The BMP Signaling Pathway Leads to Enhanced Proliferation in Serous Ovarian Cancer—A Potential Therapeutic Target

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Members of the transforming growth factor-β (TGF-β) superfamily transduce signals via SMAD proteins. SMAD2 and SMAD3 mediate TGF-β signaling, whereas SMAD1, SMAD5, and SMAD8/9 transduce bone morphogenetic protein (BMP) signals. We would like to identify the function of BMP/SMAD5 signaling in serous ovarian cancer. The protein levels of total SMAD5 and phosphorylated SMAD5 (pSMAD5) were examined by immunohistochemical analysis using clinical serous ovarian cancer samples. Following treatment with either recombinant BMP2 (rBMP2) or Dorsomorphin (DM), western blotting was performed to observe pSMAD5 protein in the cytoplasm and the nucleus, separately. Cell proliferation was detected in SMAD5 knockdown serous ovarian cancer cell lines cultured with DM or rBMP2. The impact of DM or rBMP2 on tumor growth was observed in a mouse model of serous ovarian cancer. An inverse correlation was observed between pSMAD5 levels in the nucleus and the prognosis of patients with serous ovarian cancer. The treatment of SK-OV-3 with rBMP2 stimulated pSMAD5 translocation from the cytoplasm to the nucleus, and the addition of DM inhibited this effect. The proliferation of ovarian cancer cell lines was enhanced by BMP2 and suppressed by DM via SMAD5 in vitro. In vitro and in vivo experiments clearly demonstrated BMP2-stimulated proliferation of serous ovarian cancer and inhibition of this effect by DM. Our data suggests that BMP/SMAD5 signaling plays an important role and, therefore, becomes a potential therapeutic target in serous ovarian cancer.

Key words: BMP: bone morphogenetic protein; SMAD; serous ovarian cancer

INTRODUCTION

Ovarian cancer is the most lethal malignancy of gynecological cancers. Various factors that affect biological behavior of this tumor may serve as potential therapeutic targets. Bone morphogenetic proteins (BMPs) are extracellular signaling molecules that belong to the transforming growth factor beta (TGF-β) superfamily [1,2]. By regulating target gene transcription, BMPs control various cellular processes, such as proliferation, differentiation, apoptosis, and migration [3–5]. They are well-known to play critical roles in diverse developmental phases [6,7]. In addition, BMPs have increasingly become the focus of many cancer research fields.

More than 20 distinct BMPs constitute the BMP family and belong to the larger TGF-β ligand superfamily [8]. BMPs form heterotetrameric complexes with type I and II BMP receptors (BMPRs), which triggers phosphorylation of BMP-responsive receptor-regulated SMADs (R-SMADs, namely, SMAD1, SMAD5, and SMAD8/9). R-SMADs form a complex with common-partner SMADs (Co-SMADs: SMAD4) and translocate into the nucleus, where the complex regulates transcription of its target genes [9].

Previous in vivo and in vitro studies showed that BMP2/4/7 was related to the invasion and metastasis of prostate cancer, melanoma, gastric cancer, chondrosarcoma, and bladder cancer [10–15]. Compared to normal lung tissues, BMP2 is highly expressed in 98% of lung cancer [16]. The importance of BMP signaling has also been reported in ovarian cancer, although its precise mechanisms in the carcinogenesis or progression in this tumor are still unclear. This is partly because the large number of combinations of

Abbreviations: BMP, bone morphogenetic protein; BMPRs, BMP receptors; DM, dorsomorphin; IOSE, immortalized ovarian surface epithelial cell line; pSMAD5, phosphorylated SMAD5; rBMP2, recombinant BMP2; SAM, significant analysis of microarray; TCGA, The Cancer Genome Atlas; TGF-β, transforming growth factor.

The authors declare no conflict of interest.

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Received 14 August 2014; Revised 21 November 2014; Accepted 18 December 2014

DOI 10.1002/mc.22283

Published online in Wiley Online Library (wileyonlinelibrary.com).

BMP ligands and receptors makes it difficult to define exactly which of them is the most important and could be a therapeutic target. With this in mind, we focused on SMAD5, the common intracellular signaling effector of BMP family, and analyzed if expression of this molecule was associated with the clinical outcome of the patient as well as the behavior of ovarian cancer cells. In addition, we attempted to address the possible roles of BMP signaling by inhibiting a wide-range of downstream pathways using a small molecule inhibitor of type I BMPRs, *dorsomorphin* [17,18]. The potential utility of this molecule as a molecular inhibitor of BMP signaling in treatment of ovarian cancer was also evaluated.

MATERIALS AND METHODS

Cell Lines

Human ovarian cancer cell lines (SK-OV-3, OVCA420, ovary1847, NIH: OVCAR3, Caov3, Hey and OV90) and IOSE (immortalized ovarian surface epithelial cell line) were kindly provided by Dr. Susan K. Murphy in Duke University and cultured as previously described [19].

Immunohistochemical Staining

Ovarian cancer specimens were taken from women who received primary tumor resection at the Department of Gynecology and Obstetrics of Kyoto University Hospital from 2002 to 2008. We examined serous ovarian cancer tissue samples ($n = 36$) including Stage I ($n = 3$), Stage II ($n = 1$), Stage III ($n = 26$), and Stage IV ($n = 6$). Immunohistochemical staining was carried out using the streptavidin-biotin-peroxidase method. The slices were incubated with anti-phospho-SMAD5 (ab76296, Abcam, Cambridge, UK) antibody or anti-SMAD5 antibody (ab55484, Abcam, Cambridge, UK). Two gynecological pathologists examined the immunohistochemistry slices independently without any prior information about the clinical history of these patients. Staining scores were calculated based on the staining degree (−, 0; +, 1; ++, 2; +++, 3) and proportion (<10%, 1; 10%–25%, 2; 25%–50%, 3; >50%, 4). Staining Scores were calculated as proportion × (degree −) + proportion × (degree +) + proportion × (degree ++) + proportion × (degree +++). Cases scored more than nine in nucleus were classified as pSMAD5 high expression group.

qRT-PCR

Total RNA was extracted and quantified as previously described [20]. Primers used for human samples are the following:

SMAD1, forward: 5′-CGGTTGAACAACTGTTCCTTTA-3′, reverse: 5′-AAAAAGTAACCCAGTCAGCACAA-3′;

SMAD5, forward: 5′-AGGCGACATATTGGAAAAGG-3′, reverse: 5′-TGAGGCATTCCGCATACAC-3′;

SMAD9, forward: 5′-TGCCACAGCTGATAGACATGTAG-3′, reverse: 5′-CATAGTAGGCGACCGAGCAC-3′;

BMP2, forward: 5′-CGGACTGCGGTCTCCTAA-3′, reverse: 5′-GGAAGCAGCAACGCTAGAAG-3′;

BMPR1A, forward: 5′-CAAAGCTATTTGGAGAAAATCA-3′, reverse: 5′-CGASSGCACTTGACAATGAC-3′;

BMPR1B, forward: 5′-TTTCATGCCTTGTTGATAAAGG-3′, reverse: 5′-GCTTGTTTAACTTTTTGTTTCC-3′;

BMPR2, forward: 5′-TCTTTCAGCCACAAATGTCCT-3′, reverse: 5′-TGCCATCTTGTGTTGACTCAC-3′.

