved on a 12% SDS-PAGE and subjected to Western blotting using anti-phospho Akt (Ser 473), anti-Akt and anti-ß-actin antibodies. ß-actin was used as loading control. (C) CT26 cells were transfected with BMP2 siRNA (100 nM) or non-specific siRNA. After 24 h, lysates were prepared and subjected to Western blotting with antibodies for phopho-Akt (Ser473), Akt and ß-actin. ß-actin served as a loading control.

loss E-cadherin and gain of Slug or N-cadherin staining, whereas cells expressing BMP2 siRNA exhibit a typical staining pattern for E-cadherin at sites of cell-cell contact and complete loss of Slug or N-cadherin staining. Western blot analysis confirmed the immunofluorescence data, showing induction of E-cadherin and suppression of Slug and N-cadherin in BMP2 siRNA-treated CT26 cells (Fig. 3D). Importantly, BMP2 siRNA-expressing CT26 cells are strongly blocked in formation of scattered colonies and induced compact colonies with E-cadherin in the area of cell-cell contact (Fig. 3E). To evaluate whether BMP2 signaling was still required for motility and invasiveness of CT26 cells, wound closure and the Matrigel invasion assay were performed on CT26 cells that had knocked-down BMP2 expression by siRNA. Strikingly, expression of BMP2 siRNA reversed the stimulatory effect of BMP2 on cell migration and invasiveness by a 40 and 67%, respectively, in CT26 cells compared with non-specific control siRNA-expressing cells (Fig. 3F and G), demonstrating that BMP2 signaling plays an important role in the motility and invasiveness of metastatic colon cancer cells. Taken together, these findings indicate that BMP2-activated signaling is required for both induction of EMT and maintenance of invasiveness during late-stage tumorigenesis.

BMP2-induced EMT and invasion is mediated through down-regulation of the PI-3 kinase/Akt pathway. Recently, the critical role of PI3K/Akt pathways has been suggested in osteoblast differentiation and the anti-apoptotic effect of chondrocytic cells in response to BMP2 stimulation (16). We determined if PI3K/Akt pathways were involved in the BMP2-mediated cellular response in colon cancer. Using Western blotting, we first compared the levels of total Akt expression and activated Akt in colon cancer cells treated with BMP2 and control vehicle for 120 h. We found that the phosphorylation of ser473 in Akt was slightly enhanced in SW480 and DLD-1 cells treated with BMP2 for 24 h, while it was strongly reduced in the cells that were exposed to BMP2 for up to 120 h (Fig. 4A). In CT26 cells which display the mesenchymal phenotype and are resistant to BMP2-mediated growth inhibition, phosphorylation of Akt was also reduced in cells treated with BMP2 (Fig. 4B). Importantly, transient expression of BMP2 siRNA caused a strong enhancement in the phosphorylation of Akt compared with non-specific control siRNA (Fig. 4C).

We next determined whether the kinase activity of Akt affected BMP2-induced EMT and cellular motility and invasiveness. In order to determine if overexpression of activated Akt blocked the BMP2-induced EMT and migration/invasion, CT26 cells were transfected with constitutively active myristoylated Akt (Akt^{Myr}) or control vector and then treated with BMP2 or control vehicle. Wound closure and the Matrigel invasion assay showed that ectopic expression of Akt^{Myr} completely suppressed the ability of BMP2 to stimulate migration and invasion of those cells compared with the cells transfected with control vector (Fig. 5A and B). The results by Western blotting showed that reduced expression of E-cadherin in CT26 cells completely recovered by overexpression of Akt^{Myr} (Fig. 5C). The kinase activity was confirmed by Western blotting for phopho-GSK3α/ß. To further assess the influence of Akt on BMP2-induced EMT and invasion, SW480 cells showing the BMP2-sensitive phenotype were pretreated with the PI3K/Akt inhibitor, LY294002, before BMP2 stimulation. We found that inhibition of Akt activity by incubation with LY294002 exerted a greater increase in invasiveness of SW480 cells compared to control vehicle (Fig. 5D). Western blotting also showed that induction of E-cadherin by BMP2 was suppressed in BMP2-sensitive SW480 cells when the PI3K/Akt pathway was blocked by LY294002 (Fig. 5E), suggesting that the PI3K/Akt pathway is involved in the EMT response to BMP2.