Western Blotting Analysis

Recombinant BMP2 (rBMP2) (100 ng/mL) (Sigma, St. Louis, MO), DM (5 μM) (Calbiochem, San Diego, CA) or PBS were added to SK-OV-3, OVACA420, ovary1847, OV90, and IOSE for 2 h. Cells were harvested and lysed in RIPA buffer with a protease inhibitor cocktail (EMD, Madison, WI) and a phosphatase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). A nuclear extract kit (Active Motif, Japan) was used to separate nuclear and cytoplasmic protein fractions. Protein was separated by SDS-PAGE gel and immunoblotted with anti-phospho-SMAD5 antibody (ab76296, Abcam, Cambridge, UK), anti-SMAD5 antibody (ab55484, Abcam, Cambridge, UK), anti-β-actin antibody (ab8227, Abcam, Cambridge, UK), or an anti-TFIID antibody (sc-421, Santa Cruz Biotechnology Inc., Santa Cruz, CA). The anti- β-actin and the anti-TFIID antibodies were used as the endogenous controls for total, cytoplasmic and nuclear protein, respectively. Specific proteins were visualized using ECL Plus Western Blotting Reagent (GE Healthcare Life Sciences).

Immunofluorescence

2×10^4 SK-OV-3 cells or IOSE cells were treated with PBS (control), DM (5 μM), rBMP2 (100 ng/mL) or DM (5μM) plus rBMP2 (100 ng/mL) for 2 h and then fixed and incubated with rabbit anti-phospho-SMAD5 antibody (ab76296, Abcam, Cambridge, UK) and mouse anti-Ki67 antibody (Dako, Denmark), followed by F(ab) goat anti-rabbit IgG (H + L) antibody (Alexa Fluor 555) (Invitrogen, Carlsbad, CA) (Invitrogen) and FITC-conjugated rabbit anti-mouse immunoglobulins antibody (Dako, Denmark). Prolong gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) was added for counterstaining of the nuclei. Fluorescent microscopic images were acquired using a fluorescence microscope.

Flow Cytometric Analysis of Cell Cycle

APC BrdU Flow Kit (BD Bioscience) was used to analyze the cell cycle distribution, according to manufacturer's instructions.

RNA Interference

Synthetic small interference RNA (siRNA) targeting human SMAD5 were obtained from Invitrogen

(Validated StealthTM DuoPak). The Stealth RNAi negative control (Invitrogen, Carlsbad, CA) was used as a negative control. Transfections were performed using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) with OPTI-MEM I reduced serum medium (Gibco, Life Technologyies, Grand Island, NY), according to the manufacturer's protocol.

Cell Proliferation Assay

SK-OV-3, OVCA420, and IOSE cells transfected with SMAD5 siRNA or control siRNA were seeded into a 96-well plate (Asahi Glass, Japan), followed by addition of with PBS (control), rBMP2 (100 ng/mL), DM (5 μM), or DM (5 μM) plus rBMP2 (100 ng/mL) for 24 or 48 h (n = each 18). The number of viable cells in each well was examined using the WST-1 assay (Takara Bio, Japan) following manufacturer's instructions.

In Vivo Experiments

1×10^6 SK-OV-3 cells were injected subcutaneously into contralateral axillae of CD-1 foxn/nu nude mice purchased from Japan. Mice were treated with PBS, DM (200 μg per one mouse) or rBMP2 (100 ng per one mouse) intraperitoneally every 72 h for five consecutive doses (each group $n = 10$). Mice were weighed and tumors were measured every 7 d until sacrifice.

A portion of each tumor was fixed in 10% formalin at room temperature and paraffin embedded using anti- phospho-SMAD5 antibody (ab76296, Abcam, Cambridge, UK), anti-Ki67 antibody (ab16667, Abcam, Cambridge, UK), or Cleaved Caspase-3 (Asp175) antibody (#9661, Cell Signaling Technology, Danvers, MA). For staining quantification, five sections from five tumors per treatment group were analyzed and measured from four low-power fields (×200) per section.

Microarray Analysis

Expression microarray data of serous ovarian cancers (GSE3149) was obtained from the Gene Expression Omnibus web site (http://www.ncbi.nlm.nih.gov/geo), which contains 146 tissue samples from advanced (stage III–IV) serous ovarian cancers. Gene expression microarray (GSE60135) was generated from SK-OV-3 and SK-OV-3 plus BMP2 using Affymetrix U133 Plus 2.0 gene chips (Affymetrix, Santa Clara, CA). Data generated by Affymetrix MAS 5 Gene Chip software was normalized by transformation with Robust Multichip Analysis. BMP2-stimulated gene signature that included 25 probes (23 gene symbols) was identified by SAM (http://statweb.stanford.edu/~tibs/SAM/) using R (http://www.r-project.org/) software. BMP2-stimulated gene signature predictive of overall survival was validated by single sample GSEA on an independent data set, data set 559 from The Cancer Genome Atlas (TCGA), which is composed of high-grade serous ovarian cancer samples.

Statistical Analysis

Univariate analysis for overall survival was performed and evaluated with the log-rank test, and Kaplan–Meier curves were generated from data set GSE3149. Pearson's correlation coefficient was used to analyze the linear relationship between each gene of BMPRs/SMADs signaling expression and MKI67. Univariate Cox proportional-hazard model was used to evaluate the pSMAD5 expression as a prognostic factor. Comparisons of pSMAD5 expression in the nucleus were carried out by using Student's t-test or ANOVA. Spearman's coefficient rank test was used to evaluate the correlation between Ki67 expression, pSMAD5 and Cleaved Caspase-3. All P-values < 0.05 were considered statistically significant.

RESULTS

Identification of BMP-Activated Signaling Pathway in Serous Ovarian Cancer Patients With Poor Prognosis by Gene Expression Profiling Analysis

We evaluated signature probabilities for each gene (BMPs, BMPRs, and SMADs) of the BMP signaling pathway using GSE3149 ovarian cancer data set. Among them, up-regulation of SMAD5, the direct downstream signal of the BMP ligand, but not other SMADs, BMPRs, or BMPs (data not shown), was associated with poor prognosis in serous ovarian cancer patients (Figure 1A, $P = 0.003$). Furthermore, SMAD5 was positively correlated with proliferation marker MKI67 (Figure 1B, coefficient of determination $R^2 = 0.557$, $P < 0.001$). However, SMAD1 or SMAD9 was not correlated with MKI67 (Figure 1C and D). We also investigated the correlation between levels of BMP receptors and a proliferation marker MKI67. MKI67 was positively correlated with BMPR1A, BMPR1B, and BMPR2 (Figure 1E–G; $R^2 = 0.735$, 0.410, 0.824, respectively, $P < 0.001$).

We next investigated the gene expression of BMPs/BMPRs/SMADs in multiple serous ovarian cell lines. They expressed BMP2 mRNA, while IOSE did not show BMP2 expression (Supplementary Figure 1A). BMPRs show diversity in different serous ovarian cancer cell lines, BMPR1A (five out of seven cell lines), BMPR1B (five out of seven cell lines), and BMPR2 (three out of seven cell lines) highly expressed in some ovarian cancer cells compared to IOSE (Supplementary Figure 1B–D). SMAD5 mRNA was significantly increased in most of serous ovarian cancer cell lines (six out of seven cell lines) compared to IOSE (Supplementary Figure 1E). However, SMAD1 or SMAD9 was not significantly increased in serous

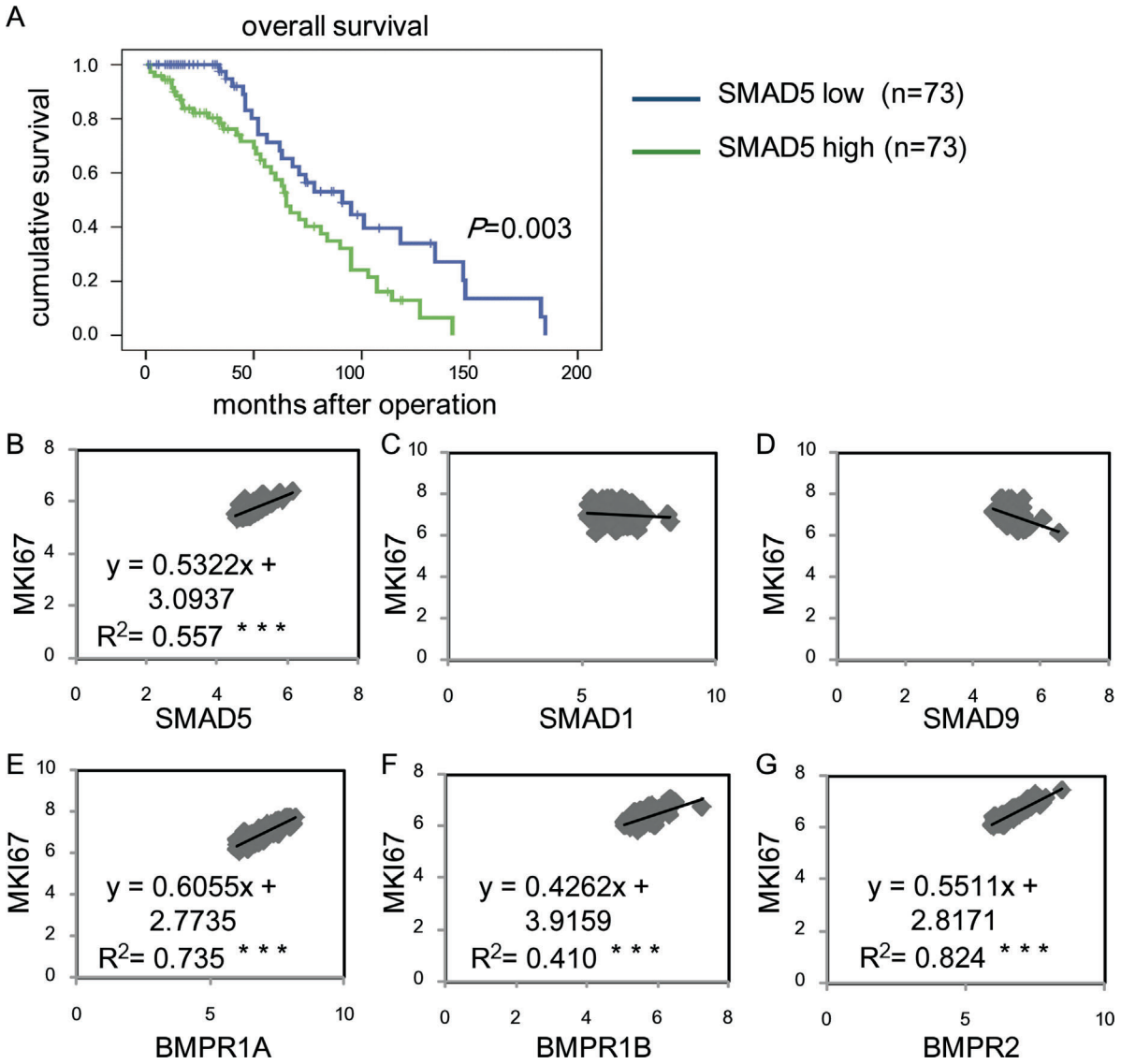

Figure 1. Activated BMP signaling pathway is in poor prognosis serous ovarian cancer patients from publically data set GSE3149. (A) Overall survival analysis of 146 patients with advanced (stage III–IV) serous ovarian cancer was according to high or low expression of SMAD5 mRNA in clinical microarray data set GSE3149. (B–H) Linear regression was demonstrating the correlation between proliferation marker MKI67 mRNA and SMAD5 mRNA (B), SMAD1 mRNA (C), SMAD9 mRNA (D), BMPR1A mRNA (E), BMPR1B mRNA (F), or BMPR2 mRNA (G) in clinical microarray data set GSE3149. ***, $P < 0.001$.

ovarian cancer lines compared to IOSE (Supplementary Figure 1F and G).

High pSMAD5 Level in the Nucleus is Associated With Poor Prognosis in Serous Ovarian Cancer Patients

Immunohistochemoical expression of total SMAD5 staining was not associated with prognosis in 36 institutional serous ovarian cancer patients (data not shown). By contrast, the survival rate of patients with high nucleus pSMAD5 expression score was significantly poorer than those with low expression score (Figure 2A and B; Supplementary Table 1). The overall survival period (mean $\pm$ SD) of patients with low and high nucleus pSMAD5 was 78.72 ± 24.27 and 48.22 ± 29.64 months, respectively. Patients died of disease (DOD) or alive with disease (AWD) had significantly higher nucleus pSMAD5 staining scores than those with no evidence of disease (NED) (Figure 2C). These data clearly demonstrate a strong inverse correlation between nucleus pSMAD5 expression and the prognosis of the patients with serous ovarian cancer. No statistically significant correlation was observed between nucleus pSMAD5 expression and various clinicopathological factors, such as primary tumor stage, lymph node metastasis, distant metastasis ($P > 0.05$).