Discussion

Although BMPs have been shown to be involved in tissue development, morphogenesis, cell proliferation and apoptosis of a variety of tissues and cells, an emerging body of evidence demonstrates that dysregulation of BMP signaling is associated with the progression of various types of cancer, including colon cancer (18-21). Up-regulation of BMPs and their receptors by tumor is an important hallmark in cancer progression, as it contributes through autocrine and paracrine

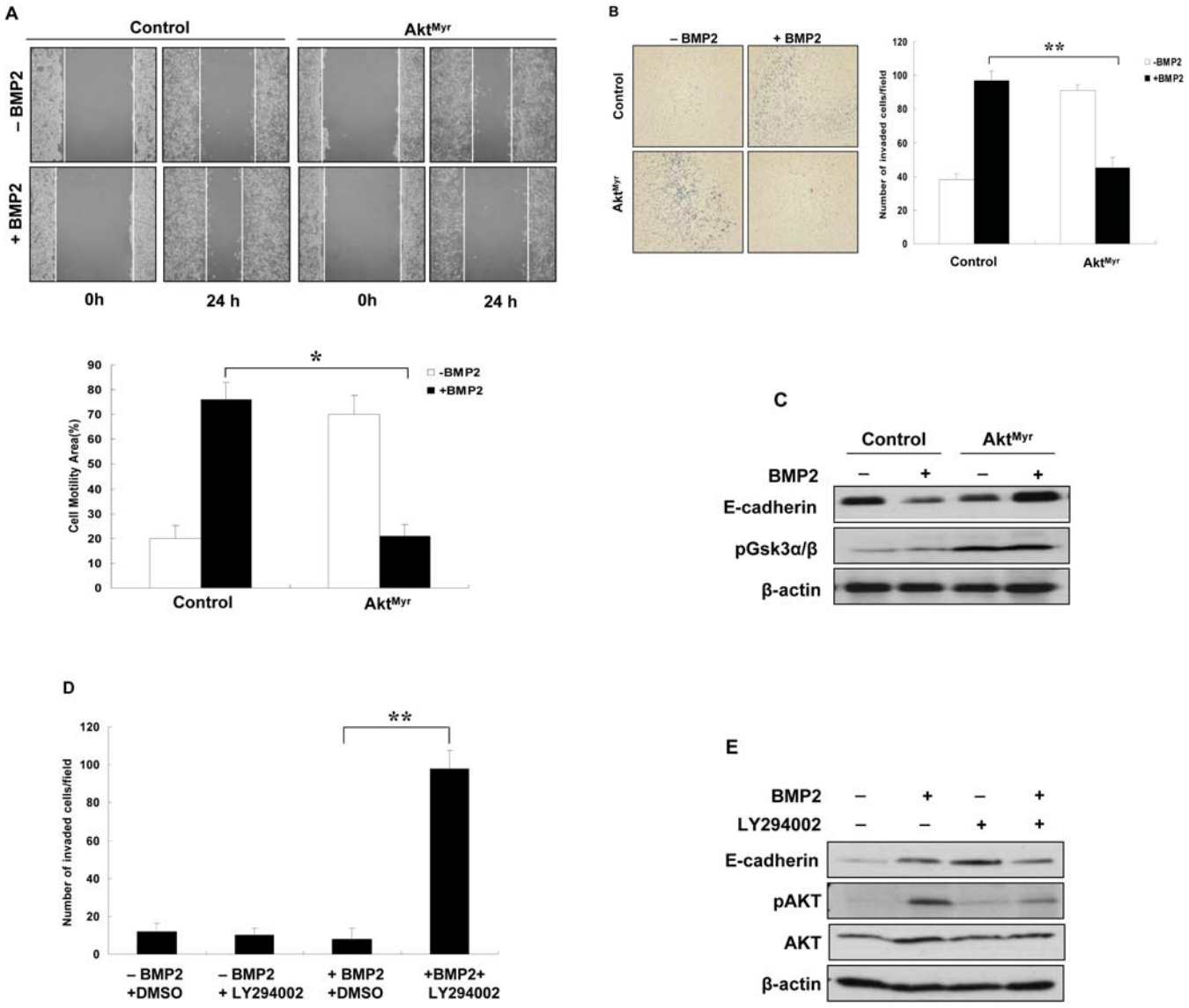

Figure 5. Invasion in BMP2-induced EMT is mediated through down-regulation of the PI-3 kinase/Akt pathway. (A) CT26 cells were transfected with 0.5 μg Akt^{Myr} or 0.5 μg control vector. Twenty-four hours after transfection, cells were wounded with a pipette tip and then treated with 100 ng/ml BMP2 (+BMP2) or control vehicle (-BMP2) in serum-free medium. Cell migration to the wound area was then monitored for 24 h post-wound and the percentage of total area covered by cells was then assessed using the NIH Image program. Bars represent the standard deviation of three independent experiments conducted in triplicate. P-values were calculated compared with control vector treated with BMP2, *P<0.05. (B) CT26 cells transfected with 0.5 μg Akt^{Myr} or 0.5 μg control vector were seeded into the upper chamber and serum-free medium treated with 100 ng/ml BMP2 (+BMP2) or control vehicle (-BMP2) was then added into the lower chamber. After 24 h of incubation, cells that migrated to the lower surface of the insert were stained with Diff-Quik Stain and counted by microscopy. Five different areas of invaded cells were counted for each data point (n=5). The data are expressed as the means of three independent experiments ± SD. P-value were calculated compared with control vector