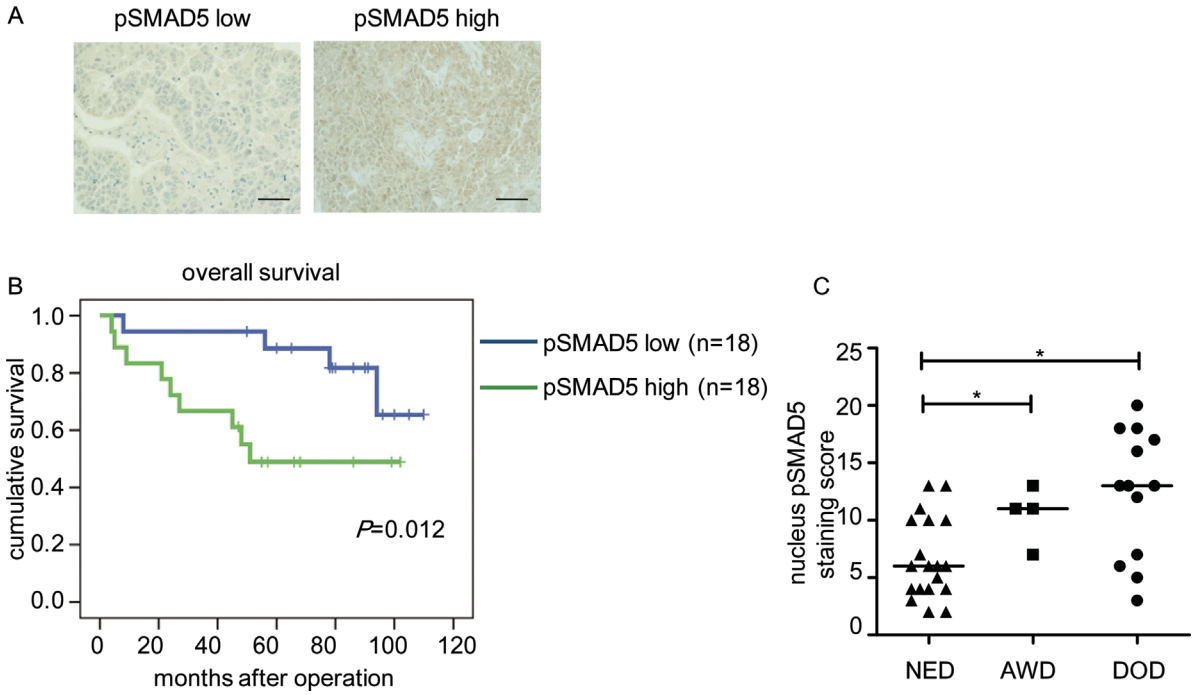

Figure 2. High phospho-SMAD5 expression in nucleus is associated with poor prognosis in serous ovarian cancer patients. (A) Low or high nucleus phospho-SMAD5 (pSMAD5) protein staining score image (original magnification ×400; scar bar, 50 μm) in tissue samples from serous ovarian cancer patients. (B) Kaplan–Meier analysis of overall survival was performed for 36 patients with serous ovarian cancer according to high and low pSMAD5 protein staining score in nucleus. (C) Nucleus pSMAD5 protein staining score in patients died of disease (DOD), alive with disease (AWD) and no evidence with disease (NED). *, $P < 0.05$.

Identification of the Effects of Recombinant BMP2 (rBMP2) and Dorsomorphin (DM) on pSMAD5 Translocation From the Cytoplasm to the Nucleus in Serous Ovarian Cancer Cell Lines

We subsequently investigated the role of the BMP signaling pathway in human serous ovarian cancer cell lines by analyzing the effects of rBMP2 protein and BMP signaling inhibitor, and a small-molecule compound, Dorsomorphin (DM), which was reported to selectively decrease the level of SMAD1/5 phosphorylation by inhibiting kinase activity of BMP type I receptor [21]. After serous cancer cell line (SK-OV-3, ovary1847, OV90, and OVCA420) and IOSE were incubated with PBS, DM, rBMP2, or rBMP2 plus DM, SMAD5 mRNA expression was not altered (Figure 3A). Then, we found that the level of pSMAD5 from whole cell extract was increased by the treatment with rBMP2 and was significantly inhibited by DM in serous ovarian cancer cell lines and IOSE(Figure 3B–D). In contrast, treatment with rBMP2 or DM did not alter total SMAD5 protein expression in serous ovarian cancer cell line and IOSE (Figure 3B and C; Supplementary Figure 2A).

Subsequently, we observed the amount of SMAD5 and pSMAD5 protein in nucleus and cytoplasm separately and identified that cytoplasm or nucleus SMAD5 was not affected by rBMP2 or DM in OVCA420, SK-OV-3, and IOSE (Figure 4; Supplementary Figure 2B and C). Treatment with DM significantly decreased both in cytoplasm and nucleus pSMAD5 expression in OVCA420, SK-OV-3, and IOSE (Figure 4). Although treatment with rBMP significantly increased cytoplasm pSMAD5 expression both in IOSE and serous cancer cell lines (OVCA420, SK-OV-3), nucleus pSMAD5 was significantly increased in serous cancer cell lines (OVCA420, SK-OV-3) (Figure 4C–F) but not in IOSE (Figure 4A and B) when treated with rBMP2. These data demonstrate that pSMAD5 in serous cancer cell lines is prone to translocation from the cytoplasm to the nucleus following BMP2 treatment.

The treatment of SK-OV-3 with rBMP2 stimulated pSMAD5 translocation and these effects of rBMP2 were inhibited by DM (Figure 5). Nucleus pSMAD5 positive cells percentage was not altered by rBMP2 or DM in IOSE (Supplementary Figure 3). Nucleus pSMAD5 staining in SK-OV-3 and IOSE cells obviously showed co-localization with Ki67 staining (Figure 5; Supplementary Figure 3).

The Effects of rBMP2 or DM on Cell Proliferation of Serous Ovarian Cancer Cell Lines are Dependent on SMAD5

When SK-OV-3 cells were incubated with rBMP2, the proportion of S-phase cells significantly increased from 10.5% to 18%; the concurrent addition of DM resulted in a 4-fold decrease of S-phase cell proportion from 18% to 3.87% (Figure 6A; Supplementary Figure 4 A).

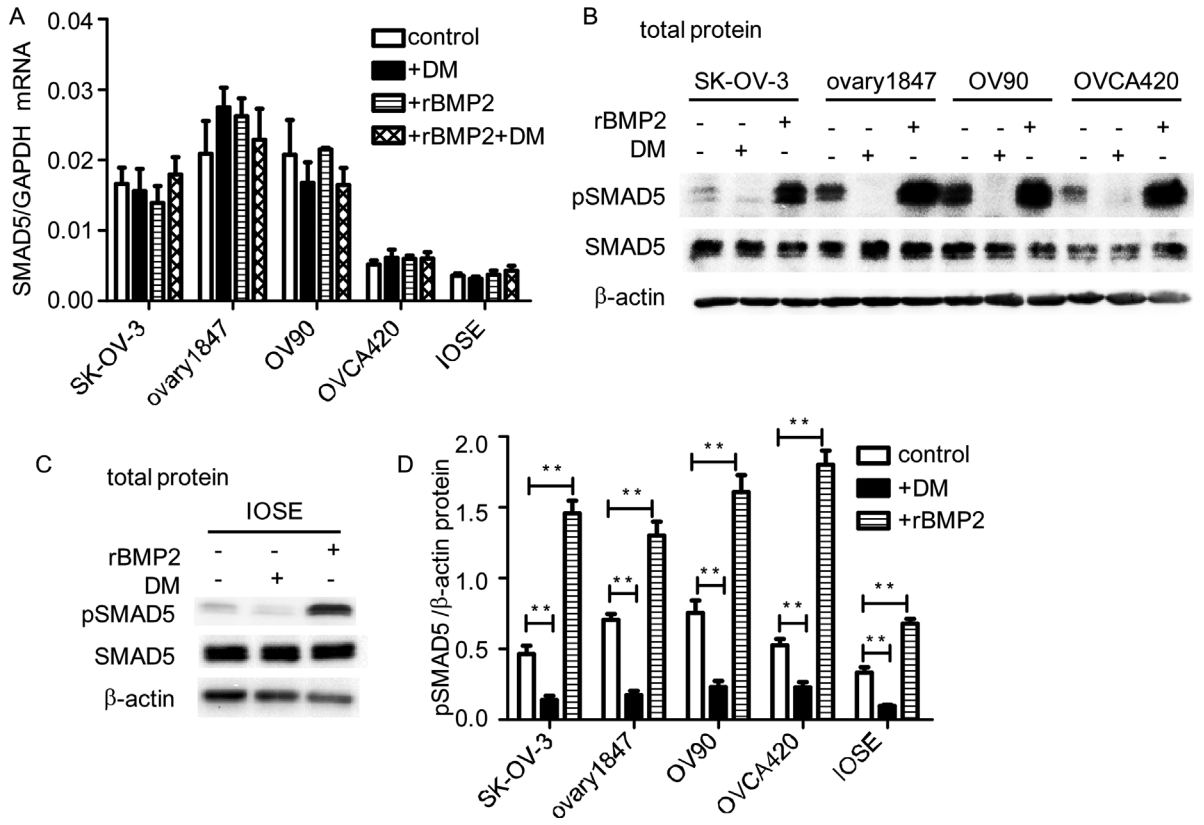

Figure 3. The effects of recombinant BMP2 (rBMP2) or Dorsomorphin (DM) are on SMAD5 or pSMAD5 expression in serous ovarian cancer cells and IOSE. (A) Serous cancer cells (SK-OV-3, ovary1847, OV90, and OVCA420) were incubated with PBS, DM (5 μM), rBMP2 (100 ng/mL), or rBMP2 (100 ng/mL) plus DM (5 μM) for 2 h. qRT-PCR was demonstrating SMAD5 mRNA expression based on three independent experiments. (B–C) Western blotting of whole cell extracted protein for pSMAD5 and SMAD5 of serous cancer cells (SK-OV-3, ovary1847, OV90, and OVCA420) (B), and IOSE (C), cultured with PBS, DM (5 μM) or rBMP2 (100 ng/mL) for 2 h. (D) Densitometric analysis of pSMAD5 in serous cancer cells (SK-OV-3, ovary1847, OV90, and OVCA420) (B) and IOSE (C), cultured with PBS, DM (5 μM) or rBMP2 (100 ng/mL) for 2 h. Statistic analysis was based on three independent experiments. **, $P < 0.01$.

Neither rBMP2 nor DM altered proliferation of IOSE (Figure 6A; Supplementary Figure 4B). This result suggests that BMP signaling promote proliferation of serous ovarian cancer, but do not promote proliferation of normal ovarian surface epithelial cells.

Then, we generated SMAD5 knockdown cell lines (SK-OV-3, OVCA420, and IOSE) (Supplementary Figure 5). Proliferation of IOSE or SMAD5-knockdown IOSE was not altered by rBMP2 or DM (Figure 6B). Proliferation of serous cancer cells (SK-OV-3 and OVCA420) was enhanced by rBMP2 and inhibited by DM (Figure 6C and D). In contrast, rBMP2 or DM did not alter proliferation of SMAD5-knockdown serous cancer cells (Figure 6C and D). These data show that the effect of rBMP2 or DM on cell proliferation of serous ovarian cancer cell lines depends on SMAD5.

The Impact of DM or rBMP2 is on Tumor Growth in Serous Ovarian Cancer Mice Model In Vivo

We investigated the in vivo effects of BMP2 and DM on subcutaneously inoculated SK-OV-3 xenografts in immune-comprised mice. Recombinant BMP2 significantly promoted tumor growth starting from 4 wk after tumor innoculation and DM significantly inhibited tumor growth at 5 wk compared to control (Figure 7A and B). However, the weight of treated mice was not affected by either DM or rBMP2 (Data not shown). Interestingly, after tumor inoculation 4 wk, the percentage of DM-treated mice tumors with necrosis was significantly higher than other groups, although DM-treated mice tumors size was smallest (Figure 7C). Moreover, Cleaved Caspase-3 staining in DM-treated mice tumors tissue was higher than other groups. (Figure 7D and H; Supplementary Table 2). These data suggest that DM induce both apoptosis and necrosis to ovarian cancer xenografts.

Nucleus pSMAD5 expression score in tumors from rBMP2-treated mice was highest and lowest in DM-treated group (Figure 7D and E; Supplementary Table 2). Lowest Ki67 staining in tumors from DM-treated mice and highest in rBMP2-treated mice was observed (Figure 7D and F; Supplementary Table 2). Positive correlation was identified between nucleus pSMAD5 and Ki67 expression in mice tumors (Figure 7G; $R^2 = 0.657$, $P < 0.01$).