treated with BMP2, **P<0.01. (C) Total lysates were harvested and subjected to Western blotting using anti-E-cadherin, phopho-GSK3α/ß and ß-actin. ß-actin served as a loading control. (D) SW480 cells treated with 100 ng/ml BMP2 alone or together with DMSO or LY294002 (10 μM) were evaluated in a performance invasion assay. The data are expressed as the means of three independent experiments ± SD. P-values were calculated compared with SW480 culture treated with BMP2, **P<0.01. (E) Lysates were prepared and subjected to Western blotting with antibodies for E-cadherin, phospho-Akt(Ser473), Akt and ß-actin. ß-actin served as a loading control.

mechanisms to tumor development, invasion and metastasis (12,22-25). However, several studies have also shown that BMP apparently plays a role as a tumor suppressor. Hardwick *et al* have reported that BMP2 inhibits the growth of normal colonic epithelial cells and several colon cancer cell lines by promoting apoptosis and differentiation (10). Despite the central role that BMP signaling plays in the development of colon cancer, little is known about the mechanism by which BMP2 can play different roles, depending on the cell type and context in which it is acting. Here, we reported that BMP signaling, acting through the Akt pathway, selectively contributes to colon cancer progression.

In early cancer progression, TGF-ß signaling leads to induction of an anti-proliferative effect, resulting in decreased cell proliferation of cancer cells. However, the increased TGF-ß expression by cancer cells provides an advantage of cancer progression through autocrine effects on the cancer cells (26). Similar to the action of TGF-ß signaling in cancer progression, our studies also showed that BMP2 signaling in SW480 and DLD-1 cells generated from a primary colon