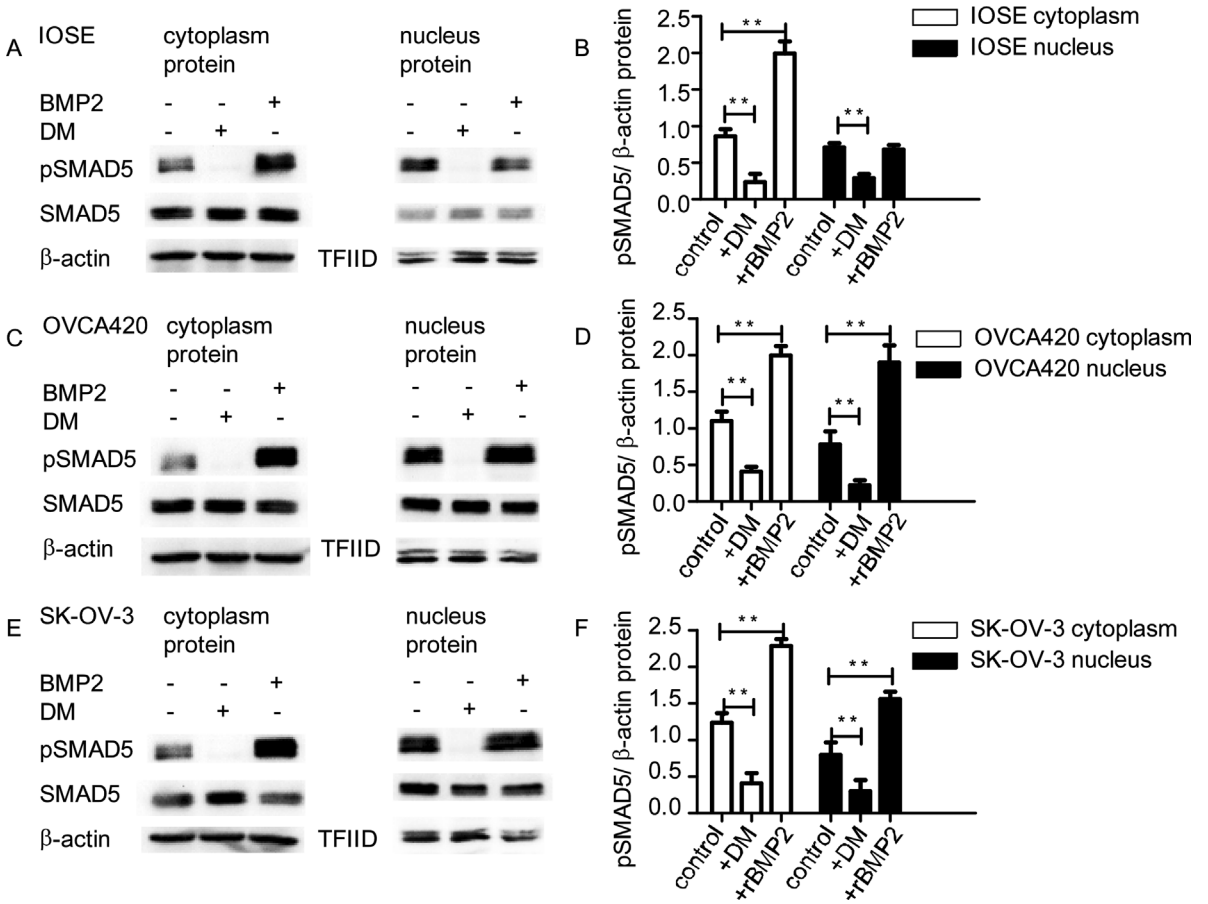

Figure 4. pSMAD5 in serous ovarian cancer cells are prone to translocation into nucleus treated with BMP2 . (A) Western blotting for cytoplasm or nucleus pSMAD5 and SMAD5 in IOSE, cultured with PBS, DM (5 μM), or rBMP2 (100 ng/mL) for 2 h. (B) Densitometric analysis of cytoplasm and nucleus pSMAD5 protein in IOSE based on three independent experiments. **, $P < 0.01$. (C) Western blotting for cytoplasm or nucleus pSMAD5 and SMAD5 in OVCA420 treated with PBS, DM (5 μM) or rBMP2 (100 ng/mL) for 2 h. (D) Densitometric analysis of cytoplasm and nucleus pSMAD5 protein in OVCA420 based on three independent experiments. **, $P < 0.01$. (E) Western blotting for cytoplasm or nucleus pSMAD5 and SMAD5 in SK-OV-3 treated with PBS, DM (5 μM), or rBMP2 (100 ng/mL) for 2 h. (F) Densitometric analysis of cytoplasm and nucleus pSMAD5 protein in SK-OV-3 based on three independent experiments. **, $P < 0.01$.

The Utility of BMP2-Stimulated Gene Signature as a Prognostic Gene Signature is Validated by TCGA Dataset

BMP2-stimulated gene signature was identified by comparing SK-OV-3 plus rBMP2 to control SK-OV-3 in GSE60135 (Supplementary Figure 6A). BMP2-stimulated gene signature was correlated with poor survival ($P = 0.033$; risk ratio 2.388) in TCGA, which is composed of 559 high-grade serous ovarian cancer samples. Each of the samples in the dataset was assigned a prognostic gene score, reflecting the similarity between its expression profile and the prognostic gene signature. Low prognostic gene score assigned using BMP2-stimulated gene signature was significantly correlated with longer patient survival (Supplementary Figure 6B).

DISCUSSION

In this study, we tried to explore the role of the BMP-SMAD pathway in the progression of ovarian cancer. In an analysis of publicly microarray data set GSE3149 of serous ovarian carcinoma, we found a significant correlation between SMAD5 mRNA expression and the prognosis of the patients. Additionally, SMAD5 expression as well as expression of BMP receptors were significantly correlated with mRNA level of Ki67, suggesting that BMP-SMAD signaling is associated with in vivo cancer proliferation. Moreover, we investigated if SMAD5 protein expression is associated with patient prognosis in 36 institutional cases. Not the levels of total SMAD5 protein, but nucleus expression of pSMAD5 was inversely correlated with outcome of the patients suggesting that active SMAD5 signal are associated with poorer prognosis. These clinical data suggest that SMAD signaling plays an important role in the biological behavior of ovarian cancer. In pancreatic ductal adenocarcinomas, SMAD protein expression in epithelial tumor cells was related to a shorter postsurgical overall survival [22]. In addition, Le Page C et al.

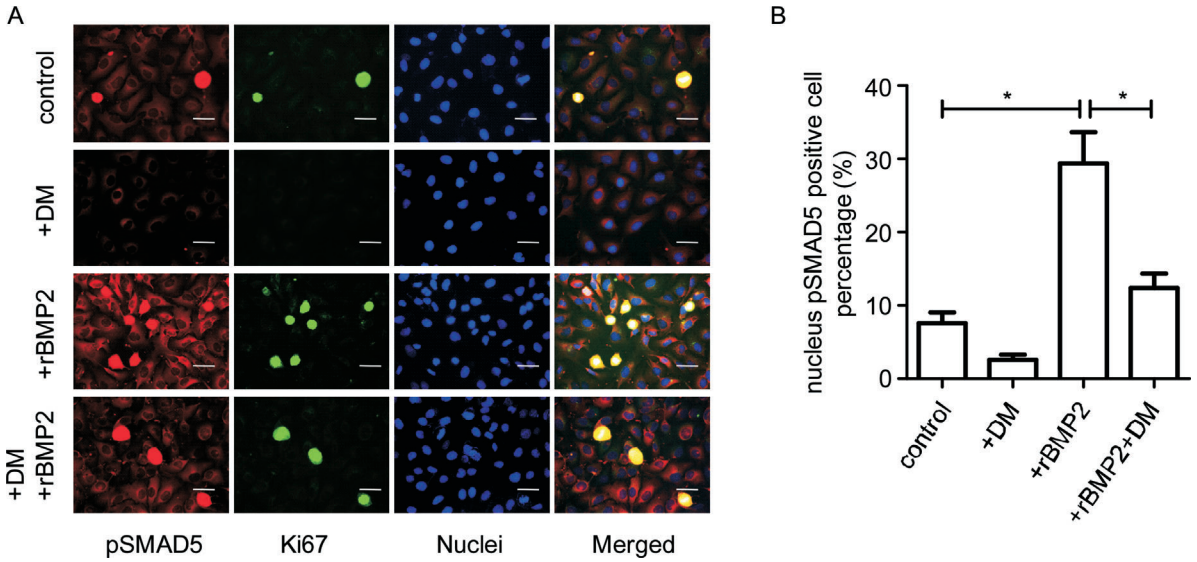

Figure 5. BMP2 stimulate pSMAD5 translocation into nucleus in SK-OV-3 and the effects of BMP2 are inhibited by DM. (A) SK-OV-3 cells were treated with PBS (control), DM (5 μM), rBMP2 (100 ng/mL). or DM (5 μM) plus rBMP2 (100 ng/mL) for 2 h. Fluorescent images were showing Alexa Fluor 555-labeled phospho-SMAD5, FITC-labeled Ki67 staining and DAPI-labeled nuclei. (Original magnification, 400×; scar bar, 50 μm) (B) Statistic analysis of nucleus pSMAD5 positive cell percentage in (A) based on three independent experiments. *, $P < 0.05$.

reported that BMP2 expression in ovarian cancer tissue was inversely correlated with patient survival [23], suggesting an important role of BMP-SMAD signaling.

We then examined whether BMP-SMAD signaling is active in serous ovarian cancer cell lines and IOSE. SMAD5 was overexpressed in most ovarian cancer cells compared to IOSE. Some cancer cells also highly expressed BMP2 mRNA, while IOSE did not show BMP2 expression. Moreover, receptors for BMP2 were highly expressed in some ovarian cancer cells. These data suggest that BMP-SMAD signaling is activated in ovarian cancer cells, partly due to expression of BMP2 in an autocrine fashion and partly as a result of increased expression of BMP receptors. The activation of the autocrine BMP loop has been reported in other malignancies, such as mouse breast cancer cells [24]. In intestinal adenoma, autocrine differentiating BMP signals serve to limit adenoma growth [25]. Additionally, autocrine or paracrine BMP7 loops regulate progression of gastric cancer [26]. Thus, the effect of the BMP autocrine loop likely differs depending on the cancer cell type and the BMP subtype.