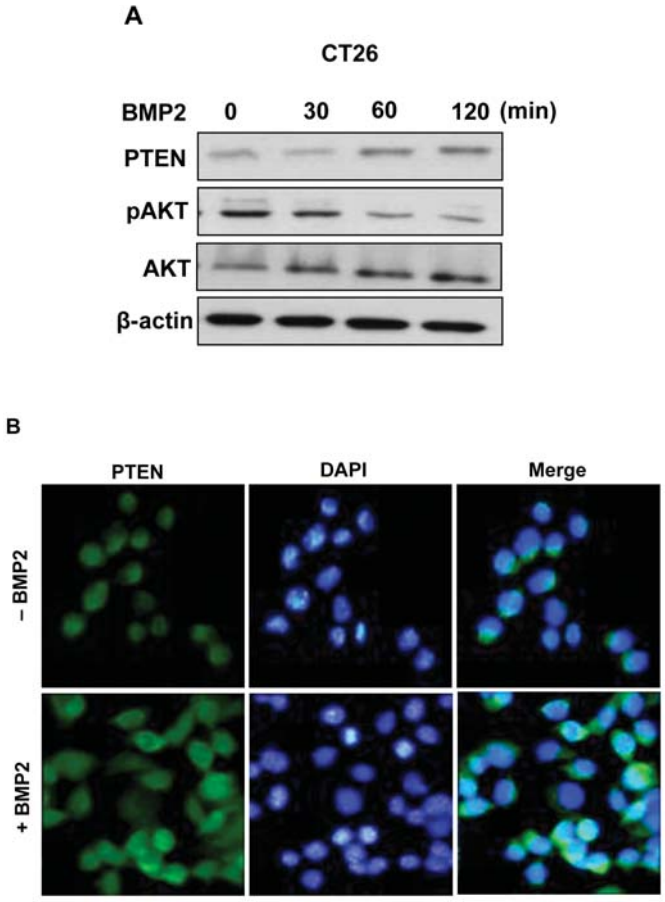

Figure 6. Treatment of CT26 cells with BMP2 produced a significant increase in cytoplasmic PTEN levels. (A) CT26 cells were serum-starved and treated with 100 ng/ml BMP2 for the times indicated. The total protein was harvested and resolved on a 12% SDS-PAGE and subjected to Western blotting using anti-PTEN, anti-phospho Akt (Ser 473), anti-Akt and anti-ß-actin antibodies. ß-actin was used as loading control. (B) CT26 cells treated with 100 ng/ml BMP2 for 30 min were fixed using 4% paraformaldehyde and processed for indirect immunofluorescence, as described. PTEN localization was determined utilizing a FITC-conjugated anti-mouse secondary antibody and nuclei were stained using DAPI.

cancer inhibited carcinogenesis by growth inhibitory response, whereas highly metastatic colon carcinoma CT26 cells displayed BMP2-resistant proliferation and survival, following BMP2 stimulation. In addition, the sustained exposure to BMP2 allowed SW480 and DLD-1 cells to cause the resistance to BMP2-mediated growth suppressive response and induce cellular motility and invasion. To determine that the altered expression in the components of BMP2 signaling is responsible for different BMP2 sensitivity, we performed RT-PCR using BMP2, BMPR-IA, or BMPR-II primers. However, prolonged treatment of these cells with BMP2 showed no change in the levels of *BMP2*, *BMPR-IA* and *BMPR-II* mRNA compared with the acute treatment (data not shown). These finding indicate that alternative signaling pathways associated with BMP2 growth regulation may allow SW480 and DLD-1 cell survival in the presence of high concentrations of BMP2. In some cellular cancer models, for example, EMT and metastasis required cooperation of BMPs with the activated oncogenes such as RAS or activated receptor tyrosine kinases (8,16,27-29). SW480 and DLD-1 cells used in the present studies express mutated *K-RAS* oncogenes. The cooperation of BMPs with the activated K-RAS might allow these cells to cause BMP2-resistant response and induce EMT and invasiveness.