Next, we tested the biological effect of BMP2 on serous ovarian cancer cells and IOSE. However, BMP2 treatment shifted the cell cycle towards the S phase and stimulated cellular proliferation, while DM, the inhibitor of BMP2 signaling, completely abrogated BMP2 induced proliferation in ovarian cancer cells. In contrast, the treatment of IOSE cells with BMP2 or DM did not alter cell proliferation, providing evidence that IOSE and ovarian cancer cells possess distinct capabilities to respond to BMP2 in accordance with the diversity of their BMP ligand and receptor expression. A previous report has suggested that BMP signaling promotes growth of primary human colon carcinomas [27]. Similarly, BMP4 promotes prostate tumor growth in bone through osteogenesis [28]. However, one report showed that BMP2 and BMP4 play tumor suppressor roles in human diffuse-type gastric carcinoma [29]. In ovarian cancer, BMP4 signaling induced epithelial-mesenchymal transition in vitro in primary human ovarian cancer cells [30,31]. There is no previous report comparing the effect of BMP on cancer tissue and its normal counterpart. Our data suggest that ovarian cancer acquires a growth advantage by augmenting its responsiveness to BMP over the course of carcinogenesis. In addition to in vitro analysis, we generated a BMP2 stimulated gene signature, which was able to serve as a prognostic factor in a large clinical database of ovarian cancer TCGA. Given that nucleus pSMAD5 protein was inversely correlated with prognosis in serous ovarian cancer, signaling downstream of BMP2 (including pSMAD5) is clinically important in terms of the behavior of clinical ovarian cancer.

To further elucidate the mechanism of BMP2-induced proliferation in ovarian cancer cells, we evaluated the changes in intracellular BMP2 signaling by assessing the amount and localization of pSMAD5 protein. Nuclear pSMAD5 was significantly increased in ovarian cancer cells, but not in IOSE, suggesting that BMP2 signaling is mainly mediated by pSMAD5 translocation into the nucleus in ovarian cancer cells. Next, we confirmed that treatment of ovarian cancer cells with BMP2 caused drastic translocation of pSMAD5 protein into the nucleus, and DM partially inhibited the observed effect. In ovarian cancer,

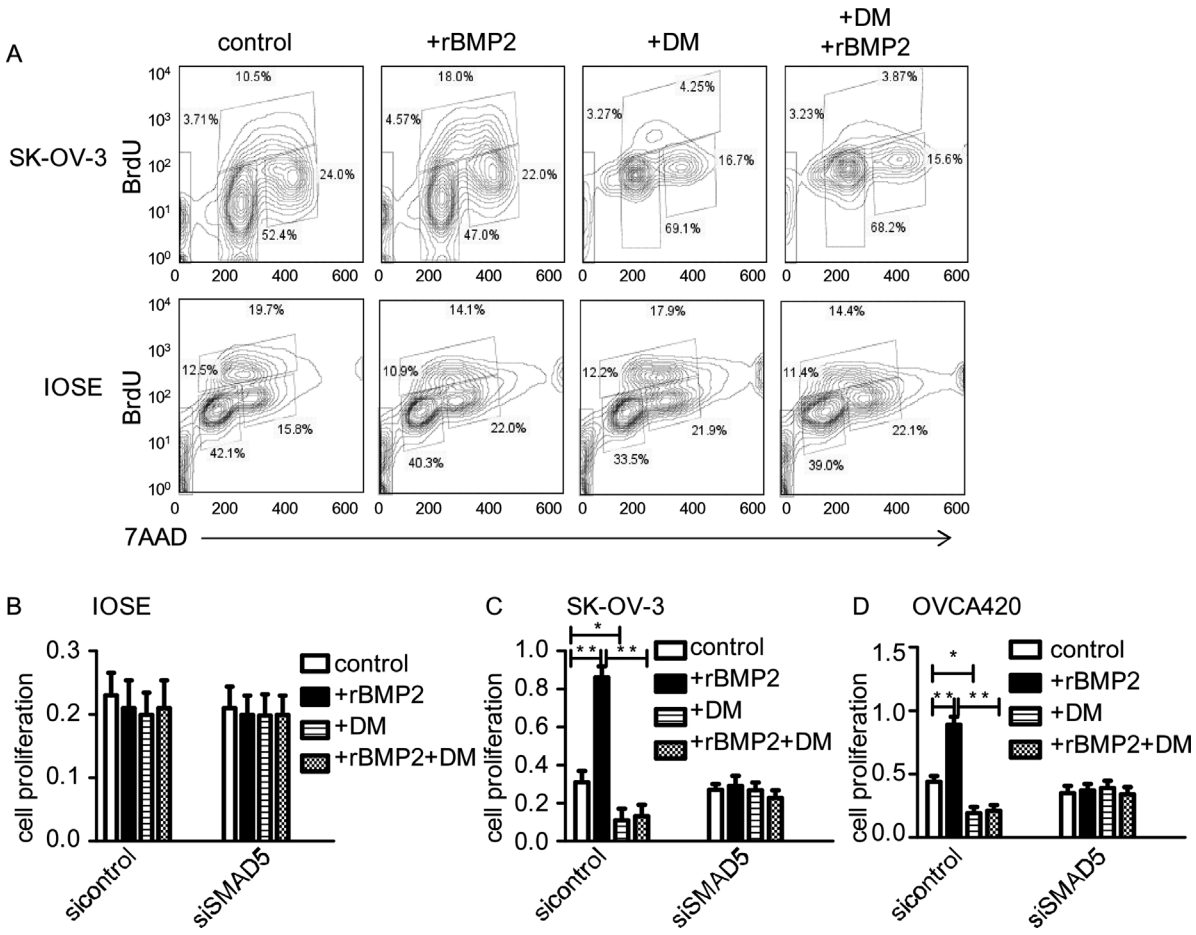

Figure 6. The effects of rBMP2 or DM on cell proliferation are via SMAD5 in serous ovarian cancer cells in vitro. (A) Analysis of cell cycle by flow cytometry for SK-OV-3 and IOSE cells, cultured with PBS, rBMP2 (100 ng/mL), DM (5 μM), or DM (5 μM) plus rBMP2 (100 ng/mL) for 48 h, then incubated with BrdU for 30 min. Subsequently, cells were fixed permeabilized, and stained with anti-BrdU and 7AAD. (B) IOSE transfected with control siRNA or SMAD5 siRNA, then cultured with PBS, rBMP2 (100 ng/mL), DM (5 μM), or DM (5 μM) plus rBMP2 (100 ng/mL) for 24 h. The numbers of viable cells were examined to analyze proliferation of cells using the WST-1 assay. (C) SK-OV-3 transfected with control siRNA or SMAD5 siRNA, then cultured with PBS, rBMP2 (100 ng/mL), DM (5 μM), or DM (5 μM) plus rBMP2 (100 ng/mL) for 24 h. The numbers of viable cells were examined to analyze proliferation of cells using the WST-1 assay. *, $P < 0.05$; **, $P < 0.01$. (D) OVCA420 transfected with control siRNA or SMAD5 siRNA, then cultured with PBS, rBMP2 (100 ng/mL), DM (5 μM), or DM (5 μM) plus rBMP2 (100 ng/mL) for 48 h. The numbers of viable cells were examined to analyze proliferation of cells using the WST-1 assay. *, $P < 0.05$; **, $P < 0.01$.

pSMAD2/3 also shows translocation following stimulation with TGF-β [32]. Since co-localization was observed in nucleus pSMAD5 protein and Ki67 staining both in ovarian cancer cells and IOSE, our studies suggest that BMP2 signaling mediated by pSMAD5 translocation may contribute to cell proliferation of ovarian cancer. BMP2 causes translocation of pSMAD5 only in ovarian cancers but not in IOSE, suggesting cancer cells appear more sensitive to BMP2 stimulation, but underlying mechanism is to be elucidated.

Indeed knockdown of SMAD5 impaired BMP2 induced proliferation and DM suppressed proliferation in serous ovarian cancer cell lines. Taken together our data indicates that BMP2 promote cell proliferation of ovarian cancer via SMAD5 signaling pathway.

Then, we examined if inhibition of BMP2 signaling by DM may have tumor-inhibitory effects. In mouse subcutaneous xenograft, the administration of DM significantly inhibited tumor growth and increased cell apoptosis and necrosis, indicating its possible utility as a targeted cancer therapy against BMP2. There are several reported ways of inhibiting BMP signaling, including direct extracellular inhibition through cysteine-knot containing proteins from the DAN-, the twisted gastrulation-, the chordin- and the noggin-family [33]. Inhibition of BMP receptors by DM or by LDN-193189 may be superior because these small molecules can block all known BMP-induced signaling cascades [34]. DM represents a potentially new therapeutic agent in treating and preventing occlusive vascular disease [34]. With its other tumor-inhibitory mechanisms [35,36], DM could be a

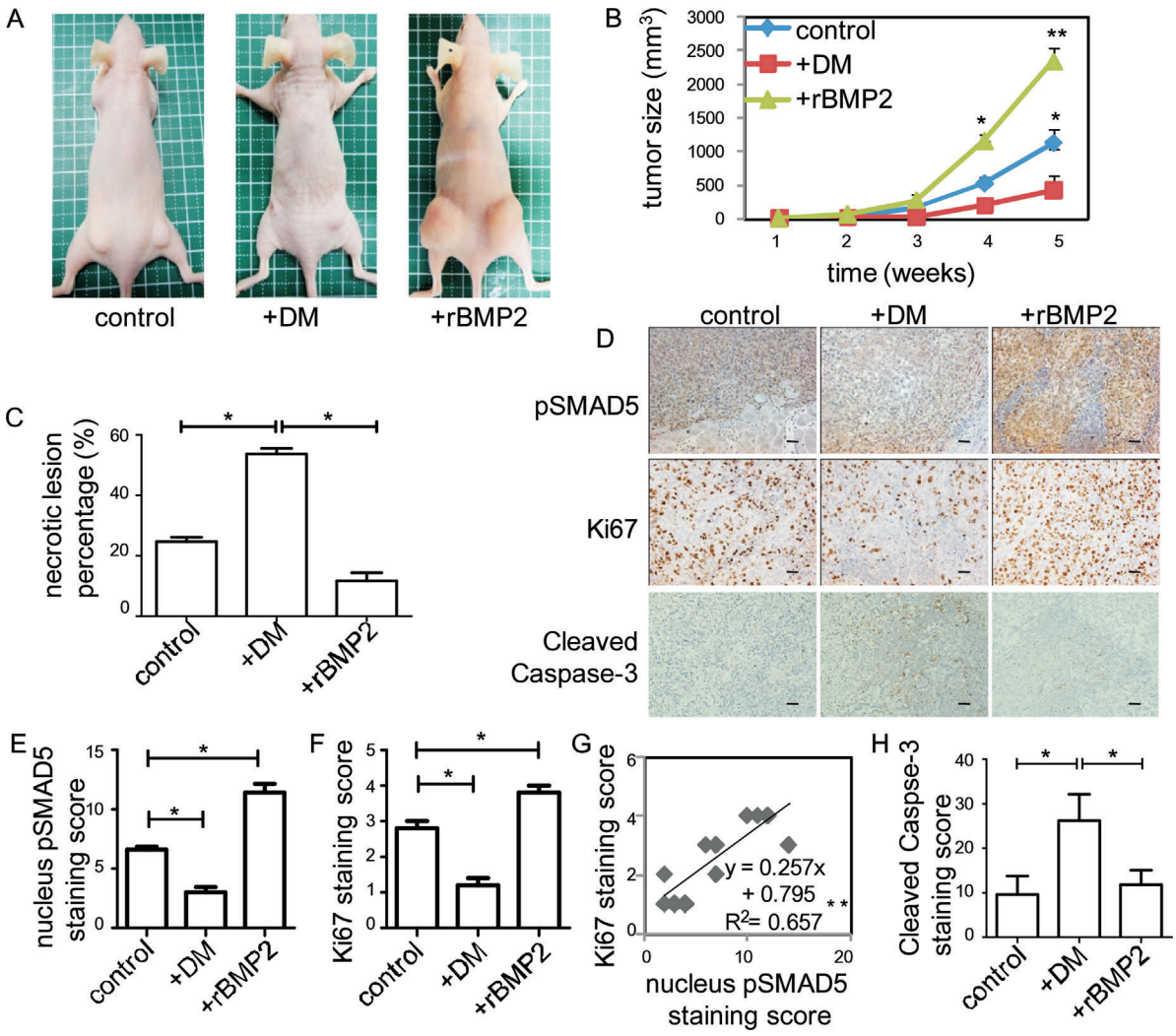

Figure 7. The impact of DM or rBMP2 is on tumor growth in serous ovarian cancer mice model in vivo. (A) SK-OV-3 cells inoculated tumors in contralateral axillae of CD-1 foxn/nu nude mice, treated with PBS (control), DM, or rBMP2 intraperitoneally every 72 h for totally five times. (B) Tumor sizes in control ($n = 10$) or DM ($n = 10$), or rBMP2 ($n = 10$) group were measured separately. **, $P < 0.01$; *, $P < 0.05$. (C) The percentage of necrosis lesion in tumors (nodules showing necrosis lesion/total tumor nodules at day 28, day 30, day 32) was analyzed in each group after tumor inoculation. *, $P < 0.05$. (D) Immunohistochemical staining pSMAD5, Ki67, or Cleaved Caspase-3 was in mice tumors tissue from each group. (Original magnification, 200×; scar bar, 50 μm). (E) Quantification analysis of nucleus pSMAD5 staining in mice tumors tissue from each group ($n = 5$). *, $P < 0.05$. (F) Quantification analysis of Ki67 staining in mice tumors tissue from each group ($n = 5$). *, $P < 0.05$. (G) Linear regression showing the correlation between nucleus pSMAD5 and Ki67 expression in mice tumors (determination coefficient $R^2 = 0.657$). **, $P < 0.01$. (H) Quantification analysis of Cleaved Caspase-3 staining in mice tumors tissue from each group ($n = 5$). *, $P < 0.05$.

promising clinical candidate for cancer treatment, including ovarian cancer.

In conclusion, we demonstrated that pSMAD5 plays a crucial role in serous ovarian cancer both clinically and biologically. In some ovarian cancers, BMP2 was expressed by cancer cells in an autocrine fashion. However, the sources of BMP in ovarian cancer cells could be more diverse. Mclean et al. reported that cancer-associated mesenchymal stem cells contribute to BMP production [37].Our data also suggest that SMAD5 expression and signaling constitute a prognostic factor in serous ovarian cancers. Moreover, the BMP-SMAD5 signaling pathway could be a potential therapeutic target in ovarian cancer, and DM may serve as a promising therapeutic candidate.

ACKNOWLEDGMENTS

The authors thank the excellent technical assistance of Yuko Hosoe and Maki Kurokawa.

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