To become a more aggressive phenotype, it is necessary for epithelial cancer cells to acquire migratory and invasive capabilities, which then leads to degradation of the basement membranes and the extracellular matrix (ECM) at the primary tumor site and establishment of metastatic colonies at distant organs. Generally, increased motility and invasion are positively correlated with EMT. EMT is a complex process that leads to loss of epithelial morphology and gain of an invasive fibroblast-like mesenchymal phenotype (30-32). At the molecular level, there is altered expression of the epithelial markers, E-cadherin and Snail/Slug family and up-regulated expression of the proteins involved in cell migration and invasion (33,34). In this study, our results showed that BMP2-resistant SW480 and DLD-1 cells established by the prolonged BMP2 exposure caused a loss of E-cadherin-mediated cell-cell contact and gain of a mesenchymal phenotype, which led to the increased cellular motility and invasiveness. Additionally, we found that blockage of BMP2 signaling by BMP2 siRNA induce MET and rendered metastatic colon cancer cells unresponsive to BMP2-mediated migration and invasion, indicating that the BMP2 induces EMT and modulates motility and invasiveness of colon cancer cells and plays an important role in promoting late-stage tumor progression.

The mechanism whereby BMP2-resistance develops in colon cancer cells initially sensitive to BMP2 treatment remains poorly understood. Here, we reported a functional correlation between the PI3K/Akt pathway and BMP2 responsiveness. We found that SW480 and DLD-1 cells showing sensitivity to the anti-proliferative effect of BMP2 expressed a high level of phospho-Akt, whereas cells resistant to BMP2-mediated growth inhibition displayed BMP2-induced EMT and invasiveness and there was little or no detectable expression of phospho-Akt. Therefore, we tested the possibility that enhanced levels of phospho-Akt in CT26 cells partially block BMP2-mediated motility and invasion by suppressing EMT 3. As shown in Figs. 4 and 5, our data show that the enforced activation of Akt by transfection of CT26 cells with Akt^{Myr} suppressed the EMT response and invasiveness by BMP2. Moreover, inhibition of PI3K/Akt activity by treatment with LY294002 in SW480 cells induced EMT and exerted enhanced motility and invasiveness of these cells. Our data thus suggest that decreased activation of Akt results in BMP2-mediated EMT and invasion and that this is followed by resistance to a BMP2 growth-inhibitory response. However, the precise mechanism by which the BMP2 signaling controls the PI3K/Akt pathway to regulate BMP2-mediated metastatic potential requires further investigation.

The PI3K/Akt pathway is negatively regulated by PTEN, which is primarily a lipid phosphatase that hydrolyzes the 3-phosphate on phophatidylinositol 3,4,5-triphosphates (PIP_3) to generate phophatidylinositol 4,5-bis (35-37). PTEN is predominantly localized to the nucleus in primary and resting cells, whereas in many types of carcinoma there is a marked reduction of nuclear PTEN. In the cytoplasm, PTEN is thought to have roles of suppressing apoptosis and regulating cell growth (38-41). It has been reported that BMP pathway regulate PTEN protein levels by decreasing PTEN's

association with the degradative pathway (42). In the present study, we also showed that treatment of CT26 cells with BMP2 produced a significant increase in PTEN protein levels (Fig. 6A). Specifically, we found that BMP2-induced PTEN was predominantly localized to the cytoplasm (Fig. 6B). These data suggest that up-regulation of cytoplasmic PTEN mediated by BMP2 might contribute to reduction of the activity of Akt and thereby further promoting tumor progression. However, further investigation is necessary to elucidate the mechanisms involved.

Overall, our findings emphasize the potential role of the PI3K/Akt pathway in BMP2-induced cellular migration and invasion. These observations improve our understanding of the mechanism by which BMP2 signaling activation occurs as it relates to the metastatic behavior of colon cancer cells, which may prove useful in identifying therapeutic molecular targets to inhibit BMP2-dependent migration and invasion.

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